Aus der Klinik für Endokrinologie, Diabetologie und Rheumatologie

der Heinrich-Heine-Universität Düsseldorf

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Molekulare Untersuchungen zu Störungen der Insulinsekretion und Insulinwirkung in der Entstehung des Diabetes mellitus Typ2

Habilitationsschrift

zur Erlangung der Venia Legendi für Innere Medizin an der

Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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2009

Meiner Familie

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I. Einleitung

Gegenwärtig erleben wir eine drastische Zunahme der Inzidenz des Diabetes mellitus. Prognosen gehen von einem weltweiten Anstieg des Diabetes mellitus von aktuell ca. 170 Mio. Menschen auf 365 Mio. Menschen im Jahre 2030 aus[1]. Der Diabetes mellitus Typ2 entsteht auf dem Boden einer Insulinresistenz und manifestiert sich beim Versagen der pankreatischen ß-Zellen. Somit sind sowohl eine gestörte Insulinwirkung als auch eine Sekretionsstörung als pathogenetische Faktoren anzusehen und damit auch Angriffspunkte für eine pharmakologische Therapie des Typ2 Diabetes mellitus.

In den letzten Jahren ist zunehmend erkannt worden, dass beim Diabetes mellitus außerdem eine Dysregulation des insulinantagonistischen Hormons **Glukagon** eine große Rolle spielt. Glukagon wird in den α-Zellen des endokrinen Pankreas gebildet, stimuliert die hepatische Glukoseabgabe und steigert den Blutzucker. Die Sekretion von Glukagon wird durch Insulin gehemmt[2]. Bereits 1975 beschrieben Unger und Orci die Rolle von Glukagon beim Diabetes mellitus: Bei Menschen mit Diabetes mellitus kommt es zu einer Hyperglukagonämie, die wesentlich zur Hyperglykämie beiträgt[3]. Eine Ursache hierfür ist möglicherweise eine verminderte Hemmbarkeit der Glukagonsekretion der α-Zellen durch Insulin. Tatsächlich zeigte sich auf zellulärer Ebene, dass eine chronische Exposition mit hohen Insulinkonzentrationen im Medium – ein Zustand, der das initiale Stadium des Diabetes mellitus Typ2 imitiert - zu einer Insulinresistenz der α-Zellen mit konsekutiver ungehemmter Glucagonfreisetzung führt[4]. Wir haben uns deshalb intensiv mit der parakrinen Wirkung von Insulin auf umliegende pankreatische α-Zellen beschäftigt. In diesem Zusammenhang konnten wir in α-Zellen die intrazelluläre Insulin-Signalkaskade zum Glukagongen charakterisieren (**Publikation 1**)[5].

Da die Hyperglukagonämie zur Blutzuckerentgleisung beiträgt, ist die Hemmung von Glukagon ein Ziel pharmakologischer Interventionen beim Diabetes mellitus. Beispielsweise können Glukagonrezeptor-Antagonisten den Blutglukosespiegel senken[6]. GLP-1 (glucagon like-peptide-1) basierte Therapiekonzepte sind bereits in breiter klinischer Anwendung: GLP-1 steigert Glukose-abhängig die Insulinsekretion und hemmt gleichzeitig die Glukagonsekretion. Interessanterweise senkt die Applikation von GLP-1 auch im komplett insulinopriven Tiermodell den Blutzucker. Dieser Effekt wird über die Senkung der Glukagonspiegel vermittelt[7].

Auch beim Menschen senkt GLP-1 den Blutzucker nicht nur über Effekte auf ß-Zellen, sondern auch durch eine Hemmung der Glukagonfreisetzung[8,9]. Wir haben außerdem die Regulation der Glukagon- und Insulingenexpression durch Thiazolodindione (Glitazone), eine weitere Gruppe oraler Antidiabetika, auf molekularer Ebene untersucht. Dabei fanden wir eine Inhibition der Glukagon- und Insulingentranskription durch den Transkriptionsfaktor Peroxisome proliferator-activated receptor (PPAR)γ und Glitazone als PPARγ-Liganden. Hierbei unterschieden sich die molekularen Mechanismen der Hemmung an den beiden Promotoren (**Publikation 2**)[10], (**Publikation 3**)[11].

Aus dem Bemühen heraus, intrazelluläre Mechanismen der **Insulinresistenz** zu verstehen, wurden in den vergangenen Jahrzehnten durch grundlagenwissenschaftliche Arbeiten wesentliche Schritte in der Insulinsignalkaskade, vor allem im Skelettmuskel, aufgeklärt. Abb.1. zeigt ein Schema der intrazellulären Ereignisse nach Bindung von Insulin an seinen Rezeptor. Der Insulinrezeptor ist ein Zellmembranrezeptor mit intrazellulärer Tyrosinkinasefunktion. Nach Bindung von Insulin kommt es durch Autophosphorylierung von Tyrosinresten zur Rekrutierung von Brückenproteinen wie IRS (insulin receptor substrate). Dies bedingt eine Bindung der PI 3-K (phosphatidylinositol 3-kinase) an den Komplex, welche PIP2 (Phosphoinositol-4,5-Biphosphat) und PIP3 (Phosphoinositol-3,4,5-Triphosphat) generiert. Dadurch wird eine weitere Kinase, die PDK-1 (phosphoinositide-dependent kinase-1), aktiviert. Die PDK-1 induziert mit der Phosphorylierung der PKB (Proteinkinse B, Akt) an Thr308 deren Aktivierung, die nach zusätzlicher Phosphorylierung an Ser473 komplett ist[12]. Die PKB ist ein zentraler Vermittler der Insulinantwort und kann – wie in Abb.1 gezeigt – über GSK-3 (glykogen-synthase kinase-3) oder FKHR-(forkhead in rhabdomyosarcoma) Transkriptionsfaktoren die Transkription von Zielgenen oder aber „direkt“ die Translokation des Glukosetransporters GLUT4 an die Plasmamembran regulieren (reviewed in **Publikation 4**[13]).
Trotz des inzwischen guten molekularen Verständnisses der Insulinsignaltransduktion in zellulären Systemen und Tiermodellen ist die Bedeutung der einzelnen Signalmoleküle für die Glukosehomöostase beim Menschen weitaus weniger gut verstanden. Wir haben deshalb in einem Kandidatengenansatz exemplarisch in einer Familie mit extremer und frühmanifestierter Insulinresistenz

Gene untersucht, die kausal mit der extremen Insulinresistenz in Zusammenhang stehen können. Wir fanden, dass die Proteinkinase B (PKB) für die Insulinwirkung in Leber und Fettgewebe beim Menschen essentiell ist (**Publikation 5**)[14].

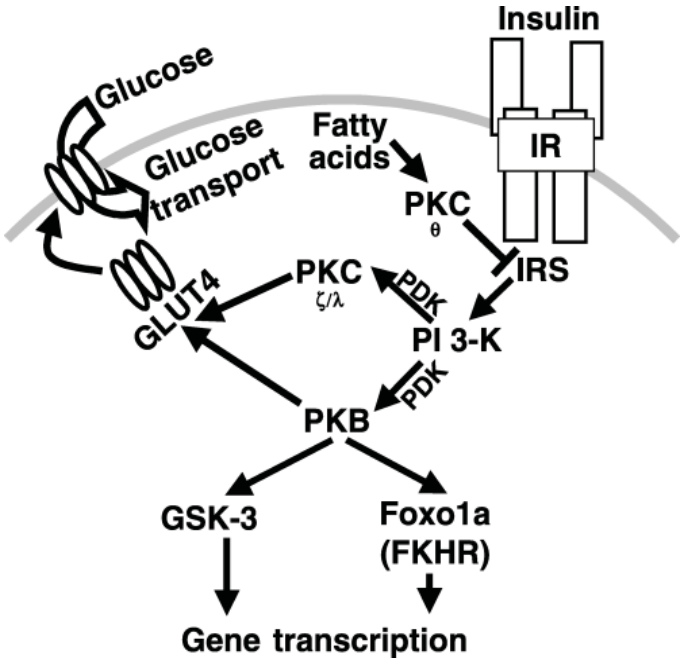

Abbildung 1: Schematische Darstellung der Insulin-Signaltransduktion: Durch Bindung von Insulin an seinen Zellmembranrezeptor kommt es zur Aktivierung der PI 3-Kinase. Die PI 3-Kinase vermittelt über die PDK die Aktivierung der PKB, welche über eine Phosphorylierung von GSK-3 oder Foxo1a Zielgene reguliert. Weiterhin induziert aktivierte PKB die Aufnahme von Glukose in die Zelle durch Rekrutierung des Glukosetransporters GLUT4 an die Plasmamembran. Weitere distal der PI 3-Kinase gelegene Zielmoleküle sind die PKCζ/λ. Eine Hemmung der intrazellulären Insulinwirkung kann durch freie Fettsäuren (FFA) erfolgen. Dies kann durch die PKCθ vermittelt werden. *Abkürzungen: IR, insulin receptor; IRS, insulin receptor substrate; PDK, phosphoinositide-dependent kinase; PI 3-K, phosphatidylinositol 3-kinase; PKB, protein-kinase B; PKC, protein-kinase C; GSK-3, glykogen-synthase kinase-3; GLUT-4, glucose transporter 4; Foxo, forkhead box protein; FKHR, forkhead in rhabdomyosarcoma.*
aus: Schinner S. et al. Diabetic Medicine. 22, 6: 674-682 (2005).

Adipositas ist der Hauptrisikofaktor für das Entstehen einer Insulinresistenz. Die Rolle des Fettgewebes bei der Entstehung der Insulinresistenz ist in den vergangenen Jahren Fokus intensivsten wissenschaftlichen Interesses gewesen (reviewed in[13, 15]). Randle und Kollegen hatten bereits in den 60er Jahren des letzten Jahrhunderts einen Grundstein für diese Zusammenhänge gelegt, indem sie die Wirkung freier Fettsäuren auf die Insulinwirkung am Skelettmuskel charakterisierten[16].

Arbeiten der letzten Jahre zeigten, dass Adipozyten nicht nur Lipide speichern, sondern auch Signalmoleküle (sogenannte Adipokine) sezernieren. Beispielhaft seien hier die Adipokine Leptin, TNFα, Resistin, Visfatin und Adiponektin genannt. Bei Adipositas liegt eine Dysfunktion von Adipozyten vor, weshalb es zur Überschwemmung des Organismus mit Fettsäuren kommt, die dann ektop – z.B. in Leber und Skelettmuskel – abgelagert werden, wo sie eine Insulinresistenz bewirken. Zudem ist das Sekretionsmuster und die Wirkung von Adipokinen bei Adipositas gestört, was ebenfalls eine Insulinresistenz hervorruft[17]. Wir haben in diesem Zusammenhang in einer klinischen Studie bei morbid adipösen Hochrisikopatienten Faktoren untersucht, die mit dem Auftreten einer gestörten Glukosetoleranz/Diabetes mellitus Typ2 (IGT/DM2) assoziiert sind und fanden signifikant niedrigere Serumspiegel von Adiponektin bei IGT/DM2 als bei metabolisch Gesunden (**Publikation 8**)[18]. Dies interpretieren wir als eine protektive Wirkung von Adiponektin gegen die Entstehung eines Diabetes mellitus Typ2 in Hochrisikopatienten.

Die Differenzierung von Fibroblasten-ähnlichen Präadipozyten zu Adipozyten (**Adipogenese**) bestimmt wesentlich die Adipozytenzahl und damit die metabolische Funktion des Fettgewebes. Wir haben deshalb in zellbiologischen Arbeiten die molekulare Regulation der Adipogenese untersucht. Hierbei konnten wir den Corepressor ETO sowie PPARγ–Coaktivator(PGC)-1ß und den Wnt-Antagonisten Dickkopf(Dkk)-1 als Regulatoren der Adipogenese charakterisieren (**Publikation 6**)[19], (**Publikation 7**)[20], (**Publikation 10**)[21].

Die **Regulation der Insulinsekretion** ist ein wesentlicher Forschungsschwerpunkt zum Verständnis der Pathomechanismen beim Diabetes mellitus Typ2: Der Großteil der insulinresistenten Patienten kompensiert die verminderte Insulinwirkung (zumindest temporär) und entwickelt keine diabetische Stoffwechsellage. Der Verlust der Insulinsekretion schreitet dann aber über eine gestörte Glukosetoleranz bis zum Diabetes mellitus Typ2 fort[22].
Die molekularen Mechanismen, über die es bei Adipositas-induzierter Insulinresistenz zur kompensatorischen Hyperinsulinämie kommt, sind bisher unvollständig verstanden. Einerseits kann der Insulinspiegel reaktiv durch minimale Schwankungen des Glukosespiegels innerhalb des Normbereichs ansteigen. Andererseits hat der BMI (Body Mass Index) einen positiven Effekt auf die

Insulinspiegel, der unabhängig von der Insulinresistenz ist[23]. Dies kann durch eine direkte Wirkung von Adipozyten auf ß-Zellen erklärt werden. In der Tat sind für verschiedene Adipokine wie Leptin oder Visfatin Wirkungen auf pankreatische ß-Zellen beschrieben worden[24, 25].

Eigene Arbeiten haben einen weiteren Signalweg in diesen Kontext gesetzt: Fettzellen produzieren Wnt-Signalmoleküle (**Publikation 11**)[26], die auf auto- und parakrinem Weg die Adipozytendifferenzierung regulieren (**Publikation 10**)[21, 27]. Zudem konnten wir zeigen, dass Adipozyten über sezernierte Wnt-Signalmoleküle die Funktion pankreatischer ß-Zellen regulieren und wahrscheinlich auch trophische Effekte auf die ß-Zellen ausüben (**Publikation 13**)[28]. Interessanterweise finden sich in einem adipösen, insulinresistenten und hyperinsulinämischen Tiermodell vermehrt Adipozytenablagerungen im Pankreas, was parakrine Wirkungen zwischen Adipozyten und ß-Zellen ermöglichen würde (**Publikation 14**)[29]. Parallel zu diesen grundlagenorientierten Arbeiten hat der Wnt-Signalweg seit 2006 große Beachtung gefunden, weil Polymorphismen in TCF7L2, einem Wnt-reguliertem Transkriptionsfaktor, die stärkste bisher beschriebene Assoziation mit dem Auftreten von Diabetes mellitus 2 beim Menschen zeigen[30-34].

II. Darstellung und Diskussion der Ergebnisse

II.a Parakrine Insulinwirkung: Regulation der Transkription des insulinantagonistischen Hormons Glukagon

Charakterisierung des Insulin-Signalwegs in pankreatischen α-Zellen

Glukagon wird von pankreatischen α-Zellen gebildet und erhöht den Blutzuckerspiegel durch eine Steigerung der hepatischen Glukosefreisetzung[35]. Die Bedeutung des erhöhten Glukagonspiegels beim Diabetes mellitus wird ersichtlich, wenn man betrachtet, dass die Hemmung der Glukagonfreisetzung im Tiermodell und im Menschen die Blutglukose senkt und zwar sowohl die Nüchtern-Glukose als auch den postprandialen Blutglukose-Anstieg[7,8].

Innerhalb der Langerhans'schen Insel liegen die insulin-produzierenden ß-Zellen in enger Nachbarschaft zu den Glukagon-produzierenden α-Zellen. Somit sind letztere hohen Konzentrationen parakrin wirkenden Insulins ausgesetzt[35]. Insulin ist ein zentraler Regulator der Glukagonsekretion und der Transkription des Glukagongens[2]. In Analogie zu Leber und Skelettmuskel wurde eine Insulinresistenz der α-Zellen als eine Ursache der Enthemmung der Glukagonfreisetzung und somit für die erhöhten Glukagonspiegel beim Diabetes mellitus postuliert. In neuesten Arbeiten hat sich *in vitro* bestätigt, dass chronische Insulinbehandlung von α-Zellen zu einer Abnahme der Hemmbarkeit der Glukagongentranskription durch Insulin führt[4].

Um die Regulation des Glukagongens durch Insulin zu verstehen, haben wir in pankreatischen α-Zellen den Insulin-Signalweg zum Promotor des Glukagongens untersucht: Durch den Einsatz von Kinase-Inhibitoren konnten wir zeigen, dass die hemmende Wirkung von Insulin auf den Glukagongenpromotor von der Aktivität der PI 3-Kinase, nicht aber von der Aktivität der Proteinkinase C (PKC), der Mitogen-activated protein kinase (MEK) oder der p70S6-Kinase abhängt. Um die Signalkaskade distal der PI 3-Kinase aufzuklären, führten wir Immunoblot- und *in vitro* Kinase-Assays durch. Diese zeigten, dass Insulin in α-Zellen zu einer PI 3-kinase abhängigen Phosphorylierung und Aktivierung der Proteinkinase B führt. Überexpression einer konstitutiv aktiven PKB-Mutante wiederum konnte die Effekte von Insulin auf den Promotor des Glukagongens vollständig imitieren: Sowohl die Insulin- als auch die PKB-Responsivität des Glukagongenpromotors gingen verloren, wenn die Bindungsstellen für den Transkriptionsfaktor Pax6 in den

Promotorelementen G1 und G3 mutiert wurden. Sofern Pax6 oder der transkriptionelle Coaktivator CREB-binding protein (CBP) mit Hilfe des GAL4 – Systems wieder an die mutierten G1- und G3-Elemente des Promotors rekrutiert wurden, konnte die Responsivität sowohl für Insulin als auch für PKB gleichermaßen wiederhergestellt werden. Diese Daten definieren das Glukagongen als ein Zielgen für die Proteinkinase B. Weiterhin implizieren sie die Regulation des Glukagongens durch Insulin durch den PI 3-Kinase/PKB-Signalweg (**Publikation 1**)[5] (s. Abb. 2).

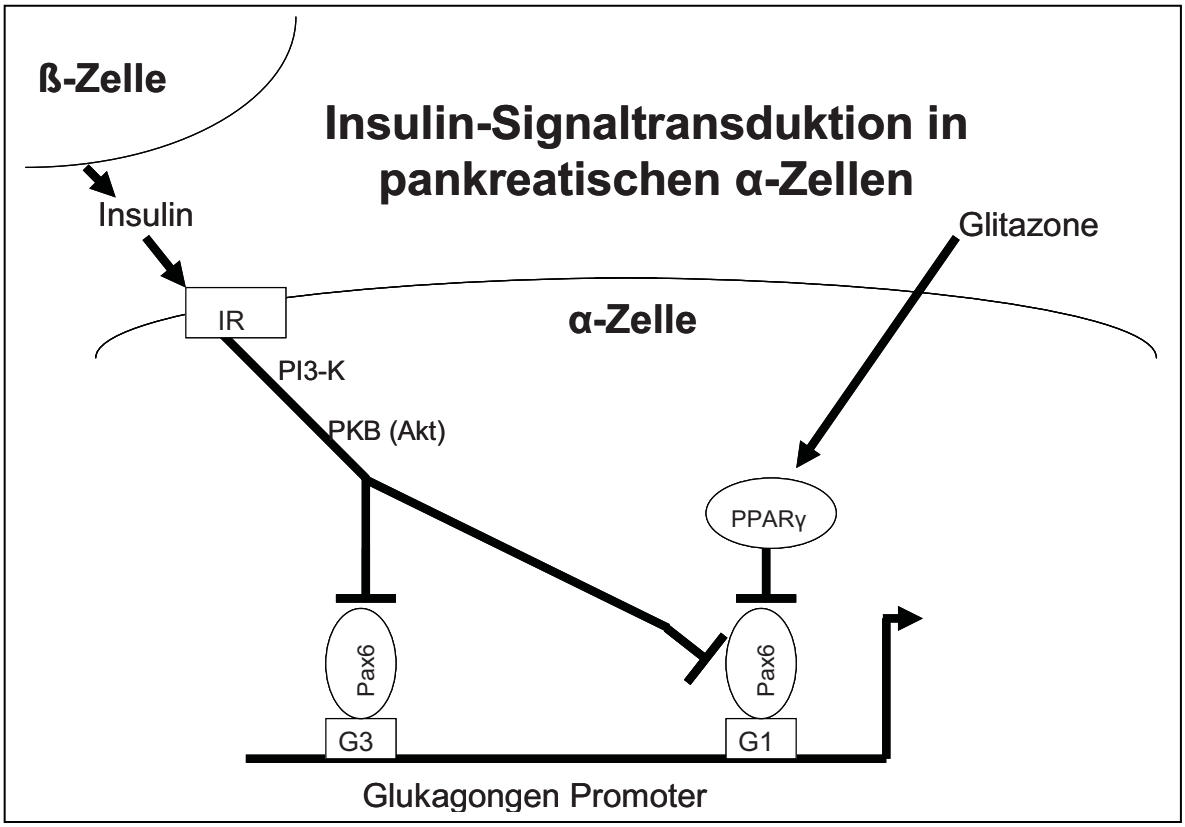

Abbildung 2: Darstellung der Insulin-Signaltransduktion in pankreatischen α-Zellen: Insulin aus benachbarten β-Zellen bindet an den Insulin-Rezeptor (IR). Dies führt zu einer Aktivierung der PI 3-Kinase und konsekutiv der Proteinkinase B. Diese reguliert den Promotor des Glukagongens über den Transkriptionsfaktor Pax6, der an die Elemente G1 und G3 im Promotor bindet. Glitazone aktivieren den nukleären Rezeptor PPARγ. Dieser hemmt die transkriptionelle Aktivität von Pax6 an G1 und hemmt somit die Transkription des Glukagongens.
Abkürzungen: IR, insulin receptor; PI 3-K, phosphatidylinositol 3-kinase; PPAR peroxisome proliferator-activated receptor

PPARγ/Glitazone imitieren die Wirkung von Insulin auf die Transkription des Glukagongens

In zahlreichen Tiermodellen und beim Menschen konnte gezeigt werden, dass der nukleäre Rezeptor PPARγ den Glukosestoffwechsel reguliert und beeinflusst[36,37,38]. Glitazone (Thiazolidindione) sind Liganden an PPARγ. Typische Vertreter dieser Stoffklasse sind Rosiglitazon und Pioglitazon. Diese Substanzen werden als orale Antidiabetika eingesetzt, deren Hauptwirkung die Verbesserung der Insulinsensitivität ist (sog. Sensitizer)[39].

PPARγ kann nach Aktivierung durch Liganden gemeinsam mit RXR (9-cis-retinoic acid receptor) als Heterodimer spezifische DNA-Consensus-Motive binden und so die transkriptionelle Aktivität von Zielgenen steigern. Diese Consensus-Motive in Zielgenen werden als PPRE (PPAR-responsive element) bezeichnet und bilden ein sogenanntes DR-1 Muster (direct repeat mit einer Base zwischen zwei Hexameren)[36]. Weiterhin kann PPARγ – wie auch andere nukleäre Rezeptoren - nach Ligandenaktivierung zu einer Hemmung von Zielgenen führen. Dies erfolgt jedoch ohne DNA-Bindung und kann bspw. durch eine Hemmung anderer Transkriptionsfaktoren geschehen, wie z.B. für STAT1, NF-κB und AP-1 gezeigt[40, 41]. Als weitere Beispiele hierfür seien die Hemmung der DNA-Bindung der Transkriptionsfaktoren NFAT und NRF2 und die konsekutive Inhibierung der NFAT- und NRF2 abhängigen Zielgene genannt[42, 43].

Glitazone zeigen an verschiedenen Geweben Insulin-ähnliche Wirkungen und PPARγ ist in α- und ß-Zellen der Langerhans'schen Insel stark exprimiert[44,45]. Deshalb haben wir untersucht, ob Glitazone über PPARγ eine direkte Wirkung auf die Transkription und Sekretion von Glukagon haben.

Wir fanden eine PPARγ abhängige Hemmung der Transkription des Glukagongens in einer α-Zell-Linie (InR1G9) durch verschiedene Glitazone (Rosiglitazon, Darglitazon und Englitazon). Parallel hierzu hemmten Glitazone die Sekretion und den Gewebsgehalt von Glukagon in primär isolierten Pankreas-Inseln der Maus.

Um den molekularen Mechanismus der Hemmung des Glukagongens durch Glitazone zu untersuchen, führten wir Mapping-Experimente zur Lokalisation Glitazon/PPARγ-responsiver Elemente im Promotor durch: In 5'Deletions-Studien fanden wir Hinweise für Glitazon/PPARγ-Responsivität im Bereich zwischen -136 und -60bp. Die 3'Deletionen grenzten den Bereich zwischen -48 und -91 ein, so dass wir zusammenfassend ein entsprechendes responsives Element zwischen -136 und -48

vermuteten. In diesem Bereich liegt das G1-Element des Glukagongenpromotors. Dieses Element enthält das sogenannte PISCES-Motiv, das für die Promotoraktivität essentiell ist[46,47]. Eine interne Mutation dieses PISCES-Motivs innerhalb des G1-Elements führte in der Tat zu einem Verlust der Hemmbarkeit des Glukagongenpromotors durch Rosiglitazon. Um zu prüfen, ob die Hemmung des Glukagongens durch eine Promotorbindung von PPARγ an das PISCES-Motiv im G1-Element vermittelt wird, benutzten wir das GAL4-System, um PPARγ an dieses Element zu rekrutieren. Es zeigte sich jedoch eine Aktivierung der Transkription, wenn PPARγ an G1 rekrutiert wird. Übereinstimmend mit Daten zu anderen negativ-regulierten Zielgenen von PPARγ (s.o.) scheint also eine DNA-Bindung von PPARγ nicht der Mechanismus zu sein, über den die Hemmmung vermittelt wird. Tatsächlich konnte die Hemmbarkeit des Glukagongens durch PPARγ wiederhergestellt werden, wenn Pax6 - ein Transkriptionsfaktor, der an das PISCES-Motiv bindet - wieder an den mutierten Promotor rekrutiert wurde. Hiermit übereinstimmend hemmte liganden-aktiviertes PPARγ die Pax6-vermittelte Transkription auch an einem heterologen Promotor. Diese Ergebnisse zeigen, dass Glitazone direkt an der pankreatischen α-Zelle die Sekretion und Transkription von Glukagon hemmen. Dies könnte ein weiterer Mechanismus sein, über den Glitazone den Blutzucker senken. Auf molekularer Ebene zeigen diese Ergebnisse zudem, dass aktiviertes PPARγ die transkriptionelle Aktivität von Pax6 hemmt (s. Abb. 2) und erweitern somit unser Verständnis von negativer Regulation von Zielgenen durch PPARγ (**Publikation 2**)[10].

<u>Die Wirkung von PPARγ/Glitazonen auf die Transkription des Insulingens</u>

In klinischen Studien konnte gezeigt werden, dass eine orale antidiabetische Therapie mit Glitazonen zu einer Senkung des Insulinspiegels führt[48, 49]. Dieser Effekt ist einerseits durch die verbesserte Insulinsensitivität zu erklären; andererseits stellte sich die Frage, ob Glitazone direkte Wirkungen auf die Insulinproduktion in pankreatischen ß-Zellen haben.

Wir fanden eine Hemmung der Insulingentranskription durch Rosiglitazon in Zellkulturen einer β-Zell-Linie (HIT): Entsprechend zeigte sich durch Rosiglitazon auch eine Hemmung der Glukose-stimulierten Insulingentranskription in primären Inseln der Maus, die ein Luciferase-Reportergen unter der Kontrolle des humanen Insulingenpromotors tragen. Wie oben dargestellt spielt eine Interaktion von PPARγ mit dem Transkriptionsfaktor Pax6 eine entscheidende Rolle für die Hemmung des

Glukagongenpromotors durch Glitazone/PPARγ. Wir haben deshalb untersucht, ob ein ähnlicher Mechanismus auch am humanen Insulingen zutrifft:
Hier zeigte sich jedoch, dass ein ”knockdown” von Pax6 mittels siRNA weder die basale transkriptionelle Aktivität noch die Hemmung des humanen Insulingenpromotors durch PPARγ/Glitazone verändert. Daraus kann geschlossen werden, dass Pax6 hier nicht die Wirkung von PPARγ vermittelt. Mapping-Experimente wiesen auf PPARγ-Responsivität im proximalen Promotorbereich in Nähe zum Transkriptionsstartpunkt (-56 bis +18) des humanen Insulingens hin. Für den hemmenden Effekt von PPARγ auf den humanen Insulingenpromotor war die DNA-Bindungsdomäne von PPARγ essentiell. Zudem ging die Hemmbarkeit des humanen Insulingens durch PPARγ verloren, wenn der proximale Promotorbereich mutiert wurde. Diese Befunde sprechen dafür, dass PPARγ – im Gegensatz zum Mechanismus am Glukagongen - durch eine direkte Bindung an den proximalen Promotorbereich die Transkription des humanen Insulingens hemmen.
Im EMSA (Electrophoretic Mobility Shift Assay) zeigte sich, dass Zellextrakte primärer Inseln an die proximale Promotorregion des humanen Insulingens binden; die Bindung ist durch ein PPAR-bindendes Oligonekleotid kompetierbar. Diese Daten implizieren, dass PPARγ aus dem Zellextrakt primärer Inseln an die beschriebene Promotorregion im humanen Insulingen bindet (**Publikation 3**)[11].
Zusammenfassend demonstrieren unsere Befunde eine direkte Hemmung der Insulingentranskription durch Glitazone/PPARγ. Dies kann - neben der Verbesserung der Insulinsensitivität - ein Mechanismus sein, der zur Senkung des Insulinspiegels unter Therapie mit Glitazonen führt.
Die direkte Hemmung der Insuligentranskription stimmt mit Daten von Bollheimer et al. und Nakamichi et al. überein, die eine Hemmung von Proinsulin bzw. von Präproinsulin mRNA-Synthese durch Glitazone beschrieben[50,51]. Demgegenüber fanden Vandewalle et al. keinen Effekt von 1µM Rosiglitazon auf die Insuligentranskription nach chronischer Behandlung von Inseln mit freien Fettsäuren[52]. Exposition mit freien Fettsäuren scheint also das Ansprechen von β-Zellen auf Glitazone zu modulieren.

II.b Molekulare Mechanismen der Insulinresistenz

Die Entstehung einer Insulinresistenz von Skelettmuskel, Leber und Fettgewebe ist ein zentraler Prozess in der Pathogenese des Diabetes mellitus Typ2 und geht der Manifestation des Diabetes mellitus meist um Jahre voraus. Zur Entstehung der Insulinresistenz tragen genetische und erworbene Faktoren bei (reviewed in **Publikation 4**[13]). Bei letzteren stehen Adipositas und Bewegungsmangel im Vordergrund. Zudem hat die Rolle subklinischer Inflammation in der Genese der Adipositas in den letzten Jahren besondere Aufmerksamkeit erlangt. Wir haben uns in den hier vorgestellten Arbeiten mit diesen verschiedenen pathogenetischen Aspekten der Insulinresistenz beschäftigt.

Die intrazellulären Kaskaden des Insulinsignalwegs sind durch grundlagenwissenschaftliche Arbeiten inzwischen auf vielen Ebenen gut charakterisiert: Der Insulinrezeptor besitzt einen extrazellulären Abschnitt (Ligandenbindungsdomäne) und intrazelluläre Tyrosinkinasedomänen. Nach Ligandenbindung kommt es zur Autophosphorylierung von Tyrosinresten, wodurch Brückenproteine – wie die Insulinrezeptorsubstrate (IRS) - rekrutiert und phosphoryliert werden. Durch die IRS wird u.a. die PI 3-Kinase an den Komplex rekrutiert und aktiviert. Diese Kinase generiert PIP2 (Phosphoinositol-4,5-Biphosphat) und PIP3 (Phosphoinositol-3,4,5-Triphosphat). Letztere bewirken eine Aktivierung der PDK-1 (Phosphatidylinositol-dependent protein kinase). Die PdK-1 phosphoryliert die PKB (Proteinkinase B) (synonym wird Akt verwendet) an Thr308, was eine weitere Phosphorylierung an Ser473 nach sich zieht (s. Abb.1). Diese Phosphorylierungen bewirken eine Aktivierung der PKB. Die zentrale Rolle der Proteinkinase B im Glukosestoffwechsel ist *in vitro* und in Tiermodellen gut untersucht: Die PKB induziert einerseits die Rekrutierung des Glukosetransporters GLUT4 an die Plasmamembran, andererseits vermittelt diese Kinase an einer Vielzahl von Zielgenen die Insulin- Effekte. Typische Substrate der PKB sind die GSK-3 (Glykogen Synthase-Kinase-3) und FKHR (Foxo) Transkriptionsfaktoren (reviewed in[12], **Publikation 4**[13]).

<u>Genetische Aspekte der Insulinresistenz</u>

Die Mehrzahl der Fälle von Diabetes mellitus Typ2 entsteht auf polygenetischer Grundlage. Es gibt jedoch auch Fälle von familiärem monogenetischen Diabetes mellitus Typ2, welche die einzigartige Möglichkeit bieten, die Rolle eines Gens im

Glukosestoffwechsel beim Menschen zu verstehen und so weiterführende pathophysiologische und potentielle therapeutische Konzepte zu entwickeln.

In einem Kandidatengenansatz in einer Kohorte mit früh-manifestierter extremer Insulinresistenz fanden wir bei einer Patientin eine mis-sense Mutation in der Proteinkinase Bβ (Akt2). Die betreffende Patientin hatte im Alter von 30 Jahren einen Diabetes mellitus Typ2 entwickelt, ohne übergewichtig zu sein. In hyperinsulinämisch/euglykämischen „Clamp"-Versuchen zeigte sich bei der Patientin eine extreme Insulinresistenz.

In der Familienanamnese fielen weitere Mitglieder mit ähnlichem klinischem Bild auf. Sowohl die Patientin als auch die betroffenen Familienangehörigen trugen die gleiche Mutation (R274H) in Akt2. Diese Position liegt in der katalytischen Domäne der Kinase. In einem errechneten Molekülmodell wurde ein Verlust der katalytischen Aktivität durch die R274H Mutation vorausgesagt. Tatsächlich bestätigte sich diese These experimentell in einem *in vitro* Kinase-Assay: Das mutante Protein konnte im Gegensatz zum Wildtyp-Protein eine typische Zielsequenz aus der GSK-3 nicht phosphorylieren. Ebenso konnte das mutante Protein den Transkriptionsfaktor FOXA2 nicht inhibitorisch phosphorylieren und bewirkte somit nicht dessen nukleäre Exklusion wie man es durch den Akt2 wildtyp sieht. Die Akt-R274H Mutante wirkte sogar dominant negativ über cotransfizierte wildtyp-Akt Plasmide.

In zellulären Differenzierungs-Assays vermittelte die Akt-R274H Mutante (wiederum im Gegensatz zum Wildtyp-Protein) keine Induktion der Adipogenese. In Übereinstimmung hiermit zeigte die Patientin eine Verminderung der Körperfettmasse um 35% (bezogen auf Gewicht und Körpergröße) (**Publikation 5**)[14]. Somit belegte diese molekulare Aufarbeitung einer familiären Insulinresistenz, dass eine monogenetische heterozygote Mutation der Akt2 ausreicht, um eine klinische Konstellation mit extremer Insulinresistenz zu induzieren und zeigte (sub-)zelluläre Mechanismen auf, dies zu erklären. Hierdurch wird die Bedeutung der Akt2 für die Glukosehomöostase beim Menschen unterstrichen.

Neben der Akt2 wurden in weiteren Molekülen des klassischen Insulinsignalwegs Mutationen beschrieben, die mit Insulinresistenz cosegregierten. Hierzu zählen der Insulinrezeptor, IRS-1 und PI 3-K, wobei für die beiden letzteren keine klaren *in vitro*-Daten für die Kausalität der Mutationen für den Phänotyp existieren (reviewed in **Publikation 4**[13]).

Insulinresistenz und Adipozytenfunktion

Die Anzahl und Funktionsfähigkeit von Adipozyten sind zentrale Einflußgrößen auf die Insulinsensitivität eines Organismus: Zum einen führt eine Verminderung, bzw. das Fehlen von Adipozyten (Lipodystrophie), zu einer Insulinresistenz von Leber und Muskel. Zum anderen liegt auch bei Adipositas eine solche Insulinresistenz vor. Ursache ist hier eine Dysfunktion von Adipozyten, die sich in morphologischen und metabolischen Veränderungen ausdrückt. Betroffen sind vor allem viszerale Adipozyten[53,54]. Adipozyten übernehmen im gesunden Organismus zwei wichtige Funktionen, nämlich die othotope Speicherung von Lipiden und die geregelte Sekretion adipozytärer Hormone (sogenannte „Adipokine“). Beide Funktionen sind sowohl beim Fehlen von Adipozyten (Lipodystrophie) als auch bei deren Dysfunktion (Adipositas) beeinträchtigt. Es folgt zum einen eine Überschwemmung des Organismus mit Lipiden (v.a. freien Fettsäuren), die dann ektop bspw. in Leber oder Skelettmuskel gespeichert werden. Die Pionierarbeiten zu diesem Komplex stammen von Randle und Kollegen, die zeigten, dass freie Fettsäuren die Glukoseaufnahme am Muskel hemmen[16]. Es gibt inzwischen zahlreiche Arbeiten (*in vitro*-Studien, Tiermodelle und Untersuchungen am Menschen), die belegen, dass eine solche ektope Lipideinlagerung zu einer Insulinresistenz des betroffenen Organs führt (reviewed in[17, 55]).

Zum Anderen produzieren dysfunktionale Adipozyten ein verändertes Muster an Adipokinen wie Leptin, TNFα, Adiponektin, Resistin oder Visfatin, was ebenfalls die Insulinsensitivität von Muskel und Leber beeinflusst (s.u.).

Aus diesen Gründen ist es von großem Interesse, Prozesse und Mechanismen zu verstehen, welche die Adipozytenzahl und -funktion determinieren. Die Differenzierung von Fibroblasten-ähnlichen Präadipozyten zu Adipozyten (**Adipogenese**) ist ein Determinator der Adipozytenzahl. Die Adipogenese wird durch eine zeitlich organisierte Abfolge der Expression von Transkriptionsfaktoren reguliert: Initial kommt es zu einer Induktion der Transkriptionsfaktoren C/EBP(C/CAAT-enhancer binding protein)ß und C/EBPδ. Diese binden die Promotoren von C/EBPα und PPARγ und aktivieren deren Transkription, wodurch die volle Differenzierung zu Adipozyten eingeleitet wird. PPARγ ist der zentrale Regulator der Adipogenese: Die Überexpression von PPARγ induziert einen Adipozytenphänotyp in Fibroblasten und das Fehlen von PPARγ macht eine Differenzierung von Fibroblasten zu Adipozyten unmöglich[56, 57].

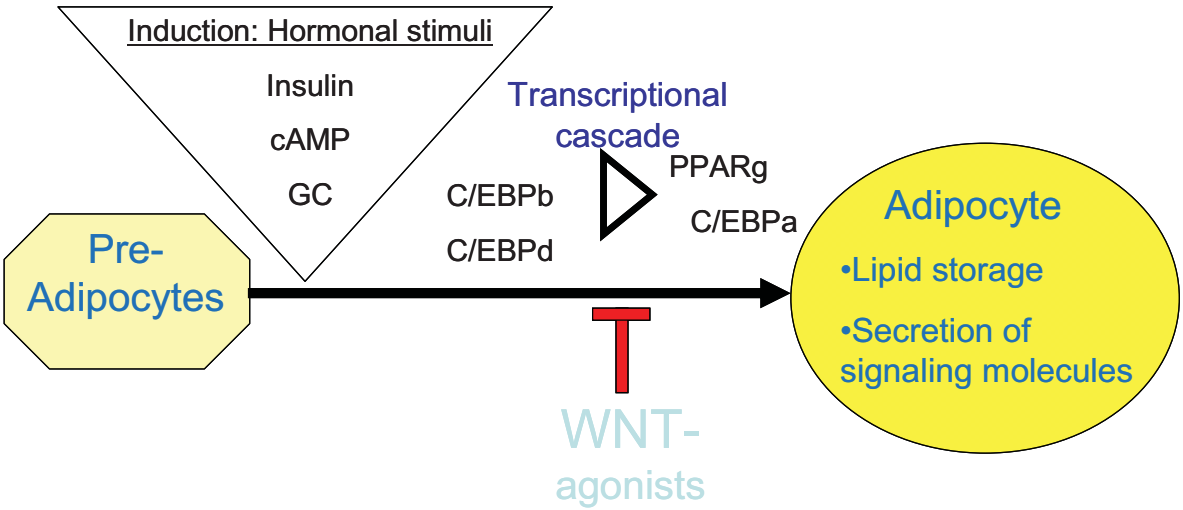

Abbildung 3: Schematische Darstellung der transkriptionellen Regulation der Adipogenese: Externe Stimuli wie Insulin oder Glucocorticoide (GC) können die Adipogenese induzieren. Initial werden die Transkriptionsfaktoren C/EBPß und C/EBPδ induziert. Diese aktivieren C/EBPα und PPARγ, wodurch schließlich das volle Differenzierungsprogramm ausgelöst wird. Wnt-Agonisten hemmen die Adipogenese.
Abkürzungen: C/EBP, C/CAAT-enhancer binding protein; PPAR, peroxisome proliferator-activated receptor; cAMP, cyclic AMP; GC, Glucocoticoid.
aus: Schinner Horm. Metab. Res. 41(2):159-63 (2009)

Wir fanden, dass die Expression des transkriptionellen Corepressor ETO in Fibroblasten durch IGF-1-Stimulation gehemmt wird. Da IGF-1 wie Insulin ein Stimulus der Adipogenese ist, haben wir die Frage untersucht, ob ETO ein Regulator der Adipozytendifferenzierung ist.

ETO als Regulator der Adipogenese

ETO wird in Fibroblasten stark exprimiert; seine Expression wird aber nach Induktion der Adipogenese sehr schnell (innerhalb von vier Stunden) herabreguliert. Übereinstimmend hiermit fand sich auch in primären maturen Adipozyten der Ratte nur eine sehr geringe Expression von ETO, während die Expression in primären Präadipozyten hoch ist. Das humane Homolog zu ETO heißt MTG8. Für MTG8 wurden vergleichbare Befunde im Rahmen der Adipogenese erhoben. Funktionell konnten wir zeigen, dass Überexpression von wildtyp ETO die Adipogenese *in vitro* hemmt, während die Überexpression einer dominant-negativen ETO-Mutante eine verstärkte Adipozytendifferenzierung bewirkte. Die Überexpression von ETO ging mit einer Hemmung der Expression von C/EBPα und PPARγ einher, wohingegen die

Expression von C/EBPß und –δ unverändert war. Dies legt eine Wirkung von ETO auf die Induktion von C/EBPα nahe. Tatsächlich konnten wir eine Hemmung der Wirkung von C/EBPß auf den C/EBPα Promotor durch ETO zeigen. ETO hemmt als Corepressor also die transkriptionelle Aktivität von C/EBPß. Weiterhin fanden wir eine Protein-Protein Interaktion von ETO und C/EBPß, die eine Hemmung der DNA-Bindung von C/EBPß bewirkte (**Publikation 6)**[19].

***PGC-1ß als neuartiger Coaktivator für PPAR*γ**

Der nukleäre Rezeptor PPARγ ist ein Schlüsselregulator des Glukosestoffwechsels und der Adipogenese. Die Induktion der Adipozytendifferenzierung durch Aktivierung von PPARγ wird als wesentlicher Mechanismus der Steigerung der Insulinsensitivität durch Glitazone als Liganden an PPARγ betrachtet[57]. PPARγ besitzt eine N-terminale liganden-unabhängige Transaktivierungsdomäne (AF-1), eine DNA-Bindungsdomäne und C-terminal eine weitere liganden-abhängige Transaktivierungsdomäne (AF-2). PPARγ bindet als Heterodimer mit RXR (9-cis-retinoic acid receptor) über seine DNA-Bindungsdomäne Konsensusmotive (TGACCT) in typischen Zielgenen. Ligandenbindung führt zu einer Konformationsänderung von PPARγ, die eine Bindung von transkriptionellen Coaktivatoren über deren LXXLL-Motiv erleichtert[36]. Transkriptionelle Coaktivatoren können prinzipiell verschiedene Transkriptionsfaktoren binden und so deren Aktivität regulieren. Die Bindung eines Coaktivators an einen Transkriptionsfaktor ist neben den Interaktionspartnern selbst auch vom zellulären Kontext und vom Promotorkontext abhängig. PPARγ kann in verschiedenen Zellen beispielsweise die Coaktivatoren SRC-1, pCIP und CBP rekrutieren[57].

In einem „yeast two-hybrid“ System wurde 1998 ein neuartiger Coaktivator für PPARγ gefunden, der als PGC (PPARγ Coactivator)-1α bezeichnet wurde[58]. PGC-1α wird bei Nagern im braunen Fettgewebe stark exprimiert und seine Expression dort ist durch Kälte induzierbar. Im Skelettmuskel wird die Expression von PGC-1α durch Kältereiz oder Aktivität und in der Leber durch Hungern induziert. Zu den wesentlichen physiologischen Endpunkten der Expression von PGC-1α gehören die Induktion der mitochondrialen Biogenese und der Transkription von uncoupling proteins (UCPs), weshalb PGC-1α eine wesentliche Rolle in der adaptiven Thermogenese zugeschrieben wird[57-59].

Wir konnten aus humaner cDNA aus Adipozyten ein Homolog zu PGC-1α klonieren und funktionell charaktierisieren, was als **PGC-1ß** bezeichnet wurde. Durch differentielles Splicing entstehen sowohl am 5'-wie auch am 3'-Ende Varianten, die zu verschiedenen PGC-1ß Isoformen führen (PGC-1ß-a und –b; sowie PGC-1ß-1 und-2). PGC-1ß wird beim Menschen vor allem in Herz- und Skelettmuskel sowie im Gehirn exprimiert, wobei PGC-1ß-1a die häufigste Isoform ist. Bei Maus und Ratte besteht eine ähnliche Gewebsverteilung, wobei sich zudem eine starke Expression im braunen Fettgewebe findet.

Die Expression von PGC-1ß war in adipösen Ratten nicht verändert. Ebensowenig wurde die Expression von PGC-1ß durch Kälte, Nahrungskarenz oder Aktivität verändert, was im Gegensatz zu PGC-1-α steht. Funktionell fanden wir eine Coaktivierung der nukleären Rezeptoren TR1ß (thyroid hormon receptor1ß), PPARα, $PPAR_{\gamma}$ und des Glucocorticoid-Rezeptors durch PGC-1ß-1a. Retrovirale Überexpression von PGC-1ß-1a in Myoblasten führte zu einem erhöhten Gehalt an Mitochondrien und zu einem gesteigerten Sauerstoffverbrauch. Diese Arbeit beschrieb die Klonierung und funktionelle Charakterisierung von PGC-1ß, einem neuen Coaktivator für nukleäre Rezeptoren. Zu den wichtigsten Prozessen, die durch PGC-1ß reguliert werden, gehört die Biogenese von Mitochondrien. Dies korrespondiert auch mit der Gewebsverteilung der PGC-1ß Expression, die in Organen mit hohem Gehalt an Mitochondrien am höchsten ist (**Publikation 7**)[20]. Im Kontext des Diabetes mellitus Typ2 spielen die Funktion und der Gehalt von Mitochondrien im Skelettmuskel eine besondere Rolle: Dysfunktion oder eine Verminderung der Menge von Mitochondrien korrelieren mit einer Insulinresistenz des Skelettmuskels[60, 61].

Adipokine, Adipositas und Insulinresistenz

1987 wurde durch die Gruppe um Spiegelman und Flier ein Sekretionsprodukt von Adipozyten identifiziert (Adipsin), das auch im zirkulierenden Blut nachgewiesen werden konnte[62]. 1994 wurde dann Leptin als Prototyp eines Adipokins kloniert[63]: Es reguliert zentralnervöse Funktionen wie die Nahrungsaufnahme, aber auch die Insulinsensitivität. Die Adipokine TNFα und Resistin können eine Insulinresistenz an Zielzellen bewirken[64, 65]. Visfatin wurde 2005 als Fettzellhormon identifiziert und besitzt insulin-ähnliche Wirkungen: Eine Arbeit aus 2005 zeigte, dass Visfatin im

Mausmodell den Insulinrezeptor binden und aktivieren und so den Glukosespiegel senken kann[66, 67].

Adiponektin, initial als Acrp30 bezeichnet, wurde 1995 erstmals beschrieben[68]. Es verbessert die Insulinsensitivität und verstärkt die Hemmung der hepatischen Gluconeogenese *in vitro* und *in vivo*[69,70]. Die Serumspiegel von Adiponektin beim Menschen korrelieren negativ mit der Fettmasse[71]. Interessanterweise zeigt sich bei der Maus, dass eine Überexpression von Adiponektin die Insulinsensitivität der Leber erhöht und die Tiere vor der Entstehung einer gestörten Glukosetoleranz nach 6-monatiger fett- und kalorienreicher Ernährung schützt[72]. Adipositas und Diabetes mellitus Typ2 sind auch durch eine chronische, subklinische Inflammation und durch erhöhte Spiegel pro-inflammatorischer Zytokine im Serum gekennzeichnet, welche die Insulinsensitivität vermindern [73-75].

Um Faktoren zu identifizieren, die in einer Hochrisikogruppe die Glukosetoleranz regulieren, haben wir in einer Kohorte morbid adipöser Patienten Inflammationsmarker und Serumspiegel von Adiponektin bestimmt und mit dem Status des Glukosestoffwechsels korreliert. In einer Gruppe von 2754 Patienten mit morbider Adipositas (BMI>40kg/m²) fand sich im Glukosetoleranztest bei 54% der Patienten eine normale Glukosetoleranz, bei 14% eine gestörte Glukosetoleranz (IGT) und bei 32% ein Diabetes mellitus Typ2 (DM2). Nachfolgend führten wir eine Fallkontrollstudie an 111 konsekutiven Patienten dieser Kohorte durch.

Zwischen Patienten mit normaler Glukosetoleranz (NGT) und solchen mit gestörter Glukosetoleranz (IGT oder DM2) fanden sich keine signifikanten Unterscheide hinsichtlich der mRNA-Expression (in Leukozyten) oder den Serumspiegeln von Inflammationsmarkern. Allerdings war der Serumspiegel von Adiponektion bei den Patienten mit normaler Glukosetoleranz signifikant höher als bei den Patienten mit gestörter Glukosetoleranz. Diese Daten zeigten zum ersten Mal eine Beziehung zwischen der Glukosetoleranz und dem Adiponektinspiegel bei morbider Adipositas. Adiponektin scheint für diese Hochrisikogruppe von Patienten einen protektiven Efffekt gegen die Entstehung einer Glukosetoleranzstörung zu haben. In dieser Studie fanden wir keine Korrelation zwischen Pro-Inflammation und einem gestörten Glukosestoffwechsel bei morbider Adipositas. Das Vorliegen morbider Adipositas scheint bereits mit einer ausgeprägten Pro-Inflammation verbunden zu sein[76]. Die Enstehung einer gestörten Gluksetoleranz auf diesem Boden scheint dann von anderen Faktoren abzuhängen, zu denen auch Adiponektin gehört (**Publikation 8**)[18].

II.c Wnt-Signaling: neuer Kandidat in der Pathogenese des Diabetes mellitus Typ2

Der Wnt-Signalweg ist ursprünglich im entwicklungsbiologischen Kontext, dann auch im Rahmen der Tumorgenese untersucht worden. Im Jahr 2000 erschien die erste Arbeit, die diesen Signalweg in eine metabolische Fragestellung einbettete: In der Arbeitsgruppe von O. MacDougald wurde gezeigt, dass Wnt-Signalmoleküle die Adipozytendifferenzierung regulieren[27]. Seitdem wurde der Signalweg im Zusammenhang der Adipogenese intensiv untersucht; weiterhin zeigten vor kurzem erschienene Arbeiten aber auch eine zentrale Rolle dieses Signalwegs in der ß-Zell-Physiologie (reviewed in (**Publikation 9**[77]). Zwei Arbeiten im Jahr 2006 identifizierten außerdem TCF7L2 – einen Transkriptionsfaktor im kanonischen Wnt-Signalweg - als stärksten Kandidaten für die genetische Prädisposition in der Entstehung des Diabetes mellitus Typ2[30,32]. Diese Arbeiten regten eine Reihe von humangenetischen Studien in unterschiedlichsten Populationen an, die den initialen Befund der Assoziation von Polymorphismen im TCF7L2-Gen mit Diabetes mellitus Typ2 bestätigten. TCF7L2 zeigt von allen untersuchten Genen die stärkste Korrelation mit dem Auftreten von Diabetes mellitus Typ2.

Der Wnt-Signalweg besteht aus extrazellulären Liganden (Wnts), Zellmembranrezeptoren (Frizzled) sowie Corezeptoren (LRP, LDL receptor-related protein). Gegenwärtig sind 19 verschiedene Wnts bekannt. Weiterhin existieren verschiedene extrazelluläre Wnt-Antagonisten, z.B. sFRPs (secreted frizzled related protein). Diese können Wnts binden und so deren Rezeptorbindung kompetieren; Dickkopf ist ein weiterer Wnt-Antagonist, der durch Bindung an LRP-Corezeptoren spezifisch den sogenannten kanonischen Wnt-Signalweg hemmt[78].

Nach Bindung von Wnts an den Frizzled Rezeptor und LRP-Corezeptoren kommt es zur intrazellulären Signaltransduktion: Im kanonischen Signalweg kommt es unter Vermittlung eines Komplexes aus Axin und APC schließlich zu einer Inaktivierung der GSK3-ß. Hierdurch wird ß-catenin, der zentrale Regulator im kanonischen Signalweg frei, transloziert in den Nukleus und coaktiviert spezifische Transkriptionsfaktoren an kanonischen Wnt-Zielgenen (s. Abb. 4). Typische Vertreter dieser Transkriptionsfaktoren sind TCF/LEF Transkriptionsfaktoren. Die nicht-kanonischen Signalwege laufen ohne ß-catenin ab und spielen nach bisherigem Kenntnisstand für metabolische Fragen eine untergeordnete Rolle.

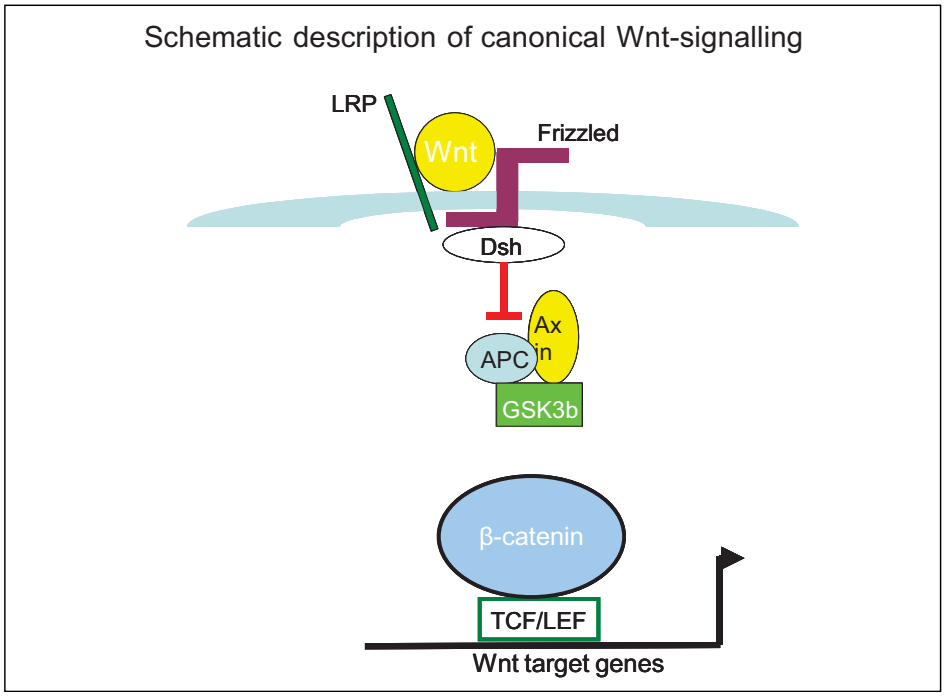

Abbildung 4: Schematische Darstellung des kanonischen Wnt-Signalwegs: Wnts binden an Frizzled Rezeptoren und LRP Corezeptoren. In der Folge kommt es zu einer Inhibierung der GSK-3β, wodurch β-catenin frei wird, in den Nukleus transloziert und dort bspw. TCF/LEF Transkriptionsfaktoren an Zielgenen coaktiviert.
Aus: Schinner S. Horm. Metab. Res. 41(2):159-63 (2009)

Wnt-Signalweg und Adipogenese

Die Differenzierung von Fibroblasten-ähnlichen Präadipozyten zu Adipozyten kann *in vitro* durch externe Stimuli wie Insulin/IGF-1, Glucokortikoide oder cAMP-Agonisten induziert werden (s. Abb. 3). Ross et al. zeigten im Jahre 2000, dass eine Hemmung des kanonischen Wnt-Signalwegs in der frühen Phase der Adipogenese eine notwendige Vorrausetzung für die Differenzierung zu Adipozyten ist (s. Abb. 3). Durch Überexpression von Wnt10b in murinen Präadipozyten wurde der Ablauf der Adipogenese und die Expression von C/EBPα und PPARγ *in vitro* gehemmt. Diese Präadipozyten blieben so in einem undifferenzierten Zustand. Auch *in vivo* bleiben nach subcutan verpflanzte Präadipozyten in der Maus undifferenziert, wenn sie konstitutiv Wnt10b überexprimierten. In Präadipozyten der Maus kommt es in der initialen Phase der Adipogenese entsprechend zu einer Herabregulation der Wnt10b Expression, was erst eine Differenzierung zu reifen Adipozyten erlaubt[27]. Auch in einem Mausmodell mit einer fettgewebsspezifischen Überexpression von Wnt10b konnte dieses Konzept bestätigt werden: Diese Mäuse zeigen eine verminderte Fettgewebsmasse unter normaler und hochkalorischer Ernährung[79].

Wir haben die Regulation und Rolle von Wnt-Signalmolekülen in der **Differenzierung humaner Präadipozyten** untersucht: Hier kommt es innerhalb der ersten 24 Stunden nach Induktion der Adipogenese zu einer verstärkten Expression von Dkk-1 (mRNA und Proteinebene). Parallel hierzu fand sich eine Herabregulation der Expression von typischen Wnt-Zielgenen in Präadipozyten (Cyclin D1, PPARδ).
Dkk-1 wird in stroma-vaskulären Zellen (Präadipozyten) stark exprimiert, aber in reifen humanen Adipozyten nur minimal. Dieses Expressionsmuster ist Spezies-spezifisch, da in murinen Präadipozyten Zellen Dkk-1 nicht detektiert wurde.
Funktionell konnten wir zeigen, dass eine Überexpression von Dkk-1 in Präadipozyten eine Hemmung des kanonischen Wnt-Signalwegs und eine verstärkte Adipogenese bewirkte: Dies zeigte sich an der verstärkten Lipideinlagerung (Oil-Red-Färbung) und der Expression adipogener Marker (PPARγ, aP2). Weiterhin fanden wir, dass die Wnt-Corezeptoren LRP5/6 in der frühen Phase der Adipogenese in ihrer Expression herabreguliert werden.
In dieser Studie wurde der Speziesunterschied zwischen muriner und humaner Adipogenese herausgearbeitet. In beiden Organismen ist initial eine passagere Herabgregulation des kanonischen Wnt-Signalings eine Voraussetzung für die Einleitung der Adipogense. In der Maus wird dies durch eine Herabregulation der Expression von Wnt-Agonisten erreicht; in humanen Präadipozyten durch eine gesteigerte Expression des Wnt-Antagonisten Dkk-1 und eine verminderte Expression der Wnt-Corezeptoren LRP5/6 (**Publikation 10**)[21].
Die Bedeutung des kanonischen Wnt-Signalwegs für die Adipogenese beim Menschen wird durch die Identifizierung von Mutationen in den Genen für Wnt10b und LRP5 und deren Assoziation mit einem adipösen Phänotyp bestärkt[80,81].

Interaktion zwischen Fettzellen und endokrinen Zellen über den Wnt-Signalweg

Nachdem die Sekretion von Wnt-Signalmolekülen durch Adipozyten gezeigt worden ist, haben wir in weiteren Schritten untersucht, ob Adipozyten über den Wnt-Signalweg auf endokrine Zellen wirken können.
In der Arbeitsgruppe Bornstein/Scherbaum war gezeigt worden, dass mature humane Adipozyten an **adrenocorticalen Zellen** *in vitro* eine Steigerung der Sekretion von Cortisol und Aldosteron bewirken. Dies geht mit einer gesteigerten Expression von StAR (steroidogenic acute regulatory protein) – dem Schrittmacherenzym der Steroidogenese - einher[82]. Wir haben im Folgenden

untersucht, ob dieser Effekt durch Wnts vermittelt wird. Zunächst fanden wir im Überstand primärer humaner Fettzellen (fat-cell conditioned-medium; FCCM) Wnt3a und Wnt10b als typische Aktivatoren des kanonischen Signalwegs in Konzentrationen von 16 bzw. 32ng/ml. Dies spiegelte sich funktionell wieder, indem Inkubation mit fat-cell conditioned-medium (FCCM) die Transkription eines Wnt-regulierten Reportergens (TOPFLASH) in adrenocorticalen Zellen induzierte. Weiterhin konnten wir den Befund einer Induktion der Sekretion von Cortisol und Aldosteron durch adrenocorticale Zellen nach Behandlung mit FCCM von Ehrhart-Bornstein et al. bestätigen: Darüber hinaus fand sich eine signifikante und konzentrationsabhängige Hemmung dieses Effektes durch Zugabe des Wnt-Antagonisten sFRP-1. Dies zeigte, dass der aktivierende Effekt von Fettzellprodukten auf die Steroidsekretion – zumindest partiell - über den Wnt-Signalweg vermittelt wird (**Publikation 11**)[26].

Zur Identifizierung weiterer Signalwege in dieser Interaktion führten wir Experimente mit verschiedenen Kinase-Inhibitoren durch. Zunächst fand sich eine Aktivierung und nukleäre Translokation von ERK1/2-MAP-Kinasen (ERK; extracellular-signal regulated kinase. MAP; mitogen-activated protein) nach Behandlung adrenaler Zellen mit FCCM. Weiterhin zeigte sich, dass der Effekt von FCCM auf die Aldosteronsekretion durch den MAPK-Inhibitor UO126 hemmbar ist, was auf eine Beteiligung des ERK1/2- MAP-Kinase-Signalwegs neben dem Wnt-Signalweg an der adipo-adrenalen Interaktion hindeutet (**Publikation 12**)[83].

Um zu untersuchen, über welche intrazellulären Signale adipozytäre Wnts eine Steigerung der Cortisol- und Aldosteronsekretion induzieren, haben wir in Reportergenassays die Transkription des StAR Genpromotors untersucht. FCCM induzierte die StAR Gentranskription und dieser Effekt war wiederum durch den Wnt-Antagonisten sFRP-1 hemmbar. Gleichzeitig fanden wir eine dosisabhängige Aktivierung des StAR Genpromotors durch Cotransfektion einer konstitutiv aktiven ß-catenin Mutante (S45A), so dass StAR als Zielgen des kanonischen Wnt-Signalwegs identifiziert wurde. Wir spekulierten, dass die Aktivierung des StAR Promotors durch ß-catenin durch den Transkriptionsfaktor SF-1 vermittelt werden könnte. SF-1 besitzt einerseits Bindungsstellen im StAR Promotor, andererseits wurde eine Interaktion zwischen SF-1 und ß-catenin am α-Inhibingen gezeigt[84]. Wir fanden eine Coaktivierung SF-1-abhängiger Transkription an einem Reportergen unter der Kontrolle multimerisierter SF-1 Bindungsstellen durch ß-catenin und durch Fettzell

konditioniertes Medium. Diese Arbeit zeigt ein *in vitro* Modell auf, um den bekannten klinischen Zusammenhang zwischen Adipositas und arterieller Hypertension mit einer Hypersekretion von Aldosteron[85, 86] zu untersuchen. Hier existiert eine direkte Interaktion zwischen Fettzellen und adrenocorticalen Zellen, die - zumindest partiell - über den Wnt-Signalweg vermittelt wird und eine Induktion der Steroidogenese und der Sekretion von Aldosteron und Cortisol bewirkt (**Publikation 11**)[26].

Klinisch ist Adipositas zudem mit einer Insulinresistenz sowie einer kompensatorischen Hyperinsulinämie und einer **Hyperplasie pankreatischer ß-Zellen** vergesellschaftet[87-91]. Wir haben deshalb *in vitro* direkte Wirkungen von Fettzellen auf ß-Zellen untersucht: Wir fanden, dass Sekretionsprodukte von Fettzellen den kanonischen Wnt-Signalweg in ß-Zellen induzieren (**Publikation 13**)[28]. Dies steht im Gegensatz zu pankreatischen α-Zellen, in denen der kanonische Wnt-Signalweg nicht aktivierbar ist[92,93]. Da Wnts Regulatoren des Zellzyklus sind, haben wir die Proliferation von ß-Zellen als einen Endpunkt untersucht und fanden eine Induktion der Proliferation von Ins-1 ß-Zellen durch Fettzellprodukte und durch exogen appliziertes Wnt3a. Auch in primären Inselzellen der Maus zeigten sich vergleichbare Resultate und in beiden zellulären Systemen war die Wirkung des Fettzellmediums durch Zugabe von Wnt-Inhibitoren hemmbar. Passend zur gesteigerten Proliferation induzierte FCCM auch die Transkription von Cyclin D1, einem Wnt-Zielgen und Zellzyklusregulator.
Parallel hierzu induzierte FCCM die Insulinsekretion in primären Inseln der Maus (**Publikation 13**)[28]. Um molekulare Hintergründe dieses Befundes zu verstehen, haben wir die Regulation des Glukokinasegens – dem Schrittmacher des Glukose-Sensings der ß-Zelle - untersucht. Zuvor hatte eine andere Arbeitsgruppe eine verminderte Glukose-induzierte Insulinsekretion und eine verminderte Expression der Glukokinase in Inseln von LRP5 knock-out Mäusen beschrieben[94]. Wir konnten eine gesteigerte Expression des Glukokinasegens nach Stimulierung primärer Inseln mit rekombinantem Wnt3a zeigen (semi-quantitative Taqman PCR). Zudem fanden wir – nur in Anwesenheit von PPARγ - eine direkte Regulation des Glukokinase Promotors durch ß-catenin (**Publikation 13**)[28]. Es ist bekannt, dass PPARγ in primären ß-Zellen stark exprimiert wird und ein aktivierender Transkriptionsfaktor am Glukokinasepromotor ist[95]. Wir schlagen deshalb ein Modell vor, in dem adipozytäre Wnts in pankreatischen ß-Zellen zu einer Stabilisierung und nukleären Translokation

von ß-catenin führen, welches dann PPARγ am Glukokinasepromotor coaktiviert und so zu gesteigertem Glukose-Sensing und gesteigerter Insulinsekretion führt (s. Abb. 5).

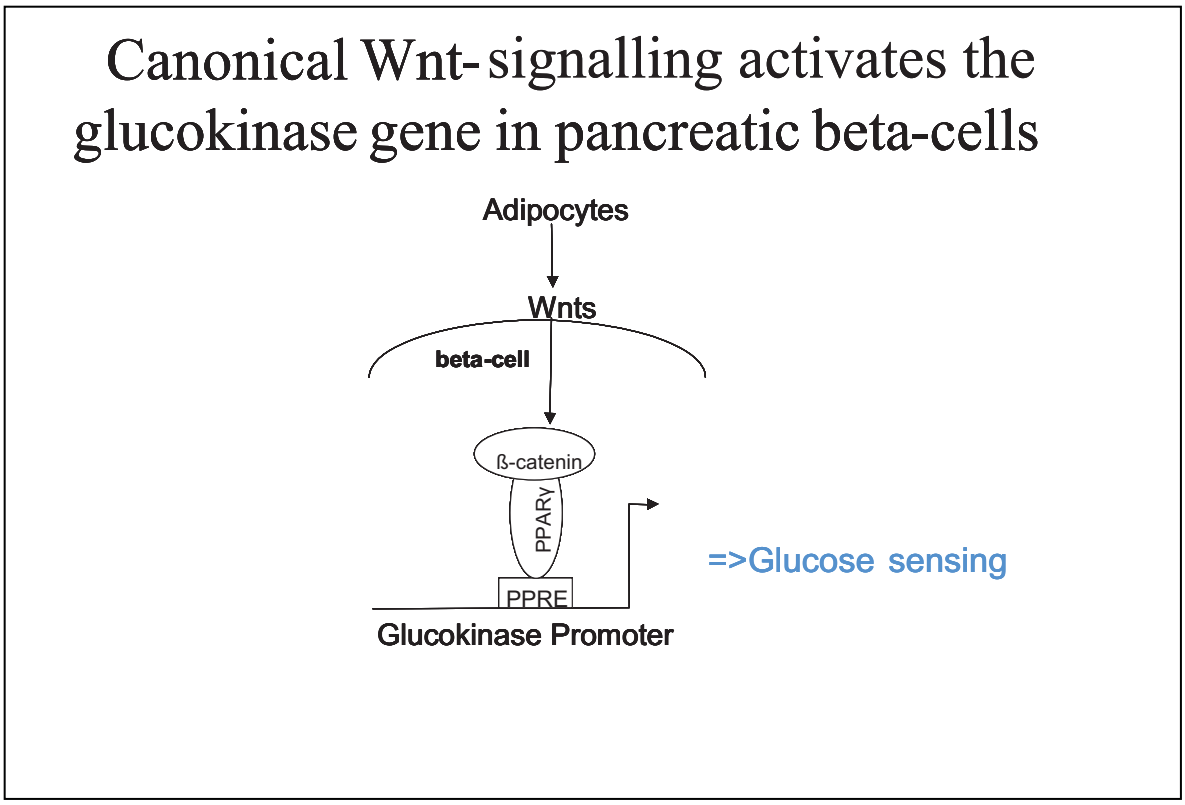

Abbildung 5: Modell der Wirkung von Wnt-Signalmolekülen an der β-Zelle. Bindung von Wnt-Liganden bewirkt eine Aktivierung von β-catenin, welches den Transkriptionsfaktor PPARγ am Glukokinase Genpromotor coaktiviert und so ein gesteigertes Glukose-Sensing bewirkt.
Aus: Schinner S. Horm. Metab. Res. 41(2):159-63 (2009)

Übereinstimmend mit unseren Befunden erschienen kürzlich weitere Arbeiten zum Wnt-Signalweg in der ß-Zell Physiologie (reviewed in **Publikation 9**[77]): Rulifson et al. konnten zeigen, dass eine Aktivierung des kanonischen Wnt-Signalwegs *in vitro* und *in vivo* zu einer verstärkten Proliferation von ß-Zellen führt[96]. Übereinstimmend hiermit führt ein knock-down von TCF7L2 zur verminderten Proliferation und Insulinsekretion von ß-Zellen *in vitro*[97]. Schließlich zeigten Liu und Habener, dass GLP-1 seine proliferativen Effekte auf ß-Zellen – zumindest partiell - über den kanonischen Wnt-Signalweg vermittelt[98].

Wie können adipozytäre Sekretionsprodukte die pankreatischen ß-Zellen in vivo erreichen?

Wnt-Signalmoleküle sind prinzipiell auch systemisch nachgewiesen worden, was endokrine Wirkungen ermöglicht[99,100]. Um die Möglichkeit parakriner Wirkungen adipozytärer Signalmoleküle wie Wnts auf pankreatische ß-Zellen zu untersuchen, haben wir die Pankreasmorphologie an einem adipösen Mausmodell untersucht (NZO, new zealand obese-mouse). Die untersuchten Mäuse waren adipös, insulinresistent und hyperinsulinämisch und stellen somit ein gutes Modell für die prädiabetische Adipositas dar. Wir fanden in den Mäusen zum einen hyperplastische Langerhans'sche Inseln. Zum anderen zeigten die Mäuse aber auch eine Zunahme von ektopen Ablagerungen von Adipozyten im Pankreas. Nach lichtmikroskopischer Quantifizierung fanden wir im Pankreas dieser Mäuse circa 20fach mehr Adipozytenmasse als in Kontrolltieren. Es fanden sich zudem Adipozyten in unmittelbarer Nachbarschaft zu Langerhans'schen Inseln, was parakrine Wirkungen ermöglichen könnte (**Publikation 14**)[29]. Diese ektope Ablagerung von Adipozyten ist ein grundsätzlich anderer Mechanismus als die ektope intrazelluläre Einlagerung von Triglyceriden in Muskel, Leber oder ß-Zellen. Denn Adipozyten können auf sie umgebende Zellverbände über aktiv sezernierte Signalstoffe wirken.

III. Zusammenfassende Betrachtung

Störungen der Insulinsekretion und Insulinwirkung bestimmen die Pathogenese des Diabetes mellitus Typ2. Die im Rahmen dieser kumulativen Habilitationsschrift vorgestellten Arbeiten leisten Beiträge zu diesen beiden Aspekten des Diabetes mellitus Typ2. Wir haben in *in vitro* Studien die Wirkung von Insulin auf Glukagon-produzierende α-Zellen, die *in natura* einem parakrinen Insulinstimulus ausgesetzt sind, untersucht. Wir konnten die Signaltransduktionskaskade in α-Zellen vom Insulinrezeptor bis zu den responsiven Elementen im Promotor des Glukagongens charakterisieren. Wir fanden weiterhin, dass Glitazone (sog. Insulinsensitizer) die Expression des Glukagongens über ähnliche molekulare Mechanismen hemmen wie Insulin. Die Hyperglukagonämie, die wahrscheinlich aufgrund verminderter Hemmung durch Insulin zustande kommt, trägt zur Hyperglykämie des Diabetikers bei und ist deshalb ein pharmakologischer Angriffspunkt in der Behandlung des Diabetes mellitus Typ2.

Weiterhin konnten wir verschiedene Aspekte der Insulinresistenz beleuchten. In einem Kandidatengenansatz identifizierten wir eine Mutation in der Proteinkinase B, einem Signaltransduktionsmolekül im Insulinsignalweg, in einer Familie mit extremer Insulinresistenz. Da diese Mutation auch *in vitro* charakteristische metabolische Störungen an Leber- und Fettzellen auslöste, ist sie als kausal für die Entstehung dieser familiären Insulinresistenz anzusehen. Durch diese Studie wurde die Bedeutung der Proteinkinase B für den Glukosestoffwechsel beim Menschen herausgearbeitet.

Zudem haben wir molekulare Mechanismen der Adipozytendifferenzierung untersucht, da eine gestörte Adipozytenfunktion eine Insulinresistenz auslösen kann. Die Funktionen der Moleküle ETO, PGC-1β und des Wnt-Signalwegs in der Adipogenese wurden in diesen Arbeiten charakterisiert.

Der Wnt-Signalweg wurde schließlich als ein neuartiger Signaltransduktionsweg zwischen Fettzellen und endokrinen Zielzellen identifiziert. Hier zeigte sich eine Wirkung von Adipozyten auf die Proliferation und Insulinsekretion an pankreatischen β-Zellen über den Wnt-Signalweg.

Zusammenfassend konnten diese Arbeiten neue Aspekte der molekularen Pathogenese des Diabetes mellitus Typ2 auf Seite der Insulinsekretion und der Insulinwirkung aufzeigen.

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V. Eigene Publikationen

PUBLIKATION 1

Schinner S, Barthel A, Dellas C, Grzeskowiak R, Sharma SK, Oetjen E, Blume R, Knepel W.

Protein Kinase B activity is sufficient to mimic the effect of insulin on glucagon gene transcription.

J. Biol. Chem. 280(8):7369-76, (2005)

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 8, Issue of February 25, pp. 7369–7376, 2005
Printed in U.S.A.

Protein Kinase B Activity Is Sufficient to Mimic the Effect of Insulin on Glucagon Gene Transcription*

Received for publication, July 28, 2004, and in revised form, November 12, 2004
Published, JBC Papers in Press, December 7, 2004, DOI 10.1074/jbc.M408560200

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Insulin inhibits glucagon gene transcription, and insulin deficiency is associated with hyperglucagonemia that contributes to hyperglycemia in diabetes mellitus. However, the insulin signaling pathway to the glucagon gene is unknown. Protein kinase B (PKB) is a key regulator of insulin signaling and glucose homeostasis. Impaired PKB function leads to insulin resistance and diabetes mellitus. Therefore, the role of PKB in the regulation of glucagon gene transcription was investigated. After transient transfections of glucagon promoter-reporter genes into a glucagon-producing islet cell line, the use of kinase inhibitors indicated that the inhibition of glucagon gene transcription by insulin depends on phosphatidylinositol (PI) 3-kinase. Furthermore, insulin caused a PI 3-kinase-dependent phosphorylation and activation of PKB in this cell line as revealed by phospho-immunoblotting and kinase assays. Overexpression of constitutively active PKB mimicked the effect of insulin on glucagon gene transcription. Both insulin and PKB responsiveness of the glucagon promoter were abolished when the binding sites for the transcription factor Pax6 within the G1 and G3 promoter elements were mutated. Recruitment of Pax6 or its potential coactivator, the CREB-binding protein (CBP), to G1 and G3 by using the GAL4 system restored both insulin and PKB responsiveness. These data suggest that insulin inhibits glucagon gene transcription by signaling via PI 3-kinase and PKB, with the transcription factor Pax6 and its potential coactivator CBP being critical components of the targeted promoter-specific nucleoprotein complex. The present data emphasize the importance of PKB in insulin signaling and glucose homeostasis by defining the glucagon gene as a novel target gene for PKB.

The pancreatic islet hormones insulin and glucagon are biologic antagonists in the regulation of blood glucose concentration. Insulin is known to increase peripheral glucose uptake and oppose hepatic glucose production. In contrast, glucagon balances the effect of insulin on blood glucose levels by increasing hepatic glucose production and opposing hepatic glucose storage (1–3). As the glucagon-producing α-cells are mainly located in the peripheral regions of the islets of Langerhans surrounding the insulin-producing β-cells, they are exposed to high concentrations of insulin (1–3). Acting directly on α-cells, insulin inhibits glucagon secretion as well as glucagon gene transcription (4). The inhibition of glucagon gene transcription by insulin depends on the paired domain transcription factor Pax6, which binds to the G1 and G3 element within the glucagon gene promoter (5). Pax6 binding to the G1 element within the glucagon promoter also confers responsiveness of the glucagon gene to thiazolidinediones, a novel class of insulin-sensitizing drugs (6). The paracrine inhibition of glucagon gene transcription by insulin is an important mechanism in the regulation of blood glucose concentrations. Consequently, in diabetic patients a relative hyperglucagonemia has been described that contributes to hyperglycemia in these patients (1). The elevated glucagon levels may be explained by the loss of insulin-mediated inhibition of glucagon synthesis and secretion.

Numerous signaling pathways downstream of the insulin receptor tyrosine kinase have been suggested to contribute to gene regulation by insulin, including PKC[1] (7), p70 S6 kinase (8), mammalian targets of rapamycin (8, 9), PI 3-kinase/PKB (10–15), glycogen synthase kinase-3 (16), a PI 3-kinase/ERK1/2 pathway (7), and a Ras/ERK/p90 ribosomal S6 kinase (RSK) pathway (17). There is strong evidence suggesting that activation of the PI 3-kinase/PKB pathway is required for the maintenance of normal glucose homeostasis. The serine/threonine kinase PKB (also called Akt) is known to be activated by phosphorylation in a PI 3-kinase-dependent manner (18, 19) and to mediate biological effects of insulin such as stimulation of GLUT4-dependent glucose transport, glycogen synthesis, and protein synthesis or the suppression of hepatic gluconeogenesis (20). Suppression of hepatic gluconeogenesis has also been suggested to be achieved, in part, by PKB-dependent repression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase catalytic subunit gene expression (10, 13), although this conclusion is controversial (14, 21, 22). In addition, activation of PKB has been found to be a central mechanism in the insulin-dependent regulation of target genes like the *IGFBP-1* gene (12), the *GLUT1* gene (23), or the fatty acid synthase gene (24). Consistent with this view, impaired PKB

* This work was supported by Deutsche Forschungsgemeinschaft Grants GRK 335 and SFB402/A3. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PKC, protein kinase C; CRE, cAMP-responsive element; CREB, CRE-binding protein; CBP, CREB-binding protein; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; IGFBP-1, insulin-like growth factor-binding protein 1; IRE, insulin-responsive element; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/ERK kinase; PI, phosphatidylinositol; PISCES, pancreatic islet cell-specific enhancer sequence; PKB, protein kinase B.

function has been found to cause insulin resistance and diabetes mellitus in animal models and humans (25, 26).

Although the paracrine effect of insulin on the glucagon-producing α-cells within the endocrine pancreas is a crucial regulator of glucagon gene transcription (4, 5) and, furthermore, although an impaired inhibition of glucagon gene transcription by insulin is involved in the pathogenesis of the relative hyperglucagonemia and consecutive hyperglycemia in diabetic patients (1, 27), the signal transduction mechanisms linking the insulin receptor to the promoter of the glucagon gene have not been characterized yet. Given the essential role of PKB in maintaining glucose homeostasis, we investigated the potential role of PKB in the insulin-dependent repression of glucagon gene transcription. We found the effect of insulin on the promoter of the glucagon gene to be mediated via the activation of PI 3-kinase. At the same time, insulin activated, in a PI 3-kinase-dependent manner, PKB, which could mimic the effect of insulin. Furthermore, the transcription factor Pax6 and the coactivator CBP were found to be critically involved in this process. These data emphasize the importance of PKB in mediating biological effects of insulin and in establishing glucose homeostasis by defining the glucagon gene as a novel target gene for PKB.

EXPERIMENTAL PROCEDURES

Materials—Insulin was from Serva (Heidelberg, Germany), and a stock solution (10 μM) was prepared in 0.9% saline containing 2 mg/ml bovine serum albumin. Genistein, Ly 294002, Ro-31-8220, PD 98059, and rapamycin were purchased from Sigma-Aldrich. Controls received the solvent only.

Plasmid Constructs—The plasmids -350GluLuc (28), -136GluLuc, 3xGluCRE-136GluLuc, 4xG3A-136GluLuc, 4xG2–136GluLuc, pT81-Luc, 4xG3A(T81)Luc, -350(mutG1/G3)GluLuc, pGAL4-CBP, pGAL4-Pax6, pGAL4-VP16 (5), pmyr-PKB, pPKB-K179M (29), and pGAL4-p300 (30) have been described previously. The plasmid pCMV-GFPtpz was purchased from Canberra-Packard (Dreieich, Germany). The expression vector encoding myr-PKB was used to generate an expression vector encoding myr-PKB-K179M by site-directed mutagenesis (QuikChange XL kit, Stratagene, La Jolla, California). The construct was confirmed by sequencing.

Cell Culture and Transfection of DNA—The glucagon-producing pancreatic islet cell line InR1-G9 was grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (28). Cells were trypsinized and transfected in suspension by the diethylaminoethyl-dextran method (28) with 2 μg of reporter gene plasmid and the indicated amount of expression vector per 6-cm dish. Cotransfections were carried out with a constant amount of DNA, which was maintained by adding the Bluescript vector (Stratagene). In all experiments, 0.5 μg of cytomegalovirus-GFP (plasmid pCMV-GFPtpz) per 6-cm dish were cotransfected to check for transfection efficiency (the relative luciferase activities presented in the figures are derived from luciferase/GFP ratios). Twenty-four hours after transfection, cells were incubated in serum-free RPMI 1640 medium containing 0.5% bovine serum albumin and antibiotics as described above. When indicated, cells were treated with insulin (10 nM) for 23 h before harvest. Kinase inhibitors were added, when indicated, 0.5 h before insulin. Cell extracts (28) were prepared 48 h after transfection. The luciferase assay was performed as described previously (28). GFP was measured in the cell extracts using the FluoroCount™ microplate fluorometer (Packard).

PKB Kinase Assay—The assay was carried out as described previously (31, 32). In brief, cells were lysed with ice-cold lysis buffer (50 mM Hepes, pH 7.6, 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM Na_3VO_4, 30 mM $Na_4P_2O_7$, 10 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, and 100 nM okadaic acid), and the enzyme activity in the immunoprecipitates obtained with PKB antibodies was assayed with [γ-^{32}P]ATP using the glycogen synthase kinase-3 peptide (GRPRTSSFAEG) as a substrate for phosphorylation. The phosphorylated peptide was separated from free [γ-^{32}P]ATP on a 40% polyacrylamide gel, and the phosphopeptide spots were quantified using a Cyclone phosphorimaging device and the OptiQuant software (Packard).

Immunoblots—The protein concentration in the cell lysates was determined using a BCA kit (Pierce). Twenty micrograms of protein per lane were analyzed by Western blotting. Detection of PKB, (phosphoserine 473 PKB) was performed with specific antibodies (Cell Signaling). Nitrocellulose membranes were controlled for equal loading and transfer by staining with Ponceau S. After incubation with the appropriate first antibody and peroxidase-coupled secondary antibody, the signal was visualized by enhanced chemiluminescence. For immunoblotting of myr-PKB and myr-PKB-K179M (Fig. 3*C*), expression vectors encoding the hemagglutinin-tagged proteins were transfected into InR1-G9 cells (3 μg), and the blots were probed with an antibody directed against the influenza hemagglutinin protein (sc-805, Santa Cruz Biotechnology, Santa Cruz, California).

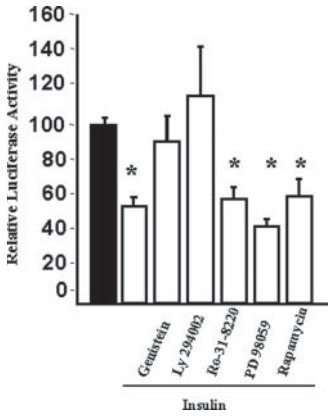

FIG. 1. **The effect of insulin on the activity of the glucagon promoter is sensitive to genistein and Ly 294002, inhibitors of tyrosine kinase and PI 3-kinase activity, respectively.** InR1-G9 cells were transiently transfected with a luciferase reporter gene under the control of the 350-bp glucagon gene promoter (plasmid -350GluLuc). The cells were incubated for 24 h in serum-free medium in the absence (*black bar*) or presence of 10 nM insulin alone (*unlabeled white bar*) or together with the indicated inhibitors (100 μM genistein, 100 μM Ly 294002, 1 μM Ro-31-8220, 100 μM PD 98059, and 1 μM rapamycin, final concentration). The luciferase activities are expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means ± S.E. of three independent experiments, each done in duplicate. *, $p < 0.05$ (Student's t test).

RESULTS

Insulin Inhibits Glucagon Gene Transcription through Activation of PI 3-Kinase—A 350-base pair fragment of the rat glucagon gene promoter was shown to confer tissue-specific gene expression (33) and to mediate the response of the promoter to cAMP, calcium, PKC, and insulin (5, 28, 34–39). To investigate the signaling mechanisms involved in the regulation of the glucagon promoter by insulin, a reporter gene construct containing 350 base pairs of the 5′-flanking region of the rat glucagon gene (construct -350GluLuc) was transiently transfected into InR1-G9 cells. Treatment of InR1-G9 cells with 10 nM insulin led to an inhibition of the glucagon gene promoter activity by 45% (Fig. 1). A comparable inhibition of the glucagon gene promoter activity by insulin was observed in the presence of 1 μM Ro-31-8220, an inhibitor of conventional PKC activity (Fig. 1). Furthermore, inhibitors of MEK (100 μM PD 98059), and p70 S6 kinase (1 μM rapamycin) did not alter the effect of insulin on the glucagon reporter gene. Ro-31-8220 (1 μM) and PD 98059 (100 μM) prevented the activation of glucagon gene transcription by a PKC-activating phorbol ester (12-*O*-tetradecanoylphorbol-13-acetate) and Raf kinase, respectively (data not shown), suggesting that these compounds effectively blocked the targeted signaling pathways. However, when inhibitors of tyrosine kinases (100 μM genistein) or PI 3-kinase (100 μM Ly 294002) were added, the inhibition of glucagon gene transcription by insulin was abolished (Fig. 1). The effect of Ly 294002 was specific, because Ly 294002 had no effect on glucagon gene transcription under basal conditions or after stimulation with a PKC-activating phorbol ester (data not shown). These results confirm that the transcription of the

glucagon gene is inhibited by insulin. Furthermore, these data suggest that both tyrosine kinase and PI 3-kinase activity, but not PKC, MEK, or p70 S6 kinase activity, are required to mediate this effect. Class 1a PI 3-kinase consists of a p110 catalytic subunit and a p85 or p55 regulatory subunit that possess two SH2 domains that interact with tyrosine-phosphorylated Tyr(P)-Met-Xaa-Met and Tyr(P)-Xaa-Xaa-Met motifs in insulin receptor substrate proteins (40, 41). Therefore, our data suggest that the proximal insulin-signaling pathway to the glucagon gene may involve the recruitment and activation of PI 3-kinase.

Insulin Leads to a PI 3-Kinase-dependent Phosphorylation and Activation of PKB in InR1-G9 Cells—PI 3-kinase may transmit multiple signals, including the activation of PKB (41). PI 3-kinase catalyzes the phosphorylation of phosphoinositides on the 3 position to produce phosphatidylinositol-3-phosphates. The formation of phosphatidylinositol 3,4,5-triphosphate is thought to recruit PKB through its pleckstrin homology domain to the plasma membrane, thereby inducing a conformational change in PKB that allows its activation by phosphorylation at two residues (Thr-308 and Ser-473) (41–43). Because the above results suggest that the inhibition of glucagon gene transcription by insulin is mediated by PI 3-kinase (Fig. 1) and because PKB is a known downstream target of PI 3-kinase signaling, we next tested the potential involvement of PKB. To study a potential role of PKB in the regulation of glucagon gene transcription, we first studied the effect of insulin on PKB activity in glucagon-producing pancreatic islet cells (Fig. 2). As demonstrated by phosphoserine-specific immunoblot, insulin induced the phosphorylation of PKB on Ser-473 in glucagon-producing pancreatic islet cells (Fig. 2*A*, *top*). This phosphorylation is required for the activation of PKB (42). To test whether insulin increases PKB activity in InR1-G9 cells, PKB was precipitated with specific antibodies, and the amount of enzymatic activity in the precipitates was then determined via the use of a synthetic peptide based on the sequence of the PKB phosphorylation site in glycogen synthase kinase-3. As shown in Fig. 2*A* (*bottom*), insulin markedly stimulated PKB activity in glucagon-producing islet cells. Stimulation of PKB activity by insulin was dose- and time-dependent (Fig. 2, *A* and *C*). Maximum stimulation of PKB activity was at ~10–100 nM insulin, resulting in a 13-fold increase of PKB activity over basal activity (Fig. 2*A*). The EC_{50} value of insulin for the stimulation of PKB activity was at ~1 nM (Fig. 2*A*), corresponding to the reported IC_{50} value of insulin for inhibition of glucagon gene transcription (0.5 nM) (5). In addition, these data relate well to the intra-islet situation *in vivo*, where α-cells are exposed to insulin concentrations ranging from 1 to 100 nM (4). When a PI 3-kinase inhibitor (100 μM Ly 294002) was added, the activation of PKB by insulin was completely abolished (Fig. 2*B*). Time course studies demonstrated an activation of PKB by insulin within minutes (Fig. 2*C*) with a sustained response over >24 h (~30% of maximum activity; data not shown), thereby providing further support for the idea that activation of PKB is involved in the insulin-dependent regulation of glucagon gene transcription in InR1-G9 cells. These results demonstrate that treatment with insulin results in a PI 3-kinase-dependent activation of PKB in the InR1-G9 cell line.

PKB Mimics the Effect of Insulin on the Glucagon Gene Promoter—Because the results obtained to date suggest that insulin both inhibits glucagon gene transcription and activates PKB activity via PI 3-kinase in pancreatic islet cells, we investigated whether activated PKB is sufficient to mimic the effect of insulin on the glucagon gene promoter. Co-expression of a constitutively active version of PKB (construct myr-PKB) (44) in InR1-G9 cells was found to inhibit the transcriptional activity of the glucagon reporter gene (Fig. 3*A*). The extent of the inhibition of glucagon gene transcription by myr-PKB (by 45%) was similar to the inhibition by insulin (Fig. 3*A*). In contrast, a catalytically inactive PKB mutant bearing a point mutation in position 179 of the kinase domain (construct PKB-K179M) (29) did not inhibit glucagon gene transcription (Fig. 3*A*). Also, a second kinase-dead form of PKB (myr-PKB-K179M), which is identical to the constitutively active myr-PKB apart from the single amino acid change in the kinase-dead form myr-PKB-K179M, did not inhibit glucagon gene transcription (Fig. 3*B*). The lack of effect of myr-PKB-K179M was not due to the decreased protein expression of the catalytically inactive mutant PKB protein (Fig. 3*C*), indicating that the effect of PKB on the glucagon promoter is specific and due to its kinase activity.

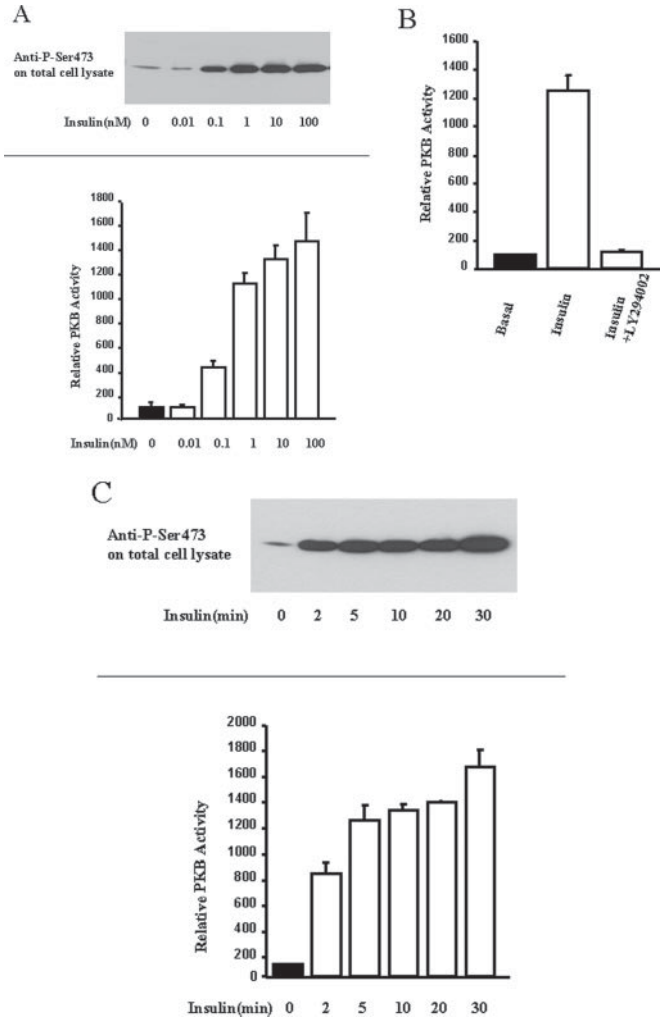

FIG. 2. **Activation of PKB by insulin in InR1-G9 cells.** *A*, concentration-response relationship. Cells were serum-starved for 2 h and then treated for 15 min with the indicated concentrations of insulin. The *top section* of the panel shows a Western blot with total cell lysates probed with antiphospho-Ser-473-PKB (*Anti-P-Ser473*) antibodies to determine the phosphorylation state of PKB. The *bottom section* shows PKB activity that was determined as described under "Experimental Procedures." *B*, the activation of PKB by insulin is sensitive to Ly 294002, an inhibitor of PI 3-kinase. Cells were incubated for 15 min in the absence or presence of 10 nM insulin with or without Ly 294002 (100 μM). *C*, time course study of the activation of PKB by insulin in InR-G9 cells. The cells were stimulated with 10 nM insulin for the indicated times. The *top section* of the panel shows a Western blot with total cell lysates probed with antiphospho-Ser-473-PKB antibodies to determine the phosphorylation state of PKB. The *bottom section* shows PKB activity that was determined as described under "Experimental Procedures." The basal PKB activity was set as 100%; values are means ± S.E. of three independent experiments.

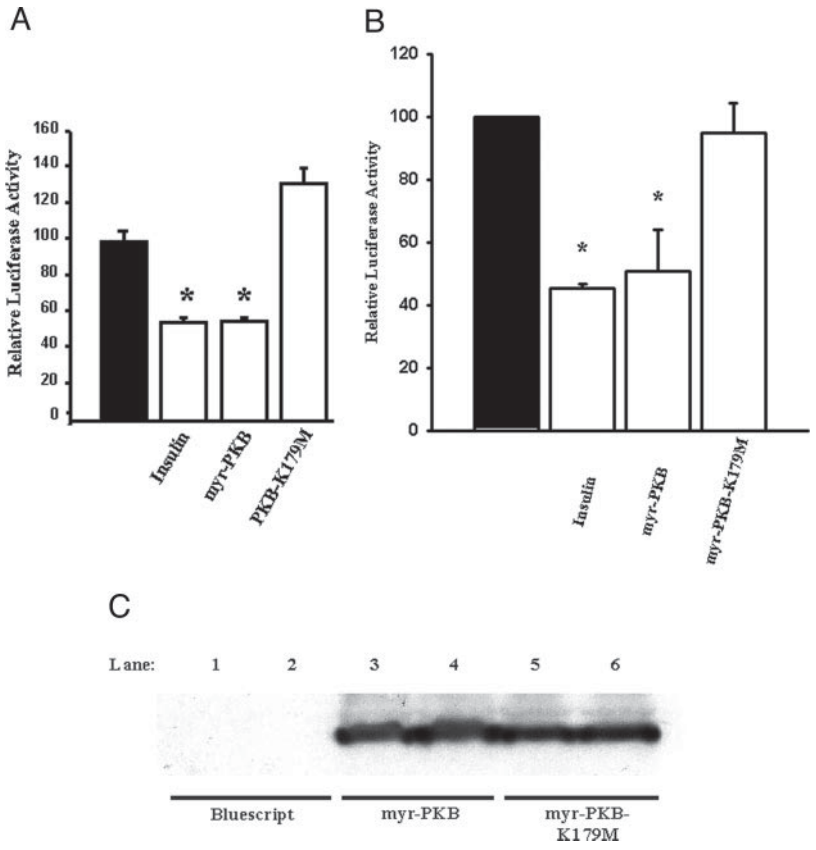

FIG. 3. **The effect of insulin on glucagon promoter activity can be mimicked by constitutively active PKB (myr-PKB) in InR1-G9 cells.** *A* and *B*, cells were transfected with the luciferase reporter gene under the control of 350 bp of the glucagon gene promoter (plasmid -350GluLuc) together with 2 μg of the construct myr-PKB, PKB-K179M (in *panel A*) or myr-PKB-K179M (in *panel B*), as indicated. The cells were grown in serum-free medium in the presence or absence (*black bars*) of 10 nM insulin. The luciferase activity is expressed as the percentage of the mean value of the activity measured in the untreated controls. Values are means ± S.E. of three independent experiments, each done in duplicate. *, $p < 0.05$ (Student's t test). *C*, Western blot for myr-PKB and myr-PKB-K179M after transfection into InR1-G9 cells. Cells transfected with the vector Bluescript served as controls.

These results show that PKB activity is sufficient to inhibit glucagon promoter activity. Because insulin stimulates PKB activity, these data also suggest that insulin may inhibit glucagon gene transcription by signaling through the PI 3-kinase-PKB pathway.

PKB, like Insulin, Targets Several Control Elements within the Glucagon Gene Promoter—The rat glucagon gene promoter contains the enhancer-like elements G2 and G3 as well as a CRE (45). The truncated glucagon gene promoter (136 base pairs) containing the proximal promoter elements G1 and G4 exhibits low transcriptional activity but is essential for proper enhancer function (45). A previous study has shown that insulin does not inhibit glucagon gene transcription through a single IRE (5); insulin responsiveness of the glucagon gene is rather conferred by interactions between a proximal promoter and more distal enhancer-like elements involving the paired domain transcription factor Pax6 (5). This finding suggests that insulin may target arrays of transcription factors at the coactivator level (5). Consistent with this view, the ligation of several enhancer-like elements (CRE, G3, G2) onto an insulin-nonresponsive minimal glucagon promoter (136 base pairs) resulted in the generation of insulin-responsive fusion genes (5). As shown in Fig. 4*A*, PKB, like insulin, can repress the expression of these CRE-, G3A-, and G2-glucagon minimal promoter constructs. The truncated glucagon promoter (-136GluLuc) was not responsive to PKB (Fig. 4*A*). The kinase-dead PKB mutant myr-PKB-K179M had no effect (not shown). When compared with -136GluLuc (100 ± 2%), basal activity of the constructs was 179 ± 8% (3xCRE-136GluLuc), 11,110 ± 397% (4xG3A-136GluLuc), and 422 ± 48% (4xG2–136GluLuc) ($n = 6$ each), as has been reported previously (5). These results indicate that PKB, like insulin, targets several control elements within the glucagon gene promoter.

It has been shown previously that G3A, but not the CRE and G2 elements, confers insulin responsiveness to a heterologous minimal thymidine kinase promoter (5). As shown in Fig. 4*B*, PKB, like insulin, inhibited the transcriptional activity of this G3A-thymidine kinase promoter construct. The kinase-dead PKB mutant, myr-PKB-K179M, had no effect (not shown). In contrast to G3A, three copies of the glucagon CRE or four copies of the G2 element did not confer PKB responsiveness to this heterologous promoter (not shown). The fact that G3A confers insulin and PKB responsiveness suggests that sequence motifs of this enhancer-like element may play a specific role in the inhibition by PKB of glucagon gene transcription. G3A contains the domain A of G3 (from −262 to −247) with the PISCES (pancreatic islet cell-specific enhancer sequence) motif (45), which is also present in the proximal promoter element G1 (45).

Inhibition of Glucagon Gene Transcription by Insulin and PKB Depends on the PISCES Motifs within the Glucagon Promoter Involving Pax6 and CBP—Within the glucagon gene promoter the PISCES motifs in G1 and G3 were shown to be essential regulatory elements in islet α-cell-specific activation of the glucagon gene and its inhibition by thiazolidinedione oral antidiabetic drugs (5, 6, 45). Furthermore, the PISCES motifs are required for the inhibition of glucagon gene transcription by insulin (5). To investigate whether the effect of PKB is conferred by the same regulatory elements as the effect of insulin, we used a mutated glucagon promoter with PISCES in G3 and G1 being converted into GAL4 binding sites (construct -350(mutG1/G3)GluLuc) (Fig. 5*A*). We found that mutation of the PISCES motifs in the glucagon promoter abolished the responsiveness of the promoter both to insulin as well as to PKB (Fig. 5*B*). Basal activity of -350(mutG1/G3)GluLuc was low (6 ± 1% of wild type) but well detectable, as has been reported previously (5). Furthermore, the lack of inhibition by insulin and PKB of -350(mutG1/G3)GluLuc was not just secondary to low basal activity, because internal deletion of the G2 element decreased basal activity of the glucagon promoter to a similar degree but did not abolish the inhibition by insulin (5)

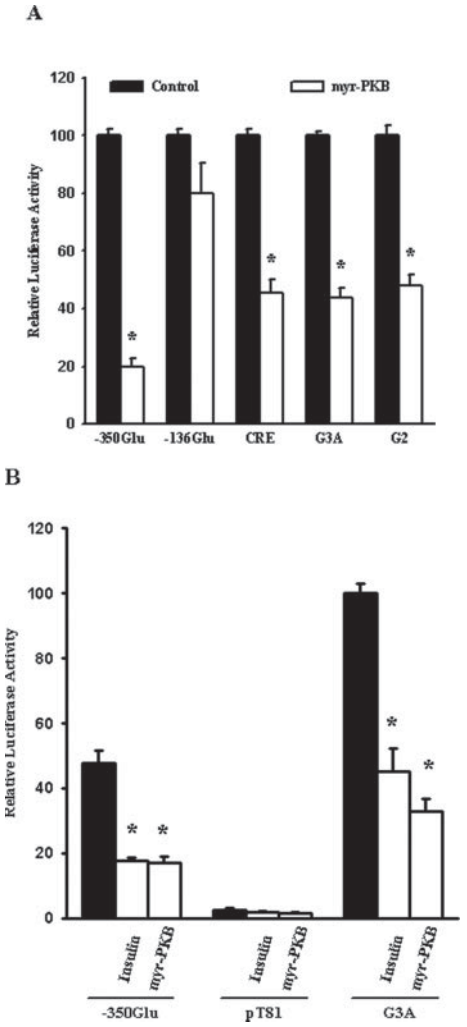

FIG. 4. **PKB, like insulin, can target several control elements within the glucagon promoter.** *A*, the enhancer-like elements CRE, G3A, and G2 of the glucagon gene confer PKB responsiveness to the nonresponsive truncated glucagon gene promoter. The plasmids -350GluLuc, -136GluLuc, 3xGluCRE-136GluLuc, 4xG3A-136GluLuc, and 4xG2–136GluLuc were transfected into InR1-G9 cells together with an expression vector encoding myr-PKB (2 μg) as indicated. Luciferase activity is expressed as the percentage of the mean value in each experiment of the activity measured in the respective control (no myr-PKB). *B*, effect of PKB on the transcriptional activity of G3A placed in front of a minimal viral thymidine kinase promoter. The plasmids -350GluLuc, pT81Luc, and 4xG3A(T81)Luc were transfected into InR1-G9 cells together with an expression vector encoding myr-PKB (2 μg) as indicated. When indicated, cells were treated with insulin (10 nM). Luciferase activity is expressed as percentage of the mean value in each experiment of the activity measured in the G3A control (no insulin, no myr-PKB). Values are means ± S.E. of three independent experiments, each done in duplicate. *, $p < 0.05$ (Student's *t* test).

and PKB (data not shown). Thus, the present data show that the inhibition of glucagon gene transcription by PKB depends on the PISCES motifs as does the inhibition by insulin. Together with the above data, this suggests that PKB is a downstream effector of the insulin signal to the glucagon gene.

The PISCES motif is known to serve as a binding site for the paired domain transcription factor Pax6 (46, 47) which is required for insulin responsiveness of the glucagon gene promoter (5). Cotransfection of the mutated glucagon promoter construct -350(mutG1/G3)GluLuc together with a GAL4-Pax6 expression vector was found to completely restore the responsiveness of the mutated reporter gene to both insulin and constitutively active PKB (myr-PKB) (Fig. 5*C*). In contrast, cotransfection of catalytically inactive mutants of PKB, PKB-K179M (Fig. 5*C*) and myr-PKB-K179M (not shown), did not affect promoter activity in the presence of GAL4-Pax6. Basal activity of -350(mutG1/G3)GluLuc was raised by GAL4-Pax6 18 ± 2-fold ($n = 6$), thus restoring wild-type activity. The expression of GAL4-VP16 also restores the basal activity of the doubly mutated glucagon promoter but does not confer responsiveness to insulin (5) or PKB (not shown). When taken together, these results suggest that Pax6 is required for responsiveness of the glucagon promoter to PKB, as it is for responsiveness to insulin.

In addition to Pax6, its potential coactivator protein, CBP, has been shown to confer responsiveness of the glucagon gene promoter to insulin (5). We therefore tested whether CBP may contribute to the effect of PKB on the transcription of the glucagon gene. In analogy to the above described results, we found that the responsiveness of the -350(mutG1/G3)GluLuc promoter to insulin and PKB was completely restored when CBP was recruited to the mutated PISCES motifs by cotransfection of a GAL4-CBP expression vector (Fig. 5*D*). Cotransfection of this amount of GAL4-CBP expression vector raised basal activity of -350(mutG1/G3)GluLuc 17 ± 1.4-fold ($n = 6$), thus achieving approximate wild-type activity (5). The cotransfection of kinase-dead forms of PKB, PKB-K179M (Fig. 5*D*) and myr-PKB-K179M (not shown), did not inhibit CBP-driven glucagon promoter activity. In contrast to CBP, the related cofactor p300 did not confer insulin and PKB responsiveness to the glucagon promoter (Fig. 5*E*). Taken together, these data indicate that activated PKB is sufficient to mimic the effect of insulin on glucagon gene transcription. In addition, the data provide strong evidence that insulin acts on the G1 and G3 element within the glucagon gene promoter via a mechanism involving the integrated action of PKB, Pax6, and the coactivator CBP.

DISCUSSION

Insulin is well known to inhibit the transcription of the glucagon gene in pancreatic islet α-cells, which is an important physiological mechanism involved in the complex regulatory process of glucose homeostasis (1, 5, 48). Thus, an impaired suppression of glucagon synthesis and secretion, as is typically found in patients with diabetes mellitus, may contribute to hyperglycemia. Consequently, it has been shown, that the inhibition of glucagon secretion reduces fasting hyperglycemia in diabetic animals and humans (27, 49). Therefore, the regulation of glucagon gene expression and the signaling mechanisms involved in this process are of particular interest because they are potential targets for novel pharmacological approaches for the treatment of diabetes. However, the signaling mechanism integrating the response of the glucagon gene promoter to insulin has not been characterized.

Recent studies emphasize the central role of PKB in regulating glucose metabolism. Both animal models using gene targeting approaches as well as human genetic studies have revealed that an impaired function of PKB is sufficient to result in a state of insulin resistance and diabetes mellitus (25, 26). Given the importance of PKB in insulin signaling and glucose homeostasis, we focused on the characterization of PKB in mediating the effect of insulin on glucagon gene transcription. Analysis of the proximal steps of insulin signaling by the use of pharmacological inhibitors revealed that the effect of insulin on the glucagon gene promoter is independent of MEK, PKC, and p70 S6 kinase but involves a process of tyrosine phosphorylation and activation of PI 3-kinase. In addition, our results demonstrate that insulin both inhibits glucagon gene transcription and activates PKB through PI 3-kinase. Furthermore, constitutively active PKB was found to mimic the inhibitory effect of insulin on the glucagon gene promoter, therefore sug-

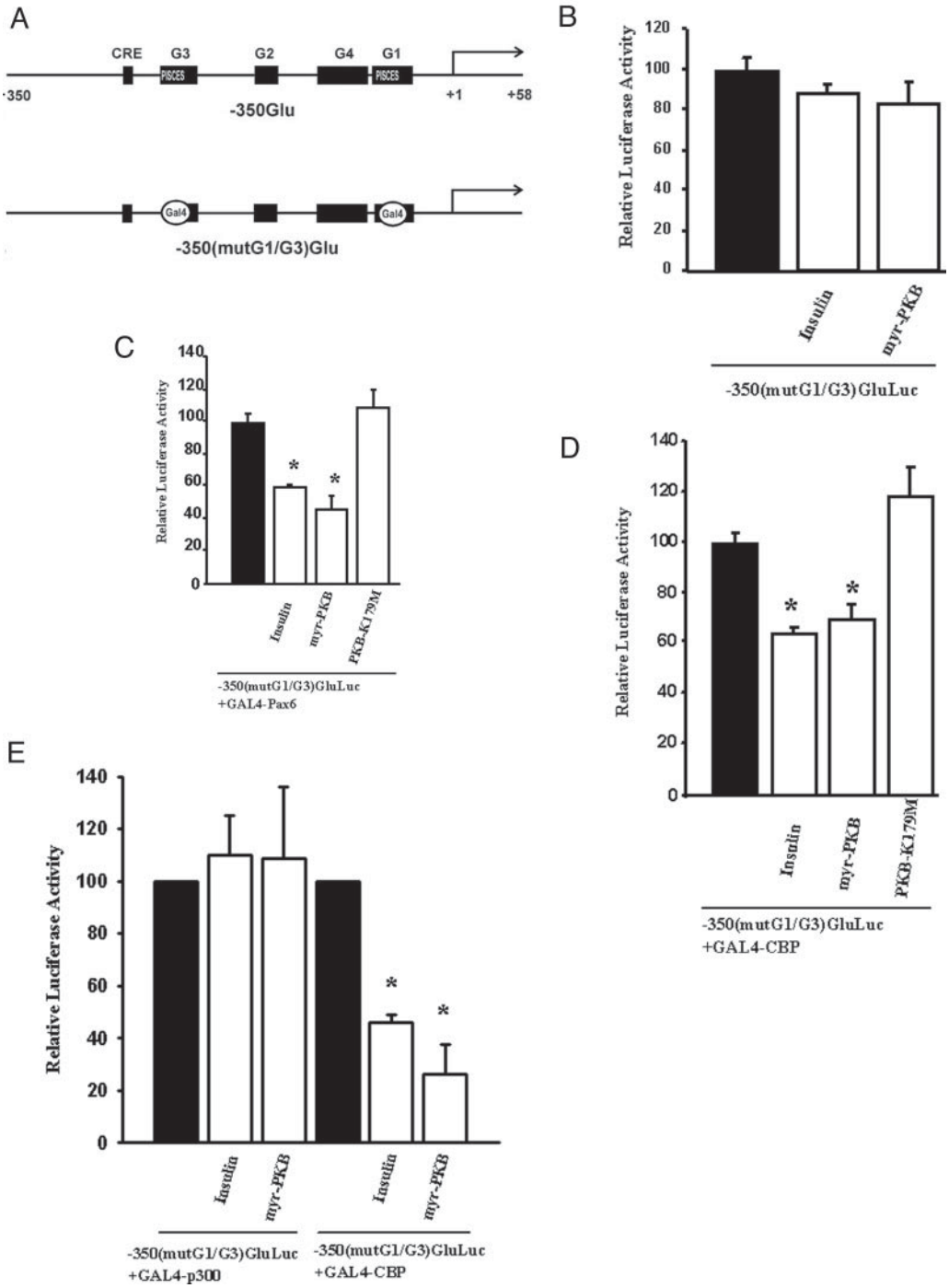

FIG. 5. **The PISCES motif of the G1 and G3 elements within the glucagon gene promoter is required for both insulin and PKB responsiveness of the glucagon gene involving Pax6 and CBP.** *A*, structural organization of the 350-bp glucagon gene promoter construct (-350GluLuc; *top*) and the glucagon gene promoter construct with mutation of the PISCES motifs within G1 and G3 into GAL4 binding sites (–350(mutG1/G3)GluLuc; *bottom*). Pax6 is known to bind to the PISCES motifs within G1 and G3. *B*, the PISCES motifs within G1 and G3 are required for the effect of insulin and PKB on glucagon promoter activity. InR1-G9-cells were transfected with the plasmid -350(mutG1/G3)GluLuc with or without 2 μg of the construct myr-PKB. The cells were kept in the presence or absence (*black bar*) of 10 nM insulin for 23 h before harvest. Luciferase activity is expressed as the percentage of the mean value of the activity measured in the controls (no insulin, no myr-PKB). Insulin and PKB had no statistically significant effect on the mutated glucagon promoter (Student's *t* test). *C*, Pax6 binding to the G1 and G3 elements restores insulin and PKB responsiveness of the glucagon gene promoter. InR1-G9 cells were transfected with -350(mutG1/G3)GluLuc together with 50 ng of the expression vector encoding the fusion protein GAL4-Pax6 and either a 2-μg control plasmid (*black bar*) or an expression vector encoding either myr-PKB or PKB-K179M, respectively. The cells were treated with insulin (10 nM) or left untreated. *D*, CBP recruited to the glucagon promoter restores insulin and PKB responsiveness of the mutated glucagon reporter gene construct. The reporter gene -350(mutG1/G3)GluLuc was transfected into InR1-G9 cells together with an expression vector (2 μg) encoding the fusion protein GAL4-CBP in combination with expression vectors encoding myr-PKB, PKB-K179M, or an empty vector as a control (*black bar*). The cells were treated with insulin (10 nM) or left untreated. *E*, GAL4-p300 does not restore the insulin and PKB response. The reporter gene plasmid -350(mutG1/G3)GluLuc was transfected into InR1-G9 cells together, as indicated, with expression vectors encoding GAL4-p300 (0.1 μg), GAL4-CBP (2 μg), and myr-PKB (2 μg). The cells were treated with insulin (10 nM) or left untreated. In each experiment, the mean luciferase activity in the respective controls (no insulin, no myr-PKB; *black bars*) was set at 100%. Values are means ± S.E. of three independent experiments, each done in duplicate or triplicate. *, $p < 0.05$ (Student's *t* test).

gesting that insulin exerts this response through activation of PI 3-kinase and PKB.

It has been suggested that PKB regulates the transcription of insulin target genes such as *GLUT-1* (23), fatty acid synthase (24), *IGFBP-1* (12), phosphoenolpyruvate carboxykinase (13), and glucose-6-phosphatase (10), although for the latter two genes the reports are controversial (14, 21, 22). Analysis of the promoters of the *IGFBP-1* and phosphoenolpyruvate carboxykinase genes has provided evidence for the critical involvement of a so-called consensus IRE (T(G/A)TTT) and transcription factors of the Foxo-family in this process (50). Other IREs have also been identified such as the serum response element and binding sites for transcription factors like AP-1, SREBP-1c, and the liver X-activated receptor (5, 50). However, the promoters of various insulin target genes, including the glucagon gene, clearly lack defined IREs (5, 51, 52). Therefore, one current concept of insulin action on gene transcription suggests that insulin may target a composition of transcription factors and coactivators independently of a particular IRE (51, 52). Mapping studies of the glucagon promoter have indicated that it is not a single DNA element that confers the insulin response to this gene but interactions between the proximal promoter element G1 and more distal enhancer-like elements, including G3 (5). Both of these control elements contain a so-called PISCES motif that has been shown to serve as a binding site for the paired domain transcription factor Pax6 (46, 47). Pax6 is a crucial regulator of glucagon gene transcription, conferring responsiveness to both insulin as well as the peroxisome proliferator-activated receptor γ to the glucagon gene (5, 6). In this study we found that the PISCES motif in G1 and G3 is essential also for the effect of PKB on the glucagon promoter. In addition, targeting Pax6 to the PISCES motif in G1 and G3 completely restored both the insulin as well as the PKB responsiveness of the glucagon promoter. Taking into consideration that Pax6 contains a putative phosphorylation site for PKB, R*X*R*XX*(S/T), within its paired-domain, it is tempting to speculate that PKB may act on the glucagon promoter by directly phosphorylating Pax6. However, using *in vitro* phosphorylation assays and Western blots, we did not find evidence for a direct phosphorylation of Pax6 by PKB (data not shown). Therefore, these data suggest that Pax6 is regulated by PKB through an indirect mechanism.

The coactivator proteins CBP and p300 are known to interact with sequence-specific transcription factors and components of the basal transcription machinery and are thought to be general integrators of the transcriptional process (53). Consistent with the view that, in many cases, insulin may regulate gene transcription independently of a particular IRE but may rather target arrays of interacting transcription factors at the coactivator level (51, 52, 54, 55), effects of insulin on CBP/p300 function have been described recently (5, 56–59). However, whether insulin alters CBP function positively, negatively, or not at all depends on the recruiting transcription factors involved as well as on the promoter context (5, 56–59). The C-terminal transactivation domain of Pax6 is proline/serine/threonine-rich and can bind to CBP/p300 (60, 61), suggesting that recruitment of CBP may be important for the distinct function of Pax6 at the glucagon promoter. Indeed, the transcriptional activity conferred by CBP (as a GAL4-CBP fusion protein) to the glucagon promoter is inhibited by insulin (5). The present study now demonstrates that PKB, like insulin, also suppresses the transcriptional activity conferred by CBP to the glucagon promoter. Similarly, insulin and PKB have been found to inhibit the activity of the transcription factor C/EBPβ through CBP at the IGFBP-1 promoter in HepG2 hepatoma cells (56). However, this effect of insulin and PKB on the IGFBP-1 promoter seems to be mediated through a PKB phosphorylation site of CBP that is conserved in p300 (Ser-1834) (56), whereas insulin and PKB responsiveness was conferred to the glucagon promoter by CBP but not p300 (this study). Interestingly, CBP is phosphorylated by insulin-induced signaling at Ser-436 in serum-starved 293T cells (59). This residue is not conserved in p300 (59). The significance of CBP-Ser-436 in the inhibition of glucagon gene transcription by insulin and PKB remains to be defined. When taken together, the present study suggests that Pax6 and CBP may be essential components of a glucagon promoter-specific nucleoprotein complex that integrates the activities of proximal promoter elements and more distal enhancer-like elements and the function of which is sensitive to insulin through PKB.

Several lines of evidence indicate that insulin acts not only on pancreatic islet α-cells to inhibit glucagon gene transcription but also acts, in an autocrine manner, on β-cells to stimulate insulin gene transcription (8, 62). This finding suggests an important functional role for the insulin receptor in glucose sensing by the pancreatic β-cell and proposes that defects in insulin signaling at the levels of both the α- and the β-cell may contribute to the observed alterations in glucagon and insulin secretion in type II diabetes mellitus. Data have been presented that show that secreted insulin acts via β-cell insulin receptors and up-regulates insulin gene transcription by signaling through the PI 3-kinase/p70 S6 kinase and calcium/calmodulin-dependent protein kinase pathways (8). By suggesting that insulin inhibits glucagon gene transcription through PKB, the data of the present study indicate that insulin regulates the glucagon and insulin genes through distinct signaling pathways in pancreatic islet α- and β-cells, respectively.

In conclusion, our results demonstrate that activation of PKB is sufficient to mimic the effect of insulin on the glucagon promoter and suggest that the inhibition of glucagon gene transcription by insulin is mediated via activation of PI 3-kinase and PKB. Furthermore, we propose a model according to which the effect of insulin and PKB on the promoter of the glucagon gene essentially requires the targeting of the transcriptional coactivator CBP and the paired domain transcription factor Pax6 to the PISCES motifs within the glucagon promoter. The present data emphasize the importance of PKB in insulin signaling and glucose homeostasis by defining the glucagon gene as a novel target gene for PKB.

Acknowledgments—Plasmids were generously provided by Thomas Franke, Columbia University, New York, NY 10032 (myr-PKB, PKB-K179M), and Anton Krumm, Fred Hutchinson Cancer Research Center, Seattle, WA 98109 (GAL4-p300).

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PUBLIKATION 2

Schinner S, Dellas C, Schröder M, Heinlein C, Chang C, Fischer J, Knepel W.

Repression of glucagon gene transcription by PPARγ though inhibition of Pax6 transcriptional activity.

J. Biol. Chem. 277(3):1941-8, (2002).

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 3, Issue of January 18, pp. 1941–1948, 2002

Printed in U.S.A.

Repression of Glucagon Gene Transcription by Peroxisome Proliferator-activated Receptor γ through Inhibition of Pax6 Transcriptional Activity*

Received for publication, October 9, 2001
Published, JBC Papers in Press, November 13, 2001, DOI 10.1074/jbc.M109718200

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The nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) is involved in glucose homeostasis and synthetic PPARγ ligands, the thiazolidinediones, a new class of antidiabetic agents that reduce insulin resistance and, as a secondary effect, reduce hepatic glucose output. PPARγ is highly expressed in normal human pancreatic islet α-cells that produce glucagon. This peptide hormone is a functional antagonist of insulin stimulating hepatic glucose output. Therefore, the effect of PPARγ and thiazolidinediones on glucagon gene transcription was investigated. After transient transfection of a glucagon-reporter fusion gene into a glucagon-producing pancreatic islet cell line, thiazolidinediones inhibited glucagon gene transcription when PPARγ was coexpressed. They also reduced glucagon secretion and glucagon tissue levels in primary pancreatic islets. A 5′/3′-deletion and internal mutation analysis indicated that a pancreatic islet cell-specific enhancer sequence (PISCES) motif within the proximal glucagon promoter element G1 was required for PPARγ responsiveness. This sequence motif binds the paired domain transcription factor Pax6. When the PISCES motif within G1 was mutated into a GAL4 binding site, the expression of GAL4-Pax6 restored glucagon promoter activity and PPARγ responsiveness. GAL4-Pax6 transcriptional activity was inhibited by PPARγ in response to thiazolidinedione treatment also at a minimal viral promoter. These results suggest that PPARγ in a ligand-dependent but DNA binding-independent manner inhibits Pax6 transcriptional activity, resulting in inhibition of glucagon gene transcription. These data thereby define Pax6 as a novel functional target of PPARγ and suggest that inhibition of glucagon gene expression may be among the multiple mechanisms through which thiazolidinediones improve glycemic control in diabetic subjects.

Peroxisome proliferator-activated receptor γ (PPARγ)[1] is a member of the ligand-regulated nuclear hormone receptor superfamily (1). Like other nuclear receptors, PPARγ comprises an amino-terminal ligand-independent transactivation domain (AF-1), a central DNA-binding domain, and a carboxyl-terminal ligand-binding domain that contains a second, ligand-dependent transactivation surface (AF-2) (1). PPARγ binds as a heterodimer with the 9-*cis*-retinoic acid receptor, RXR, to response elements in target genes to activate transcription. A typical PPRE consists of a direct repeat of hexamer half-sites, TGACCT, spaced by one nucleotide (DR-1) (1). PPAR and RXR occupy the 5′ and 3′ half-sites, respectively, and thus show a polarity in binding that is the opposite of that observed for other nuclear receptor-RXR heterodimers (1). Like other nuclear receptors, there is evidence that PPARγ-RXR require the ligand-dependent recruitment of coactivator proteins like SRC-1, GRIP-1, pCIP, CBP, p300, DRIP205, and p120 (2–5) to effectively stimulate gene transcription. This recruitment is dependent on allosteric alterations in the AF-2 helical domain. A "mouse trap" model of receptor activation has been proposed, in which the AF-2 helix closes on the ligand-binding site in response to ligand and establishes a transcriptionally active form of the receptor (3). Cocrystal studies (3, 4) indicated that two highly conserved amino acids, Glu-469 in the AF-2 helix and Lys-301 in helix 3 of the ligand-binding domain, form a charge clamp that places a helical L*XX*LL motif of SRC-1 class of coactivators into a hydrophobic pocket in the receptor. In addition to stimulation of transcription, PPARγ has been shown to be capable of also negative regulation of gene transcription (6–14).

PPARγ has been suggested to be involved in a broad range of cellular functions, including adipocyte differentiation, inflammatory responses, and apoptosis, as well as in chronic diseases such as obesity, atherosclerosis, and cancer (15, 16). Of particular importance is its role in glucose homeostasis and type 2 diabetes mellitus (15, 16). Human genetic studies support an important role of PPARγ in mammalian metabolism (15, 17, 18). Thus, dominant negative mutations in human PPARγ are associated with hypertension, severe insulin resistance, and diabetes mellitus (18). These physiologic and pathophysiologic actions suggest that synthetic PPARγ ligands may be of use in the treatment of type 2 diabetes mellitus.

Thiazolidinediones like rosiglitazone are PPARγ ligands and a new class of orally active antidiabetic drugs (19–21). They

* This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 402/A3 and by a grant from the Medical Faculty, University of Göttingen. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-activated receptor response element; PISCES, pancreatic islet cell-specific enhancer sequence; RT, reverse transcriptase; CBP, cAMP-response element-binding protein-binding protein; GFP, green fluorescent protein; RXR, retinoid X receptor.

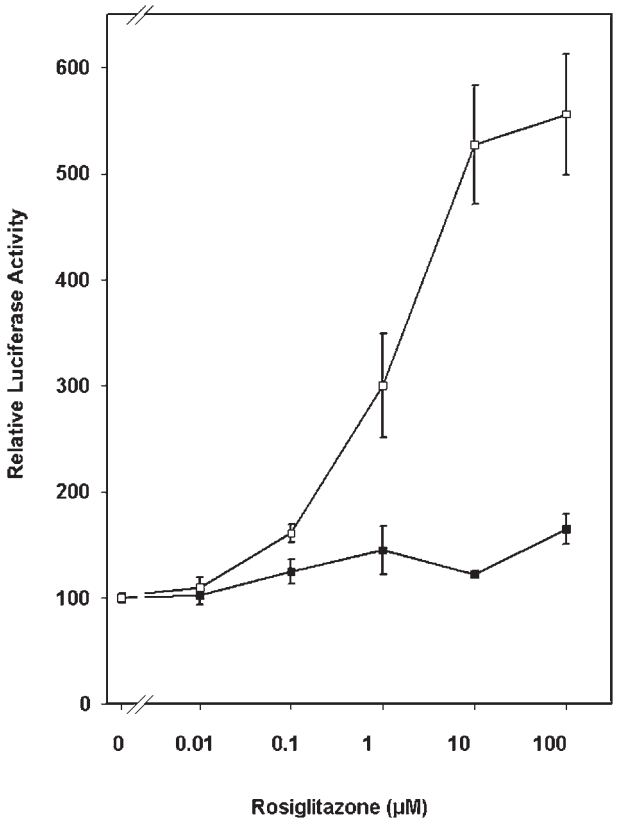

FIG. 1. **Activation of a PPAR-dependent promoter by rosiglitazone and PPARγ in InR1-G9 cells.** A luciferase reporter gene under the control of three copies of a PPRE (plasmid PPRELuc) was transiently transfected into InR1-G9 cells together with an expression vector encoding PPARγ (*white squares*) or without (*black squares*). Increasing concentrations of rosiglitazone were added 24 h before harvest. The expression of PPARγ without rosiglitazone treatment had no effect on PPRELuc transcriptional activity (data not shown). Luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means ± S.E. of four independent experiments

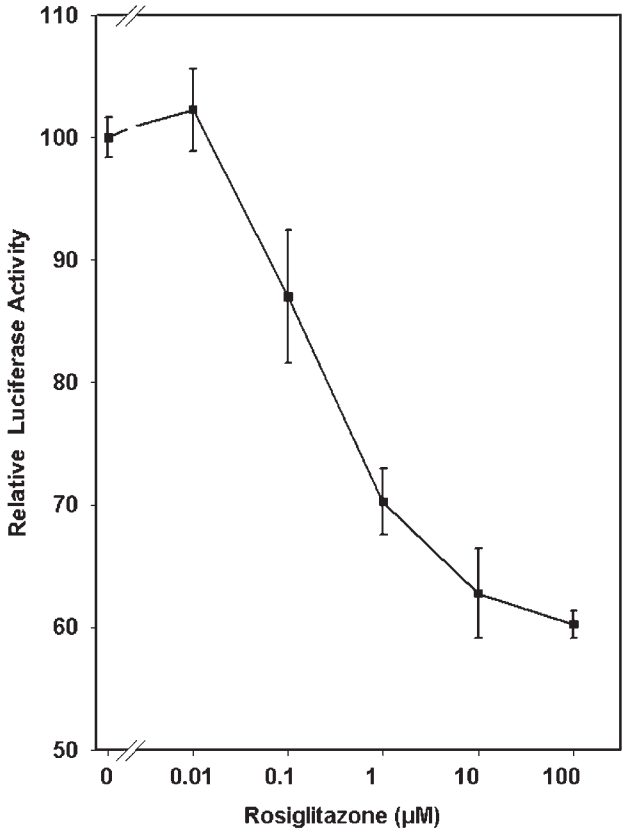

FIG. 2. **Inhibition of glucagon gene transcription by rosiglitazone and PPARγ.** Plasmid −350GluLuc was transfected into InR1-G9 cells together with pPPARγ. Rosiglitazone was added 24 h before harvest. Luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means ± S.E. of three independent experiments, each done in duplicate.

decrease hepatic glucose output and reduce insulin resistance by increasing insulin-dependent peripheral glucose disposal (19). Thiazolidinediones thereby markedly decrease plasma glucose, insulin, and triglyceride levels in animal models of type II diabetes as well as in type II diabetic subjects (19). The antidiabetic effect of thiazolidinediones requires several days of treatment and does not produce overt hypoglycemia (19). Thiazolidinediones have been shown to decrease adipocyte tumor necrosis factor α/resistin secretion and circulating free fatty acid levels; to increase basal glucose uptake in 3T3-L1 adipocytes, L6 myocytes, and human muscle cultures derived from obese type II diabetic subjects; and to stimulate glucokinase gene transcription in HepG2 cells (19–28). The mechanism of action of these drugs has nevertheless remained unknown. The correlation between *in vivo* antihyperglycemic activity and *in vitro* PPARγ activity (29) suggests that thiazolidinediones act as antidiabetic agents by regulating the transcription of a subset of genes through PPARγ. However, the target genes involved are unclear.

It has been shown recently that high levels of PPARγ are expressed in glucagon-producing α-cells of the endocrine pancreas (30–32). The pancreatic islet hormone glucagon is a biologic antagonist of insulin. The effects of glucagon on blood glucose levels balance those of insulin; glucagon increases hepatic glucose production and opposes hepatic glucose storage, whereas insulin increases peripheral glucose uptake and opposes glucagon-mediated hepatic glucose production. The metabolic consequences of abnormal α-cell function in diabetes are well defined (33–35). In addition to hyperglycemia, insulin resistance, and impaired β-cell function, relative hyperglucagonemia is a common feature of patients with type II diabetes (33–35). The elevated glucagon levels in diabetes contribute to increased hepatic glucose output and hyperglycemia (33–35). Consequently, inhibition of glucagon secretion has been shown to reduce fasting hyperglycemia in diabetic animals (36) and patients (37, 38). Effects on the expression of glucagon in pancreatic islets are therefore important aspects in the treatment of diabetes mellitus.

Because PPARγ is expressed in glucagon-producing α-cells but its function has been unknown, in the present study the effect of PPARγ and thiazolidinediones on glucagon gene transcription was investigated. PPARγ and thiazolidinediones were found to inhibit glucagon gene transcription in pancreatic islet cells. They also reduced glucagon secretion and tissue levels in pancreatic islets. Mapping studies and the use of GAL4 fusion proteins indicate that PPARγ represses in a ligand-dependent but DNA binding-independent manner transactivation by the paired domain-containing transcription factor Pax6 leading to inhibition of glucagon gene transcription. This novel action of PPARγ assigns a function to PPARγ expressed in pancreatic islet α-cells and suggests that the mechanisms through which thiazolidinediones improve glycemic control in diabetic subjects may include the inhibition of glucagon gene expression.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The plasmids pT81Luc (39), −350GluLuc (40), 5xGal4E1BLuc (41), −292GluLuc, −169GluLuc, −136GluLuc, −60GluLuc, −350/−48GluLuc, −350/−91GluLuc, −350/−150GluLuc, −350/

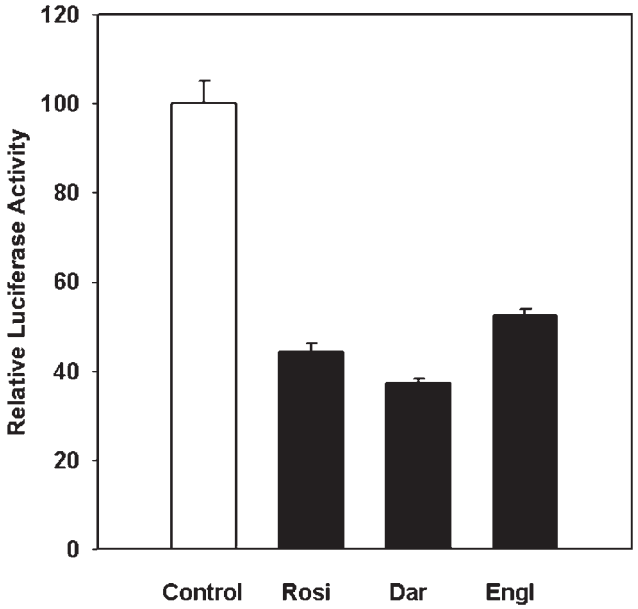

FIG. 3. **Inhibition of glucagon gene transcription by the thiazolidinediones darglitazone and englitazone.** InR1-G9 cells were transfected with −350GluLuc and pPPARγ. They were treated with rosiglitazone (*Rosi*, 10 μM), darglitazone (*Dar*, 30 μM), or englitazone (*Engl*, 100 μM) for 24 h before harvest. Luciferase activity is expressed as percentage of the mean value, in each experiment, of the activity measured in the untreated controls. Values are means ± S.E. of three experiments.

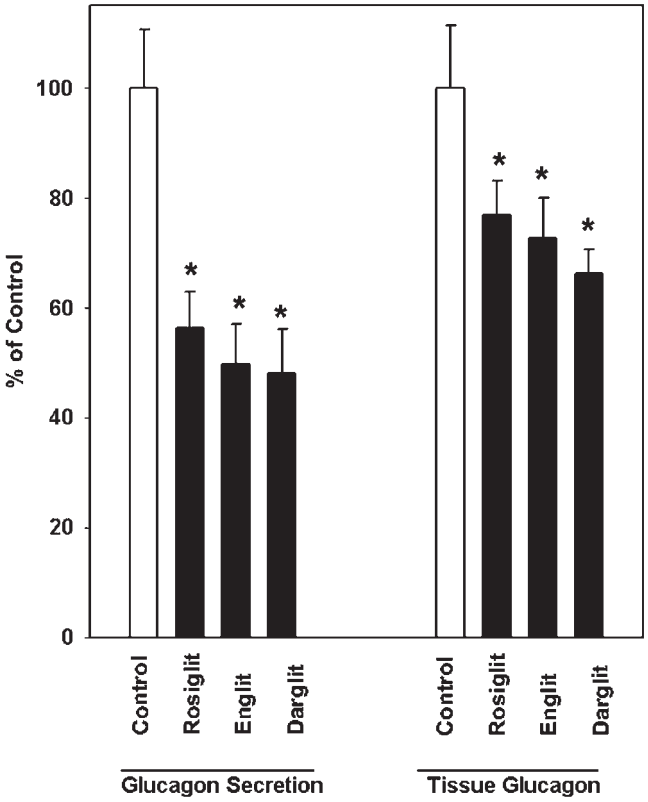

FIG. 4. **Inhibition by thiazolidinediones of glucagon secretion and glucagon tissue levels in primary pancreatic islets.** Isolated mouse pancreatic islets were treated with rosiglitazone (*Rosiglit*, 30 μM), englitazone (*Englit*, 100 μM) darglitazone (*Darglit*, 30 μM), or the solvent (control) for 48 h. Glucagon secretion and tissue levels are expressed as percentage of the mean value, in each experiment, of the levels measured in the respective control. Values are means ± S.E. of five independent experiments, each done in duplicate. *, $p < 0.005$ (Student's *t* test).

−210GluLuc, (42), −350(mutG1)GluLuc, pGAL4-Pax6 (43), PPRELuc, pPPARγ, pRXRα (44), and pGAL4-PPARγ (45) have been described previously. The plasmid pCMV-GFPtpz was purchased from Canberra-Packard (Dreieich, Germany).

RT-PCR—Total RNA was extracted from InR1-G9 cells using a commercial kit (RNeasy, Quiagen). For first strand cDNA synthesis, random hexamer primers (Amersham Biosciences, Inc.) were used. The RT enzyme was obtained from Invitrogen (Superscript II reverse transcriptase). For PCR amplification, the following primers were used: upstream primer, 5′-AGAGCTGACCCAATGGTTGC-3′; and downstream primer, 5′-ATCTCCGCCAACAGCTTCTC-3′ (EMBL/GenBank™/DDBJ accession no. Z30972) (size of the expected product: 421 bp). PCR without RT step served as control for DNA contamination. After agarose gel electrophoresis, the RT-PCR product was identified by extraction, subcloning (TA-cloning kit, Promega), and cycle sequencing (Thermo Sequenase fluorescent labeled primer cycle sequencing kit, Amersham Biosciences, Inc.; IRD-800 labeled primers, MWG Biotech, Ebersberg, Germany).

Cell Culture and Transfection of DNA—The glucagon-producing pancreatic islet cell line InR1-G9 (46) was grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were trypsinized and transfected in suspension by the DEAE-dextran method (40) with 2 μg of reporter gene plasmids and, when indicated, 1 μg of expression vector per 6-cm dish. Cotransfections were carried out with a constant amount of DNA, which was maintained by adding Bluescript (Stratagene, La Jolla). In all experiments 0.5 μg of cytomegalovirus-green fluorescent protein (GFP) (plasmid pCMV-GFPtpz) per 6-cm dish was cotransfected to check for transfection efficiency (the relative luciferase activities presented in the figures are derived from luciferase/GFP ratios). Twenty-four hours after transfection, cells were incubated in RPMI 1640 containing 0.5% bovine serum albumin and antibiotics as described above. Cell extracts (40) were prepared 48 h after transfection. The luciferase assay was performed as described previously (40). Green fluorescent protein was measured in the cell extracts using the FluoroCount™ microplate fluorometer (Packard).

Incubation of Isolated Pancreatic Islets—After the preparation of Langerhans pancreatic islets of NMRI mice (32), islets were cultured in RPMI medium supplemented with 5 mM glucose, 10% bovine serum albumin, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 48 h, glucagon levels were measured in the supernatants and the islet extracts (40) by radioimmunoassay using a commercial kit (IBL, Hamburg, Germany).

Materials—Rosiglitazone was kindly provided by SmithKline Beecham (Worthing, United Kingdom); darglitazone and englitazone (CP-72,467–02, sodium salt) was provided by Pfizer Inc. (Groton, CT). A stock solution (100 mM) was prepared in Me_2SO. Controls received the solvent only.

RESULTS

Inhibition of Glucagon Gene Transcription by PPARγ in Response to Thiazolidinediones—PPARγ was found by RT-PCR to be expressed in the glucagon-producing pancreatic islet cell line InR1-G9 (data not shown). In normal pancreatic islets, the expression of PPARγ is very high, approximately two thirds of the expression level in white adipose tissue (30). In contrast, InR1-G9 cells express low levels of PPARγ such that activation of a PPAR-dependent promoter (PPRELuc) required transfection of a PPARγ expression plasmid (Fig. 1). This cell line therefore allowed a direct assessment of the role of PPARγ in glucagon gene transcription. Similarly, low level expression of PPARγ in cell lines derived from tissues with high level expression has been reported previously (6, 13). To study the effect of PPARγ and thiazolidinediones on glucagon gene transcription, 350 base pairs of the 5′-flanking region of the rat glucagon gene were fused to the luciferase reporter gene (construct −350GluLuc) (40). This glucagon promoter fragment is sufficient to confer tissue-specific gene expression (47) and regulation of gene transcription by cAMP-, calcium-, protein kinase C-, and insulin-induced signaling pathways (40, 42, 43, 48–52). In the absence of a cotransfected PPARγ expression plasmid, treatment of InR1-G9 cells with the thiazolidinedione rosiglitazone at concentrations up to 100 μM had no effect on glucagon

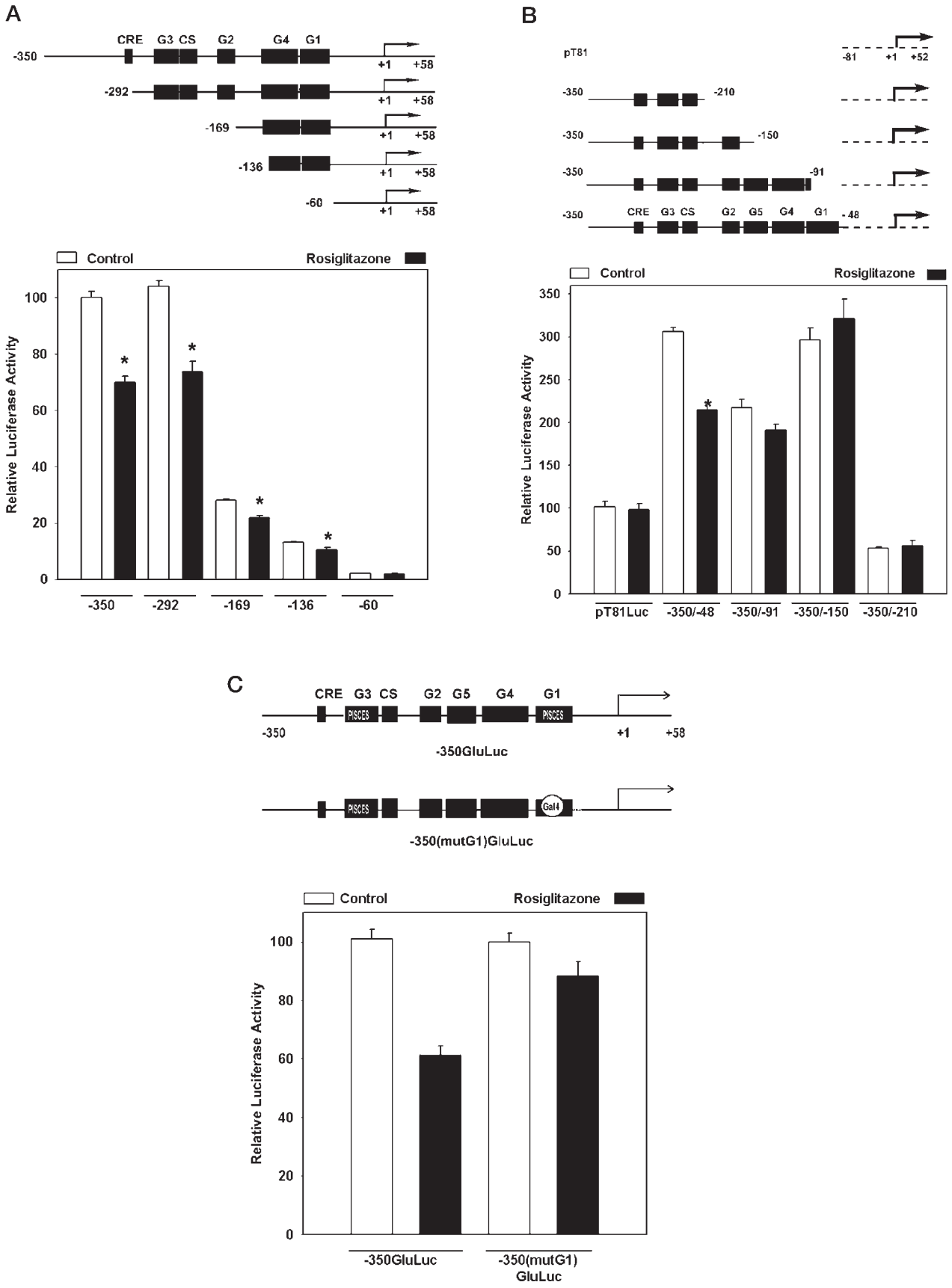

FIG. 5. **Mapping of a negative PPARγ response element in the glucagon gene promoter.** *A*, 5′-deletion analysis. After cotransfection of the indicated constructs and a PPARγ expression vector, InR1-G9 cells were treated with rosiglitazone (10 μM) or solvent (control) for 24 h before harvest. Luciferase activity in the presence of rosiglitazone is expressed as percentage of the mean value, in each experiment, of the activity measured in the −350 control. Values are means ± S.E. of three independent experiments, each done in duplicate. *, $p < 0.005$ (Student's *t* test). Control elements in the 5′-flanking region of the glucagon gene are indicated (see "Results" for explanation). *B*, 3′-deletion analysis. The indicated

promoter activity (data not shown). Additionally, the cotransfection of an expression plasmid encoding PPARγ alone had no effect on −350GluLuc activity (94 ± 3% of controls, $n = 6$). However, when a PPARγ expression plasmid was transfected into these cells, rosiglitazone inhibited glucagon gene transcription (Fig. 2). Thus rosiglitazone inhibits glucagon gene transcription by a PPARγ-dependent mechanism. Inhibition of glucagon gene transcription by rosiglitazone was concentration-dependent with an IC_{50} value of ~300 nM (Fig. 2). Cotransfection of an expression vector encoding RXRα together with PPARγ did not alter the concentration-response curve for inhibition by rosiglitazone of −350GluLuc activity (data not shown). These concentrations of rosiglitazone are similar to those that activated a PPARγ-dependent promoter (Fig. 1). The maximum inhibition of glucagon gene transcription by rosiglitazone was ~40% (Fig. 2). Like rosiglitazone, two other thiazolidinediones, darglitazone and englitazone, also inhibited glucagon gene transcription (Fig. 3). To assess the effect of thiazolidinediones in a natural context, the effect of thiazolidinediones on glucagon secretion and glucagon tissue levels was investigated in primary pancreatic islets. After 48 h of treatment with rosiglitazone, glucagon secretion from isolated pancreatic islets was inhibited by 44% (Fig. 4). Englitazone and darglitazone showed a similar inhibition (Fig. 4). Furthermore, glucagon tissue levels were significantly reduced by treatment with rosiglitazone, englitazone, or darglitazone (Fig. 4). These data indicate that PPARγ inhibits glucagon gene transcription in response to binding of thiazolidinediones. Thiazolidinediones also reduce glucagon tissue levels and secretion in pancreatic islets.

Mapping of a Negative PPARγ Response Element in the Glucagon Gene—PPARγ is known to activate transcription through DR-1 motifs (1). The inhibition by PPARγ of the glucagon reporter fusion gene −350GluLuc indicates that a negative PPARγ response element resides within 350 base pairs of the 5′-flanking region of the glucagon gene (Figs. 2 and 3). This fragment of the glucagon gene contains the enhancer-like element G2 and G3 as well as a cAMP response element (53). The truncated glucagon gene promoter (136 base pairs) containing the proximal promoter elements G1 and G4 exhibits low transcriptional activity but is essential for proper enhancer function (53). To localize more precisely the *cis*-acting DNA sequences of the glucagon gene that mediate transcriptional repression by PPARγ, a 5′/3′-deletion, and internal mutation analysis was performed.

Expression of 5′-deleted mutant plasmids in InR1-G9 cells revealed that the repression by PPARγ in response to rosiglitazone was unimpaired when the 5′ end was shortened from −350 to −169 (Fig. 5*A*). It was, if at all, only slightly diminished when the 5′ end was shortened to −136 (Fig. 5*A*). However, truncation to −60 abolished the repression by PPARγ (Fig. 5*A*). These results indicate that a DNA control element required for repression by PPARγ may have its 5′ boundary and reside between −136 and −60.

The results of the 3′-deletion analysis are shown in Fig. 5*B*. Fragments of the glucagon promoter with deletions at their 3′ ends were linked to the truncated thymidine kinase promoter (−81 to +52) of herpes simplex virus (pT81Luc). This promoter does not respond to PPARγ and rosiglitazone (Fig. 5*B*). The

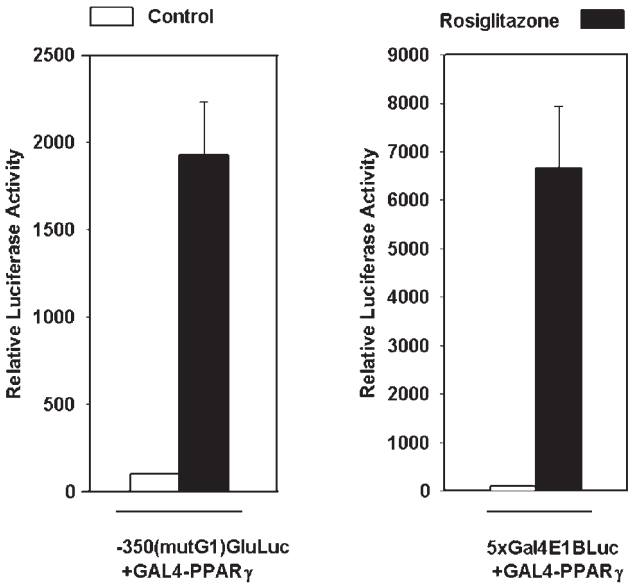

FIG. 6. **GAL4-PPARγ stimulates transcription, when anchored to the glucagon promoter.** An expression vector encoding GAL4-PPARγ was transfected into InR1-G9 cells together with −350(mutG1)GluLuc or 5xGal4E1BLuc reporter gene. Cells were treated with rosiglitazone (10 μM) or solvent (control) for 24 h before harvest. Luciferase activity in the presence of rosiglitazone is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective control. Values are means ± S.E. of four experiments.

glucagon gene 5′-flanking region from −350 to −48 conferred repression by PPARγ (Fig. 5*B*). When only sequences from −350 to −91 were fused to the viral promoter, PPARγ in response to rosiglitazone no longer inhibited gene transcription (Fig. 5*B*). This deletion eliminates the G1 element (Fig. 5*B*). Further deletion to −150 or −210 had no effect (Fig. 5*B*). These data suggest that a DNA control element required for repression by PPARγ may have its 3′ boundary and reside between −48 and −91.

Taken together, the results of the 5′- and 3′-deletion analysis suggest that a DNA control element conferring PPARγ repression to the glucagon gene may be located between −136 and −48. This region contains the G1 element (Fig. 5, *A* and *B*). The G1 element contains a PISCES motif that is essential for promoter function (43, 47, 54, 55). To examine the role of the PISCES motif within G1 in the repression of glucagon gene transcription by PPARγ, the PISCES motif in G1 was mutated (and thereby changed into a binding site of the yeast transcription factor GAL4) (Fig. 5*C*). The mutation of the PISCES motif within G1 decreased basal activity to low but detectable levels (2.5 ± 0.3% of wild type, $n = 6$) and almost abolished the repression of transcription by PPARγ in response to rosiglitazone (Fig. 5*C*). These results confirm that the PISCES motif within G1 is important for basal glucagon promoter activity. Although interpretation is difficult in view of the change in basal promoter activity, these results furthermore provide evidence that the PISCES motif within G1 is required for repression of glucagon gene transcription by PPARγ.

constructs were transfected into InR1-G9 cells together with a PPARγ expression vector. Cells were treated with rosiglitazone (10 μM) or solvent (control) for 24 h before harvest. Luciferase activity in the presence of rosiglitazone is expressed as percentage of the mean value, in each experiment, of the activity measured in the pT81 control. Values are means ± S.E. of three independent experiments, each done in duplicate. *, $p < 0.005$ (Student's t test). *C*, internal mutation. Pax6 binds to PISCES motifs within G1 and G3. Bases including the PISCES motif within G1 were mutated into a GAL4 binding site. Plasmids −350GluLuc or −350(mutG1)GluLuc were transfected into InR1-G9 cells together with pPPARγ. Cells were treated with rosiglitazone (10 μM) or solvent (control) for 24 h before harvest. Luciferase activity in the presence of rosiglitazone is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective control. Values are means ± S.E. of three independent experiments, each done in duplicate.

Inhibition of Pax6 Transcriptional Activity by PPARγ in Response to Thiazolidinediones—The 5′/3′-deletion and internal mutation studies suggest that a proximal promoter fragment containing the G1 element and the PISCES motif is required for inhibition of glucagon gene transcription by PPARγ in response to thiazolidinediones. To examine the function of PPARγ when forced to bind to this promoter region, a GAL4-PPARγ fusion protein was used (45). This GAL4-PPARγ fusion protein was transfected into InR1-G9 cells together with −350(mutG1)GluLuc, in which the PISCES motif within G1 has been mutated into a binding site of GAL4. After cotransfection of GAL4-PPARγ, rosiglitazone markedly stimulated −350(mutG1)GluLuc activity (Fig. 6, *left panel*), similar to the stimulation by rosiglitazone of a luciferase reporter gene placed under the control of multiple GAL4 DNA binding sites linked to a minimal viral E1B promoter (5xGal4E1BLuc) (Fig. 6, *right panel*). This enhancement of −350(mutG1)GluLuc transcriptional activity (Fig. 6, *left panel*) is in contrast to the inhibition of −350GluLuc transcriptional activity by PPARγ in response to rosiglitazone (Figs. 2 and 3). These results therefore suggest that the ligand-dependent transcription control domain of PPARγ stimulates transcription when anchored to the glucagon proximal promoter.

The PISCES motif has been shown to bind the paired-domain transcription factor Pax6 (55, 56). The observation that mutating the PISCES motif within G1 abolished the repression of glucagon gene transcription by ligand-activated PPARγ (Fig. 5*C*) thus raises the possibility that PPARγ may target Pax6 to inhibit glucagon gene transcription. However, because of potentially overlapping binding sites, the mutation of the PISCES motif within G1 may not only abolish Pax6 binding but also affect the binding of additional transcription factors like cdx2/3 and brain-4 (57–59). We therefore examined whether repression of the glucagon promoter by PPARγ can be restored by Pax6 recruited to the mutant glucagon promoter through the GAL4 binding site. When 1 μg of an expression vector encoding a GAL4-Pax6 fusion protein (43) per dish was transfected together with −350(mutG1)GluLuc, basal transcriptional activity of the mutant glucagon promoter was raised to a level similar to that of the wild-type promoter (Fig. 7*A*). The expression of GAL4-Pax6 also conferred repression by PPARγ in response to rosiglitazone (Fig. 7*A*). After expression of GAL4-Pax6, PPARγ inhibited the transcriptional activity of the mutated glucagon promoter in response to rosiglitazone by ~40% (Fig. 7*A*); this is similar to the inhibition by ligand-activated PPARγ of wild-type glucagon promoter activity. This effect of GAL4-Pax6 seems to be specific and also not secondary to the restoration of basal activity, because the expression of GAL4-VP16 also elevated basal activity of the mutated glucagon promoter but did not confer PPARγ responsiveness (7*A*). Likewise, when cotransfected with a reporter construct, in which multiple GAL4 binding sites had been placed in front of the truncated viral E1B promoter (5xGal4E1BLuc), GAL4-Pax6 transcriptional activity was inhibited by PPARγ upon treatment of the cells with rosiglitazone (Fig. 7*B*, *left panel*). This effect was again specific because PPARγ plus rosiglitazone had no effect on the transcriptional activity conferred by GAL4-VP16 to 5xGal4E1Bluc (Fig. 7*B*, *right panel*). This indicates that inhibition of Pax6 transcriptional activity by PPARγ in response to thiazolidinediones does not depend on the glucagon promoter context. These results thus suggest that PPARγ in response to thiazolidinediones inhibits Pax6 transcriptional activity and thereby reduces glucagon gene transcription.

DISCUSSION

PPARγ has been shown to be highly expressed in normal human glucagon-producing pancreatic islet α-cells (30), although its function has been unknown. The present study now demonstrates that PPARγ inhibits glucagon gene transcription in glucagon-producing pancreatic islet cells. This was followed by a decrease in glucagon tissue levels and secretion. This study thereby assigns a function to pancreatic PPARγ receptors, further supporting a role of PPARγ in glucose homeostasis. This action of PPARγ is ligand-dependent, because it was observed only upon adding the thiazolidinedione PPARγ ligands rosiglitazone, darglitazone, and englitazone. These synthetic compounds mimic the effect of endogenous PPARγ ligands like fatty acids and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (16). The fact that rosiglitazone inhibited glucagon gene transcription over the same range of concentrations as it stimulated through PPARγ the expression of a reporter gene directed by multiple PPARγ DNA binding sites (IC_{50} and EC_{50} values of ~300 nM and 1 μM, respectively) suggests that inhibition of glucagon gene transcription may accompany other PPARγ-mediated effects. This effect may thus be therapeutically relevant for the action of thiazolidinediones. The IC_{50} value of rosiglitazone for inhibition of glucagon gene transcription (~300 nM) is also similar to the reported affinity of rosiglitazone for PPARγ binding (K_i 214 nM) (60).

It is now well established that, after several weeks of treatment of type II diabetic patients, thiazolidinediones diminish insulin resistance and reduce hepatic glucose production rates, resulting in lowering of both fasting and postprandial blood glucose as well as insulin levels (19, 61). The fasting plasma glucagon concentrations were not significantly changed by troglitazone treatment of type II diabetic patients (61). However, the fact that plasma glucagon concentrations were maintained despite decreased plasma glucose and insulin levels (61), which should disinhibit and, thus, enhance glucagon secretion (33–35), suggests that thiazolidinedione treatment imposed an inhibition on glucagon secretion. Indeed, troglitazone inhibited the glucagon response to a meal tolerance test in type II diabetic patients (61). By demonstrating an inhibition of glucagon gene transcription and expression by thiazolidinediones, the present study shows a novel action of PPARγ and this class of antidiabetic agents. It offers a mechanism through which thiazolidinedione treatment of type II diabetic subjects could lead to decreased glucagon expression and secretion, thereby preventing a glucagon-induced increase in hepatic glucose output. The present study therefore suggests that inhibition of glucagon gene expression by thiazolidinediones could be part of the mechanisms through which these antidiabetic agents improve glycemic control in diabetic patients.

PPARγ stimulates gene transcription by binding as an RXR heterodimer to DR-1-like DNA response elements, ligand binding, and coactivator recruitment (see Introduction). The glucagon gene provides an additional example that PPARγ can also inhibit gene transcription (6–14). Several mechanisms have been described for negative regulation of gene transcription by nuclear receptors. The thyroid hormone receptor harbors ligand-independent repressor function and actively represses transcription upon binding to cognate sites within the promoter region of target genes. These active repressive function requires the recruitment of corepressor complexes that are dismissed upon ligand binding (62). PPARγ inhibits the glucagon gene by a clearly distinct mechanism, because this inhibition is ligand-dependent and does not appear to involve direct binding to the glucagon promoter (see below). The mechanism may thus be more related to the ones best established for the glucocorticoid receptor, which in many cases mediates transrepression in a DNA binding-independent manner (63–67). Similarly, inhibition of inducible nitric-oxide synthase gene transcription by PPARγ has been proposed to be achieved at least partially by

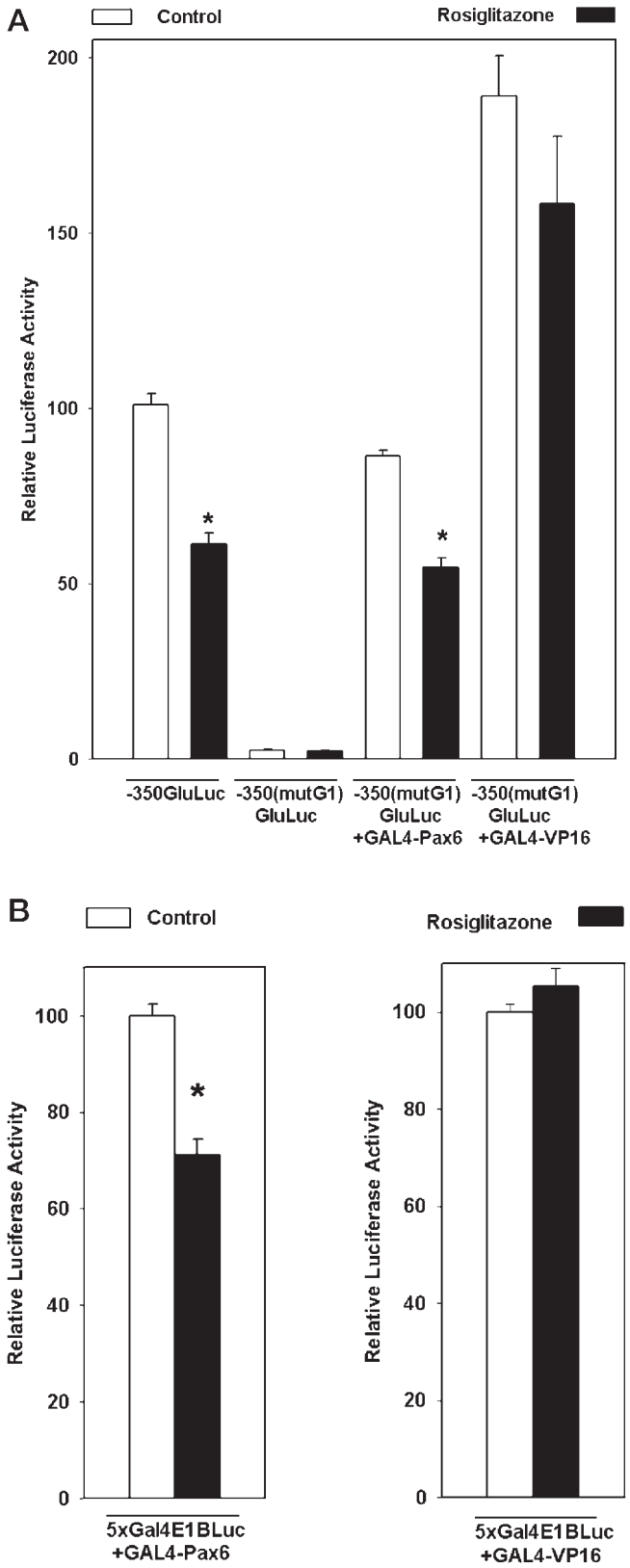

FIG. 7. **Inhibition of Pax6 transcriptional activity by PPARγ in response to rosiglitazone.** *A*, Pax6 restores basal activity and confers PPARγ responsiveness to the mutant glucagon promoter. Expression vectors encoding PPARγ and GAL4-Pax6 or GAL4-VP16 were transfected into InR1-G9 cells together with −350(mutG1)GluLuc. Cells were treated with rosiglitazone (10 μM) or solvent (control) for 24 h before harvest. Luciferase activity is expressed as percentage of the mean value, in each experiment, of the activity measured in the −350 control. Values are means ± S.E. of five independent experiments, each done in duplicate. *, $p < 0.005$ (Student's t test). *B*, Pax6 confers transcriptional activity and PPARγ responsiveness to a minimal viral promoter. Expression vectors encoding PPARγ and GAL4-Pax6 or GAL4-VP16 were transfected into InR1-G9 cells together with 5xGal4E1BLuc reporter gene. Cells were treated with rosiglitazone (10 μM) or solvent (control) for 24 h before harvest. Luciferase activity in the presence of rosiglitazone is expressed as percentage of the mean value, in each experiment, of the activity measured in the respective control. Values are means ± S.E. of five independent experiments, each done in duplicate. *, $p < 0.005$ (Student's t test).

antagonizing the activities of STAT1, NF-κB, and AP-1 without binding of PPARγ to the promoter (6, 7). By 5′/3′-deletion and internal mutation analysis as well as by using GAL4 fusion proteins, the present study provides evidence that PPARγ inhibits glucagon gene transcription by inhibition of Pax6 transcriptional activity. These results thus define Pax6 as a novel functional target of PPARγ.

Pax6 is a member of the paired box-containing genes that play important roles during development (68). Pax6 is expressed early in pancreas development defining endocrine cell lineages. Inactivation of Pax6 results in the absence of glucagon-producing α cells (69). Pax6 is expressed also in adult islets (55, 56, 70). It binds and strongly activates the glucagon promoter (43, 54–56, 71, 72). Mutational analyses further support the view that Pax6 is essential for activation of the glucagon gene in pancreatic islet cells (53). The inhibition by PPARγ of Pax6 transcriptional activity thus appears to be sufficient to explain the inhibition of glucagon gene transcription by PPARγ in response to thiazolidinediones. The glucagon promoter contains two Pax6 binding sites, a PISCES motif within the enhancer-like element G3 and another PISCES motif in the proximal promoter element G1 (53, 54, 71). The mapping experiments of the present study suggest that PPARγ responsiveness is conferred by Pax6 at the proximal promoter site, indicating that Pax6 may function differently at the two binding sites.

The present study suggests that PPARγ inhibits glucagon gene transcription through inhibition of transactivation by Pax6. Previous reports have shown that repression of gene transcription by PPARγ can involve a change in transcription factor binding. Thus, PPARγ activators were found to induce ATF3 in human vascular endothelial cells, which bound to and repressed E-selectin gene expression (9). Furthermore, inhibition of NFAT and NRF2 DNA binding was found when PPARγ agonists inhibited interleukin-2 gene transcription in T-cells and thromboxane synthase gene transcription in macrophages, respectively (13, 14). In contrast, the results of the present study indicate that inhibition of glucagon gene transcription by PPARγ does not depend on inhibition of Pax6 DNA binding but instead involves the inhibition of transactivation by Pax6, produced by PPARγ in a ligand-dependent manner without binding of PPARγ to the glucagon promoter. First, PPARγ stimulated glucagon promoter activity in response to thiazolidinediones when fused to the GAL4 DNA binding domain and anchored to the proximal promoter element G1. Second, PPARγ inhibited Pax6 transcriptional activity when Pax6 bound through a heterologous DNA-binding domain (GAL4-Pax6) to the G1 element of the glucagon gene (−350(mutG1)GluLuc) or to multiple binding sites in front of a minimal viral promoter (5xGal4E1BLuc). The mechanism of transcriptional repression of Pax6 by PPARγ remains to be defined but could include direct binding of PPARγ to Pax6 or protein-protein interactions of PPARγ with coactivators or signaling molecules. The glucocorticoid receptor was found to bind directly to transcription factors like AP-1 and NF-κB (63–67). On the other hand, single amino acid mutations in PPARγ that abolished ligand-dependent interactions with SRC-1 and CBP also abolished transre-

pression by PPARγ of the inducible nitric-oxide synthase gene (7). This suggests that, in this case transrepression by PPARγ may involve competition for limiting amounts of the general coactivators CBP and p300, which is achieved by targeting CBP through direct interaction with its NH_2-terminal domain and via SRC-1-like bridge factors (7).

When taken together, the results of the present study define Pax6 as a novel functional target of PPARγ. Our results are consistent with a model in which PPARγ in a ligand-dependent but DNA binding-independent manner inhibits transactivation by Pax6 leading to inhibition of glucagon gene transcription in pancreatic islet cells. This function of PPARγ further supports the role of PPARγ in glucose homeostasis and suggests that inhibition of glucagon gene expression and secretion could be part of the mechanisms through which thiazolidinediones like rosiglitazone improve glycemic control in diabetic subjects.

Acknowledgments—The plasmid pGAL4-PPARγ was a generous gift from Brad Henke and Steve A. Kliewer (Glaxo Wellcome, Inc., Research Triangle Park, NC). We thank C. Spinhoff for typing the manuscript.

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PUBLIKATION 3

Schinner S, Krätzner R, Baun D, Dickel C, Blume R, Oetjen E.

Inhibition of human insulin gene transcription by peroxisome proliferator-activated receptor γ and thiazolidinedione oral antidiabetic drugs.

Br. J. Pharm. 157(5):736-45, (2009)

British Journal of Pharmacology (2009), 157, 736–745

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Inhibition of human insulin gene transcription by peroxisome proliferator-activated receptor γ and thiazolidinedione oral antidiabetic drugs

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Background and purpose: The transcription factor peroxisome proliferator-activated receptor γ (PPARγ) is essential for glucose homeostasis. PPARγ ligands reducing insulin levels *in vivo* are used as drugs to treat type 2 diabetes mellitus. Genes regulated by PPARγ have been found in several tissues including insulin-producing pancreatic islet β-cells. However, the role of PPARγ at the insulin gene was unknown. Therefore, the effect of PPARγ and PPARγ ligands like rosiglitazone on insulin gene transcription was investigated.
Experimental approach: Reporter gene assays were used in the β-cell line HIT and in primary mature pancreatic islets of transgenic mice. Mapping studies and internal mutations were carried out to locate PPARγ-responsive promoter regions.
Key results: Rosiglitazone caused a PPARγ-dependent inhibition of insulin gene transcription in a β-cell line. This inhibition was concentration-dependent and had an EC_{50} similar to that for the activation of a reporter gene under the control of multimerized PPAR binding sites. Also in normal primary pancreatic islets of transgenic mice, known to express high levels of PPARγ, rosiglitazone inhibited glucose-stimulated insulin gene transcription. Transactivation and mapping experiments suggest that, in contrast to the rat glucagon gene, the inhibition of the human insulin gene promoter by PPARγ/rosiglitazone does not depend on promoter-bound Pax6 and is attributable to the proximal insulin gene promoter region around the transcription start site from –56 to +18.
Conclusions and implications: The human insulin gene represents a novel PPARγ target that may contribute to the action of thiazolidinediones in type 2 diabetes mellitus.
British Journal of Pharmacology (2009) **157,** 736–745; doi:10.1111/j.1476-5381.2009.00208.x; published online 26 March 2009

Keywords: β-cells; thiazolidindiones; human insulin gene transcription; PPARγ

Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ; RXRα, retinoid X receptor α

Introduction

The nuclear hormone receptor, peroxisome proliferator-activated receptor γ (PPARγ), is, among other functions, an important regulator of glucose homeostasis (Desvergne and Wahli, 1999). While the endogenous ligand of PPARγ is not known, there are several synthetic compounds that bind PPARγ with high affinity and activate the receptor. These include the thiazolidinedione class of oral antidiabetic drugs, which are in use for the treatment of type 2 diabetes mellitus (Desvergne and Wahli, 1999). Thiazolidinediones like rosiglitazone and pioglitazone are effective agents for the control of glycaemia in patients with type 2 diabetes (Natali and Ferranini, 2006). In addition, in a randomized controlled clinical trial including 5238 patients, pioglitazone significantly reduced by 16% a composite end point. This included death from any cause, nonfatal myocardial infarction and stroke (Dormandy *et al.*, 2005). The overall clinical benefit from use of thiazolidinediones, however, remains to be defined (Lago *et al.*, 2007; Lincoff *et al.*, 2007; Nissen and Wolski, 2007; Singh *et al.*, 2007).

When compared with other oral antidiabetic drugs like the sulphonylureas and metformin, thiazolidinediones exhibit a unique antidiabetic effect. Through PPARγ, they reduce hepatic glucose output and increase insulin sensitivity (Natali and Ferranini, 2006). Furthermore, decreases in fasting plasma insulin levels have been reported in most trials performed with thiazolidinediones (Walter and Lübben, 2005; Wajchenberg, 2007). Reductions in fasting plasma insulin

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Received 19 June 2008; revised 24 November 2008; accepted 16 January 2009

levels have been in the range of 10.7–31.8 pmol·L^{-1} with pioglitazone monotherapy and 6.6–27.2 pmol·L^{-1} with rosiglitazone monotherapy (Walter and Lübben, 2005). Thiazolidinediones also reverse the decline in the function of insulin-producing pancreatic islet β-cells in type 2 diabetes mellitus with improvements in islet architecture, insulin content, proinsulin to total immunoreactive insulin ratio and glucose-stimulated insulin secretion (Zeender *et al*., 2004; Walter and Lübben, 2005; Wajchenberg, 2007). Although these effects on insulin could be explained as the beneficial sequelae of reducing hyperglycaemia and peripheral insulin resistance, direct effects of PPARγ on β-cells may contribute as well. PPARγ serves as transcription factor for a great number of genes involved in fatty acid uptake and storage, inflammation and glucose homoeostasis in adipose tissue, skeletal muscle and liver (Kostadinova *et al*., 2005; Gervois *et al*., 2007). Furthermore, PPARγ is expressed also in pancreatic islet β-cells (Braissant and Wahli, 1998). Nevertheless, the role of PPARγ in β-cells in the regulation of the insulin gene has received little attention so far.

Therefore, the direct effect of PPARγ and thiazolidinediones on insulin gene transcription in pancreatic islet β-cells was examined in the present study. Using transfection studies in the β-cell line HIT and in primary islets of mature transgenic mice, this study demonstrates that PPARγ ligands inhibit insulin gene transcription. This study suggest that, in contrast to the glucagon gene, the inhibition of the insulin gene does not depend on promoter-bound Pax6 and is conferred by the proximal insulin gene promoter region around the transcription start site.

Methods

Plasmid constructs

The plasmids pT81Luc, –350GluLuc (Schwaninger *et al*., 1993), –410rInsLuc (Siemann *et al*., 1999), –336hInsLuc, –258hInsLuc, –222hInsLuc, –193hInsLuc, –140hInsLuc, –93hInsLuc, –56hInsLuc, –336/+18hInsLuc (Oetjen *et al*., 2007), PPRE-Luc, pPPARγ (Schinner *et al*., 2002) and pcDNA3-retinoid X receptor (RXR)α, pcDNA3-PPARγ1-475N, pcDNA3-PPARγ175-475N (Krätzner *et al*., 2008) have been described previously. The expression vector pBAT14.mPax6 was kindly provided by Dr M. German (University of California, San Francisco, CA) (Sander *et al*., 1997). The plasmid pCMV-GFPtpz was purchased from Canberra-Packard (Dreieich, Germany). To generate –336/–31P-Luc, the fragment from –336 to –31 of the human insulin gene promoter was amplified by PCR using –336hInsLuc as template and cloned in front of the heterologous minimal promoter –85rInsLuc (P) (Siemann *et al*., 1999). All constructs were verified by sequencing using the enzymic method.

Cell culture and transfection of DNA

The insulin-producing pancreatic islet β-cell line HIT-T15 (Santerre *et al*., 1981) was grown in RPMI 1640 medium supplemented with 10% foetal calf serum, 5% horse serum, penicillin (100 U·mL^{-1}) and streptomycin (100 μg·mL^{-1}). JEG-3 human choriocarcinoma cells (Oetjen *et al*., 2007) were grown in DMEM supplemented with 10% foetal calf serum, penicillin (100 U·mL^{-1}) and streptomycin (100 μg·mL^{-1}). HIT cells were trypsinized and transfected in suspension by the DEAE-dextran method (Schwaninger *et al*., 1993) with 2 μg of reporter gene plasmids and, when indicated, 1 μg of expression vector per 6 cm dish. Twenty-four hours after transfection, cells were incubated in RPMI 1640 containing 0.5% bovine serum albumin and antibiotics as described above. Thiazolidinediones were added 24 h before harvest. JEG cells were transfected by the calcium phosphate precipitation method (Oetjen *et al*., 2007) with 3 μg of reporter gene plasmid and, when indicated, 207 ng of expression vector per 6 cm dish. In all experiments (HIT and JEG), cotransfections were carried out with a constant amount of DNA, which was maintained by adding Bluescript (Stratagene, La Jolla, CA). In all experiments 1 μg (HIT) or 50 ng (JEG) of cytomegalovirus–GFP (plasmid pCMV-GFPtpz) per 6 cm dish was cotransfected to check for transfection efficiency. Cell extracts (Schwaninger *et al*., 1993) were prepared 48 h after transfection. The luciferase assay was performed as described previously (Schwaninger *et al*., 1993). For the transfection of small interference RNA (siRNA) against Pax6, cells were transfected by metafectene (Biontex, Munich, Germany) with 50 or 100 pmole per 2 cm dish of siRNA as indicated according to the manufacturer's recommendations. The following sequences were employed: siRNA 1 – GGGACCACUUCAACAGGACUCAUUU, siRNA 2 – GGAGUGAACCUGACAUGUCUCAGUA, siRNA 3 – ACCACACCUGUCUCCUCCUUCAC AU and their respective complementary strands. The efficiency of Pax6 knock down was tested by immunoblot using an antibody against Pax6 (Santa Cruz, Heidelberg, Germany). Green fluorescent protein was measured in the cell extracts using the FluoroCount™ microplate fluorometer (Packard) with a 485 nm (excitation)/530 nm (emission) filter pair.

Electrophoretic mobility shift assay

Synthetic complementary oligonucleotides (hIns PPRE wt: 5′-GGCCCAGCAGCCCTCAGCCCTCCAGGACAGGCT-3′, hIns PPRE mut: 5′-GGCCCAGCAGCAAGCATCTTGCCAGG ACAGGCT-3′) were annealed and labelled by a fill-in reaction using [α-^{32}P]dCTP and Klenow enzyme. Fifteen microlitres of islet extracts was pre-incubated with 2 μg poly dI/dC in binding buffer (20 mmol·L^{-1} HEPES, 1 mmol·L^{-1} EDTA) and when indicated with a 200-fold molar excess of the competitors (PPRE wt: 5′-GGTAAAGGTCAAAGGTCAAT-3′, PPRE mut: 5′-GGTAAAGAACAAAGAACAAT-3′) for 10 min at room temperature, followed by a 15 min incubation with the labelled oligonucleotides at room temperature. The binding reaction was subjected to electrophoresis on a 5% non-denaturing poly acrylamide gel.

Generation and analysis of transgenic mice

All animal studies were conducted according to the National Institutes of Health's guidelines for care and use of experimental animals and were approved by the Committee on Animal Care and Use of the local institution and state. The generation and analysis of transgenic mice carrying a transgene with the luciferase reporter gene under the control of the

human insulin gene promoter from –336 to +112 have been described before (Oetjen *et al.*, 2003a).

Isolation and culture of islets

Pancreatic islets from mice or transgenic mice were isolated and incubated as described previously (Oetjen *et al.*, 2003a). In short, isolated islets were pre-incubated in a humified atmosphere of 95% air/5% CO_2 for 12 h in RPMI 1640 medium containing 5 mmol·L^{-1} glucose and supplemented with 10% foetal calf serum, penicillin (100 U·mL^{-1}) and streptomycin (100 μg·mL^{-1}). Rosiglitazone was added 7 h, glucose (final concentration 20 mmol·L^{-1}) 6 h before harvest. Islet collection and extraction, as well as the measurement of luciferase activity and protein content were performed as has been described (Oetjen *et al.*, 2003a). Following this protocol, the human insulin promoter has been shown to confer a normal, physiological glucose response to reporter gene expression in isolated islets (Oetjen *et al.*, 2003a). For the electrophoretic mobility shift assay, approximately 800 islets were lysed in 150 μL lysis buffer (50 mmol·L^{-1} HEPES, 150 mmol·L^{-1} NaCl, 1.5 mmol·L^{-1} $MgCl_2$, 1 mmol·L^{-1} EGTA, 10% glycerol, 0.5% NP-40, 1 mmol·L^{-1} $NaVO_4$, 50 mmol·L^{-1} NaF, 20 mmol·L^{-1} β-glycerophosphate), passed five times through a 20 G needle, incubated on ice for 30 min and centrifuged at 4°C, 20 800× *g* for 5 min. The supernatant was used for the binding reaction.

Statistical analysis

All results are expressed as means ± SEM. Statistical significance was calculated with ANOVA, followed by Student's *t*-test. A value of $P < 0.05$ was considered significant.

Materials

Rosiglitazone was kindly provided by GlaxoSmith-Kline (Welwyn Garden City, Hertfordshire, UK). Darglitazone and englitazone (CP-72,467-02, sodium salt) were provided by Pfizer Inc (Groton, CT). RNAi was obtained from Invitrogen (Karlsruhe, Germany). Luciferin was purchased from Promega (Mannheim, Germany).

Results

Effect of PPARγ and thiazolidinediones on human insulin gene transcription in HIT β-cells

The expression of PPARγ is very high in normal pancreatic islets, approximately two-thirds of the expression level in white adipose tissue (Braissant and Wahli, 1998; Rosen *et al.*, 2003; Lupi *et al.*, 2004). In contrast, HIT cells express low levels of PPARγ as indicated by the observation that activation of a PPAR-dependent promoter (PPRE-Luc) by the thiazolidinedione rosiglitazone required transfection of a PPARγ expression vector (Figure 1). Consequently, this cell line allowed a direct assessment of the role of PPARγ in insulin gene transcription. Similarly, low-level expression of PPARγ in cell lines derived from tissues with high-level expression has been reported previously (Ricote *et al.*, 1998) including the

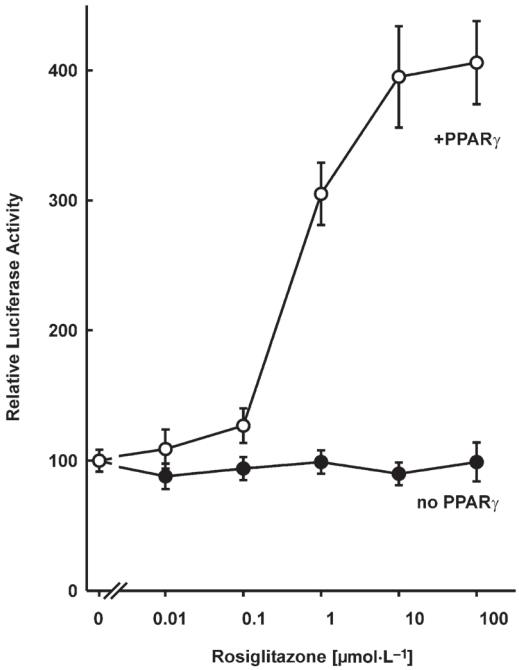


Figure 1 Activation of a peroxisome proliferator-activated receptor (PPAR)-dependent promoter by rosiglitazone and PPARγ in HIT β-cells. A luciferase reporter gene under the control of three copies of a PPARγ response element (plasmid PPRE-Luc) was transfected into HIT cells together with and without an expression vector encoding PPARγ. Increasing concentrations of rosiglitazone were added 24 h before harvest. Luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means ± SEM of three independent experiments, each in duplicate.

islet α-cell line InR1-G9 (Schinner *et al.*, 2002) and β-cell line MIN6 (Nakamichi *et al.*, 2003). To study the effect of PPARγ and thiazolidinediones on insulin gene transcription, a fragment from –336 to +112 of the human insulin gene was fused to the luciferase reporter gene (construct –336hInsLuc) (Oetjen *et al.*, 2007; 2003a). This insulin promoter fragment is sufficient to confer tissue-specific gene expression and regulation of gene transcription by cAMP-, calcium-, glucose-, calcineurin- and mitogen-activated protein kinase-induced signalling pathways (Melloul *et al.*, 2002; Hay and Docherty, 2006; Oetjen *et al.*, 2003a,b; 2007). In the absence of cotransfected PPARγ expression plasmid, treatment of HIT cells with rosiglitazone at concentrations of up to 100 μmol·L^{-1} had no effect on insulin gene transcription (data not shown). This is consistent with the reported lack of effect of rosiglitazone on insulin gene transcription in the MIN6 β-cell line (Richardson *et al.*, 2006). However, when a PPARγ expression plasmid was cotransfected, rosiglitazone inhibited insulin gene transcription (Figure 2). Consistently, rosiglitazone activated a PPRE-driven reporter gene only when PPARγ was cotransfected (Figure 1). Thus, rosiglitazone inhibits insulin gene transcription by a PPARγ-dependent mechanism. Inhibition of insulin gene transcription by rosiglitazone was concentration-dependent with an IC_{50} value of about 1 μmol·L^{-1} (Figure 2). Cotransfection of an expression vector encoding the RXRα together with PPARγ did not alter the concentration–response curve for inhibition of insulin gene transcription by rosiglitazone (data not shown). The concentrations of rosiglitazone

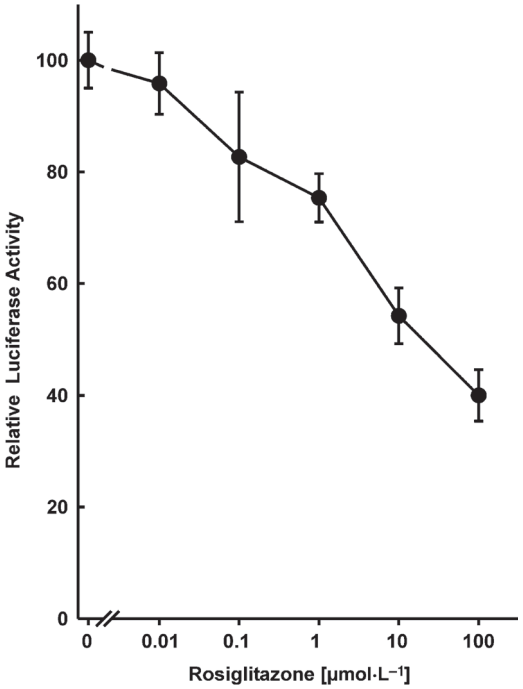


Figure 2 Inhibition of human insulin gene promoter activity by rosiglitazone and peroxisome proliferator-activated receptor γ (PPARγ). Plasmid −336hInsLuc was transfected into HIT β-cells together with pPPARγ. Rosiglitazone was added 24 h before harvest. Luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means ± SEM of three independent experiments, each in duplicate.

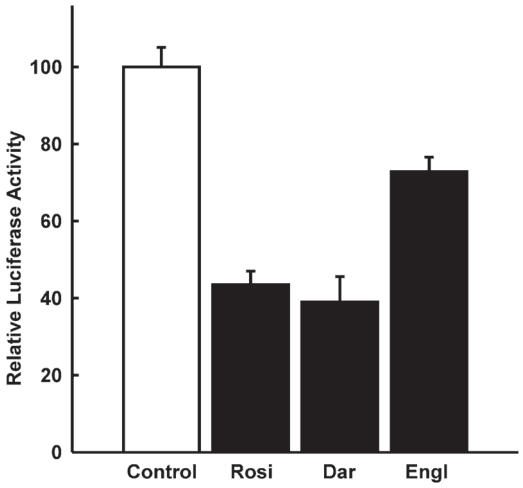


Figure 3 Inhibition of insulin gene transcription by the thiazolidinediones rosiglitazone, darglitazone and englitazone. HIT β-cells were transfected with −336hInsLuc and pPPARγ. They were treated with rosiglitazone (Rosi, 10 μmol·L^{-1}), darglitazone (Dar, 30 μmol·L^{-1}) or englitazone (Engl, 100 μmol·L^{-1}) 24 h before harvest as indicated. Luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means ± SEM of three independent experiments, each in duplicate.

that inhibit insulin gene transcription (Figure 2) are similar to those that activate the PPARγ-dependent promoter (Figure 1). The maximum inhibition of insulin gene transcription by rosiglitazone was about 60% (Figure 2). In addition to rosiglitazone, two other thiazolidinediones, darglitazone and englitazone, also inhibited insulin gene transcription (Figure 3). The specificity of the effect of PPARγ/rosiglitazone on insulin gene transcription is further supported by the lack of effect of PPARγ/rosiglitazone on CMV-promoter activity (not shown). These data indicate that PPARγ inhibits insulin gene transcription in response to the binding of thiazolidinediones.

Effect of rosiglitazone on glucose-stimulated insulin gene transcription in primary pancreatic islets

HIT cells are a well-established β-cell line and very useful in studies of insulin gene transcription (Santerre *et al.*, 1981; Melloul *et al.*, 2002; Hay and Docherty, 2006). As a tumour cell line, they may differ in critical aspects from normal β-cells. To investigate the effect of rosiglitazone in normal primary β-cells and under stimulation by glucose as the major physiological stimulus of insulin gene transcription (Melloul *et al.*, 2002; Hay and Docherty, 2006), islets of adult mice carrying a luciferase reporter transgene under the control of the human insulin gene promoter (from −336 to +112) were used. The expression of the human insulin gene within the islets of these transgenic mice has previously been shown to be regulated by glucose within the physiological concentration range (Oetjen *et al.*, 2003a). In the present study, we show that the treatment of the isolated islets with rosiglitazone abolished glucose-induced human insulin gene transcription (Figure 4).

Mapping of the PPARγ-responsive segment in the human insulin gene promoter

The transcription factor Pax6 has been shown to interact with PPARγ through its transactivation domain and to mediate the inhibition by PPARγ and thiazolidinediones of rat glucagon gene transcription (Schinner *et al.*, 2002; Krätzner *et al.*, 2008). Pax6 also binds to the rat insulin I gene promoter (Knepel *et al.*, 1991; Sander *et al.*, 1997). To examine whether Pax6 may bind to and activate also the human insulin gene promoter, hInsLuc was transfected into the heterologous cell line JEG, with and without cotransfection of an expression vector encoding Pax6. The luciferase reporter gene under the control of the rat insulin I (rInsLuc) or rat glucagon gene promoter (GluLuc) was transfected as a control. As shown in Figure 5A, Pax6 activated the rat glucagon gene promoter 117-fold and, less so, the rat insulin I gene promoter. In contrast, the activation of the human insulin gene promoter by Pax6 was only fivefold (Figure 5A). This activation appears to be non-specific, as similar slight increases were produced by Pax6 using the luciferase reporter gene under the control of promoters that lack a Pax6 binding site such as the truncated viral thymidine kinase promoter (pT81Luc) (not shown). Reduction of the cellular Pax6 content of HIT cells by siRNA did not decrease the transcriptional activity of the human insulin gene promoter (100 ± 5.6%, control: 92.3 ± 1.8% in the presence of siRNA, $n = 6$; $P < 0.05$) nor did it interfere with the inhibitory effect of rosiglitazone on human insulin gene transcription (Figure 5B). In addition, rosiglitazone inhibited human insulin gene transcription only when the overexpressed PPARγ contained its DNA binding domain, indicating

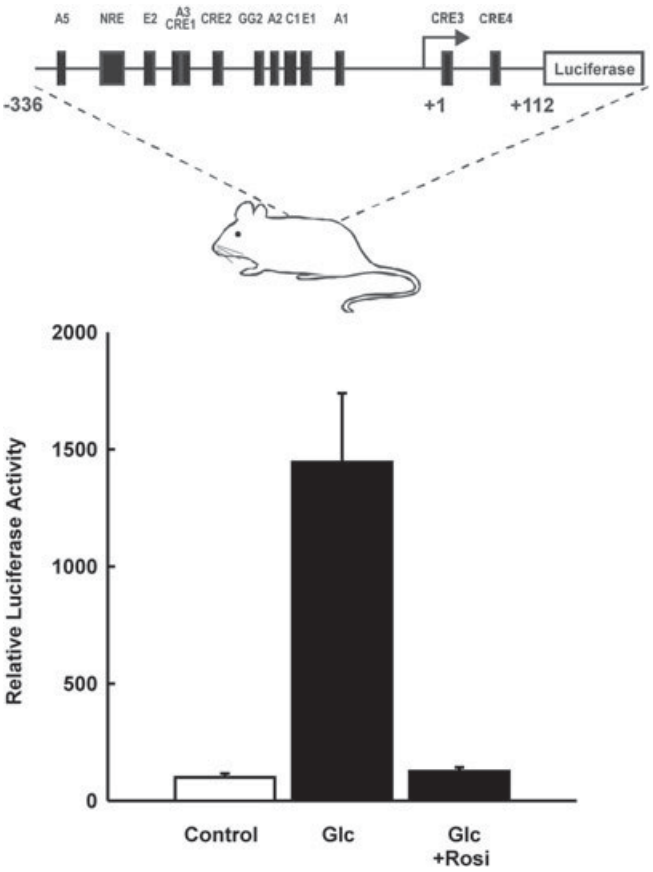
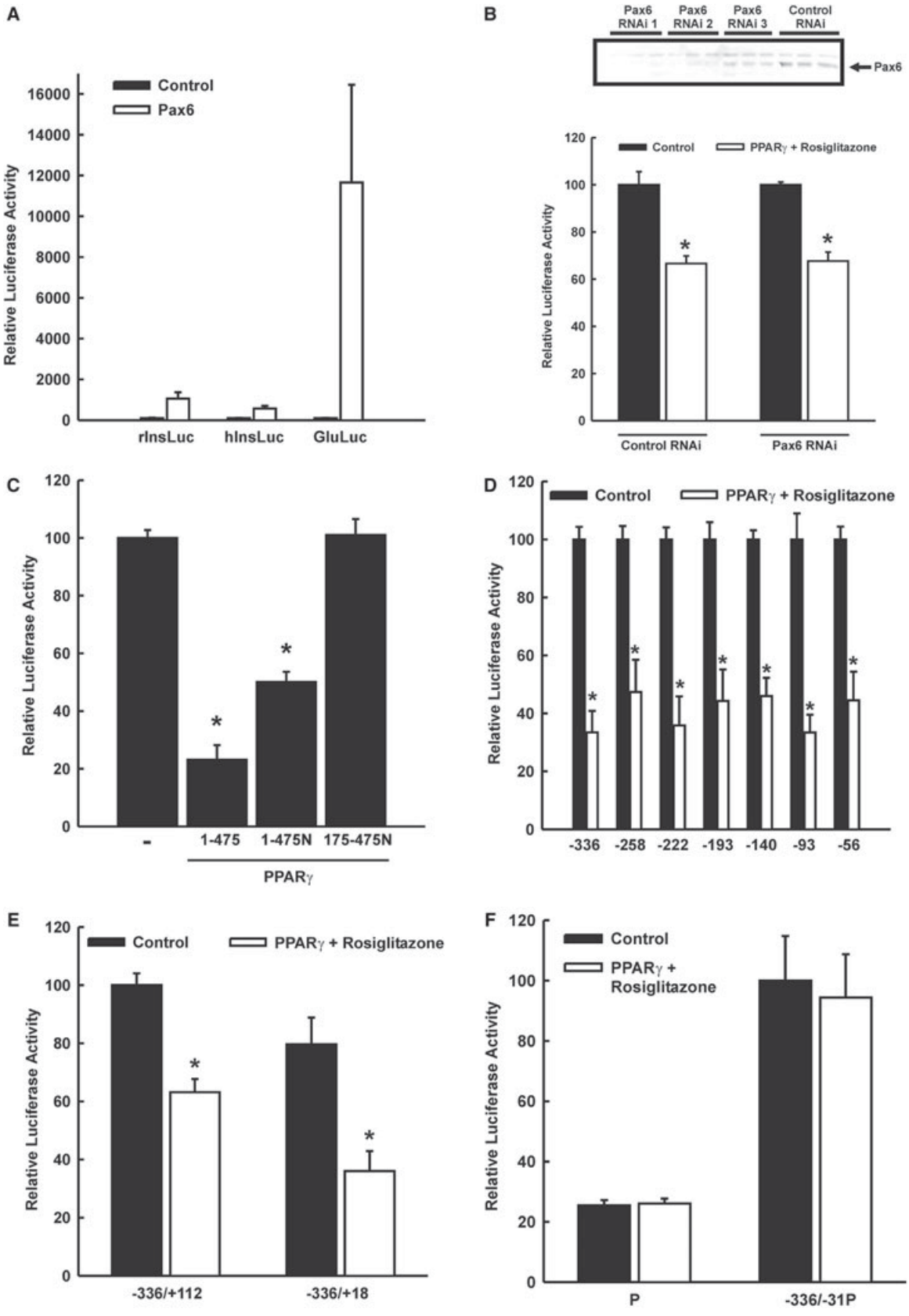


Figure 4 Effect of rosiglitazone on glucose-stimulated insulin gene transcription in normal primary pancreatic islets of transgenic mice. The upper panel depicts a scheme of the reporter gene used to generate the transgenic mice. Islets of transgenic mice were isolated and treated with glucose (Glc, 20 mmol·L^{-1}) 6 h prior to harvest with and without rosiglitazone (Rosi, 30 μmol·L^{-1}; 7 h prior to harvest). The control received 5 mmol·L^{-1} glucose only. Luciferase activity is expressed as percentage of the activity measured in the untreated controls. Values are means ± SEM of four experiments.

that binding of PPARγ to the insulin gene promoter is necessary for the inhibitory effect of the thiazoldindione (Figure 5C). These data do not support the view that PPARγ and thiazolidinediones may inhibit the human insulin gene promoter through inhibition of the transcriptional activity of promoter-bound Pax6, as is the case with the rat glucagon gene promoter (Schinner *et al*., 2002; Krätzner *et al*., 2008).

To define the cis-acting DNA sequences within the human insulin gene promoter that mediate transcriptional repression by PPARγ, a deletion analysis was performed. As shown in Figure 5D, expression of 5′-deleted mutant plasmids in HIT cells revealed that the repression by PPARγ and rosiglitazone was unimpaired when the 5′ end was shortened from –336 to –56. Basal activity was eventually reduced by 5′-deletion; for –56hInsLuc it was low but still detectable (2.4 ± 0.2% of –336hInsLuc). These results indicate that DNA sequences that allow repression by PPARγ may reside 3′ to –56. When the 3′ end of the human insulin gene promoter was shortened from +112 to +18 (construct –336/+18hInsLuc), the inhibition by PPARγ and rosiglitazone was fully preserved (Figure 5E). Bearing in mind the results of the 5′-deletion analysis, these data suggest that DNA sequences that are critical for repression by PPARγ to the human insulin gene may be located between –56 and +18. This conclusion was further supported by the lack of inhibition by PPARγ and rosiglitazone when the DNA sequences around the transcription start site of the human insulin gene promoter (sequences 3′ to –31) were replaced by a heterologous non-responsive minimal promoter (Figure 5F). The electrophoretic mobility shift assay showed that protein(s) of primary islet extracts bind in a mutation-sensitive way to the proximal sequence of the human insulin gene promoter (Figure 6). The protein complex was competed for by the consensus PPRE sequence (Desvergne and Wahli, 1999) but less so by a mutated PPRE sequence (Figure 6B), indicating that PPARγ is among the binding proteins.

Figure 5 Mapping of the segment of the human insulin gene promoter that mediates the responsiveness to peroxisome proliferator-activated receptor γ (PPARγ)/rosiglitazone. (A) The transcription factor Pax6 is known to activate the rat glucagon gene and to confer responsiveness to PPARγ. This figure shows the effect of Pax6 on promoter activity of the rat insulin I, rat glucagon and human insulin gene. Luciferase reporter genes under the control of the rat insulin I (rInsLuc), rat glucagon (GluLuc) or human insulin gene promoter (hInsLuc) were transfected into heterologous JEG cells with and without cotransfection of an expression vector encoding Pax6. Luciferase activity is expressed as percentage of the activity measured in the controls (no Pax6). Values are means ± SEM of three experiments, each done in duplicate. (B) Reduction of cellular Pax6 does not interfere with rosiglitazone-induced inhibition of insulin gene transcription. Upper panel, HIT cells were transiently transfected with three different small interference RNA (shown as RNAi 1–3, see *Methods*; 50 and 100 pmole per dish) against Pax6. Cells were harvested after 48 h and an immunoblot using a Pax6 antibody was performed. RNAi 1 and 2 decrease the content of Pax6 in HIT cells. Lower panel, HIT cells were transiently transfected with RNAi 1 (50 pmole per dish), the luciferase reporter gene under control of the humane insulin gene promoter and the expression vector for PPARγ. Cells were treated with rosiglitazone (30 μmol·L^{-1}) for 24 h or left untreated. Luciferase activity is expressed as percentage of the mean value measured in the control (without PPARγ cotransfection, without rosiglitazone). Values are means ± SEM of two independent experiments, each in triplicate; *$P < 0.05$. (C) Inhibition of insulin gene transcription by rosiglitazone depends on the DNA binding domain of PPARγ. A luciferase reporter gene under control of the human insulin gene promoter from –336 to +112 bp was transiently cotransfected into HIT cells with expression vectors for PPARγ wild type (1–475 PPARγ), PPARγ with extended carboxyl terminus by a nuclear localization signal (1–475N PPARγ), PPARγ lacking the AF-1 and the DNA binding domain and carrying at the carboxyl terminal a nuclear localization signal (175–475N PPARγ). Rosiglitazone (30 μmol·L^{-1}) was added 24 h before harvest. Luciferase activity is expressed as percentage of the mean value measured in the control (without PPARγ cotransfection, without rosiglitazone). Values are means ± SEM of two independent experiments, each in triplicate. *$P < 0.01$ versus PPARγ wild type. (D) 5′-Deletion analysis. Human insulin gene promoter-luciferase reporter genes with 5′ ends as indicated were transfected into HIT β-cells with and without cotransfection of pPPARγ and rosiglitazone treatment (30 μmol·L^{-1}). Luciferase activity is expressed as percentage of the activity measured in the respective untreated controls. Values are means ± SEM of three independent experiments, each in duplicate, *$P < 0.05$. (E) Effect of a 3′-deletion of the human insulin gene promoter from +112 to +18 on PPARγ/rosiglitazone responsiveness. The plasmids –336hInsLuc (–336/+112) or –336/+18hInsLuc were transfected into HIT β-cells with or without cotransfection of pPPARγ and rosiglitazone treatment (30 μmol·L^{-1}). Luciferase activity is expressed as percentage of the activity measured in the untreated –336hInsLuc controls. Values are means ± SEM of four independent experiments, each in duplicate, *$P < 0.05$. (F) The fragment of the human insulin gene promoter from –336 to –31 was placed in front of a heterologous nonresponsive promoter (P). The plasmids were transfected into HIT β-cells with and without cotransfection of pPPARγ and rosiglitazone treatment (30 μmol·L^{-1}). Values are means ± SEM of three independent experiments, each in duplicate.


A
Control
Pax6
Relative Luciferase Activity
16000
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GluLuc
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Pax6 RNAi 3
Control RNAi
Pax6
Control
PPARγ + Rosiglitazone
Relative Luciferase Activity
120
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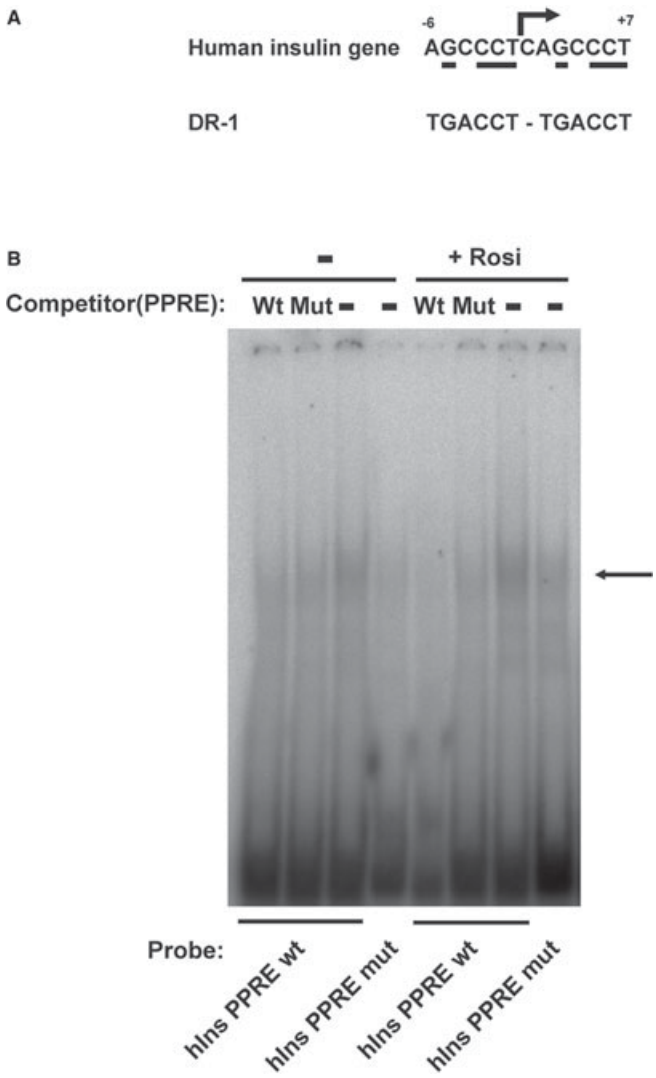

Figure 6 Protein binding to the proximal human insulin gene promoter around the transcription start site. (A) Sequence comparison between the proximal human insulin gene promoter around the transcription start site and a typical peroxisome proliferator-activated receptor γ (PPARγ) DNA binding site. PPARγ binds as a heterodimer with retinoid X receptor to response elements, which consist of a direct repeat of a hexamer half-site, spaced by one nucleotide (DR-1). The arrow indicates the transcription start site. The numbers give the first and last nucleotide relative to the transcriptional start site. The bases that match the consensus sequence are underlined. (B) Electrophoretic mobility shift assay. Islet extracts were incubated with radioactively labelled hIns PPARγ response element (PPRE) wt or hIns PPRE mut. For competition, PPRE consensus sequence (*Wt*) or its mutation (*Mut*) was used as indicated. The arrow indicates the PPARγ containing complex.

Discussion

As in adipose tissue, skeletal muscle and liver (Gervois *et al.*, 2007), PPARγ and thiazolidinediones also exert important glucose regulatory functions within pancreatic islets (Walter and Lübben, 2005; Wajchenberg, 2007). In α-cells, they inhibit glucagon gene transcription (Schinner *et al.*, 2002; Krätzner *et al.*, 2008), which may contribute to the thiazolidinediones' antidiabetic effect by reducing glucagon-dependent hepatic glucose production (Schinner *et al.*, 2002). In β-cells, PPARγ and thiazolidinediones activate the genes encoding GLUT2 (Kim *et al.*, 2000), glucokinase (Kim *et al.*, 2002) and ABCA1 cholesterol efflux transporter (Brunham *et al.*, 2007). These effects seem to allow thiazolidinediones to establish a more timely insulin secretion, as is indicated by the findings that 13 week treatment with rosiglitazone increases the ability of an oscillatory glucose infusion to programme high-frequency pulsatile insulin secretion in patients with type 2 diabetes mellitus (Juhl *et al.*, 2003), and that pioglitazone and rosiglitazone restore the first-phase insulin response to an intravenous glucose tolerance test in patients with impaired glucose tolerance and with frank type 2 diabetes mellitus (Ovalle and Bell, 2004). The present study now identifies the insulin gene as a new PPARγ target gene in pancreatic islet β-cells.

The early work on characterizing insulin gene transcription focused on the rat insulin I gene (Melloul *et al.*, 2002; Hay and Docherty, 2006). The early perception that human insulin promoter constructs would not function in transfected rodent cells proved to be unfounded (Melloul *et al.*, 2002; Hay and Docherty, 2006), and the human insulin promoter exhibited the expected pattern of activity in transgenic mice (Fromont-Racine *et al.*, 1990). Furthermore, it became apparent that rodent insulin promoters differ considerably from the human promoter, leading to the conclusion that extreme care should be taken when extrapolating rodent-based data to the human insulin gene (Melloul *et al.*, 2002; Hay and Docherty, 2006). In order to avoid these problems, the human insulin gene promoter was used in the present study.

In this study, we found human insulin gene transcription to be inhibited by PPARγ and thiazolidinediones in HIT β-cells. Rosiglitazone inhibited human insulin promoter activity also in normal primary pancreatic islets as revealed by the use of islets from transgenic mice carrying a human insulin promoter-luciferase transgene, which has been described before (Oetjen *et al.*, 2003a; 2007). The effect of thiazolidiones on insulin gene expression is still a matter for debate: a recent study showed no effect of 1 μmol·L^{-1} of rosiglitazone on insulin gene expression in human pancreatic islets after chronic fatty acid exposure (Vandewalle *et al.*, 2008). However, consistent with the present study, troglitazone reduced preproinsulin mRNA levels in primary islets (Bollheimer *et al.*, 2003), and PPARγ and pioglitazone decreased proinsulin biosynthesis as indicated by [^{3}H]leucine labelling in MIN6 cells (Nakamichi *et al.*, 2003).

The fact that rosiglitazone inhibited insulin gene transcription over the same range of concentrations as it stimulated, through PPARγ, the expression of a reporter gene directed by a well-defined PPARγ DNA binding site suggests that inhibition of insulin gene transcription may accompany other PPARγ-mediated effects. All findings, taken together, support the conclusion that inhibition of insulin gene transcription may be relevant both physiologically and therapeutically for the action of thiazolidinediones. Consistent with this view, the insulin content was significantly elevated in pancreatic islets from mice in which the expression of the PPARγ gene in β-cells was eliminated (βγKOmice), in spite of normal glucose and insulin levels in their sera (Rosen *et al.*, 2003).

PPARγ is well known to bind as a heterodimer with RXR to response elements in target genes and to activate transcription (Desvergne and Wahli, 1999; Natali and Ferranini, 2006). With the human insulin gene promoter, the present study provides another example that PPARγ can also inhibit gene transcription (Ricote *et al.*, 1998; Schinner *et al et al.*, 2002; Krätzner *et al.*, 2008). As has been first established for the glucocorticoid receptor (Reichardt *et al.*, 1998; Tuckermann *et al.*, 1999), nuclear receptors including PPARγ may mediate transrepression in a DNA binding-independent manner (Li *et al.*, 2000). Thus, the PPARγ/RXR heterodimer binds through protein–protein interaction to the transcription factor Pax6 and thereby represses the activity of this transcriptional activator, leading to inhibition of rat glucagon gene transcription in α-cells (Schinner *et al.*, 2002; Krätzner *et al.*, 2008). Pax6 is expressed also in β-cells (St-Onge *et al.*, 1997) and binds to the rat insulin I gene promoter at about –310 (Knepel *et al.*, 1991; Sander *et al.*, 1997). This Pax6 binding site may mediate the activation of the rat insulin I gene promoter when Pax6 is expressed in the heterologous JEG cells (this study). The rat glucagon gene promoter was more markedly activated by Pax6 (this study), most likely because it contains two Pax6 binding sites that interact synergistically (Knepel *et al.*, 1991; 1990; Sander *et al.*, 1997; Beimesche *et al.*, 1999; Grzeskowiak *et al.*, 2000). Nuclear protein binding to the rat insulin I gene at about –310 (Knepel *et al.*, 1991), later to be identified as Pax6 (Sander *et al.*, 1997; (Beimesche *et al.*, 1999), is not conserved in the human insulin gene (Yildiz *et al.*, 1996). The human promoter may also not contain other Pax6 binding sites, as is indicated by the low Pax6-induced activation of the human insulin gene promoter when compared with that of the glucagon gene promoter (this study). The finding that the inhibitory effect of rosiglitazone depends on the DNA-binding domain of PPARγ indicates that PPAR might directly bind to the promoter (this study). Mapping experiments revealed that DNA sequences between –56 and +18 may confer PPARγ responsiveness to the human insulin gene promoter. Importantly, this fragment contains a sequence motif from –6 to +7 with similarity to a typical PPARγ response element (Figure 6A), which consists of a direct repeat of hexamer half-sites, TGACCT, spaced by one nucleotide (DR-1) (Desvergne and Wahli, 1999). This raises the possibility that PPARγ may inhibit human insulin promoter activity by competing for binding to the region around the transcription start site with the general transcription machinery. Indeed, a complex of primary islet proteins bound to the sequence of the proximal promoter element, and this binding was competed for by additional incubation with a typical PPRE sequence but not to the same extent with the mutated PPRE sequence. Thus, although the mode of inhibition remains to be verified, the present study suggests that the mechanism through which PPARγ inhibits human insulin gene transcription differs from the one at the rat glucagon gene and may target the proximal insulin promoter around the transcription start site.

Thiazolidinediones efficiently improve glycaemic control and may reduce the risk of death from any cause in type 2 diabetic patients, with significant side effects (Dormandy *et al.*, 2005; Lago *et al.*, 2007; Lincoff *et al.*, 2007; Nissen and Wolski, 2007; Singh *et al.*, 2007). Inhibition of insulin gene transcription appears to be detrimental to these patients, who already suffer from insulin deficiency, and to be in opposition to several lines of evidence suggesting that treatment with thiazolidinediones may preserve and even improve β-cell function (Walter and Lübben, 2005; Wajchenberg, 2007). However, repression by thiazolidinediones of the insulin gene may in fact be in keeping with known thiazolidinedione effects and even be beneficial for the patients. Consequently, inhibition of insulin gene transcription is in line with and may contribute to the reductions in fasting plasma insulin levels found in most clinical trials performed with rosiglitazone, pioglitazone or troglitazone in patients with type 2 diabetes mellitus (Walter and Lübben, 2005; Wajchenberg, 2007). These reductions have so far been attributed solely to the increase in insulin sensitivity and the decrease in blood glucose concentrations (Walter and Lübben, 2005; Wajchenberg, 2007). Furthermore, insulin gene repression may protect β-cells from some of the damage induced by chronic overstimulation. Firm genetic and other lines of evidence indicate that β-cells may be especially sensitive to adverse effects of perturbed endoplasmic reticulum function (Wajchenberg, 2007). Glucose-mediated stimulation of proinsulin biosynthesis promotes some endoplasmic reticulum stress because it imposes a load on the folding and protein processing machinery of the endoplasmic reticulum. In hypersecretory states, such as insulin resistance, glucose intolerance and frank diabetes mellitus, proinsulin and other client proteins are translocated into the lumen of the endoplasmic reticulum in excess of the folding capacity of the organelle, inducing a state of severe endoplasmic reticulum stress with induction of programmed cell death. This mechanism may well contribute to the decline in β-cell function and mass in the insulin-resistant patient (Wajchenberg, 2007). Inhibition of insulin gene transcription by thiazolidinediones, we presume, decreases protein flux through the endoplasmic reticulum of the β-cell and thus reduces endoplasmic reticulum stress. Evidence of endoplasmic reticulum stress such as the accumulation of electron-dense material in the endoplasmic reticulum and distorted organelle morphology in diabetic islets are indeed reduced by thiazolidinedione treatment (Diani *et al.*, 1984; 2004; Walter and Lübben, 2005; Wajchenberg, 2007). Similarly, induction of β-cell rest by K_{ATP}-channel openers has been shown to improve β-cell function (Ritzel *et al.*, 2004). In conclusion, in this study we show an inhibition of insulin gene transcription by thiazolidinediones. Our results suggest that this inhibition is mediated by the proximal promoter region.

Acknowledgements

This work was supported by the *Deutsche Forschungsgemeinschaft* (SFB402/A3, GRK 335).

Statement of conflicts of interest

The authors state no conflict of interest.

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PUBLIKATION 4

Schinner S, Scherbaum WA, Bornstein SR, Barthel A.

Molecular mechanisms of insulin resistance.

Diabet. Med. 22(6):674-82, (2005).

DM

Molecular mechanisms of insulin resistance

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Accepted 24 September 2004

Abstract

Currently, we observe an epidemic expansion of diabetes mellitus. In subjects with Type 2 diabetes the resistance of fat, muscle and liver to insulin is the central pathophysiological event in the development of this disease. Genetic and environmental factors play a major role in this process, although the precise pathogenesis of insulin resistance and Type 2 diabetes is still largely unknown. However, recent studies have contributed to a deeper understanding of the molecular mechanisms underlying this process. In this review we therefore summarize the current developments in understanding the pathophysiological process of insulin resistance and Type 2 diabetes. Among the many molecules involved in the intracellular processing of the signal provided by insulin, insulin receptor substrate (IRS)-2, the protein kinase B (PKB)-β isoform and the forkhead transcription factor Foxo1a (FKHR) are of particular interest in this context as recent data have provided strong evidence that dysfunction of these proteins results in insulin resistance *in-vivo*. Furthermore, we have now increasing evidence that the adipose tissue not only produces free fatty acids that contribute to insulin resistance, but also acts as a relevant endocrine organ producing mediators (adipokines) that can modulate insulin signalling. The identification of the molecular pathophysiological mechanisms of insulin resistance and Type 2 diabetes is essential for the development of novel and more effective therapies to better treat our patients with insulin resistance and Type 2 diabetes.

Diabet. Med. 22, 674–682 (2005)

Keywords adipokines, diabetes mellitus Type 2, insulin resistance, PI 3-kinase, protein kinase B

Abbreviations CAP, Cbl associated protein; Cbl, casitas B-lineage lymphoma; G6Pase, glucose-6-phosphatase catalytic subunit; GLUT-4, glucose transporter 4; GS, glycogen synthase; GSK-3, glykogen synthase kinase-3; FFA, free fatty acids; FKHR, forkhead in rhabdomyosarcoma; Foxo, forkhead box protein; HK, hexokinase II; HNF, hepatocyte nuclear factor; IR, insulin receptor; IRS, insulin receptor substrate; MODY, maturity onset of diabetes in the young; PDH, pyruvate dehydroxygenase; PDK, phosphoinositide-dependent protein kinase; PEPCK, phosphoenolpyruvate carboxykinase; PFK, phosphofructokinase; PGC-1, peroxysome-proliferator activated-receptor γ coactivator-1; PIP2, phosphatidylinositol-3,4-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PI 3-K, phospho-inositide 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PPARγ, peroxysome-proliferator activated-receptor γ; PTEN, phosphatase and tensin homologue; PTP, protein tyrosine phosphatase; SH, Src homology; SHIP2, SH2-containing inositol phosphatase; src; sarcoma; TNFα, tumour necrosis factor-α; TZD, thiazolidinediones

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Introduction

Diabetes mellitus is the most common endocrine disorder, currently affecting over 170 million people world-wide and prospectively over 365 million in the year 2030 [1]. More than 90% of the diabetic patients suffer from Type 2 diabetes mellitus. Besides β-cell failure, the major pathophysiological event contributing to the development of Type 2 diabetes mellitus is resistance of target tissues to insulin. Insulin lowers blood glucose levels by facilitating glucose uptake mainly into skeletal muscle and fat tissue and by inhibiting endogenous glucose production by the liver. In insulin resistant states, these organs do not properly respond to insulin, thereby causing hyperglycaemia and a reactive increase in insulin secretion by the pancreatic β-cells. The elevated insulin levels can compensate for the poor insulin response only for a limited time, but on the other hand impair insulin resistance [2]. This vicious circle finally leads to disturbance of the fragile balance between β-cell function and peripheral insulin resistance and finally results in the clinical manifestation of Type 2 diabetes.

Development of Type 2 diabetes is a multistep process with strong genetic and environmental influences. Although the precise pathogenesis and the pathophysiological sequence resulting in insulin resistance is still largely unknown, recent studies have contributed to a deeper understanding of the underlying molecular mechanisms. In addition to classical biochemical *in-vitro* studies, the use of gene targeting approaches in mice and the analysis of naturally occurring mutations in animal models and insulin resistant patients has shed some light on the molecular dysregulations that can contribute to insulin resistance and Type 2 diabetes. The detailed understanding of these basic pathophysiological mechanisms is critical for the development of novel therapeutic strategies to treat diabetes.

Based on the current literature available from the public databases (PubMed; http://www.ncbi.nlm.nih.gov/) we will therefore summarize the recent progress contributing to our understanding of the molecular basis underlying this disease.

Insulin signalling

The insulin receptor consists of extracellular ligand binding and intracellular tyrosine kinase domains. Binding of insulin to the extracellular portion of the receptor activates its kinase activity resulting in autophosphorylation of specific intracellular tyrosine residues. This autophosphorylation step enables a variety of scaffolding proteins including insulin receptor substrate (IRS) proteins, Cbl (casitas B-lineage lymphoma) or Cbl associated protein (CAP) to bind to intracellular receptor sites and to become phosphorylated [3–7].

IRS-proteins

Based on results from specific knockout models, the most important IRS proteins in the regulation of carbohydrate metabolism appear to be IRS-1 and -2 [8]. In humans, rare mutations of the IRS-1 protein are associated with insulin resistance [9] and disruption of the IRS-1 gene in mice results in insulin resistance mainly of muscle and fat [10]. Interestingly, IRS-2 knockout mice not only show insulin resistance of muscle, fat and liver, but also develop manifest diabetes as a result of β-cell failure [11]. Therefore, it is tempting to speculate that dysfunction of IRS-2 and its downstream targets might represent a common feature of both peripheral insulin resistance and β-cell failure. Furthermore, there are data linking IRS dysfunction in skeletal muscle to adipocyte biology and lipotoxicity. For example, circulating free fatty acids (FFA) and the adipokine TNFα may increase serine phosphorylation of IRS proteins, thereby causing impaired insulin signal transduction [8]. In addition, prolonged stimulation with insulin—as commonly found in diabetic patients with hyperinsulinaemia—may result in regulated degradation of IRS protein [12].

IRS proteins are not catalytically active themselves, but harbour several interaction domains to recruit other signalling molecules like phospho-inositide-3-kinase (PI 3-kinase) to form large protein complexes at the plasma membrane.

PI 3-kinase/PKB-signalling

Downstream of IRS-proteins, the phospho-inositide-3-kinase (PI 3-kinase) is a central mediator of the effects of insulin. PI 3-kinase isoforms can be subdivided into three classes. Class Ia PI 3-kinases are thought to be the major effector of insulin signalling and activate PKB by the generation of PIP2 and PIP3. Class Ib is a G-protein-regulated kinase. Class II PI 3-kinases can be activated by insulin, but are unable to generate PIP2 and PIP3, therefore it is unlikely that they mediate common insulin-effects. Also, class III PI 3-kinases appear not to play a role in insulin signalling.

Binding of PI 3-kinase to phosphorylated sites in IRS proteins leads to activation of PI 3-kinase. The activated PI 3-kinase generates 3′-phosphoinositides [phosphatidyl-inositol-3,4-bisphosphate (PIP2) and phosphatidyl-inositol-3,4,5-trisphosphate (PIP3)] [13]. PIP2 and PIP3 bind to the phosphoinositide-dependent kinase 1 (PDK1). Therefore, these two phospholipids may attract PDK1 and the putative PDK2 to the plasma membrane. Known substrates of the PDKs are the proteinkinase B (PKB) and also atypical forms of protein kinase C (PKC) [14].

PKB (also called Akt) is a serine/threonine kinase with high homology to PKA and PKC, hence the name. So far, three different isoforms of PKB have been identified in mammals (α, β, γ). PKB is conserved from invertebrates to mammals and shows high homology among different species emphasizing its pivotal role in development, cell proliferation and metabolism [15]. PKB mediates the effects of insulin on glucose transport, glycogen synthesis, protein synthesis, lipogenesis and suppression of hepatic gluconeogenesis. PKB regulates both, glucose uptake via facilitated glucose transporters (GLUTs) and intracellular glucose metabolism in insulin sensitive tissues such as skeletal muscle [13]. Under non-stimulated conditions, PKB is

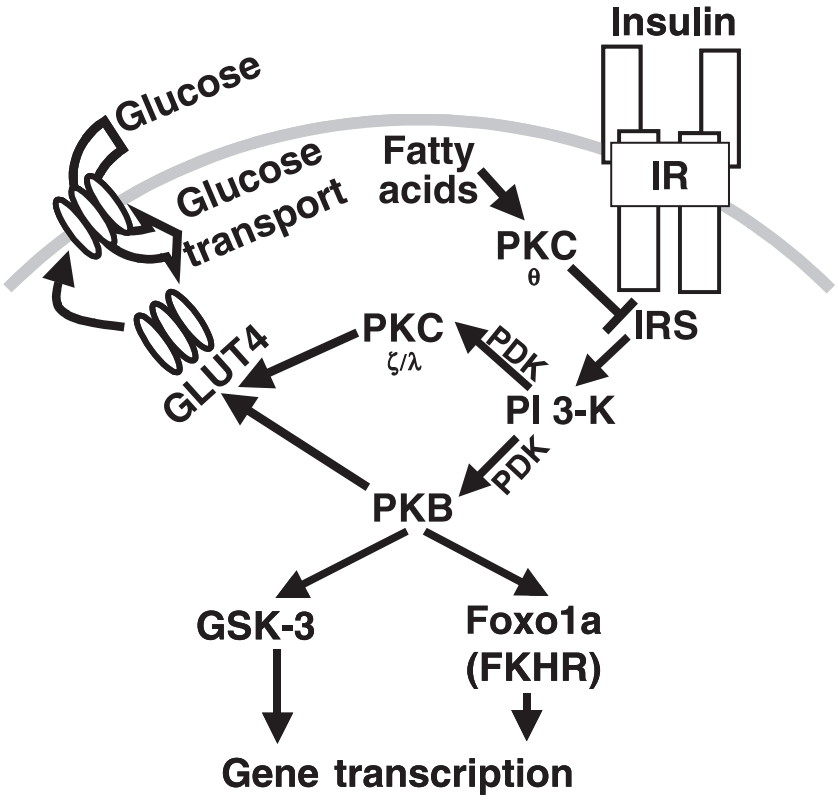

Figure 1 Schematic representation of insulin signalling as detailed in the text. Binding of insulin to the IR activates PI 3-K through IRS. Downstream of PI 3-K, the PDK mediates activation of PKB. Activated PKB can regulate transcription of target genes via GSK-3 or Foxo1a. Also, PKB regulates glucose uptake by recruiting GLUT-4 to the plasma membrane. Downstream of the IR the signalling cascade branches with PKCζ/λ as additional downstream effectors of insulin. Modulation of PKCθ activity by fatty acids may impair signalling through IRS/PI 3 kinase and GLUT4-dependent glucose transport. IR, insulin receptor; IRS, insulin receptor substrate; PDK, phosphoinositide-dependent kinase; PI 3-K, phosphatidylinositol 3-kinase; PKB, protein-kinase B; PKC, protein-kinase C; GSK-3, glykogen-synthase kinase-3; GLUT-4, glucose transporter 4; Foxo, forkhead box protein; FKHR, forkhead in rhabdomyosarcoma.

located in the cytoplasm and stimulation with insulin results in translocation of PKB to the plasma membrane, where PKB may bind to PIP-2 and PIP-3 [15,16]. At the plasma membrane, PKB co-localizes with PDK and becomes activated by phosphorylation of its two principal regulatory sites, Thr308 and Ser473. Phosphorylation of both sites is essential for the activation of PKB. PDK1 is the kinase phosphorylating Thr308, while the mechanism of phosphorylation of the Ser473 residue remains controversial and the corresponding kinase PDK2 still needs to be identified [17]. Following activation, PKB detaches from the plasma membrane to affect metabolic processes such as glycogen synthesis and glucose transport. Parts of the activated PKB also translocate through the cytoplasm into the nucleus by an unknown mechanism to affect gene expression [15,18,19]. Substrates for a direct phosphorylation by PKB include the GSK-3 (glycogen synthase kinase-3) and members of the Foxo-family of transcription factors which are critically involved in the insulin-dependent regulation of glucose homeostasis. The insulin signalling mechanisms described in this review are summarized in Fig. 1.

Protein and lipid phosphatases

Termination of the insulin signal is critical for the maintenance of metabolic control. Signalling of the insulin receptor cascade is terminated by specific phosphatases. One of the key phosphatases in this context is the protein-tyrosine-phosphatase 1B (PTP1B). For example, mice lacking the PTP1B gene exhibit increased insulin sensitivity and fail to develop insulin resistance under a high-fat diet [20]. In addition, the inhibition of PTP1B-activity by systemic application of antisense oligonucleotides specific for PTP1B improved insulin sensitivity and glycaemic control in diabetic mice [21]. Other phosphatases relevant for the termination of insulin signalling include PTEN (phosphatase and tensin homologue) which inactivates the lipid products of the PI 3-kinase and also SHIP2, an inositol 5′-phosphatase. For example, knockout mice with a homozygous deletion of the SHIP2 gene display increased insulin sensitivity and hypoglycaemia because of an inhibition of hepatic glucose production [22]. Also, antisense oligonucleotides specific for PTEN drastically improved glycaemic control in diabetic ob/ob- and db/db-mice [23]. Therefore, PTP1B, SHIP2 and PTEN can be regarded as potential therapeutic targets for the treatment of Type 2 diabetes.

Glucose metabolism

Effectors involved in insulin-dependent glucose disposal into fat and muscle

About 75% of insulin-dependent postprandial glucose disposal occurs into the skeletal muscle [24]. Regulation of insulin-sensitive glucose uptake by the PI 3-kinase/PKB pathway has been subject to intensive research over the past years. Activation of PI 3-kinase results in translocation of GLUT4 into the plasma membrane with consecutive increase of glucose transport into

muscle and fat. Accordingly, inhibition of PI 3-kinase with pharmacological inhibitors like wortmannin blunted the insulin-stimulated glucose uptake [25,26]. In addition, over expression studies with several PKB-constructs demonstrated an increase of GLUT4-mediated glucose uptake [16,27,28]. Furthermore, so called atypical PKC isoforms, namely PKCλ and -ζ, increased GLUT4-dependent glucose uptake when activated by PI 3-kinase/PDK1. Conversely, overexpression of dominant-negative mutants of these PKC forms caused an inhibition of insulin-stimulated GLUT4 translocation and glucose uptake [14,29], therefore suggesting an important role for atypical PKCs in mediating insulin-stimulated glucose uptake in muscle and fat. Recently, an alternative PI 3-kinase independent mechanism to enhance GLUT4 translocation and glucose uptake was described. According to this model, binding of insulin to its receptor finally activates the small G-protein TC10 via the scaffolding protein CAP (Cbl-associated protein) resulting in GLUT4 translocation and enhanced glucose uptake [3,4,30].

The potential role of PKB in the pathogenesis of insulin resistance has been of central interest during the recent years. However, the use of biochemical *in-vitro* assays to study the function of PKB in normal and diabetic humans has led to controversial observations in the past. For example, the function of PKB was found to be impaired in muscle and adipocytes from diabetic patients [31,32]. In a different study, no impairment of PKB activity in muscles from diabetic patients despite reduced PI 3-kinase activity could be observed [33]. This might indicate that only activation of a small fraction of cellular PI 3-kinase is required to induce full activation of PKB. Interestingly, an *in-vitro* study in human skeletal muscle strips found all three PKB isoforms in muscles from lean control subjects to be activated by insulin, whereas only the PKBα (Akt1)-isoform was activated in the muscles from obese, insulin-resistant patients [34].

Recent data from PKB knockout animal models offer a clearer answer to the question whether PKB is required for normal glucose homeostasis. While disruption of PKBα (Akt1) in mice did not cause any significant perturbations in metabolism, mice with a knock out of the PKBβ (Akt2) isoform show insulin resistance ending up with a phenotype closely resembling Type 2 diabetes in humans [35,36]. Initially, these animals have impaired insulin-mediated glucose disposal and a very prominent lack of suppression of hepatic glucose production in response to insulin. Finally, they progress to develop a relative β-cell dysfunction and consecutively manifest diabetes [36]. Consistently, recent studies on inherited insulin post-receptor mutations in humans detected a missense mutation in the kinase domain of PKBβ (AKT2) in a family of severely insulin resistant patients. This mutation co-segregated with the insulin-resistant phenotype over three generations within the family. The mutant PKB was unable to phosphorylate downstream targets and to mediate inhibition of phosphoenolpyruvate carboxykinase (PEPCK), a gluconeogenic key enzyme [37]. Therefore, these data suggest an essential role of PKBβ in maintaining glucose homeostasis.

Recently, the PPARγ co-activator-1 (PGC-1) has been recognized as playing a major role in glucose homeostasis of the organism. Work mainly by Spiegelman's group demonstrated a crucial role of PGC-1 in the regulation of GLUT4 gene expression in muscle cells [38]. Furthermore, PGC-1α may play a role in the regulation of genes involved in the process of oxidative phosphorylation which commonly show reduced expression in the muscle of diabetic patients [39]. However, regulation of hepatic glucose production is another major function of PGC-1 [40].

Hepatic glucose production

The fasting hyperglycaemia in patients with Type 2 diabetes is the clinical correlate of the increased glucose production by the liver because of insulin resistance. This is as a result of the lack of inhibition of the two key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and the glucose-6-phosphatase catalytic subunit (G6Pase). Insulin inhibits the expression of both of these enzymes at the transcriptional level [41] and it is widely accepted that this process is mediated via activation of PKB [42,43]. The promoters of both, the PEPCK- and G6Pase genes contain so called insulin-responsive elements (IRE) that are essential for the effect of insulin on the regulation of those genes. Data from *in-vitro* studies have shown that three Foxo-family transcription factors (Foxo1a, Foxo3a and Foxo4) are capable of binding to these structures [43–45] and that phosphorylation of Foxo-proteins by PKB results in transcriptional inactivation, nuclear export and consequently inhibition of target gene expression [44,46]. There is increasing evidence that Foxo-proteins are critically involved in the insulin-dependent regulation of gluconeogenic gene expression and insulin-resistance *in-vivo*. For example, the partial knockout of the Foxo1 gene in insulin-resistant mice resulted in reduced G6Pase-mRNA and insulin levels comparable with metabolically unaffected control animals [47]. Furthermore, transgenic animals with liver- and pancreatic β-cell specific expression of a constitutively active, non-insulin regulatable Foxo1 point mutant (Ser253Ala) have a diabetic phenotype [48,49]. Therefore, these results demonstrate a causal relationship between Foxo1-regulation by insulin and glycaemic control *in-vivo*. In addition, the PPARγ co-activator-1 (PGC-1), a factor integrating the effects of glucocorticoids and cAMP on gluconeogenic gene expression in the liver [40,50,51] is also regulated by PKB and Foxo1 [52], therefore providing additional evidence that PKB and Foxo1 are critical parts of the network integrating hepatic glucose production.

In addition to the Foxo-transcription factors, members of the hepatocyte nuclear factor (HNF) family of transcription factors may be involved in the regulation of glucose metabolism by insulin. For example, HNF1 enhances the effect of insulin on the promoter of the G6Pase gene via interaction with an IRE [53]. In addition, consensus sequences for HNF3 and also HNF4 have been identified in the G6Pase promoter, although the functional implications with respect to the regulation

of the promoter by insulin are unclear [54]. Knockout mice homozygous for a null-mutation of HNF3 display a phenotype with a complex impairment of glucose metabolism including persistent hypoglycaemia [55]. Recently, there is increasing evidence for an involvement of HNF4 in the insulin-dependent regulation of hepatic gene expression. For example, HNF4 is involved in the PI 3-kinase/PKB-dependent stimulation of glucokinase gene expression by insulin, an important mechanism to increase glycolysis [56]. On the molecular level, HNF4 may directly interact with Foxo1, and Foxo1 may act as an inhibitor of HNF4. In this setting, insulin stimulates HNF4-transcriptional activity by sequestrating Foxo1 from HNF4 [57]. However, although genetic defects of some HNF-transcription factors (e.g. HNF1α, HNF4α) are the basis for some forms of maturity-onset of diabetes in the young (MODY), the role of HNF transcription factors in the pathogenesis of Type 2 diabetes remains unclear.

Currently, probably the best characterized substrate of PKB is the GSK-3 (glycogen synthase kinase-3), a critical enzyme regulating glycogen synthesis. There is abundant evidence that PKB-mediated inhibition of GSK-3 is the key mechanism through which insulin promotes glycogen synthesis. The major part of glucose taken up from the blood after insulin stimulation is stored as glycogen in skeletal muscle. Dysregulated glycogen synthesis is a critical feature in diabetes mellitus as glycogen synthesis rates in diabetic patients are approximately 50% lower than in healthy subjects [58]. Under basal conditions, GSK-3 is constitutively active and phosphorylation of glycogen synthase (GS) by GSK-3 inhibits glycogen synthesis. The phosphorylation of GSK-3 by PKB results in inhibition of this kinase. Furthermore, expression of a GSK-3 mutant that is insensitive to phosphorylation by PKB results in a reduction of insulin-mediated glycogen synthesis [59]. Impaired hepatic glycogen storage and glycogen synthase activity is a common finding in insulin resistance [60,61] and polymorphisms in the glycogen synthase gene have been described in insulin resistant patients. The most frequent mutations are the so-called *Xba*I-mutations and Met416Val within intron 14 and exon 10, respectively. Currently, there are conflicting data on the correlation of these polymorphisms with insulin resistance and Type 2 diabetes mellitus [62–66]. Table 1 summarizes mutations in the insulin signalling cascade identified in humans and the potential relationship to insulin resistance.

The role of the adipose tissue in insulin resistance

Currently, there is strong evidence that dysfunction of adipose tissue plays a crucial role in the development of insulin resistance and Type 2 diabetes. Both obesity and lipodystrophy lead to insulin resistance in muscle. As the latter can be reversed by transplanting adipose tissue, fat seems to influence glucose homeostasis through different mechanisms than just by taking up glucose itself [72]. In line with these findings, the organ specific disruption of the GLUT4-gene in mouse adipose tissue results in insulin resistance of muscle and liver [73].

Adipose tissue can modulate whole body glucose metabolism by regulating levels of circulating free fatty acids (FFA) and also by secreting adipokines, thereby acting as an endocrine organ. There is also clear evidence from clinical and biochemical studies that circulating FFA can impair insulin sensitivity in muscle. Randle and colleagues were the first to show that fatty acids inhibit glucose uptake in rat heart muscle *in-vitro* [74]. Accordingly, the levels of circulating FFA are inversly correlated with insulin sensitivity in humans [75]. Randle and collegues hypothesized that the crucial mechanism was increased fatty acid oxydation by the muscle. This would lead to a backward inhibition of intracellular glucose metabolism at the level of several limiting key enzymes like PDH (pyruvate dehydroxygenase), PFK (phosphofructokinase) and HK (hexokinase II) and result in increased intracellular glucose concentrations. However, recent studies performed *in-vivo* suggested that glucose uptake rather than intracellular glucose metabolism is the rate-limiting step for fatty acid induced insulin resistance in humans [58]. This indicates a mechanism in which accumulation of intracellular fatty acids or their metabolites results in an impairment of signalling through IRS/PI 3-kinase also potentially involving PKCθ finally resulting in a reduced translocation of GLUT 4 into the plasma membrane. Consistently, it has been shown that FFAs may increase serine phosphorylation of IRS proteins, thereby impairing insulin signal transduction [8].

Recently, several groups provided strong evidence to support the concept of adipose tissue as an endocrine organ producing

Table 1 Mutations in insulin signalling molecules that are reported to be associated with insulin resistance in humans

Signalling molecule	Characterization of the mutation	Reference
IR	Ala1134Thr: markedly deficient in insulin-stimulated autophosphorylation Arg1174Gln: mutation within the intracellular receptor β-subunit	[67,68]
IRS-1	Met614Val: association with insulin resistance. No conclusive proof of causality from functional *in-vitro* data	[9]
PI3-K	Met 326Ile: association with insulin resistance not clear from current data	[69,70]
PDK	No association found yet	
PKC	No association found yet	
PKB/Akt	Arg274His: dominant negative mutation within the kinase domain	[37]
GS	Different mutations described: Met416Val, Gln71His, and Xba-mutation; controversial data on the association with insulin resistance	[62–66]
GSK-3	No association described	[71]

adipokines which modulate glucose homeostasis [76]. Currently, those most intensly discussed are tumour necrosis factor-α (TNFα), leptin, adiponectin and resistin.

TNFα is a cytokine produced by adipocytes that may contribute to the development of insulin resistance in obesity. TNFα can impair insulin action on glucose metabolism and increase lipolysis. At a molecular level, TNFα increases serine phosphorylation of IRS-1 and down-regulates GLUT4 expression, thereby contributing to insulin resistance [76,77].

The role of leptin in regulating food intake and energy expenditure is well established. Humans with leptin deficiency or leptin receptor mutations are severely obese [78,79]. In addition, leptin appears to have direct effects on insulin sensitivity and a variety of physiological processes independent of its effects on the hypothalamus. For example, leptin reverses insulin resistance in mice with congenital lipodystrophy [80]. In addition, administration of leptin to patients with this disease can increase the body fat content and reverse insulin resistance [81].

The adipokine adiponectin has insulin-sensitizing effects as it enhances inhibition of hepatic glucose output as well as glucose uptake and utilization in fat and muscle. The expression of adiponectin is decreased in obese humans and mice [82]. Thus, in humans, adiponectin levels correlate with insulin sensitivity. In addition, mice deficient in adiponectin are insulin resistant [83] and conversly, the administration of adiponectin to obese and insulin resistant mice improved insulin sensitivity [84–86]. Consistent with the idea that adiponectin acts on the peripheral target tissues of insulin, the cloning and subsequent expression analysis of adiponectin receptors has revealed that the subtype 1 adiponectin receptor is predominantly expressed in skeletal muscle, whereas the subtype 2 is mainly expressed in the liver [87,88]. Furthermore, adiponectin appears to have central effects on the regulation of bodyweight. For example, intracerebroventricular administration of adiponectin in mice increased energy expenditure and decreased bodyweight. Interestingly, the sensitivity to adiponectin was increased in leptin-deficient ob/ob mice and reduced in Agouti mice, suggesting an involvement of the melanocortin pathway in central adiponectin effects [89].

Because of its insulin-antagonistic effects, the adipocytokine resistin has attracted a lot of research interest. This is mainly based on data obtained *in-vitro* and from some animal models. Resistin decreases insulin-dependent glucose transport *in-vitro* and increases fasting blood glucose concentrations and hepatic glucose production *in-vivo* [90–93]. The latter is reflected by reduced levels of mRNA of the two key gluconeogenic enzymes glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) in livers from resistin knockout mice. Interestingly, the activity of AMP-activated protein kinase (AMPK), a kinase that is known to be involved in the regulation G6Pase- and PEPCK gene expression is also significantly increased in resistin knockout mice. These data suggest that resistin may exert at least parts of its action by inhibiting AMPK, thereby increasing hepatic glucose production [94]. However, the physiological significance of resistin in humans is less clear [95].

The finding that thiazolidinediones (TZD) act as anti-diabetic drugs by improving insulin sensitivity has generated new hope for an effective therapeutic approach to treat insulin resistance. TZDs are ligands of the nuclear peroxysomal proliferator activating receptor γ (PPARγ) which is highly expressed in white adipose tissue. One of the striking effects of PPARγ ligands is to induce adipocyte differentiation [96]. When adipocytes undergo differentiation, they express more GLUT4 glucose transporters and generate less circulating FFAs. In addition, TZDs are thought to redistribute triglycerides from skeletal muscle and liver to adipose tissue. Furthermore, those adipocytes express less TNFα which may contribute to insulin resistance in skeletal muscle [77]. Probably the clearest observation linking PPARγ to whole body glucose metabolism was that dominant negative PPARγ mutations in the ligand-binding domain cause not only hypertension, but also severe insulin resistance and diabetes in humans [97]. Furthermore, a common PPARγ polymorphism (Pro12Ala) is statistically associated with a reduced risk of developing Type 2 diabetes [98]. Although the precise mode of action of TZDs is still not clear, it is likely that they exert parts of their effect on glucose metabolism via adipose tissue and skeletal muscle.

Conclusion

In this review, we have summarized current developments contributing to our understanding of insulin resistance and to the pathogenesis of Type 2 diabetes. Among the many molecules involved in the intracellular processing of the signal provided by insulin, IRS-2, the PKB-β-isoform and the transcription factor Foxo1a have attracted particular interest, because their dysfunction results in insulin resistance *in-vivo*. It is therefore tempting to speculate on a potential role of these signalling proteins in the development of the metabolic abberations observed in patients with Type 2 diabetes. However, Type 2 diabetes is a complex polygenic disease and most of the current data are based on monogenic approaches like knockout models or clinical observations of specific point mutations, thereby limiting our current pathophysiological understanding of the disease. To complete the puzzle, it will be necessary to apply other powerful strategies like genetic linkage analysis and positional cloning approaches to this problem.

We have now increasing evidence that adipose tissue not only produces free fatty acids that contribute to insulin resistance, but also acts as an endocrine organ. It secretes a cocktail of mediators (adipokines) that may adversely affect insulin signalling, extending our classical view of lipotoxicity. Among the best-studied adipokines is TNFα which has insulin-antagonistic properties, but also leptin and adiponectin, both of which may promote insulin sensitivity. This complex network of adipogenic factors modulates the response of tissues to insulin.

The identification of signalling defects and understanding of the complex relationship of the different factors modulating insulin sensitivity is an important prerequisite for the development of novel and more specific anti-diabetic compounds.

Competing interests

None declared.

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PUBLIKATION 5

George S, Rochford JJ, Wolfrum C, Gray SL, **Schinner S**, Wilson JC, Soos MA, Murgatroyd PM, Williams RM, Acerini CL, Dunger DB, Barford D, Umpleby MA, Wareham NJ, Davies HA, Schafer AJ, Stoffel M, O'Rahilly S, Barroso I.

A family with severe insulin resistance and diabetes due to a mutation in AKT2.

Science. 304(5675):1325-8, (2004).

ments. In any case, the questions remain: What kind of elements associated with these processes would have arrived relatively early in chordate evolution and then become practically frozen in birds and mammals? And what mechanisms would underlie this, allowing them to resist virtually all further change?

Note added in proof: We recently became aware of related observations made by Boffelli *et al*. (*37*).

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39. We thank the Genome Sequencing Consortia for the human, mouse, rat, dog, chicken, and other genome sequences we used in this analysis; W. Miller, M. Diekhans, A. Hinrichs, K. Rosenbloom, D. Thomas, and the members of the University of California Santa Cruz (UCSC) browser team for providing the genome alignments and other tracks of genome annotation available on the UCSC genome browser; M. Blanchette, S. Salama, T. Lowe, M. Ares, K. Pollard, and B. Cohen for helpful discussions; A. Siepel for the neutral substitution rate analysis involving chicken and chimp; K. Roskin for the calculation of the percent identity in ancestral repeat sites for 1-Mb windows; and S. Walton for help in preparing the manuscript. G.B., W.J.K., and D.H. were supported by National Human Genome Research Institute grant 1P41HG02371 and National Cancer Institute contract 22XS013A, and D.H. was additionally supported by the Howard Hughes Medical Institute. S.S., M.P., I.M., and J.S.M. were supported by the Australian Research Council and the Queensland State Government.

Supporting Online Material
www.sciencemag.org/cgi/content/full/1098119/DC1

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19 March 2004; accepted 27 April 2004
Published online 6 May 2004;
10.1126/science.1098119
Include this information when citing this paper.

A Family with Severe Insulin Resistance and Diabetes Due to a Mutation in *AKT2*

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Inherited defects in signaling pathways downstream of the insulin receptor have long been suggested to contribute to human type 2 diabetes mellitus. Here we describe a mutation in the gene encoding the protein kinase AKT2/PKBβ in a family that shows autosomal dominant inheritance of severe insulin resistance and diabetes mellitus. Expression of the mutant kinase in cultured cells disrupted insulin signaling to metabolic end points and inhibited the function of coexpressed, wild-type AKT. These findings demonstrate the central importance of AKT signaling to insulin sensitivity in humans.

Most forms of diabetes are likely to be polygenic in origin, although a number of monogenic forms are being recognized (*1*, *2*). Although rare, these monogenic examples offer insight into the function of the affected gene in humans as well as offering important clues to understanding more common forms.

We have been screening genomic DNA from 104 unrelated subjects with severe insulin resistance for mutations in genes that are implicated in insulin signaling. We identified a missense mutation in the serine/threonine kinase gene *AKT2* in one Caucasian proband. AKT2 (also known as PKBβ) is highly expressed in insulin-sensitive tissues and is activated in response to growth factors and related stimuli (*3*, *4*), a process that requires its phosphorylation by the phosphoinositide-3 phosphate-dependent kinase activities designated PDK1 and PDK2 (*3*). The proband (Fig. 1D, iii/1) is a nonobese 34-year-old female who developed diabetes mellitus at 30 years of age. The proband, her nonobese mother, her maternal grandmother, and a maternal uncle were all heterozygous for a G-to-A substitution predicted to result in an R-to-H substitution at amino acid 274 (Fig. 1, A and B) (*5*). All were markedly hyperinsulinemic (table S1), and the mother and maternal grandmother developed diabetes mellitus in their late thirties. Three other first-degree relatives available for study were all clinically normal, with normal fasting glucose and insulin, and were homozygous for the wild-type AKT2 sequence (Fig. 1D and table S1). This mutation was not found in the genomic DNA of 1500 Caucasian control subjects from the United Kingdom.

R^{274} forms part of an RD sequence motif within the catalytic loop of the AKT2 kinase

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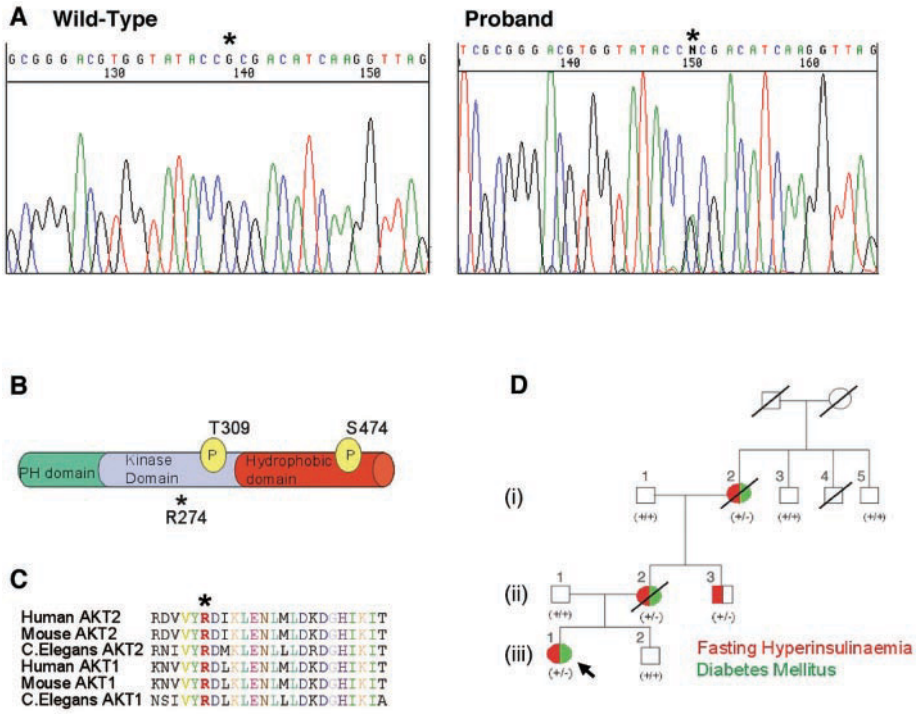

Fig. 1. Detection of a nonconservative heterozygous mutation in *AKT2* that cosegregates with severe insulin resistance. (**A**) Direct sequencing of genomic DNA from the proband (right) and a control subject (left). Asterisks indicate the heterozygous G-to-A substitution that produces H^{274}. (**B**) The location of R^{274} in relation to known functional domains and phosphorylation sites required for activation of AKT2. (**C**) R^{274} (in red and marked with an asterisk) is highly conserved across different AKT isoforms and diverse species. (**D**) A family pedigree demonstrating cosegregation of clinical phenotype (table S1) with the $R^{274}H$ mutation. All family members heterozygous for the mutation (+/−) are hyperinsulinemic, and three out of four have diabetes mellitus. All wild-type subjects (+/+) are normoinsulinemic and nondiabetic. Red denotes fasting hyperinsulinemia; green denotes diabetes mellitus.

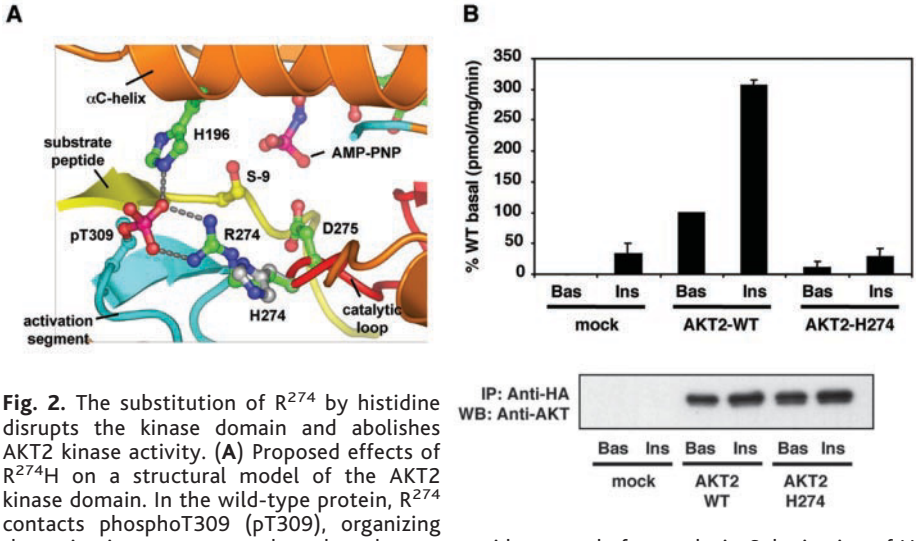

Fig. 2. The substitution of R^{274} by histidine disrupts the kinase domain and abolishes AKT2 kinase activity. (**A**) Proposed effects of $R^{274}H$ on a structural model of the AKT2 kinase domain. In the wild-type protein, R^{274} contacts phosphoT309 (pT309), organizing the activation segment to place the substrate peptide correctly for catalysis. Substitution of H for R^{274} is predicted to disrupt the conformation of both the activation segment and the catalytic loop. (**B**) Hemagglutinin-tagged HA-AKT2 and HA-AKT2H274 were immunoprecipitated from lysates of appropriately transfected CHO-T cells. Top: Enzyme activity was determined by an in vitro kinase assay. Mock-transfected cells received empty vector only. Serum-starved cells in the basal state were treated without (Bas) or with (Ins) 100 nM insulin for 10 min before lysis. Data are mean ± SEM of four experiments. Crosstide was used as the substrate. WT, wild-type. Bottom: Duplicate immunoprecipitates were also immunoblotted with an antibody to AKT2, to demonstrate equal expression of the wild-type and mutant proteins. IP, immunoprecipitate; WB, Western blot.

domain that is invariant in AKT isoforms in all species and is also highly conserved within the protein kinase family (Fig. 1C) (*6*). The RD motif includes the invariant D residue (D^{275} of AKT2) that performs an essential catalytic function in all protein kinases.

R^{274} is positioned in the core of the catalytic domain, forming critical hydrogen bonds with the phosphate moiety of phosphoT309 in the activation segment permitting correct positioning of the substrate peptide relative to the catalytic base and adenosine triphosphate (ATP) (*7*). A model of the mutant protein AKT2H274 (Fig. 2A) indicates that this mutation would disrupt the conformation of both the activation segment and the catalytic loop, misaligning the substrate peptide relative to catalytic residues and ATP and hence ablating catalytic activity. Consistent with these predictions, unlike the wild-type AKT2, AKT2H274 was unable to phosphorylate a peptide substrate based on the AKT target sequence of glycogen synthase kinase 3 in an in vitro kinase assay (Fig. 2B).

To examine the effect of the $R^{274}H$ mutation on the signaling ability of AKT2, we studied the regulation of the FOXA2 transcription factor, a substrate of AKT2 (*8*). FOXA2 activity is inhibited by phosphorylation, which leads to its exclusion from the nucleus. Treatment of HepG2 human liver cells with insulin induced the translocation of endogenous FOXA2 from the nucleus to the cytosol (Fig. 3A). Overexpression of wild-type AKT2 mimicked this effect (Fig. 3A). In contrast, in cells transfected with mutant AKT2H274, FOXA2 remained entirely nuclear (Fig. 3A). Consistent with this, although cotransfected wild-type AKT2 ablated FOXA2-mediated transcription from the phosphoenolpyruvate carboxykinase (PCK1) promoter in HepG2 cells, AKT2H274 had no effect (Fig. 3B). AKT2H274 also prevented wild-type AKT2 from inhibiting FOXA2-driven transcription from the PCK1 promoter, in a dose-dependent manner, when both AKT2 proteins were coexpressed (Fig. 3C). In the same assay, AKT2H274 also inhibited the effect of wild-type AKT1, which also contributes substantially to AKT activity in the liver (Fig. 3D) (*9*).

AKT overexpression in preadipocytes augments differentiation to adipocytes (*10*). We therefore examined the effect of AKT2H274 on adipocyte differentiation. 3T3-L1 mouse preadipocytes overexpressing wild-type AKT2 showed increased accumulation of lipid during adipogenesis (Fig. 3E), whereas cells overexpressing the mutant AKT2H274 showed markedly decreased lipid accumulation. The fatty acid transport protein aP2 is a well-defined marker of adipogenesis (*11*). Expression of aP2 was augmented by expression of wild-type AKT2 but reduced by expression of AKT2H274 (Fig. 3F). Thus,

AKT2H274 may also exert dominant-negative effects over endogenous AKT proteins during adipocyte differentiation.

To allow closer examination of the in vivo consequences of the AKT2H274 mutation, the proband underwent a two-step hyperinsulinemic/euglycemic clamp. This revealed extreme insulin resistance (fig. S1), with the glucose infusion rate remaining very low even in the second step, despite an insulin concentration of 7346 pmol/l. Examination of the effects of insulin on hepatic glucose production and peripheral glucose use revealed severe insulin resistance in this subject in both the liver and peripheral tissues (supporting online text).

Analysis of the proband's body composition revealed a –35% difference in total body fat compared to that predicted for her height and weight, consistent with both the ability of AKT2H274 to impair adipogenesis and the recently reported observation that *AKT2* knockout mice develop lipoatrophy as they age (*12*).

This family provides an example of a monogenic inherited defect in post-receptor insulin signaling that leads to human insulin resistance and diabetes mellitus. Genetic variants in *IRS1* and *PIK3R1* have been previously reported in subjects with insulin resistance and/or type 2 diabetes mellitus, but they have not clearly segregated with insulin resistance in a pedigree or seriously disrupted signal transduction (*13*, *14*). Dominant-negative mutations in peroxisome proliferator-activated receptor gamma (PPARγ) are associated with autosomal, dominant, severe insulin resistance and diabetes mellitus. However, the mechanisms whereby they result in insulin resistance are unclear and are unlikely to be due to simple impairment of insulin signal transduction (*15*).

AKT2$^{-/-}$ mice show resistance to insulin's effects on glucose metabolism in muscle and liver, and a subset of these mice go on to develop frank diabetes (*12*, *16*). In view of the abnormal fat distribution in our proband, it is of interest that atrophy of adipose tissue with age has been described in one strain of these mice (*12*, *16*). *AKT2*$^{-/+}$ mice show little metabolic phenotype, and even in (−/−) animals, the degree of insulin resistance is only moderate. This contrasts with the extreme hyperinsulinemia and insulin resistance seen in humans heterozygous for the AKT2H274 mutation. This may result from this mutant inhibiting other coexpressed AKT isoforms in a dominant-negative manner or from interference with other functions of upstream kinases such as PDK1.

Apart from lipodystrophy, there are no other overt structural or functional alterations in AKT2-expressing tissues from patients with this mutation. This suggests that, at least in those tissues where AKT2 is highly expressed, insulin signal transduction represents the major role for AKT. Frank diabetes mellitus developed in three of the four human carriers of the AKT2 mutation. In the fourth, a middle-aged male, marked hyperinsulin-

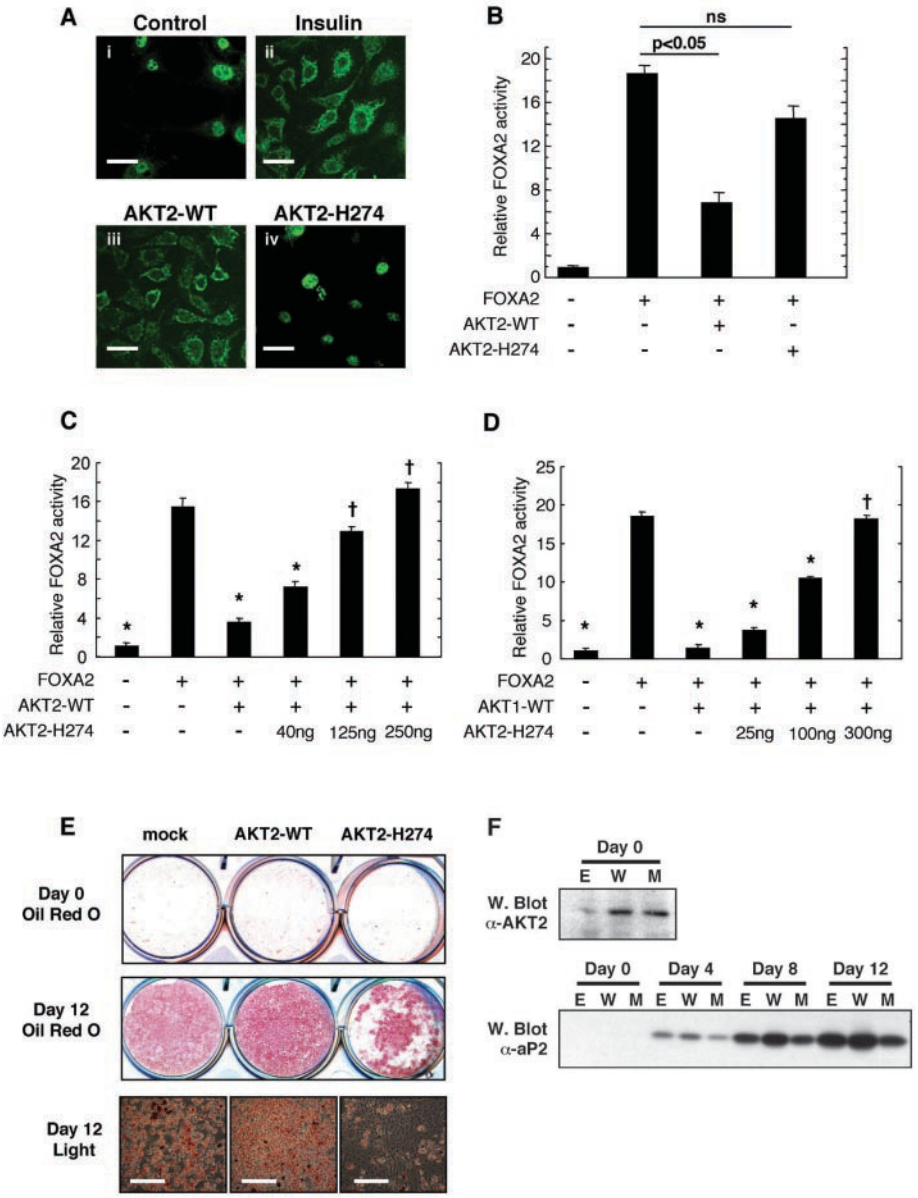

Fig. 3. Functional properties of wild-type AKT2 and AKT2H274 in cultured human liver and rodent fat cells. (**A**) HepG2 cells were treated in the (i) absence or (ii) presence of 50 nM insulin, then fixed and probed with antibodies to FOXA2 to determine intracellular localization (*8*). The same assay was performed with cells transfected with (iii) wild-type AKT2 or (iv) AKT2H274. Scale bars, 40 μm. (**B**) Luciferase activity was determined in extracts of HepG2 cells transfected with the phosphoenolpyruvate carboxykinase reporter construct, pPCK1, with or without FOXA2 and in the absence or presence of wild-type AKT2 or AKT2H274. ns, no significant difference. (**C** and **D**) Alternatively, HepG2 cells were transfected with the pPCK1 reporter construct with or without FOXA2 and either (C) wild-type AKT2 (125 ng) or (D) wild-type AKT1 (100 ng). In each case, AKT2H274 was cotransfected in increasing quantities as indicated. *, a significant difference from activity in cells transfected with FOXA2 alone ($P < 0.05$); †, no significant difference in this comparison. In all cases, data are mean ± SD of four experiments. All data were normalized to coexpressed β-galactosidase activity. (**E**) 3T3-L1 preadipocytes were stably transfected with empty vector (mock), wild-type AKT2, or AKT2H274. Top: Two-day post-confluent cells (Day 0) or cells induced to differentiate for 12 days were fixed and stained with oil red O to assess lipid accumulation. Bottom: Images of day 12 differentiated cells were obtained by light microscopy; scale bars, 200 μm. (**F**) Lysates were prepared from cells transfected with empty vector (E), wild-type AKT2 (W), or AKT2H274 (M) at 2 days post-confluence (Day 0) or at various intervals up to day 12 after the induction of differentiation. Day 0 samples were Western blotted (W.) for AKT2 expression, and all lysates were Western blotted for aP2 expression.

emia occurred simultaneously with normal glucose tolerance. Moreover, in our proband, severe hyperinsulinemia preceded diabetes by many years. Although we cannot exclude an effect of the AKT2 mutation on beta-cell function, it is clear that the major effect of this mutation was on insulin action.

Germline loss-of-function mutations in genes that encode intracellular signaling kinases are being increasingly recognized as causes of human inherited disease. Thus, *JAK3* mutations cause severe combined immunodeficiency disease (*17*), *RPS6KA3* mutations cause Coffin Lowry Syndrome (*18*), and *WNK4* mutations cause an inherited form of hypertension (*19*). The kindred described here demonstrate that AKT2 can be added to this list, the $R^{274}H$ mutation in this enzyme causing a rare form of human diabetes due to a post-receptor defect in insulin signaling. Although AKT2 mutations are unlikely to explain most common forms of diabetes, this mutant uniquely demonstrates the critical role of AKT signaling in maintaining insulin sensitivity in humans.

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20. We thank D. Alessi for AKT2 cDNA and B. Hemmings for helpful discussions. Supported by the Wellcome Trust (S.O.R., J.J.R., M.S., J.C.W., D.B.D., and A.M.U.), the U.K. Medical Research Council (S.G. and P.R.M.), the Canadian Natural Sciences and Engineering Research Council (S.L.G.), the Deutsche Forschungsgemeinschaft (S.S.), the Raymond and Beverly Sackler Foundation (S.G.), Cancer Research–UK (D.B.), and Diabetes UK (R.W.). The GenBank accession number of *AKT2* is M77198.

Supporting Online Material

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11 February 2004; accepted 16 April 2004

S-Nitrosylation of Parkin Regulates Ubiquitination and Compromises Parkin's Protective Function

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Parkin is an E3 ubiquitin ligase involved in the ubiquitination of proteins that are important in the survival of dopamine neurons in Parkinson's disease (PD). We show that parkin is *S*-nitrosylated in vitro, as well as in vivo in a mouse model of PD and in brains of patients with PD and diffuse Lewy body disease. Moreover, *S*-nitrosylation inhibits parkin's ubiquitin E3 ligase activity and its protective function. The inhibition of parkin's ubiquitin E3 ligase activity by *S*-nitrosylation could contribute to the degenerative process in these disorders by impairing the ubiquitination of parkin substrates.

Parkinson's disease (PD) is a common neurodegenerative disorder that leads to the progressive loss of dopamine (DA) neurons (*1*). The majority of PD is sporadic and is thought to be due in part to oxidative stress through derangements in mitochondrial complex–I activity (*1*–*3*). There are also rare familial causes of PD due to mutations in α-synuclein, parkin, and DJ1 (*4*–*6*). Several indices of oxidative stress are also present in PD, including increased nitrotyrosine immunoreactivity (*7*, *8*), reduced glutathione and ferritin levels, increased lipid peroxidation, and increased levels of iron (*1*, *3*, *8*).

Parkin is a ubiquitin E3 ligase that is responsible for the addition of ubiquitin on specific substrates (*9*–*11*). Mutations in parkin that lead to a loss of parkin's ubiquitin E3 ligase activity are the most common cause of hereditary PD (*12*). Parkin has a number of putative substrates, and the failure to ubiquitinate some of these substrates in the absence of functional parkin protein may play an important role in the demise of DA neurons (*13*–*17*). In addition, parkin may play a more general role in the ubiquitin proteasomal pathway by participating in the removal and/or detoxification of abnormally folded or damaged proteins (*18*). The observation that there are increased markers of nitrosative stress in PD (*7*, *8*) prompted our investigation of whether parkin could be modified by nitric oxide (NO). Here, we show that parkin is *S*-nitrosylated and that this *S*-nitrosylation markedly diminishes parkin's E3 ligase activity and protective function.

Baculovirus recombinant parkin protein (BV-parkin) and human embryonic kidney (HEK) 293 cells transfected with myc-parkin were treated with *S*-nitrosoglutathione (GSNO), and both samples were subjected to the *S*-nitrosylation biotin switch assay (Fig. 1A) (*19*, *20*). HEK293-expressed parkin was readily *S*-nitrosylated, whereas *S*-nitrosylation of BV-parkin was not detectable. The absence of *S*-nitrosylated parkin in samples treated with glutathione devoid of NO (GSH) demonstrated the specificity of this modification of parkin. A similar result was observed when we used another NO donor, NOC18, with NOC18 depleted of NO [NOC18(–NO)] as a control (Fig. 1B).

To further control for the specificity of the *S*-nitrosylation of parkin, we examined whether myc–α-synuclein, which contains no cysteines, or myc–ITCH-1, an unrelated ubiquitin E3 ligase, are *S*-nitrosylated. Under conditions that lead to *S*-nitrosylation of parkin, neither α-synuclein (Fig. 1C) nor ITCH-1 (Fig. 1D) were *S*-nitrosylated. Because parkin expressed in HEK293 cells was *S*-nitrosylated but BV-parkin was not, we wondered whether the cellular extract contained a factor that facilitated the *S*-nitrosylation of parkin. Accordingly, we subjected BV-parkin to the *S*-nitrosylation biotin switch assay in the presence or absence of HEK293 cell lysate (Fig. 1E). BV-parkin was *S*-nitrosylated only in the presence of cell lysate. The factor is likely to be proteinaceous, because denaturing the cell lysate by boiling completely blocked the *S*-nitrosylation of BV-parkin (Fig. 1E). To further confirm that parkin is *S*-nitrosylated, we used the 2,3-diaminonaphthalene (DAN) assay on HEK293 cells transfected with myc-parkin (*21*). This assay showed that parkin was *S*-nitrosylated, whereas ITCH-1 and α-synuclein were not (Fig.

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PUBLIKATION 6

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ETO/MTG8 Is an Inhibitor of C/EBP{beta} Activity and a Regulator of Early Adipogenesis.

Mol. Cell. Biol. 24(22):9863-9872, (2004).

MOLECULAR AND CELLULAR BIOLOGY, Nov. 2004, p. 9863–9872 Vol. 24, No. 22
0270-7306/04/$08.00+0 DOI: 10.1128/MCB.24.22.9863–9872.2004

ETO/MTG8 Is an Inhibitor of C/EBPβ Activity and a Regulator of Early Adipogenesis

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Received 28 January 2004/Returned for modification 26 March 2004/Accepted 30 July 2004

The putative transcriptional corepressor ETO/MTG8 has been extensively studied due to its involvement in a chromosomal translocation causing the t(8;21) form of acute myeloid leukemia. Despite this, the role of ETO in normal physiology has remained obscure. Here we show that ETO is highly expressed in preadipocytes and acts as an inhibitor of C/EBPβ during early adipogenesis, contributing to its characteristically delayed activation. ETO prevents both the transcriptional activation of the C/EBPα promoter by C/EBPβ and its concurrent accumulation in centromeric sites during early adipogenesis. ETO expression rapidly reduces after the initiation of adipogenesis, and this is essential to the normal induction of adipogenic gene expression. These findings define, for the first time, a molecular role for ETO in normal physiology as an inhibitor of C/EBPβ and a novel regulator of early adipogenesis.

Adipose tissue is a key depot for the storage of energy as triglycerides and also plays a dynamic role in the regulation of metabolism (30). Studies of obese and lipodystrophic humans and rodents demonstrate that both increased and decreased adipose tissue mass are associated with insulin resistance and abnormal glucose and lipid metabolism (17, 24, 29). Thus, tight control of adipocyte development, size and insulin-sensitivity appears to be of critical importance in maintaining whole body energy homeostasis. The process of adipogenesis requires highly organized and precisely controlled expression of a cascade of transcription factors within the preadipocyte (25, 32, 35). The rapid and transient induction of the C/CAAT-enhancer binding proteins C/EBPβ and C/EBPδ is one of the earliest steps in this process (35). These transcription factors bind to specific sequences in the promoters of C/EBPα and the nuclear hormone receptor PPARγ, inducing their expression and in turn activating the full adipogenic program of gene expression (11, 34, 47). Although the central involvement of these proteins in adipogenesis has been demonstrated in both cellular systems and knockout animals, important roles for other regulatory molecules in this highly orchestrated transcriptional program are becoming increasingly apparent (25, 35). The C/EBPs are subject to control through heterodimerization with other members of this protein family. Some of these are intrinsically active, such as C/EBPα, C/EBPβ-LAP, and C/EBPδ, whereas others appear inhibitory, including C/EBPβ-LIP, CHOP10, and C/EBPγ (33). Interaction with coactivators such as p300 and corepressors such as histone deacetylase 1 (HDAC1) and Sin3a further modulate function (12, 44). Moreover, C/EBPs are subject to regulation at the levels of transcription and translation, the latter giving rise to alternative forms from the same mRNA as occurs with the LAP and LIP forms of C/EBPβ (4, 10). Posttranslational modification by serine and tyrosine phosphorylation has also been reported for these proteins (33). In addition to regulating target gene expression in a classical fashion C/EBPs may exert nontranscriptionally mediated effects through interaction with cell cycle inhibitors (23). The multifaceted nature of both the control and the function of this family of transcription factors attests to their importance in diverse biological processes and the need for their precise regulation.

In a screen for novel genes regulated by insulin-like growth factor 1 (IGF-1) in 3T3-L1 preadipocytes we identified the transcriptional corepressor ETO/MTG8 as a transcript rapidly repressed by IGF-1 (26). Given the key role of IGF-1 as a stimulus for the conversion of these cells into terminally differentiated adipocytes and the need for tight transcriptional regulation we postulated that ETO might play a role in this process. ETO has been extensively studied in myeloid cells due to its involvement in a chromosomal translocation causing the t(8;21) form of acute myeloid leukemia (19, 46). However, ETO is also clearly detectable in brain, heart, skeletal muscle, and adipose tissue (46), and its presence in metabolically important tissues suggested to us that its hormonal regulation merited further study. ETO is considered to have no inherent DNA-binding activity. Instead, it may form complexes with DNA-bound transcription factors and recruit other corepressors such as Sin3, N-CoR, and HDACs thereby inhibiting transcriptional activity (9). To date, only the transcriptional repressors PLZF, Bcl-6, and Gfi-1 have been identified as ETO targets (5, 9), all of which are involved in hematopoiesis. The function of ETO in vivo remains obscure, although mice lacking this protein have severe abnormalities of midgut development, leading in most cases to embryonic or early neonatal death (3).

We demonstrate here a previously unknown role for ETO in

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preadipocytes as an inhibitor of C/EBPβ function. By impairing the activity of this key, early modulator of adipogenesis, ETO restrains cells from progressing through the transcriptional program to form mature adipocytes. The rapid disappearance of ETO after exposure of preadipocytes to prodifferentiative hormonal stimuli closely precedes the acquisition of DNA-binding activity by C/EBPβ and the resulting stimulation of transcription from the C/EBPα promoter. This represents not only a novel role for ETO but also defines a new mechanism for its action and reveals its importance in the regulation of adipogenesis.

MATERIALS AND METHODS

RNA isolation, real-time PCR, and Northern blot analyses. Total RNA was isolated from cultured cells by using an RNeasy kit (Qiagen) or from tissue samples by using RNA STAT-60 (AMS Biotechnology) and quantified by GeneQuant (Amersham Biosciences). Then, 10-μg portions of each sample were analyzed by Northern blotting as described previously (26). Where quantification is shown, blots were reprobed for, and values were normalized to, expression of rRNA. Elsewhere, blots are representative of at least three independent experiments.

Primer Express software (version 1.0; Perkin-Elmer Applied Biosystems) was used to design the probes and primers for real-time quantitative PCR to determine human MTG8 or murine ETO, PPARγ1, PPARγ2, aP2, or Glut4 mRNA expression. RNA was reverse transcribed, and the resulting cDNA was used in 25-μl PCRs, in which 300 nmol of forward and reverse primers/liter and 150 nmol of fluorogenic probe/liter were used. Reactions were carried out in duplicate for each sample on an ABI 7700 sequence detection system (Perkin-Elmer Biosystems) according to the manufacturer's instructions, and target values were normalized to 18S rRNA (reagents from Perkin-Elmer).

Protein analyses and immunoprecipitation. Protein samples were extracted by scraping in lysis buffer containing 1% NP-40 as described previously (26), followed by sonication. After centrifugation for 10 min at 13,000 × *g* samples of supernatant containing 30 μg of protein were denatured and analyzed by Western blotting. Green fluorescent protein (GFP)-tagged proteins were immunoprecipitated from lysates containing 150 μg of protein by incubation with agarose conjugated α-GFP antibodies for 3 h at 4°C rotating end over end. Precipitates were washed, denatured, and analyzed by Western blotting essentially as described previously. C/EBPβ or ETO was similarly immunoprecipitated by using an antibody prebound to protein G-Sepharose. All antibodies were from Santa Cruz Biotechnology.

Plasmids and mutagenesis. Full-length cDNA encoding mouse MTG8/ETO was generated from 3T3-L1 preadipocyte RNA by RT-PCR. This was subsequently cloned in frame downstream of GFP in pEGFP-C1 (Clontech) to generate a construct encoding an N-terminally tagged protein. DNA sequencing confirmed the absence of mutations. GFP-ETO-AA was generated by using a QuikChange site-directed mutagenesis kit (Stratagene). GFP-ETO and GFP-ETO-AA were subsequently subcloned into the SnaB1 site of pBabePuro. The coding regions of ETO and ETO-AA were also subcloned into pcDNA3 (Invitrogen) to produce untagged constructs. The pMT2-C/EBPβ expression vector and the pGL3-C/EBPα promoter reporter construct were generously provided by Q.-Q. Tang and M. D. Lane.

Cell culture and transfection. 3T3-L1 (42), HEK293 (28), and HepG2 (16) cells were cultured as described previously. Preadipocytes were induced to differentiate by transfer to medium containing fetal calf serum and a standard cocktail of insulin, isobutyl methyl xanthine (IBMX), and dexamethasone as previously described (42). HEK293 cells were transiently transfected with Fugene 6 reagent (Roche) according to the manufacturer's protocol. To generate retroviruses, BOSC-293 cells were similarly transfected with 5 μg of pBabePuro vectors encoding either, GFP, GFP-ETO, or GFP-ETO-AA with Fugene 6 reagent (Roche). Supernatants containing virus were collected 48 h later and used to generate stably transfected populations of 3T3-L1 cells essentially as previously described (48). Differentiating 3T3-L1 cells were assessed for lipid content by staining with oil-red O as described previously (44). To assay C/EBPβ activity, HepG2 cells were transfected with 50 ng of pGL3-C/EBPα promoter reporter construct (containing C/EBPα nucleotides −1450 to +125) (37) and 100 ng of pMT2-C/EBPβ (38) in the absence or presence of ETO constructs as indicated with Fugene 6 reagent (Roche). Where indicated, an alternative C/EBPβ responsive promoter, C/EBPwt-LUC was used in which luciferase expression was controlled by two copies of the C/EBPβ binding site of the interleukin-6 promoter cloned upstream of the adenovirus major late promoter as described previously (13). Alternatively, to measure PPARγ activity, cells were transfected with 200 ng of $(PPARE)_3TKLuc$ and 100 ng of pcDNA3-PPARγ2 with or without ETO. Activity was assayed 48 h posttransfection by using a dual-luciferase reporter assay system (Promega). Values were normalized to the activity of cotransfected pRL-TK (PPARγ) or pRL-CMV (C/EBPβ) constitutive *Renilla* luciferase reporter vectors (Promega). For immunofluorescence studies, 3T3-L1 preadipocytes were grown on glass coverslips and transiently transfected with pEGFP or pEGFP-ETO vectors by using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions.

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed essentially as described earlier (15). Briefly, cells were treated as indicated, rinsed twice in ice-cold phosphate-buffered saline (PBS), and cross-linked by using a 1% solution of formaldehyde for 10 min at room temperature. After two rinses with PBS, cells were scraped in lysis buffer (1% sodium dodecyl sulfate [SDS], 5 mM EDTA, 50 mM Tris-HCl [pH 8.1]), sonicated, and centrifuged at 14,000 × *g* for 10 min. Samples were diluted 10× in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 8.1]) supplemented with protease inhibitors and then incubated with anti-C/EBPβ antibody prebound to protein G-Sepharose at 4°C, with rotation end over end for 4 h. Precipitates were washed once in dilution buffer, once in TSE1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 150 mM NaCl), once in TSE2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl), and once in buffer 3 (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl). After a final wash in Tris-EDTA buffer, 100 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) was added to each pellet or to a 1/10 volume of the corresponding initial lysate sample (input). Samples were incubated at 65°C for 6 h, and DNA was isolated by using a Qiagen PCR cleanup kit according to the manufacturer's instructions. C/EBPα promoter DNA was assayed by using real-time PCR with probe and primers amplifying the C/EBPβ binding site at −190 bp proximal to the transcriptional start site. Values obtained from immunoprecipitated samples were normalized to those from input samples.

Immunofluorescence. At 3 days posttransfection 3T3-L1 preadipocytes were induced to differentiate for 24 h as described above. Cells were then rinsed in PBS and fixed in 3% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 and then incubated with rabbit polyclonal antibodies to C/EBPβ (Santa Cruz Biotechnology). Slides were subsequently probed with Alexa-Fluor 594 goat anti-rabbit secondary antibodies (Molecular Probes, Inc.), mounted, and analyzed by laser scanning confocal microscopy. Similarly, subconfluent 3T3-L1 cells retrovirally transfected with GFP, GFP-ETO, or GFP-ETO-AA were grown on coverslips and fixed, and images were obtained to determine subcellular localization.

Gel shift assays. ETO and C/EBPβ proteins were synthesized from cDNA templates in pcDNA3.1 by using a TNT quick-coupled transcription/translation system (Promega). Double-stranded oligonucleotide probe (5′-CAGTGGGCG TTGCGCCACGATCTCTCT-3′) was radiolabeled with polynucleotide kinase and [γ-^{32}P]ATP. Protein mixes were preincubated for 1 h at 37°C and then incubated with 0.1 pmol of radiolabeled probe in buffer containing 20 mM HEPES (pH 7.9), 50 mM KCl, 2 mM dithiothreitol, and 10% glycerol for 30 min at room temperature. Analysis of binding complexes was performed by electrophoresis on a 6% polyacrylamide gel in 0.5× Tris-borate-EDTA.

RESULTS

ETO is hormonally regulated, and its expression decreased during adipogenesis. To determine whether ETO was hormonally responsive in 3T3-L1 preadipocytes undergoing differentiation, we treated cells with a standard adipocyte differentiation cocktail containing insulin, IBMX, and dexamethasone. This led to a rapid and sustained decrease in ETO expression, such that the mRNA was significantly decreased within 4 h of treatment (Fig. 1A). Analysis of ETO expression in 3T3-F442 preadipocytes demonstrated that ETO mRNA was also highly expressed in confluent cultures and was again rapidly decreased in response to hormonal induction of adipogenesis in these cells (Fig. 1B). Using real-time quantitative PCR, we examined ETO mRNA expression over a longer time course of differentiation in 3T3-L1 cells. These data confirmed the rapid fall of ETO mRNA expression observed in the Northern blots

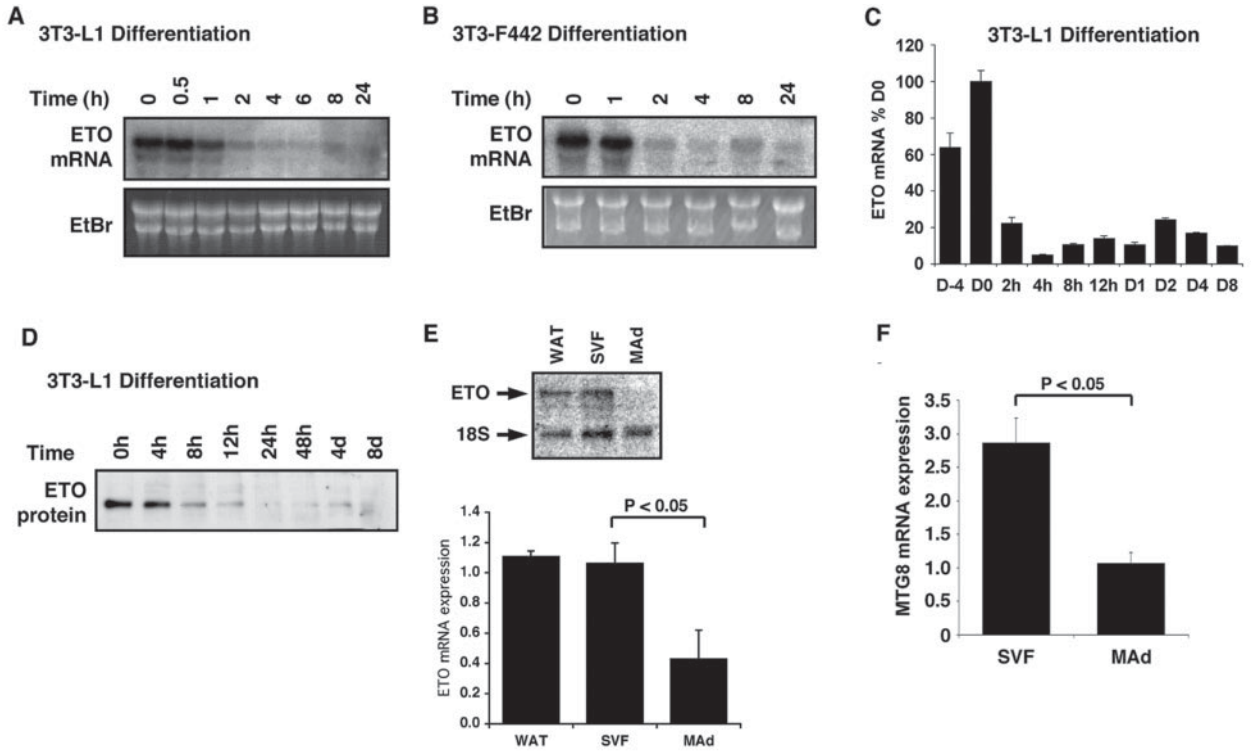

FIG. 1. The expression of ETO in preadipocytes is inhibited during adipogenesis. ETO mRNA expression was determined by Northern blotting (A and B) or real-time PCR analysis (C) of RNA samples extracted from 2-day postconfluent 3T3-L1 (A and C) or 3T3-F442 (B) preadipocytes treated for the times indicated with differentiation mixture. (D) Protein samples extracted from 3T3-L1 preadipocytes incubated with differentiation mixture for various times were Western blotted with and anti-ETO polyclonal antibody (Santa Cruz Biotechnology). (E) RNA isolated from rat whole adipose tissue (WAT), cells of the stromovascular fraction (SVF), or mature isolated adipocytes (MAd) was analyzed by Northern blotting to determine ETO expression. Blots were reprobed with a ribosomal probe to control for loading. A representative blot is shown (upper panel) along with mean data ± the SEM from four rats (lower panel). (F) RNA was isolated from cells of the stromovascular fraction (SVF) or mature isolated adipocytes (MAd) from subcutaneous human adipose tissue samples. MTG8 expression was determined by using real-time PCR and normalized to 18S mRNA. EtBr, ethidium bromide.

and demonstrated sustained inhibition of ETO expression even after 8 days (Fig. 1C). Western blot analysis of 3T3-L1 cell lysates during differentiation revealed that ETO protein expression was also rapidly inhibited such that it was almost undetectable 24 h postinduction and again remained suppressed even in the later stages of adipogenesis (Fig. 1D). Having analyzed ETO expression in cultured cells, we sought to assess its potential involvement in vivo. Consistent with previous expression studies (46), we could clearly detect ETO mRNA in rat whole adipose tissue by Northern blotting (Fig. 1E). When this tissue was fractionated, we found that the majority of the ETO mRNA was present in the stromovascular fraction in which the preadipocytes are found. ETO mRNA expression was significantly lower in the mature adipocytes, a finding consistent with our data obtained with fully differentiated 3T3-L1 adipocytes. Identical data were obtained when these samples were assayed by quantitative real-time PCR (data not shown). We further used real-time PCR to quantify the expression of the human ETO homologue, MTG8, in the stromovascular and mature adipocyte fractions of human subcutaneous adipose tissue (Fig. 1F). This gave results almost identical to those seen in the rat with 2.6-fold-higher expression in the preadipocyte-containing than the mature adipocyte fraction. These findings strongly imply that ETO has a role in the function of normal adipose tissue and that decreased expression of ETO is a feature of preadipocyte to adipocyte conversion in vivo.

ETO inhibits adipogenesis in 3T3-L1 cells, whereas dominant-negative ETO augments lipid accumulation. To explore the role of ETO in adipogenesis further, we sought to manipulate the expression of ETO in 3T3-L1 cells. In addition, we attempted to create a dominant-negative form of the molecule. It has been demonstrated that ETO functions as a dimer and is ordinarily localized to the nucleus (21, 28). However, mutation of both lysine 238 and arginine 239 to alanine in the nuclear localization sequence of ETO is known to cause the molecule to be mistargeted to the cytosol (28). We postulated that this mutant form of ETO (hereafter referred to as ETO-AA), if expressed in excess, might dimerize with and cause nuclear exclusion of wild-type ETO, thereby functioning as a dominant negative. Therefore, we generated this mutant by site-directed mutagenesis. Both wild-type ETO and ETO-AA were subsequently tagged with GFP at the N terminus, as represented in Fig. 2A, to allow visualization of the proteins in intact cells and specific identification and isolation of the transfected ETO when expressed in the presence of the endogenous protein.

To determine whether these constructs would indeed form heterodimers with endogenous ETO, we coexpressed them with untagged full-length wild-type ETO. Using an antibody

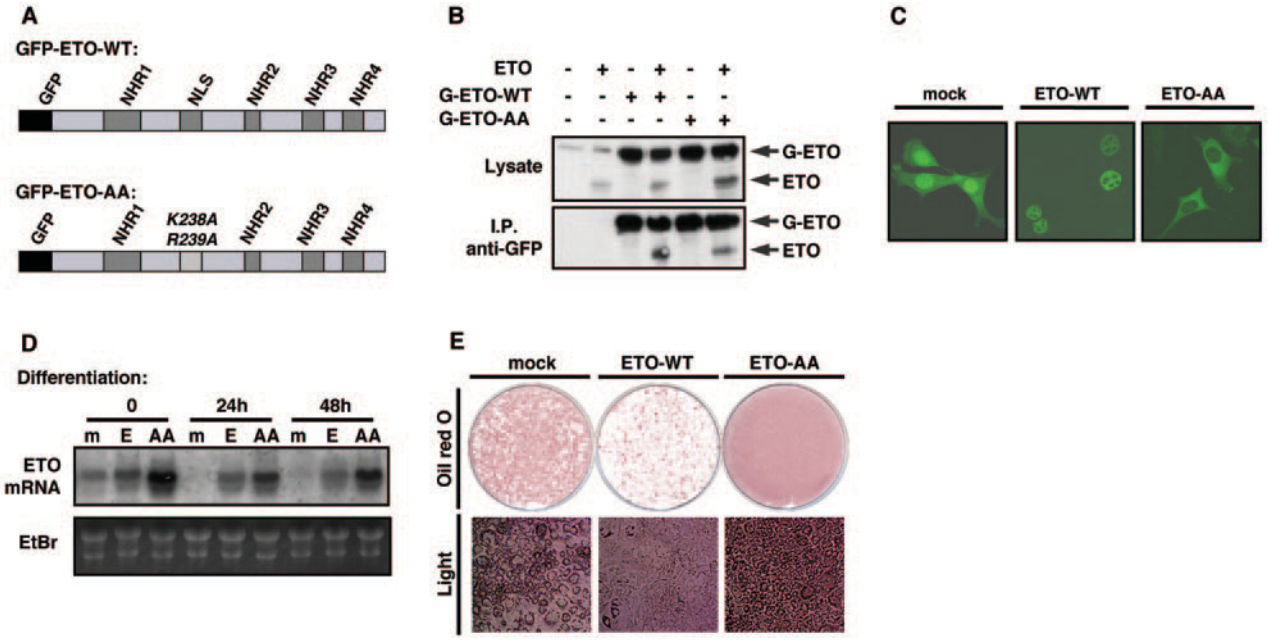

FIG. 2. ETO localizes to the nucleus and inhibits adipogenesis, whereas the mutant ETO-AA, in which the nuclear localization signal is disrupted, is targeted to the cytosol and accelerates preadipocyte differentiation. (A) The construction of N terminally GFP-tagged forms of ETO is shown. The nuclear localization sequence (NLS) was disrupted by the introduction of mutations at codons 238 and 239 to generate GFP-tagged ETO-AA. The positions of the NHR domains, involved in protein-protein interactions, are indicated. (B) GFP-ETO (G-ETO-WT) and GFP-ETO-AA were expressed in HepG2 cells in the presence or absence of untagged wild-type ETO (ETO) as indicated. ETO proteins were analyzed by Western blotting in whole-cell lysates (upper panel) or anti-GFP immunoprecipitates obtained by using agarose conjugated anti-GFP polyclonal antibody (lower panel). (C) Subconfluent cultures of 3T3-L1 preadipocytes infected with retroviruses encoding GFP alone (mock) or GFP-tagged ETO (ETO-WT) or ETO-AA were fixed in 4% paraformaldehyde and fluorescent images captured by laser-scanning confocal microscopy. (D) Two-day postconfluent 3T3-L1 preadipocytes retrovirally transfected with GFP (m), GFP-ETO (E), or GFP-ETO-AA (AA) were treated for the times indicated in the absence or presence of differentiation mixture as indicated. RNA was extracted and ETO mRNA expression determined by Northern blotting. E, 3T3-L1 cells expressing GFP, GFP-ETO or GFP-ETO-AA were differentiated for 8 days and lipid accumulation assessed by oil-red O staining (upper panels) or light microscopy (lower panels).

directed to GFP, untagged ETO coimmunoprecipitated with GFP-ETO (Fig. 2B). Coprecipitation was equally effective whether wild-type or mutant GFP-ETO was used, demonstrating that both molecules can indeed bind untagged ETO. In addition, the proportion of tagged to untagged ETO seen in the immunoprecipitates was very similar to that in the lysate samples (compare upper and lower panels), suggesting that most of the untagged ETO dimerized with the more highly expressed GFP-tagged forms.

The GFP-tagged ETO constructs were then introduced into 3T3-L1 cells by using retrovirus-mediated gene transfer. As shown in Fig. 2C, GFP-ETO was expressed almost exclusively in the nuclei of the preadipocytes, whereas the GFP-ETO-AA protein was localized instead to the cell cytosol. Mock-transfected cells were also generated expressing GFP alone, which showed a diffuse localization throughout the cell. When induced to differentiate, the cells exhibited a rapid and sustained reduction of mRNA encoding endogenous ETO (Fig. 2D). In contrast, the expression of transfected GFP-ETO or GFP-ETO-AA, which migrated with the endogenous ETO mRNA, was preserved during differentiation. Importantly, the expression levels of GFP-ETO and endogenous ETO were very similar, whereas those of GFP-ETO-AA were much higher. This pattern of expression was mimicked at the protein level (data not shown).

We next determined the effect of constitutive ETO expression on preadipocyte differentiation. As shown in Fig. 2E cells expressing GFP-ETO showed a severely impaired ability to accumulate lipid during differentiation, both when visualized by oil-red O staining (upper panels) and when visualized by light microscopy (lower panels). In contrast, identically treated cells expressing GFP-ETO-AA showed a consistent increase in lipid accumulation compared to untransfected cells.

These data indicate that loss of ETO expression is a key step in the initiation of the full adipogenic program.

ETO inhibits the expression of key proadipogenic genes in intact cells. We next examined a number of key markers of adipogenesis to define more clearly the effects of both wild-type and dominant-negative ETO expression on adipocyte differentiation. Figure 3A demonstrates that the induction of C/EBPα protein was significantly impaired in differentiating cells constitutively expressing ETO. The data from four independent time courses are shown, and the quantified data ± the standard error of the mean (SEM) are presented below a representative Western blot. Expression of both the p42 and the p30 isoforms of C/EBPα was almost entirely prevented by expression of ETO 3 days after the induction of differentiation. Conversely, expression of the mutant ETO-AA protein consistently increased C/EBPα expression at this time point. Interestingly, at the later time points, particularly 12 days postinduction, the expression of C/EBPα in ETO-expressing cells appears to reach levels approaching those in control cells.

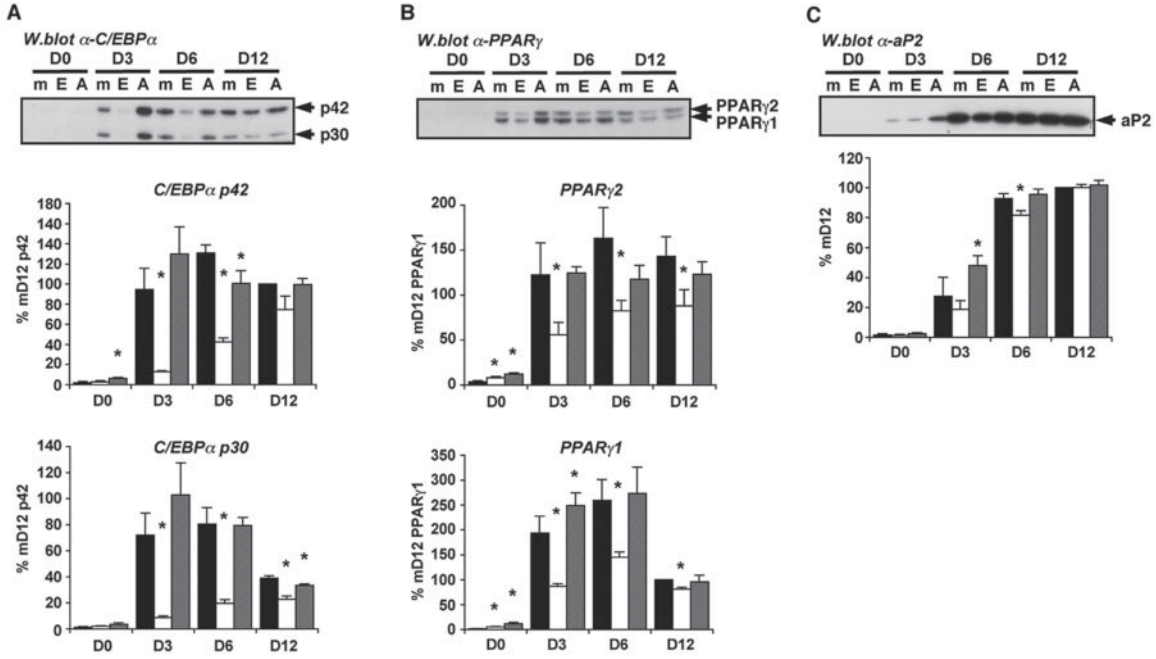

FIG. 3. ETO inhibits the expression of adipogenic proteins. Cells retrovirally transfected with GFP (■), GFP-ETO (□) or GFP-ETO-AA (▨) were induced to differentiate for 0 to 12 days (D0 to D12) as indicated. Total cell lysates were prepared and proteins analyzed by SDS-PAGE and Western blotting with antibodies to C/EBPα (A), PPARγ (B), or aP2 (C) as appropriate. In each case band intensities were quantified from four independent experiments and the mean data ± the SEM is presented below a representative blot. The data were calculated as the percentage of expression at D12 in the mock transfected cells. Asterisks indicate a statistically significant difference from the expression in mock-transfected cells at the same time point ($P < 0.05$).

Examination of PPARγ proteins in the same cell extracts revealed that induction of both $PPAR\gamma_1$ and $PPAR\gamma_2$ were also reduced by constitutive ETO expression, again particularly at the earlier time points (Fig. 3B). However, the inhibitory effect of ETO and any stimulatory effect of ETO-AA were weaker than that observed with C/EBPα. The expression of aP2 protein was also examined (Fig. 3C). Again, an overall pattern of expression similar to that seen for C/EBPα and PPARγ was observed in the three cell populations. However, the effects of ETO and ETO-AA expression were much less marked for aP2 than those seen for C/EBPα or PPARγ.

Since C/EBPα expression appeared to be most affected by ETO expression, we examined the induction of its mRNA in ETO- and ETO-AA-expressing cells during differentiation. As shown in Fig. 4A, the effect of constitutive ETO expression on C/EBPα mRNA induction was similar to that seen for its protein and again was most significant at the earlier time points. As with protein expression, the normal induction of C/EBPα mRNA after 3 days was almost entirely prevented by ETO expression, whereas ETO-AA expression led to higher levels of C/EBPα mRNA than those observed in control cells. RNA isolated from cells 3 days postinduction of differentiation were also assayed for aP2, $PPAR\gamma_2$, and Glut4 mRNA expression levels by real-time PCR (Fig. 4B). Again, ETO inhibited the induction of all three mRNAs with the greatest effects observed for Glut4, a well-characterized C/EBPα target that was both significantly lower in cells expressing ETO and higher in cells expressing ETO-AA, reflecting the effects on C/EBPα protein expression, and likely activity, in these cells.

ETO decreases the expression of C/EBPα by selectively inhibiting the activity of C/EBPβ. The C/EBP transcription factors C/EBPβ and C/EBPδ play a crucial role in the early induction of genes involved in lipid accumulation by differentiating adipocytes, and their involvement in the induction of C/EBPα and PPARγ expression has been well documented (7, 14, 47). The data obtained thus far suggested that a major effect of ETO was to inhibit C/EBPα mRNA transcription, whereas the extent of the inhibition of this gene in particular suggested that the inhibitory step might be proximal to C/EBPα induction. We therefore examined the possible involvement of C/EBPβ and C/EBPδ in the inhibition of adipogenesis by ETO. Western blotting of lysates from mock-transfected cells or cells constitutively expressing ETO revealed no significant effect of ETO on the induction of either C/EBPβ or C/EBPδ proteins (Fig. 5). In contrast, in the same lysates the expression of C/EBPα was significantly reduced during differentiation in cells expressing ETO as previously observed (see Fig. 3A).

Although C/EBPβ and C/EBPδ are both known to be capable of inducing expression from the C/EBPα promoter (31, 38), the greatest effect of ETO expression on C/EBPα induction was observed at time points up to 3 days after induction of differentiation, when C/EBPβ was also highly expressed (Fig. 5). We therefore tested whether ETO could inhibit the activity of C/EBPβ and so suppress C/EBPα expression. The presence of endogenous ETO in preadipocytes suggested that these cells would be unsuitable for examining the effects of adding exogenous ETO to a C/EBPβ activity assay. Previous studies have

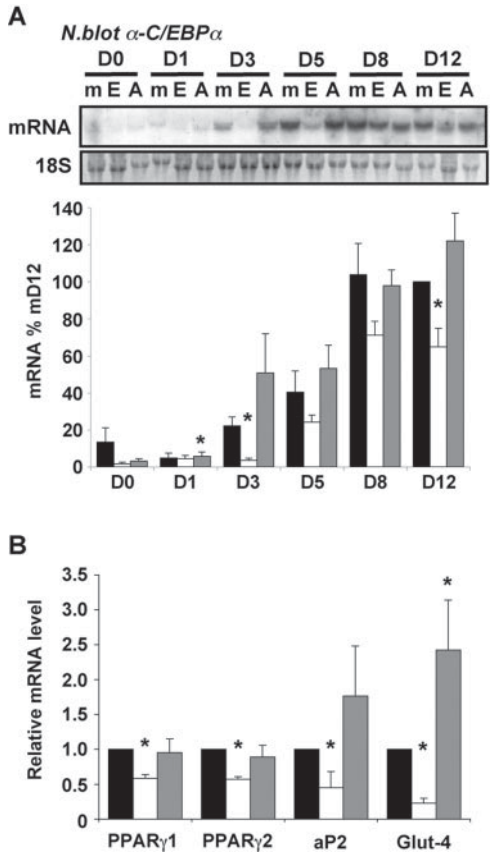

FIG. 4. ETO inhibits the induction of adipogenic gene expression. RNA was isolated from cells retrovirally transfected with GFP (■), GFP-ETO (□) or GFP-ETO-AA (▨) that had been induced to differentiate for 0 to 12 days (D0 to D8) as indicated. (A) Expression of mRNA encoding C/EBPα was determined by Northern blotting. A representative blot is shown along with 18S rRNA, which was used as a loading control and all values were adjusted accordingly. C/EBPα mRNA expression was quantified in four independent experiments, and the mean ± the SEM is shown in the lower panel. Values were expressed as percentage of those in mock-transfected cells at day 12. Asterisks indicate a statistically significant difference from mock transfected cells at the same time point ($P < 0.05$). (B) Samples from cells differentiated for 3 days were also assayed for mRNA encoding PPARγ$_1$, PPARγ$_2$, aP2, and Glut-4 by real-time PCR. The data are means ± the SEM of four independent experiments, and values are expressed relative to that in mock-transfected cells. Asterisks indicate a statistically significant difference from mock-transfected cells ($P < 0.05$).

demonstrated that ETO expression is undetectable in the liver (46) and, consistent with this, we were unable to detect ETO mRNA in the hepatocyte cell line HepG2 (data not shown). Using these cells, we were able to observe a robust activation of transcription from a luciferase-linked C/EBPα promoter construct in the presence of C/EBPβ. As shown in Fig. 6A, coexpression of ETO led to a repression of C/EBPβ activity in a dose-dependent fashion, demonstrating that ETO can indeed inhibit C/EBPβ activity toward the C/EBPα promoter. In contrast, the mutant ETO-AA was completely unable to inhibit the activity of C/EBPβ in this assay (Fig. 6B), a finding consistent with the inability of this mutant to prevent adipogenesis. To assess the specificity of these effects, we next tested whether ETO affected the transcriptional activity of PPARγ which, although also critical in the differentiation process, belongs to

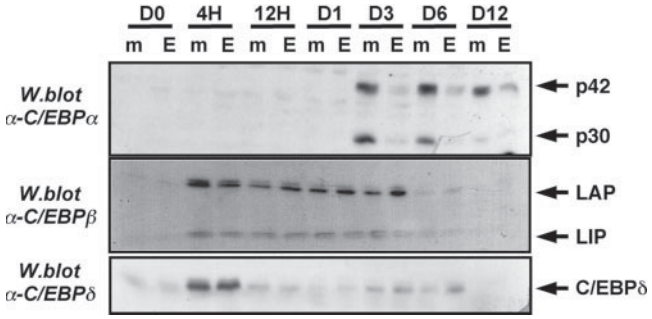

FIG. 5. ETO impairs the induction of C/EBPα but not C/EBPβ or C/EBPδ during adipogenesis. 3T3-L1 cells expressing GFP or GFP-ETO were differentiated for the times shown. Cell lysates were prepared and analyzed by Western blotting to determine the expression of C/EBPβ, C/EBPδ or C/EBPα as indicated.

a different family of transcription factors. In contrast to C/EBPβ, the activity of PPARγ was completely unaffected by ETO expression at levels that almost entirely inhibited C/EBPβ activity (Fig. 6C). To test further whether ETO acted directly to inhibit C/EBPβ activity or acted more generally to inhibit the activity of the C/EBPα promoter, we repeated our assays with an alternative minimal C/EBPβ responsive reporter construct. Using this construct, we observed almost identical inhibition of C/EBPβ activity by ETO to that seen with the C/EBPα promoter construct (Fig. 6D). In addition, no signif-

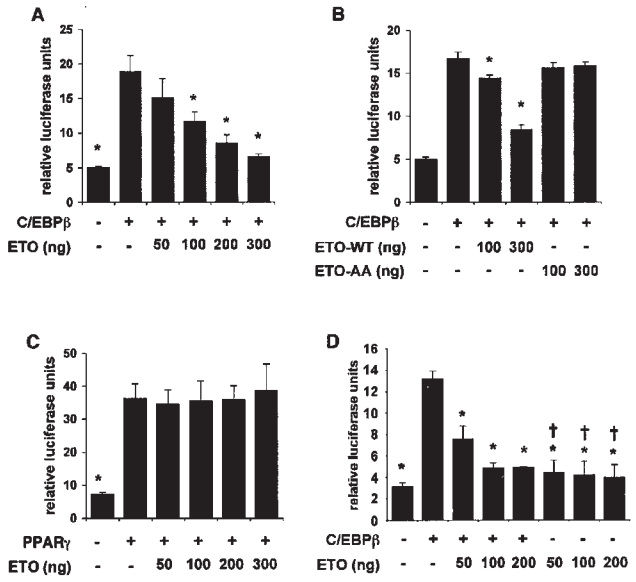

FIG. 6. ETO but not ETO-AA selectively inhibits C/EBPβ transcriptional activity. HepG2 cells were transfected with either a C/EBPα promoter-luciferase reporter construct (A and B) or a minimal C/EBP responsive reporter construct C/EBPwt-LUC (D) with or without C/EBPβ in the absence or presence of increasing quantities of ETO (A, B, and D) or ETO-AA (B) as indicated. (C) Cells were transfected with a PPRE-luciferase reporter construct alone or in combination with PPARγ and increasing quantities of ETO. In each case data shown are means ± the SEM of four independent experiments. Asterisks indicate statistically significant difference from activity in the presence of C/EBPβ (A, B, and D) or PPARγ (C) alone. In panel D, a dagger (†) indicates no significant difference from activity in cells transfected with neither C/EBPβ nor ETO.

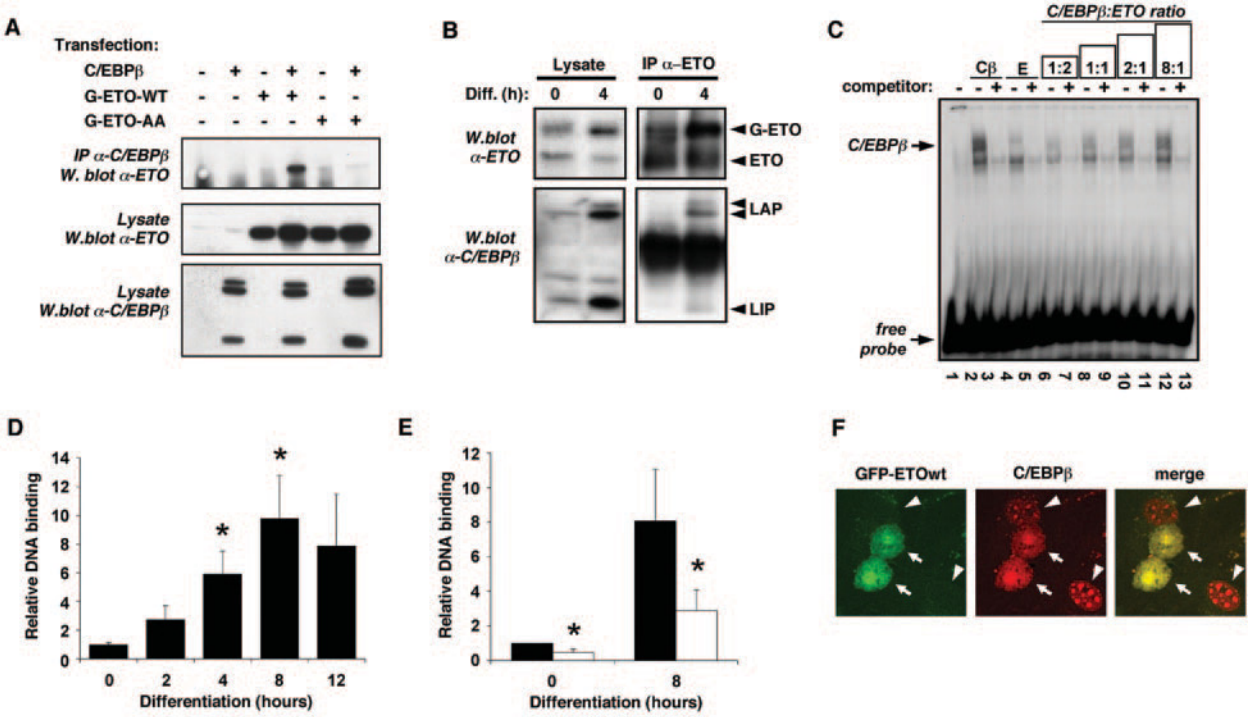

FIG. 7. ETO interacts directly with C/EBPβ inhibiting its DNA-binding activity toward the C/EBPα promoter and preventing centromeric localization during adipogenesis. (A) HEK293 cells were transfected with control vector, GFP-ETO (G-ETO-WT), or GFP-ETO-AA in the absence or presence of C/EBPβ as indicated. Anti-C/EBPβ immunoprecipitates were analyzed for associated ETO protein (upper panel), whereas corresponding cell lysates were probed for ETO (middle panel) or C/EBPβ (lower panel) by Western blotting. (B) 3T3-L1 preadipocytes expressing GFP-ETO were treated for 4 h in the absence or presence of differentiation cocktail as indicated prior to lysis. Cell lysates (left panels) or immunoprecipitates prepared by using an anti-ETO antibody (Santa Cruz Biotechnology) (right panels) were analyzed by Western blotting to detect ETO (upper panels) or C/EBPβ isoforms (lower panels). (C) In vitro transcribed/translated C/EBPβ (Cβ) and/or ETO (E) were incubated with radiolabeled DNA probe corresponding to the proximal C/EBPα binding site of the C/EBPα promoter in a gel shift assay. Various ratios of C/EBPβ to ETO were achieved by adjusting the quantity of ETO. In all lanes total protein input was kept constant by appropriate addition of rabbit reticulocyte lysate, except in lane 1, where free labeled probe was run alone. (D) 3T3-L1 preadipocytes were induced to differentiate for the times indicated and ChIP assays performed by using an anti-C/EBPβ antibody to isolate C/EBPβ-associated DNA. DNA from these immunoprecipitates corresponding to the C/EBPβ binding site in the C/EBPα promoter was quantified by using real-time PCR and normalized to DNA from a 10% sample of corresponding input lysate. The data are means ± the SEM from three independent experiments. Asterisks indicate a statistically significant difference ($P < 0.05$) from values obtained at time zero. (E) 3T3-L1 preadipocytes expressing GFP (■) or GFP-ETO (□) were differentiated for 8 h as indicated and ChIP assays performed to determine occupancy of the C/EBPα promoter by C/EBPβ as in panel D. The data are means ± the SEM from five independent experiments. Asterisks indicate a statistically significant difference ($P < 0.05$) from values obtained in GFP-transfected cells at the corresponding time point. (F) Confocal microscope images of 3T3-L1 cells transfected with GFP-ETO, grown to confluence, and differentiated for 24 h. Cells were fixed in 4% paraformaldehyde, and C/EBPβ was visualized by using anti-C/EBPβ antibody (Santa Cruz Biotechnology) and an anti-rabbit Alexa-Fluor 594 secondary antibody. Arrows indicate the nuclei of cells transfected with GFP-ETO. Arrowheads indicate cells not expressing ETO but immunostaining for endogenous C/EBPβ.

icant reduction in the basal activity of the promoter was observed when ETO was present in the absence of C/EBPβ. Since the effect of ETO is similar with two different C/EBPβ target promoters, these data strongly suggest a direct effect of ETO on the activity of this transcription factor. In addition, the lack of effect on the transactivating capacity of PPARγ argues strongly against ETO affecting the basal transcriptional machinery in these assays.

We next sought to examine more closely the mechanism of ETO's actions on C/EBPβ. To assess whether the inhibition of C/EBPβ activity by ETO involved a direct physical interaction, we coexpressed C/EBPβ with GFP-ETO or GFP-ETO-AA. As shown in Fig. 7A, we were able to immunoprecipitate GFP-ETO with an antibody specific to C/EBPβ but only when C/EBPβ was also present in the cells. However, no GFP-ETO-AA could be detected in C/EBPβ immunoprecipitates when these two proteins were coexpressed. This was not surprising given that C/EBPβ displays an almost exclusively nuclear localization, whereas GFP-ETO-AA is excluded from this cellular compartment. We also examined the interaction of these proteins in differentiating 3T3-L1 preadipocytes. ETO was immunoprecipitated from 3T3-L1 preadipocytes constitutively expressing ETO that had been induced to differentiate for 4 h (Fig. 7B). Western blotting revealed the interaction of ETO with endogenous C/EBPβ; indeed, both the LAP and the LIP isoforms were effectively coimmunoprecipitated with ETO, not only suggesting that both are susceptible to regulation by ETO but also demonstrating that the interaction between the two proteins involves the C-terminal portion of C/EBPβ that is present in all isoforms.

We next examined whether ETO affects the DNA-binding activity of C/EBPβ. The promoter of C/EBPα contains a well-characterized C/EBPβ consensus binding site 190 bp proximal to the transcriptional start site (6), and therefore we performed

gel shift assays with an oligonucleotide probe corresponding to this sequence. As shown in Fig. 7C, in vitro-translated C/EBPβ formed a stable complex with radiolabeled DNA probe, the binding of which could be competed away by using a 100-fold excess of unlabeled probe. ETO itself formed no stable complex with the DNA and, when incubated at a 2:1 ratio with C/EBPβ, almost completely prevented the latter from binding to the probe. As the ratio of ETO to C/EBPβ was decreased, the DNA-binding activity was restored in a dose-dependent fashion. These data strongly suggested that the mechanism by which ETO inhibited C/EBPβ activity involved a direct association between ETO and C/EBPβ, causing the latter to lose affinity for its target DNA sequence. To examine this effect further in intact cells, we performed chromatin immunoprecipitation (ChIP) assays in which C/EBPβ was immunoprecipitated and its associated DNA was isolated. In a novel modification of the commonly used methods in which limited cycle PCR is used to determine the relative levels of associated DNA sequences in different samples, we instead used real-time PCR to allow more accurate quantification of the extent of DNA binding. The total genomic DNA input was similarly quantified for each sample, and results from immunoprecipitated samples were adjusted accordingly. Examination of C/EBPβ binding to DNA over the first 12 h of differentiation in 3T3-L1 preadipocytes revealed that maximum binding was achieved some 8 h after induction of differentiation (Fig. 7D). We believe that this is the first assessment of these early time points of differentiation by using this method. However, the data agree well with previously published time courses assessed by gel shift analysis with nuclear extracts from 3T3-L1 preadipocytes (8), which has demonstrated strong binding of C/EBPβ to the C/EBPα promoter within 12 h of differentiation. Subsequent analysis of C/EBPβ binding to the C/EBPα promoter in cells constitutively expressing ETO revealed that C/EBPβ binding activity was significantly reduced in these cells compared to mock-transfected cells (Fig. 7E). These data demonstrate that ETO does indeed inhibit association of C/EBPβ with the C/EBPα promoter in intact cells.

In differentiating 3T3-L1 preadipocytes it is known that, although C/EBPβ is rapidly induced within 4 h, it acquires DNA-binding activity only after 12 to 24 h (39). Tang and Lane demonstrated that concomitant with the acquisition of DNA-binding activity to both the C/EBPα promoter and centromeric DNA sequences, C/EBPβ shifts from a diffuse to a punctate nuclear localization pattern, which can be clearly detected by immunohistochemistry (39). Having demonstrated that ETO could inhibit the DNA-binding activity of C/EBPβ, we next tested whether its centromeric localization during adipogenesis might be affected by ETO expression. To address this, 3T3-L1 preadipocytes were transiently transfected with GFP-ETO, grown to confluence, and then induced to differentiate for 24 h to bring about the expression and subsequent centromeric localization of endogenous C/EBPβ. Cells were then fixed and stained with antibodies to C/EBPβ, and the localization of both C/EBPβ and GFP-ETO was determined by confocal microscopy. Cells expressing GFP-ETO showed a diffuse pattern of fluorescence for this protein within the nucleus (Fig. 7F). As expected with transient transfection, some cells showed high levels of GFP-ETO expression, whereas others did not. Cells that had not been transfected with ETO showed the expected punctate centromeric pattern of C/EBPβ expression. In marked contrast, in all cells expressing GFP-ETO, C/EBPβ immunostaining was diffusely distributed in the nucleus. Of particular note was the fact that inhomogeneities in this diffuse nuclear staining pattern precisely mimicked the pattern seen with ETO-GFP, strongly suggesting an intimate colocalization of these two proteins.

These data strongly suggest that ETO associates with C/EBPβ in intact cells and is likely to contribute significantly to the delayed acquisition of DNA-binding activity of this transcription factor.

DISCUSSION

The results presented here demonstrate that ETO has an important role in the early stages of adipogenesis as an inhibitor of C/EBPβ-driven transcription. We show that ETO can directly inhibit C/EBPβ-induced transcription from the C/EBPα promoter and that this results in decreased expression of C/EBPα in intact 3T3-L1 preadipocytes. Gel shift assays, ChIP assays, and immunohistochemical data indicate that ETO causes loss of C/EBPβ binding to target DNA sequences, including the proximal C/EBP consensus site in the C/EBPα promoter. As a result the early induction of C/EBPα, normally visible within 3 days of the induction of differentiation, is entirely inhibited in cells constitutively expressing ETO, whereas a more rapid and robust increase in C/EBPα expression can be observed in cells expressing a putative dominant-negative ETO. The induction of PPARγ$_1$ and PPARγ$_2$ is also decreased in differentiating cells in which ETO expression cannot be appropriately inhibited. However, it is noteworthy that the effect is less marked that that seen for C/EBPα and that this is also true for aP2. We propose that this reflects differences in the relative individual contributions of different transcription factors to the induction of these genes. Although C/EBPβ and C/EBPδ have been identified as important regulators of C/EBPα, PPARγ, and aP2 induction during adipogenesis (36, 47), their regulation in vivo is likely to involve an array of transcriptional regulators. The data presented here are consistent with a critical role for C/EBPβ in early C/EBPα induction and an important but less essential role in the early induction of PPARγ and aP2. Pertinent to this, examination of the proximal PPARγ$_2$ promoter sequence has demonstrated that this may be effectively activated by C/EBPδ and C/EBPα but that C/EBPβ alone lacks the ability to directly stimulate transcription from this region of the promoter (11). Thus, although a role for C/EBPβ in PPAR$_2$ induction has been demonstrated in other studies (36, 47), this is probably due to an indirect effect or is mediated by more distal regions of the promoter.

It is not yet clear whether C/EBPβ represents the sole target of ETO in preadipocytes. Most of our observations in 3T3-L1 cells constitutively expressing ETO can be accounted for by inhibition of known C/EBPβ functions. However, it is possible that, given that other ETO targets, such as the zinc-finger repressor PLZF and Bcl-6, have been described (5, 9), ETO may also exert its actions through other transcription factors present in preadipocytes.

Whether or not ETO also targets other transcription factors, several pieces of evidence demonstrate that ETO does not function nonspecifically to inhibit differentiation. First, it has

no effect on the rapid expression of C/EBPβ or C/EBPδ upon induction of adipogenesis. In addition, the coexpression of ETO had no effect on the transactivating capacity of PPARγ in luciferase reporter assays or on the binding of PPARγ to target DNA probe in gel shift assays (data not shown). Finally, cells expressing ETO constitutively appear to be capable of at least partially restoring the expression of adipogenic genes such as PPARγ, C/EBPα, and aP2 at later time points in this process. Since C/EBPβ expression has by this stage subsided, we propose that it makes a less critical contribution to the expression of these genes and so the effect of ETO is attenuated. It is noteworthy that mock-transfected 3T3-L1 preadipocytes, being competent to fully differentiate, eventually accumulate equivalent levels of lipid to the more rapidly differentiating ETO-AA-expressing cells. However, cells constitutively expressing ETO fail to do so fully even when differentiated for extended periods of up to 16 days. At present, it is unclear whether this results from ETO affecting critical early C/EBPβ-mediated events, which cannot subsequently be overcome, or from modulation of other targets by ETO in mature cells. However, we have observed that Glut4 mRNA expression remains suppressed longer than other genes tested and, since glucose may represent an important substrate for lipid production in these cultured adipocytes, it is possible that this explains the lack of lipid accumulation despite almost normal levels of aP2, PPARγ, and C/EBPα.

Although ETO mRNA levels fell acutely in response to proadipogenic signals, the disappearance of ETO protein within the cells was less rapid and was absent only after 8 to 24 h of differentiation. This coincides well with the previously reported acquisition of DNA-binding activity by C/EBPβ, which lags considerably behind C/EBPβ expression (39), which is apparent within 2 h of induction of differentiation. Our data from gel shift assays, ChIP assays, and immunostaining of C/EBPβ subnuclear localization strongly suggest that not only does ETO inhibit the binding activity of C/EBPβ but that this occurs in intact cells, thus explaining the loss of induction of C/EBPβ target genes in differentiating preadipocytes constitutively expressing ETO. A similar mechanism has been described for the C/EBP homologous protein CHOP-10, a dominant-negative member of the C/EBP family of transcription factors, which heterodimerizes with C/EBPβ via leucine zipper domains (40). However, because ETO is not a member of the C/EBP family its control of C/EBPβ activity in this way represents a novel mechanism for its regulation. Since ETO has no conventional C/EBP binding domains, it is not clear which regions of the two proteins are involved in their interaction, and we are currently addressing this question. Given the colocalization of ETO and C/EBPβ that we have observed in preadipocytes, it is intriguing that ETO has been reported to reside in transcriptionally inert regions of the nucleus (2). Thus, in addition to inhibiting the DNA-binding activity of C/EBPβ per se, ETO may also actively sequester it within these regions.

Wiper-Bergeron et al. have demonstrated that, once capable of binding DNA, C/EBPβ forms a complex on the C/EBPα promoter that is inactive due to the presence of the transcriptional corepressors Sin3a and HDAC1 (44). Only once HDAC1 is degraded is transcription activated, and this may explain the additional lag between the reported binding of C/EBPβ to the C/EBPα promoter and the appearance of C/EBPα mRNA (39). Although our data demonstrate that the effect of ETO is to inhibit binding of C/EBPβ to the C/EBPα promoter, several studies have reported the binding of Sin3a and HDAC1 to ETO (1). This raises the possibility that ETO may assist in the assembly of this complex before the disappearance of ETO allows its association with the C/EBPα promoter.

ETO is selectively expressed at much higher levels in the preadipocyte than the mature adipocyte fraction of rat adipose tissue, mimicking the situation in the 3T3-L1 cells differentiated in culture. It therefore appears likely that the mechanism we have described is operative in adipogenesis in vivo. The preponderance of ETO in the preadipocyte fraction of human fat further suggests that our data are relevant to human adipose tissue development, raising the possibility that decreased ETO expression or activity may play a role in the development of obesity. Indeed, an association between obesity and a polymorphism in the 3′-untranslated region of ETO has been reported in male Pima Indians (45). Although that study failed to find any mutations in the coding sequence of the gene itself, in light of our present data one would postulate that a mutation affecting RNA stability or the expression of ETO might also predispose to obesity. Although an ETO knockout mouse model has been generated, the few mice surviving past the neonatal stage exhibit severe abnormalities in midgut development (3). The resulting nutrient malabsorption makes them unsuitable for assessing any effects on obesity. Evidently, just as decreased ETO expression may contribute to obesity, the potential involvement of ETO overexpression in syndromes of lipodystrophy also warrants examination. Thus, ETO-overexpressing mice or tissue-specific manipulation of this gene may provide insights in the future.

In addition to its role in adipogenesis C/EBPβ has been implicated in the regulation of diverse processes, including tumor development, neuronal survival, memory consolidation in the hippocampus, and myeloid differentiation (18, 22, 27, 41). Interestingly, C/EBPβ but not C/EBPα is capable of inducing myeloid differentiation of pluripotent hematopoietic progenitor cells (27). We have not investigated the effect of the leukemogenic AML1-ETO fusion protein on C/EBPβ activity. However, if it were similarly inhibitory, this may contribute to the development of AML in subjects bearing the AML1-ETO t(8;21) translocation via this mechanism. From a metabolic perspective analysis of knockout mice has revealed that loss of C/EBPβ protein throughout the body results in decreased epididymal fat mass, decreased gluconeogenesis and lipolysis during fasting, and diabetes and increased skeletal muscle insulin sensitivity (20, 36, 43). Having demonstrated its ability to inhibit C/EBPβ activity, the involvement of ETO in these fundamental C/EBPβ-regulated processes in both normal and disease states merits examination. We believe that this is likely to reveal further important physiological roles for ETO and may also suggest opportunities for therapeutic manipulation.

In summary, ETO acts in preadipocytes to inhibit C/EBPβ activity and promote the maintenance of the undifferentiated state. Its rapid downregulation by hormonal stimuli plays an essential role in coordinating the differentiation process. These findings define, for the first time, a precise function for ETO in normal cellular physiology, revealing its novel and important role in the regulation of adipogenesis.

ACKNOWLEDGMENTS

This study was supported by the Wellcome Trust (J.J.R., R.K.S., K.B.B., D.H., M.M., and S.O.R.), the Deutsche Forschungsgemeinschaft (M.L. and S.S.), the UK MRC (C.C., C.J.L., and C.M.), and the Raymond and Beverly Sackler Foundation (R.K.S.). J.K.S. is a BBSRC David Phillips Fellow.

We are particularly grateful to the members of the O'Rahilly and Siddle labs for many helpful discussions. Q.-Q. Tang and M. D. Lane generously provided C/EBPα promoter and C/EBPβ expression constructs. The C/EBPwt-LUC construct was kindly provided by S. Smola-Hess.

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PUBLIKATION 7

Meirhaeghe A, Crowley V, Lenaghan C, Lelliott C, Green K, Stewart A, Hart K, **Schinner S**, Sethi JK, Yeo G, Brand MD, Cortright RN, O'Rahilly S, Montague C, Vidal-Puig AJ.

Characterization of the human, mouse and rat PGC1 beta (peroxisome-proliferator-activated receptor-gamma co-activator 1 beta) gene in vitro and in vivo.

Biochem. J. 373(Pt 1): 155-65, (2003).

Characterization of the human, mouse and rat PGC1β (peroxisome-proliferator-activated receptor-γ co-activator 1β) gene *in vitro* and *in vivo*

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PGC1α is a co-activator involved in adaptive thermogenesis, fatty-acid oxidation and gluconeogenesis. We describe the identification of several isoforms of a new human PGC1α homologue, cloned independently and named PGC1β. The human PGC1β gene is localized to chromosome 5, has 13 exons and spans more than 78 kb. Two different 5′ and 3′ ends due to differential splicing were identified by rapid amplification of cDNA ends PCR and screening of human cDNA libraries. We show that PGC1β variants in humans, mice and rats are expressed predominantly in heart, brown adipose tissue, brain and skeletal muscle. PGC1β expression, unlike PGC1α, is not up-regulated in brown adipose tissue in response to cold or obesity. Fasting experiments showed that PGC1α, but not PGC1β, is induced in liver and this suggests that only PGC1α is involved in the hepatic gluconeogenesis. No changes in PGC1β gene expression were observed associated with exercise. Human PGC1β-1a and -2a isoforms localized to the cell nucleus and, specifically, the isoform PGC1β-1a co-activated peroxisome-proliferator-activated receptor-γ, -α and the thyroid hormone receptor β1. Finally, we show that ectopic expression PGC1β leads to increased mitochondrial number and basal oxygen consumption. These results suggest that PGC1β may play a role in constitutive adrenergic-independent mitochondrial biogenesis.

Key words: gene expression, genomic structure, isoform, mitochondrial biogenesis, peroxisome-proliferator-activated receptor-γ co-activator 1α (PGC1α), PGC1β.

INTRODUCTION

Nuclear receptor (NR) co-activators modulate transcriptional activity either by introducing enzymic modifications to the chromatin of promoter DNA or by facilitating the recruitment of the transcription machinery to specific promoters [1,2]. In addition, co-activators provide specificity by targeting tissue- and cell-type-specific subsets of NRs to specific promoters. Peroxisome-proliferator-activated receptor-γ co-activator 1α (PGC1α) is one such co-activator of the NR peroxisome-proliferator-activated receptor-γ (PPARγ), a transcription factor that plays key roles in processes such as adipogenesis [3] and energy expenditure [4]. PGC1α was described originally as a brown-fat-specific co-activator isolated through a yeast two-hybrid strategy using a PPARγ as bait and a brown fat cDNA library. Initial reports showed that PGC1α was expressed highly in brown adipose tissue (BAT) [4], and that PGC1α was the key factor that directed PPARγ towards the subset of genes that regulated a programme of adaptive thermogenesis. It was also speculated that another primary role of PGC1α was to co-activate PPARγ to induce the expression of genes leading to the differentiation of preadipocytes into brown adipocytes [4]. More recently, it has been shown that PGC1α demonstrates highly selective tissue expression and that it is capable of interacting with and co-activating other NRs, e.g. thyroid receptor (TR), hepatocyte nuclear factor (HNF)-4α [5,6], endoplasmic reticulum [7], PPARα [8], retinoid X receptors (RXRs) [9] and non-NR transcription factors such as nuclear respiratory factor 1 (NRF-1) [10] and myocyte-specific enhancer-binding factor 2 transcription factor [11], indicating that PGC1α is involved in a more broad spectrum of actions than suspected initially [12].

Regulation of the activity of NR co-activators may occur through different mechanisms including translocation between cytoplasm and nucleus, post-translational modifications, e.g. phosphorylation and acetylation, and regulated proteolysis [13]. PGC1α is also highly regulated at the transcriptional level in response to specific physiological stimuli which is unusual for a co-activator. For instance, cold exposure induces PGC1α gene expression in BAT and skeletal muscle. This is associated with stimulation of mitochondrial uncoupled respiration through the induction of uncoupling proteins [14,15] and activation of the mitochondrial biogenesis programme through its co-activating activity on NRF-1 [4,10]. PGC1α gene expression is also induced markedly during fasting in mouse hepatocytes through a mechanism that involves cAMP-response-element-binding protein phosphorylation [6]. PGC1α plays an important role in activating gluconeogenesis by co-activating HNF-4α

Abbreviations used: BAT, brown adipose tissue; CHO, Chinese-hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GFP, green fluorescent protein; GRE, glucocorticoid response element; HNF, hepatocyte nuclear factor; IRES, internal ribosomal entry; NR, nuclear receptor; NRF-1, nuclear respiratory factor 1; PERC, PGC1 (peroxisome-proliferator-activated receptor-γ co-activator 1)-related estrogen receptor co-activator; PPAR, peroxisome-proliferator-activated receptor; PRC, PGC-1-related co-activator; RACE, rapid amplification of cDNA ends; RPA, RNase protection assay; TEM, transmission electron microscopy; T_3, 3,3′,5-tri-iodothyronine; TR, thyroid receptor; RXR, retinoid X receptor.

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and glucocorticoid receptor on the phosphoenolpyruvate carboxykinase promoter [5,6]. Fasting also induces the expression of PGC1α in heart suggesting that it may contribute to fatty-acid oxidation through its co-activating action on PPARα [16,17]. Thus PGC1α is now considered a versatile co-activator [12] that plays important roles not only in adaptative thermogenesis in brown fat and skeletal muscle [4], but also in the gluconeogenic response associated with fasting [5,6].

Recently, two new co-activators have been added to this family. One of them is PGC1β [18] and the human orthologue of this is PGC-1-related estrogen receptor co-activator (PERC), which has been described as a selective co-activator of the oestrogen receptor α [19]. The second is PGC-1-related co-activator (PRC) [20], an ubiquitously expressed PGC1-related co-activator, which may play a role in modulating mitochondrial biogenesis during proliferation. This family of co-activators shares [21] maximum similarity at the N- and C-terminal regions, areas that carry most of the effector domains. Common elements in their structure are the NR boxes containing leucine-rich motifs (LXXLL domains), which mediate the interaction with hormone receptor-binding domains, an RNA-binding motif and, in PGC1α and PRC, a serine/arginine-rich domain that regulates RNA processing [22]. Recently, the presence of host cell factor motifs has also been described, which may link these co-activators with processes involving cellular proliferation/differentiation [18].

PGC1α provides the molecular basis for an integrated model of transcriptional control of metabolic-specific programmes. We speculated, independently, on the possible existence of other PGC1α homologues that may collaborate to integrate the same or other gene-specific transcription programmes. The present study was concerned with the novel human PGC1α homologue, which has also been reported as PERC and the mouse orthologue PGC1β. We report the human PGC1β cDNA sequence including several N- and C-terminal splice isoforms, which appear to be conserved in rodents. We describe the genomic structure of human PGC1β and the basis for its alternatively spliced variants. The PGC1β mRNA tissue distribution in humans, rats and mice is highly conserved and PGC1β, in contrast with PGC1α, does not seem to be regulated transcriptionally *in vivo* under the experimental conditions tested. Our results suggest that it is only PGC1α that is up-regulated in liver during fasting. *In vitro* results provide evidence that PGC1β is localized in the nucleus, where it co-activates several transcription factors. Moreover, when PGC1β is overexpressed ectopically in L6 myoblasts, there is an increased number of mitochondria and oxygen consumption suggesting that PGC1β may play a role in constitutive non-adrenergic-mediated mitochondrial biogenesis.

EXPERIMENTAL

Rapid amplification of cDNA ends (RACE) PCRs

The primer 5′-GTCACAAAGCGACCCAACTT-3′ (nt 1671–1691) was used for the 3′-RACE PCR using the human adipocyte Marathon Ready cDNA and the AP1 adaptor primer (ClonTech, Basingstoke, Hants. U.K.) in accordance with the manufacturer's instructions. Full-length cDNA and 5′ variation in human PGC1β were determined using the 5′/3′-RACE Kit (Roche Diagnostics, Basel, Switzerland). Total RNA from adult female human heart (Stratagene, Cedar Creek, TX, U.S.A.) was used for first-strand synthesis of cDNA with the primer 5′-CATCA-CAGAGCACGTCTTGAG-3′. Subsequently, an oligo(dT) primer (supplied by the manufacturer) and PGC1β-specific antisense primer 5′-CATGTAGCGTATGAGTTGCACCATC-3′ were used to amplify the sequence between exon 5 and the putative 5′ terminal, and this was followed by a further round of PCR using the oligo(dT) primer and a nested PGC1β exon 5′-specific primer 5′-GCCGAGAGGTGCTTATGTAGTTC-3′ (minimum expected size 605 bp). All PCR products were visualized on 1.2 % (w/v) agarose gel and all fragments $>$ 500 bp were purified subsequently and then cloned into pBlueScript (Stratagene) using standard techniques. Multiple clones were screened and sequenced. A second 5′-RACE using adult human subcutaneous abdominal mature adipocyte RNA for first-strand synthesis was also performed as described above (see the Results section).

Isolation of the full-length clone

We screened a human heart cDNA library master plate (Origene LHT-1001; OriGene Techologies, Rockville, MD, U.S.A.) with the PCR generated by the following oligonucleotides: 5′-GCCACTCGAAGGAACTTCAGAT-3′ (nt 2536–2558) and 5′-GGGTTAAGGCTGTTATCAATGC-3′ (nt 3003–3025 of the PGC1β-a isoform). Several wells were found to be positive and the subplates corresponding to those wells were obtained from OriGene Techologies. Clones containing PGC1β-a were identified and sequenced. This resulted in the isolation of the complete cDNA sequence for PGC1β-2a.

RNA extraction

Mice and rat RNAs were extracted from tissues using the STAT-60 method (Tel-Test 'B', Friendwood, TX, U.S.A.) or TRIzol® reagent (Gibco BRL, Invitrogen, Paisley, U.K.) according to the manufacturer's instructions. RNA concentration and integrity were determined using a spectrophotometer at 260 nm and an ethidium bromide-stained agarose gel respectively. Samples were stored at $-$ 80 °C until processed. Human RNAs were obtained from BioChain (Hayward, CA, U.S.A.).

RNase protection assay (RPA)

Probe synthesis

RPAs were performed as described previously [23–25]. The isoform-specific probes were amplified by PCR (see oligo-nucleotides and conditions in Table 1) and cloned into the vector pGEM-T easy (Promega) in the orientation 3′–5′. The identity of the sequence was confirmed by sequencing. The plasmids were linearized with *Spe*I. The ribonucleotide probe was synthesized with the T7 polymerase (Stratagene) using 300 ng of vector, 4 μl of [α-^{32}P]UTP (Amersham Biosciences), rATP, rGTP and rCTP. Probes were purified from mononucleotides by ethanol precipitation in the presence of 2.5 M ammonium acetate. Probe integrity was determined by autoradiography after electrophoresis on 4 % (w/v) polyacrylamide gel/7 % (w/v) urea-denaturing gel. The assays were done with 10 μg of RNA using 0.2–0.3 ng of PGC1α and PGC1β or 0.2–2 ng of cyclophilin-labelled probe (Ambion, Austin, TX, U.S.A.).

Solution hybridization RPA

RNA transcripts were quantified using solution hybridization RPA methods as described previously [25]. In brief, [^{32}P]UTP-labelled RNA probes were incubated with total RNA samples in a final volume of 25 μl of hybridization solution for 12–16 h at 30 °C. Samples defined as free probe include only RNA-labelled probe treated with RNases. Samples were then incubated for 1 h at 30 °C with a mixture of RNase A (40 μg/ml) and RNase

Table 1 RNAse Protection Probes

Abbreviation: WAT, white adipose tissue.

Species/name	Location in the gene	Size (bp)	Primer F (5′ → 3′)	Primer R (5′ → 3′)	Annealing temperature (°C)	Tissue of origin
Human						
Middle	Part of exon 5	300	CAAGACAAGAAGGCTCCCATGA	GCAGTAGGTGTGCATGTAGCGTA	53	WAT
C-terminus	Part of exon 10, exon 11a and part of exon 12 (52 bp of PGC-1βa)	252	AGTGTTTGGTGAGATTGAGGAGTGCGAGGT	TCATACTTGCTTTTCCCTGACGCAGGAAG	52	WAT
N-terminus	Part of exon 1a (110 bp = PGC-1β1 isoform) + 150 bp exon 2	260	ACTCCGCCGCACGCTGCAGC	GGCTGTACTGGTTGGCTTC	52	5′ RACE PCR on heart
Mouse						
C-terminus	Part of exon 10, exon 11a and part of exon 12 (49 bp of PGC-1βa)	252	GGTGTTCGGTGAGATTGTAGAGTGCCAGGT	TCGTACTTGCTTTTCCCAGATGAGGGAAG	52	Liver
Middle	Part of exon 8, exons 9, 10 and 11a	398	ATAGTCAGTGTATCTGG	CTCGTCCTGCAGCTCCTG	51	Liver
Rat						
N-terminus	56 bp 5′UTR + 204 bp exon 2 and part of exon 3	260	GCGGAAGGACTATCTCGG	CTCCATGTCAATCTGGAAG	51	Liver
C-terminus	Part of exon 10, exon 11a and part of exon 12 (59 bp of PGC-1βa)	263	AGGTGTTTGGTGAGATAGTCG	ATCCATGGCTTCGTACTTGC	53	Liver
Mouse						
PGC1α	Nucleotide 2091–2339	249	AGTTTTTGGTGAAATTGAGGAAT	TCATACTTGCTCTTGGTGGAAGC	55	Expression vector

T1 (2 μg/ml; Sigma). To inactivate the nucleases, samples were treated with 10 % (w/v) SDS (20 μl) and 10 mg/ml proteinase K (5 μl) for 20 min at 45 °C and extracted with phenol/chloroform. After precipitation with ethanol and dissolution in 7 μl of gel-loading buffer [25 % (w/v) Ficoll, 0.2 M EDTA (pH 8), 0.25 % Bromophenol Blue and 0.25 % Xylene Cyanole], samples were resolved on 5 % (w/v) non-denaturing polyacrylamide gels (0.75 mm thick) at 300 V for 2 h. Gels were mounted on 3 M (Whatman Inc., Clifton, NJ, U.S.A.) paper and dried. Protected bands were visualized by autoradiography and quantified by PhosphorImager analysis using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

In vivo experiments

Animals were housed in a temperature-controlled room with a 12 h:12 h light/dark cycle. Food and water were available *ad libitum* unless noted. All experiments were conducted in accordance with the Home Office Guidelines for the Care and Use of Laboratory Animals. Mice (C57BL6) and rats (Wistar and Zucker rats) were subjected to specific nutritional perturbation protocols as indicated in the Results section. High-fat diet experiments were performed in male rats, which had received RM1 chow pellets from Special Diet Services (Witham, Essex, U.K.) in addition to the stated diets. The low-fat diet was powdered RM1 diet from Special Diet Services (2–4 % fat) and the high-fat diet was Harlan Teklad TD 88137 (42 % fat). All animals remained on their diet for 10 weeks. Then, they were either fasted for 17–21 h or fed before being killed. Liver and heart tissues were taken. Fasting/refeeding experiments were performed in Wistar rats and C57BL6 mice. RNA from cold-exposure experiments was provided by Dr C. Y. Zhang (Harvard University Boston, MA, U.S.A.). The effect of exercise was tested using an acute endurance exercise protocol performed at East Carolina University. C57BL\6 wild-type mice were obtained from Harlan Laboratories (St. Louis, MO, U.S.A.). Twenty-six mice were randomly assigned to either a sedentary or exercised group and the latter was accustomed to treadmill running over 2 days. On the exercise day, the exercised animals were run for 3 h at a treadmill speed of 16–18 m/min at 0 % grade, rested for 1 h and then run again for 3 h. Animals were killed 12 h later by CO_2 asphyxiation and whole gastrocnemius was removed (within seconds), immediately frozen with liquid nitrogen-cooled stainless-steel tongs, and stored at − 80 °C for subsequent mRNA extraction.

In vitro experiments

Transient transfections

HepG2 cells were maintained with Dulbecco's modified Eagle's medium (DMEM), 4.5 g of glucose/l (Sigma), 10 % foetal bovine serum (FBS) and penicillin/streptomycin. Cells were split into 24-well plates and transfected 8 h later in the same medium using FuGENE (Roche, Basel, Switzerland). A ratio of 3 μl FuGENE/1 μg DNA was used for all the transfections. The transfection efficiency was estimated by co-transfecting the vector pRL-CMV (Promega) that encodes the *Renilla* luciferase gene. Fifteen hours after transfection, the medium was removed, the cells were washed once with PBS and fresh medium containing the adequate ligands was added for 30 h. Then, the cells were harvested and both firefly and *Renilla* luciferase activities were measured into 20 μl of lysate according to the manufacturer's instructions (Dual Luciferase assay; Promega). The compound GW327647 was obtained from GlaxoSmithKline (Glaxo Wellcome UK Ltd., Stockley Park West, Uxbridge, Middlesex, U.K.) and used at 100 nM as a specific PPARα ligand. The compound LG100268 (at 100 nM) was used as an RXRα agonist and BRL49653 (at 100 nM) was used as a PPARγ agonist. In the case of TRβ1, 3,3′,5-tri-iodothyronine (T_3) was used at 100 nM and 1 μM. Chinese-hamster ovary (CHO) cells were maintained in Ham-F12 medium (Sigma) plus 10 % (v/v) FBS and penicillin–streptomycin. CHO cells in a six-well plate were transfected with 1.5 μg of pGEN GFP-PGC-1β or pGEN GFP (where GFP stands for green fluorescent protein) alone. The fluorescence was assessed 48 h later under a microscope.

The HeLa cell line was transfected stably with a glucocorticoid response element (GRE)-β-galactosidase reporter construct. The resultant cell line, termed GRE 4-β-gal HeLa, was maintained in

DMEM with 10% charcoal-stripped FBS and gentamicin. The GRE-β-gal reporter cell line was transfected transiently using a calcium phosphate transfection kit (Invitrogen, Carlsbad, CA, U.S.A.) according to the standard method. A total of 20 μg of DNA was transfected per T75 flask of cells at 50% confluency. Control transfections with a luciferase-control plasmid, pGL3 (Promega), to monitor transfection efficiency and pcDNA3 carrier DNA were compared with transfections containing pcDNA3-PGC1β-1a, pGL3 and carrier pcDNA3. After 24 h, the transfected transiently cells were seeded at 1×10^5 cells/well into a 96-well plate. After 2 h, cells were stimulated with a submaximal concentration (1 nM) of dexamethasone or left unstimulated. At 48 h post-transfection, the cells were assayed for β-galactosidase and firefly luciferase using a Dual-Light kit (Tropix, Bedford, MA, U.S.A.).

Plasmid constructs

Full-length human PGC1β-1a and -2a cDNA clones were obtained by ligating the respective 5′ ends of PGC1β cDNAs generated by 5′-RACE (5′-PGC1β-1a and -2a) to the PGC1β-a 3′ isoform isolated from human heart cDNA library (as outlined above). In essence, amplicons of 412 and 578 bp incorporating an *Eco*RI restriction digest site were generated by PCR using *Pfu* turbo as polymerase and pBS-5′-PGC1β-1a and -PGC1β-1 as respective templates. These amplicons extended from exon 3 to the 5′-terminus on exon 1a, common to both PGC1β-1a and -2a. Both amplicons underwent subsequent sequential restriction digest with *Eco*RI and *Psh*AI, followed by ligation into *Eco*RI–*Psh*AI sites of pGEN-IRES-PGC1β-1a (where IRES stands for internal ribosomal entry). A further digest of both pGEN-IRES-PGC1β-1a and PGC1β-2a using an *Eco*RI–*Xba*I combination was followed by ligation into *Eco*RI–*Xba*I sites of pcDNA3, thus generating isoform-specific vectors for use in transient transfection assay systems.

To generate the retroviral expression vectors, *Eco*RI–*Xba*I inserts containing full-length PGC1β-1a and -2a were released from both pGEN-IRES-PGC1β-1a and -2a respectively, and treated with T4 DNA polymerase to generate blunt-ended fragments. A subsequent blunt-ended ligation of the respective PGC1β-1a and -2a *Eco*RI–*Xba*I fragment*s* into the *Sna*B1 site of pBabe-puromycin (a gift from Dr B. M. Spiegelman, Harvard University) was performed using standard methods. pBabe-mPGC1α was generated by initial ligation of a *Sma*I–*Not*I fragment from pSV-Sport-mPGC1α (a gift from Dr B. M. Spiegelman) with subsequent blunt-ended ligation of this fragment into the *Sna*BI site of pBabe-puromycin, as described above. All plasmid constructs were verified by direct sequencing.

Myoblast cell culture and retroviral infection

Rat L6 myoblast cell lines (a gift from Dr Justin Rochford, University of Cambridge, Cambridge, U.K.) were maintained in DMEM containing 10% FBS (Sigma). To generate cell lines stably expressing the construct of interest, these myoblasts were infected with retrovirus containing either pBabe-puromycin, pBabe-mPGC1α, pBabe-hPGC1β-1a or pBabe-PGC1β-2a as appropriate, prepared as per the methods described previously [4]. Following selection with puromycin (4 μg/ml), the residual virally infected cells were maintained in culture. To induce myotube differentiation, these stable myoblasts were grown to confluence and cultured subsequently in minimum essential medium α + Glutamix (Gibco BRL) medium with 2% (v/v) horse serum added for a period of 5 days.

Transmission electron microscopy (TEM)

Cells were prepared for ultra-structural analysis using TEM as described previously [26]. Briefly, the cells were fixed in 2.5% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h at room temperature (24 °C). After washing the cells in 0.1 M sodium cacodylate buffer, the cells were fixed in 1% (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at room temperature. The cells were then washed as before, dehydrated through a graded ethanol series and infiltrated with Agar 100 resin, before embedding and curing in Agar 100 at 60 °C for 48 h. Sections were cut on a Leica Ultracut microtome, contrasted with 3% (w/v) aqueous uranyl acetate and lead citrate, and viewed using a Philips CM 100 TEM. We used point counting to calculate the percentage of cell volume containing mitochondria [27]. Using AnalySis image analysis software (SIS, Scientific Imaging Software; Scanalytics, Fairfax, VA, U.S.A.) in conjunction with a MegaView digital camera on the TEM, a fixed 1000 μm $\times$ 1000 μm grid was placed over the images and mitochondria that lay on any intersecting points of the grid were scored. This gave the ratio of mitochondria per unit cytoplasm and, hence, the percentage of the cell containing mitochondria.

Measurement of oxygen consumption in rat L6 myoblasts

For oxygen consumption experiments, cells were detached from plates using trypsin and then centrifuged and resuspended in DMEM containing 25 mM Hepes. The suspension was transferred immediately to the 1 ml chamber of a Clarke-type oxygen electrode maintained at 37 °C. Oxygen consumption readings were recorded using Powerlab (AD Instruments, Charlotte, NC, U.S.A.). The uninhibited rate was determined by measuring the linear rate of oxygen consumption. Oligomycin (5 μg/ml) and 4 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were added sequentially to the chamber, and the oxygen consumption was recorded after each addition. Myxothiazol (5 μM)-inhibited rates were extremely low (< 1% basal) and similar for each cell type, excluding the possibility that non-mitochondrial oxygen consumption rates differed between cell lines. The myxothiazol-inhibited rate was subtracted from the basal, oligomycin and FCCP rates. Once measurements were complete, cells were collected from the chamber and counted using a haemocytometer (Neubauer Improved; Weber Scientific, Hamilton, NJ, U.S.A.), taking the average of two counts of over 50 cells.

RESULTS

Characterization of human, mouse and rat PGC1β cDNA and protein

From the Incyte LifeSeq Gold database, we identified a cDNA sequence derived from a human adipose tissue library with 30% homology to the C-terminus of human and mouse PGC1α (Incyte clone ID no. 1956431). This clone was shown subsequently to be similar to PERC [19] and to be orthologous to the mouse gene PGC1β [18]. As this Incyte clone was incomplete, and also to identify potential new 3′-splice variants of PGC1β, a 3′-RACE PCR analysis was conducted. A 1.5 kb PCR product was amplified from human adipose tissue cDNA and sequenced. This 3′-RACE product was found to be similar but not identical at the most 3′-end when compared with the original Incyte clone (Figure 1A). Consequently, we determined that at least two C-terminal variants of human PGC1β exist and these have been termed PGC1β-a and -b based on sequence homology with

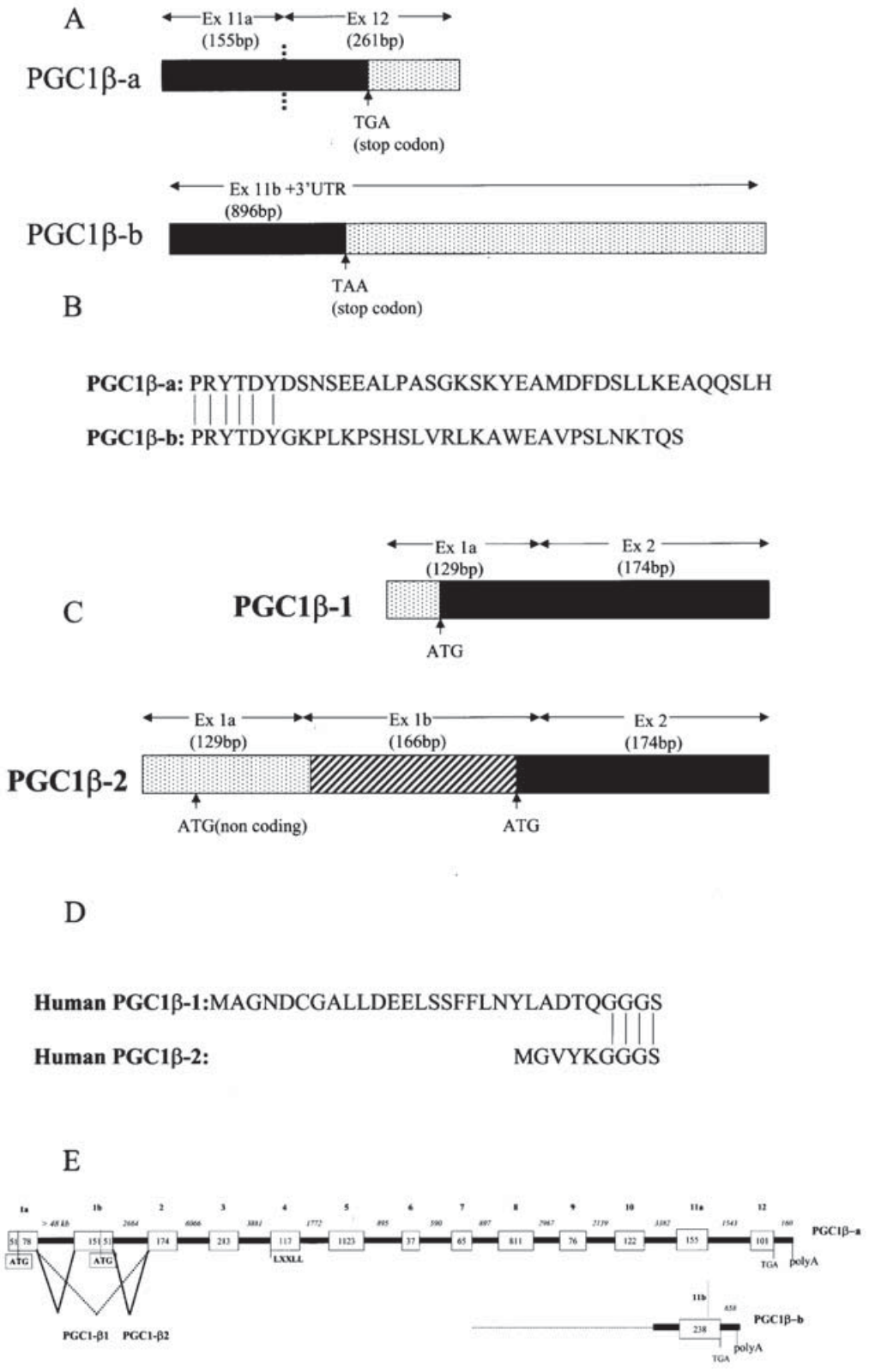


Figure 1 Characterization of the human PGC1β cDNA and protein

(**A**) Schematic diagram outlining the 3′-isoform cDNA sequences of PGC1β identified using 3′-RACE PCR; the coding region is represented by solid boxes, the non-coding region of exons 11b and 12 are both represented by dotted boxes. Alternative splice site of exon 11 is illustrated by the broken line. (**B**) Alignment of amino acid sequences of the C-terminus of PGC1β-a and -b. (**C**) Schematic diagram illustrating the 5′-isoform cDNA sequences of PGC1β-1 and -2 isolated using 5′-RACE PCR; coding region is represented by solid boxes, the non-coding region of exon 1a by the dotted box and the non-coding region of exon 1b by the hatched box. Note that the translational start site in exon 1a is not in-frame in the longer PGC1β-2 isoform. (**D**) Alignment of the amino acid sequences of the N-termini of PGC1β-1 and-2. (**E**) Putative genomic organization of PGC1β gene. Exons are represented as open boxes and introns as solid black lines. The size of the introns are indicated in italics above the introns. The size (bp) of the exons are indicated in the boxes. The LXXLL motif is positioned in exon 4.

PGC-1α (see below). Figure 1(B) illustrates that at the extreme C-terminus, PGC1β-a encodes 33 amino acids not found in PGC1β-b, whereas the latter isoform encodes 27 amino acids, which differ with respect to PGC1β-a. Moreover, sequence alignment reveals no similarity between the two isoforms at this site.

Since the expression of PGC1β was more abundant in heart (see below), we screened a human heart cDNA library for PGC1β clones. A clone containing the putative full-length cDNA of PGC1β-a was isolated and sequenced. We also proceeded to characterize the N-terminus of PGC1β using 5′-RACE PCR on human heart and adipose-tissue total RNA. Direct sequencing of cDNAs identified by 5′-RACE (see the Experimental section) revealed two distinct isoforms of PGC1β with variation at the N-terminus. More specifically, all clones isolated during the analysis using total RNA from human heart represented a single isoform (PGC1β-1), whereas the analysis using RNA from human subcutaneous mature adipocytes identified a second, longer cDNA (PGC1β-2) containing an extra 166 nt sequence, in addition to identifying the PGC1β-1 isoform (Figures 1C and 1D).

In conjunction with the 5′- and 3′-RACE, we utilized the NCBI BLAST program to search for PGC1β cDNA sequence homologues in the human genome database and located an exact match on a chromosome 5 BAC clone (AC008545, clone CTC-500L4). Thus a putative full-genomic structure of human PGC-1β gene was determined and this indicated that the gene consists of 13 exons and spans more than 78 kb (Figure 1E). Only exon 1a could not be found on the chromosome 5 BAC clone, suggesting that intron 1a is greater than 48 kb. Moreover, this genomic organization suggests strongly that exon 1b can be spliced out, generating the PGC1β-1 isoform, whereas PGC1β-a and -b are generated by alternative splicing of exon 11. Interestingly, the putative translational start site for PGC1β-1 is also present in the PGC1β-2 sequence, although in the latter it does not provide an open reading frame. However, PGC1β-2 does feature a second downstream in-frame translational start site (Figure 1C). The PGC1β-1 transcript generates a protein of 1023 and 1017 amino acids when paired with the a and b 3′-isoforms respectively, whereas the longer PGC1β-2 transcript actually encodes a protein 21 amino acids shorter than PGC1β-1 when paired with the same respective C-termini (3′a, 1002 and 3′b, 996 amino acids). Overall, the N-termini of PGC1β-1 and -2 differ with respect to each other by 26 amino acids (Figure 1D).

Although there is homology between PGC1α and PGC1β over the whole molecule, the greatest degree of homology is found at the N- and C-termini. The N-terminus of PGC1α contains an activation domain, which includes two LXXLL motifs. Human PGC1β shares between 40 and 50% identity with the first 180 amino acids of PGC1α. Whereas this region of human PGC1β contains two LXXLL motifs, in rat and mouse there is an additional LXXLL motif in the N-terminus. The C-terminus of PGC1α is thought to contribute to this RNA-binding activity of the molecule. There is a high degree of homology (50%) between PGC1α and PGC1β in this region, particularly for the PGC1β-a isoform. In addition to the NR interaction domains, PGC1β also contains two polyglutamine regions, a serine-rich motif and a putative host cell factor-binding site.

Homology searching using the human PGC1β cDNA sequence identified a rat clone in the Incyte ZooSeq database that had a high level of homology to PGC1β. Further sequencing of this clone led to the identification of a full-length cDNA for rat PGC1β that corresponded to the PGC1β-2a isoform. At the protein level, the human PGC1β had 78% identity with the mouse and rat. Mouse and rat shared 93% identity. The RNA recognition motif in the C-terminus is the most conserved domain.

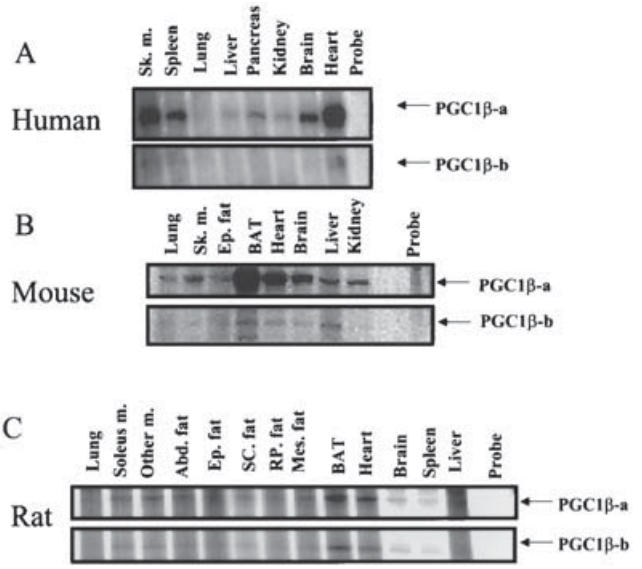


Figure 2 Tissue distribution of PGC1β

PGC1β gene expression was analysed in human (**A**), mouse (**B**) and rat (**C**) using a C-terminal RNase protection probe that allows the distinction between the 3′ variants PGC1β-a and -b. Aliquots of 10 μg of RNA were used to assay for the expression of PGC1β in these tissues. Sk.m., skeletal muscle; Ep. fat, epididymal fat; Mes. fat, mesenteric fat; RP. fat, retro-peritoneal fat; SC. fat, sub-cutaneous fat; Abd. fat, abdominal fat; m., muscle.

In vivo studies

Tissue distribution of PGC1β in human, mouse and rats

The tissue distribution of PGC1β in human, mouse and rat was assessed by RPA. PGC1β is expressed predominantly in human heart, skeletal muscle and brain (Figure 2A) although it can be detected at low levels in most other tissues, including kidney, pancreas, liver and spleen. By using a probe comprising part of exon 11a, the exon specific to the PGC1β-a isoform, as well as a region common to both isoforms, we confirmed that the PGC1β-a isoform is more abundant than PGC1β-b (Figure 2A) in human. Using a similar approach (a probe spaning exons 1a and 2), we observed that PGC1β-1 mRNA was the predominantly expressed isoform (results not shown). These results indicate that the most common isoform in human tissues is PGC1β-1a.

The pattern of expression of PGC1β in rodents and humans is conserved. Mouse PGC1β is expressed at high levels in heart, BAT and brain although it can be detected at low levels in most tissues (Figure 2B). Similar to humans, mouse PGC1β-a is much more abundant than PGC1β-b (Figure 2B). Rats also share a similar tissue distribution; however they differ in that PGC1β-a and -b isoforms are expressed at comparable levels (Figure 2C).

In vivo regulation of PGC1β gene expression in rodents

We explored whether expression of PGC1β may be regulated *in vivo* by pathophysiological-specific conditions known to affect the expression of PGC1α. Using the obese Zucker ($-/-$) and lean ($+/-$) rat models, we observed that obesity is not associated with changes in PGC1β mRNA expression or in the relative amount of its specific isoforms (Figure 3A).

Since it was suggested previously that induction of PGC1β in liver during fasting may be important for hepatic gluconeogenesis [18], we investigated the effects of fasting on PGC1β mRNA

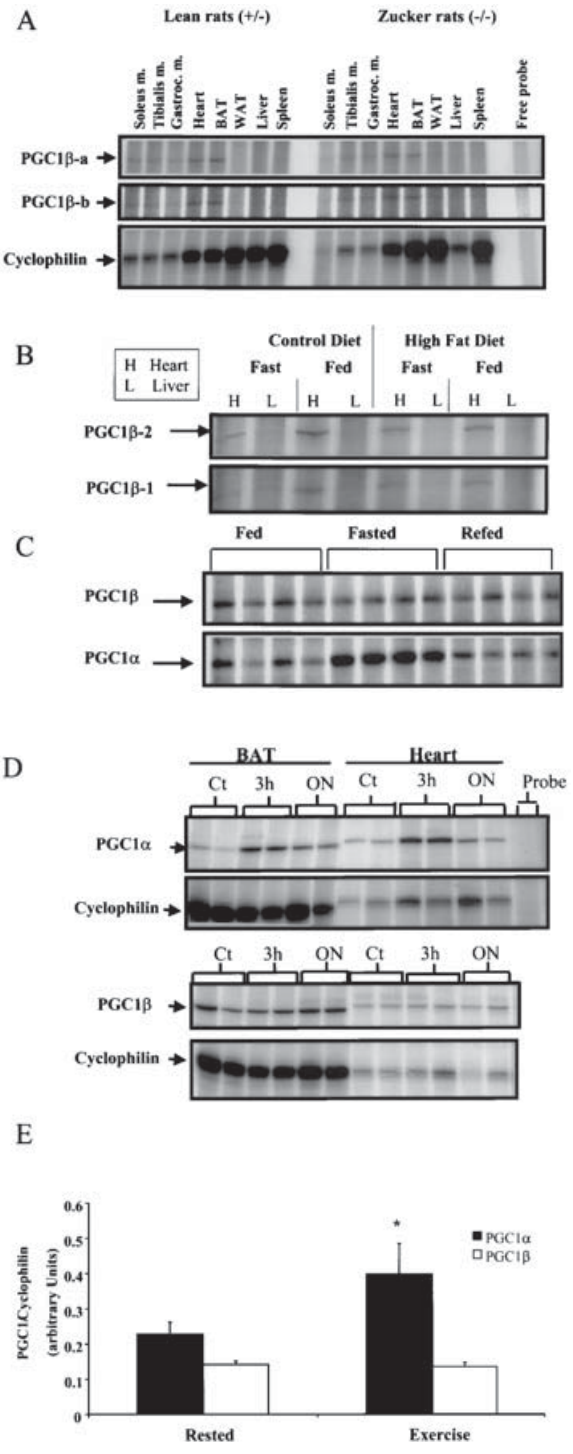


Figure 3 Regulation of PGC1β gene expression *in vivo*

(**A**) Analysis of PGC1β gene expression in tissues from lean and obese rats using an RPA probe that allows discrimination of the C-terminal isoforms ($n = 2$). WAT, white adipose tissue; m., muscle. (**B**) Effect of 24 h fasting on PGC1β gene expression in liver and heart of rats that have been previously on normal or high-fat diet. We illustrate this effect using a probe located at the N-terminus that allows discrimination between the 5′ variants PGC1β-1 and -2 ($n = 3$ animals per condition). (**C**) Comparison of PGC1α and PGC1β gene expression in liver ($n = 4$, per condition) following 24 h fasting/refeeding. The results presented in this Figure were obtained using an RPA probe covering a region common to all isoforms of PGC1β. (**D**) Effect of cold exposure [3 h and overnight (ON)] on PGC1α and PGC1β mRNA expression in mice detected by RPA. At least two animals per group and condition were analysed. (**E**) Effect of acute exercise on PGC1α and PGC1β gene expression in mouse gastrocnemius muscle. $n = 13$ mice per group and condition (*level of significance, $P < 0.05$).

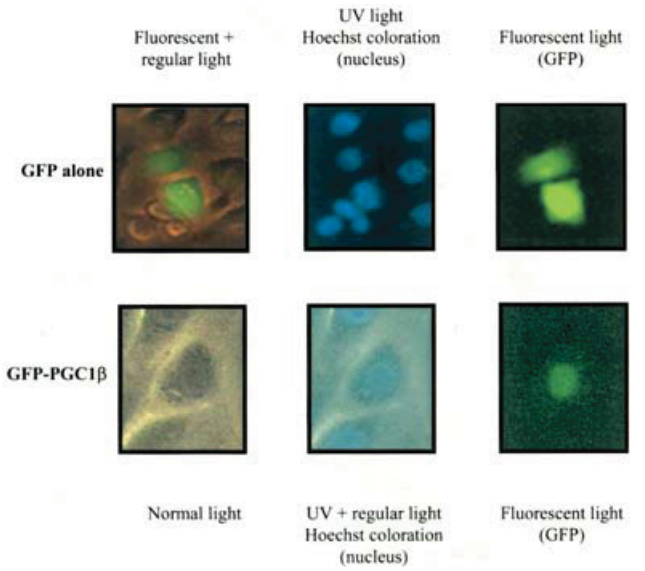


Figure 4 Cellular localization of PGC1β

Fluorescence images taken from CHO cells expressing GFP alone or GFP-PGC1β (green fluorescence). The superposition of the Hoechst coloration and the green fluorescent light in the case of the GFP-PGC1β construct confirms that PGC1β is localized into the nucleus.

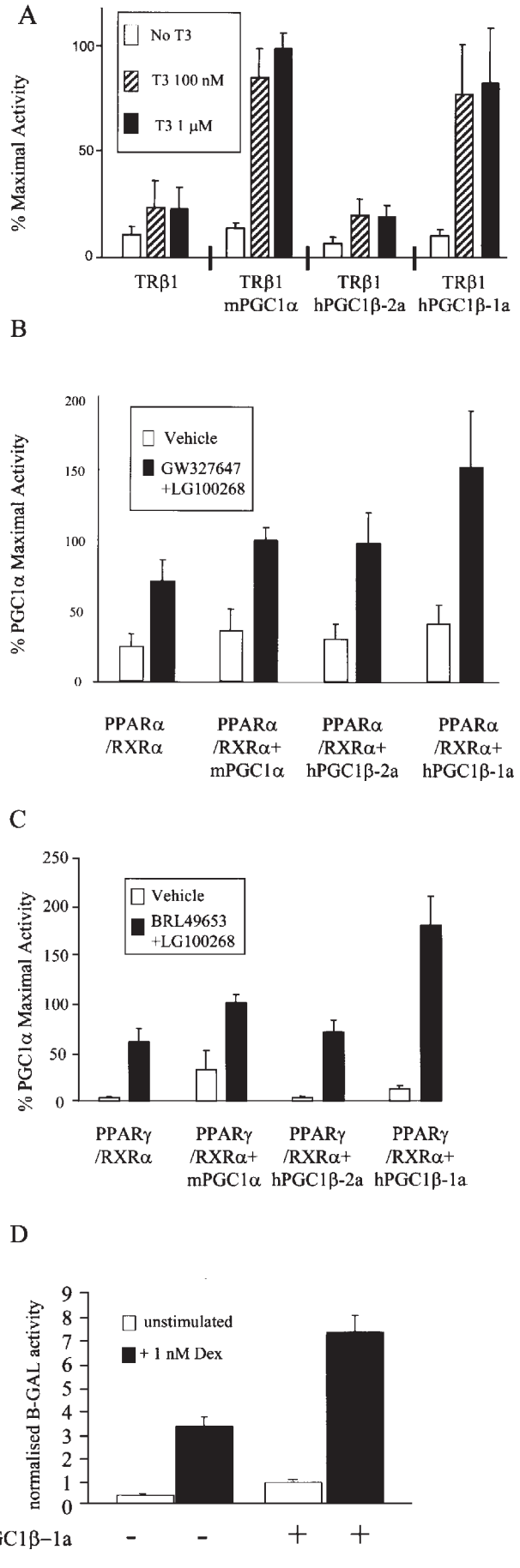
expression in rodent tissues. Initially, we examined the expression of PGC1β-1 in heart and liver of Wistar rats either fed or fasted for 17–21 h before tissue collection. We performed this experiment in animals fed previously with either a high-fat (42 %) or low-fat diet (2–4 %) for 10 weeks. No differences in PGC1β between fed and fasted rats (Figure 3B) were observed. Given the possibility that differences with previous reports were due to different technical approaches, we performed Northern blots using probes for PGC1β and PGC1α. Unlike PGC1α, the expression of PGC1β was not up-regulated by fasting in rat liver (results not shown). To determine whether there were species-related differences, we simultaneously measured PGC1α and PGC1β mRNA expressions in liver RNA from mice fasted for 24 h (Figure 3C). We confirmed that PGC1α isoform is markedly induced in liver during fasting, whereas PGC1β gene expression was unaltered during fasting in mice.

Next we examined the effect of exposure to cold on PGC1α and PGC1β expressions. Mice were exposed to cold (4 °C) for 3 h or overnight. As expected, the expression of PGC1α was up-regulated by exposure to cold in BAT and heart (Figure 3D). Conversely, the expression of PGC1β in those tissues was not altered by exposure to cold (Figure 3D). Finally, we evaluated whether acute endurance exercise may have any impact on the expression of PGC1α and PGC2β genes. We observed that whereas PGC1α mRNA was induced in exercised mice the expression of PGC1β was not affected (Figure 3E).

In vitro studies

Cellular localization and co-activation studies

Full-length cDNAs of PGC1β-1a or -2a were cloned in fusion with the GFP protein and transfected into CHO cells. Whereas cells transfected with GFP control plasmid showed green fluorescence signal in the cytoplasm, cells transfected with PGC1β-1a/GFP fusion plasmid showed green fluorescence restricted to the nucleus indicative of PGC1β-1a protein expression (Figure 4). Similar results were obtained for PGC1β-2a (results not shown).



We tested whether PGC1β could co-activate TRβ1, PPARα/RXRα and PPARγ/RXRα in HepG2 cells and compared the results with PGC1α. We used either a TR response element or the PPAR response element of the acyl-CoA oxidase gene cloned upstream of the luciferase gene to assess the transactivation efficiency of TRβ1 or PPARα/γ respectively. PGC1α and PGC1β-1a were capable of co-activating TRβ1, leading to a 4-fold increase in transactivation in the presence of T_3 at either 100 nM or 1 μM concentration (Figure 5A). Conversely, PGC1β-2a did not exert any co-activation effect despite producing a protein, which was targeted to the cell nucleus according to the GFP signal. Co-activation studies of PPARα (Figure 5B) or PPARγ (Figure 5C) and their corresponding ligands showed that PGC1a had a positive effect although less than the one observed with TRβ1 (1.5-fold) and that PGC1β-1a was a stronger co-activator than PGC1α (2-fold). Unlike PGC1α, PGC1β-1a or -2a did not exert any active transcriptional function in the absence of NRs. To explore whether PGC1β may co-activate the glucocorticoid receptor we used HeLa cells transfected stably with a GRE-β-galactosidase reporter construct. Co-transfection of these cells with PGC1β-1a increased the galactosidase response to dexamethasone (Figure 5D).

Ectopic expression of PGC1β-1a induces mitochondrial biogenesis and increases basal oxygen consumption in L6 myoblasts

Retroviral vectors were utilized to express both mPGC1α and hPGC1β-1a in rat L6 myoblasts and investigate the potential role of PGC1β in regulating mitochondrial biogenesis. TEM on thin sections prepared from these cell lines revealed an obvious proliferation of mitochondria in hPGC1β-1a cells relative to the empty vector control cells (Figure 6A). An increase in mitochondrial number was also evident in mPGC1α expressing myoblasts, although to a lesser intensity when compared with the hPGC1β-1a cells (Figure 6A). The overall mitochondrial morphology and structure did not appear to differ between the various cell lines. The enhanced mitochondrial number is also reflected in a quantitative assessment of cell mitochondrial density (based on calculations from ten randomly selected cells for each myoblast line; performed in duplicate experiments), which indicates clearly that hPGC1β-1a and mPGC1α expressions have a 2.7- and 2.0-fold higher cell mitochondrial density respectively when compared with controls (Figure 6B). Moreover, the hPGC1β-1a and mPGC1α-induced mitochondrial biogenesis is further supported by the observed >2.0-fold increase in basal oxygen consumption in the respective cell lines relative to non-expressing controls (Figure 6C). The increase in basal oxygen consumption of

Figure 5 Co-activation studies

(A–C) HepG2 cells were transiently co-transfected with FuGENE (Roche) using a ratio of 3 μl for 1 μg of DNA. The human pcDNA3-PGC1β-1a or -2a constructs were used. The mouse pcDNA3-PGC1α construct was used as a comparison. The pRL-CMV vector (Promega) was co-transfected as a normalized control. Luciferase assays were performed 40 h after transfection using the Dual Luciferase Assay system. In the case of TRβ1, T_3 was used at 100 nM or 1 μM. For PPARα, the compound GW327647 was used at 100 nM. The RXRα agonist was the compound LG100268 (at 100 nM) and the BRL49653 was used as a PPARγ agonist at 100 nM. These results represent five independent experiments ($n=5$). m, mouse; h, human. (**D**) GRE 4-β-gal HeLa cells transfected transiently using the calcium phosphate method with a luciferase control plasmid, pGL3 (Promega), to monitor transfection efficiency. pcDNA3 carrier DNA was compared with transfections containing pcDNA3-PGC1β-1a, pGL3 and carrier pcDNA3. After 2 h, cells were stimulated with a submaximal concentration (1 nM) of dexamethasone (Dex) or left unstimulated. At 48 h post-transfection, the cells were assayed for β-galactosidase and firefly luciferase using a Dual-Light kit (Tropix). These results represent four independent experiments ($n=4$).

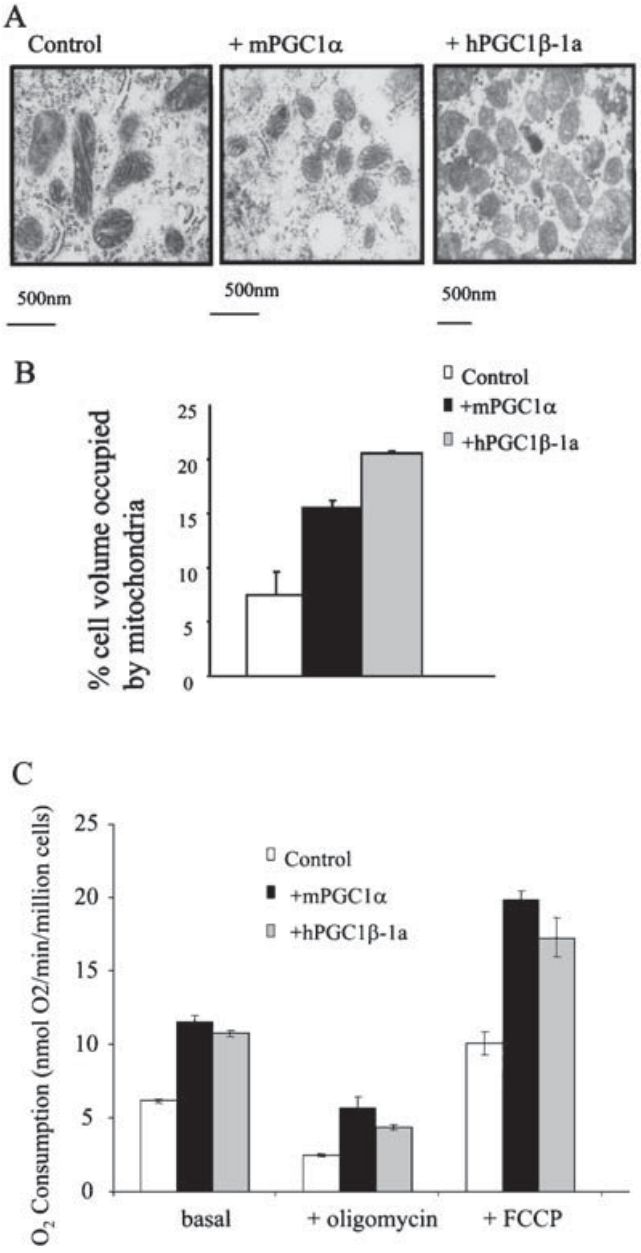


Figure 6 Electron microscopy and oxygen consumption studies

(**A**, **B**) Ultrastructural analysis using TEM of cells ectopically overexpressing PGC1α, PGC1β-1a or empty vector. The percentage of cell volume containing mitochondria was calculated using AnalySis image analysis software (SIS, U.S.A.) in conjunction with a MegaView digital camera on the TEM and a 1000 μm $\times$ 1000 μm grid placed over the images. Scale bar, 500 nM. (**C**) Oxygen consumption in myoblasts expressing mPGC1α and hPGC1β-1a. Oxygen consumption rates were measured for myoblasts retrovirally expressing an empty expression vector (white bars), mPGC1α (black bars) or hPGC1β-1a (grey bars). Oxygen consumption was measured in the absence of inhibitors (basal) or in the presence of oligomycin and FCCP. Rates were normalized to cell number and measurements made in triplicate. Results are expressed as means $\pm$ S.E.M. These results represent three independent experiments, with two or three repeats of each condition per experiment.

hPGC1β-1a and mPGC1α-expressing cells was preserved in the presence of both oligomycin and FCCP (Figure 6C). This implies strongly that increased mitochondrial density and/or electron-transport chain activity is responsible for the observed increase in oxygen consumption.

DISCUSSION

We have cloned the full-length human, mouse and rat PGC1β/PERC, a homologue of PGC1α co-activator, and characterized the multiple isoforms *in vivo* and *in vitro*. PGC1α has received recent attention as a co-ordinator of transcription factors involved in metabolic responses such as adaptive thermogenesis [4], fatty-acid oxidation [8], mitochondrial biogenesis [10], muscle fibre-type-specific differentiation [28] or gluconeogenesis [6] in rodents. Given the importance of PGC1α as a master integrator of all these complex metabolic programmes, we investigated the potential metabolic roles of this new homologue, PGC1β.

The human PGC1β gene, also reported as PERC [19], consists of 13 exons spanning a genomic region of at least 78 kb on chromosome 5. Exon sizes ranged from 37 nt (exon 6) to 1123 nt (exon 5). The human PGC-1β protein shares several motifs with PGC1α: (i) an RNA recognition motif located at the C-terminus encompassing an RNA-binding region found in proteins having a role in the pre-mRNA maturation and splicing. Such a role has been demonstrated for PGC1α [22] and is likely to be retained by PGC1β; (ii) two polyglutamate tracts found usually in cofactors as proteins rich in polar amino acids can bind to these tracts. Interestingly, neither PGC1α nor PRC carry such motifs but PERC does [19] and (iii) two LXXLL motifs, necessary for interactions between the co-activators and liganded NRs, also found in PGC1α. It is worth noting that there are only two LXXLL motifs in the human PGC1β gene compared with three in the mouse and rat PGC1β genes and one in the mouse and human PGC1α gene and this may lead to species-specific functional differences.

PGC1β, similar to PGC1α, is expressed predominantly in brown fat, heart, brain and muscle, important metabolic tissues, characterized by their high mitochondria content. PGC1β is also expressed at low levels in most tissues. Interestingly, the amount of PGC1β in a specific tissue appears to be proportional to its mitochondrial content. We have identified several PGC1β isoforms resulting from the alternative splicing of exons located at the N- and C-termini. The isoform, which we have named PGC1β-1a, was the most common isoform in humans and mice and corresponded to the isoform identified initially by Lin et al. [18]. The other isoforms were also ubiquitously expressed, although at much lower levels, in humans and mice. However, in rats, the spliced form PGC1β-2b was expressed at a level comparable with PGC1β-1a, suggesting that these alternative isoforms may be more relevant in rodent species.

The regulation of PGC1β and PGC1α gene expression *in vivo* was markedly different. The PGC1α gene expression is highly regulated at the transcriptional level. In fact, it has been shown to be markedly induced in specific tissues by cold exposure [4], adrenergic stimulation [29], fasting [5,6,16] or exercise [30,31]. In contrast, we have shown that none of these conditions produced changes in the expression of PGC1β or changes in the relative expression of its specific isoforms. Our preliminary experiments clearly identify PGC1α as the only isoform up-regulated in response to exercise, suggesting that, specifically, PGC1α may be responsible for the muscle mitochondrial oxidative metabolic adaptation to training [32]. As a whole, these results indicate that only PGC1α appears to be regulated transcriptionally, at least under the current experimental protocols. However, there is still the possibility that, similar to PGC1α, in the white adipose tissue of the 4E-BP1-knockout mice [33], PGC1β may be regulated mainly at the translational level. Thus our results show that PGC1β, like other co-activators, does not seem to be regulated transcriptionally *in vivo*. The lack of transcriptional regulation, together with its relatively widespread tissue

distribution, suggests that PGC1β may fulfil a more general role, such as maintaining the mitochondria biogenesis necessary to cover basal energy requirements of a tissue. Conversely, PGC1α, which is regulated transcriptionally by pathophysiological stimuli related to acute increased energy demands, states of high fatty-acid oxidation and gluconeogenesis may be more relevant as a co-ordinator of acute metabolic adaptations to increases in energy demand.

Our studies *in vitro*, in agreement with the PERC studies [19], showed that human PGC1β isoforms localize to the nucleus. We provide evidence that PGC1β-1a, the most abundant isoform, co-activates several NRs including the TR, PPARα and PPARγ. Also our results indicate that PGC1β-1a does co-activate the glucocorticoid receptor. The other 5′ isoform PGC1β-2a did not co-activate these transcription factors suggesting the possibility that the N-terminus may include important domains for the co-activation of these transcription factors. This observation is particularly interesting since the main transactivation domains of PGC1α have been mapped to the N-terminal 120 amino acids [21,34]. Although we have not been able to study other transcription factors, studies from other laboratories have confirmed that the mouse PGC1β, and specifically the isoform homologous with the human PGC1β-1a, also co-activates the oestrogen receptor α [19], HNF-4α and glucocorticoid receptors [18]. Interestingly, Lin et al. [18] showed that mPGC1β exerts a potent co-activating effect on NRF-1, a key transcription factor involved in mitochondrial biogenesis. Altogether, these results suggest that like PGC1α, PGC1β may also have a broad spectrum of actions. Given its predominant expression in tissues that express high levels of PPARα and mitochondria, it would be expected that the effect of PGC1β would involve promotion/maintenance of fatty-acid oxidation and mitochondrial biogenesis. Our co-activation studies also showed that PGC1β potentiates the transcription from the thyroid hormone receptor β1, an effect that was strong, particularly compared with the other NRs we studied. Thus it is likely that PGC1β may be an important co-activator integrating some of the actions of thyroid hormone in energy expenditure and mitochondrial biogenesis. The widespread action of thyroid hormones and the relative wide tissue distribution of PGC1β give support to this possibility.

In an attempt to clarify the function of PGC1β, we over-expressed PGC1β in myoblast cells using a retrovirus-mediated gene transfer strategy. The initial characterization of these cells has shown that overexpression of PGC1β is associated with an increase in the number of mitochondria, which correlated with increased oxygen consumption. The basal rate of oxygen consumption is however a complex function of mitochondrial density, rate of cellular ATP turnover and the rates at which protons are actively pumped across the inner mitochondrial membrane (by the electron-transport chain) and passively leak back across it (e.g. via uncoupling proteins). We eliminated the contribution of ATP turnover to oxygen consumption using oligomycin, which prevents ATP formation by blocking proton translocation through the ATP synthase. Differences in passive proton leak were also removed using the uncoupler FCCP, which dissipates the mitochondrial protonmotive force. Since the approx. 2-fold increase in basal oxygen consumption of hPGC1β and mPGC1α-expressing cells was preserved in the presence of both oligomycin and FCCP, this implies strongly that increased mitochondrial density and/or electron-transport chain activity is responsible for the observed increase in oxygen consumption, corroborating the TEM results. These results suggest that like PGC1α, PGC1β may also play a role in mitochondrial biogenesis. Thus it is likely that PGC1α and PGC1β may represent complementary regulatory mechanisms of a mitochondrial biogenesis programme. Based on these results, we hypothesized that whereas PGC1α may co-ordinate the response to the energy demands generated during acute metabolic situations (e.g. cold, hypoglycaemia), PGC1β may act more as a co-ordinator to promote the mitochondrial biogenesis necessary to meet the basal requirements of a specific tissue. This process may take place by co-activating the transcriptions factors involved in the basal mitochondrial biogenesis, and involving probably the programme of thyroid hormone action.

In summary, we have identified and characterized the human PGC1β/PERC cDNA and genomic sequences including several minor alternative splice isoforms. The latter might be more relevant functionally in rodents. The tissue distribution of PGC1β mRNA is conserved among species and, contrary to PGC1α, does not seem to be regulated transcriptionally *in vivo* raising questions about the role of PGC1β in adaptive thermogenesis or gluconeogenesis. Finally, we provide evidence that over-expression of PGC1β is associated with increased number of mitochondria and oxygen consumption, suggesting that PGC1β may play a role in constitutive non-adrenergic-mediated mitochondrial biogenesis. Future experiments will characterize, in detail, the metabolic characteristics and molecular mechanisms modulating mitochondrial biogenesis and fatty-acid metabolism in these cell lines.

We thank Catrin Williams, Alex Kvist and Quentin Descat for excellent technical assistance. Research in A.V.-P.'s and S.O'R's laboratories was funded by Wellcome Trust. A.M. was supported by a Marie Curie Fellowship and V.C. by a Wellcome Trust fellowship. C.L. and K.G. were funded by MRC fellowships, and S.S. was funded by Deutsche Forschunsgemeinschaft (DFG).

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Received 3 February 2003/26 March 2003; accepted 4 April 2003
Published as BJ Immediate Publication 4 April 2003, DOI 10.1042/BJ20030200

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PUBLIKATION 8

Schinner S, Kempf K, Overmann H, Rotthoff T, Schott M, Rose B, Scherbaum WA, Herder C

Association of Impaired glucose metabolism in morbid obesity with hypoadiponectinaemia.

Exp. Clin. Endocrinol. Diabetes. 116 Suppl 1:S64-9 (2008)

Association of Impaired Glucose Metabolism in Morbid Obesity with Hypoadiponectinaemia

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Key words
- adiponectin
- inflammation
- obesity
- diabetes mellitus
- peripheral blood

Abstract

Background: Increased circulating levels of cytokines and chemokines and decreased adiponectin levels are associated with impaired glucose tolerance (IGT) and type 2 diabetes mellitus (T2DM). As obesity is the major risk factor for T2DM it is not clear why many patients with morbid obesity remain normoglycaemic and if this protection can be attributed to a lower grade of inflammation or higher adiponectin levels.

Materials and methods: Glucose tolerance of morbidly obese patients (n = 2 754, body mass index ≥ 40 kg/m^2) was assessed by oral glucose tolerance tests. In a case-control design we compared levels of eight immune mediators and adiponectin from patients with IGT/T2DM (n = 52) and normal glucose tolerance (NGT; n = 59). Gene expression in peripheral blood was determined by quantitative RT-PCR, and serum concentrations of immune mediators and adiponectin were measured by ELISA and bead-based multiplex technology.

Results: About 54 % of the patients in our morbidly obese cohort were normoglycaemic, while 14 % were diagnosed with IGT and 32 % with T2DM. There was no statistically significant difference in mRNA expression or serum levels of proinflammatory markers. Interestingly, we could demonstrate an association of NGT with higher adiponectin levels (p = 0.039). Adiponectin levels were negatively correlated with interleukin (IL)-6 and macrophage chemoattractant protein (MCP)-1, but independent the other immune mediators.

Conclusions: We found an association of lower adiponectin levels with IGT/T2DM, but no further increase in inflammatory markers in morbid obesity. This suggests that in addition to chronic, low-grade inflammation, adiponectin is an important factor in the development of, or protection against, T2DM in obesity.

received 03.03.2008
accepted 07.04.2008

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DOI 10.1055/s-2008-1081490
Exp Clin Endocrinol Diabetes 2008; 116 (Suppl.1): S64–S69

ISSN 0948-146X

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Introduction

Obesity is often accompanied by insulin resistance and predisposes to the development of type 2 diabetes mellitus (T2DM) [1,2]. In the last decades, there has been an alarming rise in the prevalence of obesity [1,3], and also the incidence of patients with morbid obesity has increased [4]. Morbid obesity, as defined by a body mass index (BMI) of ≥ 40 kg/m^2, confers a substantially (> 93-fold) higher risk for the development of T2DM in women as compared to a BMI of 21 kg/m^2 [5].

Both obesity and T2DM are associated with chronic, subclinical inflammation [6–9]. The obese state is characterised by an increase in adipocyte number and cell size ([10] and references therein) as well as by invasion of leukocytes into the adipose tissue [11–13]. Consistently, the serum levels of the pro-inflammatory cytokine and adipokine IL-6 are elevated in obesity, impaired glucose tolerance (IGT) and T2DM and negatively related to insulin sensitivity [14–20]. Similar observations were found for other cytokines like interleukin (IL)-18 and transforming growth factor (TGF)-β and for chemokines including monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, IL-8, interferon-γ-inducible protein (IP)-10 and regulated upon activation, normal T-cell expressed and secreted (RANTES) [21–27]. In line with this, circulating leukocytes of obese subjects are activated [9,28] and express pro-inflammatory markers [29]. This immune activation is directly relevant for the development of insulin resistance as insulin signalling is impaired under pro-inflammatory conditions [2,30]. However, adipose tissue also releases anti-inflammatory

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and insulin-sensitising factors. The most important adipokine that links obesity with glucose metabolism may be adiponectin [31,32]. In animal and human studies, adiponectin levels are decreased in obesity, insulin resistance and T2DM [26,33–36] and normalise when obesity and insulin sensitivity improve [37]. Moreover, insulin sensitivity and glucose metabolism can be improved by application of adiponectin [38–40].

So far, it is unclear which factors determine, or protect against, the development of impaired glucose metabolism in obese and therefore high-risk individuals and if this protection might be related to lower inflammation or higher adiponectin levels. Therefore, we determined the proportion of patients with IGT or T2DM in an obesity outpatient clinic cohort and investigated in a case-control study whether the gene expression levels or circulating concentrations of cytokines, chemokines or adiponectin are associated with the prevalence of impaired glucose metabolism in morbidly obese subjects.

Methods

Study subjects and determination of glucose tolerance state

The study population comprised patients (n = 2754) of an obesity outpatient clinic between 1961 and 2006. The inclusion criterion for the participation in this study was a BMI of ≥ 40 kg/m^2. Glucose tolerance state was determined by a standardised 75 g oral glucose tolerance test (OGTT; DextroOGT; Roche Diagnostics, Mannheim, Germany) after overnight fasting, and venous blood was drawn before and 2 h after oral glucose challenge. IGT and T2DM were diagnosed according to the current guidelines of the American Diabetes Association [41]. Body weight was measured in light clothing and waist circumferences at the minimum abdominal girth (midway between the rib cage and the iliac crest) by trained investigators. Analyses of fasting serum parameters were performed by routine clinical procedures. A consecutive subgroup of those patients (n = 111) who were referred to the outpatients clinic between January 2004 and December 2005 were included in a case-control study in which mRNA expression in peripheral blood and serum levels of inflammation markers and adiponectin were compared between patients with IGT or T2DM (n = 52) and NGT controls (n = 59). The study was performed according to the rules of the Declaration of Helsinki, and all study patients gave informed written consent.

Gene expression analysis

Blood withdrawal, RNA isolation and reverse transcription was performed as described before [6]. Quantitative real-time PCR was performed in optical reaction tubes (Applied Biosystems, Foster City, CA, USA) in 96-well microtiter plate format using the ABI PRISM 7700 Sequence Detector System (Perkin Elmer, Foster City, CA, USA). 1 ng cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 10 ng for IL-18, 50 ng for intercellular adhesion molecule (ICAM)-1 and 100 ng for IL-6, macrophage migration inhibitory factor (MIF) and tumour necrosis factor (TNF)-α, 1x QuantiTect SYBR Green PCR Master Mix (Qiagen), 33 μM sense and antisense primer each (GAPDH: 5′-ACACCCACTCCTCCACCTTTG-3′; 5′-CATACCAGGAAATGAGCTTGACAA-3′ ; IL-18: 5′-GCAGTCTACACAGCTTCGGGA-3′; 5′-TGCGACAAATAGTTTGTTGCG-3′; ICAM-1: 5′-ACGCCTCCCTGAACCTATCC-3′; 5′-CCAGGGCCGGTAGGTGTAG-3′ ; MIF: 5′-AGCCCGGACAGGGTCTACA-3′; 5′-TCTTAGGCGAAGGTGGAGTTG-3′; TNF-α: 5′-AGGCGGTGCTTGTTCCTCA-3′, 5′-GTTCGAGAAGATGATCTGACTGCC-3′; IL-6: 5′-CTGACCCAACCACAAATGCC-3′, 5′-CATGTCCTGCAGCCACTGG-3′) were combined in a 25-μl reaction and incubated for 10 min at 95 °C followed by 40 cycles with 30 sec at 95 °C, 30 sec at 55 °C, 45 sec at 72 °C and 2 min at 25 °C. Each well was screened for fluorescent dye every 7 seconds, and signals were regarded as positive (threshold cycle) if the fluorescence intensity exceeded 10x the standard deviation of the baseline fluorescence. Arbitrary units of gene expression were determined by (i) comparing the threshold cycle for the immune parameter in each sample with the threshold cycle for the housekeeper GAPDH in the same sample and (ii) assessing the ratio of the sample threshold cycles with the threshold cycles of a standardised reference RNA (Stratagene, Amsterdam, The Netherlands) using Pair Wise Fixed Reallocation Randomisation Test (http://www.gene-quantification.info).

ELISA and bead-based multiplex analyses

Serum samples were stored at −80 °C until analysis of immune markers. Adiponectin, IL-6, TGFβ and RANTES were measured using Quantikine (adiponectin, RANTES), Quantikine HS (IL-6) and DuoSet (TGFβ1) ELISAs from R&D Systems (Wiesbaden, Germany) [26]. Serum levels of IL-18, MCP-1, MIP-1α, IL-8 and IP-10 were quantified using a bead-based multiplex assay on a Luminex 100 analyser (Luminex Corporation, Austin, TX) as described [26]. Fluorescent xMAP COOH microspheres were purchased from Luminex Corporation. Recombinant proteins were obtained from MBL (Nagoya, Japan; IL-18), R&D Systems (MCP-1, MIP-1α), NIBSC (Potters Bar, UK; IL-8) and BD Biosciences (Heidelberg, Germany; IP-10). Antibody pairs were purchased from MBL (IL-18), R&D Systems (MCP-1, MIP-1α, IL-8) and BD Biosciences (IP-10).

Statistical analysis

For comparison of clinical data we used two-sided unpaired t-test or Wilcoxon test. Dichotomous variables were compared by Fisher's exact test. For comparison of gene expression and serum levels of inflammation markers and adiponectin from patients with IGT/T2DM or NGT, multiple linear regression models adjusting for sex, age and BMI were calculated. The associations between adiponectin and other markers were also assessed using multiple linear regression models. Variables that did not show normal distribution (i.e. cytokines, chemokines, adiponectin, triglycerides) entered the models as ln-transformed values. The level of significance was 0.05. For data analysis SAS statistical package version 8.2 TS2MO (SAS Institute, Cary, NC, USA) was used.

Results

Impaired glucose metabolism in a cohort of morbidly obese patients

About 14% of the patients in our morbidly obese cohort with a BMI > 40 kg/m^2 (n = 2754) were diagnosed with IGT and 32% with T2DM, while about 54% had NGT. Of this cohort 111 consecutive patients were included in a case-control study in which gene expression levels in peripheral blood and serum levels of inflammation markers were compared between a group of patients with IGT or T2DM (n = 52) and NGT controls (n = 59). Patients with IGT/T2DM were older and showed higher levels of

Table 1 Clinical characteristics of morbidly obese patients in the case-control study

Variable	NGT (n = 59)	IGT/T2DM (n = 52)
sex [n] (male/female)	14/45	16/36
age [years]	40.3 ± 11.4	52.2 ± 12.4***
BMI [kg/m^2]	47.3 ± 7.2	45.7 ± 4.7
waist circumference [cm]	126 ± 18	131 ± 16
systolic blood pressure [mmHg]	138 ± 18	143 ± 17
diastolic blood pressure [mmHg]	90 ± 12	89 ± 14
total cholesterol [mg/dl]	209 ± 34	206 ± 39
LDL cholesterol [mg/dl]	154 ± 25	147 ± 30
HDL cholesterol [mg/dl]	53 ± 11	51 ± 13
triglycerides [mg/dl]	134 (1.57)	174 (1.60)**
γ-glutamyl transferase [U/l]	25 ± 13	40 ± 30***

Data are shown as mean ± SD or as geometric mean and standard deviation factor for triglycerides. Dichotomous variables were compared by Fisher's exact test. For comparison of continuous variables we used two-sided unpaired t-test or Wilcoxon test for triglycerides. **, $p<0.01$; ***, $p<0.001$. NGT, normal glucose tolerance; IGT, impaired glucose tolerance; T2DM, type 2 diabetes mellitus; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein

Table 2 Serum levels of inflammation markers and adiponectin in morbidly obese patients in the case-control study

	NGT (n = 59)	IGT/T2DM (n = 52)
cytokines		
IL-6 [pg/ml]	2.76 (2.36)	2.77 (1.68)
IL-18 [pg/ml]	123 (1.61)	139 (1.61)
TGF-β [ng/ml]	41.0 (1.32)	41.4 (1.34)
chemokines		
MCP-1 [pg/ml]	195 (1.53)	224 (1.54)
MIP-1α [pg/ml]	32.4 (2.85)	44.0 (2.27)
IL-8 [pg/ml]	13.39 (3.76)	19.93 (4.08)
IP-10 [pg/ml]	346 (1.58)	387 (1.53)
RANTES [ng/ml]	51.3 (1.49)	45.1 (1.55)
adiponectin		
adiponectin [μg/ml]	7.00 (1.78)	6.45 (1.86) *

Values are given as geometric means and standard deviation factors. Both groups were compared using multiple linear regression models based on ln-transformed values of immune mediators or adiponectin with adjustment for age, sex and BMI. *, $p = 0.039$

triglycerides and γ-glutamyl transferase (GGT) than controls with NGT, but did not differ in BMI, waist circumference, blood pressure and cholesterol levels (**Table 1**).

Adiponectin, but not cytokine or chemokine mRNA expression or serum levels differ between morbidly obese IGT/T2DM and NGT patients

In order to investigate if lower levels of subclinical inflammation may protect morbidly obese patients from the development of T2DM, we determined the mRNA expression levels of IL-6, IL-18, TNF-α, MIF and ICAM-1 in peripheral blood leukocytes from subjects with IGT/T2DM and NGT as well as serum levels of the cytokines IL-6, IL-18, TGF-β and the chemokines MCP-1, MIP-1α, IL-8, IP-10 and RANTES. In addition, we also measured serum levels of the adipokine adiponectin.

We found no statistically significant difference in mRNA expression (median and interquartile range of NGT vs. IGT/T2DM) for IL-6 [0.74 (0.40; 1.16) vs. 0.61 (0.41; 0.86), respectively], IL-18 [0.66 (0.44; 1.57) vs. 0.77 (0.50; 1.59)], TNF-α [4.96 (2.13; 6.48) vs. 4.29 (2.53; 6.23)], MIF [1.05 (0.79; 1.20) vs. 0.89 (0.58; 1.28)] or ICAM-1 [0.29 (0.18; 0.59) vs. 0.33 (0.18; 0.43)] as determined by marker expression divided by expression of GAPDH relative to standard reference. Moreover, no difference was found regarding serum levels of the eight tested inflammatory markers between the two groups (**Table 2**). However, serum levels of adiponectin were significantly lower ($p = 0.039$) in patients with impaired glucose metabolism than in NGT controls (**Table 2**).

Hypoadiponectinemia is inversely associated with IL-6 and MCP-1, but independent of the other tested immune markers in morbidly obese patients

In order to determine if adiponectin levels are associated with clinical or inflammation markers in morbidly obese patients, multiple linear regression models with different degrees of adjustment were calculated. As covariables, we included age, sex, BMI and glucose tolerance state because these factors are known as determinants of adiponectin and cytokine/chemokine levels. **Table 3** shows that adiponectin levels were significantly higher in women, positively correlated with age and HDL cholesterol and negatively correlated with GGT in the unadjusted analyses and after adjusting for age, sex, BMI and glucose tolerance state. We found no statistically significant correlations between adiponectin and most of the cytokines or chemokines investigated here. However, there was a robust inverse correlation between adiponectin and IL-6 in all models and an inverse correlation between adiponectin and MCP-1 in analyses that adjusted for age and sex or for age, sex and BMI (**Table 3**).

Discussion

In this study we identified about 14% of the patients in our morbidly obese cohort of 2 754 patients with BMI ≥ 40 kg/m^2 to have IGT, 32% to have T2DM and 54% to be normal glucose tolerant. In a case-control design we found that patients with IGT or T2DM had significantly increased levels of serum triglycerides and GGT, but there was no statistically significant difference in mRNA expression in peripheral blood or serum levels of inflammatory markers compared to NGT controls. Moreover, we could for the first time demonstrate an association of glucose tolerance with adiponectin serum levels in morbidly obese patients, with significantly lower serum levels for adiponectin in patients with impaired glucose metabolism compared to controls. Adiponectin levels were inversely correlated with IL-6 and MCP-1, but unrelated to six other inflammatory mediators suggesting that chronic subclinical inflammation and hypoadiponectinemia are largely independent from each other in morbid obesity.

Obesity is associated with a pro-inflammatory state [11–13], and a similar state of immune activation can be observed in T2DM [8, 42], as circulating leukocytes from T2DM patients are activated [9, 29] and levels of cytokines and chemokines are systemically elevated in serum and plasma of diabetic and prediabetic subjects [8, 42–45]. Nevertheless, not all morbidly obese patients develop T2DM and it is only poorly understood which factors may confer protection. In our cohort we saw no further increase in inflammation in study participants with IGT/T2DM compared to NGT. This finding could be due to the fact that the immune activation due to IGT/T2DM is less pronounced than the overriding impact of morbid obesity and therefore more difficult to detect. The comparison with other samples that were measured in the same laboratory and with the same assays indicates that in particular IL-6, IL-8 and IP-10 levels were higher that in normal-weight or overweight adults [43]. Our data there-

Table 3 Association between ln-transformed serum adiponectin levels (ng/ml) with clinical and inflammation markers in multiple linear regression models

Variable	β unadjusted	β adjusted for age and sex	βadjusted for age, sex and BMI	β adjusted for age, sex, BMI and glucose tolerance state
clinical parameters				
sex	**0.481*****	**0.469***** (a)	**0.464***** (a)	**0.440***** (a)
age [years]	**0.013****	**0.013**** (b)	**0.013***** (b)	**0.017***** (b)
BMI [kg/m^2]	0.011	0.014	n/a	0.012
waist circumference [cm]	−0.004	0.002	−0.001	0.000
systolic BP [mmHg]	0.002	−0.005	−0.004	−0.005
diastolic BP [mmHg]	0.000	−0.003	−0.003	−0.003
total cholesterol [mg/dl]	**0.004***	0.003	0.003	0.002
LDL cholesterol [mg/dl]	0.000	0.000	0.001	0.000
HDL cholesterol [mg/dl]	**0.017****	**0.013****	**0.013****	**0.012***
triglycerides [mg/dl]	−0.116	−0.129	−0.121	−0.074
γ-glutamyl transferase [U/l]	−0.006*	−0.006**	−**0.006****	−0.006*
cytokines and chemokines				
IL-6 [pg/ml]	−**0.168***	−**0.209****	−**0.252*****	−**0.257*****
IL-18 [pg/ml]	0.050	−0.010	0.002	0.025
TGF-β [pg/ml]	−0.340	−0.315	−0.340	−0.318
MCP-1 [pg/ml]	−0.197	−**0.253***	−**0.251***	−0.224
MIP-1α [pg/ml]	0.028	−0.030	−0.024	−0.005
IL-8 [pg/ml]	0.019	−0.020	−0.012	−0.005
IP-10 [pg/ml]	0.171	0.030	0.027	0.041
RANTES [pg/ml]	0.069	−0.011	−0.050	−0.072

The table gives regression coefficients β from multiple linear regression models. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Variables that did not show normal distribution (i.e. serum levels of cytokines, chemokines, adiponectin, triglycerides) entered the models as ln-transformed values. BP, blood pressure. (a) Not adjusted for sex; (b) Not adjusted for age; n/a, not applicable

fore confirm that morbid obesity is associated with a significant, albeit subclinical immune activation.

In our study population we found that high adiponectin levels were associated with female sex, higher age, higher HDL cholesterol and lower GGT levels which is in line with other studies that described the association between adiponectin and risk factors for T2DM or cardiovascular disease ([46] and references therein). We did not detect an inverse association with BMI and waist circumference, that has been found in population-based studies or in studies including lean and obese subjects, probably because of our selection of patients with morbid obesity (BMI >40 kg/m^2) as crucial inclusion criterion and thus a rather narrow BMI range. Interestingly, the IGT/T2DM group showed increased serum levels of GGT compared to controls. GGT activity is increased in non-alcoholic fatty liver disease that is frequent in obesity and considered the hepatic manifestation of the metabolic syndrome [47]. Elevated GGT levels have been identified as predictors of T2DM in overweight cohorts [48,49].

Importantly, we saw lower adiponectin levels in IGT/T2DM patients than in NGT which points towards a role of hypoadiponectinemia in the development of T2DM. Although there is an intense crosstalk between adipocytes and immune cells as evident by the negative regulation of adiponectin expression in adipose tissue by TNF-α and other immune mediators [34,37], as well as suppression of adhesion molecule expression on endothelial cells by adiponectin [50], circulating levels of adiponectin and cytokines or chemokines are only weakly correlated. In a population-based study we showed that hypoadiponectinaemia and a proinflammatory state are largely independent of each other [36], and these findings are mostly confirmed by our data from morbidly obese patients. In contrast to our previous data however, we found an inverse association between adiponectin and IL-6 as well as MCP-1. Both associations are plausible as circulating levels of IL-6 as well as *IL-6* gene variants are predictors of T2DM [17–20,51], although it needs to be mentioned that the link between IL-6 and insulin resistance is still controversial [52]. In addition, MCP-1 impairs insulin signalling, induces insulin resistance and is also an independent predictor of incident T2DM [45,53,54].

Our study has several limitations. Our selection of consecutive study participants led to a group of patients with IGT/T2DM who were significantly older than the NGT controls. It is possible that a proportion of NGT subjects will progress to T2DM in the future so that differences between both groups may be underestimated. However, this limitation cannot be expected to have caused our positive finding regarding the association of hypoadiponectinaemia and IGT/T2DM. We did not have data on anti-inflammatory medication for all patients so that this factor was not included in the regression models. Among the strengths are the facts that the association between adiponectin, inflammation and glucose tolerance has to the best of our knowledge not been described in patients with morbid obesity before, that we used OGTTs to ascertain the status of both cases and controls and that we included comprehensive measurements of multiple proinflammatory factors both on the level of gene expression in peripheral blood as well as circulating levels in order to characterise the state of immune activation.

In conclusion, we report that in morbidly obese patients, lower adiponectin levels are associated with impaired glucose metabolism, whereas we found no significant differences for proinflammatory cytokines and chemokines. Adiponectin levels were inversely related with IL-6 and MCP-1, that predict incident T2DM in population-based studies, but not associated with six other immune mediators. It is therefore tempting to hypothesise that disturbances of adiponectin expression and/or action are crucial for the question whether or not patients with morbid obesity progress to T2DM and that adiponectin appears to be

more relevant than and also mostly independent of chronic, low-grade inflammation in this high-risk group of patients.

Acknowledgements

The work was supported by the German Federal Ministry of Health and Social Security and the Ministry of Science and Research of the State North Rhine-Westphalia. We thank Prof. Dr. Stephan Martin (German Diabetes Centre) for valuable discussions and Karin Röhrig (German Diabetes Centre) for excellent technical assistance.

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PUBLIKATION 9

Schinner S: Wnt Signaling and the Metabolic Syndrom

Horm. Metab. Res. 41(2):159-63 (2009)

Wnt-signalling and the Metabolic Syndrome

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Key words
- beta-cells
- diabetes mellitus type 2
- insulin secretion
- Wnt

Abstract

The Wnt-signalling pathway plays a well-established role in embryogenesis and tumourigenesis. However, recent data puts Wnt-signalling in the context of metabolic disease. In vitro and in vivo data characterised the role of Wnt-signalling molecules in the regulation of adipocyte differentiation (adipogenesis). Furthermore, Wnts play a pivotal role in regulating pancreatic beta-cell function and mass. In addition, studies found polymorphisms within the gene encoding TCF7L2, a Wnt-regulated transcription factor, to contribute an increased risk to develop type 2 diabetes mellitus in humans. This review will summarise recent aspects of Wnt-signalling in these pathophysiologic events and discuss the contributions of dysregulation in Wnt-signalling to features of the metabolic syndrome.

Abbreviations

C/EBP	CCAAT-enhancer-binding proteins
Dkk	Dickkopf-proteins
FABP	fatty acid binding protein
FCCM	fat cell-conditioned medium
GLP-1	glucagon-like-peptide-1
GSK-|3	glycogen synthase kinase 3
LEF	lymphoid enhancer-binding factor
LRP	LDL receptor-related protein
PPAR-γ	peroxisome proliferator-activated receptor γ
sFRP-1	secreted Frizzled-related protein 1
StAR	steroidogenic acute regulatory protein
TCF	T cell-specific transcription factor

Introduction

Wnts are secreted signalling molecules and ligands on cell surface receptors called Frizzled and LRP5/6. Upon ligand binding, intracellular signalling cascades are activated resulting in the induction of either noncanonical or canonical Wnt-signalling, the latter involving the transcriptional coactivator beta-catenin. Activated beta-catenin translocates to the nucleus where it is able to coactivate various transcription factors, including TCF/LEF.

The Wnt-signalling pathway plays a well-established role in embryogenesis and tumourigenesis. However, recent data puts Wnt-signalling in the context of metabolic disease. In vitro and in vivo data characterised the role of Wnt-signalling molecules in the regulation of adipocyte differentiation (adipogenesis). In human and murine preadipocytes, a downregulation of canonical Wnt-signalling is a prerequisite in order to initiate adipogenesis. Furthermore, a pivotal role in regulating pancreatic beta-cell function and mass has been attributed to the Wnt-signalling pathway. Wnts induce glucose-stimulated insulin secretion and beta-cell proliferation. Interestingly, there appears to be another indirect link between Wnt-signalling and beta-cell function: canonical Wnt-signalling regulates the transcription of the proglucagon gene, eventually leading to GLP-1 expression. In addition, the intracellular effects of GLP-1 on pancreatic beta-cells seem to be (at least partly) mediated by canonical Wnt-signalling.

In 2006, two studies found polymorphisms within the gene encoding TCF7L2, a Wnt-regulated transcription factor, to contribute an increased risk to develop type 2 diabetes mellitus in humans. Since then, a number of genetic studies have confirmed that TCF7L2 is the gene with the strongest known association with type 2 diabetes mellitus in various populations. This review

received 24.09.2008
accepted 09.12.2008

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DOI 10.1055/s-0028-1119408
Horm Metab Res 2009;
41: 159–163

ISSN 0018-5043

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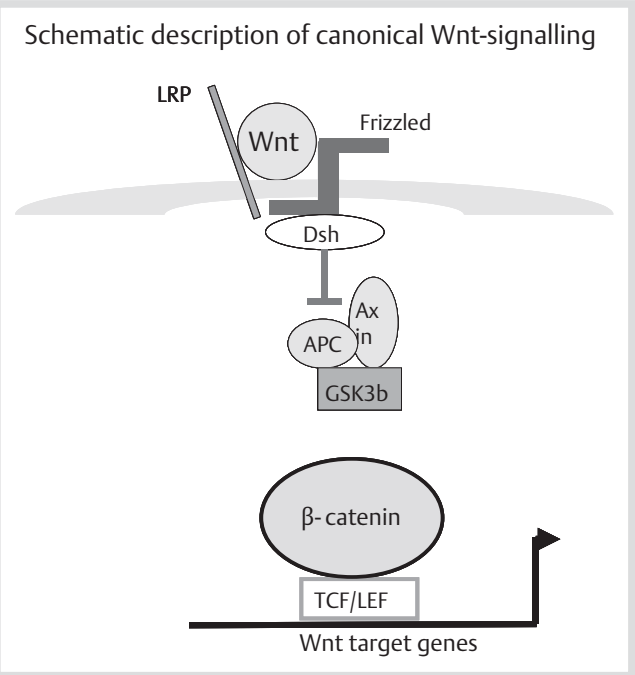

Fig. 1 Schematic description of canonical Wnt-Signalling. Binding of Wnts to the Frizzled receptor and LRP coreceptors induces canonical Wnt-signalling. This leads to an inhibition of GSK3b, eventually allowing beta-catenin to translocate to the nucleus to coactivate transcription factors like TCF/LEF on target genes.

will summarise recent aspects of Wnt-signalling in these pathophysiologic events and discuss the contributions of dysregulation in Wnt-signalling to features of the metabolic syndrome.

The Wnt-signalling Pathway

The Wnt-signalling pathway consists of extracellular ligands (Wnts), secreted antagonists (e.g., sFRPs and Dickkopf-proteins), seven transmembrane cell surface receptors (Frizzled) and co-receptors (e.g., LRP5/6), and intracellular signalling molecules, among which beta-catenin is pivotal. Currently, 19 different Wnts and 9 different Frizzled receptors are characterised.

Intracellular Wnt-signalling branches into the canonical (beta-catenin) and the noncanonical pathways. In this review we will focus on canonical Wnt-signalling.

In the absence of Wnt ligands, beta-catenin is constantly phosphorylated and thereby inactivated by GSK-3. Ligand binding to both the Frizzled receptor and the LRP coreceptor leads to an inactivation of GSK-3, which in turn can no longer phosphorylate beta-catenin. This leads to a stabilisation and nuclear translocation of beta-catenin. Subsequently, beta-catenin coactivates transcription factors among which TCF (T-Cell Factor)/LEF (Lymphoid Enhancer Factor) transcription factors are of particular interest [1] (see **Fig. 1**).

Wnt-signalling Regulates Adipocyte Differentiation

The number and function of adipocytes affects insulin resistance and various metabolic functions by altered hormone secretion from adipocytes. The recruitment of preadipocytes to the adipocyte lineage is one determinator of the number of adipocytes. Preadipocytes respond to endocrine, paracrine, and autocrine signals in order to undergo a regulated differentiaton into mature adipocytes. Well-known hormonal stimuli to initiate adipogenesis are insulin, glucocorticoids, and cAMP-activators. These stimuli induce a transcriptional cascade within preadipocytes involving the transcription factors C/EBPbeta and C/EBPdelta and eventually C/EBPalpha and PPARgamma. As a consequence, preadipocytes undergo the differentiation programm and express markers of mature adipocytes [2].

However, a key publication from Ross and colleagues in 2000 showed that overexpression of Wnt10b in mouse preadipocytes inhibited the expression of C/EBPalpha and PPARgamma and kept preadipocytes in an undifferentiated state in vitro and in vivo. Conversely, inhibition of Wnt-signalling was sufficient to facilitate spontaneous differentiation of adipocytes and transdifferentiation of myoblasts into adipocytes [3].

Mouse models supported this concept: Transgenic overexpression of Wnt10b under the control of the FABP4 promoter led to a tissue specific expression of Wnt10b in white adipose tissue (WAT). These mice were found to have less adipose tissue on normal chow diet. Furthermore, they are resistant to diet-induced obesity [4] or genetically-induced obesity on an ob/ob background [5].

Comparing these data generated from murine systems, we found cultured human preadipocytes to show very similar responses to activation or inactivation of canonical Wnt-signalling: Functional inactivation of canonical Wnt-signalling facilitates adipocyte differentiation in both species. However, inactivation of Wnt-signalling in mouse is caused by a drop in Wnt10b expression, while a transient increase in the expression of the Wnt-antagonist Dkk-1 and a decrease in LRP expression appears to mediate the inactivation of this pathway in human preadipocytes in vitro [6].

Human genetic studies have revealed an association of a heterozygous loss of function mutation (C256Y) in the Wnt10b gene with obesity. In functional assays this mutant protein showed an impaired function [7]. Similarly, two SNPs in the LRP5 gene were associated with obesity [8]. It is important to note, that these genetic alterations offer an interesting proof of concept for the role of Wnt-signalling in human adipogenesis. However, these mutations are extremely rare and will not account for the major part of obesity observed.

Taken together, preadipocytes and mature adipocytes secrete Wnt-signalling molecules in a timely regulated and autocrine and paracrine manner, thereby determining the recruitment of preadipocytes to the adipocyte differentiation process (see **Fig. 2**).

Adipocytes Act on Metabolic Target Cells through Wnt-signalling Molecules

Adipocytes act as endocrine glands and stimulate target cells through different pathways. Adipocytes secrete adipokines among which leptin and adiponectin are probably best characterised [9,10]. Preadipocytes and mature adipocytes regulate their respective functions through auto- and paracrine-acting Wnt-signalling molecules. Therefore, one might expect adipocytes to act on nonadipose target cells through Wnts as well. In fact, mature human primary adipocytes were found to secrete Wnt10b and Wnt3a and to induce canonical Wnt-signalling in target cells [11]. In adrenocortical cells this stimulation of

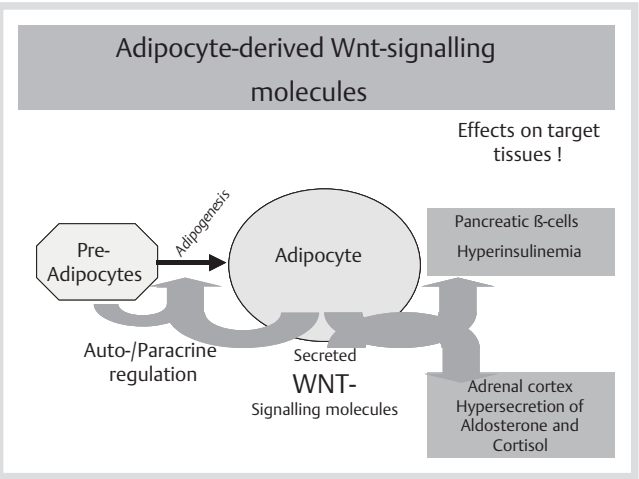

Fig. 2 Model of autocrine and paracrine Wnt actions on adipogenesis and on endocrine target cells.

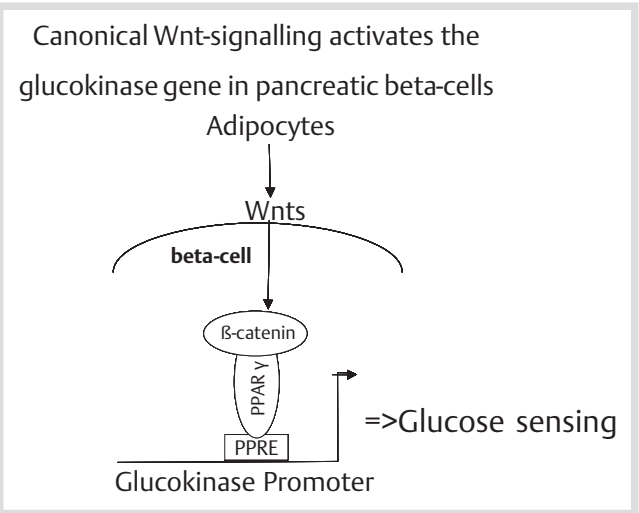

Fig. 3 Canonical Wnt-signalling activates the glucokinase gene promoter. Adipocyte-derived Wnts induce canonical Wnt-signalling in pancreatic beta-cells. As a consequence, beta- catenin coactivates PPARgamma at the glucokinase gene promoter.

canonical Wnt-signalling led to an induction of SF-1-mediated transcription and to the stimulation of transcription of the gene encoding StAR. The Wnt-signalling pathway is known to regulate crucial aspects of adrenal development and function [12, 13].

Eventually, adipocyte-derived Wnts stimulated aldosterone and cortsisol secretion from adrenocortical cells [11] (see **Fig. 2**).

The canonical Wnt-signalling pathway is inactive in pancreatic alpha cells [14]. In contrast, it is intact in pancreatic beta-cells and activated in response to adipocyte-conditioned medium. Upon stimulation with adipocyte-secreted products pancreatic beta-cells showed increased proliferation and augmented insulin secretion. On the molecular level, adipocyte-derived Wnts stabilise beta-catenin, which is able to coactivate PPARgamma-mediated transcription on the glucokinase gene promoter. Glucokinase is the key regulator of glucose-sensing in pancreatic beta-cells, thereby offering a model for the adipocyte-induced hypersecretion of insulin [15] (see **Fig. 3**).

Wnt-signalling and Beta-cell Function

In addition to the adipocyte-beta-cell interaction described above, very recent papers have assigned a role for Wnt-signalling in essential beta-cell functions: Rulifson and colleagues found recombinant Wnt3a protein to induce regulators of cell cycle control (cyclin D2 and Pitx2) in pancreatic beta-cells. Furthermore, they have described an increased proliferation rate in cultured beta-cells. Consequently, overexpression of active beta-catenin induced proliferation markers and increased beta-cell mass in a transgenic mouse model in vivo and inhibition of Wnt-signalling decreased beta-cell expansion in vivo [16].

Shu et al. knocked down TCF7L2, a Wnt-regulated transcription factor, which is associated with an increased incidence of type 2 diabetes mellitus (see below), and found this to decrease the proliferation of primary human beta-cells in vitro [17].

In a whole animal-model a knock-out of the LRP5 gene led to impaired glucose tolerance in mice. The isolated LRP5 deficient islets showed impaired glucose-stimulated insulin-secretion due to a defect in glucose sensing [18].

In extension to these findings, a recent paper investigated the effect of GLP-1 (glucagon-like-peptide-1) on Wnt-signalling in pancreatic beta-cells. GLP-1 is secreted from intestinal L-cells and stimulates insulin-secretion as well as beta-cell proliferation. Liu and Habener found out that canonical Wnt-signalling mediates the proliferative effect of glucagon-like-peptide-1 (GLP-1) on pancreatic beta-cells [19]. However, there might also be another indirect link between GLP-1, Wnt-signalling, and beta-cell proliferation: GLP-1 is transcribed from the pro-glucagon gene in intestinal L-cells. Canonical Wnt-signalling has been found to regulate the transcription of the pro-glucagon gene through TCF transcription factors [14, 20]. This suggests that altered Wnt-signalling might affect the entero-insular axis at two different levels: Firstly at the level of GLP-1 expression and secondly at the level of GLP-1 action on pancreatic beta-cells.

Wnt-signalling and Type 2 Diabetes Mellitus in Human Genetic Studies

In 2006, two reports demonstrated an association of polymorphisms in the gene encoding TCF7L2 with an increased risk to develop type 2 diabetes mellitus. Grant et al. showed a 2.4-fold increase of type 2 diabetes mellitus in homozygous carriers of the mutated TCF7L2 variant. A similar study by Florez and colleagues confirmed the association of TCF7L2 polymorphisms with type 2 diabetes mellitus: They showed that progression from impaired glucose tolerance to diabetes mellitus is positively associated with polymorphisms in TCF7L2 [21–23]. These initial studies have been confirmed in large whole-genome scans and in numerous ethnic groups [24–29]. From these studies TCF7L2 is known to be the most powerful genetic contributor to type 2 diabetes mellitus at present. However, from the data available at this stage we do not fully understand the mechanisms through which TCF7L2 variants affect glucose metabolism. There is clear evidence that TCF7L2 regulates insulin secretion rather than insulin action. The pathophysiological background gathered from data in in vitro and from animal studies suggest that TCF7L2 may have effects on a) GLP-1 secretion, b) GLP-1 action within pancreatic beta-cells, and c) GLP-1-independent beta-cell function, namely proliferation and glucose-stimulated insulin secretion. There have also been a few

studies in humans trying to unravel the role of TCF7L2: Subjects carrying TCF7L2 polymorphisms showed a blunted insulin response to both oral and intraveneous glucose load [30]. Another study investigated the effect of TCF7L2 polymorphisms on GLP-1 levels in patients and found no difference in GLP-1 levels, but in glucose-stimulated insulin secretion between carriers and noncarriers [31]. These data suggest defects within the beta-cell in subjects carrying TCF7L2 polymorphisms rather than defects in GLP-1 secretion.

Future Perspectives

Further studies are needed in order to translate the evidence from these basic science studies to clinical medicine. Based on the finding that Wnts stimulate beta-cell proliferation it is of course tempting to speculate that activation of Wnt-signalling might be a target to preserve beta-cell mass in diabetes mellitus. In addition, studies in mice revealed the Wnt-signalling pathway as a target to drive the differentiation of mesenchymal stem cells towards myoblasts versus adipocytes. This implies the option to modulate metabolic features. However, there are no such clinical options available at this stage and not even in the near future, but Wnt-signalling appears to be a promising candidate for novel approaches for the treatment of diabetes mellitus and metabolic disease.

Conclusions

The Wnt-signalling pathway is a crucial regulator of adipocyte differentiation and adipocyte function. Adipocytes secrete Wnt-signalling molecules that can act on metabolic target cells. In addition, canonical Wnt-signalling modulates glucose-sensing in pancreatic beta-cell and eventually insulin-secretion. Furthermore, in vitro data and in vivo data from animal studies confirmed the role of Wnt-signalling in cell-cycle regulation and beta-cell proliferation. These experimental approaches suggest a role for Wnts in maintaining glucose homeostasis mainly by modulating insulin release. This hypothesis is supported by recent data from human genetic studies demonstrating that polymorphisms in TCF7L2, a Wnt-regulated transcription factor, are the strongest genetic variants known at present to contribute a risk for type 2 diabetes mellitus.

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PUBLIKATION 10

Christodulides C, Laudes M, Cawthorn WP**, Schinner S,** Soos M, O`Rahilly S,Sehti JK, Vidal-Puig AJ.

The Wnt antagonist Dickkopf-1 and its receptors are coordinately regulated during early human adipogenesis.

J. Cell. Sci. 119, 2613-2620, (2006).

The Wnt antagonist Dickkopf-1 and its receptors are coordinately regulated during early human adipogenesis

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Accepted 9 March 2006
Journal of Cell Science 119, 2613-2620 Published by The Company of Biologists 2006
doi:10.1242/jcs.02975

Summary

Secretion of Wnts by adipose cells has an important role in the control of murine adipogenesis. We present the first evidence that a Wnt antagonist, Dickkopf 1 (Dkk1), is secreted by human preadipocytes and promotes adipogenesis. *DKK1* mRNA increases six hours after onset of human adipogenesis and this is followed by an increase in Dkk1 protein. With further differentiation, the mRNA and protein levels progressively decline such that they are undetectable in mature adipocytes. The transient induction in DKK1 correlates with downregulation of cytoplasmic and nuclear β-catenin levels, this being a surrogate marker of canonical Wnt signalling, and Wnt/β-catenin transcriptional activity. In addition, constitutive expression of Dkk1 in 3T3-L1 preadipocytes promotes their differentiation, further supporting the functional significance of increased Dkk1 levels during human adipogenesis. Concomitant downregulation of the Dkk1 receptors *LRP5* and *LRP6* is likely to potentiate the ability of Dkk1 to inhibit Wnt signalling and promote differentiation. Notably, Dkk1 is not expressed in primary murine preadipocytes or cell lines. The involvement of Dkk1 in human but not murine adipogenesis indicates that inter-species differences exist in the molecular control of this process. Given the public health importance of disorders of adipose mass, further knowledge of the pathways involved specifically in human adipocyte differentiation might ultimately be of clinical relevance.

Key words: Adipocyte, Adipogenesis, Wnt, Dickkopf 1, LRP5, Human

Introduction

Induction of preadipocyte differentiation in vivo is influenced by a balance of positive and negative factors (MacDougald and Mandrup, 2002). Whereas part of the signal is endocrine in nature, local signals originating from both preadipocytes and mature adipocytes are also important. Understanding the signals that regulate the balance between growth of existing and differentiation of new adipocytes might provide novel strategies to prevent obesity and related metabolic complications.

Wnts are a family of lipid-modified secreted glycoproteins comprising 19 members in humans (Miller, 2002; Willert et al., 2003). They act in autocrine and paracrine fashions to direct pattern specification during embryonic development and adult tissue remodelling (Logan and Nusse, 2004; Taipale and Beachy, 2001; Wodarz and Nusse, 1998). Wnts exert their effects by signalling through multiple pathways to regulate cell differentiation, cell growth and apoptosis. In the canonical pathway, binding of Wnts to Frizzled receptors and low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) co-receptors inhibits the activity of glycogen synthase kinase-3β (GSK-3β) (Hagen et al., 2004; Hagen and Vidal-Puig, 2002). Inactivation of GSK-3β leads to hypophosphorylation of β-catenin, which accumulates in the cytoplasm and translocates to the nucleus, where it binds to the lymphoid enhancer-binding factor/T-cell-specific transcription factor (LEF/TCF) family of transcription factors to activate Wnt target genes.

Wnt signalling can be modulated by extracellular antagonists (Jones and Jomary, 2002; Kawano and Kypta, 2003). Secreted Frizzled-related proteins (SFRPs), Wnt inhibitory factor (WIF) and Cerberus directly bind and sequester Wnt proteins from their receptors. By contrast, Dickkopf (Dkk) proteins, such as Dkk1, inhibit Wnt signalling by binding as high-affinity antagonists to LRP5/6 co-receptors, thus preventing Wnt-induced Frizzled-LRP5/6 complex formation (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001). In addition, Dkk1 also interacts with another receptor class, Kremen1 or 2 (Krm1/2) to inhibit LRP6 synergistically (Davidson et al., 2002; Mao et al., 2002). The Dkk family has at least four members – Dkk1, Dkk2, Dkk3 and Dkk4. However, not all Dkks inhibit Wnt signalling. Like Dkk1, Dkk4 can cooperate with Krm2 to inhibit Wnt/β-catenin signalling through binding and sequestering LRP6. Dkk2 can either stimulate or inhibit Wnt signalling in a context-dependent manner. By contrast, the function of Dkk3 is less clear; there is no evidence that it can bind either of these receptors or modulate Wnt/β-catenin signalling (Kawano and Kypta, 2003).

Recently, evidence has emerged for the involvement of Wnts in murine adipogenesis (Bennett et al., 2002; Ross et al., 2000).

It has been shown that Wnt signalling, probably mediated by WNT10B, prevents differentiation of the 3T3-L1 murine preadipocyte cell line by inhibiting expression of the master adipogenic transcription factors CCAAT/enhancer binding protein-α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ). Reciprocally, disruption of extracellular or intracellular Wnt signalling results in spontaneous adipogenesis. Furthermore, transgenic mice expressing *Wnt10b* under the control of the fat-specific *Fabp4* promoter have reduced body fat content without lipodystrophic diabetes (Longo et al., 2004). To date, no study has directly addressed the relevance of Wnt signalling in human adipogenesis. In the present study, we have identified *DKK1* as a novel gene that is significantly upregulated during early human adipogenesis. In addition, we present evidence suggesting that coordinate regulation of Dkk1 and its receptors might facilitate human adipocyte differentiation by inhibiting Wnt signalling.

Results

DKK1 mRNA and protein is transiently upregulated in early human adipogenesis

While interrogating microarray expression profiles of early human adipogenesis, we identified Dkk1, a secreted Wnt antagonist, as one of the most upregulated transcripts six hours following onset of differentiation. Given that Wnt signalling has been shown to inhibit murine adipocyte differentiation, we reasoned that Dkk1 might be an important endogenous inducer of human adipogenesis. To verify the microarray expression data, we analysed *DKK1* gene expression during differentiation of subcutaneous preadipocytes from eight additional, unrelated subjects using quantitative real-time reverse transcription (RT)-PCR. As illustrated in Fig. 1A, we confirmed that *DKK1* mRNA is present in confluent preadipocytes and its expression increases six hours following induction of differentiation. Thereafter, *DKK1* mRNA levels progressively decline.

To confirm that the changes in *DKK1* mRNA levels were translated to changes in Dkk1 protein, we performed western blot analysis on identically treated samples (Fig. 1B). As with the expression of *DKK1* mRNA, Dkk1 protein was also found to be upregulated transiently, peaking 12 hours following onset of differentiation (Fig. 1A,B). Furthermore, the induction of Dkk1 protein correlated with downregulation of both cytosolic and nuclear β-catenin, which is a downstream effector of canonical Wnt signalling (Fig. 1C,D). The induction of Dkk1 protein also correlated with reduced expression of cyclinD1, PPARδ and ID2, known transcription targets of Wnt/β-catenin (Fig. 1E). These results suggest that increased expression of Dkk1 during human adipogenesis is associated with inhibition of canonical Wnt signalling.

DKK1 gene and protein expression is restricted to the stromal-vascular fraction of human adipose tissue

We next examined the distribution of *DKK1* mRNA in human adipose tissue. As shown in Fig. 2A, *DKK1* mRNA was expressed in stromal-vascular cells and was essentially absent in mature adipocytes in both subcutaneous and omental

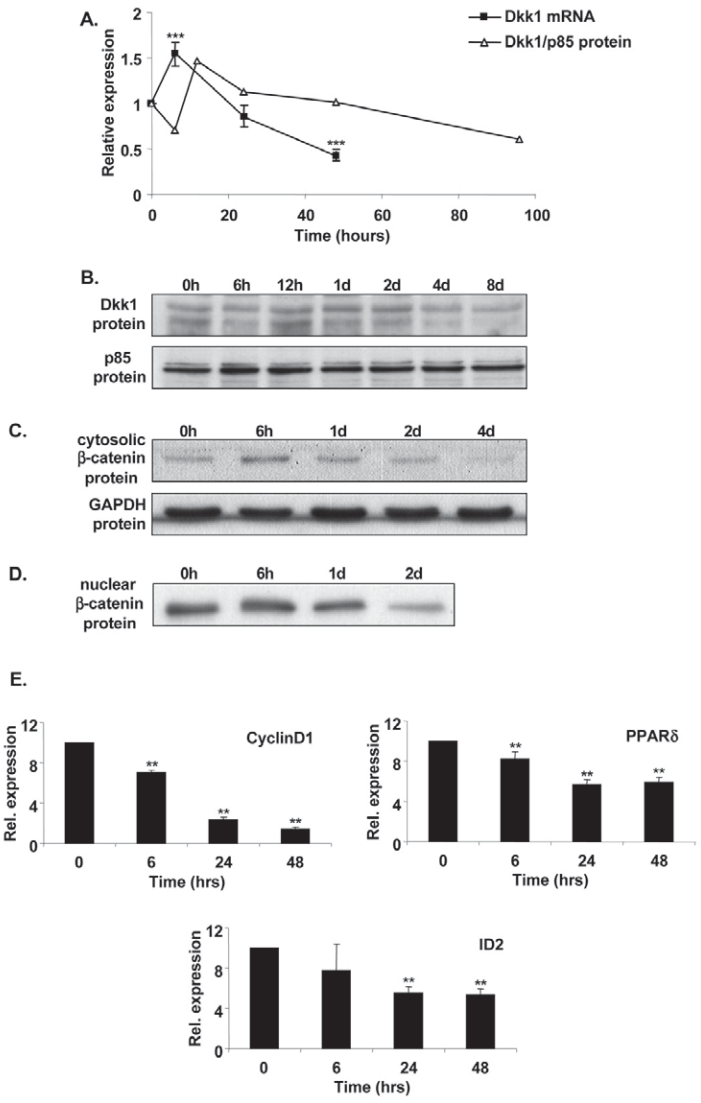

Fig. 1. *DKK1* mRNA and Dkk1 protein expression is transiently upregulated during human adipogenesis and correlates with inhibition of canonical Wnt signalling. (A) *DKK1* mRNA and protein expression during human adipogenesis. Subcutaneous preadipocytes from eight unrelated subjects were differentiated in vitro and total RNA extracted at the time points indicated. *DKK1* mRNA levels were determined by real-time RT-PCR. A plot of the ratio of Dkk1 to p85 protein obtained by densitometry (see Fig. 1B) is also shown alongside the RNA data for comparison. Results are expressed as fold difference relative to baseline (time 0). (B-D) Dkk1 and β-catenin protein expression during human adipogenesis. Subcutaneous preadipocytes were differentiated in vitro and whole-cell lysates (B), cytosolic (C), or nuclear extracts (D) were obtained at the time points indicated and subjected to SDS-PAGE and western blot analysis. p85 PI 3-kinase and GAPDH were used as loading controls. Results are representative of at least two independent experiments. 0h indicates onset of differentiation (3 days post-confluence); 6h, 12h, 1d, 2d, 4d, 8d respectively indicate 6 hours, 12 hours, 1 day, 2 days, 4 days and 8 days post-induction of differentiation. (E) Expression of Wnt target genes during human adipogenesis. CyclinD1, PPARδ and ID2 mRNA levels were determined by real-time RT PCR using RNA from six of the subjects used in A. Rel., relative. $^{**}P<0.01$, $^{***}P<0.001$.

adipose depots. Similarly, Dkk1 protein was present in stromal-vascular cells (Fig. 2B). Parallel experiments were also conducted on murine samples. However, in this species, we could not detect *Dkk1* gene expression in stromal-vascular cells and mature adipocytes (Fig. 2C) or during differentiation of murine primary cultures (our unpublished data). *Dkk1* transcript was also undetectable in 3T3-L1 preadipocytes (Fig. 2C), although the same primer pair gave a positive signal when whole mouse embryo cDNA was used as PCR template. Similarly, using a mouse-specific antibody, we could not detect Dkk1 protein in murine stromal-vascular cells or 3T3-L1 preadipocytes. Nevertheless, the same antibody, which crossreacts with human protein, readily detected recombinant human (r)Dkk1 protein and human (h)Dkk1 protein ectopically expressed in 3T3-L1 cells (see below). Hence, adipose expression of Dkk1 appears to be species specific.

Other Dkk family members are downregulated during adipogenesis

Given the lack of Dkk1 expression in murine adipose cells, we examined whether other members of the Dkk family are present in mouse adipose tissue and regulated during adipogenesis. Fig. 3A shows that both *Dkk2* and *Dkk3* are expressed in primary preadipocytes and 3T3-L1 cells, and to a lesser extent in mature adipocytes. *Dkk4* was not detected in 3T3-L1 or primary preadipocytes (our unpublished data). The expression of *Dkk2* and *Dkk3* during differentiation of 3T3-L1 cells was also analysed using quantitative real-time RT-PCR. As shown in Fig. 3B, both *Dkk2* and *Dkk3* mRNA are rapidly downregulated within six hours of induction of differentiation and thus do not show the same profile as *DKK1* during human

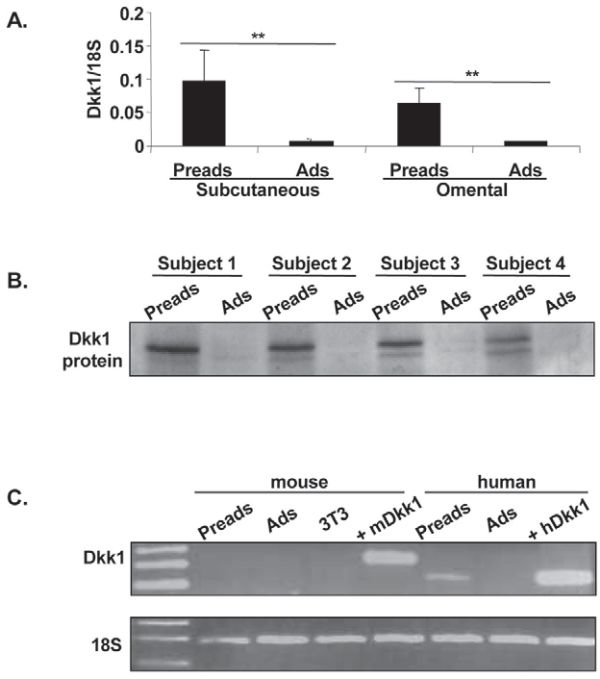

Fig. 2. *DKK1* mRNA and protein expression is restricted to the stromal-vascular fraction of human adipose tissue. (A) *DKK1* mRNA levels were measured using real-time RT PCR in subcutaneous and omental stromal-vascular cells and mature adipocytes from eight unrelated subjects. (B) Dkk1 protein expression in vivo. Western blot analysis of whole-cell lysates from subcutaneous stromal-vascular cells and mature adipocytes from four unrelated subjects. (C) *DKK1* mRNA expression in human and mouse adipose tissues. Total RNA was extracted from stromal-vascular cells and mature adipocytes from four mice (pooled samples), confluent 3T3-L1 cells and one human subject, and RT-PCR for mouse and human *DKK1* was performed. RNA isolated from whole mouse embryo and 3T3-L1 cells stably overexpressing human *DKK1* were used as positive controls for mouse and human PCRs, respectively. Data were normalised using 18S control. Preads, preadipocytes; Ads, adipocytes; 3T3, 3T3-L1 cells; m, mouse; h, human; +, positive control; ***P*<0.01.

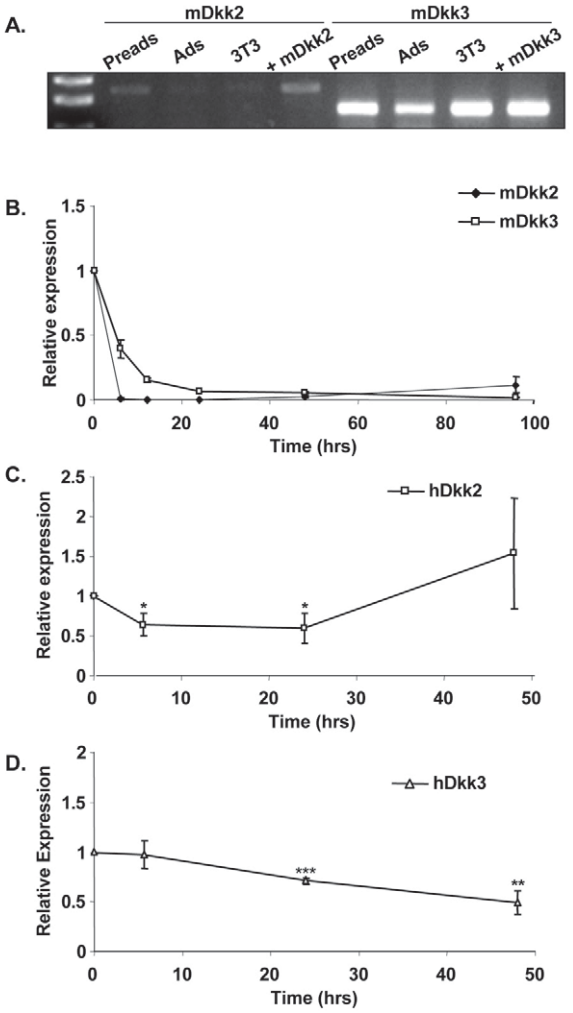

Fig. 3. Expression of *DKK2* and *DKK3* during adipogenesis. (A) Expression of *Dkk2* and *Dkk3* mRNA in mouse adipose tissue was determined using RNA obtained in Fig. 2C. (B) 3T3-L1 preadipocytes were induced to differentiate and total RNA extracted at the time points indicated. *Dkk2* and *Dkk3* mRNA levels were measured using real-time RT-PCR. Results are expressed as fold difference relative to the baseline (time 0). All results the mean ± s.e.m. of four independent experiments. (C) Human subcutaneous preadipocytes from six unrelated subjects were differentiated in vitro and total RNA extracted at the time points indicated. mRNA levels of *DKK2* and *DKK3* were determined by real-time RT-PCR. Results are expressed as fold difference relative to baseline (time 0). Preads, preadipocytes; Ads, adipocytes; 3T3, 3T3-L1 cells; m, mouse; h, human; +, positive control. **P*<0.05, ***P*<0.01, ****P*<0.001.

adipogenesis. This suggests that Dkk2 and Dkk3 are not substitutes for Dkk1 in murine adipogenesis. *DKK2* and *DKK3* mRNAs are also downregulated during human adipogenesis, albeit with different kinetics (Fig. 3C,D).

Ectopic expression of hDkk1 inhibits Wnt signalling in 3T3-L1 preadipocytes

To assess the functional consequences of Dkk1 upregulation, we generated 3T3-L1 cells constitutively expressing hDkk1. After confirming hDkk1 protein expression in our Dkk1 cell line (Fig. 4A), we determined whether hDkk1 could inhibit Wnt signalling in these cells. Both control (empty vector) and hDkk1-expressing preadipocytes were induced to differentiate, and cytosolic protein extracts collected at the times indicated (Fig. 4B). Ectopic expression of hDkk1 led to constitutive inhibition of Wnt signalling, as demonstrated by decreased levels of cytosolic β-catenin throughout differentiation in these cells. This was further confirmed with promoter assays using the luciferase reporter construct TOPflash (Fig. 4C). This reporter contains multiple TCF-binding sites and is a read-out for Wnt/β-catenin transcriptional activity. Control and hDkk1-expressing cells were transfected with TOPflash 8 hours after induction of differentiation and luciferase activity was determined at 48 hours. Overexpression of hDkk1 led to a significant reduction in promoter activity, confirming that hDkk1 inhibits canonical Wnt signalling in differentiating 3T3-L1 preadipocytes.

Ectopic expression of hDkk1 in 3T3-L1 preadipocytes promotes adipogenesis

We next determined whether inhibition of Wnt signalling by constitutive expression of hDkk1 affected adipogenesis. Canonical Wnt signalling is rapidly suppressed upon induction of differentiation of 3T3-L1 cells in response to 1-methyl-3-isobutylxanthine (IBMX) (Bennett et al., 2002; Moldes et al., 2003). As shown in Fig. 4D,E, when cells were

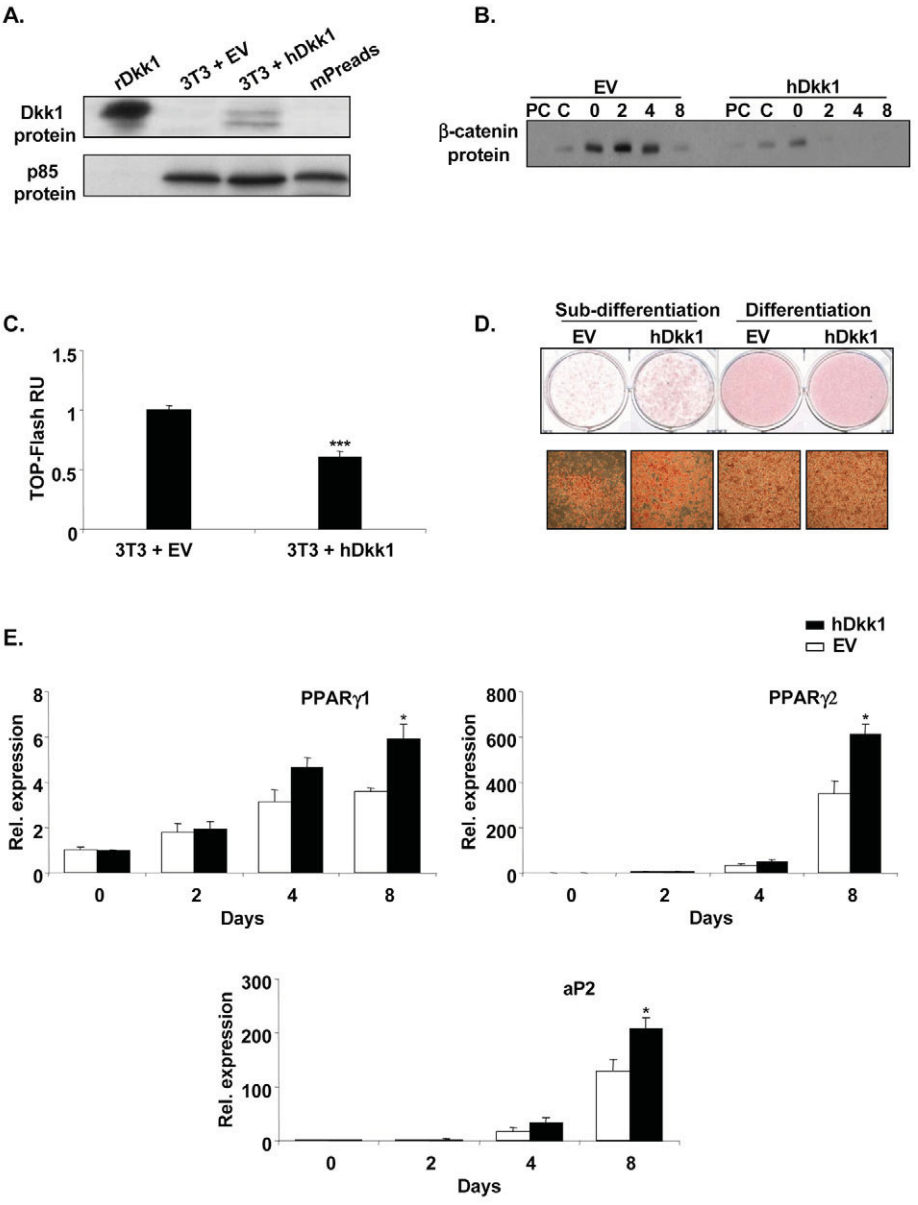

Fig. 4. hDkk1 inhibits Wnt signalling and promotes differentiation of 3T3-L1 preadipocytes. (A) Western blot analysis of whole-cell lysates from 3T3-L1 preadipocytes expressing empty vector (EV) or human Dkk1 (hDkk1) and pooled stromal-vascular cells from four mice (mPreads). Human recombinant Dkk1 protein (rDkk1) was used as positive control, and loading efficiency was assessed using p85 PI 3-kinase. (B) Western blot analysis of cytosolic β-catenin levels during differentiation of control and hDkk1-expressing 3T3-L1 preadipocytes. (C) Effect of hDkk1 on TOPflash reporter activity in 3T3-L1 cells expressing empty vector (EV) or hDkk1. Results are expressed as fold difference relative to EV control. All results are the mean ± s.e.m. of three independent experiments. (D) Oil-Red O staining of EV and hDkk1-expressing 3T3-L1 adipocytes. Cells were induced to differentiate for 8 days using either differentiation medium lacking IBMX (sub-differentiation) or the full differentiation cocktail. (E) Effect of hDkk1 on adipogenic gene expression. Control and hDkk1-expressing 3T3-L1 cells were differentiated sub-maximally using DI and total RNA extracted at the time points indicated. PPARγ1, PPARγ2 and aP2 mRNA levels were measured using real-time PCR. Results are expressed as fold difference relative to the basal (time 0) value for control. All results are the mean ± s.e.m. of three independent experiments. All comparisons were made against control using Student's *t* test. PC, pre-confluent; C, confluent; 0, onset of differentiation (2 days post-confluence); 2, 4, 8 indicate 2, 4 and 8 days post-induction of differentiation respectively; RU, relative units; 3T3, 3T3-L1 cells; hDkk1, human Dkk1. **P*<0.05, ****P*<0.001.

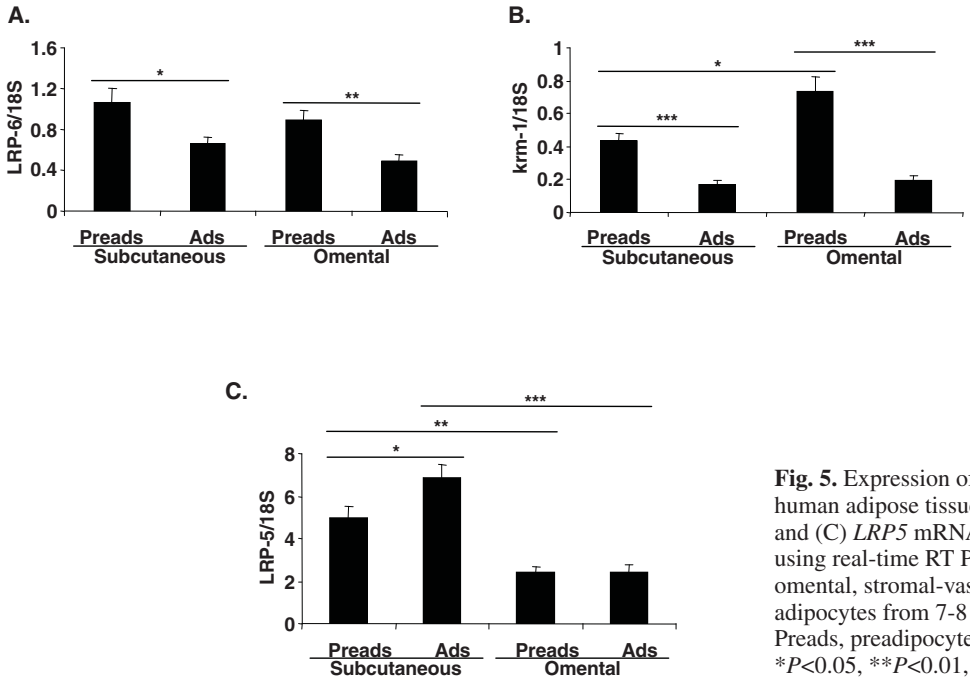

Fig. 5. Expression of Dkk1 receptors in human adipose tissue. (A) *LRP6*, (B) *KRM1* and (C) *LRP5* mRNA levels were measured using real-time RT PCR in subcutaneous and omental, stromal-vascular cells and mature adipocytes from 7-8 unrelated subjects. Preads, preadipocytes; Ads, adipocytes. **P*<0.05, ***P*<0.01, ****P*<0.001.

induced to differentiate sub-maximally by omitting IBMX from the differentiation cocktail, ectopic expression of hDkk1 led to increased lipid accumulation and expression of the adipogenic markers PPARγ1, PPARγ2 and aP2, compared with control. With maximal stimulation using 1-methyl-3-isobutylxanthine, dexamethasone and insulin (MDI), both cell lines differentiated to the same extent (Fig. 4D). Our results demonstrate that Dkk1 can facilitate differentiation of 3T3-L1 cells through inhibition of canonical Wnt signalling.

Dkk receptors are present in human adipose tissue and are transiently downregulated during human adipogenesis

Dkk1 uses a network of receptors to inhibit Wnt signalling. Hence, we investigated the presence of these receptors in human adipose tissue. Like *DKK1*, *LRP6* and *Krm1* are predominantly expressed in stromal-vascular cells from both subcutaneous and omental adipose depots (Fig. 5A,B). By contrast, *LRP5* is present at similar levels in mature adipocytes and stromal-vascular cells (Fig. 5C).

Given that human preadipocytes express the necessary repertoire of receptors required to respond to Dkk1, we assessed whether *KRM1*, *LRP5* and *LRP6* gene expression was also regulated during the early stages of human preadipocyte differentiation. Fig. 6A and Table 1 show that there was little change in expression of *KRM1* mRNA throughout the 48-hour time course. However, the levels of *LRP5* mRNA were significantly and transiently repressed six hours following treatment of human preadipocytes with adipogenic medium (Fig. 6B). A similar trend was observed for *LRP6* (Fig. 6B and Table 1). Our results demonstrate that Dkk receptors are expressed in human adipose tissue. Furthermore, *DKK1* and *LPR5/6* are reciprocally regulated during early human adipogenesis.

Discussion

We interrogated a microarray database for genes upregulated during the early stages of human adipogenesis and identified *DKK1* as a transcript that was significantly induced within six

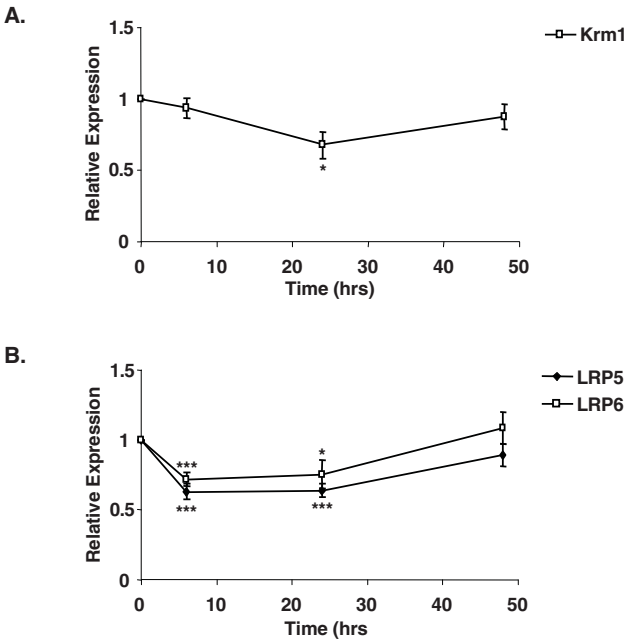

Fig. 6. Expression of Dkk1 receptors is regulated during early human adipogenesis. Subcutaneous preadipocytes from eight unrelated subjects were differentiated in vitro and total RNA extracted at the time points indicated (see Table 1). mRNA levels of (A) *KRM1* and (B) *LRP5* and *LRP6* were determined by real-time RT PCR. Results are expressed as fold difference relative to baseline (time 0). **P*<0.05, ****P*<0.001.

Table 1. Expression of Dkk1 receptors is regulated during early human adipogenesis

Gene (Rel. expr.)	0 hours	6 hours	24 hours	48 hours
KRM1	1	0.94±0.07	0.68±0.09*	0.87±0.09
LRP5	1	0.63±0.06***	0.64±0.05***	0.89±0.08
LRP6	1	0.72±0.05***	0.75±0.1*	1.09±0.11

Subcutaneous preadipocytes from eight unrelated subjects were differentiated in vitro and total RNA extracted at the time points indicated (see Fig. 6). mRNA levels of the genes *KRM1*, *LRP5* and *LRP6* were determined by real-time PCR. Results are expressed as fold difference relative to baseline (time=0). Rel. expr., relative expression. **P*<0.05, ****P*<0.001.

hours of differentiation. Given the evidence from studies on murine preadipocytes suggesting that Wnt signalling functions as an early adipogenic switch (Bennett et al., 2002; Ross et al., 2000), we investigated the role of Dkk1, a secreted Wnt antagonist, in human adipogenesis. Here, we demonstrate that Dkks and their receptors are expressed in human preadipocytes and stromal-vascular cells, indicating that they might modulate human adipogenesis in vitro and in vivo. Our in vitro studies also show that Dkk1 expression is transiently upregulated in human preadipocytes following adipogenic stimulation and this correlates with inhibition of canonical Wnt signalling. In addition, we observe coordinate downregulation of Dkk receptors *LRP5* and *LRP6* that is likely to potentiate the ability of Dkk1 to inhibit Wnt signalling.

We also show that constitutive expression of Dkk1 can modulate the adipogenic program as demonstrated by its ability to promote differentiation of 3T3-L1 preadipocytes. This is likely to occur through inhibition of canonical Wnt signalling as it is accompanied by a reduction in cytosolic β-catenin levels, leading to reduced TCF promoter activity. Furthermore, the pro-adipogenic effect of Dkk1 is most pronounced when IBMX, an agent that suppresses Wnt signalling within hours of onset of differentiation of 3T3-L1 cells (Bennett et al., 2002), is omitted from the differentiation cocktail. Given that Dkk1 is necessary for re-entry of mesenchymal stem cells into the cell cycle (Gregory et al., 2003) and considering that Wnt signalling was shown to block differentiation of 3T3-L1 cells in part through inhibiting mitotic clonal expansion (Ross et al., 2002), it is possible that Dkk1 promotes adipogenesis by driving this process.

We considered adopting a loss-of-function approach to substantiate our findings. However, the surprising lack of Dkk1 expression in murine models made this approach unfeasible. The absence of Dkk1 from murine adipose tissue indicates that its proadipogenic role might be species specific. We considered the possibility that other members of the Dkk family could be involved in the regulation of murine adipogenesis. In this respect, both mouse stromal-vascular cells and 3T3-L1 preadipocytes express *Dkk2* and *Dkk3* but not *Dkk4*. However, both *Dkk2* and *Dkk3* are downregulated following differentiation of murine as well as human preadipocytes. Thus, it seems unlikely that these Dkk family members are direct substitutes for Dkk1 in murine adipogenesis.

LRP5, *LRP6* and *KRM1*, the receptors of Dkk1, are highly expressed in stromal-vascular cells. Both, *LRP5* and *LRP6* are downregulated upon induction of differentiation. LRP6 has been shown to 'titrate' the ability of Dkk1 to inhibit Wnt signalling in vitro in HEK 293 T cells (Bafico et al., 2001; Mao et al., 2001) and in vivo (MacDonald et al., 2004). Hence, it is likely that downregulation of *LRP5/6* at a time when *Dkk1* expression is induced would enhance the ability of Dkk1 to inhibit Wnt signalling (Bafico et al., 2001; Mao et al., 2001). Furthermore, as the changes in *DKK1*, *LRP5* and *LRP6* levels occur simultaneously, it is likely that these events are coordinated to decrease Wnt signalling in human preadipocytes undergoing differentiation.

Adipocytes are derived from mesenchymal stem cells. These precursors can also differentiate into a variety of other cell types including osteoblasts (Nuttall and Gimble, 2004; Prockop et al., 2003). A key signal thought to regulate the balance between these lineages is canonical Wnt signalling. In addition to inhibiting adipogenesis, recent studies have demonstrated that Wnt/β-catenin signalling is required for osteoblast lineage differentiation. Specifically, it has been shown that loss-of-function mutations in LRP5 cause the autosomal recessive osteoporosis-pseudoglioma syndrome (OPPG) (Gong et al., 2001). Conversely, patients expressing gain-of-function mutations in LRP5 as a result of impaired affinity and antagonism by Dkk1 exhibit high bone density (Ai et al., 2005; Boyden et al., 2002; Little et al., 2002; Van Wesenbeeck et al., 2003; Zhang et al., 2004). Whether such patients or indeed mice engineered to carry mutations in LRP5/6 exhibit adipose phenotypes is as yet unreported. Furthermore, increased production of Dkk1 by myeloma cells inhibits the differentiation of osteoblast precursor cells in vitro and is associated with the presence of lytic bone lesions in patients with multiple myeloma (Tian et al., 2003). As ours is the first report to suggest that a secreted Wnt antagonist, Dkk1, might promote human adipocyte differentiation, we hypothesise that, through inhibition of Wnt signalling, Dkk1 might direct human mesenchymal stem cell fate towards adipogenesis while preventing commitment to the osteoblast lineage.

In summary, we have shown that Dkk1, a Wnt antagonist, is secreted by human preadipocytes and promotes adipogenesis in vitro by inhibiting Wnt signalling. Coordinated downregulation of Dkk1 receptors *LPR5* and *LRP6* might potentiate the pro-adipogenic action of Dkk1. In vivo, Dkk1 is appropriately expressed in the stromal-vascular fraction of human adipose tissue and might function as an endogenous trigger for recruiting new preadipocytes into the program of adipocyte differentiation. Our results also show that, although Wnt inhibition is a mechanism promoting adipocyte differentiation in humans and mice, there are inter-species differences in the molecular control of adipogenesis. Given the public health importance of human obesity, specific knowledge of the molecular pathways unique to human adipogenesis should aid better understanding of disease pathogenesis and guide the development of effective therapies.

Materials and Methods

Human adipocyte and preadipocyte isolation

Adipose tissue samples were obtained from subjects undergoing elective open-abdominal or thigh surgery. All subjects were fasted for 6 hours prior to the operation and all underwent general anaesthesia. Cambridge Research Ethics Committee approval was obtained and all patients gave their informed consent. Adipose tissue biopsies were placed in PBS (Sigma-Aldrich) and processed within 30 minutes. Samples were finely diced and digested in collagenase solution [Hank's balanced salt solution containing 3 mg/ml type II collagenase (Sigma-Aldrich) and 1.5% bovine serum albumin] at 37°C for 1 hour. Subsequently, the digest was filtered through a stainless steel mesh and centrifuged at 400 ***g*** for 5 minutes to separate mature adipocytes from the stromal-vascular cells.

Human preadipocyte culture

The cell pellet containing the stromal-vascular fraction was treated with red cell lysis buffer (154 mmol/l NH_4Cl, 10 mmol/l $KHCO_3$, 0.1 mmol/l EDTA) for 5 minutes at room temperature and centrifuged (5 minutes, 400 ***g***). The pellet was then re-suspended and cultured in DMEM:Hams F12 (1:1) medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin at 37°C in a humidified incubator at 5% CO_2. Cells were passaged 2-3 times before being grown to confluence. Differentiation was induced in 3-day post-confluent monolayers, by adding serum-free differentiation medium [DMEM:Hams F12 (1:1), 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 33 μM Biotin, 17 μM panthothenic acid, 10 μg/ml human apotransferrin, 0.2 nM tri-iodothyronine, 100 nM cortisol, 500 nM insulin] supplemented with 10^{-7} M BRL 49653 and 10^{-7} M LG 100268. For the first 3 days of culture, 0.25 mM 1-methyl-3-isobutylxanthine (IBMX) was also added to the medium. Differentiation medium was replaced after 3 days on the first occasion and thereafter every 2 days.

Culture, differentiation and infection of 3T3-L1 preadipocytes

3T3-L1 cells were cultured and differentiated into adipocytes as described previously (Nugent et al., 2001). Human *DKK1* cDNA was a kind gift from S. E. Millar (University of Pennsylvania, Philadelphia, USA). Human Dkk1-overexpressing and control 3T3-L1 cell lines were generated using the pBabe-Puro retroviral vector system as described previously (Xu et al., 1999). Cells were kept in puromycin-containing medium except during differentiation experiments.

RT-PCR

Total RNA was prepared using the RNeasy Mini Kit (Qiagen) and 500 ng RNA was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase and random hexamer primers (Promega). The cDNAs provided templates for human and mouse *DKK* PCRs using Taq DNA polymerase (Invitrogen) and the following primers: human *DKK1* (forward 5′-CGGGAATTACTGCAAAAATGGA-3′, reverse 5′-GCACAGTCTGATGACCGGAGA-3′); mouse *Dkk1* (forward 5′-GTACTG-CTCCAGCCCCAGC-3′, reverse 5′-GAGGCAGACGGAGCCTTCTT-3′); mouse *Dkk2* (forward 5′-CAGTCACTGAGAGCATCCTCA-3′, reverse 5′-CCTGATGG-AGCACTGGTTTGC-3′); mouse *Dkk3* (forward 5′-CGAGAGGTGGAGGAGC-TGATG-3′, reverse 5′-GTCTCCGTGCTGGTCTCATTG-3′). The cycle conditions were as follows: denaturation, 30 seconds at 95°C; annealing, 30 seconds at 62°C (human and mouse *Dkk1*) or 50°C (mouse *Dkk2* and *Dkk3*); elongation, 30 seconds at 72°C. PCR synthesis was quantified at 40 cycles. Data were subsequently normalised using 18S control. For 18S PCRs, cDNA samples were diluted 1:10 and amplified using a two-step PCR as follows: denaturation, 15 seconds at 95°C; annealing and elongation, 1 minute at 60°C. PCR synthesis was quantified at 18, 20, 22, 24, 26 and 28 cycles in a test reaction to ensure that the quantitative PCR amplification was in the linear range.

TaqMan® quantitative real-time reverse transcription PCR

RNA preparation, reverse transcription and conditions for TaqMan real-time reverse transcription (RT)-PCR were performed as described previously (Sewter et al., 2002). Primers and probes were designed using Primer Express software (Applied Biosystems) and sequences from the GenBank database, and are available upon request. In the case of *LRP5* and *LRP6*, TaqMan reagents were specifically designed to amplify transcripts coding for full-length receptors only. Primers and probes for 18S and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) internal controls were purchased from Perkin Elmer.

Western blot analysis

Cells were washed with cold PBS and scraped into either lysis buffer or hypotonic lysis buffer to obtain cytosolic protein extracts for immunoblots of β-catenin as described (Culbert et al., 2001; Laudes et al., 2004). For immunoblots of Dkk1 in stromal-vascular cells and mature adipocytes, adipose tissue samples were processed as outlined above and the cell pellet containing the stromal-vascular fraction or the floating mature adipocytes were solubilised in lysis buffer. After centrifugation at 4°C at 10,000 ***g*** for 10 minutes, equal amounts of protein were dissolved in Laemmli buffer, heated to 95°C and separated by SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membranes (Millipore) and immunoblotted with the following antibodies according to the manufacturer's instructions: anti-human Dkk1 (AF 1096; R&D Systems), anti-mouse Dkk1 (AF 1765; R&D Systems), anti-β-catenin (Transduction Laboratories), anti-p85 phosphoinositide 3-kinase (PI 3-kinase; kindly provided by K. Siddle, University of Cambridge, Cambridge, UK) and anti-GAPDH (Abcam). All secondary antibodies were purchased from DAKO (Denmark) and used as 1:5000 dilutions in PBS + 1% milk or BSA.

Nuclear extracts

Nuclear extracts were prepared using the NucBuster™ protein extraction kit (MERCK Biosciences) as recommended by the manufacturer. Briefly, cells were washed with, and subsequently scraped into, 200 μl of cold PBS. After centrifugation at 4°C at 500 ***g*** for 5 minutes, cell pellets were resuspended in 150 μl of NucBuster extraction reagent 1 for 5 minutes on ice to release nuclei. The nuclei were harvested by centrifugation (16,000 ***g*** for 5 minutes at 4°C) and resuspended in 75 μl of NucBuster extraction reagent 2 for 5 minutes on ice. Nuclear extracts were separated from cell debris by centrifugation (16,000 ***g*** for 5 minutes at 4°C).

Gene promoter reporter assay

Human Dkk1-overexpressing and control 3T3-L1 cells were seeded in 24-well plates (5×10^4 cells per well) and grown to confluence. At 2 days post-confluence, cells were induced to differentiate, and 8 hours later transfected with 1 μg/well of the TOPflash promoter-reporter gene construct using FuGENE® (Roche Applied Science). To correct for transfection efficiency, 10 ng/well pRL-CMV (Promega) was co-transfected. 48 hours after onset of differentiation, cells were lysed in passive lysis buffer (Promega) and luciferase activity was determined by luminometry (EG Berthold) using the dual luciferase reporter assay (Promega).

Statistical analysis

All results are presented as mean ± s.e.m. Unless otherwise stated, statistical significance was assessed using the Mann-Whitney test (*P<0.05, **P<0.01, ***P<0.001).

We thank the Addenbrooke's hospital surgeons for generously providing adipose tissue biopsies and T. Hagen and M. López for useful advice and discussions. C. Christodoulides and W. Cawthorn are sponsored by the Medical Research Council. M. Laudes and S. Schinner are sponsored by Deutsche Forschungsgemeinschaft. This research is funded by the Wellcome Trust Integrative Physiology Program (A.V.P., S.O'R.), MRC Career Establishment Award (A.V.-P.), and BBSRC David Phillips Fellowship (J.K.S.).

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PUBLIKATION 11

Schinner S, Willenberg HS, Krause D, Schott M, Lamounier-Zepter V, Krug AW, Ehrhart-Bornstein M, Bornstein SR, Scherbaum WA.

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Int. J. Obes. 31(5):864-70, (2007).

International Journal of Obesity (2007) 31, 864–870
 0307-0565/07 $30.00
www.nature.com/ijo

ORIGINAL ARTICLE

Adipocyte-derived products induce the transcription of the StAR promoter and stimulate aldosterone and cortisol secretion from adrenocortical cells through the Wnt-signaling pathway

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Context: Obesity is associated with hypersecretion of cortisol and aldosterone and a high prevalence of arterial hypertension. At the cellular level, a direct effect of adipocytes on the expression of the steroidogenic acute regulatory (StAR) protein, a regulator of cortisol and aldosterone synthesis, and on aldosterone and cortisol secretion has been shown. However, the molecular mechanisms mediating this effect are not known.
Objective: Wnt-signaling molecules are secreted by adipocytes and regulate the activity of SF-1, a key transcription factor in adrenal steroidogenesis. Therefore, we investigated whether adipocytes stimulate adrenal steroidogenesis through the activation of Wnt-signaling.
Results: Using immunohistochemistry, we detected the expression of frizzled and β-catenin in the adult human adrenal cortex. Transient transfection of a Wnt-dependent reporter-gene into adrenal NCI-H295R cells showed an induction of Wnt-mediated transcription to 308% after treatment with human fat cell-conditioned medium (FCCM). This finding was paralleled by an induction of StAR promoter activity (420%) by FCCM. The induction of StAR promoter activity by FCCM was inhibited by 49% when Wnt-signaling was blocked by the soluble Wnt-antagonist secreted Frizzled-Related-Protein-1 (sFRP-1). Overexpression of a constitutively active mutant of β-catenin induced the transcription of the StAR promoter (440%). β-Catenin and FCCM induced SF-1-mediated transcription at a SF-1-driven reporter gene (420 and 402%, respectively). Furthermore, the secretion of aldosterone and cortisol by NCI-H295R cells induced by FCCM was significantly inhibited by the Wnt-antagonist sFRP-1.
Conclusion: These data indicate that the Wnt-signaling pathway is one of the mechanisms mediating the effects of fat cells on adrenal StAR transcription and aldosterone and cortisol secretion.
International Journal of Obesity (2007) **31**, 864–870. doi:10.1038/sj.ijo.0803508; published online 9 January 2007

Keywords: adrenal cortex; Wnt-signaling; StAR; hypertension

Introduction

Obesity is associated with a high risk of arterial hypertension[1] and is characterized by hypersecretion of the adrenocortical hormones aldosterone and cortisol.[2–6] Both aldosterone and cortisol increase arterial blood pressure.[2,7,8] The obesity-induced increase in arterial blood pressure is inhibited by aldosterone receptor antagonists,[9] and aldosterone levels correlate well with obesity, suggesting an effect of adipocytes on aldosterone production.[2] Accordingly, our recent studies showed stimulation of adrenal steroidogenesis by adipocytes *in vitro*. Thus, fat-cell-derived products were shown to increase aldosterone and cortisol secretion in adrenocortical cells.[10] However, the molecular mechanisms mediating the effects of adipocyte-secretory products on adrenocortical cells are not understood.

Adipocytes are endocrine glands and as such produce a variety of adipocytokines, for example, leptin, adiponectin and TNFα (reviewed by Bays *et al.*[11]), but none of those have so far been shown to mediate the effects of fat cells on steroidogenesis that have been described previously.[10]

In addition, adipocytes secrete Wnt-signaling molecules.[12,13] Wnts are extracellular ligands on transmembrane-receptors

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Received 14 May 2006; revised 2 October 2006; accepted 7 October 2006; published online 9 January 2007

called frizzled and on low-density lipoprotein-coreceptors. Binding of ligand eventually leads to stabilization and nuclear translocation of the co-activator protein β-catenin.[14]

β-Catenin co-activates T-cell factor/lymphoid enhancer factor transcription factors on canonical Wnt target-genes, and can also interact with a variety of different transcription factors. These include SF-1, a key regulator of adrenal steroidogenesis.[15–17] SF-1 binds to consensus sequences (AGGTCA) within the promoter of the gene encoding steroidogenic acute regulatory protein (StAR).[18] StAR activity is the rate-limiting step in steroidogenesis by regulating the intramitochondrial cholesterol transfer.[19] Therefore, in this study, we investigated whether adipocyte-derived products increase StAR transcription and aldosterone and cortisol secretion through Wnt/β-catenin signaling.

Materials and methods

Human tissues

Tissue samples of human white adipose tissue were obtained from healthy (20- to 35-year-old) women undergoing surgical mammary reduction ($n=10$). The patients were free of metabolic or endocrine diseases. The body mass index range of the donors was between 21.4 and 29.2 kg/m^2 (25.4 ± 2.8, mean $\pm$ s.d.). Informed consent was obtained from the patients after the nature of the procedure was explained and the study was approved by the ethical committee of the Heinrich-Heine-University Düsseldorf, Germany (study number 2292).

Light microscopy and immunohistochemistry of human adult adrenals

Paraffin-embedded sections of human normal adult adrenal glands were immuno-stained with a rabbit polyclonal antibody to frizzled 1–10 (raised against a recombinant protein corresponding to amino acids 301–400 of human frizzled-2; Santa Cruz Biotech, Santa Cruz, CA, USA) or with a monoclonal mouse antibody to human β-catenin (Chemicon Int., Temecula, CA, USA) at room temperature. After 1 h, the sections were washed three times in phosphate-buffered saline and then incubated with the respective secondary antibody (EnVision System, DakoCytomation, Hamburg, Germany) and exposed to aminoethylcarbazole (red staining) or diaminobenzidine (brown staining) and eventually counterstained with hematoxylin.

Plasmid constructs

The plasmid TOPFLASH, a canonical Wnt reporter-gene, is commercially available (Upstate, Cell signaling solutions, Charlottesville, VA, USA). The plasmids for wild-type β-catenin and the constitutively active S45A mutant,[20] the SF-1 expression vector and the SF-1-driven reporter-gene (-65-Luc),[21] as well as the StAR-Luc reporter-gene[22] have been described previously. The plasmid pRL-TK was purchased from Promega (Mannheim, Germany).

Cell culture and transfection of DNA

The human adrenocortical cell-line NCI-H295R has been described previously.[23,24] NCI-H295R cells were cultured in Dulbecco's modified Eagle's Medium (DMEM)/F12 supplemented with insulin (66 nM), hydrocortisone (10 nM), 17β-estradiol (10 nM), transferrin (10 μg/ml), selenite (30 nM), 100 U/ml penicillin, 100 μg/ml streptomycin and 2% fetal bovine serum.[10] Cells were detached by using Accutase (PAA Laboratories, Cölbe, Germany) and transiently transfected with Fugene 6 reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol.

Cotransfections were carried out with a constant amount of DNA that was maintained by adding the vector pcDNA3 (Invitrogen, Karlsruhe, Germany). In all experiments, 0.5 μg per well of Renilla luciferase reporter-gene (plasmid pRL-TK) were cotransfected to check for transfection efficiency (the relative luciferase activities presented in the figures are derived from firefly/renilla ratios). We transfected 0.5 μg of the firefly reporter-gene per well. When indicated, cells were incubated with fat cell-conditioned medium (FCCM), or the respective control media 24 h before harvest. The luciferase assay was performed as described previously.[25]

Fat cell-conditioned medium

The isolation of adipocytes and preparation of FCCM have been described previously.[10] In brief, adipose tissue samples of 20–60 g wet weight were obtained from surgical mammary reductions and immediately transported to the laboratory in DMEM/Nutrient Mix F12 (DMEM/F12, Life Technologies, Karlsruhe, Germany) supplemented with 2% bovine serum albumin (BSA), 100 U/ml penicillin and 100 μg/ml streptomycin. After removal of fibrous material and blood vessel the adipose tissue was minced and digested in Kreb's Ringer Bicarbonate buffer (KRB) containing 2% BSA and 120 U/ml collagenase type I from clostridium histolyticum (Sigma-Aldrich, Taufkirchen, Germany) in a shaking water bath for 45–60 min at 37°C. Then, the digested tissue was filtered through nylon gauze (250 μm) and washed with KRB containing 0.1% BSA. For culturing, 2 ml of isolated floating adipocytes (containing approximately 12 million adipocytes) were transferred into culture flasks (Becton Dickinson, Heidelberg, Germany) containing 5 ml of cell culture medium (DMEM/F12 containing 15 mmol/l *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid and 2.5 mmol/l L-glutamine, supplemented with 1.125 g/l $NaHCO_3$, 100 U/ml penicillin and 100 μg/ml streptomycin). Cells were kept at 37°C in a humidified atmosphere of 5% CO_2/95% air and cultured for 24 h. After 24 h, the conditioned medium was collected, carefully avoiding the lipid floating on the top, and kept frozen at $\times$20°C until used. In addition, we quantified the proportion of contaminating cells by using primary antibodies against fibroblasts, endothelial cells (CD 31-antibody) and leukocytes (CD 45-antibody). These antibodies derive from mouse and were obtained from DAKO (Hamburg, Germany). Counterstaining was performed by

using a secondary anti-mouse horseradish peroxidase (HRP)-linked antibody (DAKO, Hamburg, Germany). DMEM/F12 medium without incubation with adipocytes was used as control medium for the treatment of NCI-H295R cells.

Quantification of aldosterone and cortisol secretion by radioimmunoassay
After incubation of NCI-H295R cells with FCCM or the respective control medium, aldosterone and cortisol in the supernatant of the cells were measured using a commercial radioimmunoassay (RIA) according to the manufacturer's protocol (DPC Biermann, Bad Nauheim, Germany).

Wnt-3a and Wnt-10b enzyme-linked immunosorbent assay
Ninety-six-well plates were coated over night at 4°C with 100 μl FCCM or recombinant Wnt-3a or Wnt-10b protein (R&D Systems, Wiesbaden, Germany), respectively. The supernatant was removed and blocking buffer (0.1% Tween-20, 3% BSA/tris-buffered saline (TBS)) was added for 90 min at 37°C. Then, the plate was washed three times with a washing buffer (0.5% Tween-20/TBS) and the Wnt-3a or Wnt-10b antibody (R&D Systems, Wiesbaden, Germany) was added for 2 h at room temperature. Afterwards, the plate was washed three times before the secondary antibody followed by the streptavidin-HRP was added.

Statistical analysis
All data are presented as means $\pm$ s.e.m. Statistical analysis was performed using Student's *t*-test. Significance was assumed at a *P*-value of less than 0.05.

Results

Wnt-signaling molecules are expressed in human adult adrenals
It has been shown previously by reverse transcription-polymerase chain reaction (PCR) that frizzled receptors are expressed in human adrenals.[26] Here, we demonstrate the expression of β-catenin and frizzled in adult human adrenals at the protein level using immunohistochemistry. We used a rabbit polyclonal antibody to detect the frizzled subtypes 1–10 (raised against a recombinant protein corresponding to amino acids 301–400 of human frizzled-2). The staining in response to incubation with the Frizzled antibodies were most intense in the zona glomerulosa (Figure 1, left panel).

Adipocyte-derived factors induce Wnt-signaling and StAR promoter activity in adrenocortical cells
NCI-H295R cells are a well-established model to study steroidogenesis in adrenocortical cells.[23,24] In this study, we show that secretory products from mature human adipocytes induce Wnt-signaling in adrenocortical NCI-H295R cells. As demonstrated in Figure 2, the transcription of a Wnt reporter-gene (TOPFLASH) is induced to 308% as compared to controls ($P<0.05$) after transfection of TOPFLASH into adrenocortical NCI-H295R cells and incubation with FCCM for 24 h. We quantified the number of contaminating cells in the fat cell culture and we found 10 fibroblasts per 1 million adipocytes and detected no endothelial cells or leukocytes (data not shown).

Fat cell products have been shown to induce StAR transcription as assessed by quantitative TaqMan PCR.[10] In line with these findings, we show an induction of a StAR reporter-gene after transfection into NCI-H295R cells and treatment with FCCM (379% of control, $P<0.05$) (Figure 3a). Our previous work showed that the candidate adipocytokines leptin, adiponectin and TNFα could not mimic the stimulatory effect of FCCM on steroidogenesis.[10] Here, we show for the first time, that fat cell secretory products contain Wnt-signaling molecules. We found high levels for both Wnt-10b (mean: 32 ng/ml; range: 16–66 ng/ml) and Wnt-3a protein (mean: 16 ng/ml; range: 11–26 ng/ml) in fat cell-conditioned media from five different patients (data not shown).

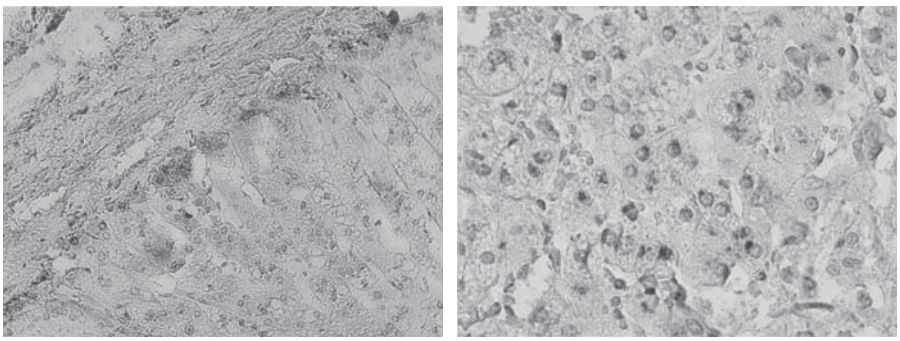

Figure 1 Frizzled and β-catenin are expressed in human adult adrenals. pan-Frizzled and β-catenin antibodies were used for immunohistochemistry of adult human adrenals. The sections were thereafter counterstained with hematoxylin. We found staining for both frizzled and β-catenin antibodies in human adult adrenals. Frizzled (left panel, magnification $\times$ 20), β-catenin (right panel) (magnification $\times$ 40).

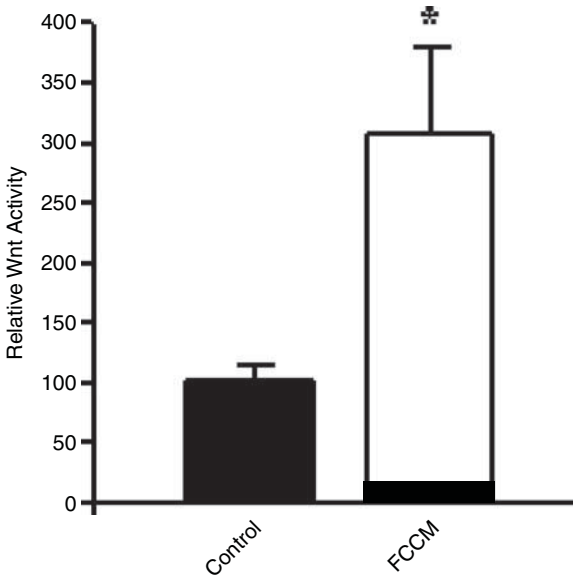

Figure 2 Adipocyte-derived factors induce Wnt-signaling in adrenocortical NCI-H295R cells. NCI-H295R cells were transiently transfected with 0.5 μg of the TOPFLASH reporter-gene and treated with FCCM or the respective control medium. Twenty-four hours after stimulation, the cells were harvested and luciferase activity was determined. The luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means$\pm$s.e. of three independent experiments, each carried out in triplicate. *$P<0.05$ (Student's *t*-test).

We hypothesized that these Wnt-signaling molecules secreted by adipocytes are candidates to mediate the stimulatory effect of FCCM on StAR transcription. To test this hypothesis, we inhibited Wnts within the FCCM by using the antagonist secreted Frizzled-Related-Protein-1 (sFRP-1) (10 ng/ml). We found a significant inhibition by 49% ($P<0.05$) of the FCCM-induced StAR-promoter activation after inhibition of Wnt-signaling (Figure 3a), demonstrating that Wnt-signaling is required to mediate the full effect of fat-cells on StAR transcription.

Furthermore, we studied whether canonical Wnt-signaling activates the transcription of the StAR promoter through β-catenin in adrenocortical NCI-H295R cells. Cotransfection of a constitutively active mutant of β-catenin (S45A)[20] led to a dose-dependent activation of StAR promoter activity with a maximal activation of 442% as compared to controls ($P<0.05$) (Figure 3b). These data define StAR as a target gene for canonical Wnt-signaling in adrenocortical cells.

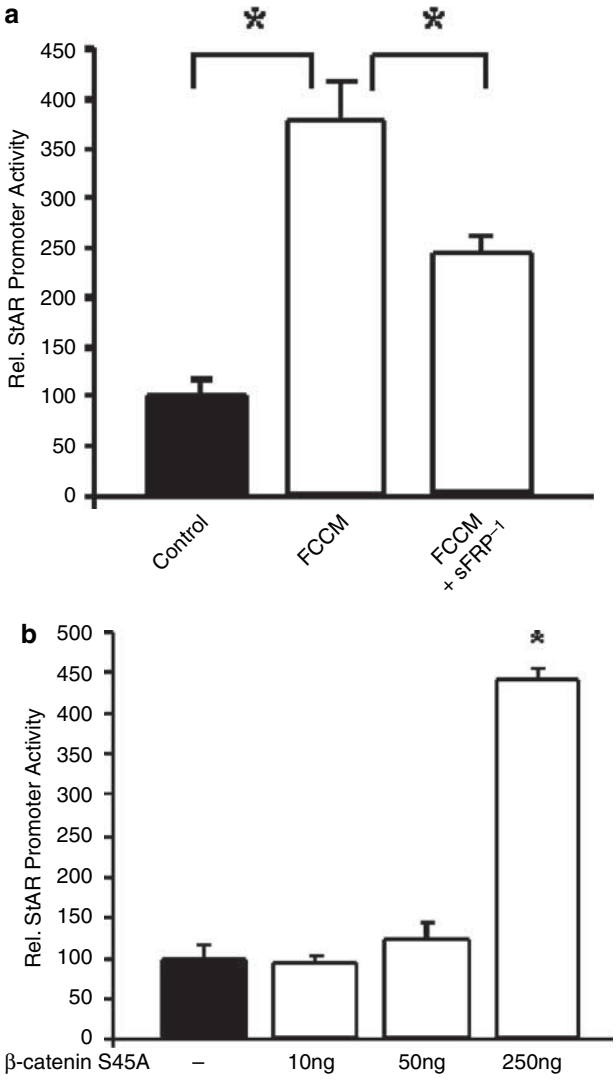

Figure 3 Adipocyte-derived factors induce StAR gene transcription through Wnt-signaling in adrenocortical NCI-H295R cells. (**a**) NCI-H295R cells were transiently transfected with 0.5 μg of the StAR reporter-gene (–966Luc) and treated with FCCM or the respective control medium. The Wnt-antagonist sFRP-1 (10 ng/ml) was added to the FCCM when indicated. (**b**) β-Catenin activates StAR promoter activity in adrenocortical cells. NCI-H295R cells were transiently transfected with 0.5 μg of the StAR reporter-gene (–966Luc). Increasing amounts of β-catenin S45A were cotransfected when indicated. Twenty-four hours after stimulation, the cells were harvested and luciferase activity was determined. The luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means$\pm$s.e. of three independent experiments, each carried out in triplicate. *, $P<0.05$ (Student's *t*-test).

Adipocyte-derived factors and β-catenin induce SF-1-mediated transcription in adrenocortical cells

We investigated the effect of β-catenin and FCCM on SF-1 transcriptional activity in adrenocortical cells. The transcription factor SF-1 is a crucial regulator of the transcription of the gene encoding StAR[19] and synergises with β-catenin at the α-inhibin promoter in placental carcinoma cells.[15] Here, we demonstrate that β-catenin co-activates SF-1-mediated transcription in adrenocortical cells (420%, $P<0.05$) (Figure 4). FCCM induces Wnt/β-catenin-signaling in adrenocortical cells (Figure 2). Therefore, we tested the effect of FCCM on SF-1-transcriptional activity. The effect of FCCM on SF-1-mediated transcription was comparable (stimulation to 402%, $P<0.05$) to the stimulation induced by β-catenin (420%) (Figure 4). These data suggest a

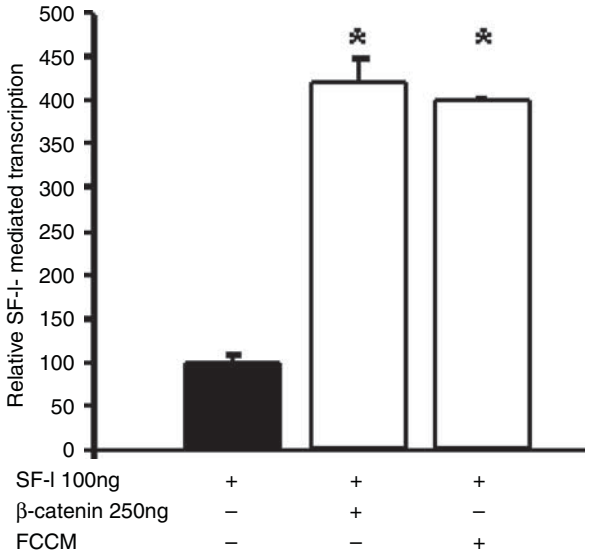

Figure 4 Wnt/β-catenin and FCCM co-activate SF-1-transcriptional activity. NCI-H295R cells were transiently transfected with 0.5 μg of a reporter-gene driven by multimerized SF-1 binding sites (-65Luc) and an expression vector encoding SF-1. β-Catenin (250 ng) S45A were cotransfected when indicated. Cells were treated with FCCM when indicated. Twenty-four hours after stimulation, the cells were harvested and luciferase activity was determined. The luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means $\pm$ s.e. of three independent experiments, each done in triplicate. *$P<0.05$ (Student's *t*-test).

stabilization of β-catenin by adipocyte-derived Wnts to co-activate the transcription factor SF-1.

Adipocyte-derived factors induce aldosterone and cortisol secretion through Wnt-signaling

As shown above FCCM induces StAR gene transcription and this effect is inhibited by a Wnt-antagonist (Figure 3a). StAR-activity is the rate-limiting step in steroidogenesis;[19] therefore, the effect of FCCM through Wnt-signaling on aldosterone and cortisol secretion by FCCM was investigated. After NCI-H295R cells were treated with FCCM for 24 h, aldosterone and cortisol in the supernatant were measured by RIA. In this study, we confirm the stimulation of cortisol and aldosterone secretion by NCI-H295R cells in response to FCCM. As shown in Figure 5a, FCCM induced cortisol secretion (180% of untreated controls, $P<0.05$) and this effect was dose-dependently inhibited by the Wnt-antagonist sFRP-1 with a significant inhibition ($P<0.05$) using sFRP-1 at a concentration of 10 ng/ml. Similarly, FCCM stimulated aldosterone secretion (250% of untreated controls) and this effect was significantly inhibited ($P<0.05$) with the maximal dose of sFRP-1 (Figure 5b). These results suggest that Wnt-signaling is one of the mechanisms, through which fat cells stimulate adrenal aldosterone and cortisol secretion.

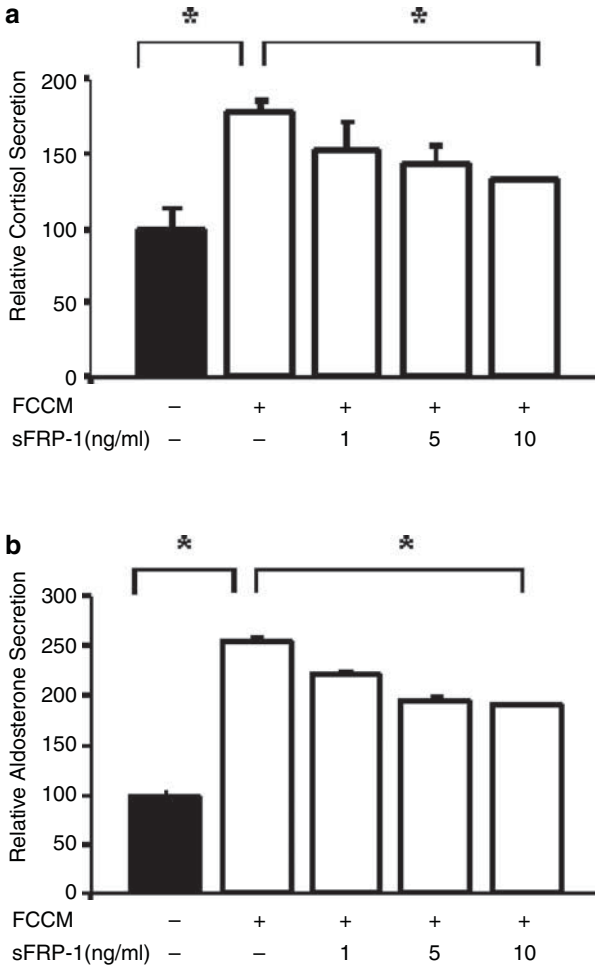

Figure 5 Adipocyte-derived factors induce aldosterone and cortisol secretion in adrenocortical cells through Wnt-signaling. NCI-H295R cells were treated with FCCM or the respective control medium. The Wnt-antagonist sFRP-1 was added to the FCCM at the concentrations indicated. Twenty-four hours after stimulation, the supernatant was removed and cortisol (**a**) and aldosterone (**b**) in the supernatant was measured using a RIA. The hormone concentration is expressed as percentage of the mean value of the concentration measured in the untreated controls. Values are means $\pm$ s.e. of three independent experiments, each carried out in triplicate. *$P<0.05$ (Student's *t*-test).

Discussion

Currently, we are observing an alarming increase in obesity and related diseases such as type 2 diabetes mellitus or arterial hypertension in western countries.[27,28] Obesity is characterized by hypersecretion of aldosterone and cortisol, both of which are known to increase blood pressure. Notably, the increased aldosterone levels in obesity do not always correlate with increased plasma renin levels.[2,5,29,30] The precise mechanisms of obesity-induced hypersecretion of these hormones are not fully understood but the correlation of aldosterone levels with body fat mass in

humans[2,3] as well as our previous *in vitro* work suggest a direct effect of adipocytes on adrenal steroidogenesis.[10] Thus, adipocyte-derived factors stimulate aldosterone and cortisol secretion by adrenocortical cells.[10] However, the signaling pathways involved have not been identified yet.

Starting with the cloning and characterization of leptin[31] the role of adipose tissue as an endocrine organ has been established.[11,32] Consistently, over the following years a number of adipocyte-derived signaling molecules have been characterized. Among them leptin, adiponectin, IL-6 and TNF-α have been subject to intensive studies (reviewed by Bays *et al*.[11]). However, none of those are involved in the adipose–adrenal interaction leading to the increased steroidogenesis described previously.[10]

Wnt-signaling molecules are expressed in adipocytes[12,13] and furthermore, here, we demonstrate the secretion of Wnt-10b and Wnt-3a by adipocytes. Therefore, we speculated that Wnt-signaling can mediate effects of adipocytes on adrenal steroidogenesis. Wnts are ligands on seven-transmembrane receptors called frizzled.[14] Here, we demonstrate the expression of frizzled receptors on human adult adrenals at the protein level (Figure 1). Wnts regulate adrenal development.[33,34] Thus, ablation of Wnt-4 in mice leads to reduced aldosterone synthesis.[34] However, over the recent years, it has become evident that Wnt-signaling also controls metabolic functions in the adult organism. These include glucose-sensing in insulin-producing β-cells[35] and the differentiation of adipocytes.[12,13] In this study, we show an interaction between adipocytes and adrenocortical cells through canonical Wnt-signaling *in vitro* (Figure 2). Adipocytes are found within the adrenal cortex, allowing paracrine interactions with adrenocortical cells.[10] However, Wnt-signaling molecules were also shown to be secreted into the bloodstream and to elicit systemic effects.[36,37] Therefore, systemic effects of adipocyte-derived Wnts on adrenocortical cells *in vivo* cannot be ruled out and are subject to further studies.

In this study, we confirm the regulation of StAR gene expression by fat cell products (Figure 3a). Furthermore, we demonstrate that the adipocyte-induced activation of StAR promoter activity can be inhibited when Wnts in the FCCM are blocked (Figure 3a). Consistently, β-catenin activates the StAR gene promoter in adrenocortical cells (Figure 3b), thereby defining StAR as a target gene for canonical Wnt-signaling in adrenocortical cells.

In line with our findings on the transcriptional regulation of the StAR gene, we confirm that adipocytes stimulate aldosterone and cortisol secretion by adrenocortical cells (Figure 5a and b). Interestingly, this effect can be inhibited after blocking Wnt-signaling (Figure 5a and b), indicating that adipocyte-derived Wnts contribute to the effect of fat cells on aldosterone and cortisol secretion. Notably, the effect of adipocytes on StAR gene transcription and on hormone secretion was not completely inhibited when Wnt-signaling was blocked (Figure 3a, Figure 5a and b). These results suggest that other signaling pathways in addition to Wnts are involved in the adipocyte-induced activation of StAR. Given the vast endocrine activity of adipocytes, it is not surprising that the combination of several signaling molecules rather than one single pathway mediates the effects of adipocytes on adrenocortical cells. It will be of scientific and clinical interest to identify further signaling molecules involved in the adipo–adrenal interaction.

β-Catenin is a central player in canonical Wnt-signaling, and studies by Gummow *et al*.[15] and Jordan *et al*.[17] have shown a functional synergy between β-catenin and the transcription factor SF-1. SF-1 is an orphan nuclear receptor and regulates the transcription of steroidogenic genes, including StAR.[18] Functionally, β-catenin has been shown to co-activate SF-1-mediated transcription on the α-Inhibin promoter and on an artificial reporter-gene driven by multimerized SF-1-binding sites.[15,17] Co-immunoprecipitation assays suggest a direct physical interaction between β-catenin and SF-1 to be one mechanism through which β-catenin co-activates SF-1.[15] In extension to these findings, here, we demonstrate that β-catenin co-activates SF-1-mediated transcription on a multimerized SF-1-driven reporter-gene in adrenocortical cells (Figure 4). This effect can be mimicked by FCCM (Figure 4), suggesting that FCCM acts on the StAR promoter through stabilization of β-catenin and subsequent co-activation of the transcription factor SF-1.

In conclusion, our findings demonstrate an interaction between adipocytes and adrenocortical cells through the Wnt-signaling pathway, resulting in increased StAR promoter activity and aldosterone and cortisol secretion. At the molecular level, fat cell products and activated β-catenin stimulate SF-1-transcriptional activity, suggesting that this is one mechanism of Wnts to regulate StAR transcription. This adipo–adrenal interaction through Wnt-signaling might represent a molecular link between obesity and arterial hypertension.

Acknowledgements

The β-catenin expression vector (β-cat S45A) has been provided by Dr Thilo Hagen (Wolfson Digestive Diseases Centre, University of Nottingham, UK). The plasmids for -65-Luc, pSF-1 and –966Luc (StAR Promoter) were generous gifts from Dr KL Parker (South-western Medical Center, University of Texas, Dallas, USA) and Dr D Stocco (Texas Tech University, Lubbock, USA), respectively. This work was supported by a grant from the Eberhard-Igler-Stiftung and Walter-Clawitter-Stiftung to S Schinner and HS Willenberg and by a grant from the Deutsche Forschungsgemeinschaft (EH 161/4-1) to M Ehrhart-Bornstein and SR Bornstein.

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PUBLIKATION 12

Krug AW, Vleugels K, **Schinner S**, Lamounier-Zepter V, Ziegler CG, Bornstein SR, Ehrhart-Bornstein M.

Human adipocytes induce an ERK1/2 MAP kinases-mediated upregulation of steroidogenic acute regulatory protein (StAR) and an angiotensin II — sensitization in human adrenocortical cells

Int. J. Obes. 31(10):1605-16 (2007)

International Journal of Obesity (2007) 31, 1605–1616

0307-0565/07 $30.00
www.nature.com/ijo

ORIGINAL ARTICLE

Human adipocytes induce an ERK1/2 MAP kinases-mediated upregulation of steroidogenic acute regulatory protein (StAR) and an angiotensin II — sensitization in human adrenocortical cells

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Objectives: Hypertension is a major complication of overweight with frequently elevated aldosterone levels in obese patients. Our previous work suggests a direct stimulation of adrenal aldosterone secretion by adipocytes. Owing to aldosterone's important role in maintaining blood pressure homeostasis, its regulation in obesity is of major importance. One objective was to determine the signaling mechanisms involved in adipocyte-induced aldosterone secretion. In addition to a direct stimulation, a sensitization toward angiotensin II (AngII) might be involved. The second objective was to determine a possible adipokines-induced sensitization of human adrenocortical cells to AngII.

Design: Human subcutaneous adipocytes and adrenocortical cells, and the adrenocortical cell line NCI-H295R were used. Adrenocortical cells were screened for signal transduction protein expression and phosphorylation. Subsequently, steroidogenic acute regulatory protein (StAR), cAMP response element-binding protein (CREB), cAMP and phosphorylated extracellular regulated kinase were analyzed by Western blot, enzyme-linked immunosorbent assay, quantitative PCR, reporter gene assay and confocal microscopy to investigate their role in adipocyte-mediated aldosterone secretion.

Results: AngII-mediated aldosterone secretion was largely increased by preincubating H295R cells with adipocyte secretory products. StAR mRNA and StAR protein were upregulated in a time-dependent way. This steroidogenic effect was independent of the cAMP-protein kinase A (PKA) pathway as cellular cAMP was unaltered and inhibition of PKA by H89 failed to reduce aldosterone secretion. However, CREB reporter gene activity was moderately elevated. Upregulation of StAR was accompanied by ERK1/2 MAP kinase activation and nuclear translocation of the kinases. Inhibition of MAP kinase by UO126 abolished adipokine-stimulated aldosterone secretion from primary human adrenocortical and H295R cells, and inhibited StAR gene activity. Adipokines stimulated steroidogenesis also in primary human adrenocortical cells, supporting a role in human physiology and/or pathology.

Conclusions: Adipokines induce aldosterone secretion from human adrenocortical cells and sensitization of the cells to stimulation by AngII, possibly mediated via ERK1/2-dependent upregulation of StAR activity. This stimulation of aldosterone secretion could be one link between overweight and inappropriately elevated aldosterone levels.
International Journal of Obesity (2007) **31,** 1605–1616; doi:10.1038/sj.ijo.0803642; published online 24 April 2007

Keywords: hypertension; aldosterone; fat cell; adrenal glands; extracellular regulated kinases

Introduction

Overweight and obesity have reached epidemic proportions with dramatic medical consequences in western countries. Obesity is a major risk factor for lipid abnormalities, atherosclerosis, type II diabetes mellitus, certain types of cancer and high blood pressure.[1] Although the association between obesity and elevated blood pressure is unquestioned, the pathogenetic mechanisms are poorly under-

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Received 23 June 2006; revised 13 March 2007; accepted 20 March 2007; published online 24 April 2007

stood. Leptin and insulin, the renin–angiotensin–aldosterone system, activation of the sympathetic nervous system, peroxisome proliferator-activated receptor gamma (PPARγ) activation and endothelial dysfunction are some of the pathogenetic factors discussed.[2–4] Interestingly, aldosterone plasma levels are frequently elevated in obese hypertensive patients.[5–9] However, plasma renin levels are not always increased, indicating renin-independent aldosterone synthesis in obesity-associated hypertension.[10] Aldosterone is the most potent mineralocorticoid, enhancing renal sodium and water retention, thus increasing blood pressure; long-term elevation of blood pressure strongly depends on increased circulating blood volume.[11,12] Furthermore, the pathophysiological role of aldosterone is now known to extend beyond the regulation of salt and water homeostasis. Aldosterone induces cardiovascular dysfunction including cardiac fibrosis, vascular inflammation and endothelial dysfunction, independently of its effects on water and electrolyte balance.[13–16]

Aldosterone is produced by the outer zone of the adrenal cortex, the zona glomerulosa. These cells differ from the other adrenocortical zones in respect to the wide variety of hormones and other molecules influencing aldosterone synthesis and secretion.[10,17] It is not absolutely certain which of these potential regulators adjust aldosterone secretion in humans. Therefore, the reasons for inappropriately elevated aldosterone levels, which are a major reason for hypertension including obesity hypertension might be manifold and are often not clear.

We demonstrated recently that adipocyte secretory products stimulate steroid secretion from human and bovine adrenocortical cells with a predominant effect on aldosterone secretion. This could provide a new link between adipose tissue and aldosterone-induced adverse cardiovascular events, such as cardiac fibrosis and high blood pressure. This stimulation of aldosterone secretion was independent of adipocyte angiotensin II (AngII) and other recently defined adipokines.[18,19]

In addition to a direct stimulation of aldosterone secretion, inappropriate stimulation of aldosterone secretion might be owing to a sensitization of adrenocortical zona glomerulosa cells toward stimulation by AngII. Therefore, one aim of this study was to determine whether adipocytokines induce sensitization of human adrenocortical cells to AngII as an additional link between adipose tissue and increased aldosterone secretion.

Owing to the important role of aldosterone in maintaining electrolyte homeostasis, defining how its secretion is regulated under different physiological and pathological conditions is of major importance. Thus, a further aim of this study was to determine the signaling mechanisms involved in fat cell-induced aldosterone secretion using human adrenocortical NCI-H295R cells. A first screen of these cells using a protein kinase array for signal transduction protein expression and phosphorylation (Figure 4) indicated the involvement of mitogen-activated protein kinases (MAPK) such as ERK1/2. In addition, it has been shown recently that in cultured rat glomerulosa cells, AngII increases protein synthesis including the expression of the steroidogenic enzymes and steroidogenic acute regulatory protein (StAR) via the p42/p44 MAPK and p38 MAPK pathways,[20] and the stimulation of hormone secretion in aldosterone-producing glomerulosa cells.[21] Therefore, the role of ERK1/2 MAPKs in fat cell-induced aldosterone secretion was determined in human H295R adrenocortical cells and isolated human adrenocortical cells in primary culture.

Methods

The study was approved by the Local Ethical Committee.

Isolation of adipocytes and preparation of adipocyte-conditioned medium (ACM)

This was performed as described previously.[18] Briefly, tissue specimens from white human subcutaneous adipose tissue were obtained from healthy women aged 30–54 years undergoing surgical mammary reduction. All of the women were otherwise healthy and free of metabolic and endocrine diseases. The body mass index (BMI) range of the donors was between 23 and 33.2 (28.4 ± 1.4, mean $\pm$ s.d.). Immediately after surgical removal, adipose tissue samples of 20–150 g wet weight were transported to the laboratory in Dulbecco's modified Eagle's medium (DMEM)/Nutrient Mix F12 (DMEM/F12; MP Biomedicals/Gibco, Karlsruhe, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin. The tissue was dissected free from fibrous material and blood vessels, minced into small pieces and digested in Krebs Ringer Bicarbonate buffer (KRB) containing 100 U/ml collagenase type I from *clostridium histolyticum* (Sigma-Aldrich, Munich, Germany) in a shaking water bath for 45–60 min at 37°C. The digested tissue was filtered twice through nylon gauze (250 μm) and washed with KRB. Two millilitres of isolated floating adipocytes were cultured in 6-well culture plates (Nunc, Corning, Acton, MA, USA) containing 2.5 ml cell culture medium (DMEM/F12 containing 15 mmol/l *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and 2.5 mmol/l L-glutamine supplemented with 1.125 g/l $NaHCO_3$, 100 U/ml penicillin, and 100 μg/ml streptomycin). Cells were kept at 37°C in a humidified atmosphere of 5% CO_2/95% air and cultured for 24 h. The conditioned medium was then collected by filtration and kept frozen at −70°C.

NCI-H295R cells

Cells were grown in DMEM/F12 containing 15 mmol/l HEPES and 2.5 mmol/l L-glutamine, supplemented with 1.125 g/l $NaHCO_3$, insulin (66 nmol/l), hydrocortisone (10 nmol/l), 17β-estradiol (10 nmol/l), transferrin (10 μg/ml), selenite (30 nmol/l), penicillin (100 U/ml), streptomycin (100 μg/ml) and 2% fetal bovine serum (FBS). NCI-H295R

cells were grown in 75-cm^2 flasks (Becton Dickinson, Heidelberg, Germany) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The medium was changed every 2 days, and cells were subcultured every 7 days using Accutase (PAA Laboratories, Pasching, Austria) for cell detachment. Cells used for experiments were subcultured from 70 to 80% confluent stock cultures in 48 or 96-well culture plates (Nunc) at a density of 140 000 cells/cm^2 for 96 or 120 h. Cells were then treated as described below.

Primary human adrenocortical cells
Three normal human adrenals from patients who had been unilaterally nephrectomized for renal carcinoma were analyzed in this study. Immediately after surgery, the adrenals were transferred to prechilled phosphate-buffered saline (PBS) and kept on ice until further treatment. Adrenals were dissected mechanically, and dispersed cells were obtained by digestion with collagenase (0.1% wt/vol) and deoxyribonuclease (0.01% wt/vol) as described previously.[22] The isolated cells were cultured in DMEM/F12 containing penicillin (100 U/ml), streptomycin (0.1% wt/vol) and 10% fetal calf serum (FCS) at 37°C under 5% CO_2.

Incubation of NCI-H295R cells and human primary cells
NCI-H295R cells were incubated with the respective stimulation medium for 15 min to 24 h. Human primary cells were incubated with the respective stimulation medium for 24 h. ACM was supplemented with insulin (66 nmol/l), hydrocortisone (10 nmol/l), β-estradiol (10 nmol/l), transferrin (10 μg/ml), selenite (30 nmol/l), penicillin (100 units/ml) and streptomycin (100 μg/ml). After incubation, culture medium was collected and kept frozen at –20°C until assay.

Aldosterone measurement
Aldosterone in the incubation medium was measured by direct specific RIA (DSL-8600; Diagnostic Systems Laboratories, Webster, TX, USA) according to the manufacturer's protocol.

cAMP measurement
cAMP in the incubation medium was measured by enzyme-linked immunosorbent assay (ELISA; CM 581001; IBL Immuno-Biological Laboratories, Hamburg, Germany) according to the manufacturer's protocol.

Protein isolation
NCI H295R cells were washed twice with ice-cold PBS and lysed in ice-cold lysis buffer (Sigma-Aldrich) for 30 min at 4°C. Insoluble material was removed by centrifugation at 12 000 *g* for 15 min at 4°C.

Western blot analysis
Western blot analysis was performed as described previously.[23,24] Briefly, samples were matched for protein content, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a nitrocellulose membrane. Subsequently, membranes were immunostained with rabbit anti-phospho-ERK1/2 antibody (1:1000; Cell Signalling, Danvers, MA, USA), anti-ERK1/2 antibody (1:1,000; Cell Signalling) or anti-StAR antibody (1:1000; Santa Cruz Technology, Santa Cruz, CA, USA). The bound primary antibody was visualized using the Western Breeze Chemiluminescent Immunodetection Kit (Invitrogen, Karlsruhe, Germany).

Quantification of phospho-ERK1/2 by ELISA
Quantification of total ERK1/2 and ERK1/2 phosphorylation by ELISA was performed as described previously.[23,24] NCI H295R cells were seeded in 96-well plates (140 000 cells/cm^2; Nunc). After stimulation as indicated below, the cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature and washed three times with PBS containing 0.1% Triton X-100 (PBS/Triton). Endogenous peroxidase was quenched with 1% H_2O_2 and 0.1% Azide in PBS/Triton for 20 min. Cells were washed three times with PBS/Triton, blocked with 5% FCS in PBS for 1 h, and incubated overnight with the primary antibody (1:5000; Cell Signalling) in PBS containing 5% bovine serum albumin (BSA) at 4°C. The next day, cells were washed three times with PBS/Triton for 5 min, incubated with peroxidase-conjugated mouse anti-rabbit secondary antibody (1:10 000) in PBS containing 5% BSA for 1 h at room temperature, and washed with PBS/Triton three times for 5 min and twice with PBS. After that, the cells were incubated in 100 μl SuperSignal ELISA Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA) for 5 min at room temperature. The resulting luminescence was detected using a multiwell reader (Mithras LB 940; Berthold, Bad Wildbad, Germany). Subsequently, the relative number of cells in each well was determined using crystal violet staining of the nuclei, allowing normalization of ERK1/2 phosphorylation for cell number.[23] Briefly, the cells were washed twice with PBS/Triton and twice with PBS. After drying the wells for 5 min, 100 μl of crystal violet solution was added for 30 min at room temperature. Subsequently, the cells were washed four times with demineralized water, and 100 μl of 1% sodium dodecyl sulfate solution was added and incubated on a shaker for 1 h at room temperature. Finally, the absorbance was measured at 595 nm.

Phospho-antibody screening
NCI H295R cells were rinsed in PBS, lysed by adding 1 ml ice-cold lysis buffer (20 mmol/l Mops, pH 7.0, 2 mmol/l ethylene glycol bis(â-aminoethylether)-*N,N,N',N'*,-tetraacetic acid (EGTA), 5 mmol/l ethylenediaminetetraacetic acid (EDTA), 30 mmol/l sodium fluoride, 40 mmol/l β-glycerophosphate,

10 mmol/l sodium pyrophosphate, 2 mmol/l sodium orthovanadate, 1 mmol/l phenylmethylsulfonyl (PMS), 3 mmol/l benzamidine and 0.5% Nonidet P-40). Cells were sonicated twice for 15 s, and the homogenate was subjected to ultracentrifugation for 30 min at 75 000 *g*. The protein concentration of the supernatant fraction was measured by using a commercial Bradford Assay (Bio-Rad, Munich, Germany) and boiled for 4 min at 100°C in SDS-PAGE buffer (31.25 mmol/l Tris–HCl, pH 6.8, 1% SDS, 12.5% glycerol, 0.02% Bromophenol Blue and 1.25% β-mercaptoethanol). The levels of 78 protein kinases were analyzed in control and ACM-treated cells by the commercial Kinetworks protein kinase screening system (KPKS 1.2; Kinexus Bioinformatics Corporation, Vancouver, Canada) as described online at www.kinexus.ca.

Reporter gene assay

NCI H295R cells were plated at a density of 90 000 cell/cm^2 in 96-well plates (Nunc) in 100 μl of media, then incubated overnight. Culture medium was replaced with 100 μl of fresh culture medium (2% FBS) 1.5 h before transfection with Fugene 6 reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. Co-transfections were carried out with a constant amount of DNA, which was maintained by adding the vector pcDNA3 (Invitrogen). pCRE-secretory alkaline phosphatase (SEAP) (40 ng; Clontech, Mountain View, CA, USA) or 0.5 μg of the StAR-firefly construct[25] per well were co-transfected together with 2 ng pRL-TK (Promega Corporation, Madison, WI, USA) in H295R cells. Cells were incubated with ACM for 3–24 h. Transcription factor activity of cAMP response element (CRE) was measured using the GreatEscAPe SEAP fluorescence detection kit (Clontech) in the supernatant of the cell cultures. Firefly luciferase activity and renilla luciferase activity were assayed with Luciferase Assay System (Promega). Renilla luciferase activity was used to normalize the SEAP activity and firefly luciferase activity, respectively. Each transfection was performed in triplicate and repeated 2–3 times with different cell culture passages and ACM preparations.

RNA extraction and first-strand cDNA synthesis

Total RNA from NCI H295R cells was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA (3 μg) was reverse transcribed with 200 U of M-MLV Reverse Transcriptase (Promega Corporation), using Oligo(dT)$_{15}$ primers (Promega) according to the manufacturer's instructions.

Quantitative PCR

StAR mRNA levels were determined by using the LightCycler FastStart DNA Master Plus SYBR Green I kit (Roche Applied Science). The amplification protocol consisted of a denaturation step at 95°C for 7 min, followed by 40 cycles with a 95°C denaturation step for 10 s, 65°C annealing for 5 s and a 72°C extension for 13 s. The following primers were used for specific amplification: StAR, forward primer: 5′-TGGCTGGAAGTCCCTCTAAGACCAA-3′; reverse primer: 5′-TTGCAGGCTTCCAGTAGGGATTCTC-3′, and β-Actin, forward primer: 5′-GCCGTCTTCCCCTCCATCGTG-3′; reverse primer: 5′-GGAGCCACACGCAGCTCATTGTAGA-3′. PCR products were analyzed by melting curve analysis. A negative control (no template) was measured in each of the PCR runs. The cDNA copy number of StAR was calculated in relation to the amplification product amount of internal standard. Quantity standard curves were generated applying dilutions over six log scales (10^3–10^8) per capillary of the pCRII-TOPO vector (Invitrogen) containing StAR-PCR-fragment and determined by the Light Cycler quantification software version 3.5.3 (Roche Applied Science). Mean $\pm$ s.e. of three individual experiments are illustrated.

Confocal imaging

NCI H295R cells were seeded onto glass coverslips (16 000 cells/cm^2). After 96 h, cells were incubated with ACM or control media for 3 h. Fixation was performed in 4% paraformaldehyde/PBS at room temperature. After five times washing in PBS, cells were permeabilized with 0.5% Triton X100/PBS on ice for 5 min, followed by three washes with PBS. Cells were then blocked with 5% FBS/PBS for 30 min at room temperature, followed by incubation with primary antibodies (phosphorylated extracellular regulated kinase (pERK), 1:500 in 5% FBS/PBS) at 4°C overnight. After five times washing in PBS + 0.2% Tween20 (Wash Buffer), the cells were incubated with FITC-conjugated secondary antibodies in 5% BSA/PBS (1:500) at room temperature for 60 min followed by washing in Wash Buffer (5 $\times$) and PBS. Coverslips were then mounted in Vectashield Mounting Media with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA, USA). Confocal microscopy was performed with Olympus FV-1000 confocal microscope with a 60 $\times$ objective lens. Images were analyzed using the FV10-ASW 1.3 viewer software.

Materials

H-89 was from Biomol (Hamburg, Germany), U0126 and AG1478 were from Calbiochem (EMD Biosciences Inc., Darmstadt, Germany). Unless otherwise stated, all other materials were from Sigma (Munich, Germany).

Statistics

The data are presented as mean $\pm$ s.e.m. Significance of difference was tested by paired or unpaired Student's *t*-test or analysis of variance as applicable. Differences were considered significant for values of $P<0.05$. Cells from at least two different passages were used for each experimental

series. Experiments were repeated 2–3 times with at least 2–4 different fat cell (ACM) preparations. *n* represents the number of tissue culture dishes investigated.

Results

The effect of ACM on basal and AngII-stimulated aldosterone release was characterized in isolated human adrenocortical cells in primary culture and a human adrenocortical cell line (NCI-H295R). ACM stimulated aldosterone secretion in the cell line as well as in primary human adrenocortical cells (Figure 1a). ACM-mediated aldosterone secretion was comparable to maximal stimulation of the cells with AngII (10^{-7} mol/l) and forskolin (FSK, 10^{-5} mol/l). FSK is used to activate the cyclic AMP-dependent, protein kinase A-mediated pathway as NCI-H295R cells only poorly express receptors for adrenocorticotropic hormone (ACTH).[26] ACM-mediated aldosterone secretion was dose-dependent (Figure 1b) as shown by dilution of ACM. In addition to this direct stimulation of aldosterone secretion, ACM sensitized H295R cells to their natural stimulus, AngII, the major regulator of aldosterone secretion *in vivo* – 24 h preincubation with ACM potentiated aldosterone secretion mediated by 10^{-9}–10^{-5} mol/l AngII (Figure 1c).

K^+ and AngII-mediated aldosterone secretion in glomerulosa cells depends on *de novo* synthesis of StAR.[27,28] StAR translocates cholesterol from the outer to the inner mitochondrial membrane, where cholesterol is converted to pregnenolone in a process mediated by cytochrome *P*450 side-chain cleavage enzyme.[29] After incubating H295R cells with ACM or AngII, aldosterone secretion, StAR mRNA levels and StAR protein levels increased in a time-dependent manner (Figure 2a and b and Table 1). The cAMP-protein kinase A (PKA)–CRE pathway is the key regulatory pathway for trophic steroidogenesis as well as StAR expression in steroidogenic cells; cAMP directly effects PKA activity, leading to enhanced phosphorylation and subsequent nuclear translocation of transcription factor cAMP response element-binding protein (CREB). CREB is a main modulator in steroidogenic enzyme and StAR regulation.[30] Therefore,

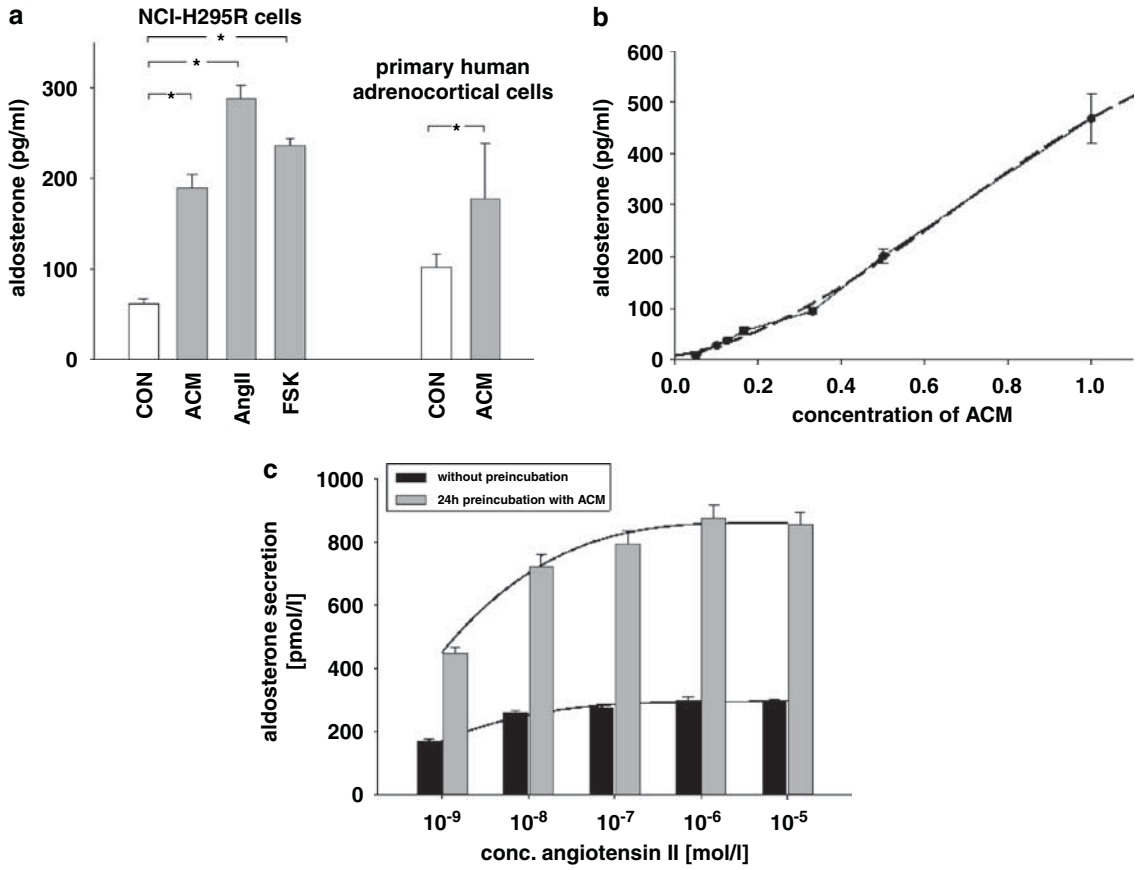

Figure 1 Effect of 24 h ACM treatment on human adrenocortical cells. (**a**) ACM-induced aldosterone secretion in primary culture of isolated human adrenocortical cells and human NCI-H295R cells, comparable to maximal stimulation with AngII (10^{-7} mol/l) and FSK (2×10^{-5} mol/l). (**b**) Dilution of ACM reveals dose-dependency of ACM-induced aldosterone secretion from NCI-H295R cells. (**c**) NCI-H295R cells were preincubated for 24 h with ACM versus control medium, and then stimulated at varying AngII concentrations for 24 h. $n = 6$–9 for all plotted values. ACM, adipocyte-conditioned medium; AngII, angiotensin II.

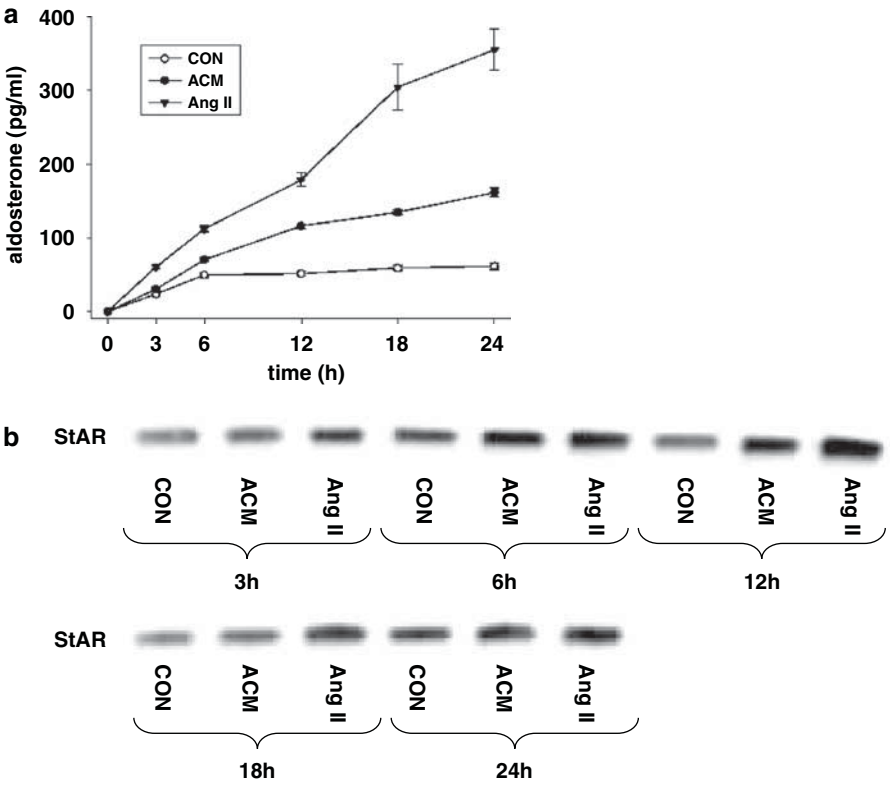

Figure 2 Effect of ACM and AngII treatment on aldosterone secretion, StAR mRNA and StAR protein expression in NCI-H295R cells. (**a**) ACM and AngII increased aldosterone secretion in a time-dependent manner. (**b**) StAR protein, as indicated by Western blot analysis, was elevated in a time-dependent way, with a maximum at 12 h, similar to StAR mRNA as shown in Table 1. Representative blot from three independent experiments. All experiments were repeated 2–3 times with at least 2–4 different fat cell (ACM) preparations. ACM, adipocyte-conditioned medium; AngII, angiotensin II; StAR, steroidogenic acute regulatory protein.

Table 1 Effect of ACM and AngII treatment on StAR mRNA in NCI-H295R cells

	3 h	*6 h*	*12 h*	*18 h*	*24 h*
ACM	120±6	186±25	352±29	204±10	129±13
Ang II	118±21	227±81	310±124	233±64	112±21

Abbreviations: ACM, adipocyte-conditioned medium; AngII, angiotensin II; StAR, steroidogenic acute regulatory protein. StAR mRNA (% of control) increased in a timely manner with maximal levels after 12 h. $n = 3$–5 for all given values. All experiments were repeated 2–3 times with at least 2–4 different fat cell (ACM) preparations.

we tested for the possible involvement of this signaling cascade in the ACM-mediated aldosterone synthesis and upregulation of StAR observed. ACM did not influence cAMP production in adrenocortical NCI-H295R cells (Figure 3a). As expected, FSK (2×10^{-5} mol/l), but not AngII (10^{-7} mol/l), induced cAMP production. Accordingly, pharmacological inhibition of PKA by H89 (10^{-5} mol/l) did not influence ACM-mediated aldosterone secretion after 24 h (Figure 3b). CREB transcriptional activity was elevated only relatively modest (approximately 190% of controls) in 24 h ACM-treated cells compared with FSK-treated cells (Figure 3c).

In our search for possible signaling cascades that mediate the ACM-mediated StAR upregulation, a screening of NCI-H295R cells was performed using the Kinexus protein Kinase screens, KPKS-1.2A and KPKS-1.2B. These revealed a significant increase in the levels of phosphorylated extracellular regulated kinase (ERK) 1 and ERK2 after 1 h stimulation of the cells with ACM. Total ERK1/2 protein remained unchanged (Figure 4a–c). The results from this screen were confirmed by Western blot analysis (Figure 4c). Moreover, phosphorylation of raf-1, an upstream mediator of ERK1/2 was also increased (data not shown). ACM-mediated activation of ERK1/2 was assessed by phospho-specific ELISA and Western blot analysis. ACM stimulated ERK1/2 phosphorylation in a time and dose-dependent manner (Figure 5a and b). The ACM-stimulated increase in phospho-ERK1/2 was completely blocked by inhibition of MEK, a direct upstream mediator of ERK, by UO126 (10^{-5} mol/l) (Figure 5c). AngII (10^{-7} mol/l) activated ERK1/2 in a similar time range as did ACM (Figure 5d). Immunoflorescence imaging with confocal microscopy was used to evaluate the effect of ACM on ERK1/2 in adrenocortical cells (Figure 6): after 3 h stimulation with ACM ERK1/2 phosphorylation was highly increased and the kinase translocated into the nucleus, indicating ERK-

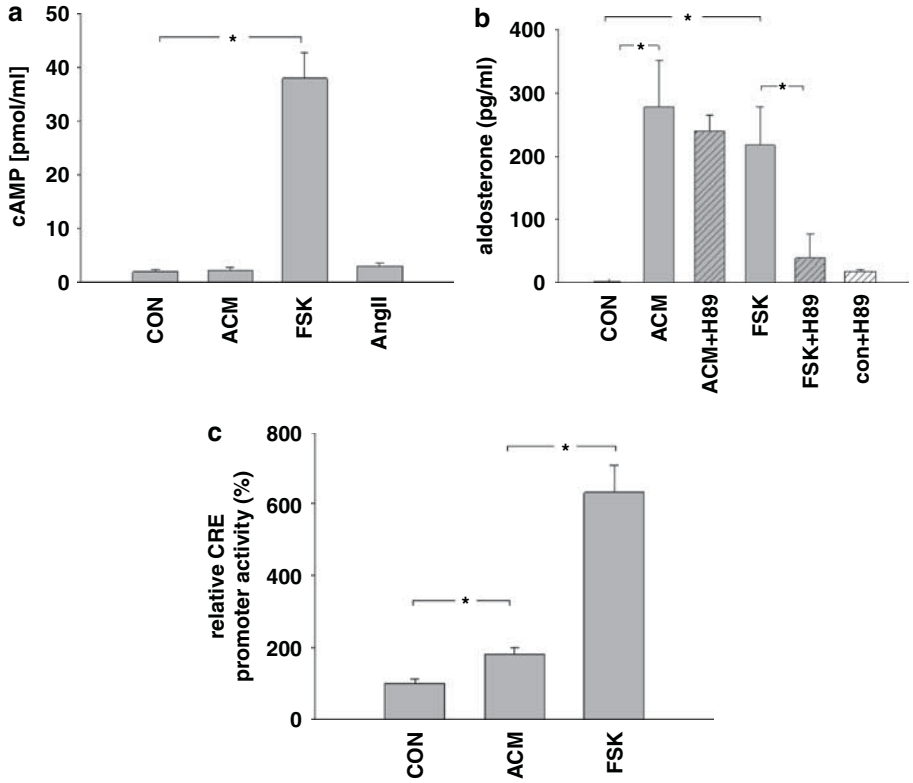

Figure 3 Influence of 24 h ACM treatment on the cAMP/PKA/CREB signaling cascade in NCI-H295R cells. (**a**) cAMP production, measured in the cell supernatant, was not elevated after treatment of NCI-H295R cells with ACM. As expected, cAMP concentrations were elevated after treatment with FSK, 2×10^{-5} mol/l, but not AngII (10^{-7} mol/l). (**b**) Inhibition of PKA by H89 (10^{-5} mol/l) did not significantly reduce ACM-mediated aldosterone secretion from NCI-H295R cells. FSK-mediated aldosterone production could be blocked by H89. $n = 6$–9 for all plotted values with 2–4 different fat cell (ACM) preparations. (**c**) Relative CRE promoter activity was elevated by ACM (~190%), FSK served as a positive control. $n = 8$ for all plotted values. ACM, adipocyte-conditioned medium; AngII, angiotensin II; CREB, cAMP response element-binding protein; FSK, forskolin; PKA, protein kinase A.

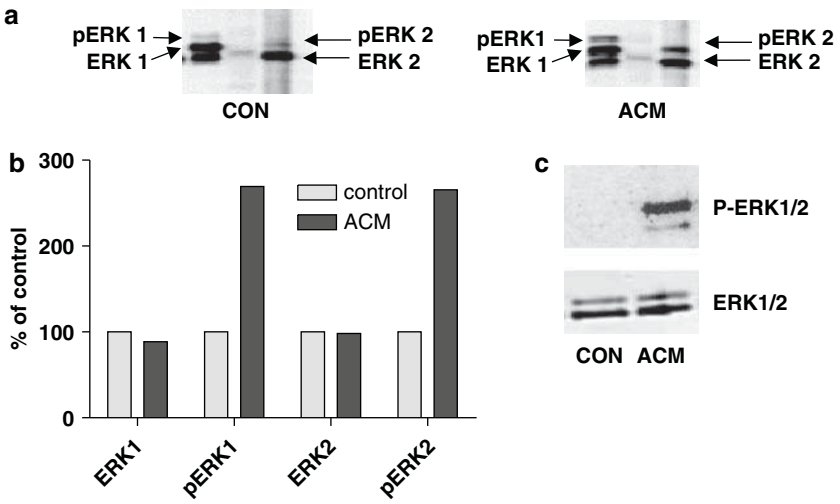

Figure 4 Screening for ACM-responsive protein kinases using phospho-site screening for 78 protein kinases (KPKS-1.2) in NCI-H295R cells. (**a**) Basal conditions (left) and conditions after incubation of NCI H295R cells in ACM for 1 h (right). Out of the 78 protein kinases screened, the identity of ERK1, pERK1, ERK2 and pERK2 are indicated. (**b**) Quantification of the protein kinase screen by Kinexus Inc. using a high-sensitivity imaging system (Bio-Rad Fluor-S Max Multi-Imager) in combination with quantization software (Bio-Rad Quantity One) to quantify and analyze the chemiluminescent samples. Data were converted to percentage increase over basal. (**c**) Confirmation of the results by Western blot analysis; total ERK1/2 was unchanged, pERK1/2 was elevated after 24 h incubation of H295R cells with ACM. ACM, adipocyte-conditioned medium; pERK, phosphorylated extracellular regulated kinase.

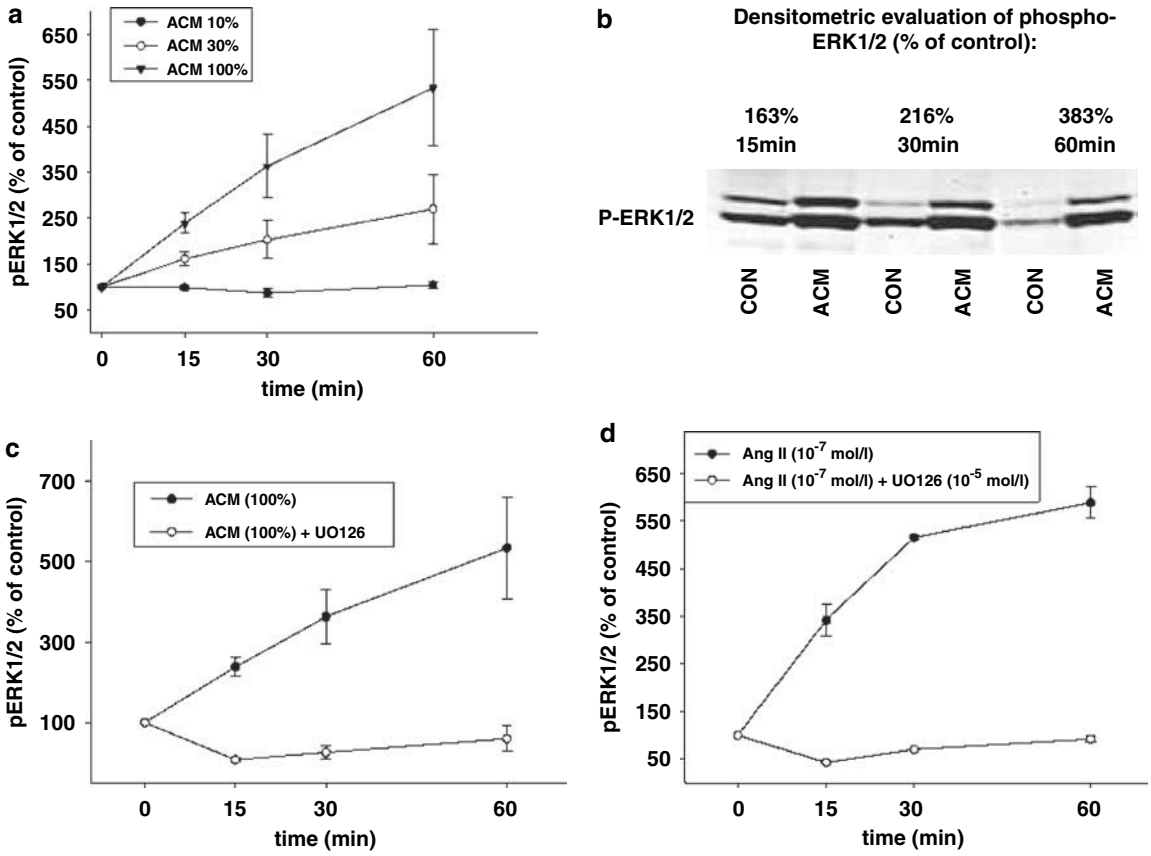

Figure 5 Influence of ACM on ERK1/2 phosporylation in NCI-H295R cells. (**a**) Phospho-specific ERK1/2 ELISA shows that adipocyte-stimulated ERK1/2 phosphorylation occurred in a time-dependent way. (**b**) Western Blot analysis confirmed these results. The figure shows a representative blot from three independent experiments. (**c**) Time course for ACM-stimulated ERK1/2 phosphorylation. (**d**) Time course for AngII (10^{-7} mol/l)-stimulated ERK1/2 phosphorylation. MAPK (MEK) was inhibited by UO126 (10 μmol/l); $n = 8$–12 for all plotted ELISA values, incubation times were 15–60 min. ACM, adipocyte-conditioned medium; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular regulated kinase; MAPK, mitogen-activated protein kinases.

mediated transcriptional activity. Also, UO126 completely abolished ACM-induced mineralocorticoid secretion from primary adrenocortical cells as well as NCI-H295R cells (Figure 7a). Total cellular protein content was not influenced by ACM or by treatment with UO126. AngII-induced aldosterone secretion was MAPK-dependent, and was blocked by MEK inhibition (Figure 7b). Next, we asked whether activation of ERK1/2 is directly involved in ACM-mediated upregulation of StAR. UO126 largely reduced ACM-stimulated StAR promoter activity after 24 h (Figure 7c). Accordingly, StAR mRNA and StAR protein expression is expected to be suppressed, as indicated by the lack of aldosterone secretion.

Discussion

Our previous *in vitro* studies showed that human fat cells secrete factors that enhance adrenocortical steroidogenesis, with a predominant effect on aldosterone secretion in a human adrenocortical cell line. In this study, steroidogenesis was also stimulated in freshly isolated primary human adrenocortical cells, supporting the role of this interaction in human physiology and/or pathology.

This stimulation of adrenocortical steroidogenesis involves upregulation of StAR, a key factor in steroidogenesis mediating the transfer of cholesterol from the outer to the inner mitochondrial membrane in a rate-limiting step.[29] Treating NCI-H295R cells with ACM for up to 24 h increased StAR mRNA levels and StAR protein expression with a maximum after 12 h. A similar biphasic effect of AngII on StAR expression with a peak after 3 h followed by a decline has been observed in bovine adrenocortical cells.[31] Adipokines in our study had the same biphasic effect with a peak after 12 h in NCI H295R cells, although the active component in the ACM has been shown to be independent of AngII released from adipocytes.[18]

The cAMP/protein kinase A (PKA)–CREB pathway is the major signaling cascade regulating steroidogenesis as well as

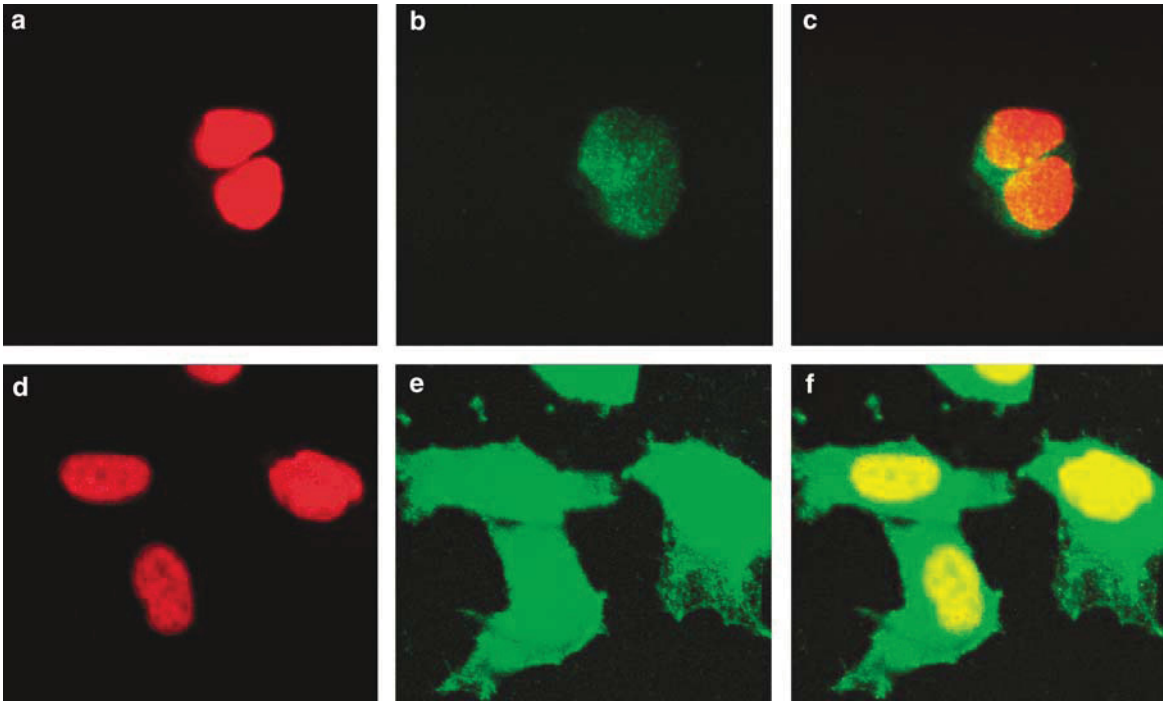

Figure 6 Confocal microscopy of ERK1/2 phosphorylation and translocation into the nucleus of H295R cells after 3-h treatment with ACM. (**a** and **d**) Red color indicates nuclear staining, (**b** and **e**) green color indicates pERK1/2, C/F: merge (**a–c**) control cells; (**d–f**) after 3 h treatment with ACM ERK1/2 phosphorylation was significantly increased (**e**) and ERK1/2 translocated into the nucleus (**f**) as indicated by yellow color. ACM, adipocyte-conditioned medium; pERK, phosphorylated extracellular regulated kinase.

StAR expression.[30] In addition, PKA is considered to be an important positive regulator of StAR activity by post-translational phosphorylation.[32] We, therefore, investigated the effect of ACM on the cAMP/PKA/CREB signaling cascade. ACM-mediated aldosterone production was not accompanied by cAMP production, and inhibition of PKA failed to reduce ACM-induced aldosterone synthesis. These findings indicated a cAMP-independet mechanism in ACM-mediated StAR expression and aldosterone synthesis. However, CREB transcriptional activity was enhanced by ACM, although relatively modest compared to stimulation by FSK, indicating an alternative, cAMP-independent activation mechanism of CREB.[33]

Several recent studies have indicated the involvement of other signaling cascades than the cAMP/PKA/CREB pathway in steroidogenesis and in the upregulation of steroidogenic enzymes and StAR, including the MAPK ERK1/2 pathway. However, disagreement exists with regard to the role of MAPK in steroidogenesis. It seems to differ in a tissue and species-specific manner.[34,35] For example, FSH and LH are known to stimulate steroid production in ovarian cells and enhance ERK1/2 activation.[36,37] However, inhibition of ERK activation enhanced agonist-induced steroid production in granulosa cells.[34] AngII stimulated ERK1/2 activity in bovine adrenal glomerulosa cells[38] and H295R cells (present study, and Watanabe *et al*.[39] and Natarajan *et al*.[40]). In the study of Natarajan *et al*.,[40] inhibition of ERK1/2 did not prevent AngII-mediated aldosterone synthesis. However, our data as well as other studies[41] showed that MAPK (MEK) inhibition prevented AngII-mediated aldosterone synthesis, indicating a major role for ERK1/2. Phosphospecific ELISA and Western Blot analysis demonstrated a time-dependent activation of ERK1/2 in ACM-treated cells. Furthermore, ERK1/2 translocated into the nucleus further indicating transcriptional activity of ERK1/2. A direct involvement of ERK1/2 in acute StAR regulation has been shown in rat Leydig cells,[35] indicating that MAPKs may play a role in the upregulation of StAR protein[42] observed in our system. We, therefore, tested the influence of MAPK (MEK) inhibition by UO126 on ACM-induced StAR gene reporter activity. Indeed, inhibition of MAPK (MEK) strongly reduced StAR transcriptional activity. This strongly supports a crucial role for ERK1/2 in fat cell-mediated upregulation of StAR, possibly providing the basis for sensitization of the cells to other stimuli, such as AngII. Even though the cAMP-independent influence on steroidogenesis is considered to be relatively low ($<5\%$), many of these cAMP-independent stimulators such as MAPK are able to sensitize the cells and potentiate the steroidogenic effect of other stimuli.[30] For example, IGF1 can enhance steroidogenesis only marginally in Leydig cells when compared with LH/human chorionic gonadotropin (hCG). However, IGF and other factors upregulate StAR expression and thereby enhance the steroidogenic responsiveness toward hCG, independent of cAMP signaling.[43–45]

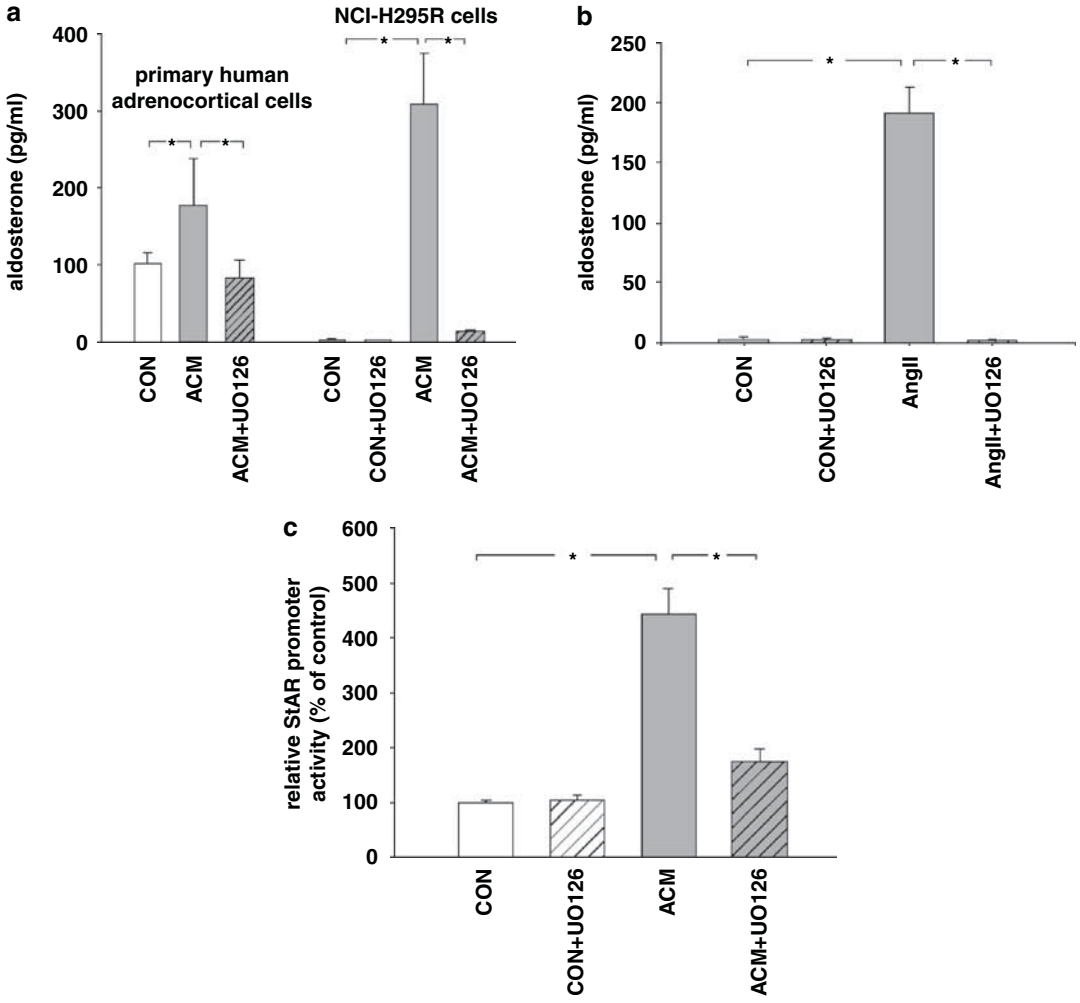

Figure 7 Effects of MAPK inhibition on 24 h ACM-induced aldosterone secretion and StAR promoter activity in adrenocortical cells. (**a**) Inhibition of MAPK (MEK) by 10 μmol/l UO126 prevented ACM-mediated aldosterone secretion in primary human adrenocortical cells and NCI-H295R cells. (**b**) AngII-induced aldosterone production in NCI-H295R cells also depended on MAPK, as indicated by inhibition with UO126. (**c**) MAPK inhibition reduced 24 h ACM-stimulated StAR promoter activity in H295R cells; UO126 alone had no effect on basal StAR promoter activity. $n = 6$–9 for all plotted values. All experiments were repeated 2–3 times with at least 2–4 different fat cell (ACM) preparations. ACM, adipocyte-conditioned medium; MAPK, mitogen-activated protein kinases.

Adipocytes produce and secrete a wide variety of active components with endocrine or paracrine activity.[46] The adipokines that produce the aldosterone-stimulating effects are heat sensitive and the activity can be reconstituted after ammonium precipitation indicating the involvement of protein.[18] The activity consists of at least two factors: filtration based on molecular mass revealed an active fraction (molecular mass >50 kDa) representing 60% of the activity[18] and an inactive fraction of lower molecular mass (<3 kDa; data not shown). The combination of the two fractions increased the recovery rate to >90%, indicating the involvement of at least two factors.

In this study, AngII-mediated aldosterone secretion was largely enhanced after preincubation of adrenocortical cells with ACM. Hence, we speculate that sensitization of adrenocortical cells to AngII may induce a renin-independent increase in aldosterone secretion in obesity. Interestingly, we could show recently that hypertensive obese patients had an increased aldosterone/renin ratio compared with normotensive obese patients, suggesting a low renin aldosteronism in these patients.[47]

Taken together, our data show that adipokines directly stimulate aldosterone secretion in primary human adrenocortical cells and H295R cells via an upregulation of StAR. These adipokines do not act via the cAMP/PKA signaling pathway but instead via the ERK1/2 MAPK signaling pathway. In addition, adipocyte secretory products pronouncedly potentiate AngII-stimulated aldosterone secretion. These mechanisms may play a role in the often observed link between obese patients and hypertension.

Acknowledgements

We thank Dr Mechthild Weiße-Lögering, Department of Plastic Surgery, Klinikum Pirna GmbH, Pirna and her team for their support in obtaining adipose tissue samples, Uta Buro for excellent technical assistance with quantitative PCR, and Jan Peychl, Max Planck Institute of Molecular Cell Biology and Genetics Dresden for technical assistance with confocal microscopy. This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG grant EH161/4–1 to MEB and SRB), a MedDrive grant (to AWK), and the Grimmke Foundation (to AWK).

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PUBLIKATION 13

Schinner S, Ülgen F, Papewalis C, Schott M, Woelk A, Vidal-Puig A, Scherbaum WA

Regulation of Insulin Secretion, Glucokinase Gene Transcription and β-cell Proliferation by Adipocyte-derived Wnt-Signaling molecules.

Diabetologia 51(1):147-54. (2008)

Diabetologia (2008) 51:147–154
DOI 10.1007/s00125-007-0848-0

ARTICLE

Regulation of insulin secretion, glucokinase gene transcription and beta cell proliferation by adipocyte-derived Wnt signalling molecules

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Received: 26 July 2007 / Accepted: 30 July 2007 / Published online: 10 November 2007

Abstract
Aims/hypothesis Adipocytes secrete signalling molecules that elicit responses from target cells, including pancreatic beta cells. Wnt signalling molecules have recently been identified as novel adipocyte-derived factors. They also regulate insulin secretion in pancreatic beta cells and the cell cycle. The aim of this study was to investigate the effect of adipocyte-derived Wnt signalling molecules on insulin secretion and beta cell proliferation.
Methods Human adipocytes were isolated to generate fat cell-conditioned medium (FCCM). Ins-1 cells were stimulated with FCCM and transiently transfected with reporter genes. Proliferation assays using [^{3}H]thymidine incorporation were carried out in Ins-1 cells and primary islet cells. Insulin secretion from primary islets was assessed by radioimmunoassay. Gene expression in primary islets was assessed by Taqman PCR.
Results Treatment with human FCCM increased the transcription of a T cell-specific transcription factor reporter gene (TOPFLASH) in Ins-1 cells (241%, $p<0.05$). FCCM induced the proliferation of Ins-1 cells (1.8 fold, $p<0.05$) and primary mouse islet cells (1.6 fold, $p<0.05$). Antagonizing Wnt signalling with secreted Frizzled-related protein 1 (FRP-1) inhibited the proliferative effect induced by Wnt3a and FCCM on Ins-1 cells by 49 and 41%, respectively. In addition, FCCM led to a twofold ($p<0.05$) induction of cyclin D1 promoter activity in Ins-1 cells. Furthermore, FCCM stimulated insulin secretion (204% of controls, $p>0.05$) in primary mouse islets, and this stimulation was inhibited by sFRP-1. At a molecular level, canonical Wnt signalling induced glucokinase gene transcription in a peroxisome proliferator-activated receptor γ-dependent fashion, thereby defining the glucokinase gene as a novel Wnt target gene.
Conclusions/interpretation Taken together, these data show that adipocyte-derived Wnt signalling molecules induce beta cell proliferation and insulin secretion in vitro, suggesting a novel mechanism linking obesity to hyperinsulinaemia.

Keywords Adipocyte · Beta cells · Islet · Obesity · Wnt signalling

Abbreviations

FCCM	fat cell-conditioned medium
HNF	hepatocyte nuclear factor
LEF	lymphoid enhancer-binding factor
LRP	LDL receptor-related protein
sFRP-1	secreted Frizzled-related protein 1
PPARγ	peroxisome proliferator-activated receptor γ
TCF	T cell-specific transcription factor
TCF7L2	transcription factor 7-like 2

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Introduction

Obesity is associated with insulin resistance, hyperinsulinaemia and beta cell hyperplasia [1–5]. Hyperinsulinaemia can result from either islet hyperplasia or hypersecretion of

insulin from the individual beta cell. Hyperinsulinaemia compensates for insulin resistance to maintain normoglycaemia. However, hyperinsulinaemia per se deteriorates insulin resistance [6] and is thought to trigger the progression of the metabolic syndrome [7, 8]. The mechanisms linking obesity and insulin resistance to hyperinsulinaemia and beta cell hyperplasia are incompletely understood [3]. Adipocytes are hormonally active and adipocytokines are able to elicit a response from beta cells [5]. Studies have shown that adipocytes secrete Wnt signalling molecules to regulate adipocyte differentiation [9–11]. In a previous study we demonstrated that Wnt3a and Wnt10b are secreted by mature human adipocytes and regulate hormone secretion from adrenocortical cells in vitro [12].

Wnts are ligands for serpentine Frizzled receptors and LDL receptor-related protein (LRP) coreceptors. Upon ligand binding to both receptors, canonical Wnt signalling is activated, resulting in a stabilisation of β-catenin. Subsequently, β-catenin coactivates the transcription factors of the T cell-specific transcription factor (TCF) and lymphoid enhancer-binding factor (LEF) families on canonical Wnt target genes [13]. Common genetic variation within the gene encoding transcription factor 7-like 2 (*TCF7L2*) has recently been shown to be associated with an increased risk of type 2 diabetes in humans. However, the mechanisms through which TCF7L2 affects glucose metabolism are not clear [14, 15]. Frizzled receptors and LRP coreceptors are expressed in pancreatic beta cells [16–18].

Wnts induce cell proliferation in a variety of tissues, including embryonic pancreatic endocrine cells [18]. In addition, genetic ablation of *Lrp5* causes impaired insulin secretion in mice, and this occurs in concert with decreased expression of the genes for glucokinase and HNF transcription factors, which regulate glucose sensing in beta cells. Wnt ligands stimulate insulin secretion in vitro [17]. Furthermore, a recent paper by Rulifson et al. [19] demonstrated that Wnt signalling regulates the proliferation of pancreatic beta cells in vitro and in vivo.

The aim of this study was to investigate whether Wnt signalling molecules secreted by adipocytes act on beta cells to regulate insulin secretion and beta cell proliferation.

Methods

Plasmid constructs The TOPFLASH plasmid is a TCF luciferase reporter plasmid. It contains two sets of three copies of the TCF binding site upstream of the thymidine kinase minimal promoter. The plasmid FOPFLASH contains mutated TCF binding-sites and serves as a negative control. Both plasmids are commercially available (Upstate, Cell signalling solutions, Charlottesville, VA, USA). The constitutively active mutant of β-catenin, S45A, was generated by T. Hagen (Wolfson Digestive Diseases Centre, University of Nottingham, UK) as described previously [20]. The cDNA bearing the Ser45→Ala mutation has been inserted into the pcDNA3 expression vector (Invitrogen, Karlsruhe, Germany). The glucokinase reporter gene contains a luciferase reporter under the control of the rat glucokinase promoter, spanning −1003/+196 of the beta cell-specific gene [21]. The expression vector for PPARγ contains the full-length cDNA transcript of the gene, as described previously [22]. The cyclin D1 reporter gene contains a luciferase reporter under the control of the promoter (spanning −1745/+134) of the human cyclin D1 gene [23].

The plasmid pRL-TK (Promega, Mannheim, Germany) serves as an internal control for transfection efficiency. The vector contains a cDNA encoding *Renilla* luciferase under the control of the herpes simplex virus thymidine kinase promoter.

Cell culture and transfection of DNA The rat insulinoma cell line Ins-1 has been described previously [24]. Ins-1 cells were cultured in RPMI 1640 medium containing 11 mmol/l glucose supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin [24]. Cells were trypsinised and transiently transfected with Fugene 6 reagent (Roche, Grenzach-Wyhlen, Germany) according to the manufacturer's protocol.

Co-transfections were carried out with a constant amount of DNA, which was maintained by adding the vector pcDNA3 (Invitrogen, Karlsruhe, Germany). For each transfection experiment 0.5 μg of the *Renilla* luciferase reporter gene (plasmid pRL-TK) and 0.5 μg of the firefly reporter gene were added to each well to check for transfection efficiency (the relative luciferase activities presented in the figures are derived from firefly/*Renilla* ratios. Where indicated, cells were incubated with fat cell-conditioned medium (FCCM), conditioned-medium from Wnt3a secreting L cells or the respective control media 24 h before harvest. The luciferase assay was performed as described previously [25, 26].

Proliferation assays The proliferation assay has been described previously [27, 28]. In brief, Ins-1 cells were resuspended in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin and 5×10^4 cells were cultured in round-bottomed, 96 well culture plates. Where, indicated sFRP-1 (R&D Systems, Minneapolis, MN, USA) was added at a concentration of 10 ng/ml. The proliferation activity was determined by [^{3}H]thymidine uptake. Cells were pulsed with 37 MBq [^{3}H]thymidine per well (Amersham Pharmacia Biotech, Braunschweig, Germany) 18 h before harvesting. After harvesting, thymidine incorporation was assessed using 25 μl of a β-scintillation cocktail (Perkin Elmer, Rodgau-

Jugesheim, Germany) and a micro scintillation counter (Trillux, Wallach, Germany). Cellular proliferation was expressed relative to the respective control medium.

Human tissues Tissue samples of human white adipose tissue were obtained from women (20–35 years of age) undergoing surgical mammary reduction (n=10). The patients were otherwise healthy and free of metabolic or endocrine diseases. The BMI range of the donors was between 21.4 and 29.2 kg/m^2 (25.4±2.8, mean±SD). Consent was obtained from the patients after the nature of the procedure was explained, and the study was approved by the ethics committee of the Heinrich-Heine-University Düsseldorf, Germany (study number 2292).

FCCM The isolation of adipocytes and preparation of FCCM have been described previously [29]. In brief, adipose tissue samples of 20–60 g wet weight were obtained from surgical mammary reductions and immediately transported to the laboratory in DMEM/Nutrient Mix F12 (DMEM/F12, Life Technologies, Karlsruhe, Germany) supplemented with 2% BSA, 100 U/ml penicillin and 100 μg/ml streptomycin. After removal of fibrous material and blood vessels, the adipose tissue was minced and digested in Krebs Ringer bicarbonate buffer (KRB) containing 2% BSA and 120 U/ml collagenase type I from *Clostridium histolyticum* (Sigma) in a shaking water bath for 45–60 min at 37°C. The digested tissue was then filtered through nylon gauze (250 μm mesh) and washed with KRB containing 0.1% BSA. For culturing, 2 ml of isolated floating adipocytes was transferred into culture flasks (Becton Dickinson, Heidelberg, Germany) containing 5 ml of cell culture medium (DMEM/F12 containing 15 mmol/l HEPES and 2.5 mmol/l L-glutamine, supplemented with 1.125 g/l $NaHCO_3$, 100 units/ml penicillin and 100 μg/ml streptomycin). Cells were kept at 37°C in a humidified atmosphere of 5% CO_2/95% air and cultured for 24 h. The conditioned medium was subsequently collected, carefully avoiding the lipid floating on the top, and kept frozen at −20°C until used. DMEM/F12 medium incubated without adipocytes was used as control medium for the treatment of Ins-1 cells. The glucose concentration in the control medium and the FCCM was adjusted to 11 mmol/l prior to the treatment of Ins-1 cells or primary beta cells.

Wnt3a-conditioned medium from L cells The preparation of conditioned medium from Wnt3a-secreting L cells has been described previously [30]. L cells expressing Wnt3a were obtained from the American Type Culture Collection (CRL-2647; contact through LGC Promochem, Wesel, Germany). Conditioned medium from wild-type L cells served as a negative control.

Preparation of mouse islets Wild-type mice (C57BL/6, 6 months old) were used as donors. The mice were obtained from the animal facility of the University Hospital Düsseldorf and the study was conducted in accordance with the Principles of Laboratory Care. Mice were killed and islets were isolated using the intraductal collagenase digestion technique as described previously [31]. Islets were purified, handpicked and thereafter incubated overnight with FCCM or the respective control medium (see above). Glucose concentrations in the FCCM and control medium were adjusted to 11 mmol/l before treatment of the islets. The next day, insulin in the supernatant was measured using a radioimmunoassay kit.

The generation of a single cell culture has been described previously [31]. In brief, islets were dissociated into single cells by trypsinising in Hanks' balanced salt solution and thereafter cultured in RPMI 1640 medium (GIBCO BRL, Karlsruhe, Germany) containing 4.5 mmol/l glucose supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Semiquantitative TaqMan PCR Pancreatic islets from 6-month-old wild-type mice (C57BL/6) were isolated and treated with recombinant Wnt3a protein (10 ng/ml) (R&D Systems) for 24 h. Total RNA was extracted using the RNeasy MiniKit (Qiagen, Hilden, Germany) including a DNase I digestion step (New England Biolabs, Ipswich, UK) and reversely transcribed with the random-primed first-strand cDNA Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. For negative control reactions, the reverse transcription step was omitted. β-Actin was used as an internal control. The glucokinase-specific primers were selected using the software PrimerExpress (PE Applied Biosystems, Foster City, CA, USA) (glucokinase: forward: 5′-CAC-AAT-GAT-CTC-CTG-CTA-CT-3′, reverse: 5′-TTC-TGC-ATC-TCC-TCC-ATG-TA-3′; β-actin: forward: 5′-CCT-GAA-CCC-TAA-GGC-CAA-CCG-3′, reverse: 5′-GCT-CAT-AGC-TCT-TCT-CCA-GGG-3′). Semiquantitative TaqMan PCR was carried out using an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems) as follows: 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 58°C for 1 min. All experiments were carried out in triplicate and average cycling threshold (C_t) units were obtained as the average of the results. Relative quantification of the glucokinase expression was performed using the comparative C_t method in separate tubes. All data are expressed as the means±SD of three independent experiments as described previously [32].

Statistical analysis All data are presented as means±SEM. Statistical analysis was performed using Student's t test. Significance was assumed at a p value of less than 0.05.

Results

Adipocyte-derived factors induce Wnt signalling in pancreatic beta cells Wnts have been discovered as novel autocrine and paracrine factors secreted by adipocytes [9, 11, 12]. In addition, Wnt signalling molecules and Frizzled receptors are expressed in the endocrine pancreas of both mice and humans [12, 16, 33] and regulate insulin secretion [17] and beta cell proliferation [19]. In contrast, the canonical Wnt signalling pathway cannot be activated in glucagon-producing alpha cells [34, 35]. We investigated canonical (i.e. β-catenin) Wnt signal transduction in insulin-producing Ins-1 cells. As shown in Fig. 1a, co-transfection of a constitutively active mutant of β-catenin (S45A) [20] activates the transcription of a canonical TCF reporter gene (TOPFLASH). The activation is dose-dependent with a maximum effect using 500 ng of the β-catenin mutant (225±27%, $p<0.05$).

To investigate the functional interaction between adipocytes and beta cells through the Wnt signalling pathway we used FCCM to treat Ins-1 cells. We found a concentration-dependent induction of a TCF reporter gene (TOPFLASH) after incubation with FCCM for 24 h (Fig. 1b). TOPFLASH transcription was increased to 136±3% of the control level ($p<0.05$) by FCCM diluted 1:10 and to 262±16% ($p<0.05$) by undiluted FCCM. This effect was comparable to the maximum activation seen by co-transfecting the β-catenin mutant S45A. FCCM had no significant effect on a mutated TCF reporter plasmid (FOPFLASH; Fig. 1b). Thus, our data show an interaction between adipocytes and insulin-producing cells through the Wnt signalling pathway in vitro.

Adipocyte-derived factors induce beta cell proliferation through Wnt signalling We assessed the effect of Wnts and FCCM on Ins-1 proliferation by [^{3}H]thymidine incorporation. Figure 2b shows that conditioned medium from L cells containing Wnt3a, an activator of canonical Wnt signalling, increases the proliferation of Ins-1 cells to 207±27% of the control level. Similarly, treatment of Ins-1 cells with FCCM leads to an increase in proliferation to 181±11% of the control level ($p<0.05$; Fig. 2a). Similarly, treatment of Ins-1 cells with FCCM leads to an increase to 181±11% in cell proliferation ($p<0.05$; Fig. 2a). To test whether the proliferation seen in the presence of FCCM is mediated through the Wnt signalling pathway we used the soluble Wnt antagonist sFRP-1 to treat Ins-1 cells, together with FCCM or Wnt3a-conditioned medium from L cells. The proliferative effects of Wnt3a-conditioned medium from L cells and FCCM were both inhibited by the Wnt-antagonist sFRP-1. After adding 10 ng/ml of recombinant sFRP-1 to the respective conditioned medium, the FCCM-mediated proliferation was inhibited by 42% (Fig. 2a) and the Wnt3a-mediated proliferation was inhibited by 49% (Fig. 2b). Treatment with sFRP-1 in the absence of FCCM had no effect on basal Ins-1 proliferation (Fig. 2a,b). Similar results were obtained in primary murine islet cells. FCCM increased islet cell proliferation to 164% of the level with

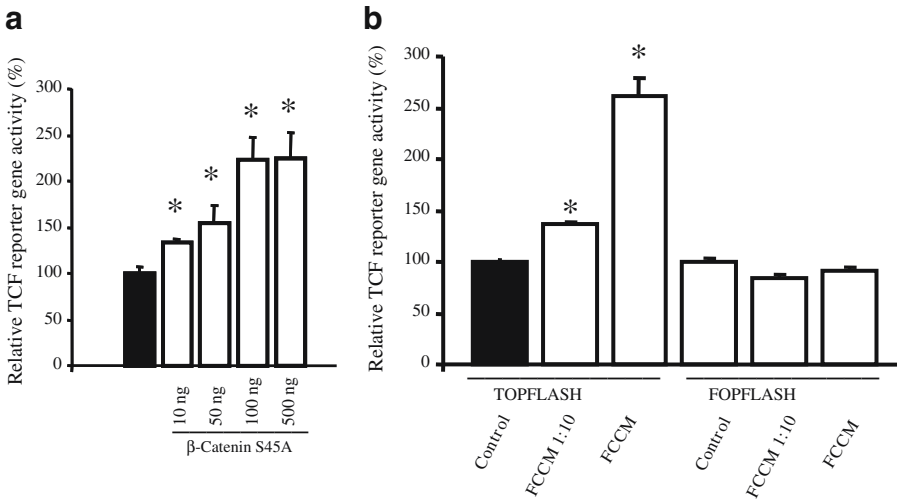

Fig. 1 Adipocyte-derived factors induce Wnt signalling in Ins-1 beta cells. **a** Intact canonical Wnt signalling in Ins-1 beta cells. Activation of a luciferase reporter gene under the control of TCF-binding-sites (TOPFLASH) in response to increasing amounts of a constitutively active mutant of β-catenin (S45A) in Ins-1 cells. **b** Activation of a TCF reporter gene in Ins-1 beta cells by FCCM. Ins-1 cells were transfected with the TOPFLASH reporter gene and treated with FCCM or the respective control medium. The FOPFLASH reporter gene bearing mutated TCF binding sites showed no response to FCCM and served as a negative control. The luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means±SE of three independent experiments, each performed in triplicate. *$p<0.05$ vs untreated control (Student's t test)

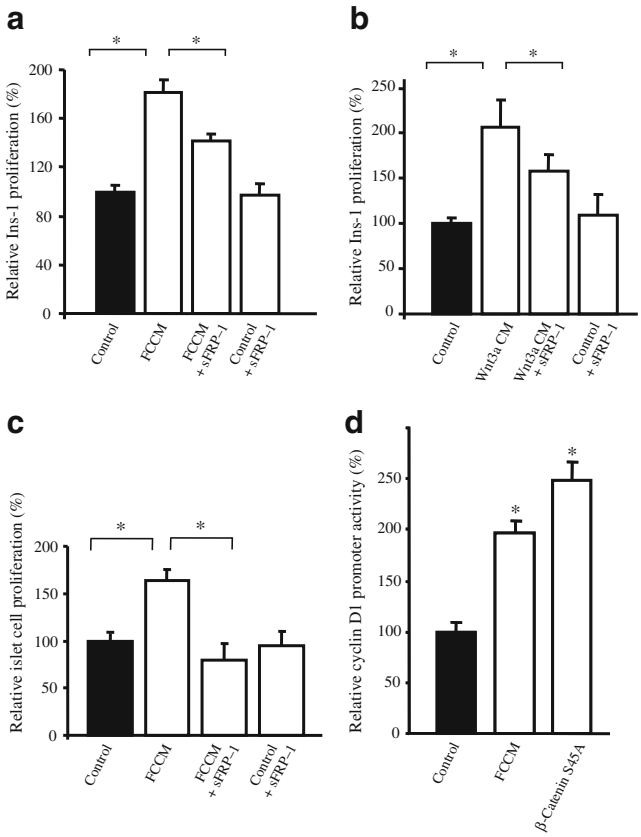

Fig. 2 Adipocyte-derived Wnts induce Ins-1 and beta cell proliferation. **a** FCCM induces the proliferation of Ins-1 beta cells. Ins-1 cells were incubated in the presence of FCCM or the respective controls. FCCM-induced proliferation of Ins-1 cells can be inhibited by antagonising Wnt signalling, as assessed by treatment with recombinant sFRP-1 (10 ng/ml) where indicated. sFRP-1 had no effect on unstimulated Ins-1 proliferation. Proliferation is expressed relative to cells treated with control medium. **b** Wnt3a-conditioned medium induces the proliferation of Ins-1 cells. Cells were treated with Wnt3a-conditioned medium (Wnt3a CM) from L cells or the respective controls. Wnt3a-induced proliferation can be inhibited by adding sFRP-1 (10 ng/ml). sFRP-1 had no effect on unstimulated Ins-1 proliferation. **c** FCCM induces the proliferation of primary murine islet cells. Islet cells were incubated in the presence of FCCM or the respective controls. sFRP-1 (10 ng/ml) was added where indicated. FCCM induced the proliferation of primary mouse islet-cells. sFRP-1 had no effect on unstimulated islet cell proliferation. The proliferation was expressed relative to cells treated with control medium. Values are means±SE of three independent experiments, each performed in triplicate. *$p<0.05$ (Student's t test). **d** Adipocyte-derived factors induce the transcription of a cyclin D1 reporter gene in Ins-1 beta cells. Co-transfection with β-catenin S45A and FCCM both induced the transcription of a luciferase reporter gene under the control of the cyclin D1 promoter. The luciferase activity is expressed as a percentage of the mean value of the activity measured in the untreated controls. Values are means±SE of three independent experiments, each performed in triplicate. *$p<0.05$ vs control (Student's t test)

control medium and this effect was abolished by adding sFRP-1 to FCCM (Fig. 2c). These data demonstrate that activation of Wnt signalling is one mechanism through which FCCM induces Ins-1 proliferation.

We explored the molecular mechanisms involved in the proliferative effect of FCCM on Ins-1 cells through Wnts by investigating the transcriptional regulation of the cyclin D1 reporter gene by fat cell products. The promoter of the gene encoding cyclin D1 contains binding sites for TCF/LEF transcription factors, which are known to be coactivated by β-catenin upon activation of canonical Wnt signalling [23]. Cyclin D1 promotes the transition from G1 to S phase by inhibiting the retinoblastoma protein (Rb) and thereby induces proliferation in multiple cell types, including pancreatic endocrine cells [36]. Consequently, overexpression of cyclin D1 has been shown to induce beta cell proliferation in vitro and in vivo [36, 37]. We questioned whether activation of Wnt signalling in Ins-1 beta cells by fat cell products could induce cyclin D1 reporter gene transcription. Transient transfection studies using a cyclin D1 luciferase fusion gene and an expression vector for β-catenin S45A into Ins-1 cells confirmed the regulation of the cyclin D1 promoter by β-catenin. Co-transfection of β-catenin S45A led to an increase in cyclin D1 transcription to 249±18% of the control level (Fig. 2d). In addition, we found that FCCM induced cyclin D1 promoter activity (197±11%, $p<0.05$; Fig. 2d). These results demonstrate the activation of the cyclin D1 promoter in response to fat cell products and suggest that this is one mechanism through which adipocytes induce the proliferation of beta cells.

FCCM stimulates insulin secretion in primary murine islets Islets were isolated from adult (6 months old) wild-type (C57BL/6) mice and transferred into primary culture. After incubation with FCCM for 24 h, insulin secretion from primary islets was stimulated to 204% of the control level and this effect was abolished after adding 10 ng/ml sFRP-1 to the FCCM (Fig. 3a). These data demonstrate that adipocyte-derived Wnts make a major contribution to the induction of insulin secretion in pancreatic cells by FCCM.

Wnt/β-catenin signalling activates glucokinase gene transcription in the presence of PPARγ To explore the molecular mechanisms underlying the stimulatory effect of FCCM (Fig. 3a) and Wnt signalling [17] on insulin secretion we tested whether glucokinase is a direct target gene for canonical Wnt signalling. Figure 3b shows that the transcription of a glucokinase reporter gene [21] is induced by an active β-catenin mutant (S45A) after co-transfection of a plasmid encoding PPARγ. In contrast, β-catenin has no effect on glucokinase gene transcription in the absence of exogenous PPARγ (Fig. 3b). Accordingly, we found that incubation with recombinant Wnt3a protein (10 ng/ml) increased glucokinase mRNA expression in primary mouse islets (Fig. 3c), as assessed by semiquantitative PCR. These results define the glucokinase gene as a novel target gene

for canonical Wnt signalling involving the transcription factor PPARγ.

Discussion

Our data presented here indicate that adipocyte-derived Wnts are, at least in part, responsible for mediating beta cell proliferation in vitro. This is suggested by the finding that the proliferative effect of FCCM on beta cells is inhibited when the Wnt signalling pathway is blocked (Fig. 2a,c). In line with this, we here demonstrate the induction of the cyclin D1 promoter, containing TCF/LEF consensus-sites [23], by fat cell secretory products (Fig. 2d). We used human FCCM as an established in vitro model to mimic the effect of fat cells on target cells [12, 29]. FCCM contains a variety of metabolites and signalling molecules, including known adipocytokines such as leptin, adiponectin, IL-6, TNF-α and resistin. However, these adipocytokines are not thought to mediate beta cell proliferation [5, 38, 39]. In addition to these known adipocytokines, Wnt signalling molecules are secreted by adipocytes [9, 12]. In line with our current study, there is evidence that the Wnt signalling pathway regulates glucose sensing in pancreatic beta cells [17] and prenatal and postnatal beta cell development in mice [18, 19].

Beta cell number is determined by their replication, neogenesis and apoptosis [5]. Animal studies in rodents revealed that the increased replication of beta cells is the major mechanism for the increased beta cell number seen in non-diabetic obesity [40, 41]. Also, in humans, increased body weight is paralleled by an increase in beta cell mass [1, 2]. However, the mechanisms through which the increase in beta cell number is achieved appears to be different between species: In humans, neogenesis was found to be the major mechanism for the increase in beta cell mass in non-diabetic-obesity [4]. The data from our current study using rodent beta cells supports these previous findings as we find adipocyte-derived products to induce the proliferation of beta cells.

Our findings prompt the following question: how do adipocyte-derived Wnts reach pancreatic islets? Wnt signalling molecules are able to elicit paracrine as well as systemic effects on target tissues [42, 43]. Besides intra-abdominal and subcutaneous depots, adipocytes are also found within the pancreas [44]. Therefore, paracrine as well as endocrine stimulation of islets by adipocyte-derived Wnts appears to be possible and further studies are needed to distinguish between these mechanisms.

Hyperinsulinaemia can be caused by islet hyperplasia as well as by hypersecretion of insulin from the individual beta cell. Wnt signalling molecules have been found to regulate insulin secretion [17]. However, the underlying molecular mechanisms are not fully understood. Fujino et al. [17] found a decrease in genes that regulate glucose sensing in pancreatic beta cells in *Lrp5* knockout mice. In this study we demonstrate the induction of insulin secretion by fat cell products and show that this effect can be inhibited by blocking Wnt signalling (Fig. 3a), suggesting

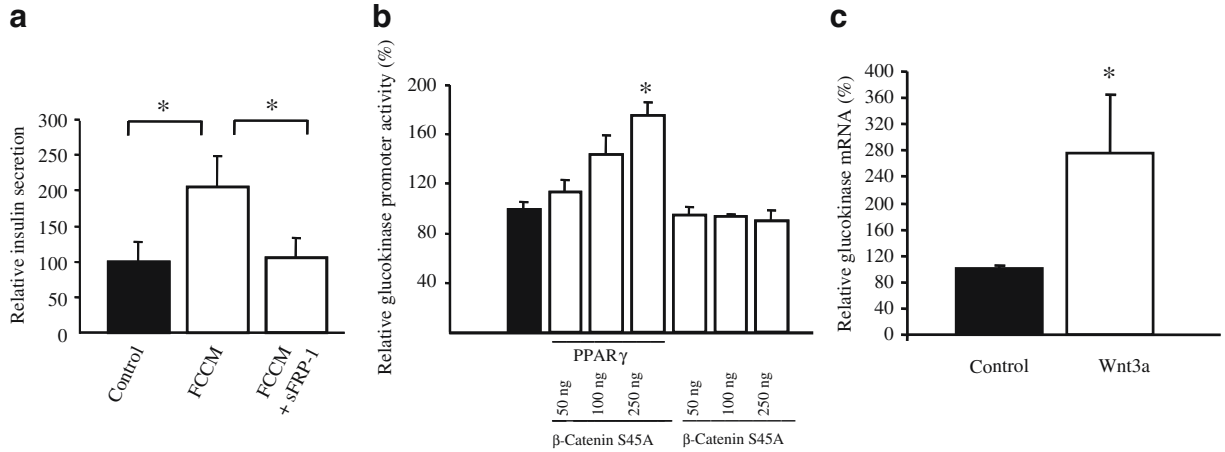

Fig. 3 Canonical Wnt signalling induces insulin secretion and glucokinase gene transcription. **a** FCCM stimulates insulin secretion through Wnts. sFRP-1 (10 ng/ml) inhibited the stimulation by FCCM. Values are means±SE of three independent experiments, each performed in triplicate. *$p<0.05$ (Student's t test). **b** Canonical Wnt signalling activates glucokinase reporter gene transcription in the presence of PPARγ. Ins-1 cells were transiently transfected with a luciferase reporter gene under the control of the glucokinase promoter. Co-transfection of increasing amounts of β-catenin S45A activated glucokinase promoter activity only in the presence of PPARγ. The luciferase activity is expressed as percentage of the mean value of the activity measured in the untreated controls. Values are means±SE of three independent experiments, each performed in triplicate. *$p<0.05$ vs control (Student's t test). **c** Recombinant Wnt3a (10 ng/ml) increases glucokinase mRNA expression in primary mouse islets. Mouse islets were isolated and treated with recombinant Wnt3a. mRNA expression was assessed by semi-quantitative Taqman PCR. Values are means±SE of three independent experiments, each performed in triplicate. *$p<0.05$ vs control (Student's t test)

that fat cells increase insulin secretion at least in part through the activation of Wnt signalling. On a molecular level, we identified the glucokinase gene promoter as a direct target for β-catenin in beta cells. Interestingly, the effect of β-catenin requires the presence of PPARγ (Fig. 3b). PPARγ is highly expressed in primary islets in alpha and beta cells [45]. This is in contrast to our previous findings in pancreatic endocrine cell lines, in which we demonstrated the loss of PPARγ expression [22]. Thus, Ins-1 cells are an excellent tool to investigate PPARγ-mediated effects without the need to knockdown the gene for this protein. PPARγ binds to its consensus motif within the glucokinase promoter [46, 47]. Here, we demonstrate a functional interaction between β-catenin and PPARγ in insulin-producing cells (Fig. 3b). We suggest that activated β-catenin coactivates PPARγ-mediated transcription at the glucokinase promoter, thereby defining the glucokinase gene as novel target gene for canonical Wnt signalling.

In conclusion, our data show that adipocyte-secreted products act on beta cells through the Wnt signalling pathway, resulting in an activation of cyclin D1 transcription and increased beta cell proliferation. In addition, fat cell products, through activation of canonical Wnt signalling, stimulate insulin secretion and glucokinase gene transcription in vitro. These data suggest a novel mechanism for obesity-induced beta cell hyperplasia and hyperinsulinaemia.

Acknowledgements This work was supported by a grant from the Medical Faculty of the Heinrich-Heine-University Düsseldorf to S. Schinner. The reporter gene plasmid for cyclin D1 was a generous gift from R. Pestell (Lombardi Comprehensive Cancer Center, Department of Oncology, Georgetown University, Washington, DC, USA). The β-catenin expression vector (S45A) was provided by T. Hagen (Wolfson Digestive Diseases Centre, University of Nottingham, UK). The β-glucokinase reporter gene was provided by Y. H. Ahn, Seoul University, South Korea. Ins-1 cells were provided by J. Seissler (Klinikum der Universität München, Innenstadt, Germany).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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PUBLIKATION 14

Ülgen F, Scherbaum WA, Partke HJ, Bornstein SR, **Schinner S**

Intrapancreatic Adipocyte Deposition in a Mouse Model for the Metabolic Syndrome

Horm. Metab. Res. 40(7):507-9 (2008)

Intrapancreatic Adipocyte Deposition in a Mouse Model of the Metabolic Syndrome

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received 29.08.2007
accepted 13.02.2008

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DOI 10.1055/s-2008-1062722
Published online:
April 14, 2008
Horm Metab Res 2008;
40: 507–509

ISSN 0018-5043

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Introduction

Obesity is a major risk factor for the development of insulin resistance and type 2 diabetes mellitus [1]. However, due to reactive hyperinsulinemia only a minority of obese and insulin-resistant individuals develop overt diabetes mellitus. The mechanisms causing hyperinsulinemia are not fully understood, but several studies have demonstrated that adipocytes are able to secret signaling molecules to act on β-cells [2]. In line with this, our previous findings have identified Wnt-signaling molecules derived from adipocytes to regulate β-cell proliferation and insulin secretion [3]. However, it is not yet clear whether local intrapancreatic adipocyte depots exist, possibly allowing for paracrine actions of adipocyte-derived hormones. Therefore, the aim of this study was to investigate the presence of local adipocyte deposition within the pancreas of wild-type (C57BL/6) and New Zealand Obese (NZO) mice, the latter representing a model for the metabolic syndrome [4].
In this study we found the deposition of ectopic adipocytes in the pancreas of obese, hyperinsulinemic NZO mice. Furthermore, we showed the proximity of adipocytes to pancreatic islets, potentially allowing for paracrine effects.

Materials and Methods

Animal treatment

New Zealand Obese (NZO) mice exhibit a polygenetic syndrome of obesity, hyperphagia, insulin resistance, and hyperinsulinemia resembling the human metabolic syndrome. The animals were fed a standard laboratory diet with free access to food and water. For our study we used hyperinsulinemic normoglycemic mice that had been characterized before with regard to blood glucose and serum insulin levels [4]. As a control we used age-matched wild-type mice (C57BL/6). Male mice were sacrificed at the age of 52 weeks and the pancreas was excised immediately after exsanguination.

Immunohistochemistry of pancreata of NZO mice and wild-type mice

The pancreata were fixed in paraformaldehyde (4% solution in phosphate-buffered saline) and embedded in paraffin. For immunohistochemistry, serial sections of the pancreas were deparaffinized, unmasked, and pre-incubated with 5% normal swine serum. The sections were exposed to the guinea pig insulin antibody (DAKO Diagnostika, Hamburg, Germany) and an HRP-linked secondary antibody (Envision System, DAKO Diagnostika, Hamburg, Germany) and counterstained with hematoxylin. The pancreas sections of NZO and wild-type mice were exposed to fatty acid binding protein 4 (FABP4) antibody (R&D Systems, Germany) and a rabbit anti-goat secondary antibody (DAKO Diagnostika, Hamburg, Germany) following counterstaining with hematoxylin. The adipogenic cells were assessed by using oil red O (Sigma, Germany) staining. The frozen sections were rinsed in PBS and fixed in 4% paraformaldehyde for five minutes at room temperature followed by incubation in 60% isopropanol for five minutes. Briefly, slides were stained with oil red O in 60% isopropanol for 10 minutes, and the staining was differentiated with 60% isopropanol and counterstained with hematoxylin for one minute. After several washes in distilled water, the stained slides were coverslipped for microscopy. The lipids were stained in red and the nuclei were stained in blue.

Adipocyte measurement in NZO mice and wild-type mice

The quantification of adipocytes within the pancreas via light microscopy was done by measuring the area of sectioned pancreas tissue covered

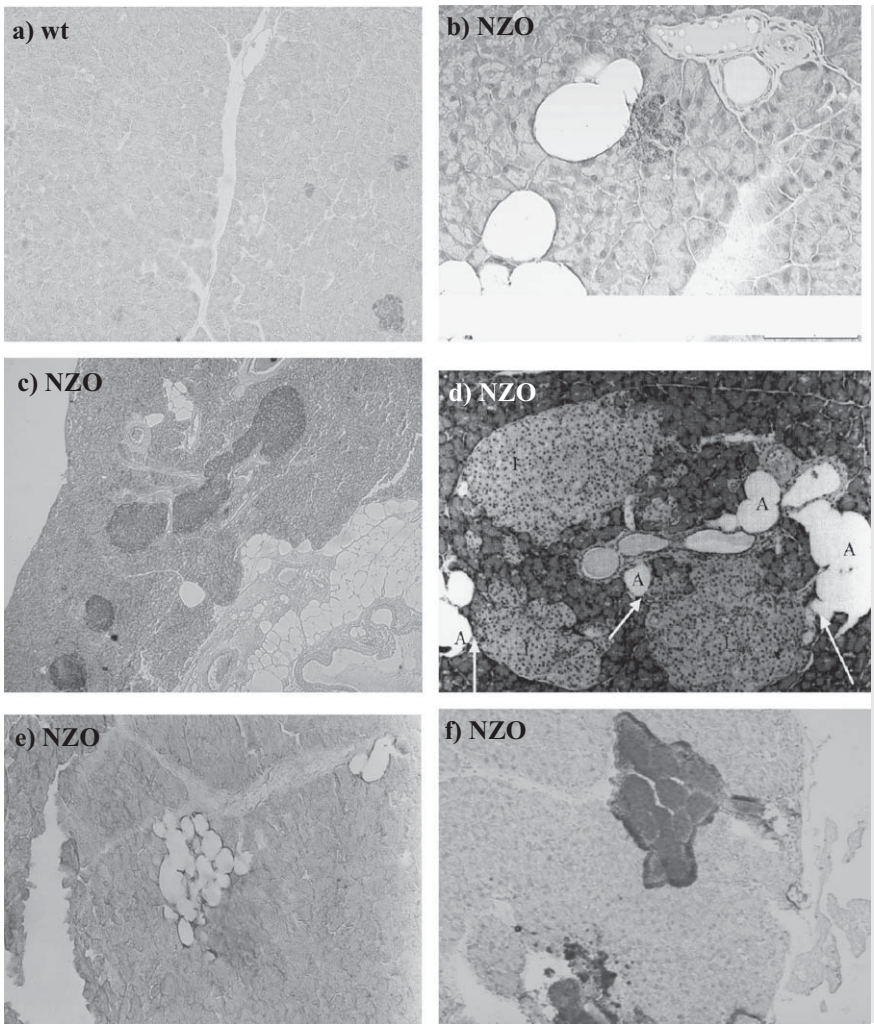

Fig. 1 Islet morphology in pancreas of wild-type (C57BL/6) and New Zealand Obese (NZO) mice. Comparison of the islet morphology and intrapancreatic adipocytes of 52-week-old wild-type (wt) and hyperinsulinemic NZO mice. Pancreas sections were immunostained for insulin and stained with hematoxylin. Wild-type mice showed no significant adipocyte deposition within the pancreas (**a**). NZO mice displayed islet hyperplasia (**c**) and adipocyte deposition within the pancreas and close proximity to islets (**b–d**). Pancreas sections of NZO mice were immunostained for FABP4 and stained with hematoxylin (**e**). The sections of NZO mice pancreas were oil red O stained and counterstained with hematoxylin (**f**). Panels **a**, **c**, and **e**: 10× magnification; Panels **b**, **d**, and **f**: 20× magnification. A: adipocyte; I: islet.

by adipocytes compared to the total area of pancreas tissue (Lucia G, Nikon GmbH, Düsseldorf, Germany).

Statistical analysis

All data are presented as means ± SEM. Statistical analysis was done using a Student's *t*-test. Significance was assumed at a p-value of less than 0.05.

Results

Intrapancreatic adipocyte deposition in a mouse model of the metabolic syndrome

New Zealand Obese mice are a polygenetic model for the metabolic syndrome. The cluster of mice that we analyzed were obese and euglycemic due to hyperinsulinemia [4]. When the pancreata of these mice were analyzed with respect to islet morphology and intrapancreatic adipocyte mass, we found islet hyperplasia (**Fig. 1c**) as a typical feature of obesity [5]. However, NZO mice showed a massive increase in adipocyte number compared with age-matched (52 weeks) wild-type mice (C57BL/6) (**Fig. 1a**). In addition, apposition of adipocytes around islets was seen (**Fig. 1b,d**), suggesting paracrine effects of adipocytes on islets. Intrapancreatic adipocytes of NZO mice showed a positive staining for FABP4, indicating that they are functionally active (**Fig. 1e**). Additional oil red O staining supports the evidence of functionally active adipocytes (**Fig. 1f**). We quantified adipocytes within the pancreas section by measuring the relative area of the pancreas covered by adipocytes and found an 18-fold increase in adipocyte deposition in NZO mice (1821 ± 223 %) compared with wild-type mice (100 ± 27 %) ($p < 0.05$) (**Fig. 2**). These data show an obesity-associated deposition of adipocytes within the pancreas with close proximity to islets, suggesting paracrine effects of adipocyte-secretory products on islets.

Discussion

Obesity is associated with arterial hypertension [6], insulin resistance, and normal blood glucose levels as long as hyperinsulinemia compensates for impaired insulin action. The underlying mechanisms linking obesity with hyperinsulinemia are, however, incompletely understood. On the one hand, slightly elevated, although still normal, blood glucose levels might contribute to elevated insulin levels [7]. On the other hand, adipocytes are able to produce a variety of signaling molecules [1], some of them potentially acting on pancreatic β-cells. In fact, our previous findings have demonstrated that adipocytes can act on pancreatic β-cells to regulate proliferation and insulin secretion through the Wnt-signaling pathway *in vitro* [3]. Therefore, the question arises how adipocyte-derived factors can reach pancreatic islets. Some adipocyte-derived factors are able to elicit systemic effects on target tissues [8,9], suggesting that

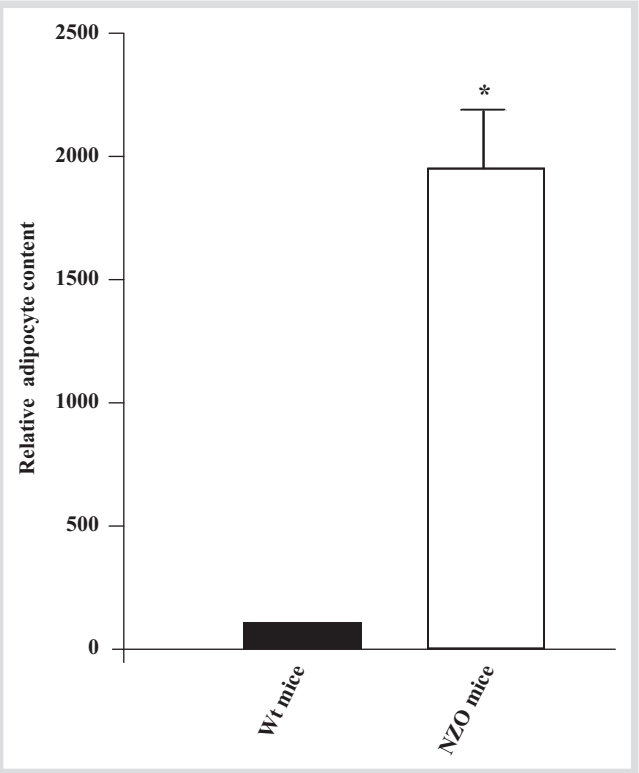

Fig. 2 Quantification of intrapancreatic adipocyte depots in NZO mice. The content of adipocytes in wild-type (Wt) mice and NZO mice within the pancreas was assessed by quantifying the area of sectioned pancreas tissue covered by adipocytes compared with the total area of pancreas tissue. The relative adipocyte content is expressed as the percentage of the mean value in wild-type mice (n = 5). Values are means ± SE of three independent experiments, each done in triplicate. *$p < 0.05$ (Student's *t*-test).

secretory products from intra-abdominal adipocytes might reach endocrine pancreatic cells via the blood stream.

Furthermore, there is a report indicating a correlation between intrapancreatic adipocyte deposition and body weight in humans [10]. However, this study did not take into consideration the islet morphology (hyperplasia), the body mass index, or the metabolic state—especially blood glucose and insulin values—of the patients.

Therefore, here we investigated pancreas morphology in a mouse model of nondiabetic hyperinsulinemia, which resembles the pre-diabetic metabolic syndrome in humans. In this study we demonstrated that increased adipocyte deposition develops together with islet hyperplasia in this hyperinsulinemic obese mouse model. In addition, adipocytes were found in close proximity to pancreatic islets. The detection of FABP4 in intrapancreatic adipocytes is an indicator of vital adipocytes. These findings raise the possibility of paracrine actions of intrapancreatic adipocytes on pancreatic endocrine cells.

Acknowledgments

This work was supported by a grant from the Medical Faculty of the Heinrich-Heine-University, Düsseldorf, to Dr. S. Schinner. The authors would particularly like to thank A. Tries for the technical assistance.

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A2. Curriculum vitae

Name: Dr. med. Sven Schinner

Geburtsdatum: 14. August 1972, Geburtsort: Hameln

Familienstand: verheiratet mit Sabine Zieseniß-Schinner

2 Kinder: Florian und Linus

Ausbildung

1992 Abitur, Albert-Einstein-Gymnasium, Hameln

1992-1999: Studium der Medizin, Universität Göttingen und Padua (Italien)

1995-1997 Promotionsarbeit, Abteilung Histologie (Prof. W. Götz), Universität Göttingen: „Eine Immunhistochemische Studie über das IGF-System in Pankreas- und Pinealtumoren MSV-SV-40 transgener Mäuse“.

Juli 1999 Promotion zum Dr. med.

Februar 2009: Facharzt für Innere Medizin

Juni 2009: Anerkennung als Diabetologe DDG

Berufliche Stationen

August 1999 bis Dezember 2001 Arzt im Praktikum / Assistenzarzt,
Abteilung Molekulare Pharmakologie (Prof. W. Knepel), Universität Göttingen.

Januar bis Dezember 2002: wiss. Mitarbeiter, Department of Clinical Biochemistry (Prof. S. O’Rahilly), University of Cambridge, Großbritannien

Januar bis Dezember 2003: wiss. Mitarbeiter, Deutsche Forschungsgemeinschaft.

Januar 2004 bis März 2009: Assistenzarzt, Klinik für Endokrinologie, Diabetologie und Rheumatologie, (Prof. W.A. Scherbaum) Universitätsklinik Düsseldorf

Seit April 2009: Oberarzt, Klinik für Endokrinologie, Diabetologie und Rheumatologie, (Prof. W.A. Scherbaum) Universitätsklinik Düsseldorf

Preise / Förderungen/ Mitgliedschaften

Dr.-Werner-Fekl-Forschungspreis der Dt. Gesellschaft für Klinische Ernährung (2008)

Jühling-Preis (Diabetologie) der Heinrich-Heine-Universität Düsseldorf (2006)

Mitgliedschaften: „American Diabetes Association“, „Endocrine Society“, EASD (European Association for the Study of Diabetes), Deutsche Gesellschaft für Endokrinologie, Deutsche Diabetesgesellschaft, Deutsche Gesellschaft für Innere Medizin, Deutschen Adipositas-Gesellschaft, Nordrhein-Westfälische Gesellschaft für Endokrinologie und Diabetologie

Forschungsförderung: Deutsche Forschungsgemeinschaft; Igler/Clawitter Stiftung; Forschungskommission HHU Düsseldorf; Forschungskommission Universität Göttingen; Stiftung Altersforschung Düsseldorf

Danksagung

Bei diesem langfristigen Vorhaben hatte ich große Unterstützung.
Zunächst möchte ich meiner Familie danken. Meine Frau Sabine und meine Kinder Florian und Linus hatten viel Geduld und haben mir auf allen Etappen der letzten Jahre wichtige Anregungen verschiedenster Art gegeben und mir sehr geholfen.
Meine Eltern haben mich in allen Dingen meiner Ausbildung – wie auch sonst – sehr unterstützt.
Bei meinen wissenschaftlichen und klinischen Lehrern möchte ich mich für Ihre Unterstützung bedanken: Prof. Knepel in Göttingen hat mir das Rüstzeug für zell- und molekularbiologisches Denken und Arbeiten mitgegeben. Prof. Steve O'Rahilly und Dr. Antonio Vidal-Puig haben mir in der Zeit in England Wege zu klinisch-orientierter Forschung aufgezeigt. In Düsseldorf hat mich Prof. Werner A. Scherbaum stets konsequent unterstützt und mir damit die Möglichkeit gegeben, wissenschaftliche Eigenständigkeit neben klinischer Tätigkeit aufzubauen. Auch Prof. Stefan R. Bornstein bin ich für die Anregungen und die fruchtbare gemeinsame Etappe in Düsseldorf zu Dank verpflichtet. Schließlich hat Prof. Scherbaum mich als klinischer Lehrer durch die internistische, diabetologische und endokrinologische Ausbildung begleitet. Ihm bin ich für die Schaffung von Freiräumen und die Stimulation meines wissenschaftlichen Weges sehr dankbar.
Schließlich möchte ich Kollegen und Freunden in Düsseldorf für klinische und wissenschaftliche Unterstützung danken. Hier sind insbesondere PD. Dr. Holger S. Willenberg und Prof. Dr. Matthias Schott zu nennen.
Mein besonderer Dank gilt den engagierten technischen Mitarbeitern und Doktoranden, die im Labor die Projekte mitgetragen haben.