Therapeutic Options in the Treatment of Diabetes

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Meiner Familie

„Ein Gelehrter in seinem Laboratorium ist nicht nur ein Techniker, er steht auch vor den Naturgesetzen wie ein Kind vor der Märchenwelt.“

- Marie Curie

Zusammenfassung

Die Entstehung von Insulinresistenz in peripheren Organen wie dem Skelettmuskel stellt eine frühe Störung in der Pathogenese des Typ 2 Diabetes dar. Seit einigen Jahren ist bekannt, dass Adipositas und Insulinresistenz in enger Verbindung miteinander stehen und Übergewicht ein großer Risikofaktor für die Ausbildung eines Typ 2 Diabetes ist. Das Fettgewebe ist nicht nur ein reiner Fettspeicher, sondern vielmehr ein sekretorisch aktives Gewebe, das neben freien Fettsäuren auch Adipokine wie Adiponektin, TNFα, IL-6 oder MCP-1 sezerniert. Mehrere Studien konnten belegen, dass verschiedene Fettgewebsprodukte an der Auslösung von Insulinresistenz im Skelettmuskel beteiligt sind. Unser Labor arbeitet mit einem Modell, bei dem mit Adipozyten-konditioniertem Medium Insulinresistenz in Skelettmuskelzellen ausgelöst wird. Mit Hilfe dieses Modells konnte gezeigt werden, dass MCP-1 im Adipozyten-konditionierten Medium vorhanden ist und zu einer Störung der Insulinwirkung im Skelettmuskel führt.

Seit kurzem ist bekannt, dass im Fettgewebe auch Endocannabinoide wie Anandamid und 2-AG synthetisiert werden. Diese Substanzen gehören zum Endocannabinoidsystem, welches u.a. an der zentralen Regulation der Nahrungsaufnahme beteiligt ist. Eine Blockade dieses Systems führt zu verminderter Nahrungsaufnahme, weshalb Cannabinoid Typ 1 Rezeptor Antagonisten wie Rimonabant als Medikament zur Behandlung der Adipositas entwickelt wurden. In Studien hat sich gezeigt, dass neben der zentralen Wirkung auch Effekte auftreten, deren Ursachen peripher bedingt sein müssen. In der vorliegenden Arbeit konnte gezeigt werden, dass Cannabinoid Typ 1 Rezeptor Antagonisten in der Lage sind, die durch Adipozyten-konditioniertes Medium induzierte Insulinresistenz im Skelettmuskel zu verhindern. Die Wirkung des Adipozyten-konditionierten Mediums kann durch Behandlung der

Zellen mit Anandamid nachgeahmt werden. Ähnlich wie nach Behandlung mit Adipozyten-konditioniertem Medium kommt es zu einer deutlichen Störung des Insulinsignals. Damit ist der Nachweis gelungen, dass wahrscheinlich auch das Endocannabinoidsystem eine Rolle bei der Entstehung der Insulinresistenz spielt.

Ein weiterer Themenbereich dieser Arbeit befasst sich mit der Frage, ob Insulinresistenz ein umkehrbarer Prozess ist. Aus klinischen Studien ist bekannt, dass sich durch Gewichtsabnahme die Insulinsensitivität der Patienten verbessert und die Serumspiegel von Adiponektin, TNFα und IL-6 normalisieren. In unserem Modellsystem verursacht die Inkubation mit Adipozyten-konditioniertem Medium nicht nur eine Störung des Insulinsignals in den Skelettmuskelzellen, sondern führt auch zu Veränderungen des sekretorischen Profils und der Expression verschiedener myogener Marker wie myoD, Myogenin und MHC. Nach Entzug des Adipozyten-konditioniertem Mediums kommt es zwar zur Normalisierung des Insulinsignals, aber die Sekretion von IL-8 und MCP-1 sowie die verminderte Expression von Myogenin bleiben auch nach 48 h noch gestört. Die Daten zeigen somit, dass es nur zu einer partiellen Reversibilität der Insulinresistenz kommt und die Zellen längerfristige Störungen aufweisen.

Der letzte Aspekt der Arbeit greift die Frage der potentiell mitogenen Wirkung von Insulinanaloga auf, die in der Therapie von Typ 1 und 2 Diabetes eine große Rolle spielen. Besonders die Sicherheit von Insulin Glargin wird aufgrund seiner erhöhten Affinität zum IGF-1 Rezeptor viel diskutiert. Die Ergebnisse dieser Arbeit zeigen, dass die proliferative Wirkung von Insulin und Insulinanaloga abhängig vom Expressionslevel des IGF-1 Rezeptor/IRS-1 Signalweges ist, so dass das individuelle Expressionsniveau als kritische Determinante des mitogenen Potenzials von Insulinanaloga fungiert. Weiterhin wurde gezeigt, dass die proliferative Wirkung von Insulin Glargin hauptsächlich über den IGF-1 Rezeptor vermittelt wird. Dies wird durch Daten aus siRNA-

Experimenten belegt, in denen die Expression des IGF-1 Rezeptors um 95 % reduziert wurde und so die proliferative Wirkung von Insulin Glargin auf das Niveau normalen Insulins gesenkt werden konnte.

Die vorliegende Arbeit greift somit verschiedene Aspekte der Entstehung und Therapie von Insulinresistenz und Typ 2 Diabetes auf. Ein wichtiger Beitrag ist dabei die Erkenntnis, dass ein überaktives Endocannabinoidsystem, wie es bei Adipositas und Typ 2 Diabetes bekannt ist, eine Rolle bei der Entwicklung von muskulärer Insulinresistenz spielt. Ferner konnte gezeigt werden, dass eine Insulinresistenz, ausgelöst durch verschiedene Sekretionsprodukte der Adipozyten, nur partiell reversibel ist und die Zellen längerfristig geschädigt werden. Im letzten Teil der Arbeit wurde nachgewiesen, dass die proliferierende Wirkung von Insulin und Insulinanaloga auf glatte Muskelzellen abhängig ist vom Proteinniveau des Insulin- und IGF-1 Signalweges. Da die verstärkte mitogene Aktivität von Insulin Glargin ausschließlich über den IGF-1 Rezeptor vermittelt wird, sind weitere Untersuchungen bezüglich eines potentiellen Risikos nötig.

Summary

The induction of insulin resistance in peripheral organs like skeletal muscle is an early defect in the pathogenesis of type 2 diabetes. It has been known for some years that obesity and insulin resistance are closely associated and that adiposity is a high risk factor for the development of type 2 diabetes. Adipose tissue is not only a fat storage organ, but also represents an active secretory tissue which produces free fatty acids and adipokines such as adiponectin, TNFα, IL-6, or MCP-1. Several studies have shown that adipose-derived factors are involved in the induction of insulin resistance. Our laboratory works with a model which uses adipocyte-conditioned medium to induce insulin resistance in skeletal muscle cells. Based on this model it was shown that MCP-1 is able to impair insulin signalling in skeletal muscle.

Recently, it has been found that adipocytes synthesise endocannabinoids like anandamide and 2-AG. These substances are part of the endocannabinoid system which is involved in the central regulation of food intake, and it was shown that blocking the endocannabinoid system results in reduced food intake. Therefore, cannabinoid type 1 receptor antagonists like rimonabant have been developed as a therapy to treat obesity. Studies revealed that part of the effects observed with rimonabant is the result of peripheral action. By using our model of adipocyte- conditioned medium-induced insulin resistance, it is shown here that rimonabant is able to prevent the impairment of insulin signalling and action in skeletal muscle cells caused by adipocyte-conditioned medium. Stimulation of skeletal muscle cells with anandamide mimics adipocyte-conditioned medium resulting in comparable disturbance of insulin signalling. Therefore, the results indicate that the endocannabinoid system as a novel player is also involved in the complex pathways leading to skeletal muscle insulin resistance.

A further topic addressed in this work is the question whether insulin resistance is a reversible process. Clinical investigations showed that reduction of body weight and therefore also fat mass is associated with improvements of insulin sensitivity and normalisation of adiponectin, TNFα and IL-6 serum levels. In our model, incubation with adipocyte-conditioned medium results not only in impairment of insulin signalling and insulin action but also alters the secretory profile of skeletal muscle cells and the expression of several myogenic markers like myoD, myogenin and myosin heavy chain. Withdrawal of adipocyte-conditioned medium normalises the insulin signalling, however the secretion of IL-8 and MCP-1 as well as the decreased expression of myogenin remains disturbed. Therefore, these data provide evidence that insulin resistance is only partially reversible and that longer lasting defects occur in the cells.

The final part concerns the safety of insulin analogues regarding their potential mitogenic effects. Specially, insulin glargine is discussed in terms of a possible risk due to its higher affinity towards the IGF-1 receptor. The results of our study implicate that the proliferative effect of insulin and insulin analogues depends on the expression level of the IGF-1 receptor /IRS-1 pathway and that the individual expression level may function as a critical determinant of the mitogenic potency of insulin analogues. Furthermore we show that the proliferative effect of insulin glargine is mainly mediated by IGF-1 receptor. This is indicated by analysis using a siRNA approach which allowed the reduction of IGF-1 receptor expression by 95 % and thus reduced the stimulation of Akt and DNA synthesis by insulin glargine to a level identical to that of insulin.

In summary, the work presented here covers several aspects of the development and therapy of type 2 diabetes. An important issue is the finding that an overactivated endocannabinoid system, which is known in conditions like obesity and type 2 diabetes, plays a role in the development of skeletal muscle insulin resistance. Furthermore it could be shown that skeletal muscle insulin resistance caused by

secretion products of adipocytes is only partial reversible and that muscle cells display longer lasting damages. The final part of the work demonstrates the dependence of the growth-promoting activity of insulin and insulin analogues on the protein level of the insulin and IGF-1 signalling pathway. Since the enhanced growth-promoting activity of insulin glargine is exclusively mediated by IGF-1 receptors further studies on a potential safety risk of these molecules are urgently required.

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Chapter 1

General Introduction

1.1 Type 2 diabetes and insulin resistance

1.1.1 Pathophysiology of type 2 diabetes

Diabetes mellitus describes a group of metabolic diseases which are characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. The vast majority of cases of diabetes falls into two categories, type 1 or type 2 diabetes. The cause for type 1 diabetes is an absolute deficiency of insulin. This form of diabetes, accounting for only 5-10 % of patients with diabetes, results from a cellular-mediated immune destruction of the pancreatic β-cell which commonly occurs in childhood or adolescence, but can occur in any age since the rate of β-cells destruction is quite variable [1]. Therefore, patients require insulin therapy for survival. The much more prevalent type 2 diabetes (90-95 % of patients with diabetes) is caused by a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. An elevated level of blood glucose, which is sufficient to cause functional and pathological changes in various tissues but without clinical symptoms, may be present for a long period before diabetes is diagnosed. During this prediabetic period, abnormalities in carbohydrate metabolism can be detected by measurement of plasma glucose in the fasting state or after an oral glucose load. Individuals with type 2 diabetes have a relative (rather than absolute) insulin deficiency, and at least initially do not require

insulin treatment to survive. In most cases it is possible to achieve adequate glycaemic control with weight reduction, exercise and/or oral glucose lowering drugs [1].

The current classification and diagnostic criteria of diabetes are based on guidelines of the American Diabetes Association (ADA) from 1997, and were adopted 1998 from the World Health Organisation (WHO) and 2000 from the German Diabetes Association (DDG). According to these guidelines, diabetes is diagnosed when 1) the fasting plasma glucose (FPG) is ≥ 126 mg/dl (7.0 mmol/l), or 2) symptoms of hyperglycaemia occur and the casual plasma glucose is ≥ 200 mg/dl (11.1 mmol/l), or 3) the 2-hour plasma glucose is ≥ 200 mg/dl (11.1 mmol/l) during an oral glucose tolerance test (OGTT) [2]. An intermediate group of patients, whose glucose levels are although not meeting the criteria for diabetes too high to be considered as normal, is referred to as having "pre-diabetes". They are defined as having fasting plasma glucose levels ≥ 100 mg/dl (5.6 mmol/l) but < 126 mg/dl (7.0 mmol/l) or 2-hour values in the OGTT of ≥ 140 mg/dl (7.8 mmol/l) but < 200 mg/dl (11.1 mmol/l). Impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) are risk factors for future diabetes as well as cardiovascular diseases and are associated with the metabolic syndrome which describes a cluster of several metabolic abnormalities [1]. The criteria defining the metabolic syndrome according to the WHO are 1) abdominal obesity (waist circumference > 88 cm in women and > 102 cm in men), 2) serum triglycerides ≥ 150 mg/dl, 3) high density lipoprotein cholesterol level ≤ 50 mg/dl in women and ≤40 mg/dl in men, 4) blood pressure ≥ 135/88 mm Hg, and 5) fasting blood glucose ≥ 110 mg/dl. In most cases, type 2 diabetic patients display a combination of obesity, dyslipidemia, hypertension and insulin resistance, whereas obesity is the risk factor which is most closely associated with the development of type 2 diabetes. In that way, beside genetic predisposition, the influence of environmental factors like overweight, physical inactivity

and smoking plays an important role in the development of type 2 diabetes.

The prevalence and incidence of diabetes, mainly type 2 diabetes, is dramatically increasing worldwide. According to the Centers for Disease Control and Prevention (CDC), an estimated 20.8 million people in the United States, which are about 7 % of the population, had diabetes in 2005. The number of people with diagnosed diabetes increased from 5.6 million to 15.8 million from 1980 through 2005. The number of adults aged 18-79 with newly diagnosed diabetes almost tripled from 493,000 to 1.4 million in the same time period [3]. The latest ADA report estimates that today approximately 17.5 million people in the U.S. have diagnosed diabetes and additionally 6.6 million people live with undiagnosed diabetes. This means over 24 million people in the U.S. suffer from diabetes [4]. The economic burden of this disease and its costs for the society are enormous. In the U.S., the total estimated cost of diabetes in 2007 were 174 billion dollars. According to the ADA, people with diagnosed diabetes, on average, have medical expenditures that are approximately 2.3 times higher than what expenditures would be in the absence of diabetes [4].

In Germany, the total prevalence of diabetes is also raising rapidly. While 6.9 % of the population were treated for diabetes in 2001, the number increased to 7.6 % in 2004, which means ~6.4 million people had diagnosed type 2 diabetes [5]. Furthermore, studies showed a raise of prevalence with age. 4-10 % of adults aged 40-59 are affected by diabetes, while among people age 60 and above, 18-28% have diabetes [5]. Results from the KORA (Cooperative Health Research in the Region of Augsburg) Survey 2000 showed that about 40% of the population aged 55 to 74 years in the Augsburg region had disturbed glucose tolerance or diabetes, whereas half of the total cases with diabetes were undiagnosed [6]. An emerging problem is the increasing number of obese children and adolescents which are diagnosed with type 2 diabetes. A study with 520 subjects aged 9-20

revealed that 6.7 % had an abnormal carbohydrate metabolism, 1.5% had already developed a type 2 diabetes [7]. At present, a few thousands of children and adolescents are affected by type 2 diabetes but a further increase should be expected since the number of overweight and obese children and adolescents is also raising [5,8].

Type 2 diabetes is a heterogeneous disease characterised by chronic hyperglycaemia, which causes microvascular, macrovascular and neuropathic complications at later stages. As outlined above, type 2 diabetic patients often show various comorbidities like hypertension, hyperlipidemia and obesity. For example, 44 % of the average population, but 77 % of type 2 diabetic patients are affected by hypertension [9]. Comorbidities influence each other and affect the same organ systems. Especially the blood vessels of heart, brain and the lower extremities as well as the microvasculature of retina and kidney are affected and raise the risk of cardiac and renal failure, myocardial infarction, stroke, retinopathy and amputation. Therefore, therapeutic interventions for diabetic patients aim to tightly control the blood sugar level to prevent and/or delay the progression of late complications. The “diabetes control and complication trial” [10] and the “United Kingdom prospective diabetes study” [11] have shown that this can be achieved with an intensified insulin therapy which mimics the physiological profile of insulin secretion as good as possible in pre- and postprandial conditions. Various preparations of human insulin and different insulin analogues with specific properties are currently in use, and the development of further improved insulins and new drugs is ongoing.

The most important pathophysiological feature of type 2 diabetes, namely insulin resistance, occurs long before clinical symptoms appear. The development of insulin resistance in peripheral tissues like liver, fat and skeletal muscle is an early event during the progression of type 2 diabetes. Skeletal muscle is one of the major insulin-sensitive organs and accounts for about 80% of insulin-stimulated glucose disposal [12]. Therefore, one important

focus of research in the area of diabetes is to understand the normal insulin signalling cascade in skeletal muscle as well as the multiple mechanisms involved in the disturbance of insulin action and hence the development of skeletal muscle insulin resistance. Based on this knowledge it will be possible to develop new strategies to improve insulin sensitivity and/or prevent insulin resistance.

1.1.2 Insulin signalling in skeletal muscle

The action of insulin is essential to maintain glucose homeostasis and to regulate the metabolism of carbohydrates, lipids, and proteins. Since its discovery, its first use in diabetic therapy and the identification of its structure, insulin and its signalling properties are in the focus of diabetes research.

Insulin is a polypeptide hormone of 5.8 kDa which is secreted by pancreatic β-cells. It is synthesised as preproinsulin with 110 amino acids and processed to proinsulin by cleavage of its signal peptide

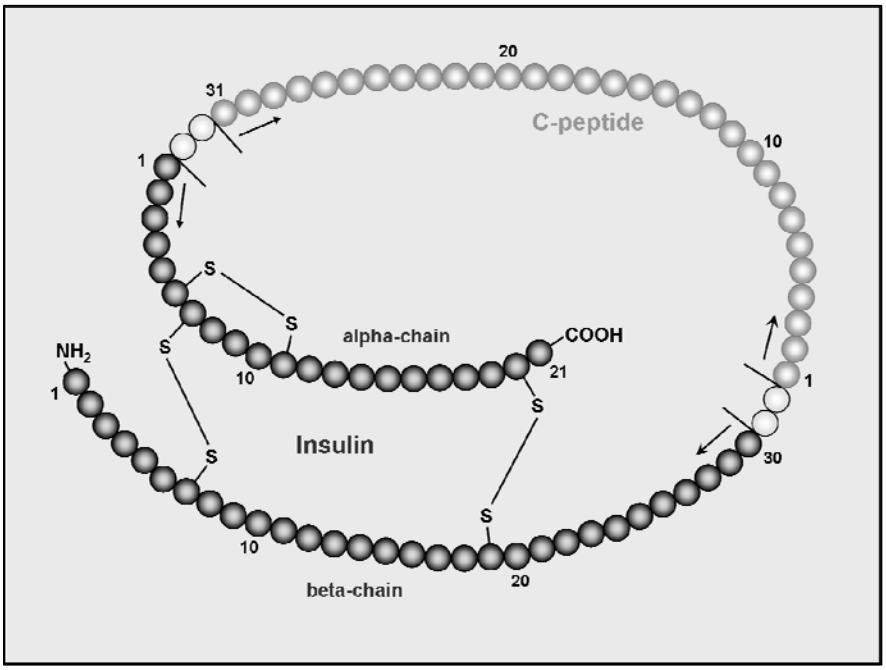

Figure 1.1 Structure of proinsulin. Cleavage of C-Peptide and formation of disulfide bonds at the indicated sites generate the mature insulin molecule.

during insertion into the endoplasmatic reticulum (ER). Here, proinsulin gains its appropriate tertiary structure via folding and the formation of disulfide bonds. The native protein transits through the lumen of the ER and the Golgi cisternae to the trans face of the ER. At the trans Golgi reticulum (TGR), proinsulin is packed into clathrin-coated vesicles which disassociate from the TGR. While these vesicles mature to secretory granules, proinsulin is processed to insulin and C-peptide through cleavages at both junction sites of the connecting segment peptide by prohormone convertases 1/3 and 2 [13,14]. The mature insulin, consisting of α-chain and β-chain, is stored in vesicles and secreted upon stimulation of the β-cells by gut hormones like GLP-1 (incretin effect) and by a postprandial increase of plasma glucose level. Distributed to its target tissues by blood circulation, insulin causes glucose uptake, stimulation of glycogen and fatty acid synthesis, inhibition of gluconeogenesis and lipolysis [15]. Beside its metabolic activity, it also regulates a variety of processes such as RNA and DNA synthesis, cell growth, and differentiation. The effects of insulin are mediated by binding to the insulin receptor (InsR). This receptor is an integral membrane protein and belongs to the receptor tyrosine kinase family. It consists of two disulfide-linked heterodimers, each of them containing an α- and a β-subunit. The α-subunit is entirely extracellular while the β-subunit extends through the plasma membrane. Both are linked by a single disulfide bond. The extracellular domain of the insulin receptor is composed of, from the N-terminus to C-terminus, a leucine-rich repeat domain (L1), a cystein-rich region (CR), a second leucine-rich repeat domain (L2), and three fibronectin type III domains (FnIII-1, FnIII-2, FnIII-3). The second FnIII domain contains a 120 residue insert domain (ID) which includes the furin cleavage site that generates the α- and β-subunit of the mature receptor. The transmembrane region of the β-subunit passes through the membrane and is followed by the juxtamembrane region, the tyrosine kinase domain and the C-tail [16]. The binding sites for insulin are located on the α-subunits. The current model of

insulin binding proposes that each α-subunit contains two different binding sites (Site 1 and Site 2), located on two different regions. Ligand binding to Site 1 involves the central β-sheet of L1, the central modules of CR and the CT peptide. Site 2 comprises the loops at the junction of FnIII-1 and FnIII-2. Binding of insulin to the low-affinity binding site (Site1) on either of the α-subunits is followed by a second binding event between the bound insulin and the second site (Site 2) of the other α-subunit. In this high-affinity state, the insulin molecule bridges Site 1 and 2´ on one side of the receptor dimer or the corresponding sites of pair (Site 1´ and 2). Furthermore, the binding

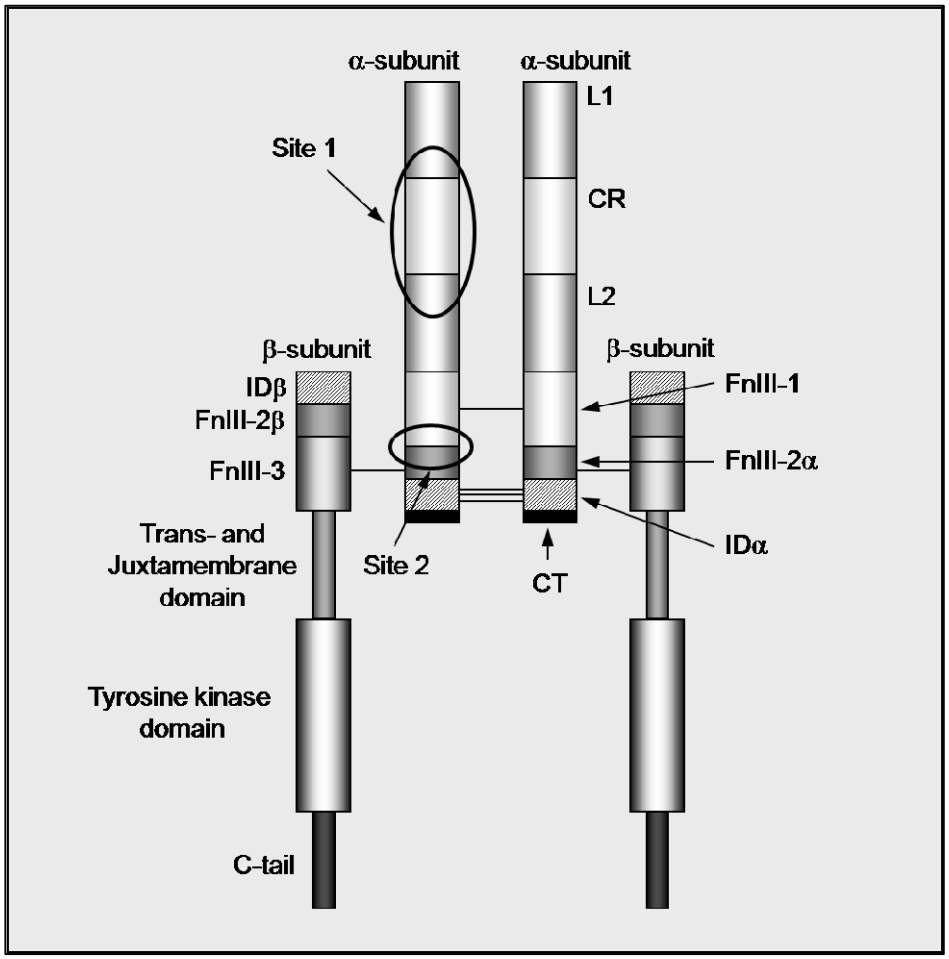

Figure 1.2 Structure of the mature insulin receptor. The receptor consists of two α- and two β-subunits which are connected by disulfide bonds. The proposed binding sites of the ligand are indicated by circles. CR, cystein-rich region; CT, C-terminus; FnIII, fibronectin type III domain; ID, insert domain; L, leucin-rich repeat domain.

sites display negative cooperativity. Two insulin molecules can not bridge both Site 1 - Site 2´ and Site 1´ - Site 2 pairs at the same time [17,18]. Before binding of insulin, the α-subunit inhibits the tyrosine kinase activity of the β-subunit. Insulin binding induces a conformational change, activates the tyrosine kinase and leads to autophosphorylation of several regions of the intracellular β-subunit including Tyr_{1146}, Tyr_{1150} and Tyr_{1151} in the regulatory loop. Autophosphorylation of these three tyrosine residues activates the kinase 10 to 20-fold, mutations in this region progressively reduce insulin-stimulated kinase activity and result in a parallel loss of biological activity. The activated tyrosine kinase directly phosphorylates several substrates on their tyrosine residues including insulin receptor substrate (IRS) proteins 1-4, Cbl, and isoforms of Shc [15]. The phosphorylated tyrosines in these proteins serve as docking sites for proteins that contain Src-homology-2 (SH2) domains. These SH2-proteins are either adapter molecules, like the p85 regulatory subunit of PI(3)K, or they are enzymes themselves, including the phosphotyrosine phosphatase SHP2 and the cytoplasmic tyrosine kinase Fyn. Substrate binding to these SH2-proteins can regulate their activity, or in some cases their subcellular location [15]. Among the IRS proteins, IRS1 and IRS2 are the most important regarding insulin-stimulated GLUT4 translocation. Furthermore, glucose metabolism is mainly regulated through IRS1/2 mediated pathways and further downstream by the mitogen-activated protein kinase (MAPK) and PI(3)K pathways [19].

The PI(3)K plays a crucial role in the metabolic action of insulin such as stimulation of glucose transport by translocation of GLUT4 vesicles to the membrane, and glycogen and lipid synthesis. PI(3)K consists of a catalytic p110 subunit and a p85 regulatory subunit that includes two SH2 domains which interact with tyrosine-phosphorylated motifs in IRS proteins. Activated PI(3)K catalyses the phosphorylation of phosphoinositides to generate PI(3)P, PI(3,4)P2 and $PI(3,4,5)P_3$, which then recruit the phosphoinositide-dependent

serine/threonine kinase 1 (PDK1) and Akt/protein kinase B from the cytoplasm to the membrane. Akt is phosphorylated by the rictor-mTOR complex on Ser473 [20] and by PDK1 on Thr308 [21], which leads to its full activation and regulates the activity of many downstream targets. Among others, Akt phosphorylates components of the GLUT4 translocation complex, protein kinase C (PKC) isoforms, and glycogen synthase kinase 3 (GSK3). Phosphorylation of GSK3 leads to its inactivation and thus activates glycogen synthesis [15].

One important effect of insulin is to stimulate the recruitment of GLUT4 vesicles to the cell surface to enhance glucose uptake. In unstimulated muscle cells, over 90 % of GLUT4 is located intracellularly and only 4–10 % resides at the plasma membrane, the result of slow exocytosis and fast endocytosis. Insulin stimulation causes a net gain in surface GLUT4 that peaks within 10–15 min based on an increase of GLUT4 exocytosis rate and a smaller reduction in its endocytosis. The fusion of GLUT4 vesicles with target membranes is mediated by specific SNARE proteins: the v-SNARE protein vesicle associated membrane protein 2 (VAMP2) which is localized at the GLUT4 vesicle, and the t-SNARE proteins syntaxin 4 and synaptosome-associated 23-kDa protein (SNAP23) which are concentrated on the plasma membrane. Interaction of the SNARE complex leads to the fusion of vesicles and plasma membrane [22].

1.1.3 Insulin resistance in skeletal muscle

Insulin resistance is an early defect in the pathogenesis of type 2 diabetes and describes the inability of insulin to correctly stimulate insulin signalling. Type 2 diabetic patients and glucose-tolerant first-degree relatives have been reported to display defects in glucose metabolism [23-25].

The insulin resistant muscle exhibits several defects in the insulin signalling cascade, including reduced insulin-stimulated tyrosine phosphorylation of InsR, IRS-1, and activation of PI(3)K. At

the level of the receptor, InsR phosphorylation is reduced [26] or unchanged [27] in skeletal muscle from non-obese type 2 diabetic patients compared to non-diabetic subjects. Furthermore, the InsR phosphorylation is impaired in skeletal muscle from morbidly obese subjects [28] or women with gestational diabetes mellitus [29]. Skeletal muscle from insulin-resistant subjects show various post-receptor defects such as reduced IRS-1 phosphorylation and PI(3)K activity [27,30,31]. Controversial data have been reported regarding defective Akt activation in the muscle of type 2 diabetic patients. Some studies reported significant reductions of insulin-stimulated Akt phosphorylation [32,33], others observed no differences in phosphorylation or enzymatic activity of Akt between controls and type 2 diabetic patients [30,34]. A possible explanation may be the fact that three isoforms of Akt exist, each with isoform-specific functions as revealed by knockout studies. Recently, it has been reported that the activities of Akt2 and Akt3, but not Akt1, are decreased in skeletal muscle biopsies from insulin-resistant morbidly obese participants [35]. In myotubes from type 2 diabetic patients isoform-specific alterations of Akt phosphorylation occur in response to insulin, with decreased Ser473 phosphorylation on Akt2 and decreased Thr308 phosphorylation on Akt1 [36]. Defective atypical PKC activation also contributes to skeletal muscle insulin resistance in subjects with impaired glucose tolerance and type 2 diabetes [37,38]. Finally, GLUT4 translocation to the plasma membrane is impaired in muscle of diabetic patients [39,40]. In type 2 diabetic patients, glucose transport defects are not related to changes in GLUT4 expression level [27,41]. However, for obese patients a decreased level of GLUT4 was observed [42]. The general impairment of GLUT4 trafficking in insulin resistance was shown by studies using other stimuli than insulin such as hypoxia. Here, the hypoxia-induced (insulin-independent) increase in cell surface GLUT4 content and glucose transport was severely blunted in skeletal muscle of type 2 diabetic patients [40].

Besides data from clinical studies, various knockout animal and cell models were used to study possible mechanisms involved in the development of insulin resistance. Whole body InsR knockout causes early neonatal death in mice [43], while tissue-specific InsR knockout has diverse effects on whole body or specific tissue insulin sensitivity. The muscle-specific InsR knockout mice (MIRKO) have normal glucose homeostasis despite insulin resistance in muscle but display elevated fat mass, serum triglycerides, and free fatty acids, indicating an altered fat metabolism which is associated with type 2 diabetes [44]. Knockout of InsR in pancreatic β-cells results in a selective loss of insulin secretion in response to glucose and a progressive impairment of glucose tolerance [45]. Loss of insulin signalling due to liver-specific InsR knockout (LIRKO) results in insulin resistance, severe glucose intolerance, and a failure of insulin to suppress hepatic glucose production and to regulate hepatic gene expression [46]. On the other hand, fat-specific disruption of the InsR gene (FIRKO) protects mice against obesity and obesity-related glucose intolerance, and increases longevity [47,48].

Deletion of IRS-1 results in insulin resistance and growth retardation, but no diabetes is developed because insulin secretion increases to compensate for the mild resistance to insulin. IRS-2 knockout impairs both peripheral insulin signalling and pancreatic β-cell function. IRS-2-deficient mice show progressive deterioration of glucose homeostasis because of insulin resistance in liver and skeletal muscle and a lack of β-cell compensation for this insulin resistance [49]. Regarding the role of Akt, isoform-specific knockout mice were generated to help understand the role of each isoform in normal physiology. Akt2-deficient mice display hyperglycaemia and an impairment of insulin action in liver and skeletal muscle. Hence, Akt2 is considered to be essential for the maintenance of glucose homeostasis and the control of insulin metabolic actions [50]. Finally, complete or tissue-specific (muscle or fat) GLUT4 deficiency also leads to insulin resistance. Heterozygous GLUT4 knockout mice show

moderate glucose intolerance, while homozygous whole-body GLUT4 knockout (GLUT4-null) mice have only mild perturbations in glucose homeostasis. Muscle-specific ablation of GLUT4 results in a profound reduction of basal glucose transport, severe insulin resistance and glucose intolerance [51]. Mice with adipose tissue-selective deletion of GLUT4 display glucose intolerance and hyperinsulinaemia, and develop insulin resistance in muscle and liver [52].

Despite these detailed insights into the function of various components of the insulin signalling cascade, the complete molecular pathways leading to insulin resistance and further development of type 2 diabetes are far from being understood.

1.2 The endocannabinoid system - involvement in the regulation of metabolism

1.2.1 The endocannabinoid system - overview

The endocannabinoid system is a complex network involved in various physiological processes. Its discovery was prompted by studies with tetrahydrocannabinol (THC), the psychoactive compound of Cannabis sativa, which led to the characterisation [53,54] and molecular cloning of the G protein-coupled cannabinoid receptors type 1 (CB1R) and 2 (CB2R) [55]. Further research demonstrated the existence of endogenous ligands, the so called endocannabinoids, as

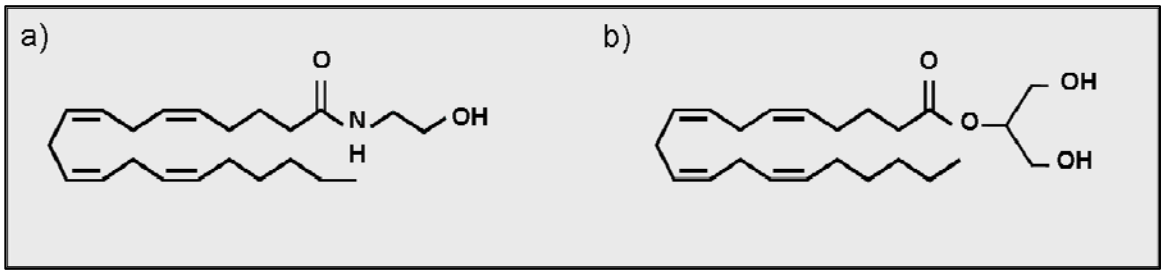

Figure 1.3 Structure of AEA (a) and 2-AG (b)

well as the complex enzymatic machinery for their synthesis, release, transport and degradation. The endocannabinoids are derivatives of arachidonic acid which are conjugated with ethanolamine or glycerol. Arachidonoylethanolamide (AEA, anandamide) and 2-arachidonoylglycerol (2-AG) are the most intensively studied compounds among the endocannabinoids, and different pathways are involved in their synthesis and release.

The formation of AEA is carried out by a specific phospholipase D (PLD) which cleaves *N*-arachidonoyl-phosphatidylethanolamine (NAPE), the precursor of AEA. The Ca^{2+} dependent biosynthesis of NAPE is processed by the enzyme *N*-acyltransferase (NAT), its activity being enhanced by phosphorylation through the cAMP-dependent activity of protein kinase A [56,57]. The activity of PLD is regulated by depolarization or by activation of G-protein coupled receptors like ionotropic glutamate *N*-methyl-*D*-aspartate (NMDA) receptors or metabotropic receptors of major neurotransmitters including dopamine, glutamate and acetylcholine. 2-AG formation is also Ca^{2+} dependent, but it is synthesised via a different pathway. The activation of phosphatidylinositol-specific phospholipase C (PLC) generates diacylglycerol by cleavage of membrane phospholipids,

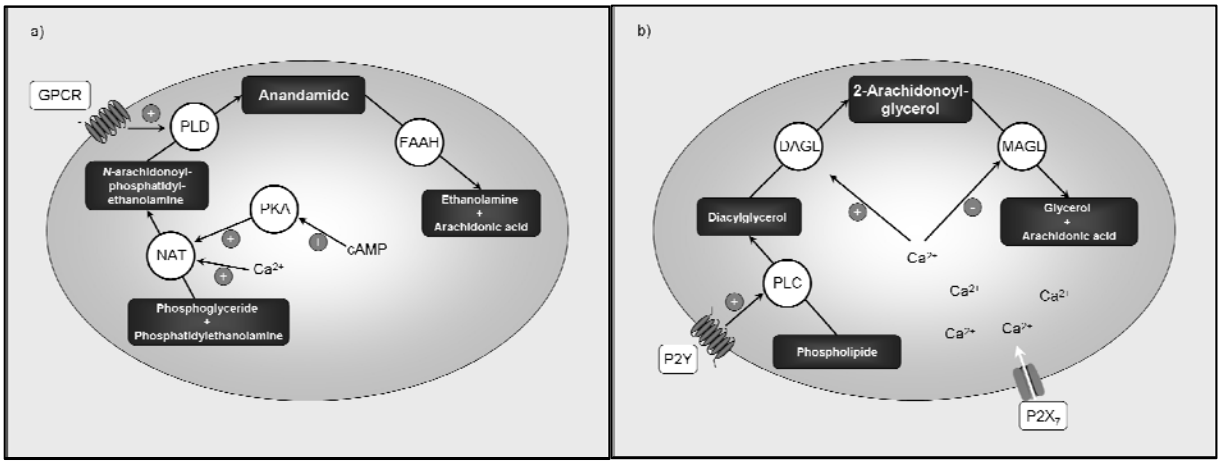

Figure 1.4 Schematic overview of the main synthesis pathway of anandamide (a) and 2-arachidonoylglycerol (b). DAGL, diacylglycerol lipase; FAAH, fatty acid amide hydrolase; GPCR, G-protein coupled receptor; MAGL, monoacylglycerid lipase; NAT, N-acyltransferase; PKA, protein kinase A; PLC, phospholipase C; PLD, phospholipase D; P2Y/P2X, purinergic receptors.

which is further processed to 2-AG by diacylglycerol lipase (DAGL). The activation of metabotropic P2Y purinergic receptors coupled to the PLC and DAGL pathway systematically increases the production of 2-AG [57,58], whereby the contribution of ionotropic purinergic receptors like P2XT boosts 2-AG formation. These receptors, when activated, allow large quantities of extracellular calcium to enter the cells and thus enhancing the activity of DAGL while inhibiting the 2-AG degrading enzyme monoacylglycerol lipase (MAGL) [59].

After synthesis, the endocannabinoids are released in the extracellular space and act as retrograde messengers at presynaptic CB1R (e.g. regulation of neurotransmitter release) or target the receptors in an autocrine/paracrine manner [60]. The signals induced by the endocannabinoids are terminated very rapidly by a transporter-mediated uptake [61] and subsequent degradation. The intracellular hydrolysis of AEA to arachidonic acid and ethanolamine is catalysed by the integral membrane enzyme fatty acid amide hydrolase (FAAH) [62]. 2-AG can also be inactivated by FAAH, but it is mainly hydrolysed by monoacylglycerid lipase (MAGL) to glycerol and arachidonic acid [63].

The endocannabinoids exert their action by binding to specific receptors. Beside the two major cannabinoid receptors, CB1R and CB2R, pharmacological studies have revealed the existence of other endocannabinoid targets including the transient receptor potential vanilloid type 1 (TRPV1) [64] and at least two non-CB1, non-CB2 cannabinoid receptors [60,65]. The molecular characterisation of CB1R and CB2R have shown that they are members of the superfamily of seven-transmembrane-spanning (7-TM) receptors, which have a protein structure defined by an array of seven membrane-spanning helices with intervening intracellular loops and a C-terminal domain that can associate with G proteins. Cannabinoid receptors are associated with G proteins of the Gi/o family and signal transduction via Gi inhibits adenylate cyclase in most tissues and cells [66]. Therefore, activation of CB1R and CB2R leads to inhibition

of cAMP production, resulting in a decrease in protein kinase A-dependent phosphorylation processes as well. Furthermore, these receptors regulate the activity of ion channels, resulting in inhibition of calcium influx through N, P/Q and L type calcium channels, and activation of inwardly rectifying potassium channels [65]. Stimulation of CB1R and CB2R leads to phosphorylation and activation of p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK and c-Jun N-terminal kinase (JNK) as signalling pathways to regulate nuclear transcription factors [66,67]. They have also been shown to be coupled to the phosphatidylinositol 3-kinase pathway, to the focal adhesion kinase, to ceramide signalling and to nitric oxide (NO) production [60].

The endocannabinoids exhibit different binding properties and intrinsic activity at CB1R and CB2R. AEA is a partial agonist at both receptors, but has a higher affinity for the CB1R. Its intrinsic activity at CB1R is 3-40 fold higher than at CB2R [60,65]. 2-AG acts as a complete agonist at CB1R and CB2R, and exhibits higher relative intrinsic activity than AEA at both receptors. Like AEA, 2-AG has marginally higher affinity for CB1R than CB2R [65].

The expression of CB1R mRNA has been demonstrated in central and peripheral nerve tissue (mainly located in terminals of neurons and glial cells) [53,57,68,69] as well as in various peripheral tissues like fat [70-74], liver [75], endocrine pancreas [76-78], skeletal muscle [79], in the reproductive system [80], some glandular systems and in the microcirculation [60,81]. The CB2R was initially described in immune cells and multiple lymphoid organs. Meanwhile it is known that the CB2R is also present in non-immune cells such as skeletal muscle cells [79], endocrine pancreas [77], adipose tissue [74], and bone [82].

1.2.2 Dysregulation of the endocannabinoid system in obesity and hyperglycaemia

In recent time is has become clear that the endocannabinoid system plays an important role for the control of energy homeostasis and body weight at both the level of food intake as well as peripheral control of metabolism. This is based on studies in $CB1R^{-/-}$ mice and/or in models of pharmacological blockade of CB1R showing reduced food intake and body weight to a higher extent than predicted from the inhibitory effect on energy intake [71,83]. Several studies in animal models as well as in humans have supplied evidence that in conditions of obesity and hyperglycaemia the endocannabinoid system is overactivated. This overactivity occurs in the hypothalamus as well as in peripheral tissues (liver, pancreas, adipose tissue) and involves altered levels of endocannabinoids and/or CB1R expression.

In patients, who are obese or hyperglycaemic due to type 2 diabetes, circulating levels of AEA and 2-AG are increased and elevated levels of 2-AG are found in visceral adipose tissue [84-86]. Genetically and diet-induced obese animal models show elevated levels of endocannabinoids in hypothalamus, adipose tissue, liver and endocrine pancreas [75,84,87]. Additionally, the endocannabinoid level might be influenced by the fatty acid composition of the food due to availability of biosynthetic precursors [88-91]. Very recently, Matias et al. [92] showed that the level of endocannabinoids during high-fat diet changes tissue-specifically and depending on the composition and duration of the diet.

Several studies revealed, that the expression of CB1R mRNA is elevated in adipose tissue [70], skeletal muscle [93] and liver [75] of diet-induced obese rodents. In humans, controversial data regarding CB1R expression in obese vs. lean patients exist. While Pagano et al. [73] found an increased expression of CB1R mRNA in visceral fat

tissue of obese patients, other working groups [85,86,94] reported decreased levels in visceral and subcutaneous fat tissue compared to lean patients. Another study carried out by Lofgren et al. [95] did not find any association between subcutaneous or visceral adipose CB1R mRNA level and body weight. Additionally, the mRNA expression of FAAH, the enzyme responsible for degradation of AEA, was shown to be decreased in adipose tissue of obese humans [85,86,94]. In mice on high-fat diet the hepatic FAAH activity was reduced more than 5fold with apparently unchanged AEA synthesis [75].

In summary, these data provide evidence that in condition of obesity and hyperglycaemia, the endocannabinoid system is upregulated. Its involvement in the regulation of food intake and energy balance makes it a promising target for pharmacological strategies in the treatment of obesity. The development and characterisation of the first specific CB1R antagonist SR141716 (rimonabant) by Sanofi [96,97] as well as the generation of CB1R knock out mice strains [98-100] allowed detailed investigations of the interplay between the endocannabinoid system and metabolism.

1.2.3 Antagonising the endocannabinoid system as therapeutical approach

Feeding studies with $CB1R^{-/-}$ mice revealed several interesting observations. On standard diet, these mice stay lean and have a lower body weight and adiposity compared to wild type controls at the age of 20 weeks, while plasma insulin and leptin levels are reduced. On high-fat diet, $CB1R^{-/-}$ mice are resistant to diet-induced obesity and do not display hyperphagia or develop insulin resistance [101].

Animal models of diet-induced obesity are used to analyse the effect of blocking CB1R in terms of several metabolic parameters. Treatment with rimonabant induces a transient reduction of food intake, a marked but sustained reduction of body weight and

adiposity, it corrects insulin resistance and lowers plasma level of leptin, insulin, and free fatty acid [83]. Additionally, rimonabant treatment increases serum adiponectin levels, significantly reduces triglycerides and low-density lipoprotein cholesterol (LDLc), and increases the HDLc/LDLc ratio [102].

The safety and efficiency of rimonabant to treat obesity and associated disorders in humans were investigated in 4 clinical studies (RIO-Lipids, RIO-North America, RIO-Europe and RIO-Diabetes; RIO = rimonabant in obesity). The results show that rimonabant treatment significantly reduces body weight and improves multiple cardiometabolic risk factors such as waist circumference, HbA1c, adiponectin, HDL cholesterol, and triglycerides [103-107] in overweight or obese non-diabetic and diabetic patients.

The data gained in animal and human studies clearly indicate that the effects of rimonabant are not only based on weight loss alone, and that peripheral effects contribute to the overall improvement of metabolic parameters following CB1R blockade. Understanding the role of the endocannabinoid system in the progression of obesity, insulin resistance and type 2 diabetes is an important requirement for the development of future therapeutic options. Thus, current research in this field addresses the question which consequence CB1R activation or CB1R blockage has for the metabolism of peripheral cell types and tissue like liver, adipose tissue, pancreas and skeletal muscle. Therefore, one focus of this work was to investigate the role of the endocannabinoid system in the induction of insulin resistance in human skeletal muscle.

1.3 The prediabetic state - intervention by lifestyle modifications

1.3.1 Therapeutic potential of lifestyle modifications

Once an elevated blood glucose level is detected and prediabetes or type 2 diabetes is diagnosed, the overall aim of therapy is to achieve normoglycaemia and thereby reducing the overall incidence of diabetic complications, such as cardiovascular, renal, and retinal disease, and the excess mortality associated with these complications. Studies have shown that lifestyle modifications are an effective way to nearly normalise the impaired glucose metabolism and prevent or delay the development of type 2 diabetes [108-111].

Lifestyle intervention therapies comprise weight management, dietary modification and increase of physical activity. Each strategy alone or in combination improves several metabolic parameters. It has been shown that caloric restriction and weight loss improved glycaemic control and insulin sensitivity [112-114]. Furthermore, weight reduction increased high molecular weight adiponectin level [115] and decreased MCP-1, IL-6, IL-18 and TNFα level [116-119]. Results from the Finish diabetes prevention study indicate that increasing physical activity may substantially reduce the incidence of type 2 diabetes in high-risk individuals [120]. Toledo et al. showed that combined intervention of weight loss and physical activity in previously sedentary obese adults is associated with enlargement of mitochondria and an increase in the mitochondrial content in skeletal muscle [121]. Additionally, the obesity associated decrease in the ability of skeletal muscle to oxidise lipids is ameliorated by exercise training [122].

1.3.2 Diet and pharmacological therapy

Weight reduction can be achieved by different ways such as diet therapy, pharmacotherapy and/or surgical treatment. A variety of diet plans for weight loss are available, including very-low-calorie or low-calorie diets, low-fat or very-low-fat diets, moderate-fat/low-calorie diets, or low-carbohydrate/high-protein diets. For example, a low-calorie diet (1000-1500 kcal/d) provides approximately 8 % loss of initial body weight at 4 to 6 months. A very low-calorie diet (< 800 kcal/d) results in 15 % to 20 % loss of initial body weight at 4 months, mediating improved glycaemic control. However, after 1 year a very low-calorie diet provides an equal amount of weight loss compared with a low-calorie diet, and the significance of improved glycaemic control is lost because of poor compliance and subsequent weight regain [123].

In pharmacotherapy, there are only two FDA-approved drugs available for the long-term treatment of obesity, orlistat and sibutramine hydrochloride. Orlistat, first approved in 1998, is a gastric and pancreatic lipase inhibitor that reduces dietary fat absorption by around 30 %. It reduces weight by around 3 kg on average and decreases progression to diabetes in high-risk patients. The major adverse effects observed under therapy are gastrointestinal. Sibutramine is a centrally acting monamine-reuptake inhibitor that mainly acts to increase satiety. It was approved in the USA in 1997 and in the European Union in 1999. Its use results in mean weight losses of 4-5 kg, but it is associated with increases in blood pressure and pulse rate. Common side-effects include insomnia, nausea, dry mouth, and constipation [124]. Rimonabant, the first CB1R antagonist, was approved in 2006 by the EMEA, while the FDA denied the approval due to potential psychiatric side-effects. Compared with placebo, rimonabant produced a 4.9 kg greater reduction in body weight in trials with one-year results. Improvements in waist circumference, high-density

lipoprotein cholesterol, triglyceride levels and systolic and diastolic blood pressure were also reported [125]. The most frequent adverse events are nausea, dizziness, diarrhoea, insomnia and psychiatric disorders (mainly depression) [124].

1.3.3 Bariatric surgery as option to treat morbid obesity

Despite recent scientific advances, no currently recommended dietary program or medication results in long-term weight loss of more than 10% of body weight for the vast majority of people who attempt these interventions [126]. Therefore, surgical intervention is an important weight loss option for patients with a BMI above 40 kg/m^2. Bariatric surgery is highly effective in obtaining weight reduction in up to 60% of the excess weight [127]. With respect to durable weight reduction, bariatric surgery is the most effective long-term treatment for obesity with the greatest chances for amelioration and even resolution of obesity-associated complications.

Several techniques are available today which reduce stomach size and/or cause malabsorption. A common procedure to reduce stomach size is the placement of an adjustable gastric band resulting in the creation of a small pouch at the top of the stomach. Another widespread technique is gastric bypass surgery, which first divides the stomach into a small upper pouch and a much larger, lower "remnant" pouch and then re-arranges the small intestine to allow both pouches to stay connected to it. Several different ways to reconnect stomach and intestine are used, and the proximal Roux-en-Y gastric bypass (RYGB) and the biliopancreatic diversion (BPD) are the most commonly employed gastric bypass techniques. The RYGB consists of a small restrictive gastric reservoir associated with early satiety, coupled with a Rouxen-Y loop to provide an element of malabsorption. Excess weight loss greater than or equal to 50% is maintained in approximately 85% of the patients at 2 years and 60%

at 5 years. The BPD confers a large component of malabsorption with only the distal 50 cm of common channel as an area for absorption of nutrients. The BPD is the most effective bariatric procedure to date resulting in a mean excess body weight loss of approximately 80% which is maintained [123].

Recent evidence shows that bariatric surgery for severe obesity is associated with decreased overall mortality [128,129]. Weight loss induced by surgery has been shown to improve cardiovascular risk factors, cardiac structure and function, and the clinical course of established cardiovascular disease [130]. As an additional benefit of surgical treatment for morbid obesity, resolution of type 2 diabetes has been observed. In these subjects glycaemic control often occurs long before a significant weight loss is observed [131,132]. The Swedish Obese Subjects study showed that patients treated with a surgical procedure for obesity experienced 47 % resolution of diabetes in comparison to 17 % in the conservatively treated, matched-control group. The 2-year incidence of type 2 diabetes was reduced 30fold [123,133]. Guidone et al. reported that very early after the operation (1 and 4 weeks) the patients displayed complete reversion of type 2 diabetes with full normalisation of the insulin-mediated whole-body glucose uptake, net improvement of the β-cell glucose sensitivity, significant decrease of GIP plasma concentration and increase of GLP-1 plasma levels as well as significant reduction of the circulating levels of leptin [134]. The exact mechanism of the dramatic effect of surgical procedures for obesity on NIDDM remains unknown. The operation may affect the enteroinsular axis by diverting nutrients away from the proximal gastrointestinal tract and by delivering incompletely digested nutrients to the ileum. This, in turn, enhances the secretion of GLP-1 in the transposed ileum, while the exclusion of the duodenum and jejunum might be responsible for the downregulation of GIP and of other gut hormones involved in insulin sensitivity regulation [134].

Taken together, the results from several lifestyle intervention studies showed that insulin resistance is a reversible process which can be handled without additional anti-diabetic pharmacological treatment. One important question is how pronounced the reversibility occurs at the level of skeletal muscle. Pender et al. analysed cultured human skeletal muscle cells obtained from obese nondiabetic patients, and showed that insulin-stimulated glucose transport and downstream insulin receptor signalling events were not different compared to non-obese controls [135]. This indicates that insulin resistance might be a reversible feature that can be acquired with obesity. However, cultured human skeletal muscle cells obtained from obese type 2 diabetic patients displayed defective insulin-stimulated glucose transport, demonstrating that insulin resistance was retained in culture [136,137]. Hence, using our model of adipocyte-induced insulin resistance in human skeletal muscle cells, the reversibility of insulin resistance and underlying mechanisms were investigated to gain a more detailed view on these processes.

1.4 Pharmacotherapy of type 2 diabetes

Despite the potential of lifestyle modification, it is often necessary to support the adjustment of blood glucose level by pharmacological intervention. The stepwise therapy usually starts with oral anti-diabetic drugs which have different action properties. Since the ability of pancreatic β-cells to produce insulin continually decreases until it completely stops, therapy with oral anti-diabetics is effective only for a limited period and finally insulin therapy is required for survival.

1.4.1 Oral anti-diabetic drugs

One of the most widely used substances is the biguanide metformin. It inhibits the hepatic glucose production, increases tissue insulin sensitivity, and has beneficial effects on lipid profile and several cardiovascular risk factors [138]. In the UKPDS, overweight and obese patients randomised to initial monotherapy with metformin experienced significant reductions in myocardial infarction and diabetes-related deaths [139]. One important feature of metformin is the decrease of blood glucose level without causing overt hypoglycaemia; additionally it does not promote weight gain. Metformin does not stimulate insulin secretion and therefore the clinical efficiency in patients with type 2 diabetes requires the presence of insulin. The mechanism of action involves indirect activation of AMPK, which may be due to inhibiting complex I of the respiratory chain and thus increasing the cellular AMP:ATP ratio [140-142]. Furthermore, it was shown that metformin protects pancreatic beta cells from lipotoxicity [143,144].

The second group of insulin sensitisers are the thiazolidinediones (e.g. rosiglitazone, pioglitazone), which improve whole body insulin sensitivity by multiple actions on gene regulation. They act as agonists of the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ), thereby modulating the expression of a range of insulin-sensitive genes. Stimulation of PPARγ promotes differentiation of pre-adipocytes, lipogenesis and fatty acid uptake in adipose tissue, increases glucose-uptake via GLUT4 in skeletal muscle and reduces the rate of gluconeogenesis in the liver. Furthermore, thiazolidinediones reduce the production and activity of TNFα [145]. Like metformin, thiazolidinediones are anti-hyperglycaemic drugs and require the presence of insulin to generate a blood glucose lowering effect. Thiazolidinediones have a site-specific effect on the differentiation of pre-adipocytes, which is markedly

enhanced in subcutaneous fat and may be due to the higher expression of PPARγ in the subcutaneous compared to visceral fat. Several studies have demonstrated that the weight gain observed in therapy with thiazolidinediones is associated with a fat redistribution, e.g. an increase in subcutaneous adipose tissue and concomitant decrease in visceral fat content, which is related to improvements of hepatic and peripheral tissue sensitivity to insulin [146-148].

Another group of compounds used in diabetes therapy are substances which stimulate the secretion of insulin. Sulfonylureas like glibenclamide, and glinide such as epaglinide and nateglinide, occupy a specific site on ATP-sensitive Kir6.2 potassium channels leading to its closure, depolarisation of the membrane and subsequent opening of calcium channels, which results in exocytosis of insulin. Thereby glinides bind to a site distinct from the sulfonylurea binding site. Sulfonylureas can cause hypoglycaemia since release of insulin is initiated even when glucose concentrations are below the normal threshold for glucose-stimulated insulin secretion. Some concerns about the use of sulfonylureas as first-line treatment of type 2 diabetes arise from the hypothesis that hypersecretion may contribute to the progressive destruction of the β-cell [149]. This is supported by data from the "A Diabetes Outcome Progression Trial" (ADOPT) study [150], showing that at 5 years glibenclamide treatment resulted in a 34 % frequency of failure of monotherapy treatment compared to 21 % and 15 % for the metformin and rosiglitazone-treated group, respectively. Furthermore, Maedler et al. showed *in vitro* that chronic treatment of β-cells with sulfonylureas can cause apoptosis [151].

A more recently developed therapeutic approach to treat type 2 diabetes uses the so-called incretin effect, which is mediated by glucose-dependent insulinotropic peptide (GIP) and glucagon like peptide-1 (GLP-1). Incretin mimetics like exenatide or liraglutide were developed, which bind to the pancreatic GLP-1 receptor. Unlike GLP-1, exenatide is not a substrate to dipeptidyl peptidase-4 (DPP-4) and

has a prolonged half-life compared to endogenous GLP-1. Exenatide has been shown to improve glycaemic control by several mechanisms. It stimulates glucose-dependent insulin secretion, promotes synthesis of proinsulin via cAMP and proteinkinase A, suppresses glucagon secretion, delays gastric emptying, and reduces food intake. Additionally, it has been shown to increase β-cell mass by stimulating neogenesis and proliferation of β-cells, and by suppressing apoptosis [152,153]. Alternatively, DPP-4 inhibitors like sitagliptin or vildagliptin were developed to increase the active levels of incretin hormones in the body. Similar to GLP-1 analogues, glycaemic control is improved by stimulating pancreatic insulin secretion and suppressing glucagon production, while no weight gain is observed [154-156].

1.4.2 Insulins

Type 2 diabetic patients usually require insulin at a later stage, after secondary failure to oral antidiabetic drugs. For therapy, numerous insulin preparations with certain properties are available today. While insulin extracted from porcine or bovine pancreas lost its importance in the last years, human insulin produced by microorganisms as well as genetically engineered insulin analogues are widely used in therapy today.

Regular human insulin has an onset of action half to one hour after subcutaneous injection, reaches a peak after 2 to 6 hours and has a duration time of up to 8 hours. It has a strong affinity for self-association and thus, the hexameric form is the most prevalent form in a solution of regular human insulin. To be absorbed, the hexamer must dissociate to a dimer and further to a monomer. The monomeric form can then be absorbed into the capillary system surrounding the subcutaneous reservoir. The rate-limiting step in the absorption of regular insulin is dissociation to the monomeric form [157]. The

pharmacokinetic and pharmacodynamic profile presents several problems for the patient. The insulin injection must be carefully timed to 30 minutes or more before a meal. Hypoglycaemia between meals can occur, because in many cases and depending on the dose it may peak for 4 to 6 hours after the injection, when glucose levels may already be low.

To delay the onset of action, insulin can be coupled to zinc (= lente insulins) or protamine (= NPH insulin), resulting in intermediate/long-acting human insulins. These insulins reach peak concentrations 4 to 10 hours after injection and the duration of action is 10 to 20 hours. When used as basal insulins, two major problems occur: they have a definite peak effect, and their duration of action make more than one daily injection necessary [157]. Thus, available intermediate/long-acting human insulin preparations are unable to provide a stable continuous baseline insulin level and furthermore, they display considerable inter- and intrasubject variations in their bioavailability [158].

Molecular genetic techniques have allowed the design and development of new drugs based on the molecular structure of human insulin with altered amino acid sequence or other modifications. These insulin analogues show various improvements compared to conventional insulin preparations due to their modified action profile and are grouped in short-acting and long-acting insulins. The advantages of short-acting preparations compared to conventional insulin preparations are faster onset of action and short duration time. Long-acting insulin analogues possess structural changes which delay the onset of action, allowing slow and continuous absorption into the systemic circulation and elongating the duration. This results in a time-concentration profile which closely resembles the normal insulin basal level and leads to physiological basal glycaemic control with less nocturnal hypoglycaemia compared to conventional insulin preparations.

Insulin lispro (=Humalog®) has been the first genetically engineered rapid-acting insulin analogue and was approved for clinical use in 1996. Its structure differs from human insulin only in the B-chain where proline at position 28 and lysine at position 29 are reversed. Exchange of these amino acids results in an insulin molecule with a greatly reduced capacity for self-association in solution. The effect is a faster absorption, higher peak serum level and shorter duration of action when compared with regular human insulin [159]. Insulin lispro acts within 15 minutes, reaches a peak after 1 hour and declines within 2 to 4 hours after subcutaneous injection [159,160]. It has been studied in comparison to regular human insulin in numerous clinical trails and major improvements in postprandial glucose levels with a lower rate of hypoglycaemic events have been achieved [161-165].

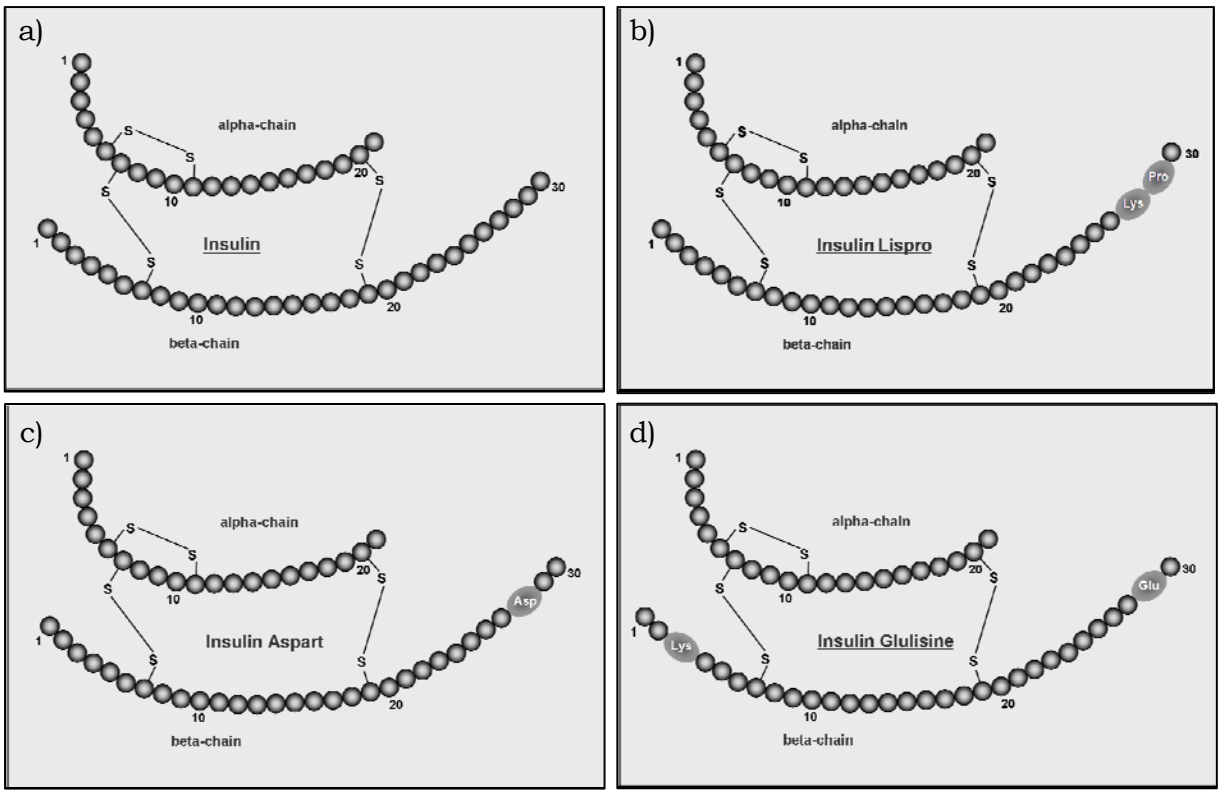

Figure 1.5 Structure of human insulin (a) and the rapid-acting insulin analogues insulin lispro (b), insulin aspart (c), and insulin glulisine (d)

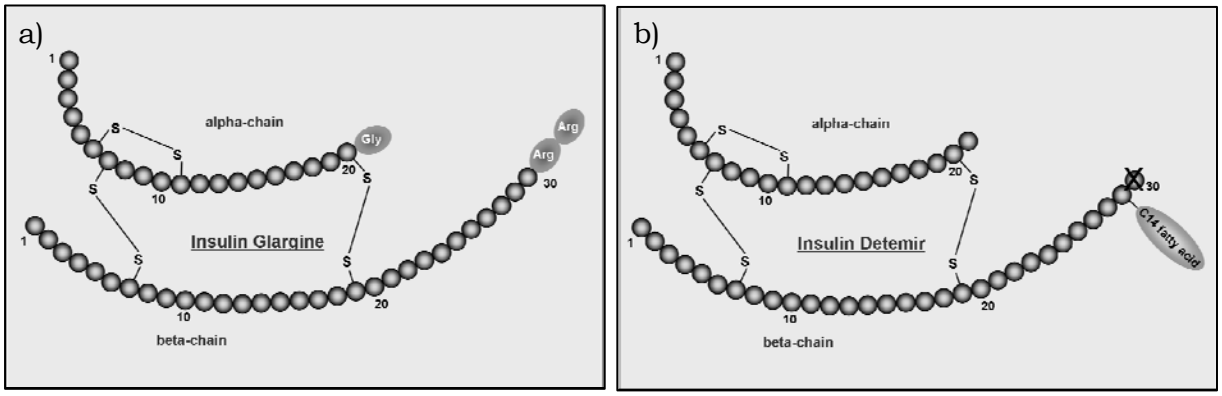

Figure 1.6 Structure of the long-acting insulin analogues insulin glargine (a) and insulin detemir (b)

Insulin aspart (=NovoRapid®) is structurally similar to human insulin except the substitution of proline at position 28 in the B-chain with the charged aspartic acid. Thereby the tendency of self-association of the molecule is reduced and it can be absorbed twice as fast as human insulin [166]. Insulin aspart has a shorter duration of action and the postprandial glucose control is improved compared to regular insulin [166,167]. Furthermore, it shows a better glycaemic control when administered directly before a meal compared to regular human insulin injected 30 minutes before a meal [168].

Insulin glulisine (=Apidra®) is the most recent rapid-acting insulin analogue and is available for clinical use since 2004. Two substitutions in the B-chain characterise this artificial molecule: asparagine at position 3 is substituted by lysine and lysine at position 29 by glutamic acid. As with insulin lispro and aspart, these modifications reduce the formation of hexamers and enhance the absorption from subcutaneous depots. The therapeutic efficiency of insulin glulisine for type 1 and 2 diabetic patients has been confirmed in various studies [169,170].

Insulin glargine (=Lantus®) was the first available long-acting insulin analogue with modifications in both chains. Asparagine at position 21 in the A-chain is substituted by glycine and the B-chain has been elongated at the C terminus by addition of two arginine residues. These modifications result in an insulin molecule with an

isoelectric point shifted towards neutral, rendering it soluble at pH 4 and significantly decreasing its solubility at physiological pH. This readily causes precipitation of insulin glargine after injection into subcutaneous tissue, stabilisation of insulin hexamers, delay of their dissociation and it allows steady absorption into the circulation [171]. The effect is a constant level of insulin glargine serum concentration without pronounced peaks and significantly elongated duration of action. The onset of action is approximately 2 hours after injection [172]. The pharmacological features of insulin glargine provide a much more physiological basal glucose control with a reduced tendency for hypoglycaemic events than e.g. NPH insulin or ultralente. For type 2 diabetes, the clinical efficiency of insulin glargine to reduce blood glucose and HbA1c levels was shown to be equal or higher compared to NPH insulin but was constantly associated with less nocturnal hypoglycaemia [173-177].

The molecular structure of insulin detemir (=Levemir®) is based on a completely novel principle of delaying the onset of action. It is characterised by acylation of a C14 fatty acid (myristic acid) to the lysine residue at position 29 and deletion of the last threonine (position 30) in the B-chain [178]. The protracted action is achieved by delayed resorption due to both increased self-association and reversible albumin binding at the injection site. Further retention of insulin detemir occurs in the circulation where albumin binding causes buffering of insulin concentration [179]. This results in a flat and protracted pharmacodynamic profile [180]. Insulin detemir provides slow absorption and a prolonged and consistent metabolic effect of up to 24 hours in type 1 and 2 diabetic patients. It has a more predictable, protracted, and consistent effect on blood glucose than NPH insulin, with less intrapatient variability in glycaemic control than NPH insulin or insulin glargine. Furthermore, the clinical benefit of less body weight gain than NPH insulin in patients with type 2 diabetes is provided by therapy with insulin detemir [181,182].

Despite the described advantages, some questions concerning the safety of insulin analogues remain open. Insulin and IGF-1 receptors display >50% of amino acid sequence homology and even >84% in the tyrosine kinase domain and both ligands are able to bind to both receptors. Because of the high degree of similarity, insulin/IGF-1 hybrid receptors can form by heterodimerization [183]. The abundance of these hybrid receptors was shown in a range of mammalian tissues [184-187]. Furthermore, it is known that modifications of the insulin molecule in the B10 and B26-B30 region can alter the affinity towards the IGF-1 receptor [188], and there are controversial debates concerning the question whether the InsR, IGF-1 receptor and/or hybrid receptors play a role in mediating the biological effects of insulin analogues. Therefore, a main objective was to analyse the proliferative effects of insulin, AspB10, glulisine, lispro, aspart and glargine in human primary cells, and to investigate the role of the IGF-1 receptor in mediating the proliferative effects of insulin and insulin analogues.

1.5 Objectives

The high prevalence of obesity and diabetes draws much attention to research in this field. Understanding the underlying mechanisms of induction and progression from obesity-induced prediabetic state towards type 2 diabetes are prerequisites for the development of effective therapeutic strategies. A new player connecting both metabolic disorders is the endocannabinoid system, which recently came into focus. Interesting results regarding e.g. weight management and glycaemic control are achieved by blocking the CB1R, and we just begin to understand the implication of an overactivated endocannabinoid system as observed in obesity. One objective of the present work was to analyse the role of CB1R stimulation in the induction of insulin resistance in human skeletal

muscle cells. Furthermore, the question whether blocking the skeletal muscle CB1R with specific antagonists can prevent insulin resistance induced by adipocyte-conditioned medium (CM) was addressed.

Lifestyle intervention therapy has been shown to prevent or delay the onset of type 2 diabetes and to improve several metabolic parameters. There are evidences that the metabolic changes associated with weight gain and impaired glucose tolerance are reversible, but there are also reports of retained insulin resistance in cultured human skeletal muscle cells, as described above. Therefore, the model of CM-induced insulin resistance in human skeletal muscle was used to analyse changes in signalling, protein expression and cytokine secretion after withdrawing CM to gain insight in the processes which may underlay reversion of insulin resistance in skeletal muscle.

The third objective addressed insulin analogues, which play an important role in the therapy of type 1 and type 2 diabetes. Because of their changed structure compared to human insulin, safety questions were raised and are still discussed with special regard to insulin glargine due to its higher affinity to the IGF-1R. Therefore, the proliferative properties of insulin and all insulin analogues except insulin detemir were studied. By using siRNA technology, the role of IGF-1R in mediating the mitogenic effects of insulin analogues was analysed.

Chapter 2

Study 1

CB1 Receptors in Human Skeletal Muscle Cells Participate in the Negative Crosstalk between Fat and Muscle†

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† Diabetologia, in revision

Abstract

Aims/hypothesis The use of specific cannabinoid receptor type 1 (CB1R) antagonists like rimonabant is a novel approach to treat obesity and related metabolic disorders. Recent data suggest that endocannabinoids are also produced by human adipocytes. Here we studied the potential involvement of endocannabinoids in the negative crosstalk between fat and muscle.

Methods The protein level of CB1R in human skeletal muscle cells (SkM) during differentiation was analysed using Western Blot. SkM were treated with adipocyte-conditioned medium (CM) or anandamide (0.1-10 µmol/l) in combination with the CB1R antagonists rimonabant or AM251, and insulin-stimulated Akt phosphorylation

as well as insulin-stimulated glucose uptake was determined. Furthermore, signalling pathways of CB1R were investigated.

Results We revealed that CB1R protein content increases in SkM during differentiation and reaches amounts comparable to differentiated human adipocytes. Incubation of SkM with CM or AEA impaired insulin stimulated phosphorylation of Akt by 60% and up to 40%, respectively. Pretreatment of cells with the CB1R antagonists rimonabant or AM251 reduced the effect of CM by about one half, while the effect of AEA could be prevented completely. The reduction of insulin-stimulated glucose uptake by CM was completely prevented in the presence of rimonabant. Short-time incubation with AEA activated ERK1/2 and p38 MAPK with no effect on NFkB.

Conclusions/interpretation Our results show that the CB1R system may play a role in the development of insulin resistance in human SkM. The results obtained with CM support the notion that adipocytes may secrete factors which are able to activate the CB1R. Furthermore, we identified two stress kinases in the signaling pathway of AEA which are known to play a role in the development of insulin resistance.

Keywords Adipocyte-conditioned medium, Anandamide, Cannabinoid receptor type 1, Endocannabinoids, Human skeletal muscle cells, Insulin resistance, Rimonabant

Abbrevations

2-AG	2-arachidonoylglycerol
AEA	anandamide
AMPKα1	AMP-activated protein kinase alpha 1
BSA	bovine serum albumine

CB1R	cannabinoid type 1 receptor
CM	adipocyte-conditioned medium
ECL	enhanced chemiluminescence
ERK1/2	extracellular regulated kinase 1/2
FAAH	fatty acid amide hydrolase
GLUT4	glucose transporter 4
HRP	horseradish peroxidase
MCP-1	monocyte chemoattractant protein-1
p38 MAPK	p38 mitogen activated protein kinase
PDK4	pyruvate dehydrogenase kinase 4
SkM	skeletal muscle cells
SREBP-1c	sterol regulatory element binding protein-1c
TBS	tris-buffered saline
TNFα	tumor necrosis factor α

Introduction

The endocannabinoid system is a complex network involved in multiple physiological processes. Among other aspects, it has been shown to be important for control of energy homeostasis and body weight [1-5], learning and memory [6], drug addiction [7], and modulation of pain [8,9]. The characterization [10,11] and molecular cloning of the G protein-coupled cannabinoid receptors type 1 (CB1R) and 2 (CB2R) [12], has led to further intensive research for their natural ligands. These ligands are derived from arachidonic acid and are conjugated with ethanolamine or glycerol. Among the endocannabinoids, arachidonoylethanolamide (AEA, anandamide) and 2-arachidonoylglycerol (2-AG) are the most intensive studied compounds. They are synthesized and released by different pathways

and act as retrograde messengers at presynaptic CB1R (e.g. regulation of neurotransmitter release) or target the receptors in an autocrine/paracrine manner [13]. The signals induced by the endocannabinoids are terminated very rapidly by a transporter-mediated uptake [14] and subsequent degradation. The fatty acid amide hydrolase (FAAH) is responsible for degradation of AEA [15], while 2-AG is mainly hydrolyzed by monoacylglycerid lipase [16].

Data from animal and clinical studies have shown that in the obese state due to impaired energy balance the endocannabinoid system is over-activated. Genetically and diet-induced obese animal models show elevated levels of endocannabinoids in hypothalamus, adipose tissue, liver and endocrine pancreas [17-19]. In obese or hyperglycemic, type 2 diabetic patients, circulating levels of AEA and 2-AG are increased and elevated levels of 2-AG are found in visceral adipose tissue [17,20,21]. Furthermore, it is known from the $CB1R^{-/-}$ mouse model that pharmacological targeting of CB1R seems to be a promising approach of treating obesity and related metabolic disorders. $CB1R^{-/-}$ mice are resistant to diet-induced obesity, they remain lean on high-fat diet without changes in metabolic or hormonal profile, and they do not develop a fatty liver [18,22].

Obesity is one of the major components of the metabolic syndrome and a strong risk factor for the development of type 2 diabetes. It is associated with increased circulating plasma levels of free fatty acids and triglycerides which contribute to insulin resistance in peripheral tissues like skeletal muscle [23]. Furthermore, it has become clear by now that adipose tissue is highly active in secreting a large number of factors like cytokines and inflammation-related proteins [24,25]. Previous work in our laboratory has shown *in vitro* that adipocyte-conditioned medium directly impairs insulin signaling in human myotubes [26,27]. Recently, it has been shown that adipocytes are able to bind, synthesize and degrade endocannabinoids and that the endocannabinoid systems is also involved in the differentiation

process of adipocytes [17,28-30]. However, the impact of the endocannabinoid system on the metabolism of skeletal muscle has been investigated less intensively. A study from Cavuoto et al. [31] analysed mRNA expression of genes regulating energy metabolism in skeletal muscle which were incubated for 24 h with endocannabinoids. A second study was published by Liu et al. [32] showing that in a genetically obese mouse model one week of treatment with rimonabant was able to increase basal glucose uptake. To the best of our knowledge, so far no study directly assessed whether a link between activation of skeletal muscle CB1R and the induction of insulin resistance may exist. Therefore, we aimed to investigate whether endocannabinoids might be involved in the negative crosstalk between fat tissue and skeletal muscle and which signalling pathways may play a role.

Material and Methods

Material Reagents for SDS-PAGE were supplied by GE Healthcare Bio-Sciences (Uppsala, Sweden) and by Sigma (Munich, Germany). Anandamide (AEA) and AM251 were delivered by Tocris Biosciences (Bristol, UK), rimonabant (Rim) was kindly provided by Sanofi-Aventis (Frankfurt, Germany). 2-deoxy-D-^{14}C-glucose and L-^{14}C-glucose was purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). Liquid scintillation Aqua safe 300 plus from Zinsser Analytic GmbH (Frankfurt, Germany) was used for glucose uptake assays. The phosphatase and protease inhibitor cocktail tablets were from Roche (Mannheim, Germany). The following antibodies were used: anti-CB1R (Dianova, Hamburg, Germany), anti-phospho Akt (Ser473), anti-Akt, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-phospho-p38 MAPK (Thr180/Tyr182), anti-phospho-NFkB (Ser536) (Cell Signaling Technology, MA, USA), anti-α-tubulin (Calbiochem/Merck, Darmstadt, Germany), and HRP-conjugated anti-rabbit and anti-

mouse IgG (Promega, Mannheim, Germany). The whole cell lysates from human hippocampus and human spinal cord were provided by Abcam (Cambridge, UK). Culture media was supplied by Gibco (Berlin, Germany). Primary human skeletal muscle cells (SkM) and supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). Adipocyte-conditioned medium (CM) was generated as described previously [33]. All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

Culture of human skeletal muscle cells Primary human SkM of six healthy donors (caucasian, male, 9 y and 5 y (M9, M5); female, 10 y, 48 y, 49 y, and 37 y (F10, F48, F49, F37)) were supplied as proliferating myoblasts and cultured according to the protocol of Promocell (Heidelberg, Germany). For an individual experiment, myoblasts were seeded into six-well culture dishes and cultured in α-modified Eagle´s/Ham´s F-12 medium containing SkM growth medium supplement pack up to near confluence. The cells were then differentiated and fused by culture in α-modified Eagle´s medium for six days.

The differentiated cells were incubated with adipocyte-conditioned medium (CM) or 0.1-10 µmol/l AEA for 24 h to induce insulin resistance. Afterwards, the cells were stimulated with 100 nmol/l insulin for 10 minutes, lysed and processed as described below. To block the effects of CM and AEA, cells were pre-incubated with CB1R antagonists Rim (1 µmol/l) or AM251 (10 µmol/l) for 2 h before adding CM or AEA. To analyse short-term effects of AEA, differentiated SkM were incubated with 10 µmol/l or 1 µmol/l AEA for 5, 10, 30 and 60 minutes. 10 min and 30 min incubation with TNFα (5ng/ml) was used as positive control. Afterwards, the cells were lysed and processed as described below.

Glucose transport assay Differentiated SkM were pre-incubated with 1 µmol/l rimonabant for 2 h, then CM was added and the incubation was continued for 24 h. Thereafter, the cells were stimulated with 100 nmol/l insulin for 30 minutes. Then, 2-deoxy-D-^{14}C-glucose (0.25 µCi/ml per well) was added and the uptake was measured for 2 h. The experiment was terminated by transferring the dishes to an ice-bath and repeated washing with ice-cold 0.25 µmol/l cytochalasin B. Cells were lysed with 1 mol/l NaOH, and acetic acid was used to neutralise the lysates. The radioactivity of the cell material was counted in a liquid scintillation counter (Beckman, Munich, Germany). Values were corrected for non-specific uptake, as measured after incubation with L-^{14}C-glucose.

Immunoblotting For analysis of the protein level of CB1R lysates were prepared from SkM of several donors at different days of differentiation, starting from myoblasts (day 0) to myotubes (day 6). For comparison and as a control, lysates of differentiated human adipocytes as well as commercially available whole cell lysates of human hippocampus and human spinal cord were used.

Lysates were prepared with ice-cold lysis buffer containing 50 mmol/l Hepes (pH 7.4), 1% (vol./vol.) Triton X-100, phosphatase and protease inhibitor cocktail. After incubation for 2 h at 4°C, the suspension was centrifuged at 10,000 x g for 20 minutes. 10 µg protein (for detection of CB1R) or 5 µg protein (for detection of pAkt, pERK1/2, pp38 MAPK, and pNFkB) was loaded per sample, separated by SDS-PAGE using horizontal gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in TBS containing 0.1% (vol./vol.) Tween-20 and 5% (wt/vol.) non-fat dry milk or 5% (wt/vol.) BSA and were then incubated overnight with the appropriate antibodies. After repeated washing, the membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The Immobilon HRP substrate from Millipore (Billerica, MA, USA) was used for enhanced

chemiluminescence (ECL) detection. The signals were visualized and evaluated on a LumiImager work station using image analysis software (Boehringer Mannheim, Mannheim, Germany).

Presentation of data and statistics Statistical analysis was carried out by ANOVA. All statistical analyses were done using StatView software (SAS Institute Inc., Cary, NC, USA). A p value of less than 0.05 was considered to be statistically significant. Corresponding significance levels are indicated in the figures. Data are presented as mean ± SEM.

Results

Protein level of CB1R increases during differentiation of human skeletal muscle cells We analysed the protein level of CB1R in human SkM of several donors during differentiation (Fig. 1) and detected a band at approximately 53-kDa by Western blotting, a molecular mass associated with a low glycosylation form of CB1R [20]. The protein level of the receptor increased during differentiation from myoblasts to myotubes and reached an unexpectedly high level at day 6 comparable to that found in differentiated human adipocytes, at least in some muscle donors. The CB1R protein level in the hippocampus lysate was even lower compared to fully differentiated SkM. Also, we observed differences in protein level between the analysed SkM donors. Our results suggest that CB1R indeed plays a relevant role in this peripheral tissue.

Partial prevention of CM-induced insulin resistance by CB1R antagonists rimonabant and AM251 To investigate whether activation of the cannabinoid system plays a role in the induction of insulin resistance by CM, we aimed to block CB1R by incubation with the specific CB1R antagonist rimonabant as well as with AM251, an analog of rimonabant [34,35]. Treatment of differentiated SkM with

CM impaired insulin-stimulated activation of Akt by ~ 60%, as shown in Fig. 2c, although insulin stimulation still produced a significant increase of Akt phosphorylation compared to basal. By pre-incubation with 1 µmol/l Rim or 10 µmol/l AM251 the inhibiting effect of CM on Akt activation was significantly reduced to ~ 20%.

Nevertheless, preincubation with Rim or AM251 could not completely abolish the effect of CM on activation of Akt. This indicates that adipocyte-conditioned medium contains additional factors which take part in the induction of insulin resistance and do not stimulate the CB1R. The incubation with CM, Rim or AM251 did not alter the level of Akt expression, as shown in Fig. 2b.

Rimonabant prevents CM-induced impairment of insulin-stimulated glucose uptake To further analyse the effect of CM in combination with Rim at a level downstream of the signalling cascade, we assessed 2-deoxy-D-^{14}C-glucose (2-DOG) uptake. As shown in Fig. 3, insulin induced a ~ 1.8fold increase of 2-DOG uptake under control conditions. Incubation of cells with CM for 24 h significantly impaired the insulin-stimulated glucose uptake to 1.5fold over basal without a significant change in basal uptake rates. Treatment of SkM with Rim surprisingly resulted in a shift of 2-DOG uptake to a lower level due to a significantly reduced basal uptake rate (Fig. 3). The reason for this shift is currently not known, and analysis of GLUT1 expression revealed no difference after incubation with rimonabant compared to control. Importantly, insulin stimulation caused a ~2fold increase in 2-DOG uptake in Rim-treated cells, which was not significantly affected by the presence of CM (Fig. 3). These data indicate that Rim is able to prevent CM-induced insulin resistance of glucose uptake in human skeletal muscle cells.

Insulin resistance can be induced by the endogenous cannabinoid AEA To further investigate the role of CB1R in insulin resistance, we stimulated the receptor by incubating differentiated SkM with the

endogenous cannabinoid AEA. As shown in fig. 4a and 4c, incubation with AEA decreased the insulin-stimulated phosphorylation of Akt in a concentration dependent manner, with a maximal reduction by ~ 40 % when using AEA at 10 µmol/l. The Akt protein level was not changed at any concentration (Fig. 4b).To assure that the effect of AEA on Akt activation is mediated by the CB1R, we used the specific CB1R antagonist AM251. Treatment of SkM with 10 µmol/l AM251 did neither change Akt activation after insulin stimulation (Fig. 5a,c) nor expression of the enzyme (Fig. 5b). Incubation with 1 µmol/l AEA for 24 h reduced insulin-stimulated Akt phosphorylation significantly to ~ 80% of control. By preincubation with 10 µmol/l AM251 for 2 h, the impaired activation of Akt was prevented (Fig. 5).

AEA activates MAP kinases in skeletal muscle cells We were interested in analysing possible signalling pathways transmitting the effects of AEA and examined whether activation of ERK1/2, p38 MAPK and NFkB might be involved. As a positive control, cells were stimulated with 5 ng/ml TNFα for 10 and 30 min. After incubation with 10 µmol/l AEA for up to 60 min, we observed a strong phosphorylation of ERK1/2 as well as of p38 MAPK (Fig. 6a). For ERK1/2, significant phosphorylation was detected after 5 min (~ 2fold of control), reached a peak at 10 min (~ 3.8fold of control) and rapidly declined thereafter. While ERK2 phosphorylation reached basal level after 30 min, ERK1 phosphorylation was still significant different from control after 60 min (~ 1.7fold of control, Fig. 6b). The activation of p38 MAPK was even more prominent with a ~ 7fold stimulation of phosphorylation compared to control after 5 min (Fig 6c). As for ERK1/2, a peak of phosphorylation was reached after 10 min AEA stimulation followed by a decline afterwards. Nevertheless, after 60 min of incubation p38 MAPK was still prominently activated compared to control. Incubation of differentiated SkM with AEA did not activated NFkB, as shown in the lower panel of fig. 6a. In contrast, TNFα produced a very prominent activation of the NFkB

pathway in addition to stimulating both ERK1/2 and p38 MAPK (Fig. 6a).

Discussion

The over-activation of the endocannabinoid system in human and animal obesity has been shown by various reports. In our model of insulin resistance we could prevent the perturbing effects of CM on insulin action in human skeletal muscle cells by incubation with the CB1R antagonists Rim or AM251. This observation prompted us to further investigate whether endocannabinoids produced by adipocytes could participate in the crosstalk between adipose tissue and skeletal muscle. Several studies have revealed now that endocannabinoids are synthesized in adipose tissue. Gonthier et al. [28] isolated mature adipocytes from human white subcutaneous adipose tissue and identified 2-AG and AEA as products of these cells. In fat biopsies from lean and obese patients Pagano et al. [29] demonstrated the expression of the cannabinoid receptors and the enzymes for cannabinoid synthesis and degradation in visceral and subcutaneous fat. Additionally, the involvement of the endocannabinoid system during adipocyte differentation has been shown in murine 3T3 cell lines [17,30]. Taken together, these data show that adipose tissue is able to produce and secrete endogenous cannabinoids which may take part in the negative crosstalk between fat and muscle tissue to induce insulin resistance. However, incubation with AM251 and Rim could not completely restore insulin-stimulated Akt phosphorylation. Since CM is a mixture of various factors secreted by the adipocytes, it is likely that other mechanisms than CB1R activation are also involved in the induction of insulin resistance. For example, MCP-1 was identified as one component of CM and it has been shown to impair insulin-stimulated Akt phosphorylation and glucose uptake in human myotubes [33].

Our results obtained in the glucose uptake assay regarding the decreased basal glucose uptake after Rim treatment were unexpected and can not be explained by decreased GLUT1 expression. Pagano et al. [29] studied glucose uptake in human fat cells under the influence of Win 55.212 alone or in combination with Rim. They observed a tendency for reduced basal and insulin-stimulated glucose uptake in Rim-treated cells compared to control. This difference did not reach significance but is consistent with our data. On the other hand, our results are in contrast with a study reported by Liu et al. [32]. They measured an increase of basal glucose uptake in isolated soleus muscle of Lep^{ob}/Lep^{ob} mice after one week of treatment with Rim compared to vehicel-treated control. However, their experimental approach was more indirect and did not analyse the direct effect of Rim on skeletal muscle. Before isolation of the muscle, this tissue was not only a target of Rim during the one week treatment period but is also affected by other physiological changes which are secondary due to Rim. For example, it has been shown by Bensaid et al. [36] that Rim increases adiponectin expression in adipose tissue of obese rats, and circulating adiponectin is known to promote glucose uptake in skeletal muscle [37]. Very recently, Migrenne et al. reported that the improvement of insulin sensitivity in diet-induced obese mice due to rimonabant treatment indeed requires adiponectin while the effect on body weight was independent of adiponectin [38]. Therefore, the effect of Rim on basal uptake may be due to secondary rather than primary effects. Furthermore, they did not analyse insulin-stimulated glucose uptake which would be interesting in the light of possibly improved insulin sensitivity due to Rim. In our study we used cultured human SkM to directly investigate the impact of CB1R antagonists on CM-induced impairment of insulin-stimulated processes like Akt phosphorylation and glucose uptake. We show here that pre-treatment of SkM with CB1R antagonists prevents the negative effects of CM on insulin-stimulated glucose uptake, supporting the notion that CB1R antagonists may exert beneficial

effects in the periphery [36,39,40], including skeletal muscle. Additional evidence for the benefit of CB1R antagonists stems from Cavuoto et al. [31], who reported that the expression of genes involved in regulating the energy metabolism in skeletal muscle is modified in response to AM251. The observed changes in PDK4 and AMPKα1 expression are consistent with increased glucose uptake and utilisation and an overall increase in metabolic capacity. Opposing effects of endocannabinoid action are observed in adipocytes in which stimulation of the CB1R resulted in increased glucose uptake and GLUT4 translocation. These effects could be antagonized by treatment with rimonabant [29]. Furthermore, in mice treated with the CB1R agonist HU-210 the hepatic mRNA levels of the lipogenic transcription factor SREBP-1c, acetyl CoA carboxylase-1 and fatty acid synthase were increased as well as de novo fatty acid synthesis [18].

Expression of CB1R mRNA has been demonstrated for various peripheral tissues in humans and rodents [41]. We show here the protein level of CB1R in human skeletal muscle cells (SkM) of different donors by Western blot. Moreover, we revealed that during differentation from myoblasts to myotubes CB1R protein increases similar to the increase observed in differentiating adipocytes [30]. Surprisingly, fully differentiated SkM contain a high level of CB1R protein similar to differentiated human adipocytes. Based on this finding we suggest that skeletal muscle tissue is highly sensitive to endocannabinoids. In addition, it is known that the mRNA expression level of the CB1R is elevated in adipose tissue [36], skeletal muscle [42] and liver [18] of diet-induced obese rodents. In humans there are controversial data regarding CB1R expression in obese vs. lean patients. While Pagano et al. [29] found increased expression of CB1R mRNA in visceral fat tissue of obese patients, Engeli et al. [20] and Bluher et al. [21] reported decreased levels in visceral and subcutaneous fat tissue compared to lean patients. Another study carried out by Lofgren et al. [43] did not find any association between

subcutaneous or visceral adipose CB1R mRNA levels and body weight. The reason for these varying results are not clear and further investigations on the regulation of cannabinoid receptors type 1 in humans are needed. Beside the expression of CB1R, the regulation of expression and activity of enzymes involved in synthesis and degradation must also be considered. In obese humans the mRNA expression of FAAH in adipose tissue was shown to be decreased [20,21] and hepatic FAAH activity in mice on high-fat diet was reduced more than 5fold while anandamide synthesis appeared to be unchanged [18]. These findings may indicate that the increasing endocannabinoid levels may be due to reduced degradation. Whether and how the modified levels of endogenous cannabinoids are involved in the regulation of CB1R expression is currently not known. Nevertheless, high protein level of CB1R sensitizes tissues to the action of endocannabinoids. Therefore it may be speculated that increased endocannabinoid levels due to chronical energy overload affect primarily high sensitive tissues like hippocampus, adipose tissue and skeletal muscle.

In obese animals and humans the level of serum and tissue endocannabinoids are known to be elevated. In obese patients with type 2 diabetes the circulating levels of AEA and 2-AG are increased 2-3fold [17]. Therefore, we used AEA for stimulation of skeletal muscle CB1R and found a concentration dependent impairment of insulin-stimulated Akt phosphorylation. This effect could be completely abolished by pre-incubation with the CB1R antagonist AM251. Interesting insights into possible mechanisms underlying the action of AEA are provided by the activation of the stress kinases ERK1/2 and p38 MAPK. Both kinases are known to play a role in the development of insulin resistance. The involvement of ERK1/2 in the induction of insulin resistance by MCP-1 was shown by Sell et al. [33] who used the ERK1/2 specific inhibitor PD98059 and were therefore able to prevent MCP-1-induced insulin resistance. The involvement of p38 MAPK in TNFα-induced insulin resistance is described for

skeletal muscle cells [44] as well as for endothelial cell [45]. Additionally, Koistinen et al. [46] reported aberrant p38 MAPK signalling in skeletal muscle of type 2 diabetic patients. Our results also show that the pro-inflammatory NFkB signalling pathway seems not to be involved in the mechanism by which AEA may induce insulin resistance.

In summary, obesity develops as a consequence of continuous energy overload and has been shown to be associated with an over-activated endocannabinoid system which influences the metabolic activity of a variety of tissues such as liver and fat. The resulting increase in triglycerides, free fatty acids and adipokines like MCP-1 as well as the endocannabinoids AEA and 2-AG promote the development of insulin resistance in peripheral tissues like skeletal muscle. We demonstrate here for the first time that activation of CB1R may play a role in the induction of insulin resistance in human skeletal muscle. While activation of ERK1/2 and p38 MAPK are probably involved in this process, AEA signalling is independent of the NFkB pathway. The results obtained with CM support the notion that adipocytes may secrete factors which are able to activate the CB1R. Hereby, our results add an additional component to the complex mechanisms that lead to the development of insulin resistance in skeletal muscle.

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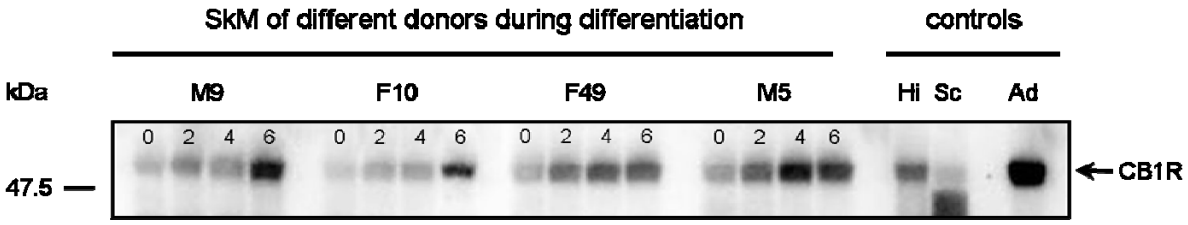

Fig. 2.1 Protein level of cannabinoid receptor type 1 (CB1R) in human SkM during differentiation. 10 µg protein of whole cell lysates from SkM of the donors M9, F10, F49 and M5, and of human hippocampus (*Hi*), human spinal cord (*Sc*), and differentiated human adipocytes (*Ad*) were resolved by SDS-PAGE, blotted and immunodetected with anti-CB1R antibody using the ECL system. The numbers indicate the day of differentiation, thereby day 0 represents myoblasts.

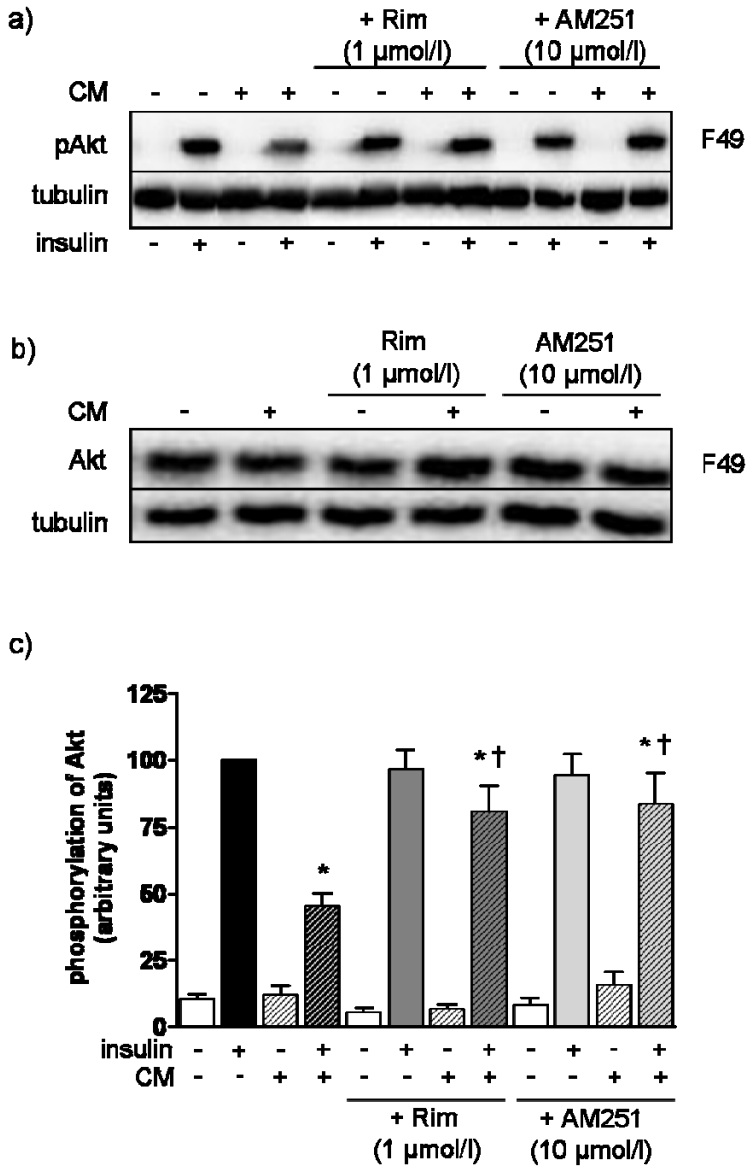

Fig. 2.2 Partial blocking of CM-induced insulin resistance by Rim and AM251. Differentiated SkM were pre-incubated with 1 µmol/l Rim or 10 µmol/l AM251 for 2 h. Then, adipocyte-conditioned medium (CM) was added and incubation was continued for 24 h. Afterwards cells were stimulated with 100 nmol/l insulin for 10 minutes and lysed. Whole cell lysates were resolved by SDS-PAGE, blotted and immunodetected with a phosphospecific Akt antibody using the ECL system. a) Representative Western blot of donor F49 showing phosphorylation of Akt after insulin stimulation. b) Representative Western blot of donor F49 showing

expression of Akt after indicated treatment. c) Quantification of Akt phosphorylation after indicated treatments. All data were normalised to the level of α-tubulin expression and are expressed relative to the insulin-stimulated control. Results represent mean values obtained from two different donors. The data are presented as means±SEM, $n \geq 4$. *Asterisk* represents $p<0.05$ vs non CM-treated insulin-stimulated control. *Dagger* represents $p<0.05$ vs CM-treated insulin-stimulated control.

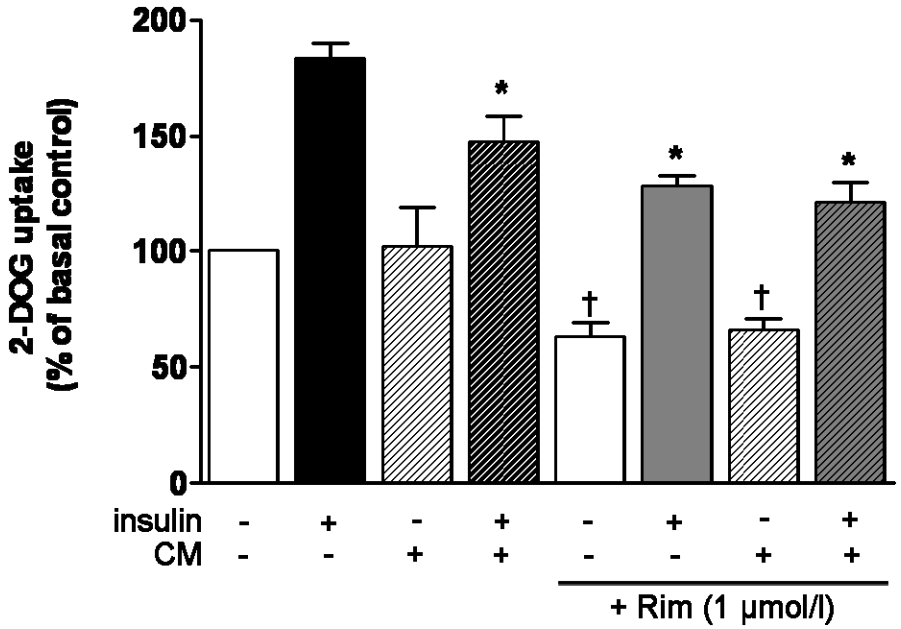

Fig. 2.3 Insulin-stimulated 2-deoxy-D-^{14}C-glucose uptake. Differentiated SkM (donor F49) were pre-incubated with 1 µmol/l Rim for 2 h. Then, adipocyte-conditioned medium (CM) was added and incubation was continued for 24 h. Afterwards, cells were stimulated with 100 nmol/l insulin for 30 minutes and 2-DOG uptake was measured for 2 h. The measured radioactivity of the cell lysates was corrected for non-specific uptake. The data are expressed relative to the basal glucose uptake of untreated controls. Results represent mean values ± SEM of at least 3 different experiments. *Asterisk* represents $p<0.05$ vs non CM-treated insulin-stimulated control. *Dagger* represents $p<0.05$ vs non CM-treated basal control.

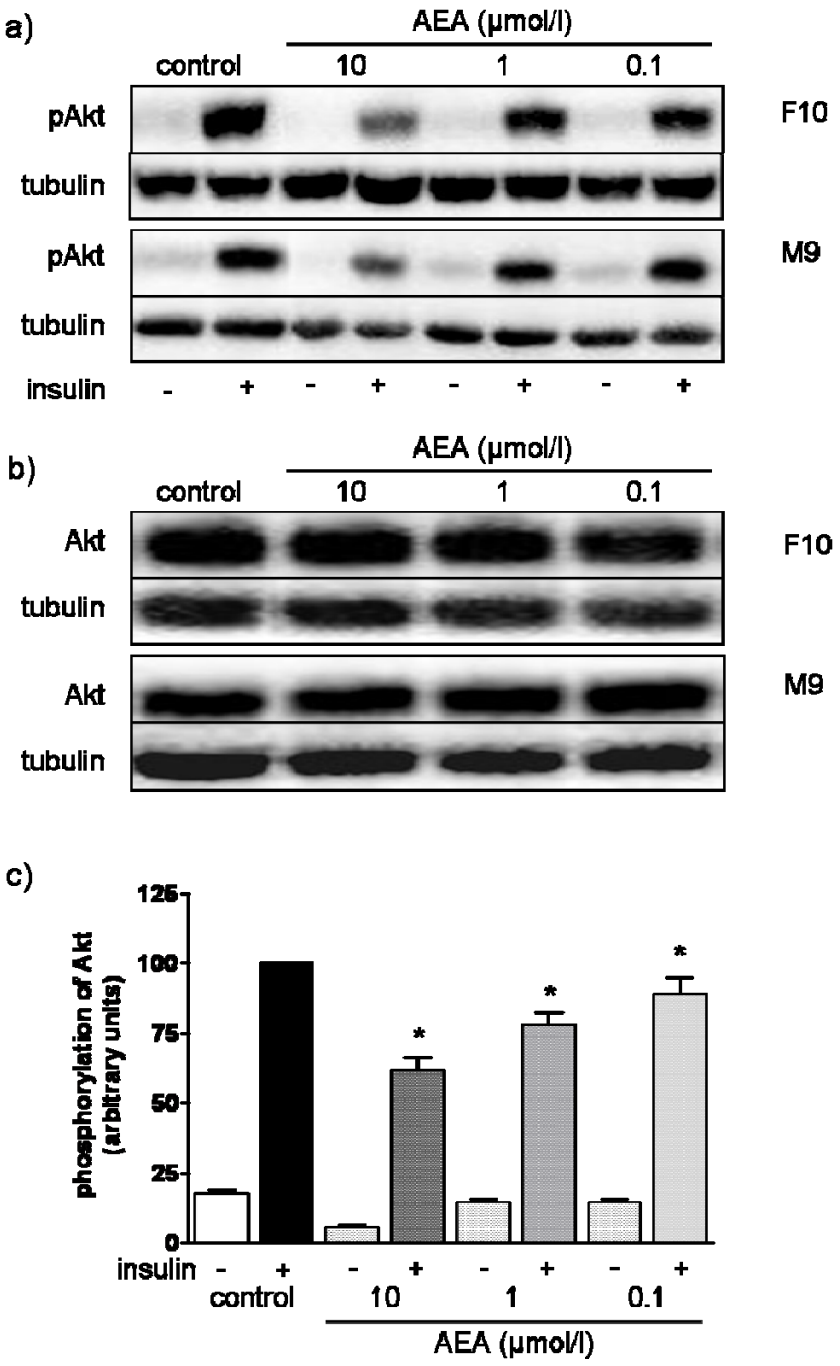

Fig. 2.4 Induction of insulin resistance by AEA. Differentiated SkM were incubated with 0.1-10 µmol/l AEA for 24 h. Afterwards, cells were stimulated with 100 nmol/l insulin for 10 minutes, lysed and processed as described in fig. 2. a) Representative Western blots of donor F10 and M9 showing phosphorylation of Akt after insulin stimulation. b) Representative Western blots of donor F10 and M9 showing expression of Akt. c)

Quantification of Akt phosphorylation after indicated treatments. All data were normalised to the level of α-tubulin expression and are expressed relative to the insulin-stimulated control. Results represent mean values obtained from three different donors. The data are presented as means±SEM, $n \geq 12$. *Asterisk* represents $p<0.05$ vs insulin-stimulated control.

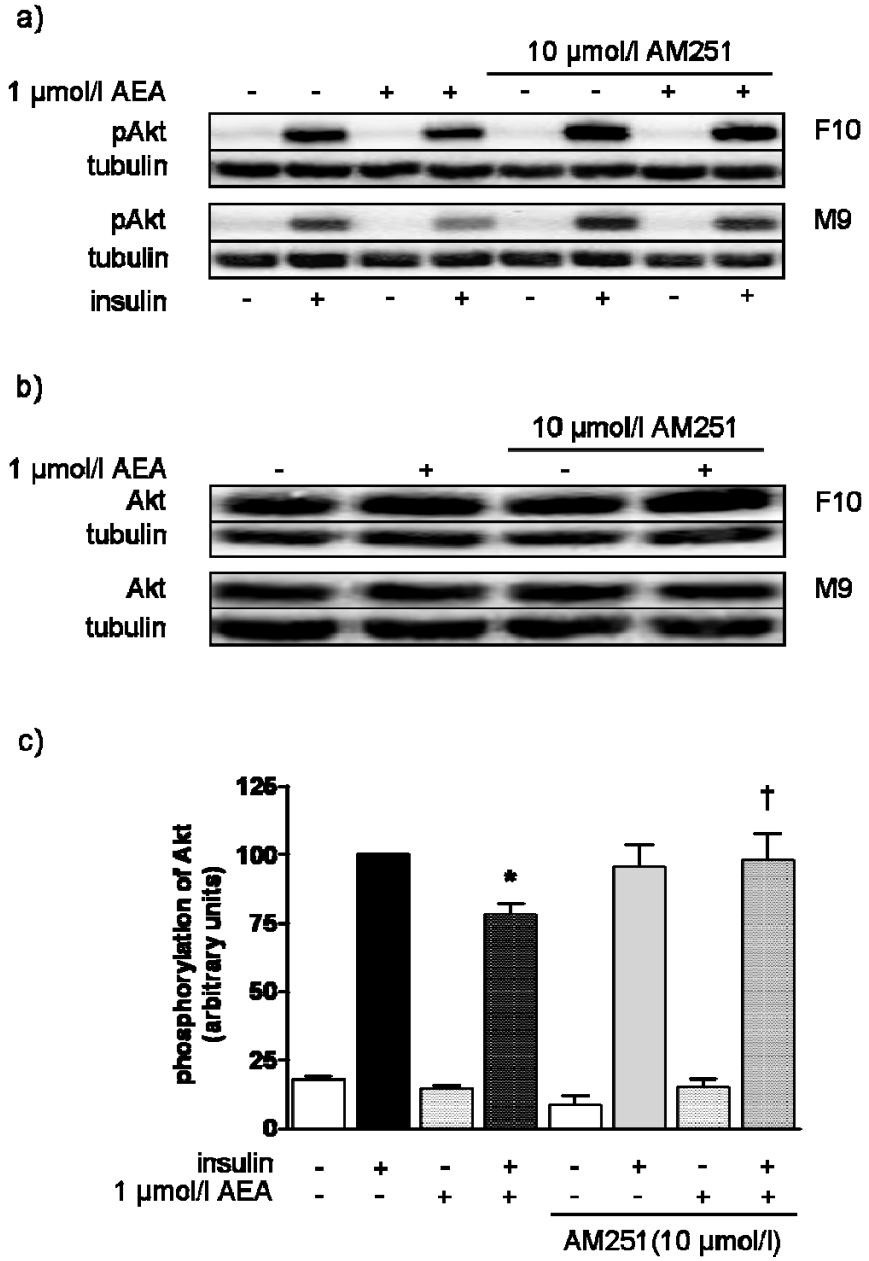

Fig. 2.5 AM251 prevents the effect of AEA. Differentiated SkM were pre-incubated with 10 µmol/l AM251 for 2 h. Then, 1 µmol/l AEA was added and incubation was continued for 24 h. Afterwards, cells were stimulated with 100 nmol/l insulin for 10 minutes, lysed, and processed as described in fig. 2. a) Representative Western blots of donor F10 and M9 showing phosphorylation of Akt after insulin stimulation. b) Representative Western blots of donor F10 and M9 showing expression of Akt. c) Quantification of Akt phosphorylation after indicated treatments. All data were normalised to

the level of α-tubulin expression and are expressed relative to the insulin-stimulated control. Results represent mean values obtained from two different donors. The data are presented as means±SEM, $n \geq 8$. *Asterisk* represents $p<0.05$ vs insulin-stimulated control. *Dagger* represents $p<0.05$ vs AEA-treated insulin-stimulated situation.

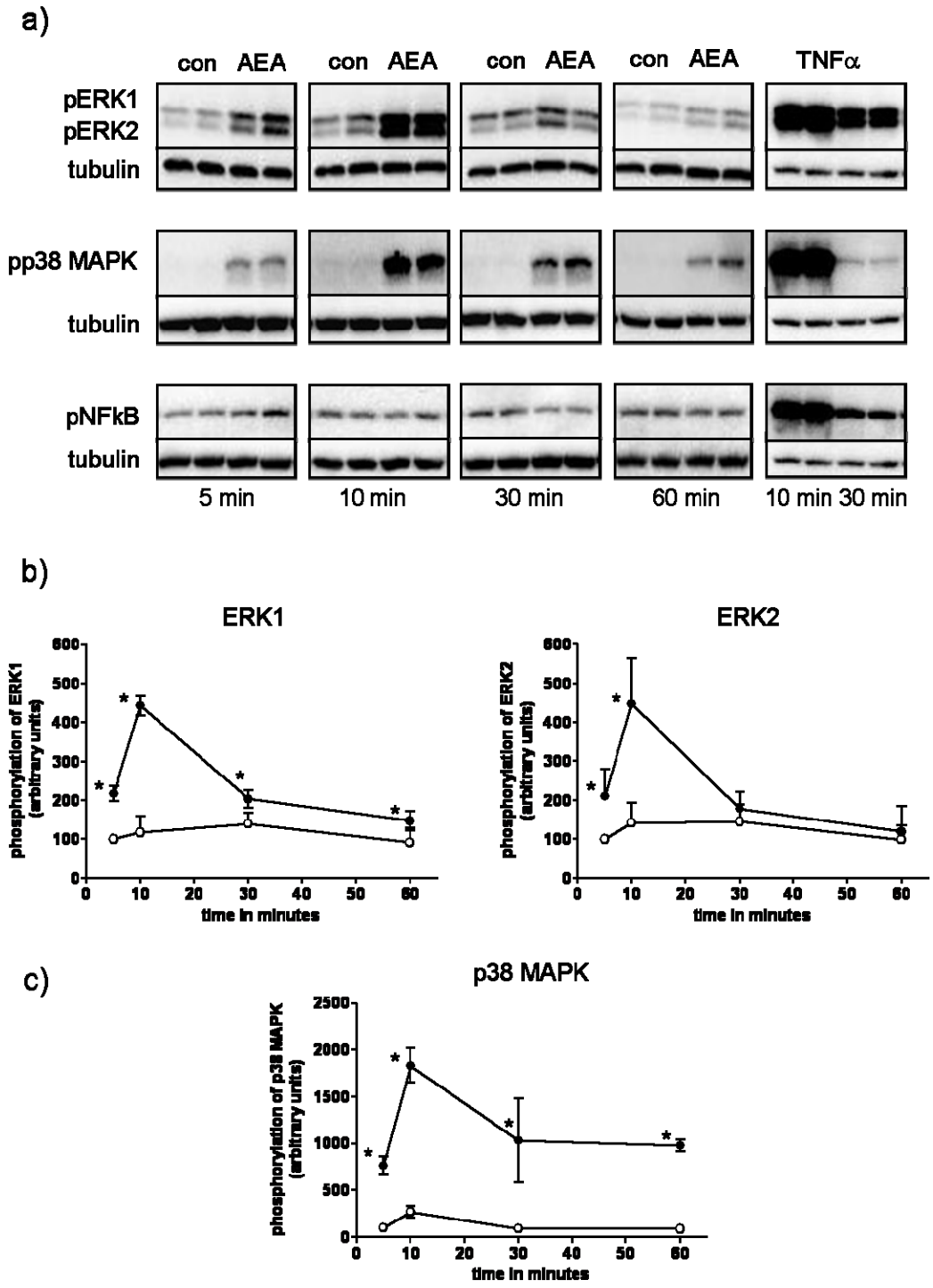

Fig. 2.6 Activation of ERK1/2, p38 MAPK, and NFkB in response to AEA treatment. Differentiated SkM were incubated with 10 µmol/l AEA or 5 ng/ml TNFα for the indicated times. Afterwards, cells were lysed and processed as described in fig. 2, using specific phospho-antibodies. a) Representative Western blots of donor F49 showing phosphorylation of

ERK1/2 (upper panel), p38 MAPK (middle panel), and pNFkB (lower panel) induced by stimulation with AEA or TNFα. Quantification of ERK1/2 phosphorylation (b) and p38 MAPK phosphorylation (c) induced by AEA stimulation from 5 to 60 min (*open circle* control, *black circle* AEA). All data were normalised to the level of α-tubulin expression and are expressed relative to the phosphorylation level of control after 5 min. Results represent mean values obtained from two different donors. The data are presented as means±SEM, $n \geq 5$ for pERK1/2 (b), $n \geq 3$ for pp38 MAPK (c). *Asterisk* represents $p < 0.05$ vs 5 min control.

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Chapter 3

Study 2

Skeletal muscle insulin resistance induced by adipocyte-conditioned medium: underlying mechanisms and reversibility†

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Abstract

Insulin resistance in skeletal muscle is an early event in the development of diabetes with obesity being one of the major contributing factors. In vitro, conditioned medium (CM) from differentiated human adipocytes impairs insulin signalling in human skeletal muscle cells but it is not known if insulin resistance is reversible and which mechanisms may underlie this process. CM induced insulin resistance in human myotubes at the level of insulin-stimulated Akt and GSK3 phosphorylation. In addition, insulin-resistant skeletal muscle cells exhibit enhanced production of reactive oxygen species and ceramide as well as a downregulation of myogenic transcription factors such as myogenin and myoD.

However, insulin resistance was not paralleled by increased apopotosis. Regeneration of myotubes for 24 or 48 h after induction of insulin resistance restored normal insulin signalling. However, the expression level of myogenin could not be reestablished. In addition to decreasing myogenin expression, CM also decreased the release of IL-6 and IL-8, and increased monocyte chemotactic protein-1 (MCP-1) secretion from skeletal muscle cells. While regeneration of myotubes reestablished normal secretion of IL-6 the release of IL-8 and MCP-1 remained impaired over 48 h after withdrawal of CM. In conclusion, our data show that insulin resistance in skeletal muscle cells is only partially reversible. While some characteristic features of insulin resistant myotubes normalise in parallel to insulin signalling after withdrawal of CM, others such as IL-8 and MCP-1 secretion and myogenin expression remain impaired over a longer period. Thus, we propose that the induction of insulin resistance may cause irreversible changes of protein expression and secretion in skeletal muscle cells.

Key words Insulin resistance; skeletal muscle; adipose tissue; cellular crosstalk; reversibility of insulin resistance

Abbreviations

CM, conditioned medium; DAF, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate; DCF, 2',7'-dichlorodihydrofluorescein diacetate; ECL, enhanced chemiluminescence; TBS, Tris-buffered saline; MCP-1, monocyte chemotactic protein-1; MHC, myosin heavy chain; MIP-1, macrophage inflammatory protein-1; NO, nitric oxide; ROS, reactive oxygen species; SDH, succinate dehydrogenase

Introduction

Obesity is one of the major risk factors contributing to the development of insulin resistance and type 2 diabetes (10). In this context, the negative crosstalk between adipose tissue and skeletal muscle is involved in early metabolic disturbances leading to insulin resistance (31, 33). Adipocytes from obese patients have a different secretion pattern as compared to lean donors with the release of pro-inflammatory factors and adipokines being increased (28). In fact, these adipose-derived molecules might be key contributors to the development of insulin resistance and other diseases such as endothelial dysfunction and atherosclerosis (36). In vitro, we were able to show that adipocyte-conditioned medium (CM) containing various adipokines induces insulin resistance in skeletal muscle cells (7, 9).

The development of insulin resistance is a reversible process. Reduction of adipose tissue mass by weight loss is a validated approach to reverse insulin resistance (11, 25). In parallel to improved insulin sensitivity, weight reduction also normalises adipokine blood level which has been demonstrated for IL-6 (5), high molecular weight adiponectin (2), monocyte chemotactic protein-1 (MCP-1) (4) and TNFα (19). It could be shown that insulin resistance disappears in cultured skeletal muscle biopsies from obese patients (3, 22) demonstrating that insulin resistance might be a reversible feature that can be acquired with obesity. However, other studies in muscle biopsies from obese and diabetic patients demonstrated that insulin resistance is retained in culture (3, 13, 39). This study was aimed at analysing reversibility of adipocyte-induced insulin resistance in skeletal muscle cells and underlying mechanisms.

Material and Methods

Material. BSA (fraction V, fatty acid free) was obtained from Roth (Karlsruhe, Germany). Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech (Braunschweig, Germany) and by Sigma (München, Germany). Polyclonal antibodies anti-phospho GSK3α/β (Ser21/9), anti-phospho-Akt (Ser473) and anti-GLUT4 were supplied by Cell Signaling Technology (Frankfurt, Germany) and anti-tubulin by Calbiochem (Darmstadt, Germany). Antibodies for myogenin came from Acris (Hiddenhausen, Germany), for MyoD from Imgenex (San Diego, CA) and the one for myosin heavy chain (MHC) from Upstate (San Diego, CA). HRP-conjugated goat-anti-rabbit and goat-anti-mouse IgG antibodies were purchased from Promega (Mannheim, Germany). Collagenase CLS type 1 was obtained from Worthington (Freehold, NJ) and culture media were obtained from Gibco (Berlin, Germany). Primary human skeletal muscle cells and supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

Culture of human skeletal muscle cells. Primary human skeletal muscle cells of four healthy Caucasian donors (male, 9 and 47 y; female, 10 and 48 y) were supplied as proliferating myoblasts (5 x 10^5 cells) and cultured as described previously (9). For an individual experiment, myoblasts were seeded in six-well culture dishes (9.6 cm^2/well) at a density of 10^5 cells per well and were cultured in α-modified Eagles/Hams F12 medium containing Skeletal Muscle Cell Growth Medium Supplement Pack up to near confluence. The cells were then differentiated and fused by culture in α-modified Eagles medium for 4 days and used for experiments.

Adipocyte isolation and culture. Adipose tissue samples were obtained from the mammary fat of normal or moderately overweight women (BMI 24.5 ± 0.9, aged between 23 and 41) undergoing surgical mammary reduction. The procedure to obtain adipose tissue was approved by the ethical committee of Heinrich-Heine-University Duesseldorf, Germany. All subjects were healthy, free of medication and had no evidence of diabetes according to routine laboratory tests. Adipose tissue samples were dissected from other tissues and minced in pieces of about 10 mg in weight. Preadipocytes were isolated by collagenase digestion as previously described (12). Isolated cell pellets were resuspended in Dulbecco's modified Eagles/Hams F12 medium supplemented with 10% FBS, seeded on membrane inserts (3.5 x 10^5/4.3 cm^2) or in a six-well culture dish, and kept in culture for 16 h. After washing, culture was continued in an adipocyte differentiation medium (DMEM/F12, 33 µM biotin, 17 µM d-pantothenic acid, 66 nM insulin, 1 nM triiodo-L-thyronin, 100 nM cortisol, 10 µg/ml apo-transferrin, 50 µg/µl gentamycin, 15 mM HEPES, 14 mM $NaHCO_3$, pH 7.4). After 15 days, 60-80% of seeded preadipocytes developed to differentiated adipose cells, as defined by cytoplasm completely filled with small or large lipid droplets. These cells were then used for generation of CM, as previously described by us (8). Briefly, after *in vitro* differentiation, adipocytes were incubated for 48 h in skeletal muscle cell differentiation medium. This conditioned medium was then harvested, centrifuged to remove any cell debris and immediately frozen in aliquots for future use. CM from 350.000 adipocytes was used to stimulate one six-well of skeletal muscle cells. In control experiments, skeletal muscle cell differentiation medium was incubated for 48 h without adipocytes and tested upon its effect on skeletal muscle. No difference in insulin signaling could be found using this medium compared to fresh skeletal muscle cell differentiation medium (data not shown).

Immunoblotting. Muscle cells were treated as indicated and lysed in a buffer containing 50 mM HEPES (pH 7.4), 1% (v/v) Triton-X, 1 mM Na_3VO_4 and Complete protease inhibitor cocktail from Roche Diagnostics. After incubation for 2 h at 4°C the suspension was centrifuged at 13,000 x g for 15 min. Thereafter 5 µg of lysates were separated by SDS-PAGE using 10% horizontal gels and transferred to polyvinylidene fluoride filters in a semidry blotting apparatus. For detection filters were blocked with TBS containing 0.1% Tween-20 and 5% non-fat dry milk and subsequently incubated overnight with the appropriate antibodies. After extensive washing, filters were incubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescene (ECL) detection using Uptilight (Interchim, France). Signals were visualised and evaluated on a LUMI Imager workstation using image analysis software (Boehringer Mannheim, Mannheim, Germany).

ELISA. ELISAs for IL-6, IL-8 and MCP-1 were purchased from Diaclone (Stamfort, CT). Undiluted samples from skeletal muscle cell supernatant were measured according to the manufacturer's protocols.

Measurement of reactive oxygen species (ROS) and nitric oxide (NO) production in skeletal muscle cells. Differentiated skeletal muscle cells were treated with CM overnight to induce insulin resistance. Then, cells were washed in PBS without Ca/Mg and used for the assay. For measurement of ROS, cells were incubated in 10 µM 2',7'-dichlorodihydrofluorescein diacetate (DCF) (Molecular Probes, Karlsruhe, Germany) solved in phenolred-free DMEM for 30 min. As a positive control, cells were treated with 0.3 % H_2O_2 for 30 min in parallel to DCF incubation. For measurement of NO, skeletal muscle cells were incubated with 10 µM 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF) (Molecular Probes) solved in phenolred-free DMEM for 30 min. As a positive control for NO

production, cells were also treated with 500 µM SNAP (Calbiochem, Darmstadt, Germany) for 30 min in parallel to DAF. Afterwards, cells were lysed in the above-mentioned lysis buffer and fluorescence measured using an excitation wavelength of 595 nm on a Fluostar-P (SLT, Salzburg, Austria).

Measurement of SDH activity in skeletal muscle cells. Differentiated skeletal muscle cells were incubated with CM for the indicated time and lysed in homogenization buffer containing 250 mM glucose, 10 mM Tris-HCl, 0.5 mM EGTA and 0.5 mM DTT. SDH activity was measured according to Pennington's method (23). Briefly, approximately 200 µg of cell lysate was incubated with 10 mM sodium succinate in 50 mM NaH_2PO_4 buffer for 20 min at 37°C. 5 mM p-iodonitrotetrazoliumviolet solved in 50 mM NaH_2PO_4 buffer was added to a final concentration of 0.5 mM for an additional 10 min at 37°C. The reaction was stopped by an ethylacetate/ethanol/trichloracid solution (5:5:1, v/v/w). Immediately after 2 min centrifugation at 13,000 x g, the supernatant was measured at 490 nm on a spectrophotometer (Beckman, Krefeld, Germany).

Measurement of apoptosis. Apoptosis was monitored by assessment of caspase 3 activity and nuclear fragmentation in skeletal muscle cells treated with CM. The DEVD-cleaving activity of the caspase 3 class of cystein proteases was determined in cell lysates using Ac-DEVD-AMC (BD Biosciences, Heidelberg, Germany) as fluorogenic substrate according to the manufacturer's protocol. The ability of cell lysates to cleave the specific caspase 3 substrate was quantified by spectrofluorometry using an excitation wavelength of 390 nm and an emission wavelength of 460 nm with a microplate reader. For detection of nuclear fragmentation, the cells were double-stained with Hoechst 33342 and propidium iodide. Skeletal muscle cells were washed twice with PBS and were stained with 10 µg/ml Hoechst

33342 and 1 µg/ml propidium iodide at 37°C for 15 minutes. Fluorescence was observed under a Leica DM IRB fluorescence microscope. At least 400 cells were counted for each experiment. Cells with condensed or fragmented nuclei were defined to be apoptotic and cells with normal-shaped nuclei were supposed to be viable.

Quantitative evaluation of ceramide. Lipids from skeletal muscle cells were extracted in chloroform/methanol/water (2:1:0.1, v/v/v) for 24 h at 48°C. Lipid extracts were applied to thin layer Silica Gel 60 plates (Merck, Darmstadt, Germany) as described earlier (38). Ceramides were resolved twice using chloroform/methanol/acetic acid (190:9:1, v/v/v) as developing system. Following development, plates were air-dried, sprayed with 8% (w/v) H_3PO_4 containing 10% (w/v) $CuSO_4$, and charred at 180°C for 10 min. Lipids were identified by their Rf value using authentic lipid samples as references. Individual lipid bands obtained by thin layer chromatography (TLC) were evaluated by photodensitometry (Shimadzu, Kyoto, Japan). Assuming constant cholesterol amounts in all samples, densitometric data obtained for ceramide were normalized to cholesterol.

Presentation of data and statistics. Statistical analysis was performed by ANOVA. All statistical analyses were done using Statview (SAS, Cary, NC) considering a P value of less than 0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

Results

CM-induced insulin resistance of insulin signaling in skeletal muscle cells is a reversible process. CM of differentiated human adipocytes impairs insulin signaling at the level of Akt in human skeletal muscle

cells (Fig. 1A). Insulin-stimulated GSK3α/β phosphorylation is only slightly decreased by CM treatment while basal phosphorylation is significantly increased leading to an insignificant insulin effect (Fig. 1B). Withdrawal of CM for 24 or 48 h reestablishes normal insulin signaling in skeletal muscle cells with Akt and GSK3α phosphorylation being similar to control and GSK3β phosphorylation being even higher than in the control situation.

Insulin resistance is accompanied by reduced expression of myogenic transcription factors in skeletal muscle cells and an irreversible downregulation of myogenin. During differentiation, skeletal muscle cells display an increased expression of myogenin, MHC and myoD which are all markers of myogenesis (Fig. 2 A-C). Analysis of myogenic transcription factors revealed that CM-treated skeletal muscle cells have significantly reduced expression of myogenin, MHC and myoD (Fig. 3A-C). Skeletal muscle cells display an increasing GLUT4 level (Figure 4A, upper panel). However, CM-treatment did not affect GLUT4 expression in differentiated myotubes (Fig. 4A, lower panel) and the cells exhibited an unaltered morphology as compared to control.cells (Figure 4B). Withdrawal of CM for 24 or 48 h reverses the downregulation of MHC and myoD while the expression of myogenin remains decreased over the whole period as compared to control (Figure 3). Thus, in spite of reestablished insulin signalling skeletal muscle cells do not normalise myogenin expression after CM-treatment and withdrawal.

CM-treated skeletal muscle cells are characterised by a partially irreversible secretory dysfunction. Skeletal muscle cells secrete various myokines including IL-6, IL-8 and MCP-1. As compared to adipocytes which secrete approximately 500 pg/ml/24 h of IL-6, skeletal muscle cells exhibit lower secretion of this cytokines with 23 ± 1 pg/ml/24 h (n = 5). Treatment with CM leads to a significantly lower IL-6 secretion during the first 24 h of regeneration of myotubes

(Fig. 5A). 48 h after CM withdrawal, however, IL-6 secretion is comparable to control cells.

IL-8 secretion is also lower in skeletal muscle cells (94 ± 12 pg/ml/24 h; n = 5) when compared to adipocytes (approximately 500 pg/ml/24 h). CM-treated skeletal muscle cells display significantly impaired IL-8 secretion over the whole regeneration period of 48 h when compared to control. This suggests that IL-8 secretion might be irreversibly disturbed in insulin-resistant myocytes (Fig. 5B).

MCP-1 is a cytokine robustly released from human adipocytes (approximately 3 ng/ml/24 h) but also secreted at low levels from myotubes (37 ± 11 pg/ml/24 h; n = 5). Induction of insulin resistance in skeletal muscle cells significantly stimulates MCP-1 secretion after 24 h of regeneration with an additional increase after 48 h (Fig. 5C).

Insulin-resistant skeletal muscle cells exhibit increased oxidative stress and decreased mitochondrial capacity but no apoptosis. ROS and NO are both potential players in the induction of insulin resistance. As presented in Fig. 6, a significant increase in both ROS and NO production was observed in skeletal muscle cells treated with CM. SDH activity was measured in whole cell lysates of skeletal muscle cells to assess oxidative capacity. CM-treatment slightly but significantly reduced SDH activity in whole cell lysates after 24 h (Fig. 7). Longer incubation with CM over 96 hours further reduced the level of SDH activity. The parallel induction of insulin resistance and oxidative stress can however not be assigned to apoptosis in skeletal muscle cells. Measurement of caspase 3 activity revealed no increase in CM-treated cells as compared to controls (1.08 ± 0.13 versus 1.06 ± 0.17 arbitrary units, significantly elevated positive control (campthotecin for 5h) 1.95 ± 0.03 arbitrary units; n = 3-4). Furthermore, nuclear fragmentation was not elevated in CM-treated cells as compared to controls (2.6 ± 0.1 % versus 2.2 ± 0.2 % apoptotic cells, significantly elevated positive control (campthotecin for 5h) 5.0 ± 1.0 % apoptotic cells; n = 3-4).

Insulin-resistant skeletal muscle cells contain higher ceramide levels. Ceramide constitutes a well-known player in insulin resistance. Fatty acids and ceramide can induce insulin resistance in skeletal muscle cells (26, 37). Analysis of lipid extracts by thin layer chromatography revealed a nearly 3-fold increase of ceramide content in insulin resistant skeletal muscle cells as compared to controls (Figure 8).

Discussion

Adipose tissue expansion and increased release of adipokines have been shown to play a crucial role in the induction of insulin resistance (14). We could demonstrate in several studies that adipocyte-derived factors can induce insulin resistance in skeletal muscle cells in vitro (7, 9, 32). The data presented here now demonstrates that CM-treated skeletal muscle cells are not only characterised by impaired insulin signalling but also by various other defects. Insulin-resistant skeletal muscle cells downregulate the expression of myogenin and display oxidative stress, lower mitochondrial capacity and higher ceramide content. Furthermore, insulin-resistant myotubes have disturbed secretion of the myokines IL-6, IL-8 and MCP-1.

In vitro differentiated skeletal muscle cells are characterised by a high abundance of the myogenic transcription factors such as myogenin and myoD. We demonstrate here for the first time that adipocyte-derived factors lead to a marked downregulation of myogenin in skeletal muscle cells. It is known from the literature that TNFα suppresses the differentiation process in C2C12 myoblasts (34) but nothing is known about its effect on differentiated cells. However, CM contains very low doses of TNFα (less than 0.02 pmol/l (7)) making it probable that another adipokine with higher concentration in CM might be the culprit for downregulation of myogenin. The loss

of myogenin in insulin-resistant skeletal muscle cells is, however, associated with a conservation of skeletal muscle phenotype as myotubes display normal morphology and GLUT4 expression. However, it cannot be completely ruled out that the downregulation of multiple markers, including myoD, MHC and SDS, points to a de-differentiation of skeletal muscle cells and it is impossible so far to speculate on the meaning of this finding for the situation in skeletal muscle in vivo.

IL-6, IL-8 and MCP-1 are known secretory products from skeletal muscle with different roles in myogenesis, exercise, inflammation and insulin sensitivity. Increased IL-6 levels are associated with insulin resistance in vivo (16) but short-term treatment of skeletal muscle cells with IL-6 can increase insulin sensitivity (40). The reported increase of IL-6 during exercise (21) makes it likely that IL-6 has completely different acute and chronic effects. As for myogenesis, IL-6 is a promyogenic factor (1) explaining the parallel decrease of myogenic markers and IL-6 secretion in the myotubes. IL-8 and MCP-1 are both pro-inflammatory chemokines being increased in serum of obese and diabetic patients (17, 29, 30). MCP-1 is a potent inducer of insulin resistance in skeletal muscle cells (32) and plays a role in myopathies (6). TNFα and INFγ have been described to induce MCP-1 transcription in myoblasts (6). While IL-8 secretion is almost completely inhibited in CM-treated skeletal muscle cells, MCP-1 release increases pointing to an inflammatory effect of CM.

SDH activity is known to be slightly but significantly reduced in skeletal muscle lysates from diabetic patients as compared to controls (20). We also observe a reduction in SDH activity in CM-treated skeletal muscle cells indicating a possible role of decreased oxidative capacity in the initiation of skeletal muscle cell insulin resistance. Notably, in diabetic patients reduced oxidative capacity in parallel to increased glycolytic activity is due to a significant alteration of skeletal muscle fiber composition.

Oxidative stress is a result of increased ROS or NO production and can lead to oxidation and damage of DNA, protein and lipids (18). Increasing ROS production as observed in our model could cause damage to mitochondria and so-called mitoptosis and explain the loss of mitochondria observed in states with increased oxidative stress such as insulin resistance and diabetes. Thus, increased ROS or NO levels could also explain decreased SDH activity in insulin-resistant skeletal muscle cells. Other work in L6 muscle cells shows that palmitate-induced insulin resistance is also characteriζed by higher levels of ROS and NO (27). However, it should be noted that fatty acids are barely detectable in CM when using a HPLC approach (data not shown). Therefore, we conclude that adipocyte-derived factors produce an increase in ROS and NO similar to that produced by fatty acids.

NO and inducible NO synthase (iNOS) are known to be increased in the diabetic state and are linked to chronic inflammation (15). However, it is not known how NO induces or exacerbates insulin resistance. In C2C12 skeletal muscle cells, the NO-donor SNAP inhibits Akt activity making it possible that an intracellular increase in skeletal muscle cell NO might contribute to insulin resistance (41). Furthermore, diabetic patients are characterized by higher blood levels of nitrates and nitrites as well as higher expression of iNOS in skeletal muscle (35). In our primary myotubes we also observed an increase in NO production after treatment with CM, which might together with ROS contribute to the development of insulin resistance. It should be noted in this context that CM-treated skeletal muscle cells are not apopototic as shown by unaltered percentage of cells with nuclear fragmentation and similar caspase 3 activity compared to controls, so that NO and ROS elevation cannot be attributed to apoptosis.

The sphingolipid ceramide is described to be a possible link between obesity and diabetes. Fatty acids and resulting higher levels of ceramide can induce insulin resistance in skeletal muscle cells (26,

37). In this study, insulin resistant skeletal muscle cells are also characteriζed by increased ceramide levels which may contribute to adipokine-induced insulin resistance and illustrate disturbed lipid metabolism.

In this study, we were able to show that adipocyte-induced insulin resistance is a reversible process in skeletal muscle cells, at least at the level of insulin signalling. However, some alterations are not fully reversible and may illustrate longer lasting damage to the myotubes by one time treatment with CM. Skeletal muscle cells display long-lasting myogenin downregulation and secretory defects of IL-8 and MCP-1. Differentiation of skeletal muscle involves a group of transcription factors including myogenin and myoD which activate muscle-specific gene expression and have each a distinct function during myogenesis (24). In our model, we observe a loss of myogenin expression with preservation of muscle phenotype. At this point, we cannot evaluate the physiological impact of the loss of myogenin. Our data clearly shows that the loss of myogenin is unrelated to early steps in insulin signalling, myotube morphology and GLUT4 expression. Certainly, our model of in vitro differentiated skeletal muscle cells has limitations as to how our findings on downregulation of myogenic markers underlie obesity-related insulin resistance *in vivo*. Future work should be aimed to relate our findings to the in vivo situation in diabetic and obese patients in this respect. In summary, we could demonstrate that adipocyte-derived insulin resistance in skeletal muscle cells impacts on various aspects of skeletal muscle cell physiology. The analysis of mechanisms involved in skeletal muscle insulin resistance and its reversibility might lead to a better understanding of this process and a possible discovery of muscular targets for the treatment of type 2 diabetes.

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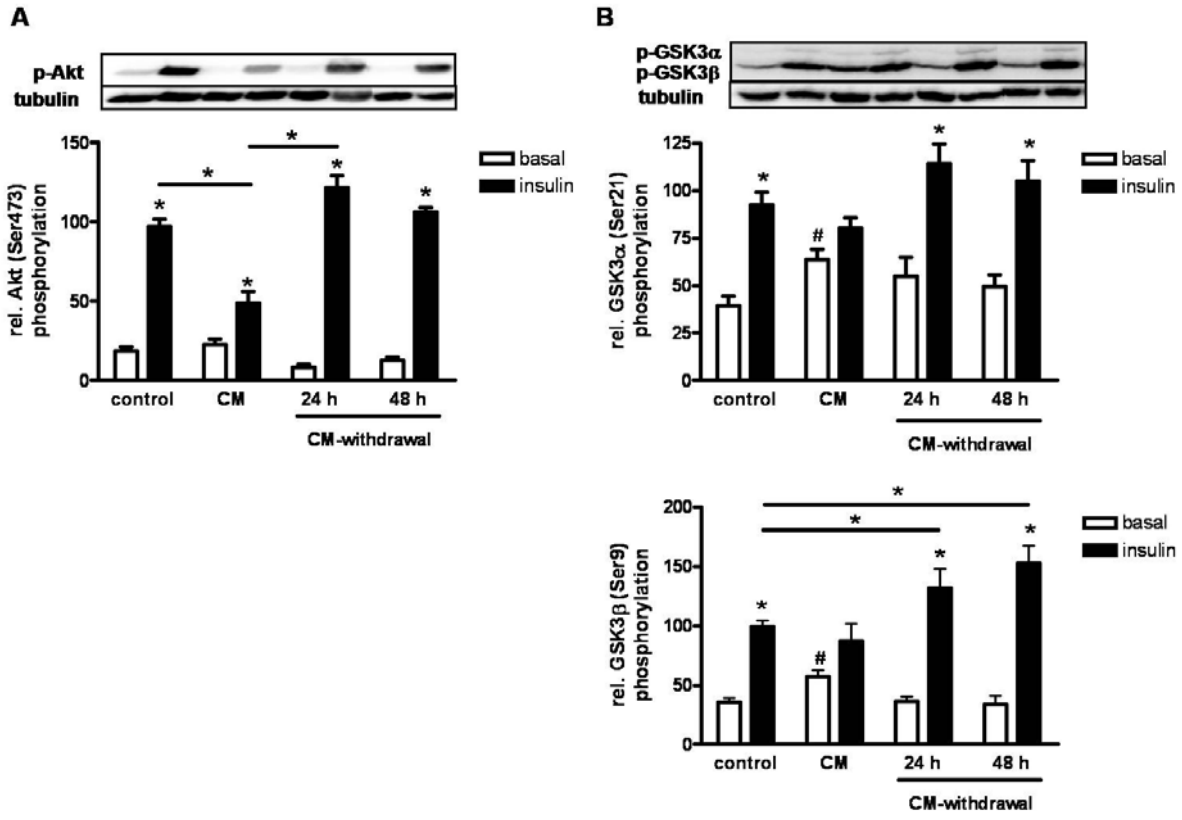

Fig. 3.1 Effect of adipocyte-conditioned medium (CM) on insulin-signalling in skeletal muscle cells. Differentiated skeletal muscle cells from 2-3 donors were treated with CM for 24 h and stimulated with insulin (100 nM, 10 min) directly or after regeneration for 24 or 48 h. 5 µg of total lysates were resolved by SDS-PAGE and blotted to PVDF membranes. Membranes were blocked with 5% milk in TBS containing 0.1% Tween-20 and incubated overnight with p-Akt (**A**) or p-GSK3 (**B**) antibodies. After incubation with the appropriate HRP-coupled secondary antibody the signal was detected by ECL. Signals were analyzed on a LUMI Imager Work Station. Data are tubulin normalised mean values ± SEM (n = 5-6).* significant insulin stimulation or significantly different from designated insulin-stimulated value, respectively. # significantly different from basal control.

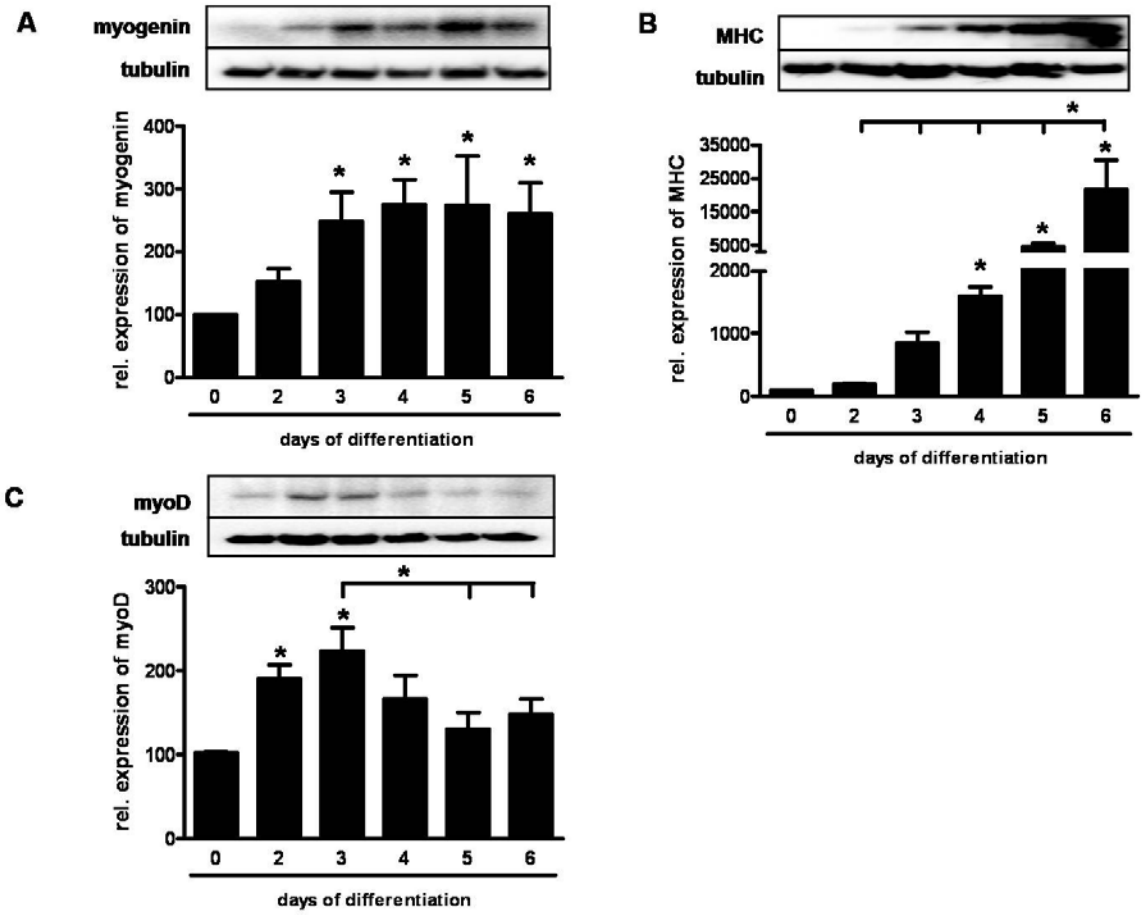

Fig. 3.2 Expression of differentiation markers in skeletal muscle cells. Myoblasts or skeletal muscle cells differentiated for 2-6 days from 4 donors were lysed and used for Western blots as described in Fig. 1. Bots were incubated overnight with myogenin (**A**), MHC (**B**) and myoD (**C**) antibodies. Data are tubulin normalized mean values ± SEM (n = 4). * significantly different from myoblasts or from designated values.

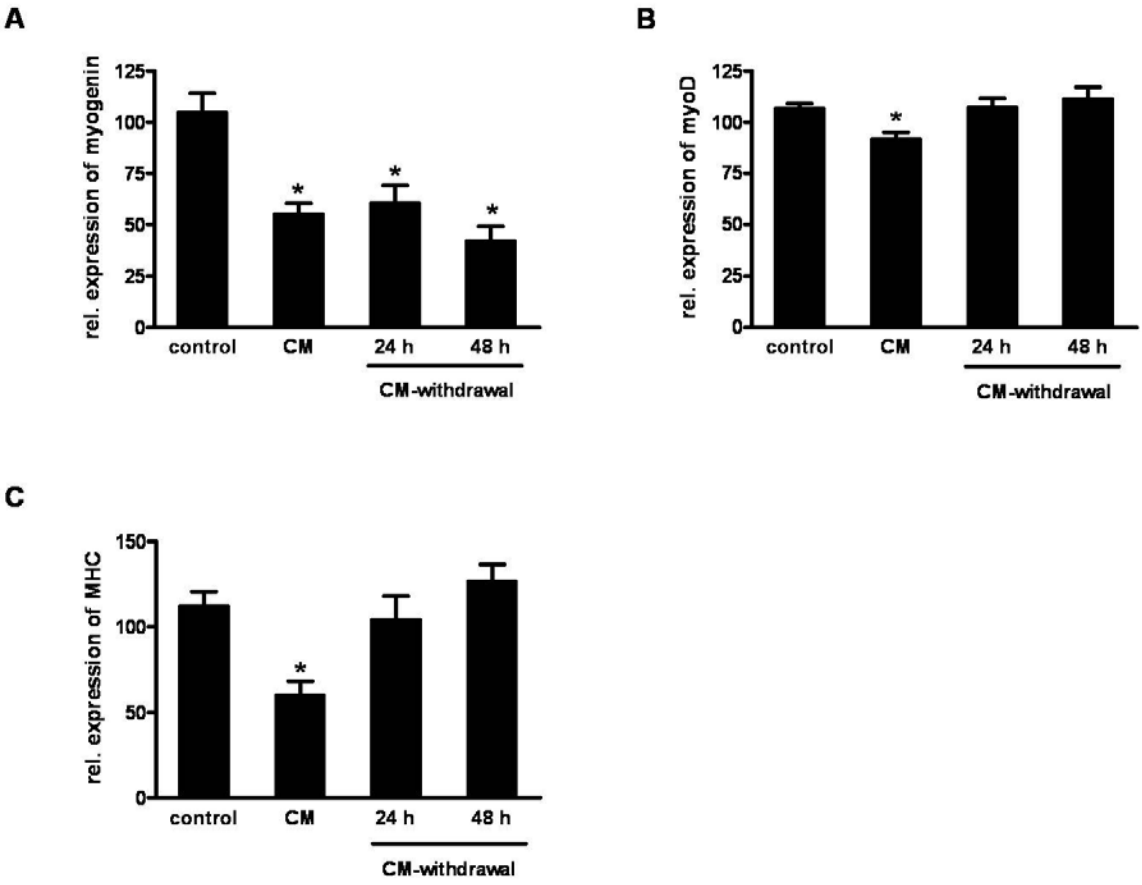

Fig. 3.3 Effect of CM-treatment and CM-withdrawal on myogenic markers. Differentiated skeletal muscle cells from 2-3 donors were treated with CM for 24 h and lysed directly or after regeneration for 24 or 48 h. Lysates were used for Western blot as described in Fig. 1 and detected with myogenin (**A**), MHC (**B**) and myoD (**C**) antibodies. Data are tubulin normalized mean values ± SEM (n = 6-12). * significantly different from control.

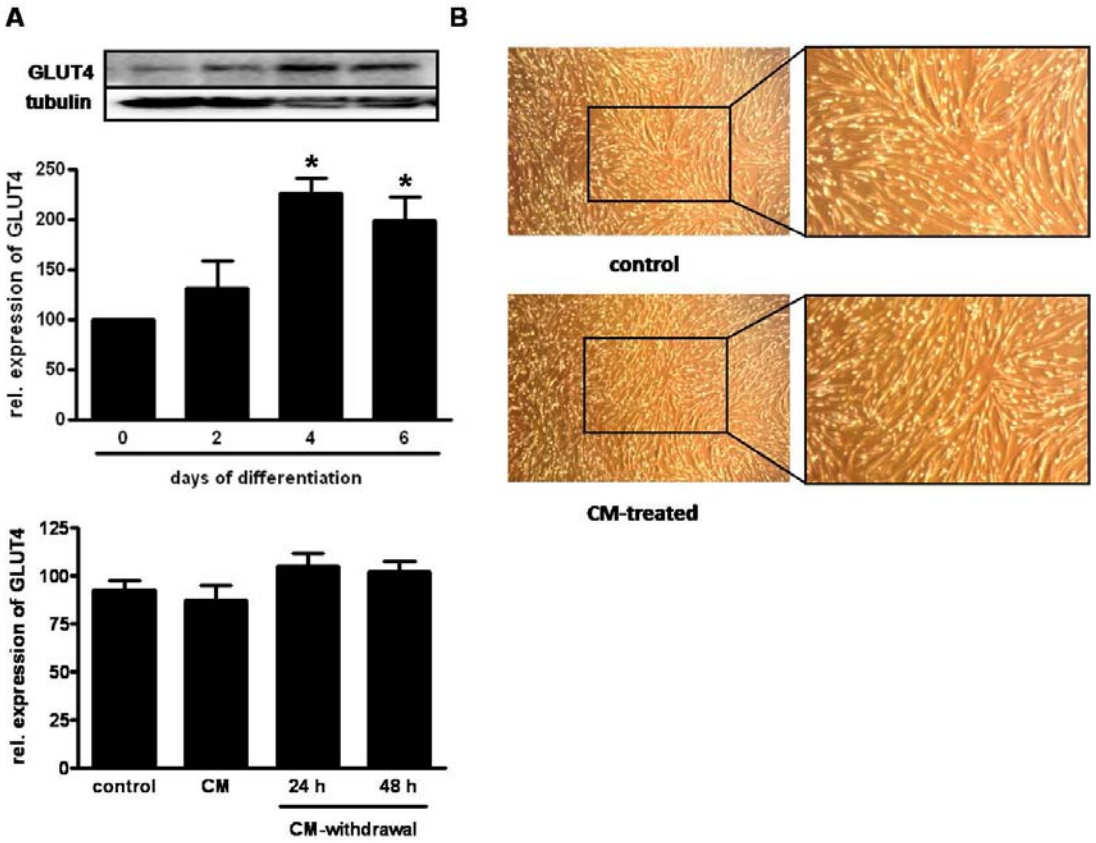

Fig. 3.4 GLUT4 expression and morphology of insulin-resistant skeletal myotubes. A: Myoblasts and differentiated skeletal muscle cells from 3 donors were analysed for GLUT4 expression during differentiation (upper panel) and differentiated skeletal muscle cells from 2-3 donors were treated with CM for 24 h (lower panel) and lysed. Lysates were used for Western blot as described in Fig. 2 and detected with a GLUT4 antibody. Data are tubulin normalized mean values ± SEM (n = 3 during differentiation and n = 6-12 for CM-treatment). * significantly different from day 0 of differentiation. **B**: Myotubes were treated with CM for 24 h and a representative micrograph showing unaltered myotube morphology in insulin-resistant skeletal muscle cells is presented. Magnification 4x.

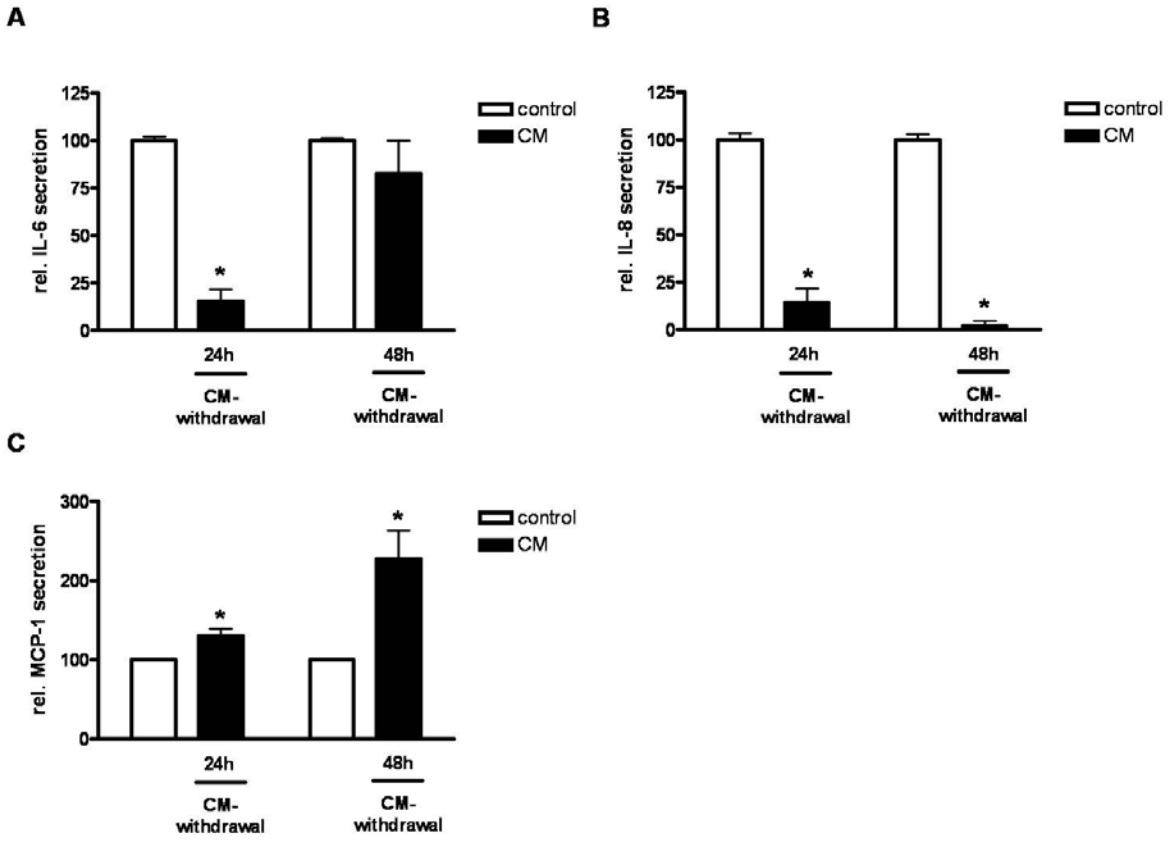

Fig. 3.5 Effect of CM-treatment on skeletal muscle cell secretion. Differentiated skeletal muscle cells from 2-3 donors were treated with CM for 24 h. After 2-times washing with PBS cells were given fresh differentiation medium for 24 h followed by medium collection. Differentiation medium was then added again for 24 h and collected for the 48h time point. IL-6 (**A**), IL-8 (**B**) and MCP-1 (**C**) secretion from the myotubes were analyzed by ELISA. Data are mean values ± SEM (n = 3-4). * significantly different from control.

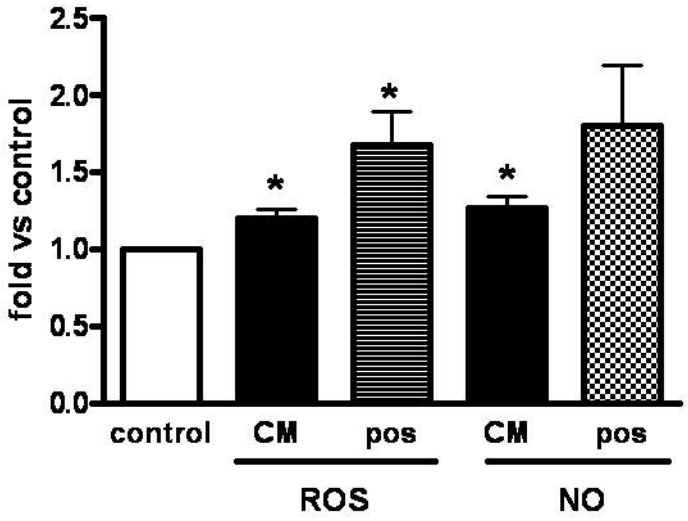

Fig. 3.6 Effect of CM-treatment on skeletal muscle ROS and NO production. Differentiated skeletal muscle cells from two donors were treated with CM for 24 h and subsequently analyzed for their capacity to produce ROS and NO as described in *Materials*. As a positive control, cells were treated 30 min prior to the beginning of the experiment with H_2O_2 and SNAP, respectively. Data are mean values ± SEM (n = 3-4). *significantly different from control.

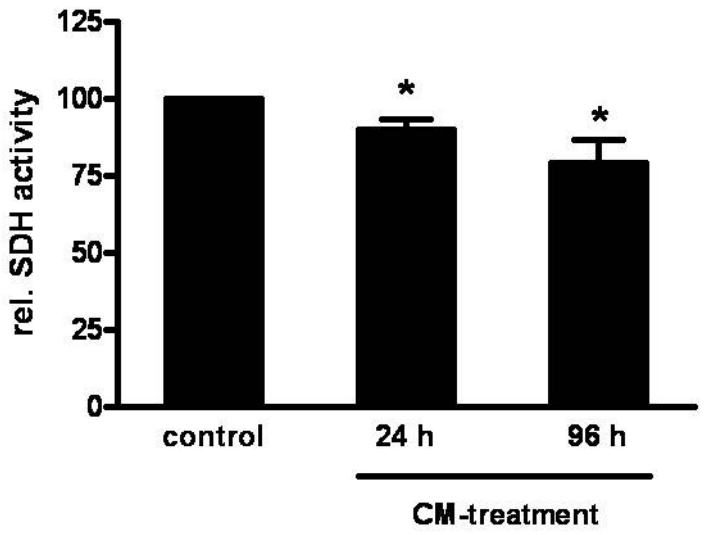

Fig. 3.7 Effect of CM-treatment on skeletal muscle SDH activity. Skeletal muscle cells from two different donors were treated with CM for 24 h or 96 h. Total cell lysates were analyzed for SDH activity as described in *Materials*. Data are mean ± SEM (n = 4). *significantly different from control.

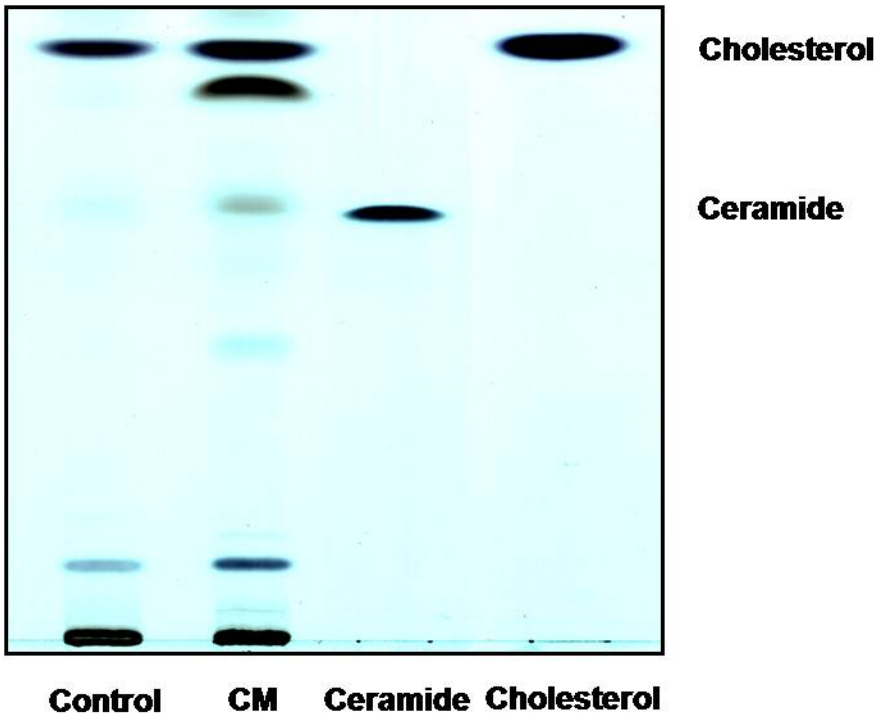

Fig. 3.8 Effect of adipocyte-conditioned medium on ceramide content in skeletal muscle cells. Cells were incubated overnight in control media or CM. Then cells were harvested and lipids extracted, separated by thin layer chromatography, and quantitatively evaluated as described in Material and Methods. Similar results were obtained in 3 different experiments.

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Chapter 4

Study 3

IGF-1 receptor signalling determines the mitogenic potency of insulin analogues in human smooth muscle cells and fibroblasts†

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Abstract

Aims/hypothesis Mitogenic activity of insulin and insulin analogues and the involvement of the IGF-1 receptor (IGF-1R) is still a controversial issue. We compared protein level of IGF-1R and insulin receptor (InsR) in fibroblasts and smooth muscle cells from healthy donors and assessed the downstream signalling and growth-promoting activity of insulin and insulin analogues.

Methods DNA synthesis was monitored in human fibroblasts and coronary artery smooth muscle cells. Using small interfering RNAs, the levels of IGF-1 and InsR were reduced by 95% and 75%, respectively

Results Enhanced mitogenic potency of insulin and insulin analogues was observed that correlated with increased level of IGF-1R and/or IRS-1. A reduction in IGF-1R levels significantly blunted stimulation of Akt phosphorylation by IGF-1, AspB10 and glargine by 72%, 58%

and 40%, respectively. Akt phosphorylation in response to insulin remained unaffected. Silencing of InsR did not significantly alter Akt phosphorylation in response to IGF-1, AspB10 and glargine. Enhanced stimulation of DNA synthesis by IGF-1 and glargine was reduced to a level identical to that of insulin by IGF-1R knockdown.
Conclusions/interpretation These data show a prominent role of IGF-1R/Akt signalling in mediating the mitogenic effects of insulin analogues. Regular insulin stimulates DNA synthesis by exclusively activating InsR, whereas insulin analogues mainly signal through IGF-1R. It is suggested that inter-individual differences in the protein level of the IGF-1R system may function as a critical determinant of the mitogenic potency of insulin analogues.

Keywords IGF-1 receptor, Insulin analogues, Insulin receptor, Mitogenic potential, Smooth muscle cells

Abbreviations

ECL	enhanced chemiluminescence
ERK1/2	extracellular regulated kinase 1/2
Fib	human dermal fibroblasts
HRP	horseradish peroxidase
IGF-1R	IGF-1 receptor
InsR	insulin receptor
siRNA	small interfering RNA
SkM	human skeletal muscle cells
SMC	human coronary artery smooth muscle cell
TBS	Tris-buffered saline

Introduction

A variety of studies have documented significant improvements in the therapy of type 1 and 2 diabetes due to the introduction of different insulin analogues into clinical use [1-7]. Advantages of short-acting artificial insulin molecules compared with conventional insulin preparations are better blood glucose control, faster onset of action and the short duration time [8]. These effects have been described for patients with type 1 and type 2 diabetes [8,9]. The long-acting insulin analogue glargine forms depots at the injection site by precipitation and is therefore slowly absorbed into the systemic circulation. In clinical studies it has been shown that treatment with glargine leads to less nocturnal hypoglycaemia, comparable or improved efficiency and minimal variability between patients compared with conventional insulin preparations [10,11].

Despite these advantages, some questions concerning the safety of insulin analogues remain open. It is known that modifications of the insulin molecule in the B10 and B26–B30 region are able to alter the affinity towards the IGF-1 receptor (IGF-1R) [12]. This has been demonstrated for AspB10 , which is known for its strong tumourigenic action [13]. Generally, the mitogenic potential of a certain insulin analogue may result from (1) an enhanced affinity towards IGF-1R [12]; (2) the time of occupancy of the insulin receptor (InsR) by this analogue [14,15]; and (3) a combination of IGF-1- and InsR-mediated processes.

Because of the high degree of similarity, insulin/IGF-1 hybrid receptors can form by heterodimerisation [16]. The abundance of these hybrid receptors has been shown in a range of mammalian tissues [17-19]. There are controversial debates ongoing concerning the question whether InsR, IGF-1R and/or hybrid receptors play a role in mediating the biological effects of insulin analogues. Kurtzhals et al. [20] used the osteosarcoma cell line Saos/B10 to determine the

mitogenic potency of insulin analogues and reported glargine to be eightfold more potent than human insulin in stimulating DNA synthesis. On the other hand, Berti et al. [21] analysed the mitogenic effect of glargine in rat fibroblasts overexpressing the human InsR and could not find differences between human insulin and glargine. Most of these studies were done in tumour cell lines or in cell systems overexpressing the human InsR and thus do not represent the normal status of primary human cells. Recently, Staiger et al. [22] reported that glargine and human insulin are not different in their proliferative effects on human coronary artery endothelial and smooth muscle cells.

In the present investigation we have tested the proliferative effects of insulin, AspB10, glulisine, lispro, aspart and glargine by measuring DNA synthesis in human dermal fibroblasts (Fib) and coronary artery smooth muscle cells (SMC) from different donors. We observed a mitogenic potency for insulin and the tested insulin analogues that correlated with a high protein level of the IGF-1R system. In vitro knockdown of IGF-1R reduced the augmented growth-promoting activity of glargine and IGF-1 to the level of that of insulin. It is suggested that the protein level of the IGF-1R system may define an individual susceptibility towards the growth-promoting effects of insulin analogues.

Materials and methods

Materials Fib, SMC, skeletal muscle cells (SkM) and the appropriate cell culture media were purchased from Promocell (Heidelberg, Germany). FCS was supplied by Gibco (Invitrogen, Carlsbad, CA, USA). The Cell Proliferation ELISA (BrdU, chemiluminescent) and protease inhibitor cocktail tablets were from Roche (Mannheim, Germany). Human IGF-1 was obtained from Biomol (Hamburg, Germany). Regular human insulin and the insulin analogues AspB10, glulisine, lispro, aspart and glargine were products of Sanofi-Aventis (Frankfurt, Germany). Reagents for SDS-PAGE were

supplied by Amersham Pharmacia Biotech (Braunschweig, Germany) and by Sigma (Munich, Germany). The following antibodies were used: anti-IGF-1Rβ (C-20; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA); anti-InsRβ, anti-α-tubulin and anti-actin (Calbiochem/Merck, Darmstadt, Germany); anti-IRS-1 (Upstate/Millipore, Billerica, MA, USA); anti-Akt, anti-phospho-Akt (Ser473) and anti-extracellular regulated kinase (ERK)1/2 (Cell Signaling Technology, Danvers, MA, USA); and horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgG (Promega, Mannheim, Germany). Greiner Bio-one (Frickenhausen, Germany) supplied the Leucosep tubes; the Biocoll solution (density 1.077 g/ml) was from Biochrom (Berlin, Germany). HiPerFect transfection reagent and non-silencing control small interfering RNA (siRNA) were obtained from Qiagen (Hilden, Germany), Silencer-validated siRNA specific for human IGF-1R and human InsR were products of Ambion (Austin, TX, USA). All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

Cell culture Primary Fib of two healthy donors (white, female, 52 and 32 years [F52, F32]) and primary SMC of four healthy donors (white, male, 40 and 21 years [M40, M21]; female, 56 and 16 years [F56, F16]) were supplied as proliferating cells (5×10^5 cells) and kept in culture according to the manufacturer's protocol. For the experiments, confluent cells between passage number 5 and 7 were used. SkM of one donor (male, 9 years) were differentiated to myotubes according to the manufacturer's protocol. Lysates were prepared as described below on day 6 of differentiation and used as the reference for comparing the various protein levels of different cell types and donors. MCF-7 and MDA-MB-231 human mammary tumour cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured as detailed by the manufacturer.

In vitro analysis of growth-promoting activity To monitor DNA synthesis, Fib or SMC were seeded in 96 well plates and allowed to attach for 24 h, followed by serum starvation for 30 h (Fib) or 24 h (SMC). Cells were then stimulated for 16 h with the different peptide hormones with the simultaneous addition of BrdU. For the analysis of cell proliferation we used the chemiluminescent BrdU ELISA kit from Roche according to the manufacturer's protocol. The signals were visualised and evaluated on a LumiImager work station using image analysis software (Boehringer, Mannheim, Germany).

Immunoblotting For analysis of the protein levels of InsR, IGF-1R, IRS-1, Akt and ERK1/2 in SMC and Fib, cells were seeded in Petri dishes and grown to confluence. Lysates were prepared with ice-cold lysis buffer containing 50 mmol/l HEPES (pH 7.4), 1% Triton X-100 (vol./vol.), 1 mmol/l Na_3Vo_4 and protein inhibitor cocktail. After incubation for 2 h at 4°C the suspension was centrifuged at 10,000 *g* for 20 min. Ten or 20 micrograms of protein were loaded per sample, separated by SDS-PAGE using horizontal gels and transferred to polyvinylidene fluoride membranes. The membranes were blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (vol.vol.) and 5% (wt/vol.) non-fat dry milk or 5% (wt/vol.) BSA and then incubated overnight with the appropriate antibodies. After repeated washing, the membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The Immobilon HRP substrate from Millipore (Billerica, MA, USA) was used for enhanced chemiluminescence (ECL) detection. The signals were visualised and evaluated on a LumiImager work station.

Isolation of peripheral mononuclear cells Fresh human whole-blood samples were collected from 16 healthy donors and diluted 1:2 with balanced salt solution. The separation of lymphocytes and peripheral mononuclear cells based on density gradient centrifugation was performed with Leucosep tubes (Greiner Bio-one) according to the

manufacturer's protocol. After centrifugation, the cell suspension was washed with 3×10 ml PBS and centrifuged for 10 min at 600 *g*. The resulting pellet was resuspended in 1 ml PBS, the cells were then plated on six well plates and incubated at 37°C for 4 h to allow the monocytes to attach. After this, cells were washed with 4×1 ml PBS to remove the lymphocytes and lysed with ice-cold lysis buffer as described above. Twenty micrograms of protein per sample were processed by SDS-PAGE and western blotting. The membrane was incubated with anti-IGF-1R overnight and processed as described above. After detection of the signal the membrane was stripped and re-incubated with anti-actin to confirm equal loading.

Silencing of IGF-1R and InsR in Fib Fib of donor F52 were seeded in six well plates and washed with fibroblast basal medium containing 1% (vol./vol.) FCS after 24 h. The siRNA was used at 20 nmol/l for IGF-1R and 10 nmol/l for InsR with HiPerFect as transfection reagent according to the manufacturer's protocol. The applied siRNA was either non-silencing RNA as negative control or siRNA specifically silencing IGF-1R (IGF-1R siRNA) or InsR (InsR siRNA). Lysates for immunoblotting were prepared 72 h after transfection and the level of IGF-1R, InsR, Akt and ERK1/2 was analysed.

After silencing IGF-1R or InsR, stimulation experiments were performed to analyse possible changes of downstream signalling pathways. Forty-eight hours after transfection with the appropriate siRNA, the medium was changed to fibroblast basal medium for 24 h starvation. Cells were then stimulated with regular insulin (100 nmol/l), IGF-1 (10 nmol/l), AspB10 (100 nmol/l) and glargine (100 nmol/l) for 15 min. Cells were washed once with ice-cold PBS and scratched into ice-cold lysis buffer containing 50 mmol/l Tris-HCl (pH 7.4), 1% NP-40 (vol.vol.), 0.25% sodium deoxycholate (wt./vol.), 150 mmol/l NaCl, 1 mmol/l EGTA, 1 mmol/l Na_3Vo_4, 1 mmol/l NaF and protein inhibitor cocktail. After incubation for 2 h at 4°C the suspension was centrifuged at 10,000 *g* for 20 min. Immunoblotting

was performed as outlined above. The membranes were blocked with TBS containing 0.1% Tween-20 (vol./vol.) and 5% BSA (wt/vol.). Incubation with anti-phospho-Akt was overnight, incubation with the secondary antibody for 1 h. After detection, membranes were incubated with anti-□-tubulin for normalisation.

To analyse the effect of IGF-1R silencing on growth-promoting activity, cells were seeded on 96 well plates and allowed to attach for 24 h. Transfection with non-silencing RNA and IGF-1R siRNA was performed as described above. After 24 h, the medium was changed to fibroblast basal medium to starve the cells for 30 h. Cells were then stimulated for 16 h with either regular insulin, IGF-1 or glargine (10 nmol/l), in the presence of BrdU, as detailed above. All experiments were performed in quadruplicate. Cells were then processed for LumiImager analysis, as outlined before.

Presentation of data and statistics Statistical analysis was carried out by ANOVA. All statistical analyses were done using StatView software (SAS Institute, Cary, NC, USA). A p value of less than 0.05 was considered to be statistically significant. Significance levels are indicated in the figures. Data are shown as means±SEM.

Results

Protein level analysis of the IGF-1R pathway in primary cells of different donors To establish a cell culture model of primary human cells with a defined abundance of IGF-1R and its downstream signalling components, we analysed the protein level of IGF-1R, InsR, IRS-1, Akt and ERK1/2 in Fib and SMC of different healthy donors. For comparison, SkM and the human breast tumour cell lines MCF-7 and MDA-MB-231 were included.

As shown in Fig. 1, Fib and SMC have remarkably lower levels of IGF-1R and InsR than the tumour cells. For IGF-1R, both cell types are more or less comparable with SkM, but we found obvious

differences between the donors. For SMC, two donors (F56, M21) showed a three- to fourfold higher level of IGF-1R compared with donor M40 and a two- to threefold higher level compared with donor F16 (Fig. 1, upper panel). For Fib donor F52 we found a twofold higher level than for F32. Furthermore, InsR was upregulated in SMC donor F56 and M21 (Fig. 1, middle panel) with M21 having the most abundant InsR level among the SMC donors. The Fib donors express much less InsR than do SkM or SMC and do not show differences in the protein level.

These individual differences were also observed further downstream at the protein level of IRS-1 (Fig. 1, lower panel). Again, donors of Fib and SMC show marked differences among themselves (Fib F32: 278±96% and F52: 648±218%; SMC M40: 126±11%, F56 289±170%, F16: 314±55% and M21: 995±247%). The differences reached statistical significance between the Fib donors and between SMC donor M21 and the other SMC donors. For Akt, the Fib donors were significantly different from each other, as also observed for some SMC donors (Fig. 2, upper panel). However, these differences were less prominent compared with IGF-1R and IRS-1 (see Fig. 1). For ERK1/2, no significant differences between the different Fib and SMC donors could be detected (Fig. 2). In subsequent experiments (see Figs 6-8), Fib F52 was used as a model of potential augmented activity of the IGF-1 signalling pathway.

Additionally, we isolated human monocytes from whole-blood samples to analyse the variation of IGF-1R protein level in a larger group of 16 donors. As shown in Fig. 3, we observed a substantial natural variation in the protein level of IGF-1R. Four donors exhibit a very prominent level of IGF-1R compared with the other donors, whereas in some other donors IGF-1R was even undetectable. There was no obvious correlation between sex, age and the level of IGF-1R protein. These data are consistent with our findings in Fib and SMC and support the notion that IGF-1R may be subject to a substantial individual variation.

Growth-promoting activity of insulin analogues in SMC and Fib In order to assess if the differences in the protein level of the IGF-1R system may be critical for the proliferative activity of insulin and insulin analogues, we monitored DNA synthesis in SMC and Fib using the incorporation of BrdU and a highly sensitive chemiluminescence immunoassay. As presented in Fig. 4a, for SMC donor F56 (high level of IGF-1R) all insulin analogues and insulin itself induced a significant stimulation of DNA synthesis, at least at 100 nmol/l. At this concentration, the growth-promoting activity of glargine was comparable with AspB10 and significantly higher than regular insulin. Similar results were found for M21, with glargine being slightly more potent than AspB10 and reaching significance compared with regular insulin at 100 nmol/l (Fig. 4b). Also, the absolute level of insulin-stimulated DNA synthesis at 100 nmol/l compared with basal was higher than in any other donor (183% vs 138% (F56), 145% (M40) and 123% (F16), Fig. 4a–d), most likely due to the high level of InsR protein (see Fig. 1). Donor F16 showed significant stimulation of DNA synthesis after treatment with 100 nmol/l insulin and all insulin analogues except aspart (Fig. 4c). For SMC M40 (Fig. 4d) neither insulin nor the insulin analogues glulisine and glargine were able to produce a significant stimulation of DNA synthesis, while for AspB10, lispro and aspart this was observed. Furthermore, in M40 and F16 (low level of IGF-1R protein) none of the tested analogues was significantly different from insulin. We therefore conclude that IGF-1R is of critical importance for the mitogenic activity of insulin analogues.

Additional evidence for this notion was obtained by measuring the dose-dependent stimulation of DNA synthesis after incubation with regular insulin, AspB10 and glargine in the Fib donors F52 and F32. In F52 (high level of IGF-1R and IRS-1) glargine was significantly more mitogenic than regular insulin at concentrations between 5 and

100 nmol/l but was not different from insulin in F32 (Fig. 5) at high concentrations of the peptide.

Silencing of IGF-1R and InsR and functional implications for the growth-promoting activity of insulin analogues To directly investigate the role of IGF-1R vs InsR in the augmented proliferative effect of insulin analogues, we established the in vitro knockdown of IGF-1R and InsR in Fib of donor F52. Figure 6 shows the quantification of protein levels of IGF-1R as well as InsR, Akt and ERK1/2 72 h after transfection. The protein level of the non-silencing RNA control was set to 100%. For IGF-1R we observed a downregulation of protein level to 4% of control levels after 72 h (Fig. 6a), while no significant changes in InsR, Akt or ERK1/2 protein levels (Fig. 6) were observed. However, the knockdown of InsR to 25% of control levels (Fig. 6b) raised IGF-1R level to 125% of the non-silencing RNA control, while Akt and ERK1/2 level remained unchanged.

We then tested the influence of IGF-1R and InsR silencing on signal transduction after acute stimulation with regular insulin, IGF-1, AspB10 and glargine (Fig. 7). The resulting phospho-signal after 15 min insulin incubation in 'no transfection' was set to 100% and all other signals were referred to it. Akt phosphorylation in response to IGF-1, AspB10 and glargine was significantly higher compared with insulin (Fig. 7a). After silencing IGF-1R, the stimulation of Akt phosphorylation by IGF-1, AspB10 and glargine was significantly reduced by 72, 58 and 40%, respectively. No changes of Akt activation were observed for insulin treatment (Fig. 7c). Reduction of InsR protein to 25% reduced the phosphorylation of Akt after acute insulin stimulation by 40% (Fig. 7d), while stimulation with IGF-1, AspB10 and glargine produced an unaltered response under these conditions.

Stimulation of DNA synthesis by regular insulin, IGF-1 and glargine in IGF-1R-silenced Fib is presented in Fig. 8. As an additional positive control we used fibroblast basal medium

supplemented with 5% FCS. Basal BrdU incorporation was set to 100% and used as reference. In the control situations, incubation of Fib with 5% FCS, IGF-1 and glargine resulted in a three- to fourfold enhanced growth-promoting activity compared with regular insulin. Silencing of IGF-1R reduced the stimulation of DNA synthesis in response to IGF-1 and glargine by 60-80% to the level comparable with that of insulin. The BrdU incorporation in response to 5% FCS and insulin itself remained unaffected.

Discussion

Proliferation and migration of smooth muscle cells in response to growth factors represents a key step in the initiation of atherosclerosis [23] and it has been shown that IGF-1 may exert a prominent stimulatory action on this process [24]. Some insulin analogues were reported to exhibit a markedly increased IGF-1R affinity and mitogenic potency, which may represent a still undefined safety risk [20] regarding atherogenesis. In the present study we have used primary human cells with a broad range of protein level of the IGF-1 signalling system. Our data identify IGF-1R as the key mediator of the augmented growth-promoting activity of insulin analogues in SMC and Fib.

A key finding of the present investigation is the observation that all tested insulin analogues and insulin itself can exhibit enhanced growth-promoting activity. Importantly, these results were strictly donor-specific and correlated to the protein level of IGF-1R, InsR and/or IRS-1. It has been shown that the level of IRS-1 and InsR varies in different tissues of healthy human individuals [25,26]. Furthermore, we found high variation of IGF-1R level in monocytes isolated from a randomly chosen group of 16 healthy donors. In our study we found that Fib and SMC with higher IGF-1R, InsR and IRS-1 level had an increased DNA synthesis when stimulated with insulin or any tested insulin analogue. The mitogenic potency of insulin itself

is much more dependent on the protein level of InsR, as observed in several donors and thoroughly supported by our IGF-1R knockout data (see Fig. 8). We therefore hypothesise that an elevated level of the IGF-1R system may function as a critical determinant of the mitogenic potency of insulin analogues. This would also imply an individual susceptibility to the mitogenic effects of insulin analogues. Clearly, our study is limited to four donors of SMC and two donors of Fib and it remains to be shown if an elevated protein level of the IGF-1R system is relevant in a larger population. On the other hand, our data obtained in monocytes lend support to this notion. Our findings are in sharp contrast to a recent publication by Berti et al. [22], in which they studied the proliferative and anti-apoptotic effects of regular insulin and glargine in human coronary artery endothelial and smooth muscle cells. Both peptides were found to be unable to stimulate DNA synthesis even at concentrations up to 100 nmol/l. It was concluded that neither insulin nor glargine may contribute to the development of atherosclerosis [22]. However, the levels of IGF-1R and IRS-1 were not determined and a detailed analysis of separate donors was not presented. Our results with SMC donor M40 and F16 (low protein level of IGF-1R) are indeed consistent with the data of Staiger et al. [22]; however, completely different findings were obtained with SMC donor F56 and M21. These considerations support our notion that the individual protein level of IGF-1R is critical for the growth-promoting activity of insulin analogues and of insulin itself. We further conclude that a potential atherogenic potency of glargine cannot be ruled out.

The molecular basis of the enhanced mitogenic potency of insulin analogues has remained a controversial issue, with data favouring either the insulin or the IGF-1R signalling pathway [27]. Using in vitro knockdown of IGF-1R and InsR, we here provide direct evidence for a key role of the IGF–InsR/Akt signalling pathway in the augmented growth-promoting activity of insulin analogues. IGF-1, AspB10 and glargine produced a prominent activation of Akt and

were significantly more potent than regular insulin. This is consistent with the well established role of Akt in the regulation of DNA synthesis, cell proliferation and cell survival [25-27]. Knockdown of IGF-1R by >95% resulted in a substantially decreased Akt phosphorylation upon treatment with IGF-1, AspB10 and glargine. The remaining phosphorylation signal is probably due to signalling through InsR. Most importantly, silencing of IGF-1R significantly reduced the effect of IGF-1 and glargine on DNA synthesis to the level identical to that observed in response to insulin. The results obtained by knockdown of InsR further support our notion that the mitogenic activity of insulin analogues is mediated by IGF-1R. Despite reducing InsR protein by 75% the phosphorylation of Akt after acute stimulation with AspB10 and glargine remained at a high level, while the insulin-stimulated phosphorylation was significantly reduced by 40%. The remaining Akt phosphorylation signal is probably due to incomplete knockdown of InsR protein. To the best of our knowledge this is the first direct demonstration that the enhanced growth-promoting activity of glargine is mediated by IGF-1R in primary human cells.

Our results also indicate that regular insulin may have a higher specificity for its own receptor than AspB10, glargine and IGF-1. This is based on the observation that silencing of IGF-1R modified neither Akt activation nor DNA synthesis in response to insulin up to 100 nmol/l, which would indicate that insulin acts exclusively through its own receptor under these conditions. This is consistent with the data of Li et al. [28], who reported that insulin at physiological concentrations selectively activates InsR and subsequently Akt, ERK1/2 and endothelial nitric oxide synthase. At high concentrations they found that IGF-1Rβ is also phosphorylated; however, despite blocking the receptor with antibodies Akt phosphorylation in response to insulin remained unchanged. We suggest that insulin analogues act through both InsR and IGF-1R. Under these conditions the affinity of a certain insulin analogue towards IGF-1R becomes

increasingly important. It has been shown that AspB10 and glargine have a six- to eightfold and insulin lispro a 1.5-fold higher affinity for IGF-1R than normal insulin [20], while aspart [29] and glulisine [30,31] have a low affinity for IGF-1R, like normal insulin. Combining an insulin analogue with increased affinity for IGF-1R with an elevated level of IGF-1R protein may be a critical safety issue which needs to be further investigated.

In summary, we show here that regular insulin exhibits a growth-promoting activity on SMC depending on the protein level of the insulin and the IGF-1 signalling pathway. The enhanced growth-promoting activity of insulin analogues is exclusively mediated by IGF-1R and further studies on a potential safety risk of these molecules are urgently required.

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

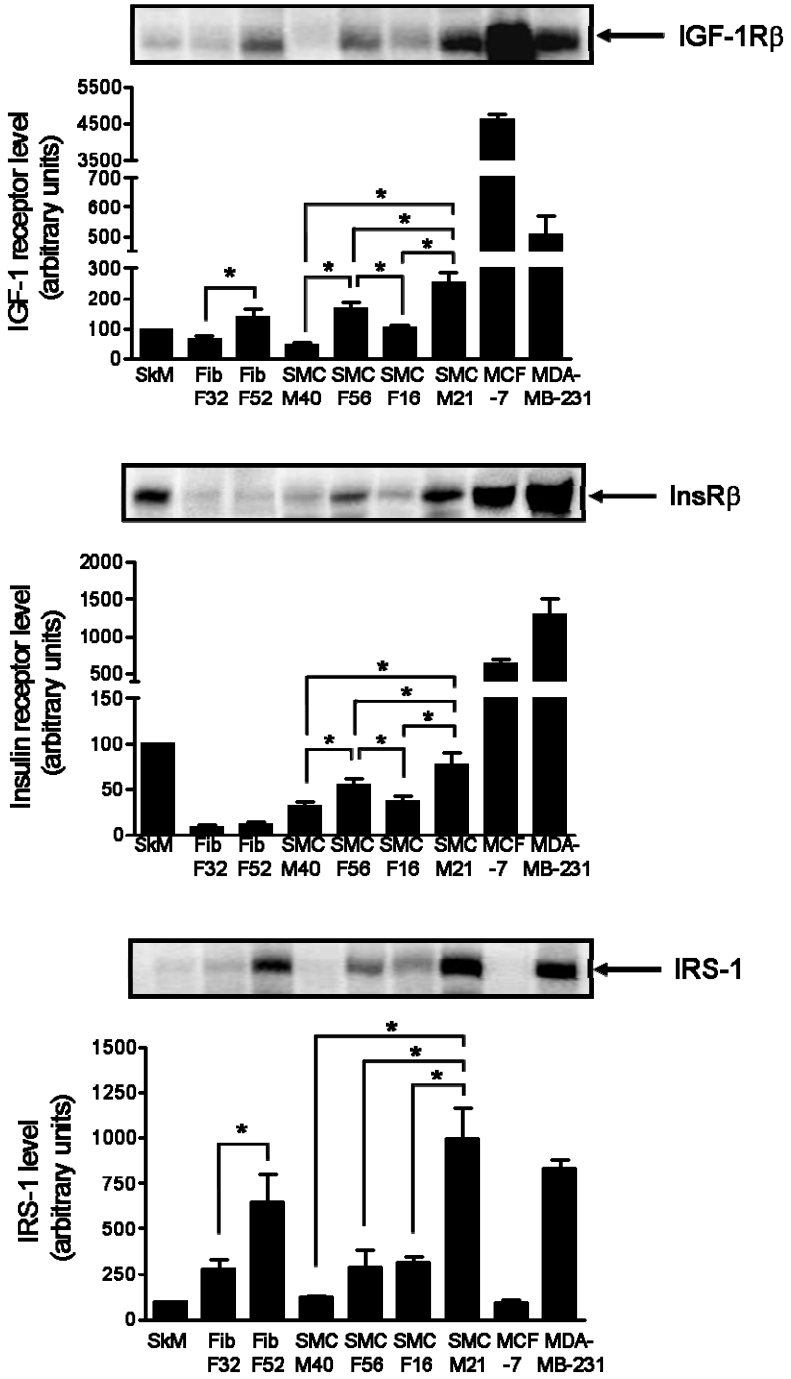

Fig. 4.1 Level of IGF-1R, InsR and IRS-1 in different cell types and donors. Whole-cell lysates were resolved by SDS-PAGE, blotted and immunodetected with antibodies against IGF-1Rβ, InsRβ or IRS-1 using the ECL system. Representative blots are shown. Quantification was performed on a LumiImager work station. Protein level of differentiated SkM was used as the reference and set to 100%. The data are presented as means±SEM, $n \geq 3$. *$p<0.05$ for difference in protein levels between donors of the same cell type.

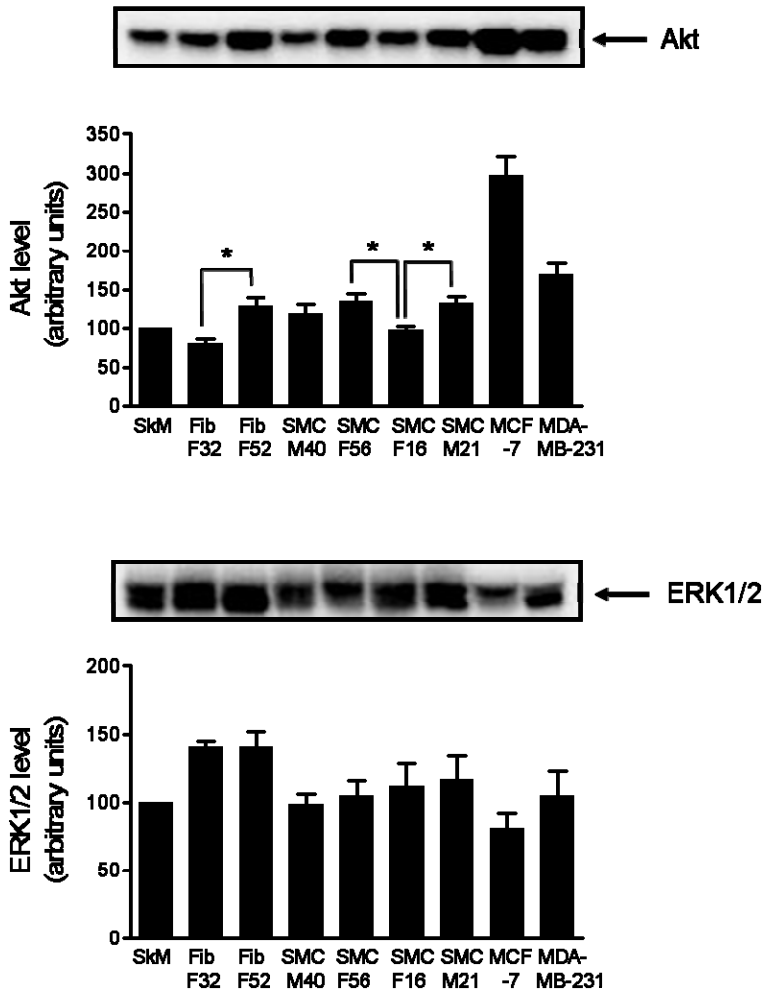

Fig. 4.2 Levels of Akt and ERK1/2 in different cell types and donors. Cell lysates were immunoblotted for Akt and ERK1/2 and quantification was performed as outlined in Fig. 1. The data are presented as means±SEM, n≥3. **p*<0.05 for difference in protein levels between donors of the same cell type.

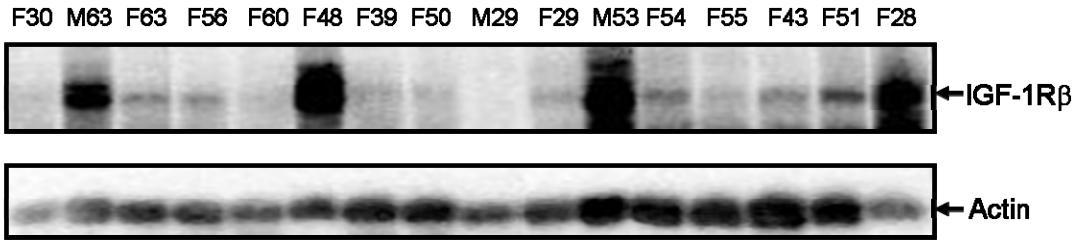

Fig. 4.3 Level of IGF-1R in human monocytes. The cells were isolated from fresh whole-blood samples of 16 randomly chosen healthy donors. Cell lysates were resolved by SDS-PAGE, blotted and immunodetected with antibodies against IGF-1Rβ and actin using the ECL system. The labels above the lanes indicate the sex (M, male; F, female) and age of the donors.

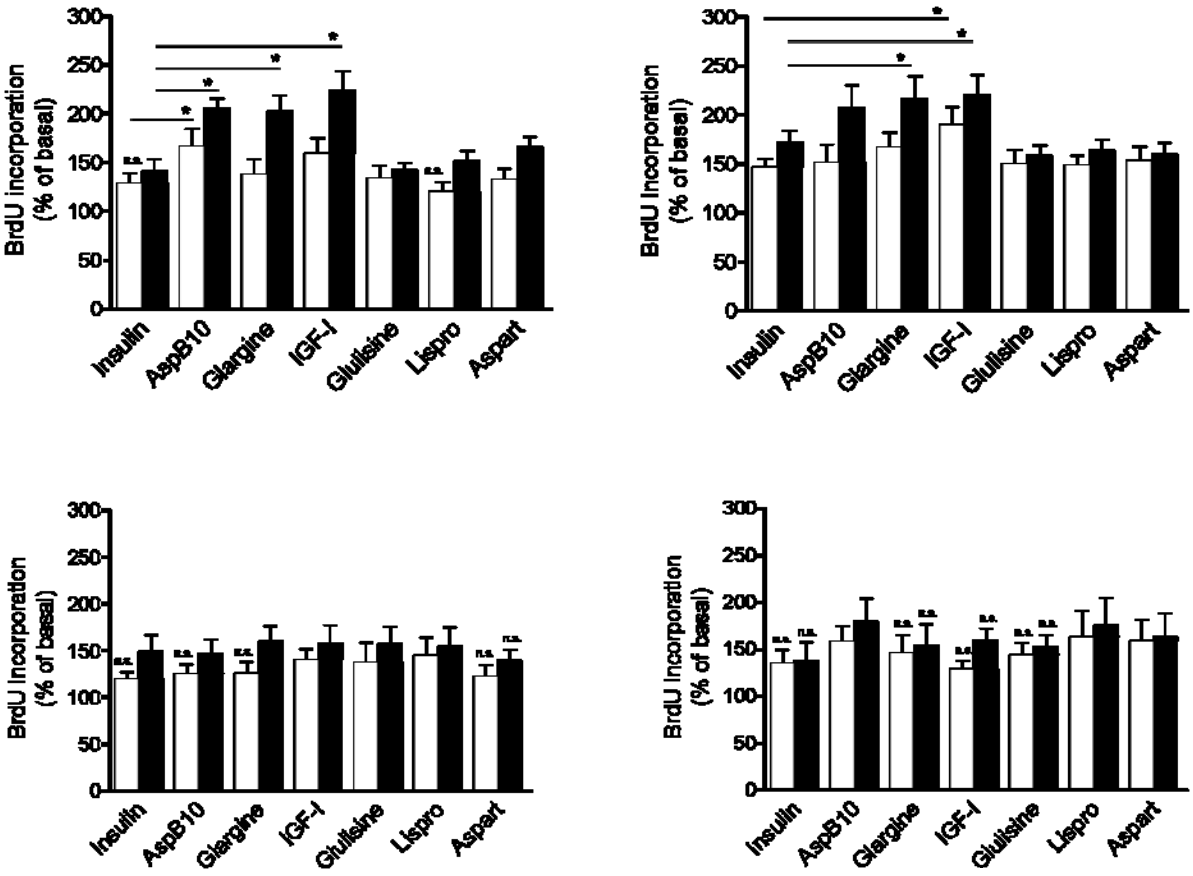

Fig. 4.4 Effect of regular insulin, AspB10, glargine, IGF-1, glulisine, lispro and aspart on the incorporation of BrdU into DNA in SMC from donor F56 (a), donor M21 (b), donor F16 (c) and donor M40 (d). Cells were serum starved for 24 h and subsequently incubated with BrdU in the absence or presence of peptide hormones for 16 h (open bars, 10 nmol/l; closed bars, 100 nmol/l. For IGF-1: open bars, 2 nmol/l; closed bars, 10 nmol/l). Cells were fixed and denatured and the incorporation of BrdU was determined using an anti-BrdU antibody and ECL detection. Signals were quantified using LumiImager software. Data are expressed relative to the basal control value, which was set as 100%. The data are presented as means±SEM, n≥7. n.s., no significant stimulation over basal; **p*<0.05 vs regular insulin.

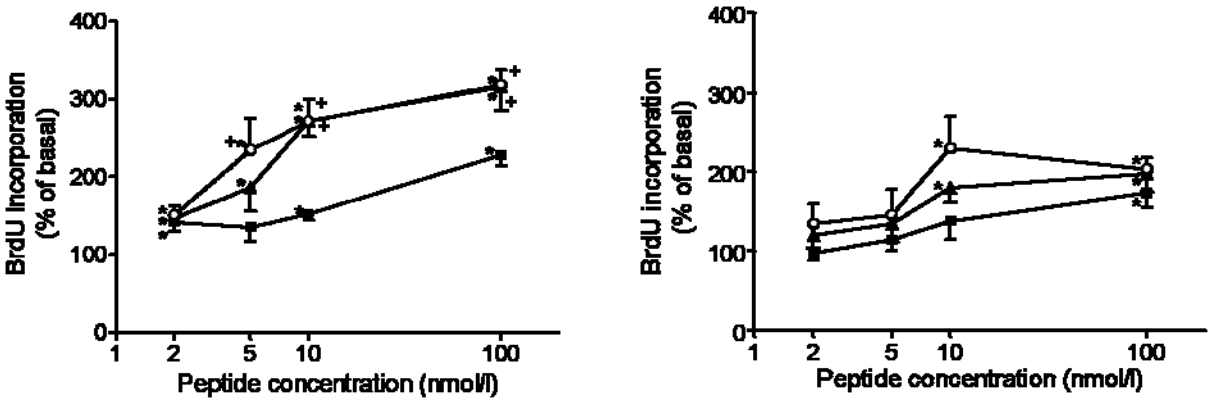

Fig. 4.5 Dose–response curve of BrdU incorporation into DNA in Fib of two different donors, F52 (a) and F32 (b), after stimulation with insulin (squares), AspB10 (triangles) or glargine (circles). Cells were serum starved for 30 h and stimulated with regular human insulin or the indicated insulin analogue. BrdU incorporation was determined as outlined in Fig. 3. The data are presented as means±SEM, n≥5. *p<0.05 vs basal; †p<0.05 vs regular insulin.

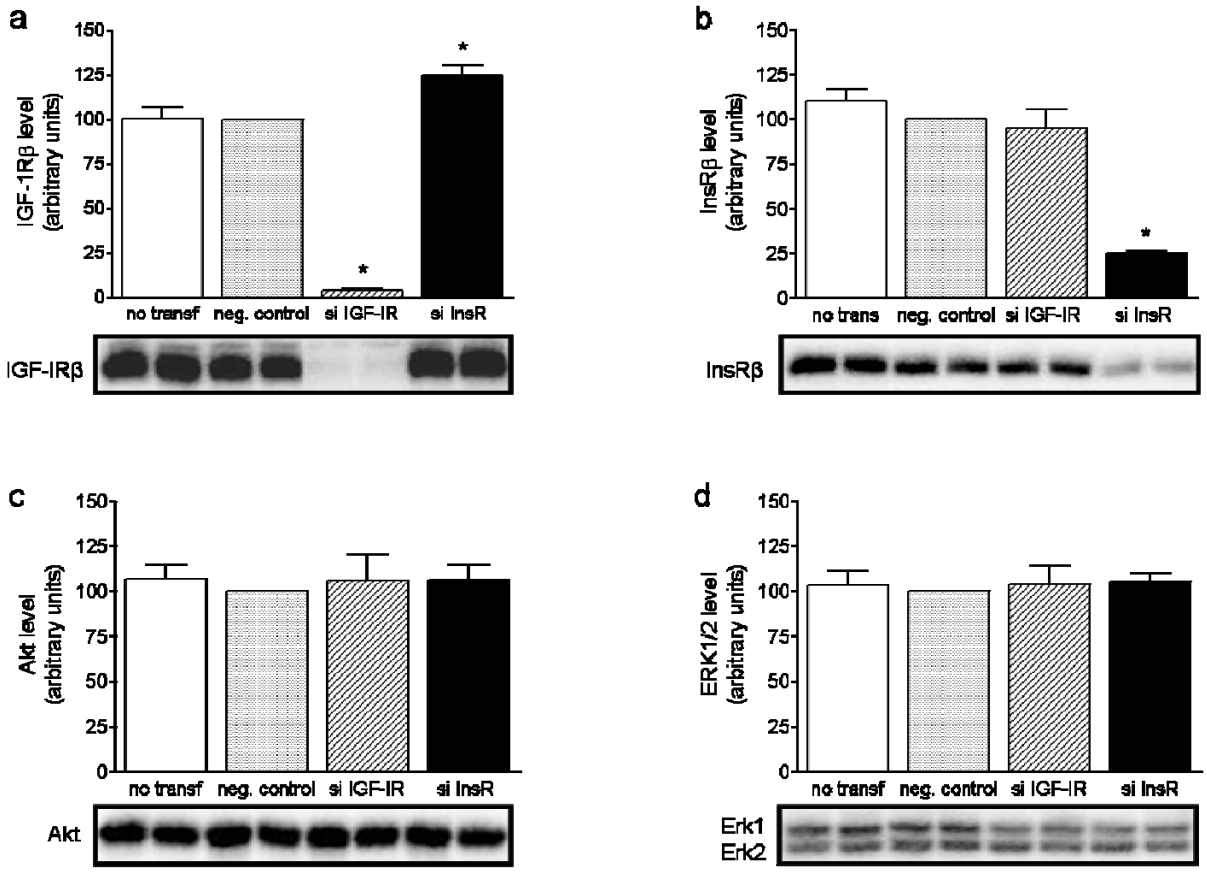

Fig. 4.6 Protein expression after silencing of IGF-1R and InsR in Fib. Fib of donor F52 were transfected with 20 or 10 nmol/l siRNA as described in Methods. Lysates were prepared 72 h after transfection, immunoblotted and analysed for expression of IGF-1R (**a**), InsR (**b**), Akt (**c**) and ERK1/2 (**d**). All data were normalised to the level of α-tubulin expression and are expressed relative to non-silencing controls. Representative western blots are shown. The data are presented as means±SEM, n≥3. no transf, no transfection; neg. control, transfection with non-silencing control RNA; si IGF-1R, transfection with 20 nmol/l IGF-1R siRNA; si InsR, transfection with 10 nmol/l InsR siRNA. *p<0.05 vs both controls.

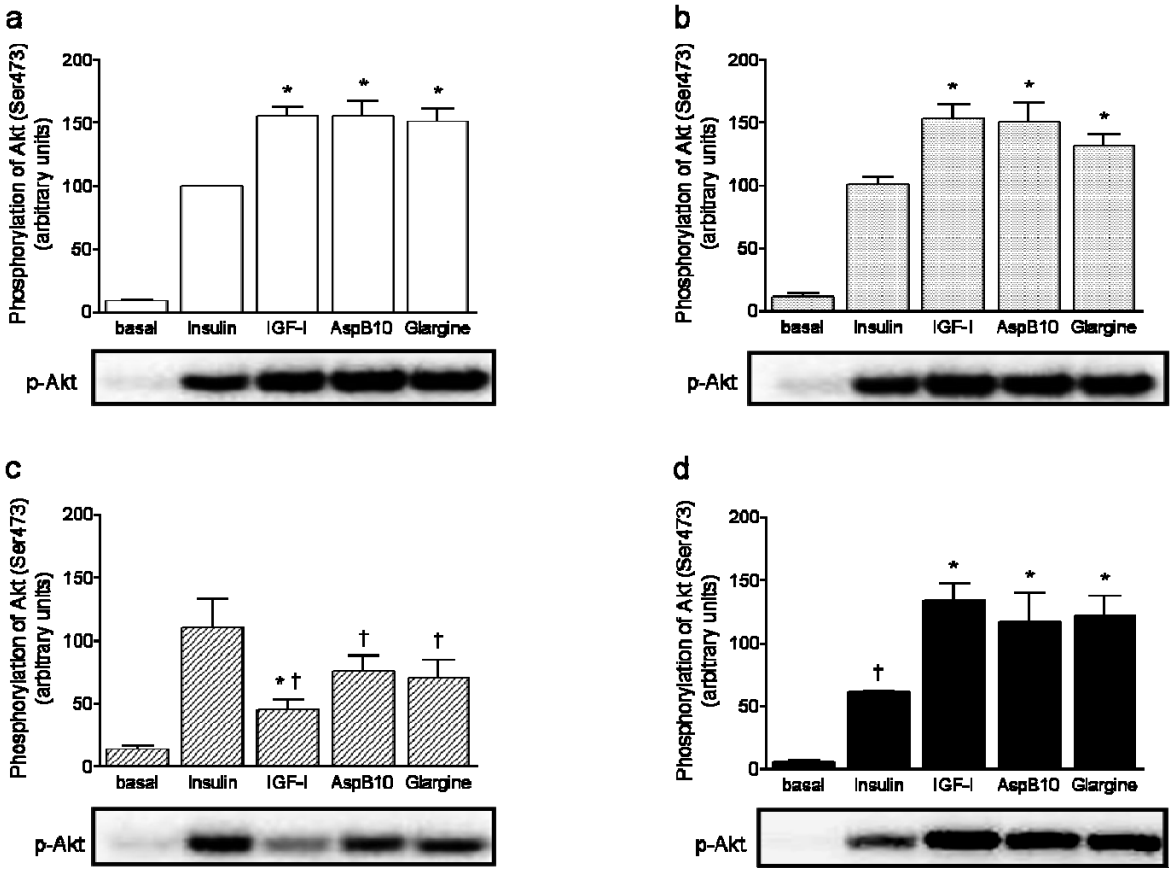

Fig. 4.7 Akt (Ser473) phosphorylation in IGF-1R- and InsR-silenced Fib after stimulation with insulin, IGF-1, AspB10 or glargine. After incubation in the absence or presence of siRNA for 72 h, the cells were stimulated for 15 min with insulin, AspB10 or glargine at 100 nmol/l or with IGF-1 at 10 nmol/l. Total cell lysates were resolved by SDS-PAGE and immunoblotted with a phospho-specific Akt antibody. Blots were stripped and reprobed with anti-α-tubulin to control for loading differences. Peptide-stimulated phosphorylation of Akt after no transfection (**a**), incubation with non-silencing control RNA (**b**), incubation with IGF-1R siRNA (**c**) or incubation with InsR siRNA (**d**). Representative western blots are shown. All data were normalised to the level of α-tubulin expression and are expressed relative to the insulin-stimulated control value (no transfection, **a**). The data are presented as means±SEM, $n \geq 3$. *$p<0.05$ vs insulin-stimulated control. †$p<0.05$ vs corresponding values in both control situations. p-Akt, phosphorylated Akt.

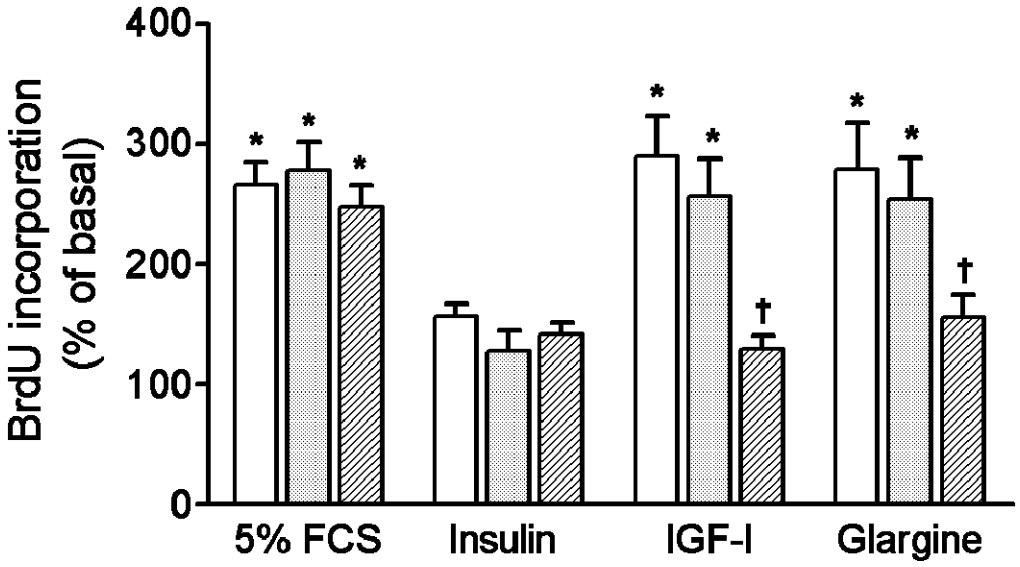

Fig. 4.8 BrdU incorporation into DNA in IGF-1R-silenced Fib after overnight stimulation with 5% FCS, insulin, IGF-1 or glargine at 10 nmol/l. Basal incorporation was set to 100%. Open bars, no transfection; dotted bars, non-silencing control RNA; hatched bars, IGF-1R siRNA. Means±SEM, $n \geq 6$. *Significantly different ($p<0.05$) from insulin-stimulated values; †$p<0.05$ for downregulation of BrdU incorporation vs no transfection and non-silencing control siRNA.

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Chapter 5

General Discussion

5.1 The endocannabinoid system as a new player in the negative crosstalk between fat and muscle

5.1.1 The endocannabinoid system in peripheral tissues – implication of its activation or blocking

While the brain endocannabinoid system takes part in regulating energy balance beside various other physiological processes, in the periphery it is engaged in control of nutrient storage and metabolism. Its overactivation due to obesity is associated with improper function of peripheral tissues.

Analysis revealed the presence of a functional endocannabinoid system in adipose tissue and data from several studies suggest that in state of imbalanced energy supply the level of endocannabinoids and CB1R expression are changed, as outlined in the introduction. The presence of CB1R was shown in human [73,74,189], mouse [70-72,190] and rat adipocytes [70,191]. Beside CB1R, the expression of CB2R could also be demonstrated in human adipocytes but its functional implication is currently not known [73,74]. In human adipose tissue, the expression of NAPE-PLD, DAGL, FAAH, and MAGL and the presence of endocannabinoids [73,189] as well as an altered expression levels of these enzymes in obese humans compared to lean controls were proven [73]. Furthermore, Gonthier et al. used isolated mature adipocytes from human subcutaneous fat to verify that adipocytes are able to synthesize 2-AG and AEA [192].

Studies in cultured mouse adipocytes revealed that the level of CB1R mRNA as well as the CB1R protein rised 3-4 fold during

differentiation [70]. Also, the amount of 2-AG increased during differentiation, which is accompanied by an elevated expression of DAGL but unchanged expression of MAGL. For AEA, a peak at day 4 was observed which goes along with a peak of NAPE-PLD at day 4 and its decrease thereafter, while FAAH expression increased from day 0 to day 12 [84]. Treatment of pre-adipocytes with CB1R agonist HU-210 accelerated the differentiation as shown by enhanced expression of PPARγ and accumulation of lipid droplets at day 8 [84]. In differentiated adipocytes the stimulation of CB1R increases glucose uptake and GLUT4 translocation [73] as well as insulin-stimulated glucose uptake [72], lipoprotein lipase activity [71], and expression of fatty acid synthase mRNA [75]. Additionally, endocannabinoids inhibit the AMP-activated protein kinase (AMPK) [193].

The described effects of CB1R stimulation were prevented by treatment with specific CB1R antagonists like rimonabant. In obese Zucker (fa/fa) rats rimonabant reduced the body weight and stimulated the expression of adiponectin in adipose tissue. In mouse adipocytes it caused overexpression of adiponectin mRNA and protein [70]. Very recently, Tedesco et al. reported a novel effect of rimonabant on mitochondrial biogenesis in white adipocytes. They demonstrated an increase of eNOS expression which resulted in an increase of mitochondrial DNA amount, mRNA levels of genes involved in mitochondrial biogenesis, and mitochondrial mass and function. The involvement of eNOS was proven by use of siRNA-mediated decrease in eNOS which blunted the effect of rimonabant [194].

In conclusion, an overactivated endocannabinoid system results in routing excess energy to the adipocytes and enhances the storage of fat, thereby increasing fat depots. These effects can be counteracted with CB1R antagonists like rimonabant. The lipogenic action of activated CB1R in adipocytes can explain in part why CB1 knockout mice fed with the same amount of food as wild-type mice still develop less fat mass [71].

The liver is the central metabolic organ and is involved in the regulation of glucose, fat and amino acid metabolism. It plays an important role in de novo lipogenesis and gluconeogenesis as well as in storage of glycogen. In fact, the liver plays an even greater role in lipogenesis than does adipose tissue [195]. Osei-Hyiaman and co-workers investigated the impact of CB1R stimulation in the liver using a diet-induced obese mouse model [75]. The presence of CB1R was confirmed by multiple methods, and after 3 weeks of high-fat diet its expression was increased compared to controls. Also, the hepatic level of AEA was increased while no change was observed for 2-AG level. This observation is accompanied by a decreased activity of FAAH while the activity of *N*-acyltransferase and the expression of FAAH remained unaltered. These changes were observed before obesity was detectable.

Stimulation of CB1R inhibits the activity of AMPK [193], induces expression of the lipogenic transcription factor SREBP-1c and its target enzymes acetyl coenzyme-a carboxylase-1 (ACC1) and fatty acid synthase, and also increases de novo fatty acid synthesis. Pretreatment of mice on high-fat diet with rimonabant reduced the rate of fatty acid synthesis. In $CB1R^{-/-}$ mice which are resistant to diet-induced obesity, the high-fat diet did not result in altered basal rate of fatty acid synthesis [75]. The results of this study suggest that the hepatic endocannabinoid system is activated during early stages of high-fat diet-induced obesity and that this may be required for the development of obesity, primarily due to an increase in de novo lipogenesis [196].

The endocrine pancreas is an important part of the regulatory network to maintain glucose homeostasis by secreting insulin in response to increasing blood glucose levels. Expression of the endocannabinoid system components in the pancreas suggests that a connection exists between the endocrine function and the endocannabinoid system. The expression of cannabinoid receptors in the pancreas was shown by several working groups [77,78,84,197], whereas the distribution of cannabinoid receptors appears to be

species-specific. A detailed study by Bermúdez-Silva [198] revealed a distinct distribution of cannabinoid receptors in human pancreas by immunofluorescence staining. CB1R expression was detected in alpha cells and in a small portion of beta cells, but not in delta cells or exocrine pancreas. On the other hand, CB2R expression was found in delta cells and exocrine pancreas, but absent in alpha and beta cells. In mice, CB1R is not expressed in beta cells but in non-beta cells, while CB2R is expressed in beta and non-beta cells. In rats, both cannabinoid receptors are expressed in beta and non-beta cells [77,198]. Also, the enzymes for synthesis and degradation of endocannabinoids are expressed in a specific pattern. Within the islet, DAGL and MAGL expression was found, and FAAH expression was detected in beta cells but not in alpha cells. NAPE-PLD was almost absent in islet but detected in acinar surrounding tissue. Beside in human islets, the enzymes of biosynthesis and degradation were also demonstrated in rat insulinoma RIN-m5F beta cells [84] and mouse pancreas [78].

Analysis of the pancreatic endocannabinoid level in diet-induced obese mice revealed an increase of AEA and 2-AG levels compared to lean controls [78,84]. Stimulating RIN-m5F beta cells with 33 mM glucose elevated both AEA and 2-AG level. When these cells were cultured under low glucose condition (13 mM), co-stimulation with 33 mM glucose and insulin prevented glucose-induced increase of the endocannabinoid level. However, cultivation in high glucose condition (33 mM) mimicking hyperglycaemia not only prevented the inhibitory effect of insulin on glucose-induced increase of the endocannabinoid level, but also enhanced the level of AEA and 2-AG *per se*.

A study in isolated mouse pancreatic islets showed, that glucose-induced Ca^{2+} oscillation and insulin secretion was prevented by stimulation of CB1R [199] and CB2R [77]. Administration of AEA to rats resulted in glucose intolerance which could be improved by treatment with CB1R antagonists [200]. Interestingly, administration of CB2R agonists resulted in improved glucose tolerance which is

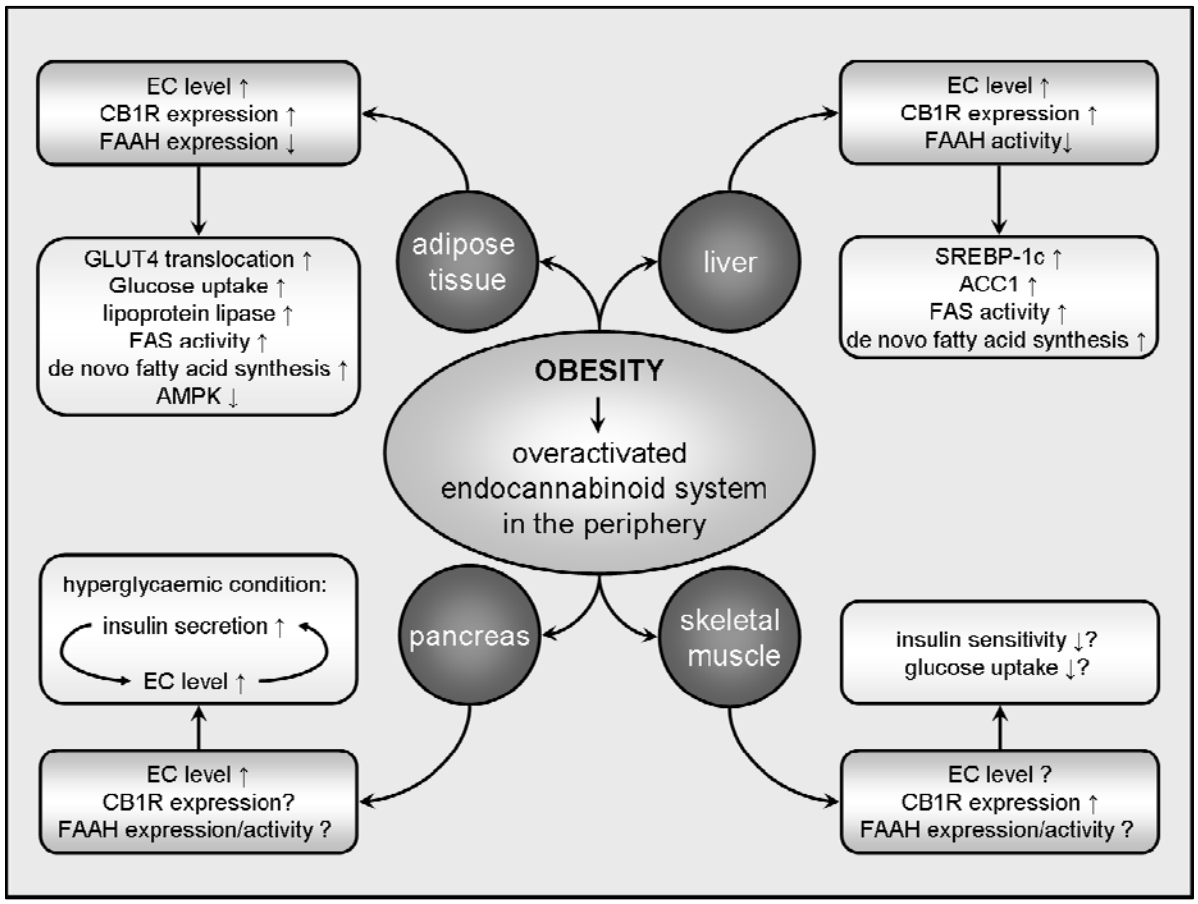

Figure 5.1 Schematic overview of the consequences of an overactivated endocannabinoid system on peripheral tissue. ACC1, acetyl coA carboxylase-1; AMPK, AMP-activated protein kinase; CB1R, cannabinoid type 1 receptor; EC, endocannabinoids; FAAH, fatty acid amide hydrolase; FAS, fatty acid synthase; SREBP-1c, sterol regulatory element binding protein-1c.

contrary to the results in mouse islets [198]. The action of CB2R on insulin release may be species-specific as also suggested by the species-specific distribution of the cannabinoid receptors in the various pancreatic cell types. In the insulinoma model, stimulation of CB1R increased insulin secretion when the cells were kept in high but not in low glucose condition [84]. In isolated human islets the stimulation of pancreatic CB1R resulted in secretion of insulin, despite the relatively low abundance of CB1R in beta cells [201]. This indicates that the result of CB1R stimulation depends on the glycaemic status, and more studies are needed to understand the role of the endocannabinoid system in the pancreas. The available data suggest, that under hyperglycaemic conditions the endocannabinoid

level in the endocrine pancreas raises and is no longer depressed by insulin. The endocannabinoid system becomes dysregulated and the subsequent overstimulation of CB1R might reinforce insulin release resulting in hyperinsulinemia which in turn starts a vicious circle by further increasing the endocannabinoid level. These findings are mirrored in the pre- and postprandial blood endocannabinoid level of lean normoglycaemic and obese hyperglycaemic subjects. While in the normoglycaemic group the AEA level decreased postprandially, in the hyperglycaemic group the level of both AEA and 2-AG increased significantly, suggesting that normal regulation of the blood endocannabinoid level is disrupted [84].

5.1.2 The endocannabinoid system and skeletal muscle

Currently, only few data are published regarding the impact of the endocannabinoid system in skeletal muscle. Cavuoto et al. reported the expression of CB1R, CB2R, and FAAH mRNA in human and rodent skeletal muscle [79]. Furthermore, CB1R mRNA was shown to be elevated in skeletal muscle of diet-induced obese mice compared to lean controls [93], while no difference in mRNA level was detected between human myotubes derived from lean or obese subjects [202]. In our lab, we detected CB1R and CB2R protein in human primary muscle cells and a differentiation-dependent increase of cannabinoid receptor protein expression.

One study investigated the effect of CB1R activation or blocking on the expression of genes involved in regulating energy metabolism in human skeletal muscle cells, which were derived from lean or obese subjects, respectively [202]. Stimulation with AEA alone did not alter the gene expression of AMPKα1, AMPKα2, PGC-1α or PDK4. The antagonist AM251 increased mRNA level of AMPKα1 and decreased PDK4, while no effects were seen in gene expression of AMPKα2 and PGC-1α. Differences between myotubes derived from lean and obese subjects were observed when antagonist and agonist were combined.

Such differences include a decrease of AMPKα1, AMPKα2 and PGC-1α mRNA in myotubes from obese subjects, but an increase of AMPKα2 gene expression and no effect on AMPKα1 and PGC-1α mRNA level in myotubes from lean subjects. These data are consistent with an overall increase in metabolic capacity within skeletal muscle following antagonism of CB1R.

Liu et al. used the genetically obese Lep^{ob}/Lep^{ob} mouse model to investigate the effects of rimonabant treatment on metabolism. They reported a 38% increase of oxygen consumption in rimonabant-treated animals compared to vehicle-treated animals as well as increased basal glucose uptake in isolated soleus muscle preparations [203]. Recently, Migrenne et al. reported that the increase of insulin sensitivity observed with rimonabant depends on adiponectin while the weight reducing effect is independent of adiponectin, as revealed by studies with adiponectin knockout mice [204]. Therefore, the effect on glucose uptake observed by Liu et al. is probably secondary due to the effect of rimonabant on the expression of adiponectin.

Therefore, our study is the first to analyse direct effects of rimonabant in human skeletal muscle cells. By using the model of adipocyte-conditioned medium to induce insulin resistance, we could prove that blocking CB1R prevents the negative consequences of CM on insulin-stimulated Akt phosphorylation and glucose uptake. Despite the fact that rimonabant decreases basal glucose uptake by a yet unknown mechanism, it restores insulin sensitivity in these cells. This effect may contribute to the improved glucose and HbA1c level and insulin sensitivity observed e.g. in the RIO-trials [205,206].

Additionally, we were able to provide evidence that chronic activation of the endocannabinoid system in skeletal muscle may play a role in the induction of insulin resistance. Incubation with AEA impaired insulin-stimulated Akt phosphorylation in a concentration-dependent manner. This effect was also observed with Win55.212-2, a synthetic CB1R agonist which differs in its structure from AEA. The

reduction of Akt activation after insulin stimulation was similar for both compounds, ~ 40 % at 10 µmol/l. Analysis of the signalling pathways revealed an activation of the stress kinases ERK1/2 and p38 MAPK by AEA. Both kinases have been shown to be involved in the development of insulin resistance [207-209].

In summary, the antidiabetic effects of rimonabant are mediated by its action on different organs and tissue such as adipose tissue and liver. As the primary tissues of glucose uptake [12] skeletal muscle is assumed a possible target as well and our data suggest that stimulation of CB1R may play a role in the induction of insulin resistance in skeletal muscle. However, while our data provide novel insights into molecular pathways leading to insulin resistance, many new questions arise from this study. No data are available whether skeletal muscle cells themselves are able to produce endocannabinoids, whether a high level of AEA may influences the differentiation from myoblasts to myotubes, or how endocannabinoids influence the secretion profile of skeletal muscle cells. Clearly, further studies are needed to fully understand the consequences of endocannabinoid system overactivation in this tissue.

5.1.3 Rimonabant & co –lifestyle drug or more?

Rimonabant is the first specific CB1R antagonist which is available for therapy, at least in Europe. Originally, it was developed as an anti-obesity drug based on findings, that central CB1R are involved in the regulation of food intake. While stimulation of these receptors promotes food intake, its inactivation by antagonist or knockout approaches reduces the consumption of food. Subsequent studies *in vitro* and *in vivo* revealed that beside central effects, peripheral effects have major contributions to the improvements of several metabolic parameters, as described before.

In 2006, rimonabant was introduced into the market as Acomplia®. At the moment, it is available in U.K., France, Germany,

Denmark, Sweden, Finland, Norway, and other countries. The drug is approved as additional therapy in combination with diet and physical activity for the treatment of obese patients (BMI ≥ 30 kg/m^2) or overweight patients (BMI > 27 kg/m^2), if they display additional risk factors such as type 2 diabetes or dyslipidemia. The most common adverse effects reported during the trials were nausea, mood alteration with depressive symptoms, depressive disorders, anxiety and dizziness. Because of concerns regarding its depressive side effects, a FDA advisory panel refused the approval of rimonabant for the U.S. market in 2007. The EMEA revised the approval last year based on new information on the psychiatric safety of rimonabant focusing on cases of depression, including suicidal ideation and suicide attempts. It was shown that depression and suicidal tendencies occur about twice as frequently with rimonabant as with placebo [210]. Therefore, the prescribing information was changed in a way that it is contraindicated to ongoing major depression or use of antidepressants and treatment is terminated if depressions are developed during therapy. Hence, one important aspect of prospective CB1R antagonists will be a better control of the psychiatric side effects. In case of taranabant, the current phase III CB1R antagonist of Merck, data are available only from two studies in healthy volunteers. As with rimonabant, taranabant treatment was associated with dose-related increased incidence of clinical adverse events, including mild to moderate gastrointestinal and psychiatric effects [211,212]. However, larger and longer-duration studies will be needed to obtain a more accurate assessment of the psychiatric profile of taranabant.

In the third quarter of 2007, the sales volume of Acomplia® reached € 21 Mio, and € 58 Mio in the first 9 month of 2007 [213]. While rimonabant is paid by the insurances as therapy for the treatment of overweight or obese type 2 diabetic patients in France and Switzerland, in Germany Acomplia® was classified as a so-called lifestyle drug by the appropriate authority (Gemeinsamer Bundesausschuss, G-BA) which means that the health insurance

companies do not pay for a therapy with Acomplia®. The reason for this decision is based on the indication as a central effective drug for treatment of overweight. The position of the G-BA is that improvements of metabolic risk factors can be achieved by weight reduction alone. Therefore, the use of rimonabant is seen primarily as an improvement of life quality since it supports weight management efforts, similar to orlistat. However, the decision does not consider data about the peripheral effects of blocking the CB1R. These data predominantly come from animal and cell culture studies, and it has been shown that the metabolic effects of rimonabant are partially independent of weight loss. The mechanisms behind these observations are not yet fully clarified, but positive effects such as improvement of insulin sensitivity, HbA1c, adiponectin, HDL cholesterol, and triglycerides have also been observed in clinical trials. Considering the enormous costs associated with type 2 diabetes and metabolic syndrome, it seems necessary to think about all possibilities to prevent or delay the progression of these diseases which also includes pharmacological therapy. Thereby, the peripheral effects of rimonabant are of important relevance. The outcomes of ongoing clinical studies will hopefully provide substantial support for the additional approval of CB1R antagonists as anti-diabetic drugs.

However, the best way to prevent disorders associated with the metabolic syndrome and type 2 diabetes is a healthy lifestyle. The worldwide raising prevalence of obesity and type 2 diabetes has pushed this topic to the awareness of public and politics. Recently, the German Government decided to start various action plans, which aim to improve the information and knowledge about appropriate food consumption and the importance of physical activity. It seems that a large part of our population needs to learn a healthier and better lifestyle, from children to adults. Hopefully, the action plans will provide useful support for a change.

5.2 Is insulin resistance reversible?

5.2.1 Link between obesity and insulin resistance

Insulin resistance is an early defect in the pathogenesis of type 2 diabetes and usually occurs long before this disease is diagnosed. It affects central and peripheral tissues such as adipose tissue, liver and skeletal muscle. Skeletal muscle represents one of the major insulin-sensitive organs and accounts for about 80 % of insulin-stimulated glucose disposal [12]. Therefore, this tissue is target of intensive research to understand the mechanisms which are involved in the development of insulin resistance, i.e. disturbed insulin signalling and insulin action.

Obesity is one of the major components of the metabolic syndrome and a strong risk factor for the development of type 2 diabetes. It is associated with increased circulating plasma levels of free fatty acids (FFA) and triglycerides which contribute to insulin resistance in peripheral tissues like skeletal muscle [214]. Originally, adipose tissue was considered to be a storage organ, an energy reservoir for triglycerides. Over the last decade it has become clear that adipocytes are also active secretory cells which release FFA by lipolysis and produce a variety of cytokines, the so-called adipokines [215,216]. Furthermore, adipose tissue is the source of key hormones for the control of body weight and secretes a range of adipokines, some of which may be directly implicated in the pathologies associated with obesity [217]. In the state of obesity, the fat depots as well as the adipocytes themselves are enlarged and characterised by an altered secretion profile of adipokines compared to lean conditions, like reduced adiponectin or increased leptin levels [216,218-221].

The association of obesity and insulin resistance is well accepted and weight gain has been shown to be a strong predictor of

the metabolic syndrome. Increased fat mass, especially visceral fat mass, was proven to lead to metabolic defects like impaired insulin sensitivity and to increase the risk for cardiovascular diseases [222]. Much effort is being made to investigate the endocrine link connecting lipid and glucose metabolism. In obese individuals, adipose tissue releases increased amounts of FFA, glycerol, hormones, pro-inflammatory cytokines and other factors that are involved in the development of insulin resistance [223]. The increased FFA flux to other tissues leads to increased triglyceride storage in these tissues, which promote insulin resistance and other adverse effects, referred to as lipotoxicity. In humans, the triglyceride content of muscle directly correlates with insulin resistance [214,224]. Several cytokines such as TNFα, resistin, IL-6 and adiponectin were identified and shown to have an impact on insulin action in skeletal muscle [225]. The list of newly discovered adipokines is rapidly growing and the influence of novel players like endocannabinoids such as AEA and 2-AG add additional components to the complex mechanisms that lead to the development of insulin resistance in skeletal muscle. The adipose-derived factors constitute a communication network between fat and other tissues which assures a balanced metabolism under normal conditions. In a state of obesity, secretion of these factors is altered resulting in disturbed communication.

5.2.2 A model to analyse the crosstalk between fat and skeletal muscle

The establishment of the co-culture model of human skeletal muscle cells and adipocytes in our laboratory allows us to directly study interactions between these two cell types [226]. Co-culture models are a generally accepted approach to investigate paracrine interaction between two cell types [227-229] and our model has already provided direct evidence for a negative crosstalk between adipocytes and skeletal muscle. Co-culture impairs insulin signalling

and insulin action in skeletal muscle cells similar to the defects observed in skeletal muscle of diabetic patients [27,32,36,39]. The cells show decreased insulin-stimulated phosphorylaton of IRS-1, Akt, and GSK3, and a markedly reduced GLUT4-translocation to the plasma membrane [226,230]. A variation of the co-culture model is the generation of adipocyte-conditioned medium (CM) which contains a complex mixture of adipokines secreted by differentiated adipoctyes during a 48 h period. CM mimics the co-culture approach and causes a similar reduction of insulin-stimulated IRS-1 and Akt phosphorylation [207,230,231].

Analysis of CM with cytokine arrays revealed that human adipocytes secrete various cytokines including IL-6, IL-8, macrophage inflammatory protein-1α/β (MIP-1 α/β), and MCP-1. MCP-1 was identified as a potential player in the negative crosstalk between fat and skeletal muscle since it was able to impair insulin signalling at doses similar to its physiological plasma concentrations. Furthermore, it significantly reduced insulin-stimulated glucose uptake in SkM [207].

The model of CM was also used to study a possible involvement of the endocannabinoid system in the development of insulin resistance. Gonthier et al. reported the production of 2-AG, AEA and the anandamide analogues *N*-palmitoylethanolamine (PEA) and *N*-oleylethanolamine (OEA) in a model of human subcutaneous adipocytes [192]. The total level of endocannabinoids produced by adipocytes were reported to be 7.05 pM for 2-AG, 9.07 pM for AEA, 7.25 pM for OEA, and 89.16 for PEA, thus PEA was the most abundant endocannabinoid in their model. PEA is known for its anti-inflammatory effects but does not bind to cannabinoid receptors [232]. It may rather potentiate AEA effects by competing with AEA for FAAH-mediated degradation [192]. This and other reports of the capability of adipocytes to synthesise endocannabinoids as well as the protective effects of CB1R antagonists against CM-induced impairment of insulin signalling and insulin action lead us to the conclusion that CM most likely also contains CB1R-activating factors.

5.2.3 Reversibility of insulin resistance

Results from several lifestyle intervention studies have revealed that insulin resistance *in vivo* is a reversible process. Weight management as a tool to reduce adipose fat mass is a validated method to restore insulin sensitivity and to normalise adipokine levels as demonstrated for IL-6 [233], MCP-1 [116], and TNFα [118]. One important question is whether the reversibility is complete at the level of SkM. To analyse the association of obesity with defects in insulin action, Pender et al. studied cultured human SkM obtained from obese nondiabetic patients which displayed impaired insulin action [135]. The results revealed no differences of insulin-stimulated signalling events like autophosphorylation of InsR and Akt phosphorylation as well as insulin-stimulated glucose transport when compared to non-obese controls. Therefore, the authors suggested that insulin resistance is an acquired feature of obesity. However, other studies carried out with cultured human SkM obtained from obese type 2 diabetic patients displayed defective insulin-stimulated glucose transport, demonstrating that insulin resistance was retained in culture [136,137]. It may be concluded that ability of SkM to restore normal insulin sensitivity *in vivo* the could depend on the duration of insulin resistance-promoting conditions like elevated level of FFA or TNFα. At a certain point irreversible damages may occur as displayed by the results of Henry et al. and Gaster et al.

We used our model of adipocyte-induced insulin resistance in human SkM to investigate the reversibility of insulin resistance and underlying mechanism to gain a more detailed view on the processes involved. Besides the previously reported defects of insulin signalling and action induced by incubation with CM [207,230,231], we demonstrated additional defects in SkM such as downregulation of myogenin, MHC and myoD, reduction of IL-6 and IL-8 secretion, and increase of MCP-1 secretion. Furthermore, ROS and NO production were elevated and SDH activity was reduced upon treatment with

CM, pointing to increased oxidative stress and reduced mitochondrial capacity.

Withdrawal of CM normalised insulin signalling, IL-6 secretion, and expression of myoD and MHC. However, myogenin level as well as disturbed secretion of IL-8 and MCP-1 were retained over the 48 h period of CM withdrawal. In summary, reversibility occurs on the level of insulin signalling whereas secretory defects remain, illustrating longer-lasting damages. These results clearly demonstrate that some features of insulin resistance are reversible while others are not.

5.3 Action of insulin analogues beyond glycaemic control

5.3.1 Role of insulin receptor, IGF-1 receptor and insulin/IGF-1 hybrid receptor

The cellular and consequently metabolic activity of insulin is initiated by binding to its receptor, thus activating a cascade of intracellular events and leading to well-known processes, e.g. GLUT4 translocation, increased glucose transport and glycogen synthesis, and inhibition of lipolysis.

The InsR is structurally very similar to the IGF-1 receptor (IGF-1R) with a homology of 45–65 % in the ligand binding domains and 60–85 % in the tyrosine kinase and substrate recruitment domains. Both receptors have different biological functions: the InsR as a central player in glucose homeostasis and the IGF-1R as a regulator of body growth. The basis for these different roles are 1) a tissue-specific distribution, i.e. InsR is expressed at high level in adipose tissue, muscle and liver, while IGF-1R is expressed in almost all tissues, and 2) small differences in the recruitment of intracellular mediators. It was shown that the InsR is more efficient in activating the IRS-1 pathway than the IGF-1R, whereas both receptors are

approximately equal in their potency to activate DNA synthesis when they are both equally expressed in a cell [234]. The high degree of similarity between InsR and IGF-1R enables the formation of insulin/IGF-1 hybrid receptors by heterodimerisation [183]. The abundance of these hybrid receptors was shown in a range of mammalian tissues [184-187]. The issue becomes even more complicated since the human InsR exists in two isoforms, isoform A (InsR-A) and isoform B (InsR-B). These isoforms are generated by alternative splicing of the primary transcript of the insulin receptor gene. They differ by 12 amino acids (residues 718 to 729) at the carboxyl terminus of the receptor´s α-subunit encoded by exon 11, which is either excluded (InsR-A) or included (InsR-B) [235]. InsR-A is the main variant expressed in foetal tissue, while InsR-B is predominantly expressed in most adult tissue [234]. InsR-A and InsR-B display several functional differences. InsR-A has a 2fold higher affinity for insulin, a faster internalisation and recycling time, an overall lower insulin signalling capacity and a 2fold lower tyrosine kinase activity [236,237]. Additionally, InsR-A is activated by IGF-2 with a high affinity while InsR-B is not [238].

The affinity of insulin/IGF-1 hybrid receptors to insulin, IGF-1, and IGF-2 depends on the InsR isoform which participate in the formation of the hybrid receptor. Hybrid receptors containing InsR-A (HR-A) bind to and are activated by IGF-1, IGF-2, and insulin. In contrast, hybrid receptors containing InsR-B (HR-B) bind to and are activated with high affinity by IGF-1, with low affinity by IGF-2, and insignificantly by insulin [239]. Therefore, the formation of hybrid receptors in cells co-expressing InsR and IGF-1R probably provides additional binding sites for IGF-1. Importantly, insulin and IGF-1 are able to bind to each other's receptor at high concentrations. However, it is still controversially discussed whether insulin triggers mitogenic effects through its own receptor, by interacting with IGF-1R or by activating insulin/IGF-1 hybrid receptors [171].

In the light of these discussions, the structural changes of the insulin molecule to create rapid- or long-acting insulin analogues

become increasingly important. It is known that modifications in the B10 and B26-B30 region are able to change the affinity towards the IGF-1R [188]. This has been demonstrated for AspB10 insulin which is known for its strong tumourigenic action [240]. Studies investigating receptor binding properties of insulin analogues have shown that AspB10 insulin and insulin glargine have a 6-8fold and insulin lispro a 1.5fold higher affinity to the IGF-1R compared to regular insulin [241], while insulin aspart [242] and insulin glulisine [243,244] have a low affinity to the IGF-1R, similarly to regular insulin. For insulin detemir a 5fold lower affinity to the IGF-1R has been found [241]. Binding to the InsR was similar to insulin for insulin aspart, insulin lispro, insulin glargine [241], and insulin glulisine [243-245], while insulin detemir displayed lower binding affinity to the InsR [241]. Implications of the increased IGF-1R affinity of insulin glargine at the cellular level are discussed below.

5.3.2 Signalling properties of insulin glulisine

Analysing the signalling properties of insulin glulisine in various *in vitro* systems revealed interesting findings. The stimulation of rat myoblasts with insulin glulisine resulted only in marginal phosphorylation of IRS-1 while a prominent phosphorylation of IRS-2 was observed similar to that after stimulation with regular insulin. This effect was even more pronounced when using primary human skeletal muscle cells, where a significantly higher IRS-2 phosphorylation compared to regular insulin occurred after stimulation with insulin glulisine [244]. The result was confirmed by Stammberger et al. in rat and human myoblasts and rat cardiomyocytes [246], and in the pancreatic beta-cell line INS1 [247]. Additionally, insulin glulisine exhibited the most prominent anti-apoptotic activity in INS1 cells compared to regular insulin, insulin aspart and insulin lispro. Since IRS-2 is crucial for the survival of pancreatic beta-cells [248,249], the strong phosphorylation of IRS-2

observed after insulin glulisine stimulation may mediate an enhanced beta-cell protective effect and therefore might serve to counteract autoimmune- and lipotoxicity-induced beta-cell destruction [247]. The results obtained in certain cell culture models are in contrast with one study performed in C57/BL6 mice [250]. Insulin glulisine or regular insulin was injected and subsequent Western blot analysis of liver and muscle tissues was carried out. These investigations detected no differences in the signalling pattern between regular insulin and insulin glulisine.

Studies on the mitogenic and metabolic potential of insulin glulisine did not reveal differences compared to regular insulin. Both were equipotent in stimulating glucose and [^{3}H]thymidine uptake in human skeletal muscle cells [243], in stimulating DNA synthesis in rat myoblasts and in increasing glucose transport, activating Akt and glycogen synthase kinase-3 in rat cardiomyocytes [244], as well as in stimulating DNA synthesis in human smooth muscle cells and fibroblasts [251].

5.3.3 Proliferative effects of insulin glargine

The increased affinity of insulin glargine towards the IGF-1R raised concerns about the safety of this insulin analogue. A study from 1998 [252] analysed the mitogenic effect of glargine in rat fibroblasts overexpressing the human InsR and could not find differences between regular insulin and glargine. The first report about an augmented mitogenic potency of insulin glargine was published by Kurtzhals et al. [241] who used the osteosarcoma cell line Saos/B10 and reported glargine to be 8fold more potent in stimulating DNA synthesis than human insulin. Given that Saos/B10 cells express a huge amount of IGF-1R (30,000 IGF-1R vs. 1,000 InsR per cell [241]), the result must be handled with care because this cell line does not represent the normal status of primary human cells. Several studies were published investigating the proliferative effects

of insulin glargine in human primary cells in comparison to regular insulin. In differentiated cultured human skeletal muscle cells obtained from non-diabetic and diabetic subjects insulin glargine was equivalent to human insulin with respect to metabolic responses and did not display augmented mitogenic effects [253]. Staiger et al. reported that insulin glargine and human insulin are not different in their proliferative effects in human coronary artery endothelial and smooth muscle cells. [254]. These results are partially in contrast to our study, in which we analysed the proliferative effects of regular insulin, AspB10 insulin, insulin glulisine, insulin lispro, insulin aspart and insulin glargine by measuring DNA synthesis in human fibroblasts and coronary artery smooth muscle cells obtained from different donors [251]. We observed that all tested insulin analogues and insulin itself can exhibit an enhanced growth-promoting activity. However, these results were strictly donor-specific and correlated to the protein level of the IGF-1R, InsR and/or IRS-1. Fibroblasts and smooth muscle cells of donors with higher IGF-1R, InsR and IRS-1 protein levels displayed an increased DNA synthesis when stimulated with regular insulin or any tested insulin analogue. However, donors of both cell types with low protein level of IGF-1R did not show increased DNA synthesis upon stimulation and this result is comparable to that of Staiger et al. Unfortunately, they did not present a detailed analysis of separate donors and the levels of IGF-1R and IRS-1 were not determined.

By using siRNA technology for *in vitro* knockdown of IGF-1R and InsR, direct evidences for a key role of the IGF-InsR/Akt signalling pathway in the augmented growth promoting activity of insulin analogues were provided. IGF-1 and the insulin analogues produced a prominent activation of Akt and were significantly more potent than regular insulin. Knockdown of the IGF-1R by more than 95% resulted in a substantially decreased phosphorylation of Akt upon treatment with IGF-1, AspB10 insulin and insulin glargine. After reducing the InsR by 75%, the Akt phosphorylation following stimulation with IGF-1 and the insulin analogues remained on a high level while the

insulin-stimulated phosphorylation was significantly reduced by 40%. Most importantly, silencing of the IGF-1R significantly reduced the effect of IGF-1 and insulin glargine on DNA synthesis to a level identical to that observed in response to insulin. The data identified the IGF-1R as the key mediator of the augmented growth-promoting activity of insulin analogues in smooth muscle cells and fibroblasts. Taken together, these results suggest that the individual protein level of the IGF-1R is critical for the growth promoting activity of insulin analogues and of insulin itself. The combination of an insulin analogue possessing an increased affinity for the IGF-1R with an elevated level of IGF-1R expression may be a critical safety issue which needs to be further investigated and a potential atherogenic potency of insulin glargine cannot be ruled out. Thus, it is of high importance to carry out further studies on a potential safety risk of insulin analogues and it should be made an effort to include all types of available tumour cell lines which are known for high expression levels of IGF-1R.

5.3.4 Insulin detemir: tissue selectivity and central effects

Although insulin detemir was not included in the study concerning the mitogenic properties, this unique insulin analogue is included in the general discussion because of several interesting features.

The basis of the protracted action profile of insulin detemir is a strong tendency to self-associate at the injection site and the slow release from albumin [179,255]. The unique structure causes its reversible binding to free fatty acid binding sites on albumin, thereby developing an equilibrium between free and albumin-bound analogue. Only the free portion of insulin detemir permeates the capillary wall, enters the circulation and binds to albumin again. Before acting at peripheral target cells, free insulin detemir has to pass the capillary wall again. Therefore, the transfer of insulin

detemir from the blood stream to the extracellular spaces of adipose tissue and muscle is limited by capillary endothelial cells [256]. In contrast, the tissue structure of the liver allows a more direct contact between the blood plasma in the sinusoids and the hepatocyte plasma membrane due to highly fenestrated endothelial cells, which line the sinusoids. Hordern and co-workers analysed whether the potential increased exposure of hepatocytes to insulin detemir may result in greater effects in the liver than in other peripheral tissues. One outcome was that insulin detemir had a greater effect on mean suppression of hepatic glucose rate of appearance when compared to equipotent doses of NPH insulin. They suggested that insulin detemir indeed has a greater effect on the liver and therefore has the potential to restore the physiological insulin gradient [256].

Another consequence of binding insulin detemir to albumin may be a relative preference for the brain. It is known that albumin directly penetrates into the cerebrospinal fluid across choroid plexus epithelial cells [257]. Additionally, insulin detemir is more lipophilic than regular insulin and likely crosses the blood-brain barrier more easily than regular insulin [258]. Albumin concentrations in the brain are 200fold lower than in the circulation [259], while in skeletal muscle the concentration is only 5fold lower [260]. This could raise the rate of unbound insulin detemir in the cerebrospinal fluid and therefore enhance the amount of free insulin detemir which can then bind to insulin receptors. Indeed, after injection of insulin detemir, Hennige et al. found an elevated level of insulin detemir in the mouse brain as well as enhanced insulin signalling in the hypothalamus and cerebral cortex [261]. Furthermore, investigations in overweight human subjects showed that despite cerebrocortical resistance to human insulin, insulin detemir was able to restore the decreased cerebral beta activity response [262].

The insulin signal in the brain serves as feed-back signal from the periphery to reduce appetite [263,264]. Transport of insulin across the blood-brain barrier is likely to be mediated by insulin receptors [265] and it has been shown in dogs that increased food

intake and weight gain during high-fat feeding is associated with reduced insulin delivery into the CNS [266]. Furthermore, neuronal knockout of InsR results in an obese and insulin-resistant mouse phenotype [267]. Taken together, the preferential activity of insulin detemir in brain and liver could potentially explain the observed weight benefit seen in therapy [268].

5.3.5 Additive effects of insulin analogues and GLP-1 receptor agonists

As outlined before, insuline glulisine has been shown to exhibit the most prominent anti-apoptotic activity in INS1 cells compared to regular insulin, insulin aspart and insulin lispro against both cytokine- and fatty acid-induced cell death [247]. Similar results regarding β-cell protective properties have been shown for glucagon-like peptide-1 (GLP-1) which enhances proliferation as well as inhibits apoptosis [269-271]. Very recently, our lab analysed the anti-apoptotic properties of a novel GLP-1 receptor agonist, AVE0010 [272]. The compound as well as exendin-4 was able to significantly reduce cytokine- and fatty acid-induced apoptosis in pancreatic β-cells by 40-50 %. However, a key finding of this investigation is that a combination of insulin glulisine, lispro, or glargine with GLP-1 receptor agonist produces an even more prominent, additive anti-apoptotic effect. Incubation of cells with this mixture was able to reduce cytokine- and fatty acid-induced apoptosis by up to 80 %. Additionally, it was shown by siRNA mediated knockdown that Akt2 signalling is not essential for the anti-apoptotic activity of GLP-1 and GLP-1 receptor agonist.

The progression of type 2 diabetes is characterised by a gradual dysfunction of the β-cell and loss of β-cell mass. Therefore, the combination of insulin analogues and incretin mimetics may represent a novel therapeutic option for the preservation of β-cell mass in type 2 diabetic patients.

5.4 Perspectives

The dissertation presented here examined several aspects of recently discussed issues concerning the development of skeletal muscle insulin resistance as well as safety aspects regarding the use of insulin analogues in therapy of type 2 diabetes. The endocannabinoid system as a novel player, which is probably involved in the complex pathways leading to insulin resistance, is certainly an exciting field to explore in the future. We are just beginning to recognise its implication in the development of obesity and type 2 diabetes. Despite the fact that compounds like rimonabant and taranabant are already in clinical use or immediately before approval, we do not yet fully understand the exact mechanisms responsible for the beneficial effects observed during treatment. Based on our data, it is planned to further analyse the consequences of endocannabinoid system activation in SkM metabolism. One important question to address is whether chronically activated CB1R may influence differentiation, expression of myogenic transcription factors or the secretion profile of SkM as observed after incubation with CM. Also, it has not been investigated to date whether SkM themselves are able to produce endocannabinoids and if so, how the secretion is regulated. The effects observed after rimonabant treatment regarding the decrease in basal glucose uptake have to be addressed as well. Furthermore, it will be interesting to study whether CB1R antagonists are able to prevent the long-lasting secretoro defects caused by CM. However, extending the endocannabinoid project will certainly provide new insights into the principles involved in the development of insulin resistance as well as bring up new questions.

Regarding the issue of insulin analogues, our study revealed that the enhanced growth-promoting activity of insulin analogues is exclusively mediated by IGF-1R. In terms of the fact that many

tumour cells overexpress IGF-1R and/or InsR-A, further studies on a potential safety risk of these molecules are urgently required. For future insulin analogues it will be necessary to closely monitor the pharmacological characteristics with careful consideration of binding properties. Our system of analysing proliferative effects in human primary cells may be useful as a screening method to evaluate potential mitogenic risks.

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List of Figures

List of Abbrevation

ACC1	acetyl coenzyme-A carboxylase-1
ADA	American Diabetes Association
ADOPT	A Diabetes Outcome Progression Trial
AEA	anandamide
AMPK	AMP-activated protein kinase
2-AG	2-arachidonoylglycerol
BPD	biliopancreatic diversion
cAMP	cyclic adenosine monophosphate
CB1R	cannaboid receptor type 1
CB2R	cannaboid receptor type 2
CDC	Centers for Disease Control and Prevention
CM	adipocyte-conditioned medium
CNS	central nervous system
CR	cystein-rich region
DAGL	diacylglycerol lipase
DDG	Deutsche Diabetes Gesellschaft
DPP-4	dipeptidyl peptidase-4
EMEA	European Medicines Agency
eNOS	endothelial nitric oxide synthase
ER	endoplasmatic reticulum
ERK1/2	extracellular regulated kinase ½
FAAH	fatty acid amide hydrolase
FAS	fatty acid synthase
FDA	Food and Drug Administration
Fib	fibroblasts
FIRKO	fat-specific insulin receptor knockout
FPG	fasting plasma glucose
GIP	glucose-dependent insulinotropic peptide
GLP1	glucagon-like peptide-1
Glut4	glucose transporter 4
GSK3	glycogen synthase kinase 3

HDLc	high density lipoprotein cholesterol
HR-A	hybrid receptor containing InsR-A
HR-B	hybrid receptor containing InsR-B
ID	insert domain
IGT	impaired glucose tolerance
IFG	impaired fasting glucose
IGF-1	insulin-like growth factor-1
IGF-2	insulin-like growth factor-2
IGF-1R	IGF-1 receptor
IL	interleukin
InsR	insulin receptor
InsR-A	insulin receptor isoform A
InsR-B	insulin receptor isoform B
IRS	insulin receptor substrate
JNK	c-Jun N-terminal kinase
LIRKO	liver-specific insulin receptor knockout
LDLc	low-density lipoprotein cholesterol
MAGL	monoacylglycerol lipase
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MHC	myosin heavy chain
MIP-1	macrophage inflammatory protein-1
MIRKO	muscle-specific insulin receptor knockout
NAPE	N-arachidonoyl-phosphatidylethanolamine
NAT	N-acyltransferase
NMDA	N-methyl-D-aspartate
NPH	neutral protamine Hagedorn
NO	nitric oxide
OGTT	oral glucose tolerance test
PDK1	phosphoinositide-dependent serine/threonine kinase 1
PDK4	pyruvate dehydrogenase kinase isoform 4
PGC-1α	PPARγ coactivator-1α
PKC	protein kinase C
PLC	phosphatidylinositol-specific phospholipase C

PLD	phospholipase D
PPAR	peroxisome proliferator-activated receptor
RIO	rimonabant in obesity
ROS	reactive oxygen species
RYGB	Rouxen-Y gastric bypass
SDH	succinate dehydrogenase
SH2	Src-homology-2
siRNA	small interfering RNA
SkM	skeletal muscle cells
SMC	human coronary artery smooth muscle cells
SNAP23	synaptosome-associated 23-kDa protein
SREBP-1c	sterol regulatory element binding protein-1c
TBS	tris-buffered saline
TGR	trans golgi reticulum
THC	tetrahydrocannabinol
TNFα	tumor necrosis factor α
TRPV1	transient receptor potencial vanilloid type 1
UKPDS	United Kingdom prospective diabetes study
VAMP2	vesicle-associated membrane protein 2
WHO	World Health Organisation

Contribution to chapter 2-4

Beside my own contribution, the results published in this thesis involve contributions from colleagues and members of the laboratory of Prof. Dr. Jürgen Eckel. In the following my specific contributions to each publication are outlined.

Chapter 2

- Analysis of CB1R expression
- Effect of rimonabant and AM251 in combination with CM on Akt phosphorylation
- Glucose uptake
- Effect of AEA on Akt phosphorylation
- Analysis of ERK1/2, p38 MAPK, and NFkB activation after AEA stimulation
- Preparation of the manuscript together with Prof. Dr. Jürgen Eckel

Chapter 3

- Preadipocyte isolation together with Henrike Sell
- Generation of CM together with Andrea Cramer
- Preparation of SkM for experiments
- Differentiation-dependend expression of myogenin, MHC, myoD
- Preparation of the manuscript together with Henrike Sell and Prof. Dr. Jürgen Eckel

Chaper 4

- Analysis of the expression of InsR, IGF-1R, IRS-1, Akt and ERK1/2
- Isolation of monocytes
- Proliferation assays in smooth muscle cells after incubation with insulin analogues
- Silencing of InsR in fibroblasts
- Analysis of signalling pathways in IGF-1R silenced fibroblasts upon stimulation with insulin, IGF-1, insulin AspB10 and insulin glargine

- supervision of practical work done by Claudia May
- Preparation of the manuscript together with Prof. Dr. Jürgen Eckel

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„Nicht das Beginnen wird belohnt, sondern einzig und allein das Durchhalten.“

- Katharina von Siena

Die vorliegende Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 08.09.2008
Kristin Eckardt