ADIPOSE TISSUE AS AN ENDOCRINE ORGAN

The role of adipocyte-derived cytokines in skeletal muscle insulin resistance

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Henrike Sell

aus Merseburg

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Referent: Prof. Dr. Jürgen Eckel
Korreferent: Prof. Dr. Lutz Schmitt

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« *Die Natur spielt immerfort mit der Mannigfaltigkeit der einzelnen Erscheinungen, aber es kommt darauf an, sich dadurch nicht irren zu lassen, die allgemeine Regel zu abstrahieren, nach der sie handelt.* »

J. W. Goethe

Zusammenfassung

Das Fettgewebe stellt zusätzlich zu seiner Funktion als Energiespeicher auch ein endokrines Organ dar. Die Zunahme der Fettgewebsmasse, besonders des viszeralen Fetts, korreliert mit einem größeren Risiko für die Entwicklung von Stoffwechselkrankheiten wie Insulinresistenz und Typ-2-Diabetes. Dieser Zusammenhang beruht vermutlich auf einer verstärkten Freisetzung von bioaktiven Proteinen aus dem Fettgewebe, den so genannten Adipokinen, die wahrscheinlich den Energiehaushalt und die Insulinsensitivität regulieren. *In vitro* konnte gezeigt werden, dass eine Ko-Kultur mit Adipozyten oder die Behandlung mit Adipozyten-konditioniertem Medium Insulinresistenz in Skelettmuskelzellen induziert, ähnlich wie man es auch im Skelettmuskel diabetischer und adipöser Patienten beobachtet. In dieser Arbeit wird mit Hilfe der Ko-Kultur von Adipozyten und Skelettmuskelzellen die Art und Regulation von Adipozytenfaktoren untersucht, die an der Erzeugung von Insulinresistenz in Skelettmuskelzellen beteiligt sind. Außerdem ist es Ziel, Mechanismen und Signalwege aufzudecken, die mit Insulinresistenz in Skelettmuskelzellen im Zusammenhang stehen.

Das Adipozytenhormon Adiponectin korreliert negativ mit Adipositas und Insulinresistenz und fungiert möglicherweise als wichtiges anti-diabetisches Agens. Gestörtes Insulinsignaling der Skelettmuskelzellen in der Ko-Kultur wird durch Behandlung mit Adiponectin verhindert. Außerdem sind Adipozyten-konditionierte Medien, die mit Adiponectin generiert wurden, nicht mehr in der Lage Insulinresistenz in Skelettmuskelzellen zu erzeugen. Da gleichzeitige Behandlung von Skelettmuskelzellen mit Adiponectin und Adipozyten-konditioniertem Medium ein normales Insulinsignaling nicht wiederherstellt, schlussfolgern wir, dass Adiponectin hauptsächlich auf die Adipozyten wirkt. Die Analyse von Adipozyten-konditioniertem Medium durch Protein Arrays ergab, dass mindestens acht verschieden Zytokine nach Adiponectinbehandlung vermindert sezerniert werden. Adiponectin aktiviert dabei die AMPK. Pharmakologische Stimulation der AMPK-Aktivität führt ebenfalls zu einer verminderten Sekretion der meisten gemessenen Zytokine. Wir schließen

aus diesen Daten, dass Adiponectin als Schlüsselregulator der Fettzellsekretion unter Beteiligung der AMPK fungiert. Die autokrine/parakrine Wirkung von Adiponectin auf die Zytokinausschüttung von Adipozyten könnte für den Schutz der Skelettmuskelzellen vor Insulinresistenz verantwortlich sein und stellt einen neuen Mechanismus der anti-diabetischen Wirkung dieses Adipokins dar.

Humane Adipozyten sezernieren verschiedene pro-inflammatorische Adipokine wie IL-6, IL-8, MIP-1α/β und MCP-1. Unter diesen Adipokinen ist lediglich MCP-1 in der Lage, in physiologischen Konzentrationen Insulinresistenz in Skelettmuskelzellen auszulösen. IL-6, IL-8 und MIP-1β induzieren nur in sehr hohen Dosierungen Insulinresistenz. Der Effekt von MCP-1 auf das Insulinsignaling der Skelettmuskelzellen ist über ERK vermittelt, da Inhibition der ERK Insulinresistenz vollständig verhindert. Unsere Daten zeigen, dass ein einzelnes Adipokin den Insulinresistenz-erzeugenden Effekt von Adipozyten-konditioniertem Medium nachahmen kann. Dies ist ein Beleg für die Beteiligung von Adipokinen im negativen Crosstalk zwischen Fettgewebe und Skelettmuskel. MCP-1 könnte einen inflammatorischen molekularen Link zwischen Adipositas und Insulinresistenz darstellen.

Skelettmuskelzellen exprimieren verschiedene Chemokinrezeptoren einschließlich CCR2, CCR4 und CCR10, die eine hohe Affinität für MCP-1 aufweisen. Die große Sensitivität der Skelettmuskelzellen für MCP-1 kann durch eine stärkere Expression von CCR2, dem Hauptrezeptor für MCP-1, im Vergleich zu Adipozyten erklärt werden. Die Expression von CCR2 nimmt mit Differenzierung der Skelettmuskelzellen ab, aber sie nimmt in insulinresistenten Skelettmuskelzellen wieder zu. Ein eindeutiger Zusammenhang zwischen der Stärke der Insulinresistenz und der Expression von CCR2 in Skelettmuskelzellen konnte jedoch nicht erbracht werden. Wir vermuten, dass die Regulation von CCR2 keinen Anteil an der Entwicklung von Insulinresistenz in Skelettmuskelzellen hat, was im Gegensatz zur herausragenden Bedeutung von CCR2 in der Fettgewebsinflammation und -insulinresistenz steht.

Zusammenfassend kann man sagen, dass die Ko-Kultur von humanen Skelettmuskelzellen und Adipozyten ein ideales Model zur Untersuchung der Adipozyten-vermittelten Insulinresistenz in Skelettmuskelzellen darstellt. Wir können zeigen, dass Adipokine die Insulinresistenz sowohl verhindern als auch auslösen können und dass die Regulation der Adipozytensekretion eine entscheidende Rolle in der Erzeugung der Insulinresistenz spielt. Die Daten dieser Arbeit illustrieren mehrere neue Aspekte des komplexen negativen Crosstalk zwischen Adipozyten und Skelettmuskelzellen.

Summary

Adipose tissue can be viewed as an endocrine organ in addition to its function in energy storage. Increased adipose tissue mass in obesity, especially in visceral adipose tissue depots, is associated with metabolic diseases such as insulin resistance and type 2 diabetes. This correlation is believed to be due to an increased release of a variety of bioactive proteins by adipose tissue, the so-called adipokines, which may regulate energy metabolism and insulin sensitivity. *In vitro*, it has been shown that co-culture with adipocytes or treatment with adipocyte-conditioned medium can induce insulin resistance in skeletal muscle cells. The degree of insulin resistance is similar to what is observed in muscle from diabetic and obese patients. In this thesis, the co-culture of adipocytes and skeletal muscle cells is used to elucidate the nature and regulation of adipose-derived factors that might participate in the generation of muscle insulin resistance. Furthermore, this work aims at revealing mechanisms and pathways involved in insulin resistance of skeletal muscle cells.

The adipocyte hormone adiponectin is negatively correlated with obesity and insulin resistance and may exert an important anti-diabetic function. Impaired insulin signaling in skeletal muscle cells is normalized upon addition of adiponectin to the co-culture. Moreover, adipocyte-conditioned medium generated in the presence of adiponectin is unable to interfere with normal insulin signaling. As concomitant addition of adiponectin and adipocyte-conditioned medium to the myocytes fail to restore normal insulin action, we propose that adiponectin primarily acts on the adipocytes. Protein array analysis of adipocyte-conditioned medium reveals that the secretion of at least eight different cytokines is diminished in response to adiponectin. At the same time, adiponectin activates AMPK. Pharmacological AMPK stimulation also decreases the secretion of most of the measured cytokines. We therefore suggest that adiponectin operates as a key regulator of adipocyte secretory function involving activation of AMPK. The autocrine/paracrine action of adiponectin on cytokine release from adipocytes may prevent the induction of skeletal muscle insulin resistance and represents a new mechanism for the anti-diabetic

effect of this adipokine.

Human adipocytes secrete various pro-inflammatory adipokines including IL-6, IL-8, MIP-1α/β, and MCP-1. Among these candidates, MCP-1 only is able to impair insulin signaling in skeletal muscle cells at doses similar to its physiological plasma concentrations. IL-6, IL-8 and MIP-1β are effective in inducing muscle insulin resistance at very high concentrations only. The action of MCP-1 on insulin signaling in skeletal muscle cells occurs via activation of ERK as inhibition of this kinase completely inhibited the insulin resistance-inducing effect of adipocyte-conditioned medium. Our data show that a single adipokine can mimic the insulin resistance-inducing effect of adipocyte-conditioned medium providing evidence for an involvement of adipokines in the negative crosstalk between adipose tissue and skeletal muscle. MCP-1 may represent an inflammatory molecular link in the relationship between obesity and insulin resistance.

Skeletal muscle cells express various chemokine receptors including CCR2, CCR4 and CCR10 which have strong affinity to MCP-1. The high sensitivity of skeletal muscle cells towards MCP-1 can be explained by a higher expression of the main MCP-1 receptor, namely CCR2, as compared to adipocytes. Expression of CCR2 is decreased during differentiation but upregulated in insulin-resistant skeletal muscle cells. However, we cannot find a clear correlation between the level of insulin resistance and CCR2 expression in skeletal muscle cells. We propose that regulation of CCR2 in skeletal muscle does not play a major role in muscle insulin resistance which is in contrast to a prominent role of CCR2 in adipose tissue inflammation and insulin resistance.

In summary, the co-culture of human skeletal muscle cells and adipocytes represents an ideal model to study various aspects of adipocyte-mediated insulin resistance in skeletal muscle. We can demonstrate that insulin resistance can be prevented and mimicked by adipokines and that adipocyte secretion may be crucial in the induction of insulin resistance. This work illustrates several novel aspects of the complex negative crosstalk between adipocytes and skeletal muscle cells.

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List of Abbreviations

Acrp30	adipocyte complement-related protein of 30 kDa
AdipoR	adiponectin receptor
AMPK	AMP-activated protein kinase
BMI	body mass index
CCR	chemokine CC motif receptor
CM	adipocyte-conditioned medium
CXCR	chemokine CXC motif receptor
ECL	enhanced chemiluminescence
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FFA	free fatty acids
FCS	fetal calf serum
gAcrp30	globular domain of Acrp30
GH	growth hormone
GLUT	glucose transporter
GRO	growth-regulated oncogene alpha
GSK	glycogen synthase kinase
HF	high fat
HGF	hepatocyte growth factor
HMW	high molecular weight
IGFBP	insulin-like growth factor binding protein
IL	interleukin
IR	insulin receptor
IRS	insulin receptor substrate
JNK	c-Jun N-terminal kinase

LADA	latent autoimmune diabetes of the adult
LMW	low molecular weight
LPS	lipopolysaccharides
MAPK	mitogen-activated protein kinase
MCM	macrophage-conditioned medium
MCP	monocyte chemotactic protein
MDC	macrophage-derived chemokine
MIP	macrophage inflammatory protein
MMW	middle molecular weight
MODY	maturity-onset diabetes of the young
NF	nuclear factor
NO	nitric oxide
OPG	osteoprotegerin
PAI	plasminogen activator inhibitor
PDK	PI3K-dependent serine/threonine kinase
PI	phosophinositol
PI3K	phosophinositol 3 kinase
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor
RBP	retinol binding protein
ROS	reactive oxygen species
sTNFR	soluble TNF receptor
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
TZD	thiazolidinedione

Chapter 1

General Introduction

1.1 Type 2 diabetes and insulin resistance

1.1.1 Pathophysiology of type 2 diabetes

Diabetes Mellitus is a metabolic disease which is characterized by hyperglycemia. In comparison to type 1 diabetes where there is an absolute lack in insulin secretion, type 2 diabetic patients are typified by their inability to compensate the loss of insulin sensitivity in peripheral tissues by increased insulin secretion. Overall, 90% of diabetes patients can be assigned to the group of type 2 diabetics [1].

Worldwide, in 2003, 194 million adults were diagnosed as diabetics (5.1% of adult population), while it is estimated that by 2025 already 333 million adults will have diabetes (6.3%) [2]. While regions such as sub-Saharan Africa where malnutrition is a far bigger problem than obesity account for the lowest diabetes prevalence in the world (Cameroon 0.8%), other parts of the world fight diabetes as the major chronic disease of their adult population (Nauru 30.2%, United Arab Emirates 20.1% prevalence). In Europe, North and Central America, there is also a high prevalence of diabetes in the adult population (Bulgaria 10%, Spain 9.9%, Austria 9.6%,United States 8.0%, Puerto Rico 13.2%) which will still increase by 50% until 2025. The largest proportional and absolute increase in diabetes will occur in developing countries (from 4.2 to 5.6% of the adult population), where 65% of all diabetic patients live today. By 2025, the adult diabetic population is expected to increase from 5.9 to 8.1% (from 35 to 73 million patients) in India. In China the same trend can be observed with 23 million diabetic patients in 2003 (2.7%) increasing to an estimated 46 million in 2025 (4.3%).

In Germany, the estimated prevalence of diabetes is one of the highest in Eu-

rope with 10.2% of the adult population being affected in 2003 and an estimation of 11.9% by 2025. In 2004, 6.4 million Germans were treated for diabetes. This number is probably far higher if one takes into account that in older patients (55-74 years old) one out of two patients with diabetes is not diagnosed according to the KORA study [3]. From an economic point of view, the yearly cost of diabetes in all diagnosed patients in Germany amounts to an estimated 16 billion Euros [4].

Type 2 diabetes is a heterogeneous disease characterized by chronic hyperglycemia which causes microangiopathic, macroangiopathic, and neuropathic complications. Late complications of the disease occur in retina, kidney, and nerves [5], while the most important pathophysiological trait, namely insulin resistance in liver and peripheral tissues such as adipose tissue and skeletal muscle, occurs earlier [6, 7]. In fact, healthy relatives of type 2 diabetic patients display several features that characterize diabetic patients even before developing overt diabetes and have a significant higher risk to become diabetic later in life [8–12].

It is generally recognized that strong genetic and environmental components influence the development of type 2 diabetes. Concerning the genetic component of the disease, only a small portion of patients have been screened. Among these patients, 5 to 10% displays MODY (maturity-onset diabetes of the young), monogenic disorders with autosomal dominant inheritance [13]. Another 5 to 10% of patients develops diabetes because of LADA (latent autoimmune diabetes of the adult) or rare genetic syndromes [14].

However the majority of diabetic patients seem to have a polygenic inheritance which acts together with environmental factors such as overweight, sedentary life style, and smoking [15]. In fact, 50% of diabetic men and 70% of diabetic women are obese and obesity predisposes strongly to the disease [16]. In addition to obese adults, type 2 diabetes arises as a serious health issue in children and adolescents with overweight or obesity [17]. Not only do all these patients have diabetes but they also display several features of the metabolic syndrome. The metabolic syndrome (also known as syndrome X or insulin resistance syndrome) is a cluster of conditions that increase the risk of heart disease and type 2 diabetes. The metabolic syndrome is present if a patient displays three out of five defined characteristics: 1. abdominal obesity (waist circumference greater than 88 and 102 cm in women and men, respectively), 2. high triglyceride levels (more than 150 mg/dl), 3. low high density lipoprotein cholesterol levels (less than 50 and 40 mg/dl for women and men, respectively), 4. high blood pressure (more than 135/85 mm Hg), and 5. high

blood sugar (more than 110 mg/dl) or existence of type 2 diabetes (definition of the International Diabetes Federation). Hence, clustering of clinical and biochemical alterations such as overweight, glucose intolerance, insulin resistance, hypertension, and dyslipidaemia is strongly associated with the development of type 2 diabetes.

The development of insulin resistance in peripheral organs such as skeletal muscle and adipose tissue is an early event in the pathogenesis of type 2 diabetes. Especially skeletal muscle is relevant for insulin resistance as it constitutes one of the major insulin-sensitive organs and contributes for about 80% of insulin-stimulated glucose disposal [18]. Therefore, normal insulin signaling in skeletal muscle as well as mechanisms of disturbed insulin action in diabetic patients or animals represent a key area of research in diabetes.

1.1.2 Insulin signaling in skeletal muscle

Insulin signaling in skeletal muscle is essential to maintain glucose homeostasis and to regulate carbohydrate, lipid, and protein metabolism. Research on insulin signaling and diabetes therapy started at the same moment in the 1920s when the anti-hyperglycemic action of insulin was described and shortly before insulin was used as the first anti-diabetes medication [19, 20]. Since then, insulin has been at the center of diabetes research and in fact it was demonstrated later on that by binding to its receptor, insulin elicits a large range of biological responses that are vital.

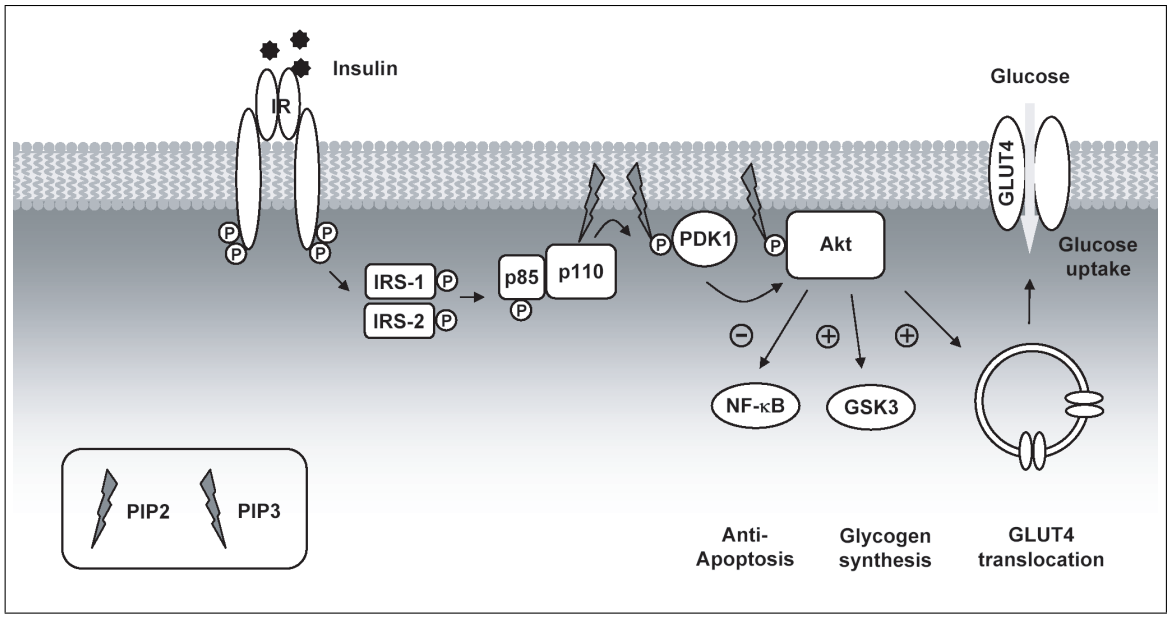
Insulin is a polypeptide hormone (5.8 kDa) that is secreted from the pancreatic β-cells. It consists of two polypeptide chains (chains A and B with 21 and 30 amino acids, respectively) that are linked together by two disulfide bridges. Insulin is secreted from the β-cells in response to a postprandial increase of the plasma glucose level and acts together with its antagonist glucagon on glucose and fat metabolism. In insulin-sensitive tissues such as skeletal muscle and adipose tissue, insulin stimulates the translocation of the glucose transporter (GLUT)4 containing vesicles to the plasma membrane leading to enhanced glucose uptake. In parallel to increasing glucose supply to the cell, insulin blocks gluconeogenesis in liver and stimulates glycogen synthesis in liver and skeletal muscle. In adipose tissue, lipolysis is reduced and lipogenesis is enhanced upon insulin stimulation. Altogether, insulin regulates glucose homeostasis by switching on glucose consuming processes and limiting glucose output from liver by gluconeogenesis [21]. Furthermore, insulin

stimulates protein biosynthesis and regulates cell growth and proliferation.

In skeletal muscle and adipose tissue, glucose uptake is regulated by the phosphatidylinositol 3 kinase (PI3K)/Akt pathway (Figure 1.1). In addition, insulin activates an alternative pathway via Cap/Cbl in adipocytes [22–24], a pathway that seems however to be less relevant for skeletal muscle [25]. In both pathways, the first step in insulin signaling is insulin binding to its receptor. The insulin receptor (IR) is a member of the receptor tyrosine kinase family and consists of two extracellular α-subunits and two transmembrane β-subunits that are linked by disulfide bridges [26, 27]. IR is expressed in a wide range of tissues and its abundance is particularly high in adipose tissue and liver [28]. Binding of insulin to its extracellular binding domain in the α-subunit induces a conformational change in the receptor molecule with subsequent activation of the tyrosine kinase activity and intermolecular trans-autophosphorylation of specific tyrosines in the catalytic β-subunit. Autophosphorylation of the IR allows then for kinase activity and interaction with adaptor proteins. IR transphosphorylates directly several substrates on their tyrosine residues including IR substrate (IRS) proteins 1-4 and Cbl. All these proteins own specific docking sites for other signaling proteins containing Src homology 2 domains [29]. In the family of IRS proteins, IRS1 and IRS2 are the most important for insulin-stimulated GLUT4 translocation. While various pathways involved in metabolism, apoptosis, and proliferation are regulated through IR activation, glucose metabolism is mainly regulated by IRS1/2 and downstream by the mitogen-activated protein kinase (MAPK) and PI3K pathways [30].

Activation of PI3K represents a key step in insulin-stimulated translocation of GLUT4. PI3K consists of a catalytic p110-subunit and a regulatory p85-subunit. In addition to allosteric regulation, the p85-subunit stimulates the translocation of PI3K to its substrates in the plasma membrane. Activated PI3K specifically phosphorylates phosphoinositol (PI) substrates to generate PI(3)P, PI(3,4)P2, and PI(3,4,5)P3. As second messengers these phospholipids recruit the PI3K-dependent serine/threonine kinase (PDK1) and Akt/protein kinase B from cytoplasm to the plasma membrane. Second messenger binding and translocation to the membrane lead to conformational changes in Akt which is subsequently phosphorylated on T308 and S473 by PDK1. Phosphorylation by PDK1 leads to full activation of Akt [31]. Akt regulates the activity of many downstream targets. Among others, Akt phosphorylates components of the GLUT4 complex, protein kinase C (PKC) isoforms, and glycogen synthase kinase (GSK)3 which in turn activates glycogen

Figure 1.1: The insulin signaling pathway



synthase upon insulin stimulation [32].

The final steps in the insulin signaling cascade lead to translocation of GLUT4-containing vesicles to the plasma membrane along the actin cytoskeleton. This process involves specific SNARE proteins that are concentrated on the membrane that interact with other SNARE proteins found on the GLUT4 vesicles [33]. In the vesicles, the v-SNARE proteins vesicle associated membrane protein 2 and 3 proteins bind to the t-SNARE protein syntaxin 4 in the plasma membrane.

1.1.3 Insulin resistance in skeletal muscle *in vivo* and *in vitro*

Insulin signaling and insulin resistance were extensively studied using various knockout mice and cell models. IR knockout making insulin signaling impossible leads to death shortly after birth in mice [34]. Humans that display mutations in the IR gene (leprechaunism) and mice with targeted IR mutations have a similar insulin resistant phenotype [35]. Mice heterozygous for a null allele of IR are insulin resistant but with some variations. Ablation of IR in different tissues has different effects on whole body or specific tissue insulin sensitivity. MIRKO mice (muscle ablation) have normal glucose homeostasis while displaying insulin resistance in muscle [36]. IR knockout in β-cells leads to pronounced insulin resistance in mice due to

loss of glucose-stimulated acute-phase insulin release [37]. NIRKO mice (brain ablation) are mildly insulin resistant and are prone to diet-induced obesity [38]. LIRKO mice (liver ablation) are hyperinsulinemic with normal blood glucose [39]. However, IR knockout in adipose tissue seems to protect from obesity, diabetes, and increases life expectancy in mice [40]. IRS1/2 ablation also leads to glucose intolerance and insulin resistance with IRS2 knockout mice displaying early and more severe insulin resistance [41, 42]. In humans, the G972R IRS1 polymorphism results in a reduced binding affinity of IRS1 to PI3K and is associated to the development of type 2 diabetes [43, 44]. Akt2 knockout in mice results in insulin resistance and type 2 diabetes-like symptoms [45] while ablation of Akt1 seems to affect normal growth but not glucose homeostasis [46]. Finally, GLUT4 ablation, either complete or tissue specific in muscle or in fat, also leads to insulin resistance. Interestingly, whole body GLUT4 knockout mice show only mild hyperglycemia together with a short lifespan [47]. Targeted disruption of GLUT4 in muscle results in insulin resistance, thus demonstrating that GLUT4-mediated glucose transport in muscle is essential to maintain glucose homeostasis [48]. Disruption of GLUT4 in adipose tissue of mice leads to secondary insulin resistance in liver and muscle, and impaired glucose tolerance [49]. Finally, ablation of syntaxin 4 expression which in turn induces inhibition of SNARE complex formation necessary for GLUT4 vesicle docking to the plasma membrane, leads to muscle insulin resistance [50]. Despite this detailed insight into the function of various components of the insulin signaling cascade, the complete molecular biology of insulin resistance leading to the development of type 2 diabetes is far from being understood, specifically in humans.

In diabetic patients where none of the above-mentioned mutations is present there is a clear deregulation of skeletal muscle insulin signaling observable [51]. Insulin resistance in this context stands for the inability of insulin to properly stimulate insulin signaling, for example in skeletal muscle of diabetic patients. Insulin-resistant muscle displays defects at several steps of the insulin signaling cascade, including decreases in insulin-stimulated IR and IRS1 tyrosine phosphorylation, and PI3K activation. In diabetic patients phosphorylation of IR is either reduced [52] or unchanged [53] in comparison to non-diabetics. As for IRS1, its phosphorylation level upon insulin stimulation is decreased in diabetics [54]. In addition, PI3K activity is inhibited in diabetic compared to non-diabetic subjects [53, 54]. The case of Akt is more complicated: one study demonstrated that insulin-stimulated pAkt is lower in diabetics [55] while several others found pAkt in muscle of diabetic patients to

be unchanged or only slightly changed [56–58]. These conflicting data might be due to different levels of insulin used in these studies and different onset of diabetes in participating patients. In first-degree relatives of diabetic patients, lower pAkt after insulin stimulation is observable in different study cohorts [59–61]. In different animal models of diabetes, insulin resistance was observed to be accompanied by lower insulin-stimulated Akt phosphorylation, for example in obese diabetic mice [62, 63] and high fat (HF) diet-induced obese rats [64]. Finally, GLUT4 translocation to the plasma membrane is impaired in muscle of diabetic patients [51, 65, 66]. In lean diabetic patients, GLUT4 expression is not altered so that lower membrane levels of GLUT4 are clearly due to defects in insulin signaling [67, 68]. However, in obese diabetics, the expression levels of GLUT4 are decreased so that this reduction in GLUT4 may also partly explain reduced GLUT4 translocation [69]. So far, all insulin-resistant patients with or without diagnosed diabetes display abnormal subcellular distribution of GLUT4 [65].

Apart from biopsy studies on insulin signaling in muscle, several studies used isolated myocytes from muscle comparing normal, obese, and diabetic patients. They were aimed at answering the important question as to whether insulin resistance is a real biochemical defect in insulin signaling or an acquired feature in skeletal muscle being in contact with increased levels of insulin, glucose or adipocyte secretory products. The data obtained are very contradictory as studies found both cases. Insulin resistance in myocytes from obese patients with insulin resistance disappears in culture and might therefore be acquired with obesity [57]. However, two studies postulated an underlying biochemical defect in insulin resistant muscle because of marked differences in myocyte insulin signaling between normal and diabetic donors [70, 71].

1.2 Obesity and its role in diabetes

1.2.1 Obesity as the main factor contributing to insulin resistance

Adipose tissue is the major organ in mammals where energy can be stored in form of triglycerides. Traditionally, the function of adipose tissue was thought to be restricted to energy storage. More recently, however, it has become evident that adipocytes are also active secretory cells capable of releasing free fatty acids (FFA) by lipolysis and of producing a variety of cytokines, the so-called adipokines [72, 73]. The link between obesity and insulin resistance is now well recognized [74] and increased adiposity, especially in the visceral region, was proven to lead to metabolic disorders such as insulin resistance and to a higher risk factor for cardiovascular diseases [75]. Many studies in humans and in various animal models have shown that obesity is strongly related to the development of diabetes [76–78].

The origin and development of adipose tissue is not entirely understood, but knowledge on fat development is central to understanding normal adipose tissue function and the negative consequences of adipose tissue expansion in obesity. Development of adipose tissue is a dynamic process that occurs throughout the whole life. In this process, the acquisition of fat cells is believed to be irreversible with apoptosis being the only possibility to reduce adipocyte number [79]. Adipose tissue mass and expansion depends on gender, diet, and localization of the adipose tissue depot. It is well recognized that women have more body fat than men with similar body mass index (BMI), a difference that is already present in newborns [80]. Obesity in both genders is also characterized by differential expansion of fat depots with men displaying abdominal fatness and women predominantly increasing fat mass in gluteal and femoral regions. These regional differences in adipose tissue expansion predispose men to have higher triglyceride, fasting glucose, and insulin levels than adiposity-matched women. Interestingly, women characterized by abdominal obesity were metabolically similar to fat mass-matched men further demonstrating fat depot-specific contribution to metabolic dysfunction. Comparison of diabetic patients to age- and BMI-matched non-diabetic patients revealed a significant higher percentage of body fat in the abdomen, the so-called visceral fat [81]. In rats, removal of visceral fat but not subcutaneous fat ameliorates insulin sensitivity [82]. A similar observation was done in women that underwent liposuction removing only subcutaneous adipose tissue without any beneficial effects on insulin sensitivity [83].

In summary, it has been demonstrated that insulin sensitivity is rather related to the location of adipose tissue than to its total amount. How exactly visceral fat impairs insulin sensitivity and what causes accumulation of visceral adipose tissue in diabetic subjects are still open questions.

Weight gain and obesity is characterized by an increase in size of individual adipocytes and by differentiation of new adipocytes increasing the total number of adipocytes. New adipocytes are known to differentiate from adipose tissue residing preadipocytes and mesenchymal progenitor cells. Very recently, it could be demonstrated that also bone marrow-derived circulating progenitor cells contribute to differentiation of new adipocytes in adipose tissue [84]. *In vitro* studies indicated that the rate of preadipocyte proliferation is very high in newborns until the age of one year and during prepuberty, but remains stable during adult life when body weight is constant. However, adults may preserve the ability to form new adipocytes at any age out of a pool of dormant precursor cells [85], but the proportion of subcutaneous adipose tissue stromovascular cells that are committed preadipocytes is reduced with obesity [86, 87]. This effect could be due to greater recruitment of preadipocytes to adipogenesis or greater preadipocyte apoptosis. Older observations argued that only in severe obesity total number of adipocytes can increase [88]. More recently, the "adipocytes life cycle hypothesis" posits that the metabolic consequences of obesity are dependent on whether adipose tissue expansion results from an increase in adipocyte size or adipocyte number [89]. There again, gender differences occur as men show adipocyte proliferation in abdominal fat depots while women show adipocyte hypertrophy and proliferation in the lower body. This may explain the different above-mentioned consequences of obesity in both genders.

Growth regulation of adipocytes does not only occur by food intake or nutrients such as fatty acids but also by hormones. Glucocorticoids [85, 86], thyroid hormones [90], and aldosterone [91] promote adipocyte differentiation. Growth hormone (GH) [92] inhibits differentiation of adipocytes and deficiency in this hormone leads to obesity [93]. However, the most important regulator of adipogenesis is peroxisome proliferator-activated receptor (PPAR)γ [94] that is also involved in glucose and lipid homeostasis. PPARγ agonists such as the thiazolidinediones (TZD) troglitazone and rosiglitazone were extensively studied because of their strong anti-diabetic action [95]. PPARγ agonists exert their primary action in adipose tissue as the expression of PPARγ is 30-times higher there than in muscle or liver [96]. Activation of PPARγ then potentiates the transcription of key genes for adipocyte differenti-

ation and lipid metabolism leading to remodeling of adipose tissue. In the absence of excess energy storage, adipocyte differentiation would result in the production of more adipocytes of smaller average size which is the case in PPARγ agonist-treated animals [97, 98]. Smaller adipocytes are generally believed to be more insulin sensitive and to have lower rate of lipolysis. However, obesity is characterized by bigger adipocytes. Bigger adipocytes, especially those in visceral depots, are highly lipolytic and thereby contribute to FFA output into circulation. Furthermore, there is a relationship between cytokine secretion and adipocyte size as large adipocytes secrete less adiponectin and higher amounts of pro-inflammatory cytokines such as tumor necrosis factor (TNF)α [99].

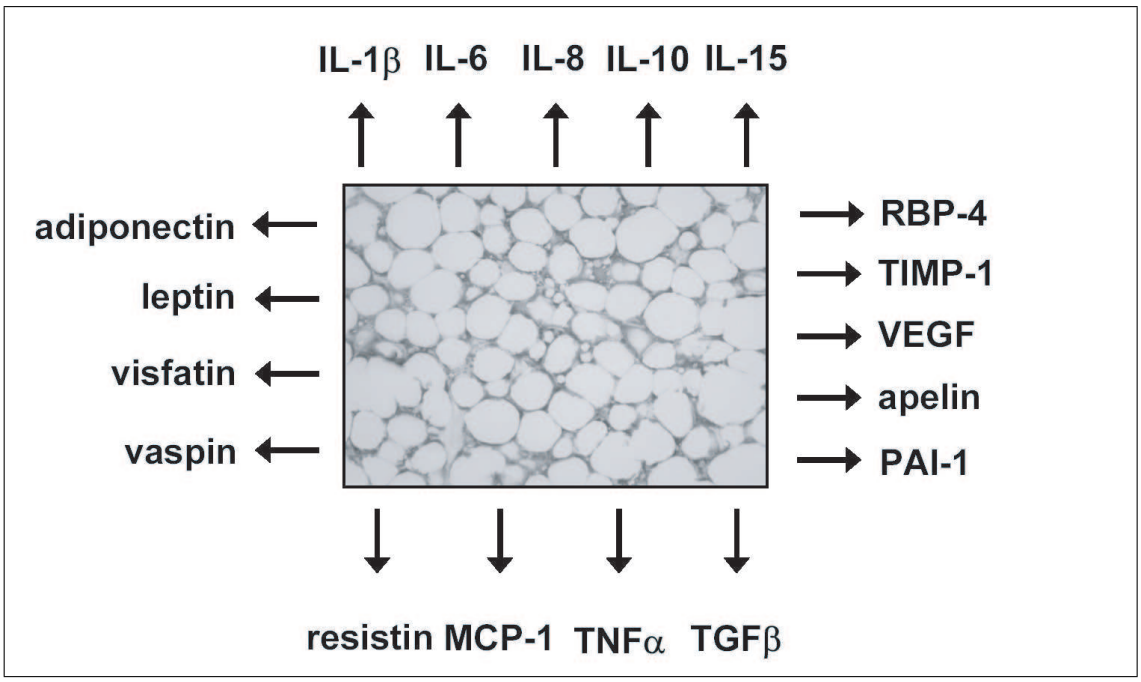
1.2.2 Adipose tissue as an endocrine organ

The association between the epidemics of obesity and diabetes has promoted research on the endocrine link between lipid and glucose homeostasis. It is well-known that elevated circulating plasma levels of triglycerides and FFA due to obesity highly contribute to insulin resistance in peripheral tissues such as skeletal muscle [74]. Moreover, several adipokines were identified and shown to influence insulin action in the muscle [100, 101] and are therefore emerging regulators of insulin sensitivity. TNFα , interleukin (IL)-6, adiponectin, and other still unknown factors might constitute the missing link between adipose tissue and insulin resistance [102]. In fact, adipose tissue is a "champion" concerning its endocrine function as it produces over 50 adipokines [103] which are released into circulation and also exert autocrine or paracrine effects in fat. Adipocyte-derived factors are significantly increased in obesity and are good predictors of the development of type 2 diabetes [104, 105]. Incidentally, the increased secretion of these factors by adipose tissue in the obese state is believed to lead to the well-known metabolic and cardiovascular complications observed in obesity. These adipokines are found to be involved in the deregulation of glucose and lipid homeostasis but also in inflammation by representing pro-inflammatory factors (Figure 1.2).

1.2.3 Adipose tissue derived factors

TNFα is upregulated in obesity of animal models [106, 107] but its role in human physiology is still controversial [108]. TNFα is mainly secreted from macrophages in adipose tissue but it is also expressed in adipocytes [109]. In rodents, it has negative

Figure 1.2: Adipokines



effects on insulin signaling including serine phosphorylation of IRS1 and the following decrease in downstream insulin signaling including PI3K [110]. Furthermore, exposure to TNFα downregulates the expression of IRS1 and GLUT4 [111]. Blocking of TNFα reestablishes normal insulin signaling in rodents but not in humans [112, 113]. Interestingly, TNFα neutralization does not reverse insulin resistance in adipose tissue, an effect that can be explained by a lack of tissue depletion of TNFα protein [114]. In humans, the relation between obesity and TNFα expression and plasma levels is well documented [106] but insulin resistance is not associated with TNFα plasma levels when adjusted for BMI [115]. Finally, TNFα plasma levels decrease upon weight loss in humans [116].

IL-6 is secreted both by adipose tissue and skeletal muscle [117] although adipose tissue contribution to IL-6 plasma levels in resting patients is considered to be more important. Therefore, IL-6 can be seen as an adipokine. IL-6 is elevated in plasma of obese patients [118] but is, as well as TNFα, not related directly to insulin resistance. *In vitro*, IL-6 has been shown to induce insulin resistance in hepatocytes and adipose tissue [119, 120]. Data from our laboratory also show induction of insulin resistance in human skeletal muscle cells in response to high doses of IL-6 [121]. However, the role of IL-6 in skeletal muscle remains controversial [118] since

there is evidence that IL-6 does not cause insulin resistance while several studies support a role of IL-6 in impaired insulin action. In short-term experiments, IL-6 might rather be involved in metabolic regulation similar to insulin supporting the notion that IL-6 may not be implicated in the induction of insulin resistance [122]. However, short-term experiments might be more relevant to the study of physical activity than to diabetes and obesity research whereas exposure of insulin responsive tissues to increased cytokine levels occurs in a more chronic way.

IL-8 is secreted from adipose tissue and its plasma levels are increased in obesity [120, 123, 124]. Since IL-8 expression is increased by pro-inflammatory cytokines such as TNFα and IL-1, it may be involved in obesity-related complications. However, a correlation between higher levels of IL-8 in obesity and increased insulin resistance is not yet established.

Increased plasminogen activator inhibitor (PAI) 1, an inflammatory mediator, has been linked not only to thrombosis and fibrosis but also to obesity and insulin resistance. PAI-1 is expressed in adipose tissue and secreted from isolated adipocytes [125]. As TNFα regulates the secretion of PAI-1 from adipocytes, increased levels of this adipokine are believed to be consequent to obesity. Clinical studies have clearly linked PAI-1 to insulin resistance and diabetes [126]. It occurs at higher levels in obese and diabetic patients and their first-degree relatives [127]. PAI-1 knockout mice are protected from HF diet-induced obesity and insulin resistance [128]. The data suggest that PAI-1 may not just increase as an inflammatory marker in response to obesity and insulin resistance, but might also be a direct player in the development of obesity and insulin resistance.

Monocyte chemotactic protein (MCP)-1 is also clearly associated to the obese state [129, 130]. MCP-1 is secreted by various cells including human adipocytes but also by cells of the vasculature upon stimulation with pro-inflammatory factors [131, 132]. The function of MCP-1 is well characterized when it comes to its role in the recruitment of monocytes and memory T lymphocytes into tissue [133]. However, MCP-1 is also involved in the development of insulin resistance as many other chemokines who have been shown to possess physiological functions going further than the simple recruitment of immune cells. For MCP-1 insulin resistance inducing capacities have been demonstrated in adipocytes [129]. Furthermore, its overexpression especially in epicardial adipose tissue is thought to increase the inflammatory stress of arteries [134]. Also, MCP-1 was shown to have angiogenic effects in endothelial cells and may therefore play a role during adipose tissue expansion and

remodeling in obesity [135].

Leptin is an adipocyte-derived hormone that plays a critical role in the regulation of body weight by controlling food intake and energy expenditure [136]. Leptin stimulates certain hypothalamic regions in the brain that regulated food intake. In peripheral tissues, it inhibits processes that lead to energy storage and stimulates energy expenditure. In addition to regulating energy balance, leptin is involved in other processes such as immune and inflammatory responses and reproduction [137]. Leptin also regulates body weight by inducing apoptosis of adipocytes [138–140]. Leptin-deficiency in the *ob/ob* mouse is accompanied by obesity, insulin resistance, and diabetes [141] supporting the notion that leptin is an important player in glucose homeostasis and probably in the pathogenesis of obesity-related metabolic diseases such as type 2 diabetes [74]. The role of leptin in the development of diabetes is however controversial. Several studies have shown that leptin treatment leads to increased insulin sensitivity and can reverse the obese and diabetic phenotypes of *ob/ob* mice and rats [142, 143]. Other studies have shown, on the contrary, that leptin impairs insulin action in hepatocytes, adipocytes, and skeletal muscle cells [144–146]. *In vivo*, leptin concentrations are higher in insulin-resistant men independent of adipose tissue mass [147]. Other research papers report leptin as a negative regulator of insulin sensitivity [148–150]. In summary, leptin acts both as an insulin sensitizer but also contributes to the insulin-resistant obese phenotype. However, there is still uncertainty about whether or not the described effects of leptin can be attributed directly to this adipocyte-derived hormone. It is probable that in the obese state, higher levels of leptin are associated to strong effects in the periphery and blunted central effects of this hormone, so that the notion of selective leptin resistance in the brain, especially with aging, emerged [151, 152]. Other research rather speaks of peripheral leptin resistance [153, 154]. Leptin resistance would explain its insulin-sensitizing role *in vivo* but not the observed effects *in vitro* making it difficult to assign to leptin a clear role in insulin resistance.

Resistin is a small polypeptide hormone produced by adipocytes and immunocompetent cells [155, 156]. It was originally proposed as a link between obesity and insulin resistance/diabetes [157]. Later, studies revealed that significant differences exist between the main sites of resistin production in rodents (adipocytes) and humans (immunocompetent cells) [155, 158]. While in rodents resistin appears to have an important role in the development of liver insulin resistance, its role in humans is less clear. In rodents, effects of resistin on skeletal muscle and adipose tissue insulin

sensitivity are thought to be secondary to its effects on liver [159, 160]. In humans, it was proposed that resistin is probably involved in the regulation of inflammatory processes rather than directly to insulin sensitivity [161]. As such, resistin emerged as a new pro-inflammatory molecule. Its expression is associated with the release of inflammatory markers such as TNFα and IL-6 [162, 163]. In human adipocytes and macrophages, resistin stimulates the secretion of TNFα via a nuclear factor (NF)-κB- and c-Jun N-terminal kinase (JNK)- dependent pathway further pointing to a pro-inflammatory character of this adipokine [164, 165].

Systematic analysis of adipose expression differences between lean and obese subjects or between different adipose depots led to the description of several new adipokines. Visfatin, formerly known as pre-B cell colony-enhancing factor, was discovered to be an adipokine in 2005 [166]. This adipokine binds to the IR and mimics insulin action in cell culture. However, visfatin plasma concentrations are much lower than those of insulin and the lack or minimal effect of its regulation by fasting and feeding raises doubts on its physiological role as insulin-sensitizing agent [167, 168]. Visfatin is expressed in visceral and subcutaneous adipose tissue and was first shown to increase in obesity but seems not to be related with parameters of insulin sensitivity in obese patients [167]. However, other studies support a role of visfatin in the pathogenesis of diabetes [169, 170] or even show a contradictory decrease in circulating visfatin levels in obese patients [171]. Taken together, clinical data on visfatin are highly contradictory and make it very difficult to speculate on the role of this adipokine. In isolated adipocytes, its expression increased by thiazolidinedione (TZD) PPARγ agonists [172] and decreased by IL-6 [173]. However, TZD have no effect on visfatin circulation levels [174]. Apelin was also recently classified as a new adipokine [175] and shown to be increased in mouse models of obesity with hyperinsulinemia but not without it. Thus, apelin seems to be insulin-regulated but whether it can bind to the IR like visfatin is not known. Parallel regulation of apelin expression and secretion by TNFα makes it difficult to speculate on the role of this adipokine in obesity or diabetes [176]. Visceral adipose tissue-derived serpin (vaspin) is also expressed in visceral adipose tissue and decreased in obesity and diabetes. This adipokine has insulin sensitizing effect in HF diet-induced obese rats and vaspin expression can be normalized by rosiglitazone [177]. However, in human biopsies, vaspin is a barely detectable adipokine whose expression is found more often in diabetic patients. Its expression increases with obesity but could not be related to insulin resistance so that it was supposed that vaspin is not an attrac-

tive candidate for research on human adipose tissue [178]. Retinol binding protein (RBP)-4 was recently described to contribute to insulin resistance *in vivo* [179, 180] but the underlying mechanism of its action remains somewhat controversial [181]. Tissue inhibitor of metalloproteinase (TIMP)-1 is another new candidate adipokine whose expression and secretion are upregulated in obese patients and *in vitro* by pro-inflammatory cytokines making it possible that TIMP-1 might play a role in maintaining adipose tissue mass in obesity [182, 183].

Adiponectin plays a special role in adipose tissue secretion. Adiponectin is the only known adipokine that is downregulated in obesity and that positively influences insulin sensitivity [184]. Adiponectin is a 30 kDa protein containing an N-terminal collagenous domain and a globular head domain that shares homology with complement factor C1q subunits. It was first identified independently in 1995 by several laboratories [185–187]. The protein was found to be exclusively produced by adipocytes and attains high plasma concentrations. In the circulation adiponectin exists in various forms of multimers due to combination of monomers at the collagen domain [188]. The three major oligomeric forms are a low molecular weight (LMW) trimer, a middle molecular weight (MMW) hexamer and a high molecular weight (HMW) 12- to 18-mer. The fact that obesity can be described by adiponectin deficiency makes adiponectin a preferred target for studying the molecular link between adipose tissue and insulin resistance. As mentioned above, adiponectin secretion is decreased in obesity [189] and low plasma levels are good indicators of insulin resistance and development of diabetes [190, 191]. Adiponectin is also significantly decreased in first-degree relatives of diabetic patients pointing to an early deregulation of adiponectin release in the pathogenesis of type 2 diabetes [10, 192]. In studies with obese and diabetic rodents, it was shown that injection of adiponectin leads to a normalized insulin sensitivity [193, 194]. Recently, the role of different multimers in insulin resistance has been found with HMW adiponectin being the most active form of the adipokine [188]. In fact, several studies could show that HMW adiponectin better correlates with insulin resistance than total adiponectin [195, 196]. Furthermore, mutations in the collagen domain of adiponectin are associated with insulin resistance and type 2 diabetes [197, 198]. In addition to insulin sensitization, known effects of adiponectin *in vitro* include anti-apoptotic action on pancreatic β-cells [199] and anti-inflammatory and vasoprotective role in vascular endothelial cells [200, 201]. Furthermore, adiponectin secretion is decreased by pro-inflammatory adipokines like IL-6 [202]. Recently, adiponectin receptors were cloned

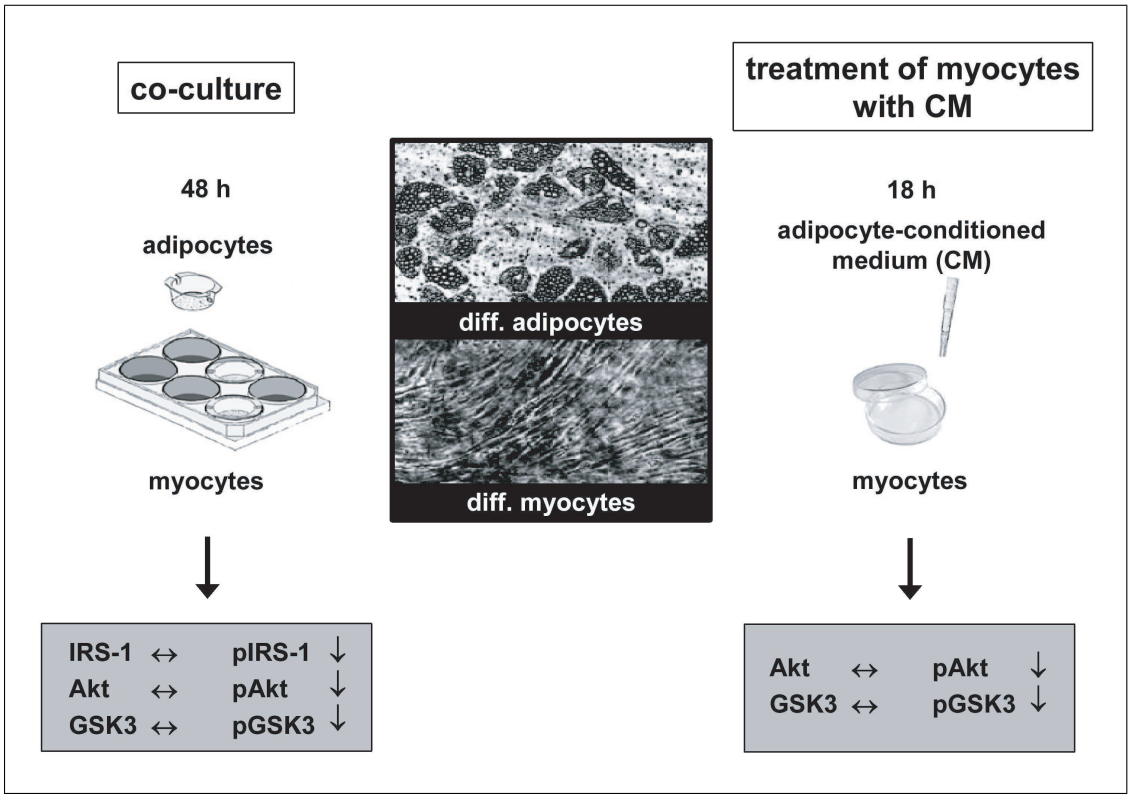
and described [203]. Two isoforms, adiponectin receptors (AdipoR) 1 and 2, are differentially expressed in different tissues - AdipoR1 ubiquitously and abundantly in skeletal muscle and AdipoR2 mainly in liver. The two receptors only share 67% homology to each other and seem to have different binding affinity for globular and full-length adiponectin.

1.2.4 Co-culture of adipocytes and skeletal muscle cells

All the facts put togehter in the previous sections have shown good evidence that a negative crosstalk between excess body fat and skeletal muscle leads to disturbances in skeletal muscle insulin signaling and finally to insulin resistance. The co-culture model of skeletal muscle cells and adipocytes was established in our laboratory to study the interaction between these two cell types [100]. Co-culture is a generally accepted approach for studies on the paracrine interaction between two cell types. Several other laboratories have used this approach to analyse the interaction between macrophages and adipocytes [204], neurons and adipocytes [205] as well as adrenocortical cells and adipocytes [206] to elucidate the role of adipocyte-derived factors for various tissues. The co-culture of skeletal muscle cells and adipocytes provided the proof of a negative crosstalk between adipocytes and myocytes. Co-culture clearly leads to insulin resistance in skeletal muscle cells similar to the defects observed in skeletal muscle of diabetic patients (Figure 1.3). Skeletal muscle cells undergoing co-culture display decreased insulin-stimulated IRS-1, Akt, and GSK3 phosphorylation.

An alternative approach to co-culture represents the use of adipocyte-conditioned medium (CM) that contains a complex mixture of adipokines. In this setting, CM mimics co-culture with reduced insulin-stimulated IRS-1 and Akt phosphorylation [121]. The insulin resistance-inducing factor was shown to be proteinase K sensitive but heat-resistant. Furthermore, the key protein(s) should be bigger than 5 kDa as demonstrated by CM fractioning.

Figure 1.3: Co-culture of skeletal muscle cells with adipocytes and treatment of skeletal muscle cells with CM



1.3 Objectives

The high prevalence of obesity and diabetes in adults demonstrates that there is a need to investigate the pathophysiological mechanisms that link both "epidemics". Especially the crosstalk between expanding adipose tissue and insulin-resistant skeletal muscle is a key issue in understanding the molecular link between obesity and diabetes. In that perspective, identifying molecules that are crucial to this crosstalk could lead to the discovery of new pharmacological treatments of diabetes and the metabolic syndrome, or of new biomarkers for the early detection of person at risk of becoming diabetic later in life. The starting point of the present work was the establishment of the co-culture model of human primary skeletal muscle cells and adipocytes. With the proof of principle of a negative crosstalk between skeletal muscle cells and adipocytes several questions appeared that are treated in this thesis.

- Adiponectin was shown to reverse insulin resistance in several animal models of diabetes but the mechanism of its action is not fully understood. The first aim of this thesis was to evaluate if adiponectin can prevent insulin resistance in skeletal muscle cells co-cultured with adipocytes. In this context, it was also necessary to assess which cell type is positively affected by adiponectin treatment and which adiponectin receptors are present to mediate these effects.

- Adiponectin acts in an autocrine/paracrine way on human adipocytes to prevent insulin resistance in skeletal muscle cells. The second objective of this thesis was to find out what soluble proteins are secreted from *in vitro* differentiated adipocytes and how the release of these factors is regulated by adiponectin.

- Analysis of the adipocyte secretion pattern unveiled a large number of adipokines that where downregulated by adiponectin, some of which may be responsible for the induction of insulin resistance. The third aim of this thesis was to study the role of adipokines in the induction of insulin resistance in skeletal muscle cells. In this regard, it was crucial to analyse if an isolated adipokine is able to induce insulin resistance or if skeletal muscle insulin resistance can only be obtained by treatment with a mixture of several adipokines with additive or

synergistic effects.

- The chemokine MCP-1 proved to be highly effective in inducing insulin resistance in skeletal muscle cells making it possible that its receptors from the chemokine CC motif receptors (CCR) family could also play a role in the induction of skeletal muscle insulin resistance. The fourth objective of this thesis was therefore to find out if regulation of CCR expression plays a role in the induction of insulin resistance in skeletal muscle cells.

Chapter 2

Study 1

Autocrine action of adiponectin on human fat cells prevents the release of insulin resistance-inducing factors[†]

Daniela Dietze-Schroeder[‡], Henrike Sell[‡], Mathias Uhlig, Marlis Koenen, and Jürgen Eckel

Institute of Clinical Biochemistry and Pathobiochemistry, German Diabetes Center, Düsseldorf, Germany

Running title: Autocrine action of adiponectin

Key words: Insulin resistance; skeletal muscle; adipose tissue; adiponectin; autocrine regulation; cellular crosstalk

Abbreviations

Acrp30, adipocyte complement-related protein of 30 kDa; AdipoR, adiponectin receptor; gAcrp30, globular domain of Acrp30; GRO, growth-regulated oncogene; GSK, glycogen synthase kinase; HGF, hepatocyte growth factor; IGFBP, insulin-like growth factor binding protein; IL, interleukin; IRS, insulin receptor substrate; MCP, monocyte chemotactic protein; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; NF, nuclear factor; OPG, osteoprotegerin; sTNFR, soluble TNF receptor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor.

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[‡]These authors contributed equally to this work.

Abstract

The adipocyte hormone adiponectin is negatively correlated with obesity and insulin resistance and may exert an important antidiabetes function. In this study, primary human skeletal muscle cells were co-cultured with human fat cells or incubated with adipocyte-conditioned medium in the presence or absence of gAcrp30 to analyze its capacity to restore normal insulin signaling in the muscle cells. Human skeletal muscle cells co-cultured with adipocytes or treated with adipocyte-conditioned medium showed an impaired Akt and GSK3 serine phosphorylation in response to insulin. Furthermore, insulin-stimulated GLUT4 translocation was reduced by adipocyte-conditioned medium. Impaired insulin signaling was normalized upon addition of gAcrp30 to the co-culture. Moreover, adipocyte-conditioned medium generated in the presence of gAcrp30 was unable to perturb insulin-stimulated Akt phosphorylation. Concomitant addition of gAcrp30 and adipocyte-conditioned medium to the myocytes failed to restore normal insulin action. Protein array analysis of adipocyte-conditioned medium indicated that the secretion of at least eight different cytokines was diminished in response to gAcrp30. We therefore suggest that adiponectin operates as a key regulator of adipocyte secretory function. This autocrine action may prevent the induction of skeletal muscle insulin resistance and may partly explain the antidiabetes action of this hormone.

Introduction

It is now well accepted that adipose tissue represents a major secretory and endocrine active organ producing a variety of factors that may regulate energy metabolism and insulin sensitivity [1]. Increased adipose tissue mass, especially in the visceral compartment, is associated with insulin resistance, hyperglycemia, dyslipidemia, hypertension, and other components of the metabolic syndrome [2, 3] and represents one of the major risk factors for the development of type 2 diabetes [4–6]. Adipocytes from obese subjects exhibit an altered endocrine function and secretory profile leading to an increased release of adipocytokines and pro-inflammatory molecules including tumor necrosis factor (TNF)α, interleukin (IL)-6, angiotensinogen, and resistin [7, 8]. Some of these factors play a key role in the induction of skeletal muscle insulin resistance in rodents [9]; however, their precise role in humans remains controversial [10].

Adiponectin or adipocyte complement-related protein of 30 kDa (Acrp30) is the only known adipocytokine for which plasma levels are decreased in obesity and type 2 diabetes [11–13]. Low adiponectin plasma levels are good indicators of insulin resistance and the development of diabetes [14, 15]. In studies with obese and diabetic rodents, it was further shown that intravenous application of adiponectin leads to normalized insulin sensitivity [16, 17]. Many studies focused on the physiological importance of adiponectin and support the notion of an antidiabetes action of this hormone. However, the cellular and molecular basis of this effect remains poorly understood, and little is known about the role of adiponectin in the crosstalk between adipose tissue and skeletal muscle. Known effects of adiponectin *in vitro* include an anti-apoptotic action on pancreatic β-cells [18] and an anti-inflammatory and vasoprotective function in vascular endothelial cells [12, 19]. Furthermore, adiponectin secretion by 3T3 adipocytes is decreased by pro-inflammatory adipocytokines like IL-6 [20].

In the present study, we took advantage of our recently described co-culture model of human adipocytes and skeletal muscle cells [10] to elucidate the role of adiponectin in the crosstalk between adipose tissue and skeletal muscle. We report here that adiponectin prevents the induction of muscle insulin resistance by reducing the release of fat cell secretory products. These factors include IL-6/-8, growth-regulated oncogene (GRO)α, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α/β, and tissue inhibitor of metalloproteinase (TIMP)-1/-2. We suggest that adiponectin acts as a key regulator of

adipocyte secretory function and that this autocrine action may contribute to the antidiabetes effect of this hormone.

Materials and Methods

Materials

BSA (fraction V, fatty acid free) was obtained from Boehringer (Mannheim, Germany). Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech (Braunschweig, Germany) and Sigma (München, Germany). The recombinant C-terminal globular domain of adiponectin and full-length adiponectin were products from Tebu (Offenbach, Germany). Polyclonal antibodies anti-phospho(p)-glycogen synthase kinase (GSK)3α/β (Ser21/9), anti-p-Akt (Ser473), and anti-Akt were supplied by Cell Signaling Technology (Frankfurt, Germany). Anti-GSK3α/β was from Stressgene (Victoria, Canada). HRP-conjugated goat anti-rabbit IgG antibody was from Promega (Mannheim, Germany). Cytokine protein arrays (RayBio® Antibody Array C Series 1000) were purchased from RayBiotech (Norcross, GA). Collagenase CLS type 1 was obtained from Worthington (Freehold, NJ), and culture media were obtained from Gibco (Berlin, Germany). The cytokines IL-6/-8, MCP-1, MIP-1α/β were purchased from Hölzel Diagnostics (Köln, Germany). Primary human skeletal muscle cells and supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). Membrane inserts for six-well culture dishes with a pore size of 0.4 μm (pore density $1.6 \times 10^6/\text{cm}^2$) and insert companion plates were supplied by FALCON. All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

Culture of human skeletal muscle cells

Satellite cells were isolated from M. rectus abdominis by enzymatic digestion with trypsin followed by a purification step with fibroblast-specific magnetic beads to prevent contamination with fibroblasts. After two passages, the myoblasts were characterized by the manufacturer (PromoCell) using immunohistochemical detection of sarcomeric myosin in differentiated cultures at 100% confluence (8 days). Primary human skeletal muscle cells of four healthy Caucasian donors (male, 9 and 5 yr [M9, M5]; female, 10 and 48 yr [F10, F48]) were supplied as proliferating myoblasts (5×10^5 cells) and cultured as described earlier [10]. 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 Eagle's/Ham's F-12 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 Eagle's medium for 4 days.

Adipocyte isolation and culture

Adipose tissue samples were obtained from the mammary fat of 13 normal or moderately overweight women (BMI 19.2-31 kg/m^2, aged between 21 and 52 yr) undergoing surgical mammary reduction. 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 ∼10 mg in weight. Preadipocytes were isolated by collagenase digestion as previously described [21]. Isolated cell pellets were resuspended in Dulbecco's modified Eagle's/Ham's F12 medium supplemented with 10% FCS, seeded on membrane inserts (3.5×10^5/4.3 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 the co-culture experiments and for generation of adipocyte-conditioned medium, as recently described [22].

Co-culture

Co-culture of human fat and muscle cells was conducted according to our recently published protocol [10]. Briefly, after *in vitro* differentiation of preadipocytes on membrane inserts, the adipocytes were washed once with PBS and then incubated for 24 h in skeletal muscle cell differentiation medium containing 1 pM insulin. Thereafter, adipocytes were washed twice with PBS and individual membrane inserts were subsequently transferred to the culture plates containing differentiated (4 days) myocytes in α-modified Eagle's medium containing 1 pM insulin. This resulted in an assembly of the two cell types sharing the culture medium but being separated by the membrane of the insert at a distance of 0.9 mm from the bottom. Co-culture was conducted for 48 h. Integrity of both cell types was routinely checked by light microscopy at the end of the co-culture period. Conditioned medium was generated by culturing adipocytes for 48 h using exactly the same protocol as de-

scribed for the co-culture followed by collection of the medium. This material was lyophilized, reconstituted, and finally added to myotubes. As a control, we used lyophilized adipocyte medium.

Assay of GLUT4 translocation

Recombinant, replication-defective adenoviral vectors were generated with the AdenoVator system from QBiogene (Heidelberg, Germany). Three days after start of differentiation, skeletal muscle cells were infected with recombinant adenoviruses encoding GLUT4myc and were used for analysis after an additional 48-h incubation. After stimulation with 10^{-7} M insulin for 30 min, GLUT4 translocation was measured based on the protocol described by Kanai et al. [23].

Primer and RT-PCR

Total RNA was extracted from differentiated human skeletal muscle cells and adipocytes by using Trizol (Roche Diagnostics, Mannheim, Germany) following the manufacturer's protocol. The reverse transcription reaction and the following PCR were performed with the One Step RT-PCR Kit (Qiagen, Hilden, Germany), as previously described [24].

Measurement of cytokine protein levels in adipocyte-conditioned medium

Adipocyte-conditioned media generated in absence or presence of 5 nM globular domain adiponectin (gAcrp30) from different fat donors ($n = 5$) were hybridized with the array membranes according to the protocol supplied by the manufacturer. Briefly, membranes were blocked by furnished blocking solution and then incubated with 1.2 ml of conditioned medium over night at 4 °C. Membranes were then washed according to the manufacturer's protocol and incubated with the mix of biotin-conjugated antibodies for 2 h at room temperature. After washing, HRP-conjugated streptavidin was added to the membranes for 1 h at room temperature. The signal was developed with detection buffers and directly detected using a LUMI Imager system (Roche Diagnostics). The signal intensity was normalized to internal positive signals on the membrane. For each fat donor, conditioned medium generated in presence of 5 nM gAcrp30 and control medium without addition of adiponectin was analyzed at the same time and cytokine signals compared. Cytokine concentrations in conditioned medium were determined with the same cytokine array. A mix of the five measured cytokines was analyzed twice using different concentrations to assure

linearity of the assay. Absolute cytokine concentrations were calculated based on the calibration curve. The level of adiponectin in adipocyte-conditioned media was determined using an ELISA kit from B-Bridge International (Sunnyvale, CA). The assay was performed as recommended by the manufacturer using duplicate samples for all determinations.

Immunoblotting

Muscle cells were treated as indicated and lysed in a buffer containing 50 mM Tris/HCl (pH 7.4), 1% (v/v) NP-40, 0.25% (v/v) sodium-deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, and protease inhibitor cocktail. After incubation for 2 h at 4 °C the suspension was centrifuged at 10000g for 10 min. Thereafter, total cell lysates were separated by SDS-PAGE using gradient (8-18%) horizontal gels and transferred to PVDF filters in a semidry blotting apparatus [25]. For detection of Akt, p-Akt, GSK3α/β and p-GSK3α/β, filters were blocked with TBS containing 0.1% Tween-20 and 5% non-fat dry milk and subsequently incubated over night with a 1:1000 dilution of appropriate antibodies. After extensive washing, filters were incubated with goat anti-rabbit or goat anti-chicken (in the case of GSK3) HRP-coupled antibody and processed for enhanced chemiluminescene detection using SuperSignal Substrate (Pierce, Rockford, IL). Signals were visualized and evaluated on a LUMI Imager work station using image analysis software (Boehringer Mannheim).

Presentation of data and statistics

Statistical analysis was carried out by using either Student's t-test or ANOVA for comparison of more than two variables. All statistical analyses were done using Prism software (Graphpad, San Diego, CA). A value of $p < 0.05$ was considered as statistically significant. Corresponding significance levels are indicated in the figures.

Results

Adiponectin ameliorates impairment of insulin signaling in skeletal muscle cells co-cultured with human adipocytes

As we were previously able to demonstrate, co-culture of human adipocytes and human skeletal muscle cells leads to a rapid disturbance of insulin signaling in the

muscle cell, as seen by a prominent reduction in the efficiency of insulin to promote insulin receptor substrate (IRS)-1/Akt phosphorylation [10]. As presented in Fig. 1A, this impairment of insulin signaling could also be detected further downstream at the level of GSK3. Co-culture conditions increased basal phosphorylation of GSK3 and concomitantly reduced the effect of insulin on this process. Thus, the fold stimulation was reduced from 2.5 ± 0.2 to 1.6 ± 0.3 and from 3.7 ± 0.9 to 1.9 ± 0.4 ($n = 12$) for GSK3α/β serine phosphorylation, respectively. Most importantly, adipocyte-conditioned medium, which mimics the co-culture condition [22], profoundly reduced insulin-stimulated GLUT4myc translocation in human skeletal muscle cells (Fig. 1B). This assay detects the movement of GLUT4 to the cell surface, a major effect of insulin in this tissue. We therefore conclude that adipocyte-derived factors impair both insulin signaling and downstream insulin action in the myocytes.

To investigate whether adiponectin has the capacity to prevent the induction of impaired myocyte insulin signaling under co-culture conditions, adipocytes and skeletal muscle cells were co-cultured for 48 h in the absence or presence of 10 nM gAcrp30. After co-culture, skeletal muscle cells were acutely stimulated with insulin, and downstream signaling was assessed at the level of the serine/threonine kinase Akt, a key mediator of insulin action on glucose transport and glycogen synthesis [26]. As reported earlier, co-culture leads to a significant reduction of insulin-induced serine phosphorylation of Akt without affecting basal phosphorylation or expression level of the kinase (Fig. 2A). As can be seen from the data, the presence of adiponectin during the co-culture period clearly prevents the impairment of insulin signaling in the muscle cells. Under these conditions, Akt serine phosphorylation after acute insulin stimulation was not significantly different from the control situation (Fig. 2A).

To check if the two cell types express the receptors for adiponectin, we analyzed the cells using RT-PCR. Our results show that *in vitro*-differentiated human skeletal muscle cells and adipocytes express both, adiponectin receptor (AdipoR)1/2 (Fig 2B). As shown in Fig. 2C, adiponectin has no direct effect on insulin-stimulated Akt-phosphorylation in skeletal muscle cells. Thus, adiponectin-mediated prevention of impaired insulin signaling in the myocytes (Fig. 2A) cannot be explained by augmented insulin responsiveness of Akt phosphorylation in these cells.

Autocrine action of adiponectin on human adipocytes prevents the release of insulin resistance-inducing factors

We next addressed the question of whether skeletal muscle cells incubated with adipocyte-conditioned medium and adiponectin are protected from impaired insulin signaling. Adipocyte-conditioned medium impaired insulin signaling in skeletal muscle cells at the level of Akt serine phosphorylation by 50-60% without affecting the expression level of this enzyme (Fig. 3). Concomitant incubation of muscle cells with adiponectin and adipocyte-conditioned medium did not prevent impairment of insulin signaling, making it likely that the results observed in the co-culture reflect adiponectin action on the fat cells.

To provide evidence for an autocrine action of adiponectin, fat cells from the same fat cell donors were cultured for 48 h in the absence or presence of 5 nM gAcrp30 and the conditioned medium was processed for experiments with myotubes. As presented in Fig. 4, impairment of insulin-stimulated Akt phosphorylation by adipocyte-conditioned medium was nearly abolished when generated in the presence of adiponectin. This experiment was repeated with the same result using full-length adiponectin. Thus, when using cells from muscle donors M9 and F48, adipocyte-conditioned medium reduced ($p = 0.006$) insulin-stimulated Akt phosphorylation to $59.5 \pm 12.5\%$ of control, whereas $92 \pm 1\%$ of insulin-stimulated control was observed with medium generated in the presence of full-length adiponectin. Furthermore, adipocyte-conditioned medium induced a significant impairment of insulin signaling downstream of Akt (69.9 ± 3.5 and $86.4 \pm 4.1\%$ [$n = 4$] of insulin-stimulated control for GSK3α/β phosphorylation, respectively). This inhibitory action was completely abolished when testing adipocyte-conditioned medium generated in the presence of gAcrp30 (99.2 ± 8.9 and $100.9 \pm 2.9\%$ of insulin-stimulated control for p-GSK3α/β, respectively). These observations suggest that adiponectin can counteract induction of skeletal muscle insulin resistance by autocrine action on adipocytes. This notion is supported by the determination of endogenous adiponectin in adipocyte-conditioned media. As presented in Table 1, a very low concentration of adiponectin was associated with inhibitory activity on insulin signaling in skeletal muscle cells. This inhibitory activity was not found in conditioned media with 10- to 20 fold higher endogenous adiponectin levels (Table 1). It is worth noting that the addition of gAcrp30 for 48 h did not alter the adiponectin release by the adipocytes. Furthermore, we observed lower MCP-1, MIP-1α/β concentrations (reduced to 40, 30, and 10%, respectively) in conditioned media with higher endogenous adiponectin that

were not used for further experiments. It should be noted that low adiponectin is unrelated to any clinical background of the donors, since adipose tissue was always obtained from healthy subjects.

Adiponectin downregulates cytokine secretion from human adipocytes

To identify adipocytokines that are secreted by differentiated human adipocytes and regulated by adiponectin, we analyzed adipocyte-conditioned medium with a cytokine protein array detecting 120 different cytokines. In conditioned medium from five different fat donors, the following cytokines were identified: adiponectin, GROα, hepatocyte growth factor (HGF), insulin-like growth factor binding protein (IGFBP)-3, IL-6/-8, MCP-1, macrophage-derived chemokine (MDC), MIP-1α/β, osteoprotegerin (OPG), soluble TNF receptor (sTNFR)-2, and TIMP-1/-2. In conditioned media from some individuals we also detected angiogenin, fibroblast growth factor-9, intercellular adhesion molecule-1, neutrophil activating peptide-2, TNFα/β and plasminogen activator receptor. To evaluate the effect of adiponectin treatment on secretion of adipocytokines, we analyzed adipocyte-conditioned medium generated in the presence of 5 nM gAcrp30 in comparison to the respective control adipocyte-conditioned medium (Fig. 5A). MDC, IGFBP-3, and HGF secretion was not affected by adiponectin treatment. However, adiponectin reduced IL-6/-8, and GROα secretion by 50-60% (Fig. 5B). Several members of the small inducible cytokine family such as MCP-1, MIP-1α/β were also significantly reduced. From the two TNF-related proteins only OPG was significantly reduced. TIMP-1/-2, two cytokines that are thought to play a role in adipocyte differentiation, were also significantly reduced in medium generated in the presence of adiponectin (Fig. 5B). A comparable reduction in adipocytokine secretion was also observed in response to 5 nM full-length adiponectin (percent of untreated control: IL-6 (52$\pm$3), IL-8 (52$\pm$14), MCP-1 (55 ± 8), MIP-1α (61 ± 15), MIP-1β (66 ± 12), OPG (53 ± 14), TIMP-1 (39 ± 7), TIMP-2 (44 ± 13); $n = 3$). The concentrations of several adiponectin-regulated cytokines in the supernatant of cultured human adipocytes are presented in Table 2. As reported earlier, a very high amount of IL-6 is released by these cells [27], whereas IL-8 and MCP-1 are present at 50- to 200-fold concentrations above the physiological level, in excellent agreement with recent observations [28, 29].

Discussion

Adipocyte-derived factors such as TNFα and IL-6 are significantly increased in obesity and are good predictors of the development of type II diabetes [30, 31]. Obesity thereby contributes to a pro-inflammatory milieu, and it is now recognized that adipose tissue functions as an endocrine organ secreting a variety of pro-inflammatory factors. Adiponectin is the only adipocytokine known to be downregulated in obesity; however, little is known about the role of adiponectin in the crosstalk between adipose tissue and skeletal muscle itself. We report here for the first time on adiponectin acting as an autocrine regulator of adipocytokine secretion of the human fat cell. By decreasing cytokine release by the adipocyte, adiponectin prevents the impairment of insulin signaling in a co-culture model of human adipocytes and skeletal muscle cells. This is in accordance with the view that the crosstalk between inflammatory and metabolic signaling pathways may elicit insulin resistance and extends this concept to human skeletal muscle. Adiponectin secretion is very low by *in vitro*-differentiated human adipocytes in accordance with studies using freshly isolated mature adipocytes in long-term culture [32]. Furthermore, we found here that the low level of adiponectin correlated with the release of insulin resistance-inducing factors by adipocytes. Therefore, the co-culture represents an ideal model of adiponectin deficiency to study the role of adiponectin in the crosstalk between fat and muscle. It is worth noting that most of our experiments were conducted using the globular head domain of adiponectin. Adiponectin prevails in serum as a trimer, hexamer or high-molecular weight form, with controversial results being reported on the biological activity of these isoforms [33]. The globular head domain of adiponectin is generated by leukocyte elastase [34] and became detectable in human plasma at low abundance when using immunoprecipitation with a globular-head specific anti-serum [35]. Furthermore, the head domain binds to AdipoR1/2, and transgenic expression protects *ob/ob* mice from diabetes [36]. However, the physiological relevance of gAcrp30 remains controversial, since other studies failed to detect it in human serum samples using non-reducing and non-heat-denaturing SDS-PAGE [37] or velocity sedimentation [38]. Nevertheless, the high effciency of gAcrp30 in animal studies highlights the potential role of this molecule for future drug development.

Earlier studies focused on adiponectin action in skeletal muscle and adipose tissue. Adiponectin was found to activate AMP kinase in skeletal muscle [39] depending on the muscle type [40]. In myocytes, adiponectin may also activate the nuclear

factor (NF)-κB pathway [41]. Furthermore, in this tissue, adiponectin treatment leads to enhanced fat oxidation and glucose transport. Adiponectin expression in adipocytes is regulated by various compounds such as TNFα and growth hormone, which also influence insulin sensitivity [42, 43], and adiponectin treatment leads to increased glucose uptake [44]. In the current investigation, we focused on the crosstalk between these two tissues, which are both critical players regarding insulin resistance and diabetes. We propose a model of autocrine/paracrine adiponectin action on adipose tissue as a pivotal determinant of muscle insulin sensitivity. In this respect, it was shown here that adiponectin had no effect on insulin signaling at the level of Akt in the muscle cells, neither alone nor in combination with adipocyte-conditioned medium, although these cells express both types of adiponectin receptors. Thus, in our model the beneficial effect of adiponectin on muscle insulin signaling is clearly adipocyte dependent since (i) it was observed upon addition to the co-culture and (ii) the insulin resistance-inducing effect of conditioned medium was ameliorated when generated in the presence of adiponectin. A paracrine effect of adiponectin on fat cell formation in bone marrow [45] and fat cell differentiation [46] was already proposed. Furthermore, adiponectin is considered an autocrine regulator of energy metabolism [47]. An autocrine regulation of cytokine secretion in adipocytes adds a new mechanism to the pleiotropic action potential of adiponectin.

In this study, we identified several cytokines exhibiting adiponectin-regulated secretion from differentiated human adipocytes. Some of these adiponectin-regulated cytokines such as IL-6/-8 and MCP-1 are known to be related to obesity and diabetes. Others such as GROα, MIP-1, and TIMP-1/-2 are related to inflammation and tissue remodeling, but their relation to adipose tissue, obesity, and diabetes is less clear. IL-6/-8 and MCP-1 are well-known to be induced in the obese state in humans and rodents [48–51]. IL-6 is expressed both by adipose tissue and skeletal muscle [52]. Elevated plasma concentrations of these adipocytokines in obese and insulin-resistant patients may contribute to the insulin-resistant state observed in obesity. Interestingly, adiponectin concentrations are inversely correlated to IL-6 plasma concentrations, insulin sensitivity, and obesity in full agreement with our findings [48]. However, the role of IL-6 in skeletal muscle remains controversial with publications both supporting and not supporting involvement of IL-6 in impaired insulin action [53]. Our recent study showed that only extremely high concentrations of IL-6 produced a slight impairment of insulin signaling in human skeletal muscle cells, making it unlikely that IL-6 alone is sufficient to induce muscle insulin resis-

tance [27]. IL-8 is secreted from adipose tissue, and its plasma levels are elevated in obesity [49, 54]. Since IL-8 expression is increased by pro-inflammatory cytokines such as TNFα and IL-1, it may be involved in obesity-related complications. The potent reduction of IL-8 secretion by adiponectin shown here supports the notion [49] that this cytokine may play a role in the induction of insulin resistance, most likely in concert with other adipocytokines.

We show here for the first time that GROα is secreted from human adipocytes. This cytokine is structurally related to IL-8 and considered to attract neutrophils to the site of inflammation and may play a role in inflammation, angiogenesis and tumorigenesis [55]. Its role in adipose tissue and the regulation of insulin sensitivity needs to be defined. MCP-1 is clearly associated to the obese state [50, 51]. Its overexpression, especially in epicardial adipose tissue, is thought to increase the inflammatory burden of arteries [56]. In adipocytes, MCP-1 expression is increased by TNFα, insulin, growth hormone, and IL-6 [57]. Treatment of 3T3-L1 adipocytes with MCP-1 was found to impair glucose uptake, indicating that this cytokine may contribute to the pathogenesis of insulin resistance [50], although the effect of MCP-1 on skeletal muscle insulin action needs to be established.

TIMP-1/-2, in combination with the matrix metalloproteinases, exert key functions in extracellular matrix remodeling. As for TIMP-1, it was shown to be strongly induced in obesity [58]. Matrix metalloproteinases and TIMP activity may be essential for adipogenesis since changes in cell-matrix interaction must accompany adipocyte hypertrophy as well as recruitment and differentiation of adipocyte precursors. This is supported by the observation that TIMP-1 knockout mice are less sensitive to the induction of obesity [59]. By decreasing secretion of TIMPs, adiponectin may decrease adipocyte hypertrophy and fat accumulation. Thus, adiponectin could directly contribute to adipose tissue remodeling by increasing the number of smaller adipocytes which are known to better retain free fatty acids and contribute to increased insulin sensitivity. MIP-1α is expressed by human adipocytes, but its secretion decreases upon differentiation [28], a feature shared with IL-8. Regulation of MIP-1α by adiponectin may also contribute to decreased adipocyte hypertrophy. Thus, in addition to regulating the release of inflammatory cytokines that may interfere with insulin signaling in the muscle cell, adiponectin may also exert its antidiabetes action by regulating fat cell differentiation and growth.

In summary, our data show that adiponectin acts as a key regulator of cytokine secretion in adipose tissue. We therefore suggest that cytokines regulated

by adiponectin may represent a molecular link between obesity and skeletal muscle insulin resistance. These adipocytokines are involved in inflammation, tissue remodeling, and angiogenesis, but their role in obesity and the development of skeletal muscle insulin resistance needs to be further analyzed.

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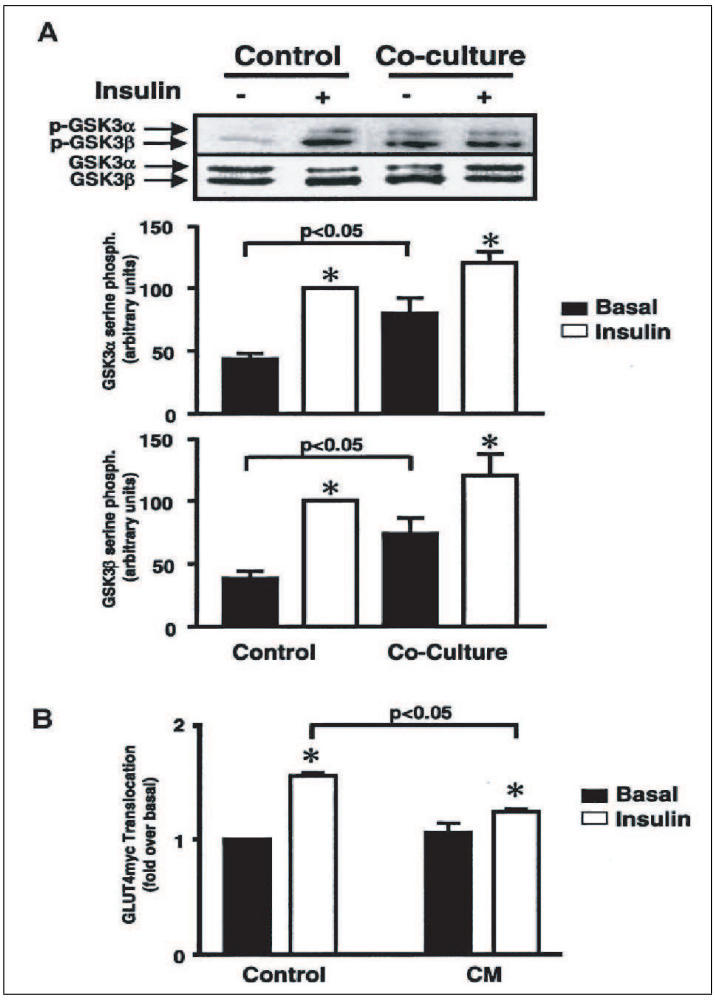
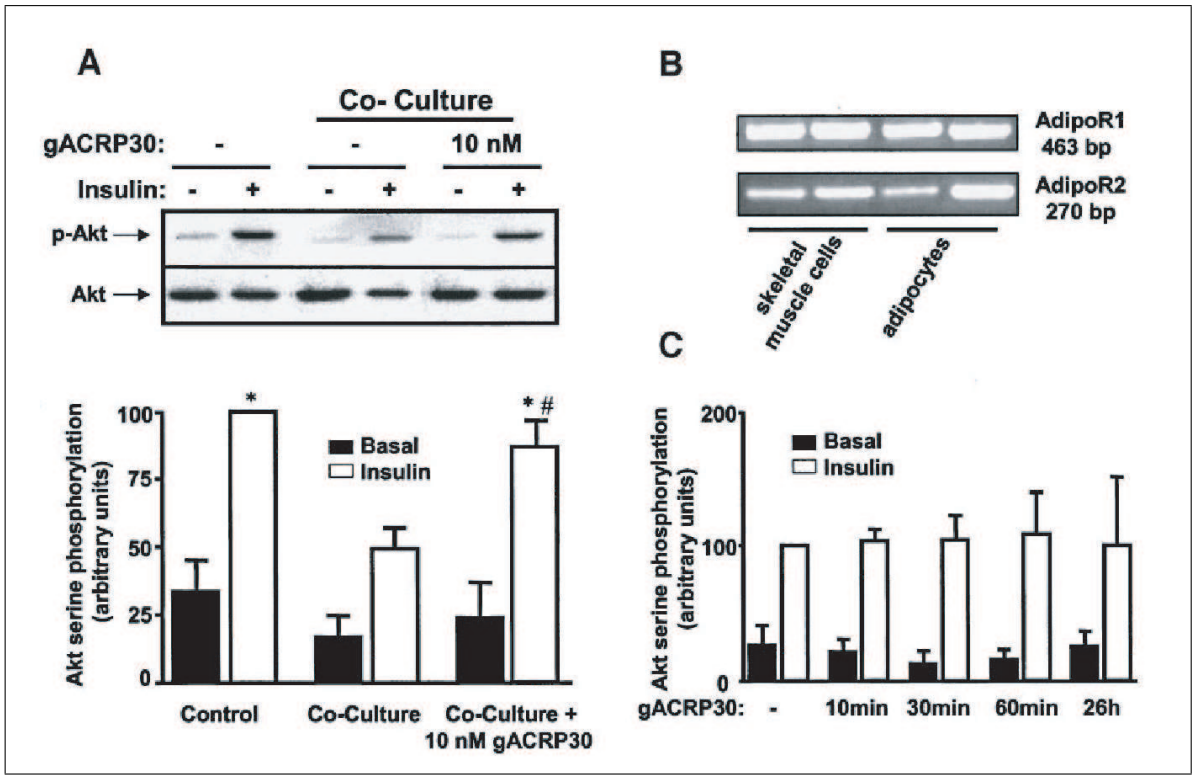
Figure 2.1: Effect of co-culture on insulin signaling and effect of adipocyte-conditioned medium on insulin-stimulated GLUT4-translocation in human skeletal muscle cells



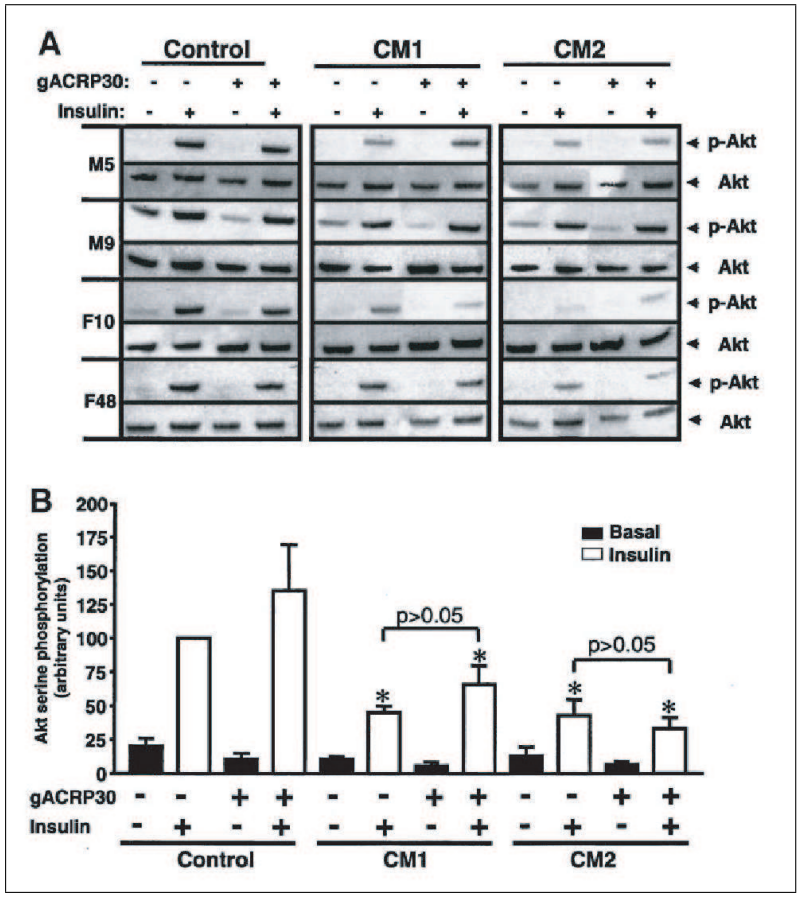
A: Skeletal muscle cells from three different donors (M5: male, 5 yr; M9: male, 9 yr; F10: female, 10 yr) were subjected to co-culture with differentiated human adipocytes and GSK3 phosphorylation in the myocytes was analyzed after acute stimulation with insulin (100 nM, 10 min). Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific GSK3 and GSK3 antibodies. Western blots were quantified on a LUMI Imager work station using image analysis software. Data are mean values ± SEM of twelve independent experiments and are normalized to GSK3 expression levels. * significantly different from corresponding basal level. **B**: Skeletal muscle cells were cultured for 18 h in absence or presence of conditioned medium from two different adipocyte donors. Translocation of GLUT4myc was assessed after acute stimulation with insulin, as outlined in *Materials and Methods*. Mean ± SEM of four independent experiments. * significantly different from corresponding basal level.

Figure 2.2: Effect of adiponectin on insulin signaling in human skeletal muscle cells subjected to co-culture with human adipocytes



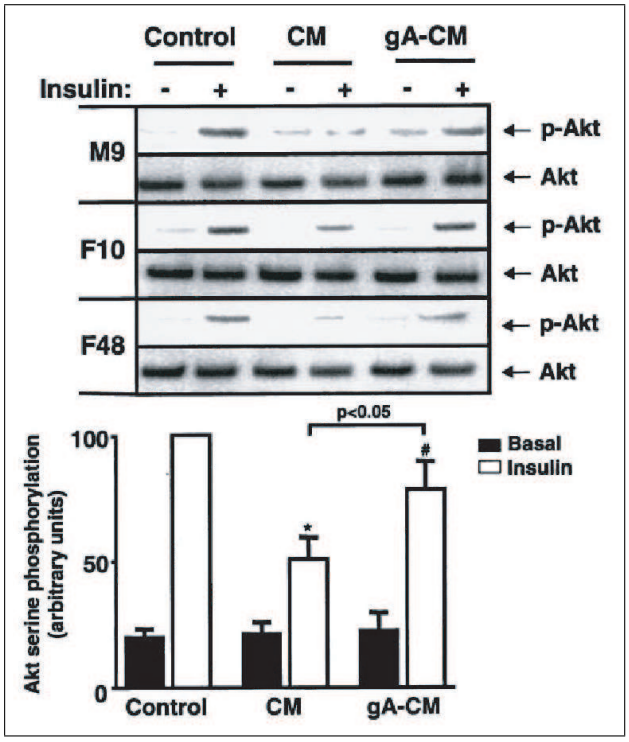
A: Human skeletal muscle cells were subjected to co-culture in the absence or presence of 10 nM gAcrp30 for 48 h and were subsequently stimulated with insulin (100 nM, 10 min). *Upper panel*: Total cell lysates (8 µg/lane) were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt antibody and Akt antibody. *Lower panel*: Western blots were quantified as described in Fig. 1. All data were normalized to Akt protein expression and are expressed relative to the insulin-stimulated control value. Data are mean values ± SEM of four independent experiments from three different muscle cell donors. * significantly different from insulin-stimulated co-culture ($p < 0.001$); # not significantly different from control ($p > 0.05$). **B**: Total RNA was extracted from differentiated human skeletal muscle cells and adipocytes and RT-PCR for AdipoR1/2 was performed as outlined in *Materials and Methods*. **C:** Skeletal muscle cells were incubated without or with 10 nM gAcrp30 for the indicated times followed by acute stimulation with insulin. Akt phosphorylation was determined as outlined above. Data are mean values ± SEM of four independent experiments from four different muscle cell donors (M5, M9, F10, F48: $n = 1$ each).

Figure 2.3: Effect of adipocyte-conditioned medium on insulin signaling in myocytes in absence or presence of adiponectin



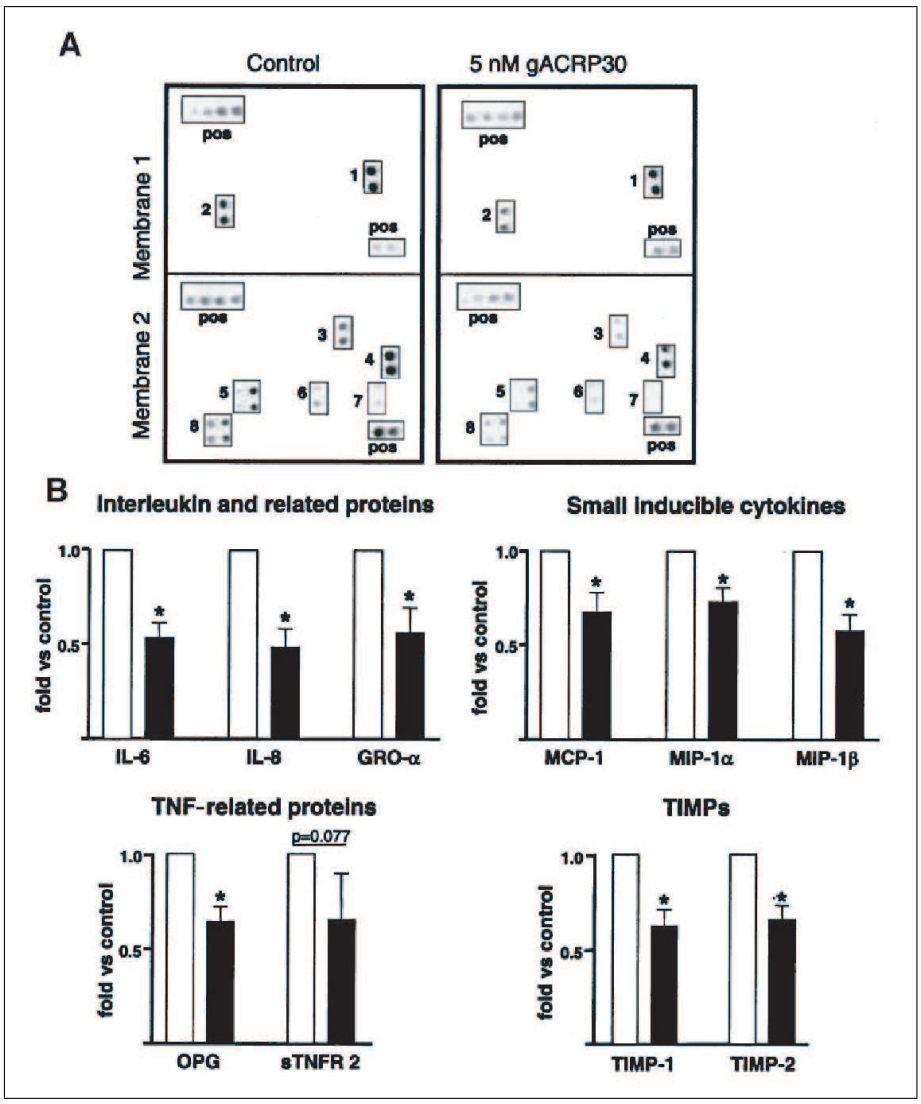
A: Myocytes from four different donors (M5, M9, F10, F48) were cultured in the presence of adipocyte-conditioned medium from two different donors (CM1, CM2) for 18 h with or without 10 nM adiponectin. After acute stimulation with insulin, total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt antibody and Akt antibody. **B**: Blots were quantified as described in Fig. 1. Data are mean values ± SEM of six independent experiments. All data were normalized to the level of Akt expression and are expressed relative to the insulin-stimulated control value. * significantly different from control ($p < 0.01$)

Figure 2.4: Analysis of adipocyte-conditioned medium generated in the presence of adiponectin



Skeletal muscle cells from three different donors were cultured under control conditions or were incubated with adipocyte-conditioned medium generated in the presence or absence of 5 nM gAcrp30. After acute stimulation with insulin, Akt serine phosphorylation and Akt expression was assessed. Data are mean values ± SEM of four independent experiments. All data were normalized to the level of Akt expression and are expressed relative to the insulin-stimulated control value. * significantly different from insulin stimulated control ($p < 0.001$), $^{\#}$ not significantly different from insulin stimulated control ($p < 0.05$)

Figure 2.5: Regulation of adipocyte cytokine secretion by adiponectin



A: Cytokine array membranes (membrane 1 and 2, each detecting 60 different cytokines) were incubated with adipocyte-conditioned medium that was generated in absence or presence of 5 nM adiponectin. After washing, the membranes were incubated with a mix of biotin-conjugated antibodies and processed for detection on the LUMI Imager system, as outlined in *Materials and Methods*. Two representative arrays with adipocyte-conditioned media from the same donor culture are presented. (1) IL-6, (2) MCP-1, (3) GROα, (4) IL-8, (5) MIP-1α/β, (6) osteoprotegerin (OPG), (7) sTNFR-2, and (8) TIMP-1/2. pos, positive control for normalization. **B**: Protein arrays were quantified using image analysis software. Data are mean values $\pm$ SEM of five independent adipocyte cultures from five different donors, each run in the absence or presence of gAcrp30. * $p < 0.05$ compared to control medium.

Table 2.1: Adiponection concentrations in adipocyte-conditioned medium

Conditioned medium	**Adiponectin concentration** (ng/ml)	**Inhibition of Akt phosphorylation** (%)
CM 1	1.13	55, ±,5 (6)
CM 2	0.43	57, ±,12 (6)
CM 3	0.83	29, ±,6 (3)
CM 4	0.74	43, ±,7 (5)
CM 5	0.74	30, ±,10 (5)
CM 6	0.80	38, ±,4 (2)
CM 7	9.06	ND
CM 8	17.40	ND

Data are mean ± SEM (no. of experiments). Conditioned medium (CM) of eight different donors was generated as outlined in *Material and Methods*. Duplicate samples were analyzed using an adiponectin ELISA kit. Inhibition of insulin-stimulated Akt phosphorylation was determined as outlined in *Material and Methods*.

Table 2.2: Cytokine concentrations in adipocyte-conditioned medium

Cytokine	**Concentration** (ng/ml)
IL-6	22.0, ±,6.0
IL-8	0.26, ±,0.08
MCP-1	31.2, ±,6.7
MIP-1α	0.20, ±,0.06
MIP-1β	1.09, ±,0.32

Data are mean values ± SEM of five adipocyte cultures. Cytokine concentrations in adipocyte-conditioned medium without addition of gAcrp30 were analyzed by protein cytokine array as detailed in *Materials and Methods*. A mix of the five cytokines was measured at different concentrations and used for calibration.

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Chapter 3

Study 2

Monocyte chemotactic protein-1 is a potential player in the negative crosstalk between adipose tissue and skeletal muscle[†]

Henrike Sell, Daniela Dietze-Schroeder, Ulrike Kaiser, and Jürgen Eckel

Institute of Clinical Biochemistry and Pathobiochemistry, German Diabetes Center, Düsseldorf, Germany

Running title: MCP-1 and muscle insulin resistance

Key words: MCP-1; insulin resistance; skeletal muscle; adipose tissue; cellular crosstalk

Abbreviations

CCR, chemokine CC motif receptor; CM, conditioned medium; CXCR, chemokine CXC motif receptor; GSK, glycogen synthase kinase; IRS, insulin receptor substrate; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; NF, nuclear factor; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor.

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Abstract

Adipose tissue is a major secretory and endocrine active organ producing a variety of bioactive proteins which may regulate energy metabolism and insulin sensitivity. In several studies, we have already shown that adipocyte-secretory products induce skeletal muscle insulin resistance. However, the precise nature of these factors has remained elusive. Human adipocytes were found to secrete various cytokines including IL-6/-8, MIP-1α/β, and MCP-1. Among these candidates, MCP-1 alone impaired insulin signaling in skeletal muscle cells at doses similar to its physiological plasma concentrations (200 pg/ml), whereas IL-6/-8 and MIP-1β were effective at very high concentrations only. In addition, MCP-1 significantly reduced insulin-stimulated glucose uptake in the myocytes. Expression analysis of chemokine receptors in skeletal muscle cells revealed the presence of CXCR1/2 and CCR1/2/4/5/10. The action of MCP-1 on insulin signaling in skeletal muscle cells occurs via ERK1/2 activation but does not involve activation of the NF-κB pathway. In conclusion, our data show that adipocytes secrete various adipocytokines that may be involved in the negative crosstalk between adipose tissue and skeletal muscle. Human skeletal muscle cells are highly sensitive toward MCP-1, which impairs insulin signaling and glucose uptake at concentrations even below that found in the circulation. However, other cytokines that are released by adipocytes impair insulin action only at supraphysiological concentrations. Therefore, MCP-1 may represent a molecular link in the negative crosstalk between adipose tissue and skeletal muscle assigning a completely novel important role to MCP-1 besides inflammation.

Introduction

Obesity is one of the most serious health hazards, especially in the Western World. Frequently, obesity is accompanied by metabolic disturbances such as insulin resistance, hyperglycemia, dyslipidemia, hypertension, and other components of the metabolic syndrome [1, 2]. Insulin resistance is a hallmark of obesity emerging early in the metabolic syndrome and is highly associated with increased visceral adipose tissue mass. The concept of adipose tissue as a major secretory and endocrine active organ producing a variety of bioactive proteins that may regulate energy metabolism and insulin sensitivity is now widely accepted [3]. Increased adipose tissue mass, especially in the visceral compartment, represents one of the major risk factors for the development of type 2 diabetes [4–6]. Adipocytes from obese subjects are characterized by altered metabolic and endocrine function leading to an increased secretion of adipocytokines and pro-inflammatory molecules including tumor necrosis factor (TNF)α, IL-6, angiotensinogen, and resistin [7, 8]. It is likely that some of these secreted molecules may be factors underlying the key association of excess body fat to insulin resistance in peripheral organs such as skeletal muscle. We recently demonstrated that skeletal muscle cells co-cultured with human adipocytes exhibit an impairment of insulin signaling and GLUT4 translocation [9, 10] and defined thereby the mechanism of a negative crosstalk between adipose tissue and skeletal muscle.

Monocyte chemotactic protein (MCP)-1 is a chemokine and member of the small inducible cytokine family and plays a crucial role in the recruitment of monocytes and T lymphocytes into tissues [11]. It is expressed by adipocytes [12] and a number of other cell types including smooth muscle and endothelial cells when exposed to inflammatory stimuli [13]. MCP-1 is overexpressed in obese rodents [14, 15] and reaches significantly higher plasma levels in diabetic patients [16]. Its overexpression, especially in epicardial adipose tissue, is thought to increase the inflammatory burden of arteries [17]. In 3T3-L1 adipocytes, MCP-1 expression is increased by TNFα, insulin, growth hormone and IL-6 [18]. Treatment of 3T3-L1 adipocytes with MCP-1 was found to impair glucose uptake, indicating that this cytokine may contribute to the pathogenesis of insulin resistance [14]. The role of MCP-1 in skeletal muscle insulin action still needs to be established.

We recently reported that the autocrine action of adiponectin ameliorates the insulin resistance-inducing capacity of adipocyte-conditioned medium (CM) concomitant with reduced secretion levels of various cytokines [10]. These cytokines

include IL-6/-8, MCP-1, and macrophage inflammatory protein (MIP)-1α/β. In the present investigation, we have assessed the effect of these cytokines on insulin signaling and downstream insulin action in primary human skeletal muscle cells. The data show that MCP-1 is a prominent inducer of insulin resistance in human skeletal muscle cells, which assigns a completely novel important role to MCP-1 besides its role in inflammation and infiltration of monocytes to adipose tissue.

Materials and Methods

Materials

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 Sigma (München, Germany). Polyclonal antibodies anti-phospho(p)-glycogen synthase kinase (GSK)3α/β (Ser21/9), anti-p-Akt (Ser473), anti-p-necrosis factor (NF)-κB (P65), anti-p-ERK1/2 (Ser536), and anti-Akt were supplied by Cell Signaling Technology (Frankfurt, Germany), anti-actin from Santa Cruz Biotechnology (Heidelberg, Germany), and anti-tubulin from Calbiochem (Merck Biosciences, Schwalbach, Germany). Anti-GSK3α/β was from Stressgene (Victoria, Canada). Antibodies for chemokine CC motif receptor (CCR)4 and CCR10 came from Imgenex (San Diego, CA) and the one for CCR2 from Alexis (San Diego, CA). HRP-conjugated goat anti-rabbit and anti-mouse IgG antibodies were from Promega (Mannheim, Germany). Cytokine protein arrays (RayBio® Custom Array) were purchased from RayBiotech (Norcross, GA). Collagenase CLS type 1 was obtained from Worthington (Freehold, NJ) and culture media were obtained from Life Technologies, Inc. (Berlin, Germany). The cytokines IL-6/-8, MCP-1 and MIP-1β were purchased from Hölzel Diagnostics (Köln, Germany) and TNFα from Sigma. Primary human skeletal muscle cells and supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). 2-deoxy-D-[1-^{14}C] glucose was purchased from Amersham Biosciences Europe (Freiburg, Germany). All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

Adipocyte isolation and culture

Adipose tissue samples were obtained from the mammary fat of normal or moderately overweight women (BMI 24.9 ± 3.5, aged between 21 and 52 yr) undergoing

surgical mammary reduction. The procedure to obtain adipose tissue was approved by the ethical committee of Heinrich-Heine-Universität (Düsseldorf, 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 ∼10 mg in weight. Preadipocytes were isolated by collagenase digestion as previously described [19]. Isolated cell pellets were resuspended in Dulbecco's modified Eagle's/Ham's F12 medium supplemented with 10% FCS, seeded on membrane inserts ($3.5 \times 10^5/4.3$ 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 adipocyte-CM, as recently described [20]. Briefly, after *in vitro* differentiation, adipocytes were incubated for 24 h in skeletal muscle cell differentiation medium containing 1 pM insulin. CM was then generated by culturing adipocytes for 48 h in the same medium followed by collection of the medium.

The purity of adipocytes in the culture was analyzed by morphological means (Fig. 1). Cells that do not differentiate most likely are preadipocytes that may contribute to the results. Isolated macrophages do not adhere to the culture dishes and are washed away. Adhesion of stromal cells is prevented by the elimination of erythrocytes by the appropriate lysis buffer during preadipocyte isolation [21]. Furthermore, the use of a two-step filtration process before seeding of cells substantially eliminates endothelial cells [22].

Measurement of cytokine protein levels in adipocyte-CM

Adipocyte-CM was hybridized with the array membranes according to the protocol supplied by the manufacturer. Briefly, membranes were blocked by the furnished blocking solution and then incubated with 1.2 ml of CM over night at 4 °C. Membranes were then washed according to the manufacturer's protocol and incubated with the mix of biotin-conjugated antibodies for 2 h at room temperature. After washing, HRP-conjugated streptavidin was added to the membranes for 1 h at room temperature. The signal was developed with detection buffers and directly detected using a LUMI Imager system (Roche Diagnostics, Mannheim, Germany). The signal intensity was normalized to internal positive signals on the membrane.

Culture of human skeletal muscle cells

Satellite cells were isolated from M. rectus abdominis by enzymatic digestion with trypsin followed by a purification step with fibroblast-specific magnetic beads to prevent contamination with fibroblasts. After two passages, the myoblasts are characterized by the manufacturer (PromoCell) using immunohistochemical detection of sarcomeric myosin in differentiated cultures at 100% confluence (8 days). Primary human skeletal muscle cells of four healthy Caucasian donors (male, 5 and 9 yr [M5, M9]; female, 10 and 48 yr [F10, F48]) were supplied as proliferating myoblasts (5×10^5 cells) and cultured as described in our earlier study [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 Eagle's/Ham's F-12 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 Eagle's medium for 4 days.

Primer and RT-PCR

Total RNA was extracted from differentiated human skeletal muscle cells using the RNeasy kit from Qiagen (Hilden, Germany). cDNA was generated with an Omniscript RT kit from Qiagen, and PCR performed with PuRe Taq Read-To-Go PCR beads from Amersham Biosciences Europe using primers shown in Table 1 [12].

Immunoblotting

Muscle cells were treated as indicated and lysed in a buffer containing 50 mM Tris/HCl (pH 7.4), 1% (v/v) NP-40, 0.25% (v/v) sodium-deoxycholate, 150 mM NaCl, 1 mM EGTA, 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 $10,000 \times g$ for 10 min. Thereafter, 8 µg of lysates were separated by SDS-PAGE using 10% horizontal gels and transferred to PVDF filters in a semidry blotting apparatus [23]. For detection, filters were blocked with TBS containing 0.1% Tween-20 and 5% nonfat dry milk and subsequently incubated over night with a 1:1000 dilution of appropriate antibodies. After extensive washing, filters were incubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescene detection using SuperSignal Substrate (Pierce, Rockford, IL). Signals were visualized and evaluated on a LUMI Imager work station using image analysis software (Roche Molecular Biochemicals, Mannheim, Germany).

Assay of glucose uptake

Recombinant, replication-defective adenoviral vectors were generated with the AdenoVator system from QBiogene (Heidelberg, Germany). Three days after start of differentiation, skeletal muscle cells were infected with recombinant adenoviruses encoding GLUT4myc [22] and were used for analysis after an additional 48 h incubation. Uptake of 2-deoxy-glucose was measured for 30 min after an acute 30 min insulin stimulus (10^{-7} M insulin) as described before [24].

Presentation of data and statistics

Statistical analysis was performed by ANOVA. All statistical analyses were done using Statview (SAS, Cary, NC) considering a value of $p < 0.05$ as statistically significant. Corresponding significance levels are indicated in the figures.

Results

Adipocyte-CM impairs insulin signaling and contains various cytokines

CM of differentiated human adipocytes impairs insulin signaling at the level of Akt and GSK3α phosphorylation, whereas GSK3β phosphorylation is modestly but significantly decreased (Fig. 2, A and B). Furthermore, CM impairs insulin action on GLUT4 translocation in primary human skeletal muscle cells, as described in several studies from our laboratory [9, 10, 20]. We concluded that adipocyte-derived factors impair insulin signaling and downstream insulin action in skeletal muscle cells. Several adipocyte-derived factors could be described to be secreted from adipocytes and could be found in CM of differentiated human adipocytes such as IL-6/-8, MCP-1, MIP-1α/β (Fig. 2C). Very recently, we demonstrated the autocrine control of the release of these adipocyte secretory products by adiponectin [10].

Human skeletal muscle cells express different chemokine CXC receptor (CXCR) and CCR

To test whether differentiated human skeletal muscle cells express chemokine receptors, we analyzed CXCR and CCR expression using RT-PCR. Our results show that human skeletal myotubes express both CXCR1/2, two important receptors for IL-8 (Fig. 3A). Furthermore, skeletal muscle cells express CCR1/2/4/5/10 mRNA.

Using Western blot analysis, the protein expression of CCR2/4/10 could also be demonstrated (Fig. 3B). These receptors mediate the action of MCP-1, MIP-1α/β, CCR2 representing the main receptor for MCP-1. Comparing the expression of these receptors between skeletal muscle cells and adipocytes, we observed that the abundance of CCR2 is significantly higher in skeletal muscle cells, but that CCR4/10 are expressed at similar levels (Fig. 3B). It is noteworthy that CCR4 protein expression is similar in skeletal muscle cells and adipocytes, whereas CCR4 mRNA is visibly more abundant in adipocytes. This might be due to different processing of CCR4 mRNA in these two cell types or different half-lives of the receptor.

Human skeletal muscle cells are highly sensitive toward MCP-1

Figure 4 shows that concentrations as low as 0.1-times the physiological plasma level (20 pg/ml) significantly reduce insulin-stimulated Akt phosphorylation in skeletal muscle cells. At a physiological dose (200 pg/ml), MCP-1 is able to decrease insulin signaling to the same extent as adipocyte-CM; however, it does not significantly increase its effect upon application of higher doses. Downstream from Akt, insulin-stimulated GSK3α/β phosphorylation is also significantly impaired by MCP-1 (Fig. 4C). However, MCP-1 does not affect the expression of Akt and GSK3α/β (Fig. 4D).

To test the effect of MCP-1 further downstream of Akt, we also measured insulin-stimulated glucose uptake. Physiological concentrations of MCP-1 as well as 10 times over physiological concentration significantly impaired insulin-stimulated glucose uptake, reducing it to about 65% of the control situation (Fig. 5). This impairment of glucose uptake is in good agreement with data obtained in adipocytes where 5 times over physiological concentration (1 ng/ml) of MCP-1 reduced insulin-stimulated glucose uptake by 25% after long-term treatment [14].

In skeletal muscle cells, MCP-1 signals via ERK1/2 (Fig. 6A). ERK has already been described as a key mediator of MCP-1 signaling in human endothelial cells and macrophages [25, 26]. In human smooth muscle cells, MCP-1-induced migration is also mediated via ERK activation [27]. Interestingly, MCP-1 does not activate the NF-κB pathway (Fig. 6A). These data contrast with our findings obtained with adipocyte-CM that was shown to contain MCP-1 [9], clearly indicating that adipocyte-derived factors other than MCP-1 must be responsible for NF-κB activation by adipocyte-CM. Significant activation of ERK1/2 with MCP-1 could be obtained with doses as low as 0.1× physiological serum concentration (20 pg/ml) in short term (10 min) and long term (overnight) exposure (Fig. 6B). This again

shows that skeletal muscle cells are highly sensitive toward MCP-1. ERK expression remains unaffected by MCP-1 (Fig. 6C).

Inhibition of ERK by the specific ERK inhibitor PD 98059 prevents the impairment of insulin signaling by MCP-1

To analyze the role of ERK in the impairment of insulin signaling by MCP-1, we preincubated the skeletal muscle cells with the specific ERK inhibitor PD 98059, and analyzed the effect of MCP-1 in skeletal muscle cells with inhibited ERK1/2. In short term experiments (Fig. 7A), we could clearly show that a preincubation with the ERK inhibitor completely blocks ERK activation by TNFα and MCP-1. In the long term, the ERK inhibitor alone has no effect on insulin signaling but can prevent the impairment of insulin signaling by MCP-1 (Fig. 7B). We conclude that ERK activation is necessary for the specific effect of MCP-1 on insulin signaling in skeletal muscle cells. A similar involvement of ERK in the impairment of insulin signaling in smooth muscle cells by angiotensin II was recently published [28], making it possible that ERK activation could play a role in signaling pathways of different cytokines that are thought to induce insulin resistance.

IL-6/-8, and MIP-1β decrease insulin-stimulated Akt phosphorylation only at very high, supraphysiological doses

When testing other cytokines present in adipocyte-CM, we were not able to find candidates to induce insulin resistance in skeletal muscle cells with a potency similar to MCP-1. IL-6 produced a slight reduction of insulin-stimulated Akt phosphorylation when used at concentrations as high as 50,000-times over circulating plasma levels (250 ng/ml) (Fig. 8A). When IL-6 is employed at lower concentrations, it is even leading to a slight but not significant increase in Akt phosphorylation. Similar observations were made in short term experiments [29] where IL-6 is acting as a metabolic stimulator similar to insulin rather than an insulin resistance inducing agent in skeletal muscle. MIP-1β produced a dose-dependent decrease in insulin signaling, but also at concentrations much higher than its circulating level of 250 pg/ml. Interestingly, MIP-1β is decreasing insulin-stimulated Akt phosphorylation by about 60% when used at 125 ng/ml, a concentration 500 times over circulating levels (Fig. 8B). Possibly, MIP-1β could play a role in restricted areas where it could reach such high concentrations. As for IL-8, this chemokine has also to be used at supraphysiological concentrations to significantly impair insulin signaling in skeletal

muscle cells making it unlikely to be a major candidate in the negative crosstalk between adipose tissue and skeletal muscle (Fig. 8C).

Discussion

Adipocyte-derived factors such as TNFα and IL-6 are significantly increased in obesity and are good predictors for the development of type 2 diabetes [30, 31]. Obesity thereby contributes to a pro-inflammatory milieu, and it is now recognized that adipose tissue functions as an endocrine organ secreting a variety of pro-inflammatory factors. In fact, adipocytokines are emerging regulators of insulin sensitivity. Prominent members of the adipocytokine family such as TNFα, plasminogen activator inhibitor-1, IL-6, and resistin have been identified to contribute *in vitro* to insulin resistance. TNFα, for example, is upregulated in obesity in animal models [32, 33], but its role in humans is controversial [34]. In rodents, TNFα increases serine phosphorylation of insulin receptor substrate (IRS)-1 and attenuates insulin signaling including phosphatidylinositol 3 kinase [35] and downregulates the expression of IRS and GLUT4 [36]. However, in humans, adipocytokines which could clearly induce insulin resistance are not known.

Adiponectin is the only adipocytokine known to be downregulated in obesity. Very recently, we reported on adiponectin acting as an autocrine regulator of adipokine secretion of the human fat cell [10]. By decreasing cytokine release by the adipocyte, adiponectin prevents the impairment of insulin signaling in a co-culture model of human adipocytes and skeletal muscle cells. Some of the adiponectin-regulated cytokines such as IL-6/-8 and MCP-1 are already known to be related to obesity and diabetes. Others such as MIP-1α/β are related to inflammation and tissue remodeling. IL-6/-8 are well-known to be induced in the obese state in humans and rodents [14, 15, 37, 38]. Elevated plasma concentrations of these adipocytokines in obese and insulin resistant patients may contribute to the insulin-resistant state observed in obesity. IL-6 is expressed both by adipose tissue and skeletal muscle [39] but its role in skeletal muscle remains controversial [29, 40]. It is shown in this study that only extremely high concentrations of IL-6 and IL-8 produced a slight impairment of insulin signaling in human skeletal muscle cells, making it unlikely that IL-6 or IL-8 alone are sufficient to induce muscle insulin resistance.

MCP-1 has already been shown to be clearly associated to the obese state in humans and rodents [14, 15, 39]. MCP-1 is secreted by various cells including human adipocytes but also cells of the vasculature when stimulated with pro-inflammatory

factors [12, 13]. MCP-1 is a well characterized chemokine when it comes to its role in the recruitment of monocytes and memory T lymphocytes into tissue [11]. However, many chemokines have been shown to possess physiological activities going far beyond the recruitment of immune cells. This is also the case for MCP-1, for which insulin-resistance-inducing capacities have been postulated in adipocytes [14]. Furthermore, MCP-1 was shown to have angiogenic effects in endothelial cells and, therefore, may play a role during adipose tissue expansion and remodeling in obesity [41]. The effect of MCP-1 in accelerating wound healing involving vessel formation also points out this angiogenic action of MCP-1 [42]. The induction of insulin resistance in skeletal muscle cells as shown in this study adds a new aspect to the role of MCP-1. Taken together with former studies, it can undoubtedly be said that MCP-1 can alter the function of tissues involved in the insulin resistant state. Skeletal muscle [43] and adipose tissue both produce MCP-1 and may, in the inflamed and obese state, increase the release of MCP-1 inducing then insulin resistance in both tissues. This adds a complete new feature to the negative crosstalk between adipose tissue and skeletal muscle pointing toward a close relationship among obesity, inflammation and diabetes, as already postulated in many publications [5, 6, 8, 44, 45].

MCP-1 has *in vivo* relevance related to diabetes and obesity, as already shown in different studies. Its expression is increased in obese mice, especially in white adipose tissue [14]. As for skeletal muscle, MCP-1 is increased in the injured state and can be induced by interferon γ [46]. In this respect, it needs to be assessed which of both MCP-1-producing tissues is contributing more to MCP-1 plasma levels that are increased in diabetic patients [16]. It is likely that skeletal muscle also contributes to increased MCP-1 plasma levels, because it has been shown to be induced in muscle of patients with inflammatory myopathies [43].

Lower MCP-1 levels due to a MCP-1 G-2518 gene variant [47] were shown to protect from the development of diabetes. As for the treatment of diabetes, it has been demonstrated that rosiglitazone [48] and exercise [49] both reduce plasma levels of MCP-1 significantly, making it possible that MCP-1 reduction is an important point in improving insulin action in diabetic patients. In hypertensive and hypercholesterolemic patients, MCP-1 could be reduced significantly by treatment with a combination of simvastatin and losartan, improving endothelial function [50]. In our study, we could show that very low concentrations of MCP-1 are effective in inducing skeletal muscle insulin resistance. This would suggest that already moderate elevation of body mass index, which is potentially associated with a slight increase in

MCP-1, may contribute to insulin resistance in skeletal muscle and possibly underlies early steps in the development of the metabolic syndrome. This question needs to be addressed in future clinical studies in order to confirm the role of MCP-1 in the development of insulin resistance.

MCP-1 is an interesting candidate that may play a role in the negative crosstalk between adipose tissue and skeletal muscle, but it is certainly not the molecule solely causing the induction of insulin resistance in skeletal muscle cells by adipocyte-CM. Because MCP-1 is highly concentrated in adipocyte-CM (31.2 ± 6.7 ng/ml) [10] and is downregulated by adiponectin to half this level that is still higher than the concentrations used in this study to induce insulin resistance in skeletal muscle, we hypothesize that adipocyte-CM must contain unknown adipocytokines that positively influence insulin action and are able to prevent induction of insulin resistance by MCP-1. These adipocytokines need to be identified in the future.

In summary, our data show that several adipocytokines might be involved in the negative crosstalk between skeletal muscle and adipose tissue. MCP-1 is a candidate of special interest because it is highly effective in inducing insulin resistance in skeletal muscle cells. Therefore, we suggest that this cytokine, which is regulated by adiponectin and which is clearly associated with the obese state and diabetes, may represent a molecular link between obesity and skeletal muscle insulin resistance. However, the possible role of MCP-1 as a connecting link between obesity and diabetes needs to be established by further studies, because cell types other than adipocytes secrete this cytokine and may contribute to its effect on skeletal muscle cells. The other adipocytokines tested in this study are involved in inflammation, tissue remodeling and, angiogenesis, but their role in obesity and the development of skeletal muscle insulin resistance needs to be further analyzed to fully understand their meaning for human physiology.

Acknowledgements

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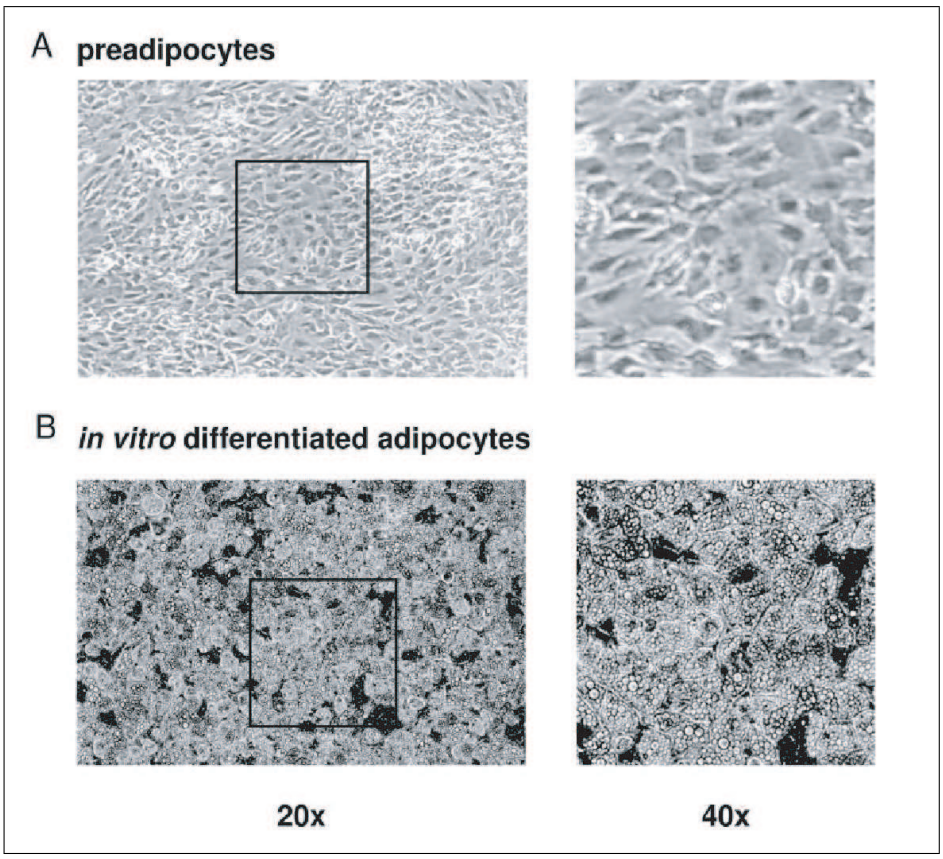
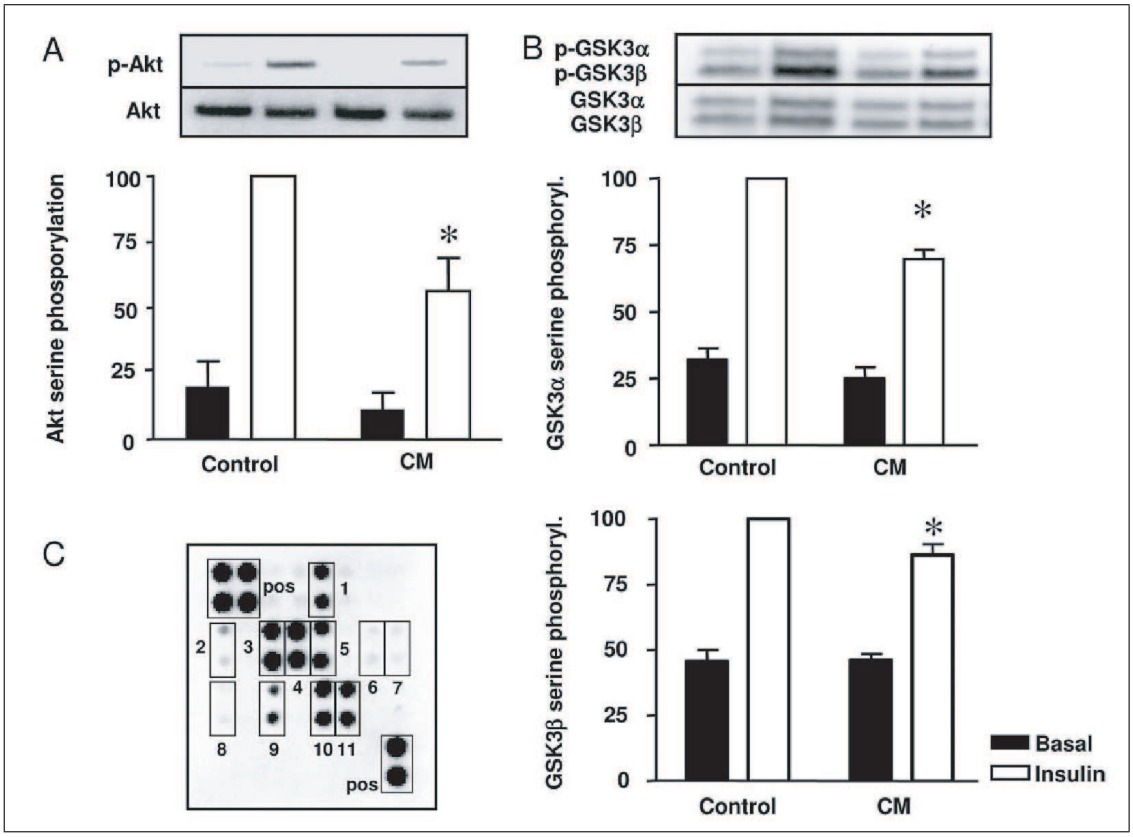
Figure 3.1: Morphological analysis of primary adipocyte cultures



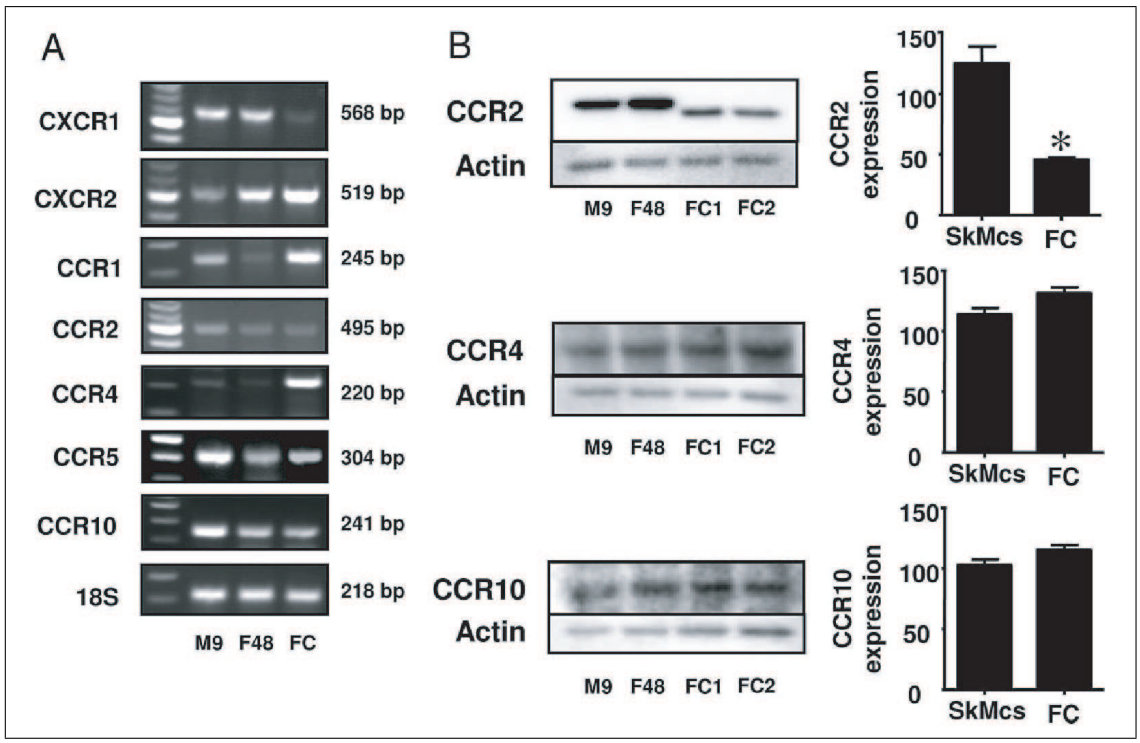
A: Light microscopy of preadipocytes one day after seeding. **B**: Micrograph of fully differentiated human adipocytes demonstrating that the majority of preadipocytes differentiates into adipocytes. A minor contribution of undifferentiated preadipocytes and small amounts of other possible contaminating cells to the results cannot be ruled out completely.

Figure 3.2: Effect of adipocyte-CM on insulin signaling in skeletal muscle cells and secretion pattern of differentiated human adipocytes



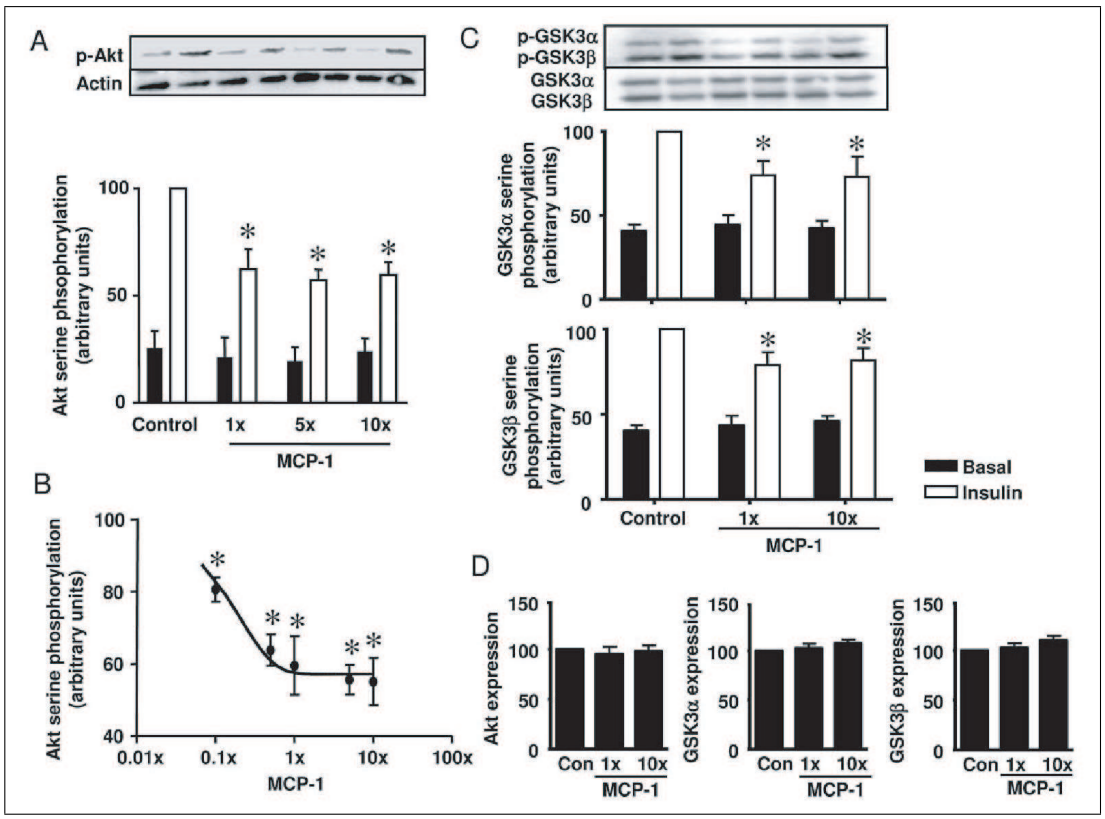
A and **B**: Akt and GSK3 phosphorylation in the myocytes were analyzed after acute stimulation with insulin (100 nM, 10 min). Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt/GSK3 and Akt/GSK3 antibodies. One exemplary Western blot is shown to illustrate the impairment of insulin signaling by adipocyte-CM. Data are mean values ± SEM of four independent experiments. All data were normalized to the level of actin expression and are expressed relative to the insulin-stimulated control value. * significantly different from insulin-stimulated control. **C**: Cytokine array membranes (each detecting 20 different cytokines) were incubated with adipocyte-CM. One representative array is presented. 1: adiponectin; 2: intracellular adhesion molecule 1; 3: IL-6; 4: IL-8; 5: MCP-1; 6: macrophage-derived chemokine; 7: MIP-1α; 8: MIP-1β; 9: osteoprotegerin; 10: tissue inhibitor of metalloproteinase (TIMP)-1; and 11: TIMP-2. pos, positive control for normalization.

Figure 3.3: Expression of chemokine receptors in human skeletal muscle cells



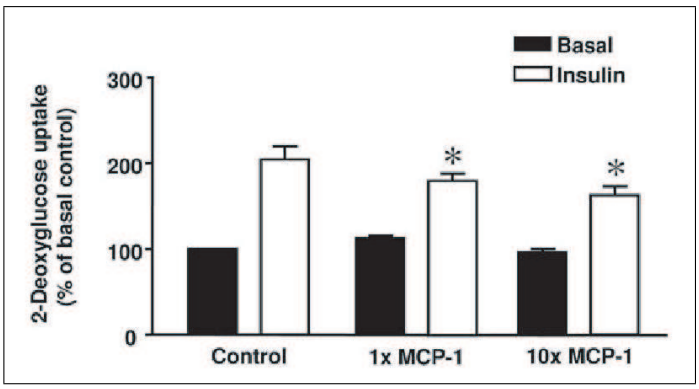
A: Total RNA was extracted from differentiated human skeletal muscle cells and adipocytes. RT-PCR for CXCR1/2, CCR1/2/4/5/10, and 18S was performed as outlined in *Materials and Methods* with at least three repeated experiments. Representative agarose gels of PCR products are shown for two skeletal muscle cell donors (M9 and F48) as well as for a fat cell control (FC). **B**: Skeletal muscle cells from two different donors (M9 and F48) and two fat cell donors (FC1 and FC2) were differentiated, and total cell lysates resolved by SDS-PAGE. Western blots for CCR2/4/10 as well as normalization for actin are shown. Data are mean values ± SEM of four independent experiments. All data were normalized to the level of actin expression and are expressed relative to the expression level of M9. * significantly different from skeletal muscle cells (SkMcs).

Figure 3.4: Effect of MCP-1 on insulin signaling in human skeletal muscle cells



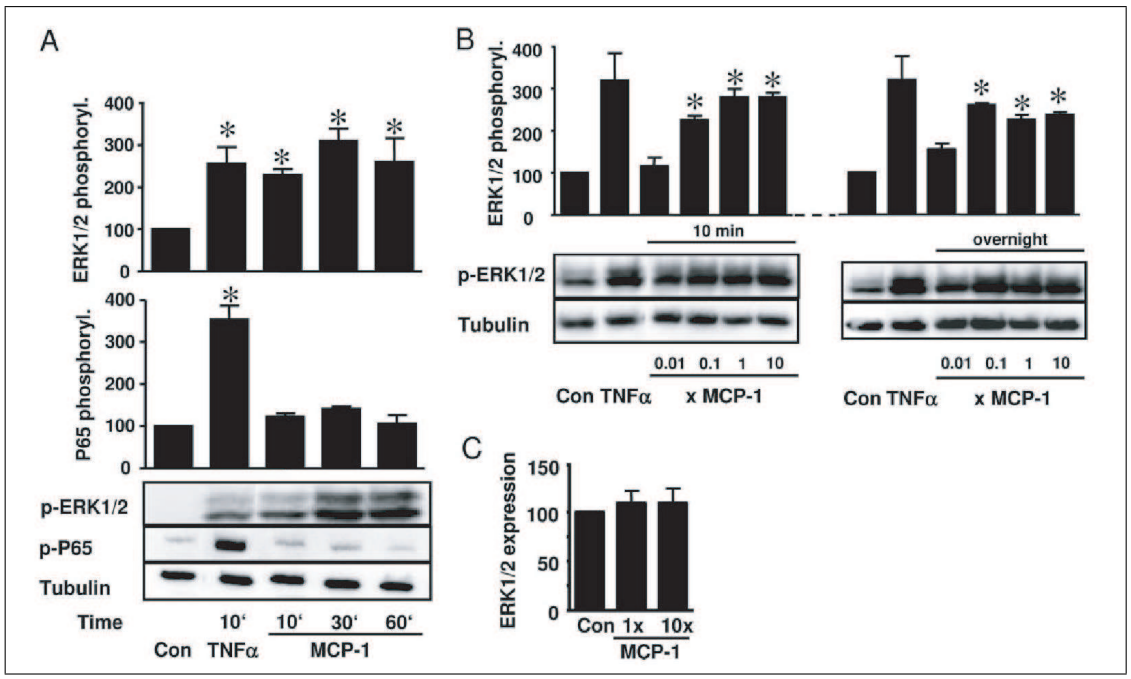
A: Myocytes from four different donors were cultured with increasing concentrations of MCP-1 (1× physiological level: 200 pg/ml MCP-1) for 18h. After acute stimulation with insulin, total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt antibody and actin antibody. Data are mean values ± SEM of five to six independent experiments. All data were normalized to the level of actin expression and are expressed relative to the insulin-stimulated control value. * significantly different from insulin-stimulated control. **B**: Skeletal muscle cells from four different donors were cultured with increasing concentrations of MCP-1 (0.1× physiological level: 20 pg/ml MCP-1). Data from five to six independent experiments are presented as a dose-response-curve of insulin-stimulated Akt phosphorylation. * significantly different from untreated control. **C**: Myocytes from two different donors were cultured as outlined in A. After acute stimulation with insulin, total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific GSK3 antibody and GSK3 antibody. Data are mean values ± SEM of four independent experiments. All data were normalized to the level of GSK3 expression and are expressed relative to the insulin-stimulated control value. * significantly different from insulin-stimulated control. D: Akt and GSK3 expression after overnight stimulation with MCP-1 relative to tubulin as a stable marker was analyzed in ten independent experiments.

Figure 3.5: Effect of MCP-1 on glucose uptake in skeletal muscle cells



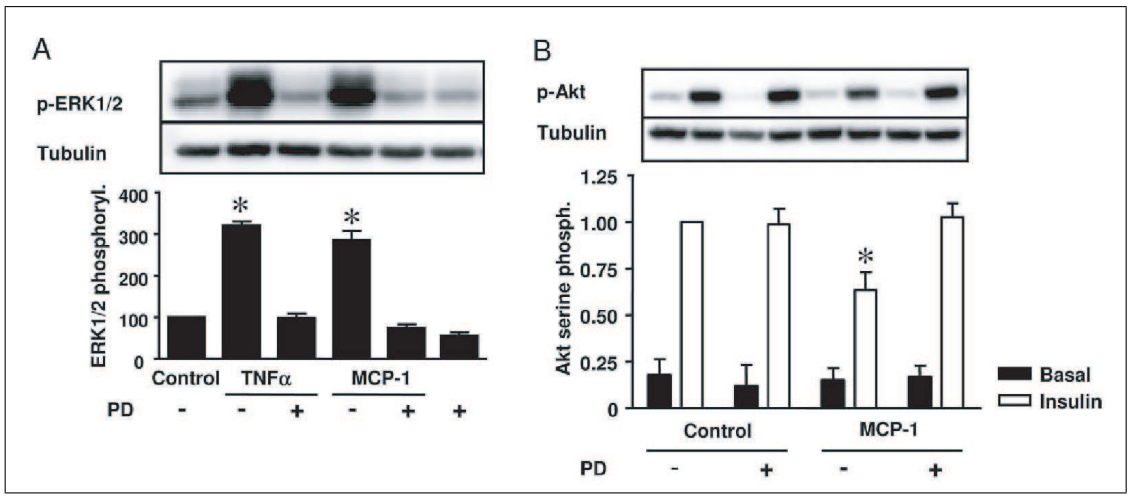
Skeletal muscle cells were first adenotransfected with GLUT4myc and then cultured for 18 h in absence or presence of a physiological level of MCP-1 (200 pg/ml) or a 10-times over physiological concentration. Glucose uptake was assessed after acute stimulation with insulin, as outlined in *Materials and Methods*. Mean ± SEM of four independent experiments. * significantly different from insulin-stimulated control.

Figure 3.6: MCP-1 signaling in skeletal muscle cells



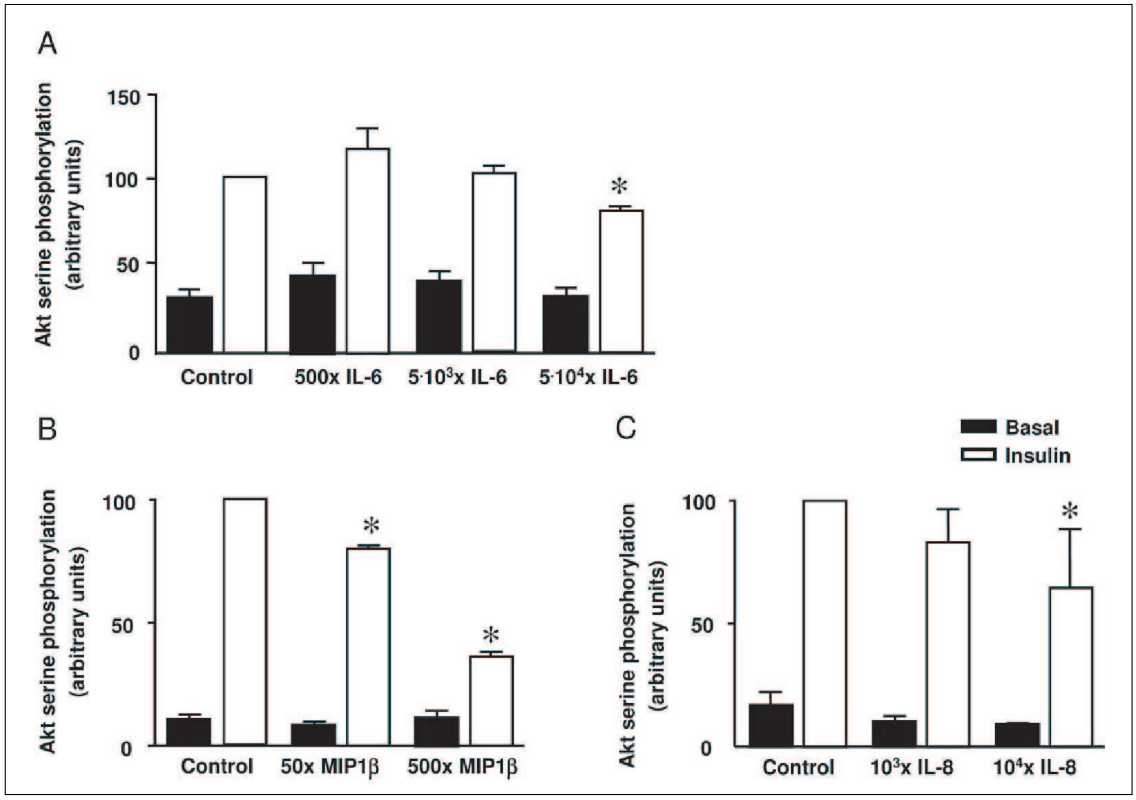
A: Skeletal muscle cells from three different donors were cultured with a 10-times over physiological concentration of MCP-1 (200 pg/ml) for the indicated time periods. As a control for ERK and NF-κB activation, 2.5 nM TNFα was used. Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific antibodies for the P65 subunit of NF-κB (p-P65) and ERK1/2 and tubulin for loading control. Representative blots are shown. Mean ± SEM of four independent experiments. * significantly different from control. **B**: Skeletal muscle cells from three different donors were cultured with increasing concentrations of MCP-1 (0.01× 2 pg/ml) for 10 min and overnight. As a control for ERK activation, a 10 min stimulus with 2.5 nM TNFα was used. Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific antibodies for ERK1/2 and tubulin for loading control. Representative blots are shown. Mean ± SEM of three independent experiments. * significantly different from control. **C**: ERK expression after overnight stimulation with MCP-1 relative to tubulin as a stable marker was analyzed in four independent experiments.

Figure 3.7: ERK inhibition prevents impairment of insulin signaling by MCP-1



A: Skeletal muscle cells from two different donors were precultured with or without 50 µM of the specific ERK inhibitor PD 98059 for 15 min before starting the treatment with MCP-1 or TNFα. Cells were then treated either with a 10-times over physiological concentration of MCP-1 (2 ng/ml) for 30 min or 2.5 nM TNFα for 10 min. Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific antibodies for the ERK1/2 and tubulin for loading control. Representative blots are shown. Mean ± SEM of four independent experiments. * significantly different from control. **B**: After pretreatment for 15 min with PD 98059 (50 µM) skeletal muscle cells from two different donors were cultured with a 10-times over physiological concentration of MCP-1 (2 ng/ml) overnight. Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific antibodies for Akt and tubulin for loading control. Representative blots are shown. Mean ± SEM of four independent experiments. * significantly different from insulin-stimulated control.

Figure 3.8: Effect of IL-6/-8 and MIP-1β on insulin signaling in human skeletal muscle cells



Skeletal muscle cells from three different donors were cultured with increasing concentrations of (**A**) IL-6 (500× over physiological level: 2.5 ng/ml IL-6), (**B**) MIP-1β (50× over physiological level: 12.5 ng/ml MIP-1β), and (**C**) IL-8 (10^3× over physiological level: 5 ng/ml IL-8). After acute stimulation with insulin, total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt antibody and actin antibody. Data are mean values ± SEM of three to four independent experiments. All data were normalized to the level of actin expression and are expressed relative to the insulin-stimulated control value. * significantly different from corresponding control.

Table 3.1: Sequences of primers used for RT-PCR

Target	**Forward primer**	**Reverse primer**
CXCR1	CCATTGCTGAAACTGAAGAGG	TTGTTTGGATGGTAAGCCTGG
CXCR2	CGAAGGACCGTCTACTCATC	AGTGTGCCCTGAAGAAGAGC
CCR1	ACGAAAGCCTACGAGAGTG	GGTGAACAGGAAGTCTTGG
CCR2	GATTACGGTGCTCCCTGTC	GCCACAGACATAAACAGAATC
CCR4	AGCACTTGTTATACTGAGCG	CCACCACGGCAAAGATCATC
CCR5	GACAAACTCTCCCTTCACTC	ACAAGTCTCTCGCCTGGTTC
CCR10	AGAGCCTGCTCCTTGCTAC	AGCCTCACCAAGACACAAC
18S	CGATGCTCTTAGCTGAGTGT	GGTCCAAGAATTTCACCTCT

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Chapter 4

Study 3

Cytokine secretion by human adipocytes is differentially regulated by adiponectin, AICAR, and troglitazone[†]

Henrike Sell, Daniela Dietze-Schroeder, Kristin Eckardt, and Jürgen Eckel

Institute of Clinical Biochemistry and Pathobiochemistry, German Diabetes Center, Düsseldorf, Germany

Running title: Adiponectin, AICAR, and troglitazone in adipocyte secretion

Key words: Cytokine, chemokine, AMPK, adipocyte, adiponectin

Abbreviations

AMPK, AMP-activated protein kinase; CM, conditioned medium; MCP, monocyte chemotactic protein; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; PPAR, peroxisome proliferator-activated receptor; sTNFR, soluble TNF receptor; TIMP, tissue inhibitor of metalloproteinase; IL, interleukin; TNF, tumor necrosis factor

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Abstract

Adipose tissue is an active endocrine organ producing a variety of cytokines and chemokines, which may be involved in the deregulation of glucose and lipid homeostasis as well as in the inflammatory state observed in obesity. We have shown previously that differentiated human adipocytes secrete a variety of cytokines which are able to induce skeletal muscle insulin resistance. However, the regulation of these factors by anti-diabetic drugs has remained mainly undefined. Secretion of IL-6/-8, MIP-1α/β, and MCP-1 by adipocytes was found to be downregulated by adiponectin. In parallel to adiponectin, the AMPK activator AICAR also decreased the secretion of most of the measured cytokines including IL-6 and MIP-1α/β but not IL-8. In contrast, the thiazolidinedione troglitazone only slightly reduced cytokine secretion despite increasing the phosphorylation of AMPK. In conclusion, we show that adipocyte secretion is strongly inhibited by the anti-diabetic adipocyte hormone adiponectin, an effect that can also be mimicked by the AMPK activator AICAR. However, the PPARγ agonist troglitazone is much less effective in reducing cytokine secretion.

Introduction

Adipose tissue is the major organ for energy storage in mammals but it also has an important endocrine function. The concept of adipose tissue as a major secretory and endocrine active organ producing a variety of bioactive proteins, which may regulate energy metabolism and insulin sensitivity, is now widely accepted [1]. It releases a variety of cytokines, the so-called adipocytokines which are involved in deregulation of glucose and lipid homeostasis but also in immunity. Increased adipose tissue mass, especially in the visceral compartment, represents one of the major risk factors for the development of type 2 diabetes [2–4]. In this state, increased secretion of adipocytokines is believed to lead to the well-known metabolic complications observed in obesity such as the development of diabetes. Adipocytes from obese subjects are characterized by altered secretory function leading to a higher release of cytokines and pro-inflammatory molecules such as tumor necrosis factor (TNF) α, interleukin (IL)-6, angiotensinogen, and resistin [5, 6]. Adipocytokines may therefore represent the missing link between adipose tissue and disturbances in other organs in the course of the metabolic syndrome.

The only adipocytokine whose secretion is decreased in the obese state is adiponectin. This protein is exclusively produced by adipocytes and attains high plasma concentrations. Accordingly, low plasma adiponectin levels are good indicators of developing diabetes. *In vitro*, adiponectin is an anti-apoptotic and anti-inflammatory factor in pancreatic β-cells and endothelial cells [7–9]. We recently demonstrated that the globular head domain of adiponectin acts as a key regulator of adipocyte secretion decreasing the release of several cytokines and thereby preventing insulin resistance in the co-culture model with skeletal muscle cells [10]. Probably, this effect occurs via AMP-activated protein kinase (AMPK) whose involvement has been identified in adiponectin signaling in various cell types, including skeletal muscle cells, endothelial cells and adipocytes [11–14]. Especially in adipocytes, AMPK is known to inhibit both lipolysis and lipogenesis by regulating enzymes involved in this process and by downregulating the expression of peroxisome proliferator-activated receptor (PPAR)γ. Accordingly, selective AMPK activators such as AICAR have also been shown to influence adipocyte secretion [15].

The metabolic effects of adiponectin and AICAR have been studied extensively but less is known regarding their effects on adipose tissue secretory function. Therefore, this study aimed to analyze cytokine/chemokine secretion of adipocytes treated with adiponectin and AICAR in comparison to the PPARγ agonist troglitazone.

This may shed some light on the significance of adipocyte secretion in the treatment of diseases that are associated to obesity such as insulin resistance and the metabolic syndrome.

Materials and Methods

Materials

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 Sigma (München, Germany). Polyclonal antibody anti-phospho(p)-AMPK was supplied by Cell Signaling Technology (Frankfurt, Germany) and anti-actin from Santa Cruz Biotechnology (Heidelberg, Germany). HRP-conjugated goat anti-rabbit and anti-mouse IgG antibodies were from Promega (Mannheim, Germany). Cytokine protein arrays (RayBio® Custom Array) were purchased from RayBiotech (Norcross, GA). Collagenase CLS type 1 was obtained from Worthington (Freehold, NJ) and culture media were obtained from Life Technologies, Inc. (Berlin, Germany). All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma. The recombinant C-terminal globular domain of adiponectin and full-length adiponectin were products from Tebu (Offenbach, Germany).

Adipocyte isolation and culture

Adipose tissue samples were obtained from the mammary fat of normal or moderately overweight women (BMI 25.4 ± 1.3, age 27.0 ± 4.1 yr) undergoing surgical mammary reduction. 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 ∼10 mg in weight. Preadipocytes were isolated by collagenase digestion as previously described [16]. Isolated cell pellets were resuspended in Dulbecco's modified Eagle's/Ham's F12 medium supplemented with 10% FCS, seeded 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 adipocytes, as defined by cyto-

plasm completely filled with small or large lipid droplets. The purity of adipocytes in the culture was analyzed by morphological means as described previously [17]. These cells were then used for generation of adipocyte-conditioned medium (CM), as recently described [18]. Briefly, after *in vitro* differentiation, adipocytes were incubated for 24 h in α-MEM containing 1 pM insulin. Conditioned medium was then generated by culturing adipocytes for 48 h in the same medium followed by collection of the medium.

Measurement of cytokine protein levels in adipocyte-CM

Adipocyte-CM was hybridized with the array membranes according to the protocol supplied by the manufacturer. Briefly, membranes were blocked by the furnished blocking solution and then incubated with 1.2 ml of CM over night at 4 °C. Membranes were then washed according to the manufacturer's protocol and incubated with the mix of biotin-conjugated antibodies for 2 h at room temperature. After washing, HRP-conjugated streptavidin was added to the membranes for 1 h at room temperature. The signal was developed with detection buffers and directly detected using a LUMI Imager system (Roche Diagnostics, Mannheim, Germany). The signal intensity was normalized to internal positive signals on the membrane.

Immunoblotting

Adipocytes were treated as indicated and lysed in a buffer containing 50 mM Tris/HCl (pH 7.4), 1% (v/v) NP-40, 0.25% (v/v) sodium-deoxycholate, 150 mM NaCl, 1 mM EGTA, 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 10,000g for 10 min. Thereafter, 5 µg of lysates were separated by SDS-PAGE using 10% horizontal gels and transferred to PVDF filters in a semidry blotting apparatus [19]. For detection, filters were blocked with TBS containing 0.1% Tween-20 and 5% nonfat dry milk and subsequently incubated over night with a 1:1000 dilution of appropriate antibodies. After extensive washing, filters were incubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescene detection using Uptilight substrate (Interchim, France). Signals were visualized and evaluated on a LUMI Imager work station using image analysis software (Roche Molecular Biochemicals, Mannheim, Germany).

Presentation of data and statistics

Statistical analysis was performed by ANOVA. All statistical analyses were done using Statview (SAS, Cary, NC) considering a value of $p < 0.05$ as statistically significant. Corresponding significance levels are indicated in the figures.

Results

Adiponectin, AICAR, and troglitazone regulate cytokine secretion by human adipocytes

In former studies from our laboratory using cultures of primary human adipocytes, we showed that CM of differentiated human adipocytes impairs insulin signaling at the level of Akt and glycogen synthase kinase 3 phosphorylation and insulin action on GLUT4 translocation in primary human skeletal muscle cells [10, 18, 20]. We concluded that adipocyte-derived factors impair insulin signaling and downstream insulin action in skeletal muscle cells. Several adipocyte-derived factors were described to be secreted from adipocytes and could also be found in CM of differentiated human adipocytes such as IL-6/-8, monocyte chemotactic protein (MCP)-1, and macrophage inflammatory protein (MIP)-1α/β. Very recently, we also demonstrated the autocrine inhibition of the release of these adipocyte secretory products by globular adiponectin [10]. We show here that full length adiponectin regulates the secretion of cytokines and chemokines by adipocytes in a similar way leading to the decreased release of IL-6/-8, MCP-1, MIP-1α/β, osteoprotegerin (OPG), soluble TNF receptor (sTNFR)-2 and tissue inhibitor of metalloproteinase (TIMP)-1/2 (Figs. 1-4).

IL-6 and IL-8 are well-known to be induced in the obese state in humans and rodents [21–23]. Both cytokines are secreted from adipocytes and increased in plasma of obese patients. Adiponectin solidly downregulates the secretion of IL-6 and IL-8 by 50%. However, AICAR led to a decreased release of IL-6 only, while having no effect on IL-8. Troglitazone does not affect the secretion of IL-6/-8 by human adipocytes (Fig.1).

Three members of the small inducible cytokine family namely MCP-1 and MIP-1α/β were also released in a significantly smaller amount from human adipocytes treated with adiponectin (Fig. 2). MCP-1 is already known to be related to obesity and diabetes [24, 25]. In a recent study, we showed that MCP-1 at physiological concentrations is able to induce insulin-resistance in skeletal muscle cells [17]. MIP-

1α/β are related to inflammation and tissue remodeling but their relationship to adipose tissue, obesity, and diabetes is less clear. In contrast to adiponectin, troglitazone induced only a marginal reduction of MCP-1 and MIP-1α while the secretion of MIP-1β remained unchanged. The strongest reduction of MIP-1α/β release was achieved by AICAR with a 70-80% decrease, respectively (Fig. 2).

The secretion of two TNF-related proteins, OPG and sTNFR-2, was significantly decreased in all three treatment regimens (Fig. 3). TIMP-1/2 combined with the matrix metalloproteinases exert key functions in extracellular matrix remodeling. Matrix metalloproteinases and TIMP activity may be essential for adipocyte hypertrophy and fat accumulation. TIMP-1 was shown to be strongly induced in obesity [26]. The release of TIMP-1/2 by human adipocytes is significantly reduced by adiponectin but not by AICAR. Furthermore, troglitazone only induced a slight reduction in TIMP-2 secretion (Fig. 3). Two cytokines not regulated by adiponectin, hepatocyte growth factor and macrophage-derived chemokine (MDC), exhibited a significantly reduced secretion in response to AICAR and troglitazone (Fig. 4).

Regulation of adiponectin secretion by globular adiponectin, AICAR, and troglitazone

The secretion of adiponectin (full length form) from adipose tissue explants is known to be regulated by AICAR [15] which could be confirmed by us for human adipocytes (Fig. 5). Troglitazone was also shown to upregulate adiponectin secretion *in vivo* [27, 28] and in 3T3 adipocytes [29, 30] which was also found in primary human adipocytes (Fig. 5). Interestingly, the globular head domain of adiponectin led to a significant decrease in the release of full length adiponectin by human adipocytes, most likely involving an autoregulatory process (Fig. 5).

As presented in Fig. 6, adiponectin, AICAR, and troglitazone significantly increased AMPK phosphorylation. After 10 min, only adiponectin and AICAR stimulated AMPK phosphorylation whereas after 30 min, the three reagents were comparable. Overnight exposure to adiponectin, AICAR, and troglitazone also results in a significant increase of AMPK activation demonstrating a prolonged effect of the treatment regimens (Fig. 6).

Discussion

Adipocyte-derived factors such as TNFα and IL-6 are significantly increased in obesity and are good predictive factors for the development of type 2 diabetes

and other associated diseases [31, 32]. Obesity is linked to biochemical changes in adipose tissue contributing to a pro-inflammatory milieu. Consequently, the role of adipose tissue has undergone a paradigm shift from a passive role in energy storage to an active function as an endocrine organ secreting a variety of pro-inflammatory factors. Prominent members of the adipocytokine family such as TNFα, plasminogen activator inhibitor-1, IL-6, and resistin have been identified to be related and to contribute *in vivo* and *in vitro* to insulin resistance and other components of the metabolic syndrome [6, 33, 34].

Adiponectin is the only adipocytokine known to be downregulated in obesity [35, 36]. As an adipocyte-derived hormone it regulates energy homeostasis and glucose and lipid metabolism. In several cell types, adiponectin leads to the activation of AMPK such as in endothelial cells where adiponectin has anti-apoptotic effects [14, 37] and in skeletal muscle where it enhances fat oxidation and glucose utilization [11, 12]. In adipocytes, adiponectin leads to increased glucose uptake without increasing the phosphorylation of insulin receptor, insulin receptor substrate-1 or Akt but activation of AMPK [13]. Very recently, we reported on globular adiponectin acting as an autocrine regulator of adipocytokine secretion of the human fat cell [10]. By decreasing cytokine release from the adipocyte, adiponectin prevents the impairment of insulin signaling in a co-culture model of human adipocytes and skeletal muscle cells. This effect of adiponectin also includes activation of AMPK as shown in this report. The secretion of adiponectin itself is known to be regulated by various compounds such as PPARγ agonists [38], ACE inhibitors [39], and AICAR [15]. The regulation of adiponectin by AICAR could be confirmed by us for human adipocytes. Also troglitazone upregulates adiponectin secretion *in vivo* [27, 28] and in 3T3 adipocytes [29, 30] which is similar in primary human adipocytes. In fact, the positive regulation of adiponectin by the above-mentioned drugs could be considered as an underlying mechanism of action.

AMPK has a pivotal role in regulating the cellular energy balance, particularly in the control of body fat stores [40, 41]. Pharmacological studies show that the AMPK activator AICAR decreases blood glucose and increases insulin sensitivity [42, 43] and is able to decrease adipose tissue mass [44]. Although it is thought that the primary site of AICAR action is muscle, we can show that AICAR also influences adipocyte secretion behavior. AICAR increases the secretion of adiponectin as already described in the literature [15] while decreasing the release of pro-inflammatory cytokines and chemokines. In this respect, it could be argued that the regula-

tory effect of AICAR on adipocyte secretion would be secondary to the increase in adiponectin secretion. However, the significant downregulation of MDC by AICAR alone and the lack of AICAR to regulate IL-8 and TIMP-1/2 could not be explained by this scenario. In the light of our findings, it might be suggested that improved insulin sensitivity by AICAR observed in former studies could partly be due to its effect on cytokine release from adipocytes.

Troglitazone has the smallest effects on cytokine release by human adipocytes. Despite increasing the secretion of adiponectin in accordance with the literature [27], this thiazolidinedione has no major effect on the release of prominent cytokines involved in obesity-related comorbidities such as IL-6 or MCP-1. PPARγ agonists are known to regulate adipocyte differentiation and to improve fat retention by transcriptional regulation of several genes involved in glucose and lipid metabolism such as lipoprotein lipase, fatty acid transporter protein, adipocyte fatty acid binding protein, fatty acyl-CoA synthase, glucokinase, and the GLUT4 glucose transporter. As a PPARγ ligand, troglitazone probably acts mainly on adipose tissue since PPARγ expression is much higher in this tissue than in skeletal muscle or liver. However, troglitazone also leads to higher insulin sensitivity in skeletal muscle and liver. It is likely that the effect of PPARγ agonists on insulin resistance in the periphery is promoted by endocrine signals from adipocytes and macrophages. Potential signals comprise free fatty acids, well-known mediators of insulin resistance linked to obesity, and adipocytokines that are overexpressed in obesity and insulin resistance. However, troglitazone leads to a modest increase in AMPK phosphorylation which is in contrast to findings with 3T3 adipocytes where effects on adipocyte secretion without activation of AMPK could be observed [30]. In summary, it might be suggested that improved insulin sensitivity by troglitazone could be due to its effect on the release of some selected adipocytokines such as adiponectin.

As already described above, the plasma concentration of many adipocytokines is altered in obesity. However, several publications working with adipose tissue explants came to the conclusion that most of the adipocytokines released by tissue explants originate from non-fat cells such as tissue matrix and stromavascular cells that are obtained after collagenase digestion of adipose tissue [31, 45]. In fact, only leptin and adiponectin were shown to be secreted at a high level by isolated mature adipocytes in these studies. In contrast to freshly prepared mature adipocytes, we can show that *in vitro* differentiated adipocytes represent active secretory cells whose secretion pattern can be influenced by the adipose-derived hormone adiponectin and

by anti-diabetic drugs involving activation of AMPK.

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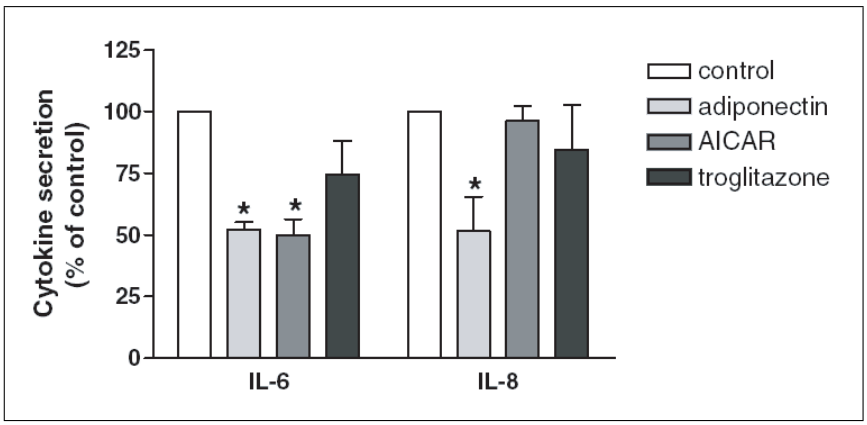
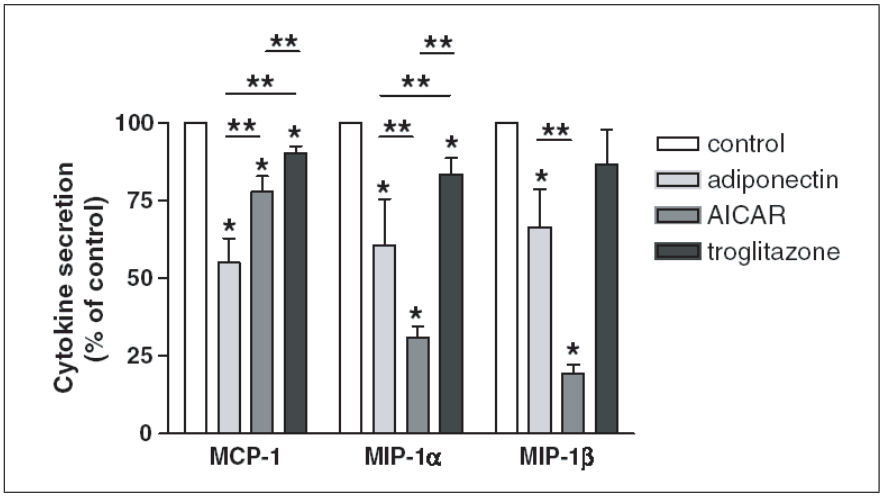
Figure 4.1: Regulation of IL-6 and IL-8 secretion by human adipocytes



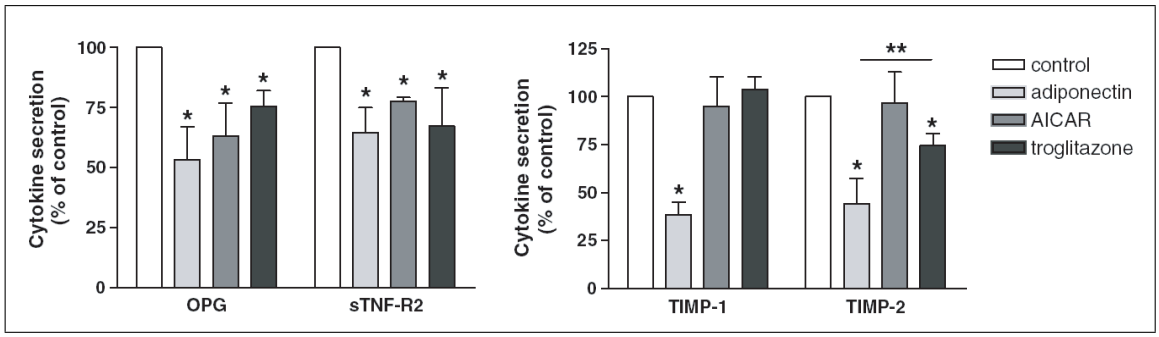
Cytokine array membranes (each detecting 20 different cytokines) were incubated with adipocyte-CM that was generated for 48 h in absence or presence of 5 nM full length adiponectin, 1 mM AICAR or 5 mM troglitazone. After washing, the membranes were incubated with a mix of biotin-conjugated antibodies and processed for detection on the LUMI Imager system, as described in *Materials and Methods*. Protein arrays were quantified using image analysis software. Data are mean values ± SEM of three independent adipocyte cultures from three different donors, each run in the absence or presence of adiponectin, AICAR, and troglitazone. * $p < 0.05$ compared to control medium.

Figure 4.2: Regulation of the secretion of small inducible cytokines by human adipocytes



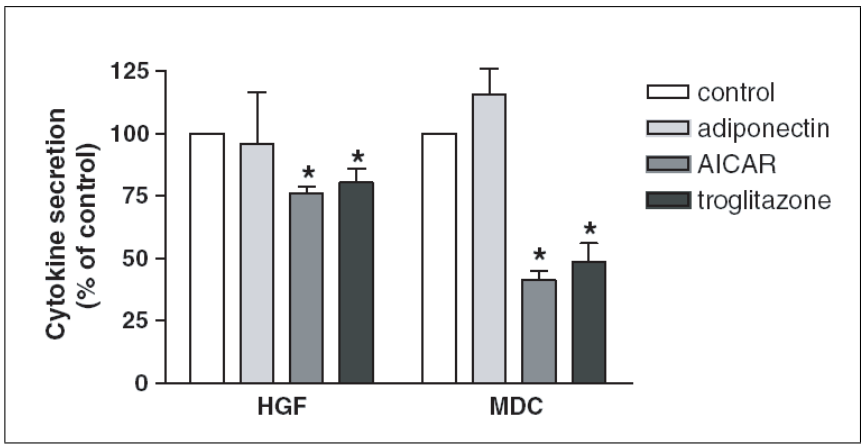
Cytokine array membranes were incubated and detected as detailed in Fig. 1. Data are mean values ± SEM of three independent adipocyte cultures from three different donors, each run in the absence or presence of full length adiponectin, AICAR, and troglitazone. * $p < 0.05$ compared to control medium, ** $p < 0.05$ compared to different treatment regimen.

Figure 4.3: Regulation of the release of TNF family members and TIMPs by human adipocytes



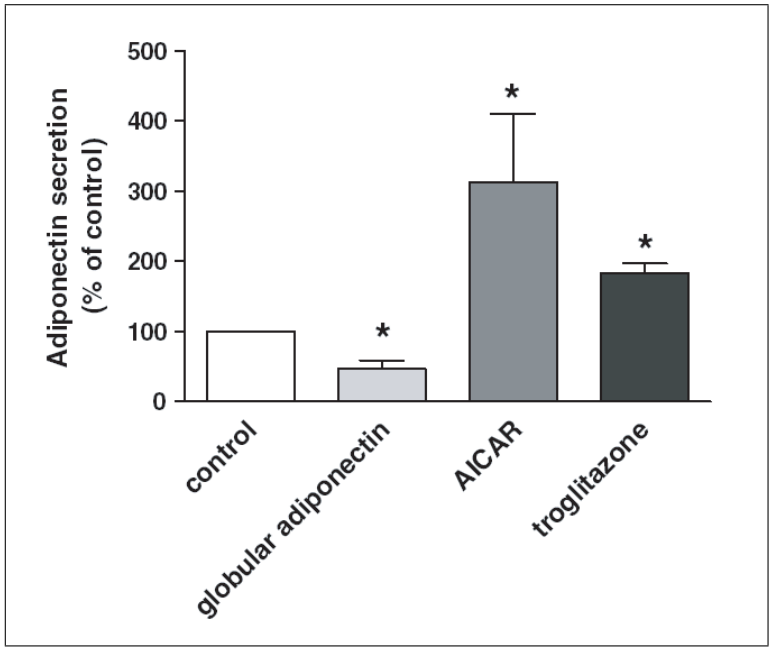
Cytokine array membranes were incubated and detected as detailed in Fig. 1. Data are mean values ± SEM of three independent adipocyte cultures from three different donors, each run in the absence or presence of full length adiponectin, AICAR, and troglitazone. * $p < 0.05$ compared to control medium, ** $p < 0.05$ compared to different treatment regimen.

Figure 4.4: Regulation of HGF and MDC secretion by human adipocytes



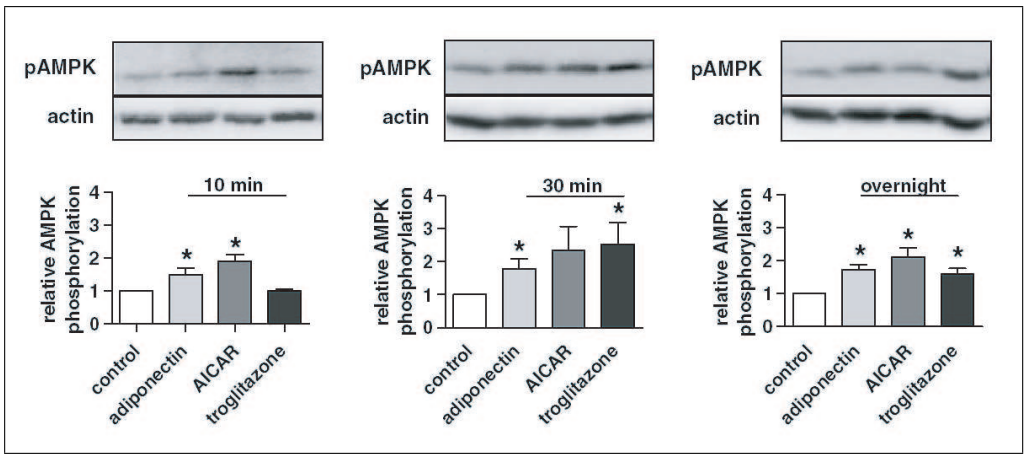
Cytokine array membranes were incubated and detected as detailed in Fig. 1. Data are mean values ± SEM of three independent adipocyte cultures from three different donors, each run in the absence or presence of full length adiponectin, AICAR, and troglitazone. * $p < 0.05$ compared to control medium.

Figure 4.5: Regulation of adiponectin secretion by the globular head domain of adiponectin, AICAR, and troglitazone



Full length adiponectin secretion was measured by cytokine arrays as described in Fig. 1. Adipocytes were incubated for 48 h with 5 nM globular adiponectin, 1 mM AICAR, and 5 mM troglitazone. Protein arrays were quantified using image analysis software. Data are mean values ± SEM of three to six independent adipocyte cultures from different donors, each run in the absence or presence of globular adiponectin, AICAR, and troglitazone. * $p < 0.05$ compared to control medium.

Figure 4.6: Activation of AMPK by adiponectin, AICAR, and troglitazone



Differentiated adipocytes from three to four different donors were cultured under control conditions or were incubated with adiponectin, AICAR or troglitazone for 10 min, 30 min, and overnight. AMPK phosphorylation was assessed as detailed in *Materials and Methods*. Data are mean values ± SEM of three to four independent experiments. All data were normalized to the level of actin expression and are expressed relative to the control. * $p < 0.05$ compared to control adipocytes.

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Chapter 5

Study 4

Expression of chemokine receptors in insulin-resistant skeletal muscle cells[†]

Henrike Sell[‡], Ulrike Kaiser[‡], and Jürgen Eckel

Institute of Clinical Biochemistry and Pathobiochemistry, German Diabetes Center, Düsseldorf, Germany

Running title: Chemokine receptors in insulin-resistant myocytes

Key words: Insulin resistance; skeletal muscle; chemokine receptor

Abbreviations

CCR, chemokine CC motif receptor; CM, adipocyte-conditioned medium; GI, high concentrations of glucose and insulin; GSK, glycogen synthase kinase; HRP, horse radish peroxidase; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein

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[‡]These authors contributed equally to this work.

Abstract

Adipokines including chemokines are able to induce insulin resistance in human skeletal muscle cells which may also be relevant for the observed link between obesity and diabetes. This study aimed to analyze the expression of chemokine CC motif receptors (CCRs) in the insulin-resistant state in human skeletal muscle cells. Differentiated skeletal muscle cells were incubated for 24-72 h with high concentrations of glucose and insulin (GI) or TNFα. In addition, myocytes were co-stimulated with monocyte chemotactic protein (MCP)-1 or adipocyte-conditioned medium (CM) and TNFα for 24 and 48 h. Treatment with GI rapidly induced insulin resistance whereas TNFα impaired insulin signaling in a more chronic fashion (48-72 h). CM and MCP-1 also induced insulin resistance that was however not increased by co-stimulation with TNFα . Expression of CCR2 was decreased during differentiation but upregulated in insulin-resistant myocytes after treatment with GI (24-72 h) and TNFα (72 h). Expression of CCR4 and CCR10 was downregulated after treatment with TNFα , MCP-1, and CM. Our data show that the expression of CCR2, CCR4, and CCR10 is differentially regulated by different insulin resistance-inducing treatments in myotubes. However, we could not find a clear correlation between the level of insulin resistance and CCR expression in myotubes. In conclusion, we propose that upregulation of CCR2 in skeletal muscle does not represent a major step leading to muscle insulin resistance.

Introduction

Type 2 diabetes is characterized by hyperglycemia and insulin resistance in peripheral tissues such as skeletal muscle [1]. Obesity is one of the major environmental factors contributing to the development of type 2 diabetes [2]. In this context, it has become evident that adipose tissue represents an active secretory organ capable of releasing a variety of cytokines such as TNFα, IL-6, and adiponectin [3]. It has been proposed that these factors might represent the missing link between adipose tissue and skeletal muscle insulin resistance [4, 5]. In fact, it is now believed that a negative crosstalk between excess body fat and skeletal muscle leads to disturbances in skeletal muscle insulin signalling and ultimately to insulin resistance [2]. Co-culture of human adipocytes and skeletal muscle cells induces muscular insulin resistance displayed by decreased IRS-1, Akt, and GSK3α/β phosphorylation, and reduced GLUT4 translocation [6].

It has been shown that adipocytes express chemokine CC motif receptors (CCR) [7] and recently this was also demonstrated for skeletal muscle cells [8]. Chemokines and their receptors exert a key role in chronic inflammation by attracting and activating T cells and monocytes [9]. They could also play a role in the induction of skeletal muscle insulin resistance where inflammation is an underlying process [10]. Consistently, we reported that the CC chemokine monocyte chemotactic protein (MCP)-1 is able to induce insulin resistance in human skeletal muscle cells [8]. However, no data is so far available on the regulation of CCRs in insulin-resistant skeletal muscle cells *in vitro*. Therefore, this study aimed to analyze the regulation of CCR expression in skeletal muscle cells during differentiation into myotubes and in the insulin-resistant state after incubation with high concentrations of glucose and insulin (GI) as well as with TNFα .

Materials and Methods

Materials

Reagents for SDS-PAGE were supplied by Amersham Pharmacia Biotech (Braunschweig, Germany) and Sigma (München, Germany). Polyclonal antibodies anti-phospho glycogen synthase kinase (GSK)3α/β (Ser21/9) and anti-phospho Akt (Ser473) were supplied by Cell Signaling Technology (Frankfurt, Germany) and anti-tubulin from Calbiochem (Merck Biosciences, Schwalbach, Germany). Antibodies for CCR4 and CCR10 came from Imgenex (San Diego, CA) and the one for CCR2

from Epitomics (Burlingam, CA). HRP-conjugated secondary antibodies were from Promega (Mannheim, Germany). Culture media were obtained from Gibco (Berlin, Germany). MCP-1 was purchased from Hölzel Diagnostics (Köln, Germany) and TNFα from Sigma. 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. Adipocyte-conditioned media (CM) were generated as detailed previously [8].

Culture of human skeletal muscle cells

Primary human skeletal muscle cells of five healthy Caucasian donors (male: 5 and 9 y (M5, M9); female: 10, 48, and 49 y (F10, F48, F49)) were supplied as proliferating myoblasts (5 x 105 cells) by PromoCell and cultured as described earlier [11]. For an individual experiment, myoblasts were seeded in six-well culture dishes (9.6 cm2/well) at a density of 105 cells per well and were cultured in α-modified Eagle's/Ham's F-12 medium containing Skeletal Muscle Cell Growth Medium Supplement Pack up to near confluence. The cells were then differentiated and fused by culture in -modified Eagle's medium for 4 days.

GI treatment (20 mM glucose, 100 nM insulin) was administered for 24, 48, or 72 h with renewal of treatment every 24 h and washed away 1 h before short term insulin stimulation (10 min) followed by cell lysis. Control cells were changed to GI medium 1 h before lysis undergoing the same washing procedure as GI-treated cells to prove efficient washing of long term insulin. TNFα treatment was administered for 24, 48 and 72 h with renewal of TNFα containing and control medium every 24 h.

Immunoblotting

Muscle cells were treated as indicated and lysed in a buffer containing 50 mM Hepes pH 7.4, 1 Triton, 1 mM Na3VO4, and Complete protease inhibitor cocktail from Roche Diagnostics (Penzberg, Germany). After incubation for 2 h at 4°C the suspension was centrifuged at 10,000 x g for 15 min. Thereafter, 5 g of lysates were separated by SDS-PAGE using 10% horizontal gels and transferred to PVDF filters in a semidry blotting apparatus. Filters were blocked with TBS containing 0.1% Tween-20 and 5% nonfat dry milk and subsequently incubated over night with a 1:1000 dilution of appropriate antibodies. After extensive washing, filters were in-

cubated with secondary HRP-coupled antibody and processed for enhanced chemiluminescene detection using SuperSignal Substrate (Pierce, Rockford, IL). Signals were visualized and analyzed on a LUMI Imager workstation using image analysis software (Boehringer Mannheim, Mannheim, Germany).

Presentation of data and statistics

Statistical analysis was performed by ANOVA. All statistical analyses were done using Statview (SAS, Cary, NC) considering a value of $p < 0.05$ as statistically significant. Corresponding significance levels are indicated in the figures.

Results

CCR2, CCR4, and CCR10 function as receptors for macrophage inflammatory protein (MIP)-1α/β and MCP-1 [9]. As presented in Fig. 1A, protein expression of CCR2 varies to some extent between the different cell donors and exhibits a differentiation-dependent decrease. CCR4 is expressed at a comparable level in all studied cell donors and shows a less prominent decrease with ongoing muscle differentiation (Figure 1B). In contrast, expression of CCR10 increased substantially with differentiation and varied to a high degree between different donors (Fig. 1C). Data for differentiation dependent expression of CCRs for individual donors is not shown but similar to pooled data.

Treatment of human skeletal muscle cells with GI leads to induction of insulin resistance within 24 h at the level of Akt and GSK3α/β phosphorylation (Fig. 2A). However, TNFα must be applied for at least 48 h to significantly reduce Akt phosphorylation (Fig. 3A). GI and TNFα exert different effects on the regulation of CCR expression (Fig. 2B and 3B). Whereas TNFα reduced the abundance of CCR4 and partly CCR10 (Fig 3B), GI had no effect on the expression of these receptors (Fig. 2B). In contrast, the level of CCR2 was significantly higher in insulin-resistant skeletal muscle cells as CCR2 was increased after treatment with GI for 24 and 72 h, and after exposure to TNFα for 72 h (Figure 2B and 3B).

Previous work from our laboratory showed that CM and MCP-1 induce insulin resistance in skeletal muscle cells [6, 17]. Since TNFα induces insulin resistance after 48 h with different effects on the expression of CCRs after 24 and 48 h, we explored whether stimulation with MCP-1 alone, or in combination with TNFα , synergizes on the induction of insulin resistance and the regulation of CCR expression. MCP-1

(Fig. 4A) alone significantly reduced Akt phosphorylation. Insulin-stimulated Akt activation was not significant after combined treatment with MCP-1 and TNFα (24 h), in contrast to the results obtained with single treatments (see Fig. 2A). Furthermore, MCP-1 treatment did not modify the expression level of CCR2, its own receptor, but MCP-1 synergizes with the TNFα stimulus in increasing CCR2 expression after 48 h (Fig. 4B). However, this increase in CCR2 is not reflected by stronger insulin resistance in this situation. As for CCR4 and CCR10, MCP-1 downregulate expression of these receptors without any additive effects of TNFα (Figure 4B). CM has similar effects as MCP-1 on insulin signaling and the expression of CCR4 and CCR10 while it has no effect on CCR2 expression with and without TNFα (data not shown).

Discussion

Treatment with GI, TNFα , MCP-1 and CM induces insulin resistance in skeletal muscle cells which is in accordance with data for L6 myotubes [12] and primary human myotubes [6, 17]. We demonstrate here that CCRs are regulated in skeletal muscle cells that were made insulin-resistant by different treatments. CCRs are generally involved in inflammatory processes such as the infiltration of immune cells into sites of chronic inflammation [9]. Since chronic inflammation is an underlying process in type 2 diabetes, these receptors might play a role in insulin resistance and type 2 diabetes [13]. Interestingly, expression of CCR4 and CCR10 are decreased in insulin-resistant human myotubes. These receptors have both high affinity for MCP-1 and MIP-1α/β , making it likely that they are involved in the negative crosstalk between adipose tissue and skeletal muscle; however, current knowledge on their specific function in pathophysiology is very limited [7]. As for CCR2, we observed an increased expression of this receptor in insulin-resistant myotubes after treatment with GI and TNFα . CCR2 is well-known to be involved in obesity and inflammation where it could be an important player during macrophage infiltration into adipose tissue. Accordingly, it was shown that CCR2 knockout mice are protected from high-fat diet-induced obesity and display reduced macrophage content in adipose tissue accompanied by improved inflammatory profile [14]. Moreover, in skeletal muscle, CCR2 is also associated with inflammatory myopathies [15] and CCR2 expression is increased in monocytes from diabetic patients [16]. In peripheral blood monoytes CCR2 is downreguated by TNFα while these receptors are induced in endothelial cells by inflammatory cytokines. Downregulation of CCR2

may serve to limit inflammation, however, this process could be disturbed in relation to metabolic derangement in diabetes [17, 18].CCR2 polymorphisms seem to be associated to type 1 diabetes and its complications Yang04CYTO but no data is so far available on type 2 diabetic patients. Downregulation of CCR2 could be observed by statins in endothelial cells and macrophages [20], by PPARγ agonists in macrophages [21], and by endocannabinoids in splenocytes [22] adding CCRs to the targets of these drugs. Now, our data show that CCR2 is upregulated by GI and TNFα in parallel to the induction of insulin resistance in skeletal muscle cells. In addition, TNFα and MCP-1 synergize to increase the expression of CCR2. However, this increase in CCR2 expression has no impact on the level of insulin resistance which was similar in myotubes treated with MCP-1 alone or in combination with TNFα . The increase in CCR2 expression might rather illustrate dedifferentiation of myotubes as described in the literature in accordance with our data on CCR2 expression decreasing with differentiation of myotubes [23]. This relationship might also be true for CCR10 which increases with differentiation in myotubes but mainly decreases in insulin-resistant myotubes.

In summary, we are not able to prove a general correlation between the level of insulin resistance and CCR2 expression in myotubes. We propose that CCR2 is more likely involved in the crosstalk between adipocytes and macrophages in adipose tissue. However, the finding that CCR2 is increased in insulin-resistant myocytes makes it an interesting candidate for further research in diabetic and obese patients. Future experiments could be aimed to study if hyperglycemia and hyperinsulinemia or high TNFα blood levels lead to increased levels of CCR2 in skeletal muscle with some relevance *in vivo*.

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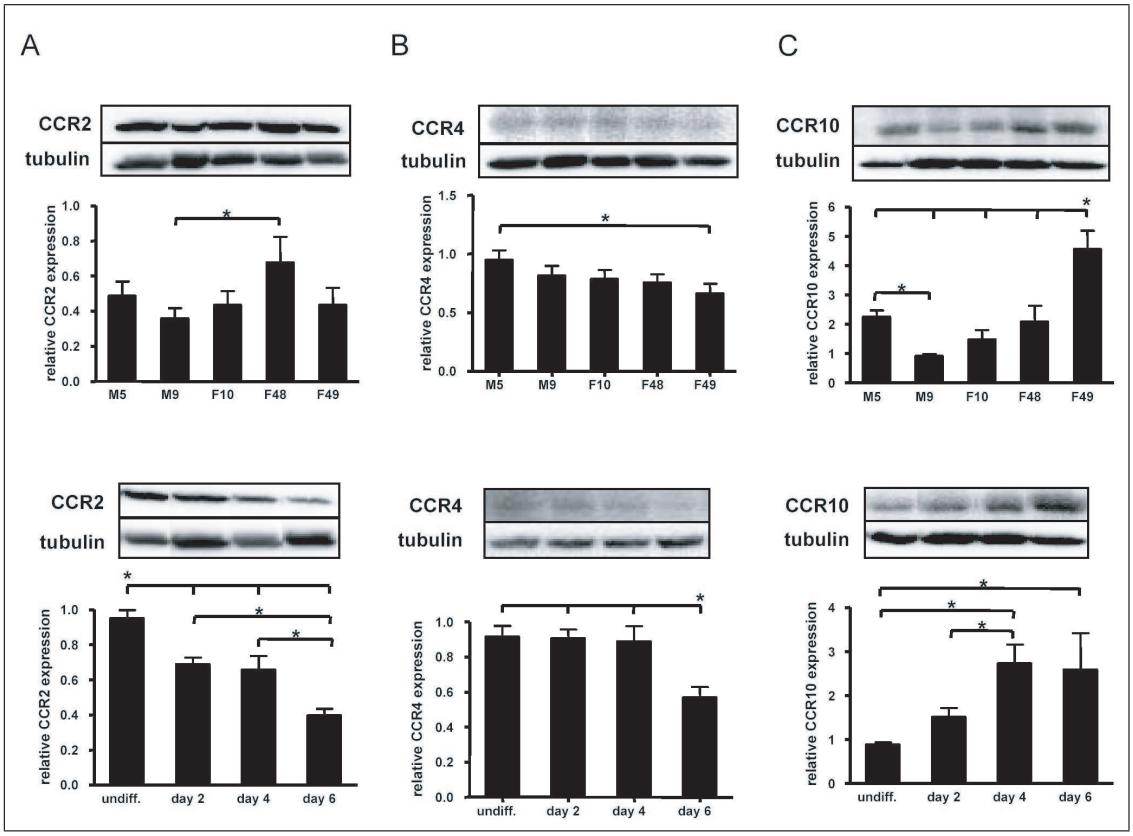
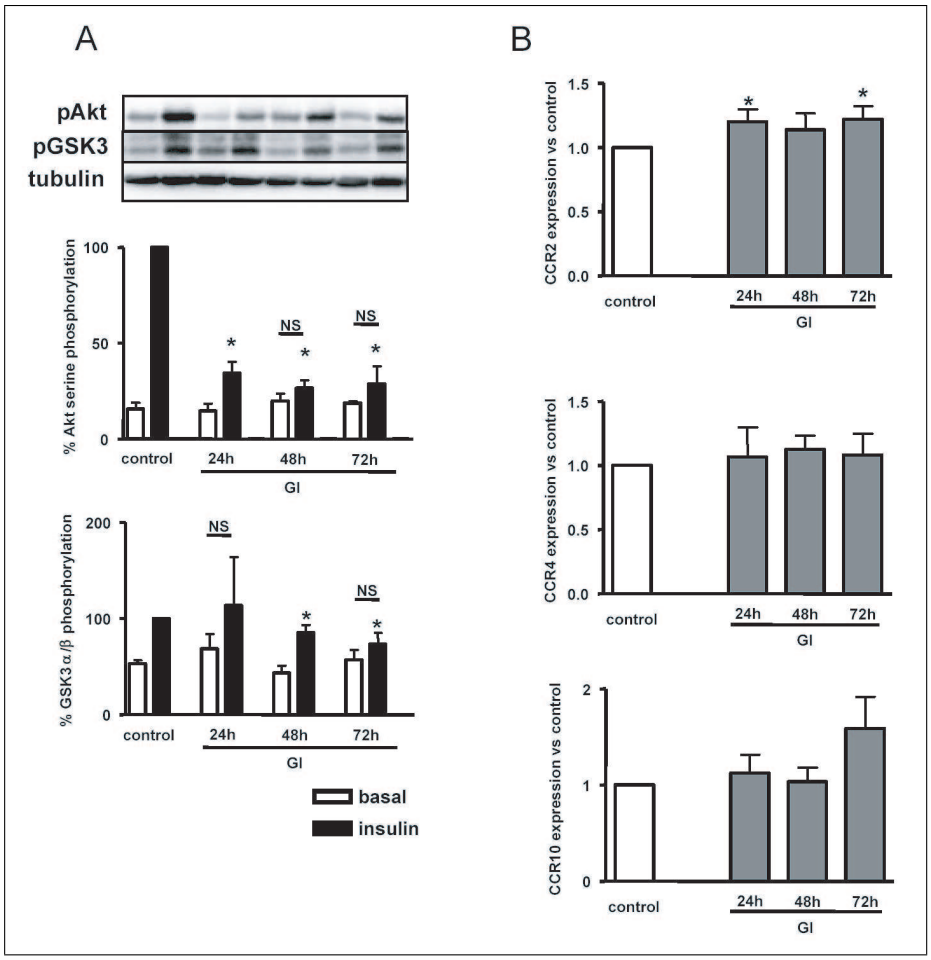
Figure 5.1: Effect of donor and differentiation state on the expression of CCR2, CCR4, and CCR10 in primary human skeletal muscle cells



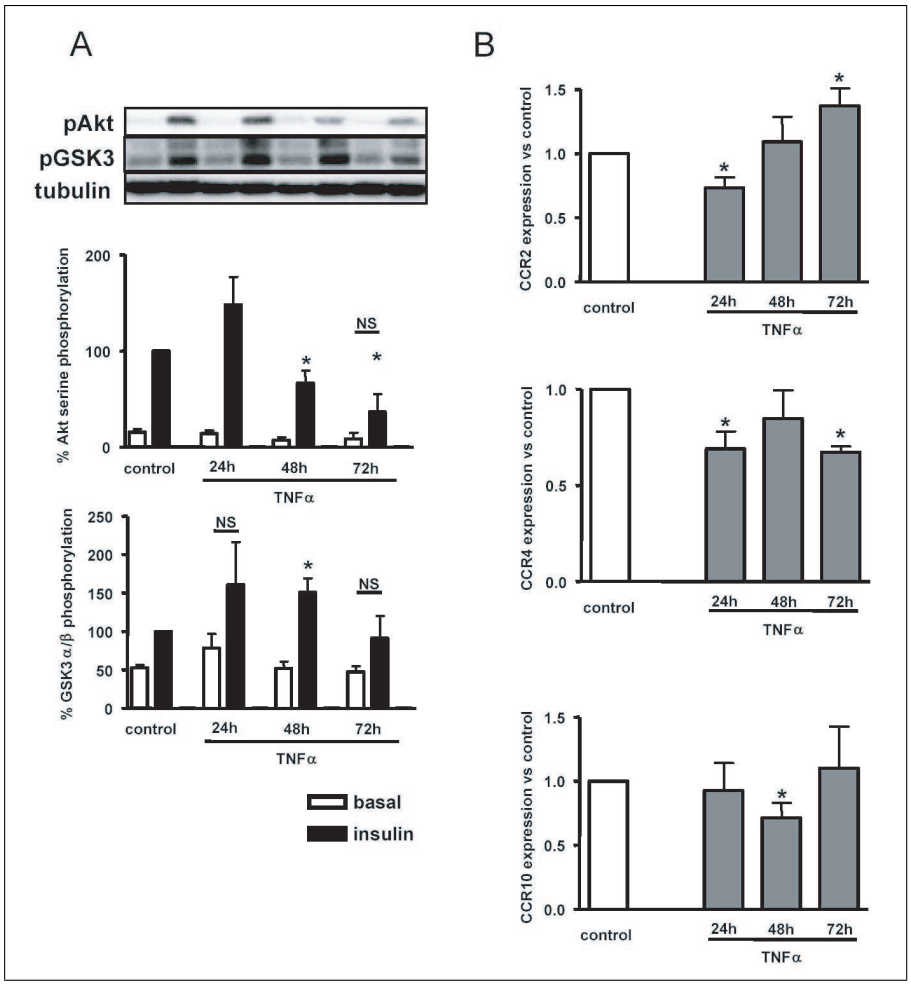
Skeletal muscle cells of five different skeletal muscle cell donors (male: 5 and 9 y (M5, M9); female: 10, 48, and 49 y (F10, F48, F49), passage 4) were differentiated over 6 days. Total cell lysates were resolved by SDS-PAGE and immunoblotted with antibodies for CCR2 (panel **A**), CCR4 (panel **B**), and CCR10 (panel **C**). Blots were reprobed for tubulin as loading control. Exemplary Western blots are shown. Data are mean values ± SEM of two different experiments in duplicates. All data were normalized to the level of tubulin expression and are expressed relative to undifferentiated myocytes from M5. * significantly different from designated donor or differentiation state, at $p < 0.05$.

Figure 5.2: Effect of GI on insulin signalling and expression of CCRs in differentiated human skeletal muscle cells



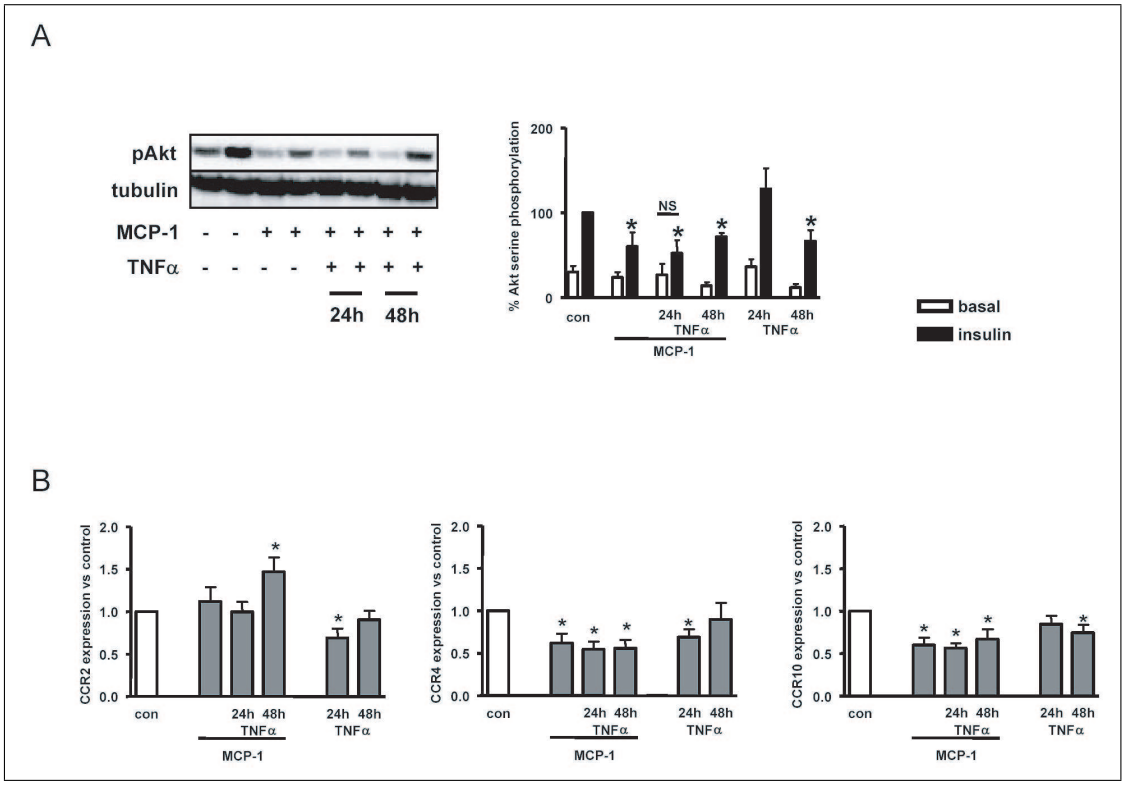
Skeletal muscle cells were incubated with GI as detailed in *Materials and Methods* for the designated periods. **A**: Akt and GSK3α/β phosphorylation in the myocytes were analyzed after acute stimulation with insulin (100 nM, 10 min). Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt/GSK3α/β and tubulin antibodies. One exemplary Western blot is shown. Data are mean values ± SEM of 3-4 independent experiments. All data were normalized to the level of tubulin expression and are expressed relative to the insulin-stimulated control value. *significantly different from insulin-stimulated control; NS, no significant insulin stimulation. **B**: Skeletal muscle cells were analyzed for expression of CCR2, CCR4, and CCR10. Data are mean values SEM of 5-7 independent experiments. All data were normalized to the level of tubulin expression and are expressed relative to control. * significantly different from control, at $p < 0.05$.

Figure 5.3: Effect of TNFα on insulin signalling and expression of CCRs in differentiated human skeletal muscle cells



Skeletal muscle cells were incubated with 2.5 nM TNFα for the designated as detailed in *Material and Methods*. **A**: Akt and GSK3α/β phosphorylation in the myocytes were analyzed after acute stimulation with insulin (100 nM, 10 min). Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt/GSK3α/β and tubulin antibodies. One exemplary Western blot is shown. Data are mean values ± SEM of 3-4 independent experiments. All data were normalized to the level of tubulin expression and are expressed relative to the insulin-stimulated control value. * significantly different from insulin-stimulated control; NS, no significant insulin stimulation. **B**: Skeletal muscle cells were analyzed for expression of CCR2, CCR4, and CCR10. Data are mean values ± SEM of 5-7 independent experiments. All data were normalized to the level of tubulin expression and are expressed relative to control. *significantly different from control, at $p < 0.05$.

Figure 5.4: Effect of co-incubation with TNFα on MCP-1 induced insulin resistance and CCR expression



Skeletal muscle cells were incubated with 2.5 nM TNFα for 24 or 48 h and were co-treated with 0.2 nM MCP-1 for the last 18 h overnight. **A**: Akt phosphorylation in the myocytes was analyzed after acute stimulation with insulin (100 nM, 10 min). Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt and tubulin antibodies. One exemplary Western blot is shown. Data are mean values ± SEM of 3-4 independent experiments. All data were normalized to the level of tubulin expression and are expressed relative to the insulin-stimulated control value. *significantly different from insulin-stimulated control; NS no significant insulin stimulation. **B**: Skeletal muscle cells were analyzed for the expression of CCR2, CCR4, and CCR10. Data are mean values ± SEM of 5-7 independent experiments. All data were normalized to the level of tubulin expression and are expressed relative to control. *significantly different from control, at $p < 0.05$

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Chapter 6

General Discussion

6.1 Adipocytes as secretory active cells

Adipose tissue and its main cell type, adipocytes, have a secretory function by releasing both metabolites and adipokines. While the release of metabolites, mainly FFA, from adipose tissue and adipocytes was known for a long time, an endocrine function was proposed only recently with the discovery of leptin in 1994. Since then, various studies revealed that expanded adipose tissue displays a different secretory function in obesity. Basically, increased secretion of pro-inflammatory cytokines together with adiponectin deficiency best describes adipose tissue in obesity. This is also the case for *in vitro* differentiated human adipocytes used in the presented studies. These adipocytes secrete various cytokines some of which like growth-regulated oncogene (GRO)α and osteoprotegerin (OPG) were first described by us to be adipokines. GROα is a chemokine of the CXC subfamily and is structurally related to IL-8. It was mainly shown to promote growth of various tumors [207] but also inflammation and angiogenesis [208]. The description of GROα as an adipokine was followed by further research in other laboratories demonstrating that GROα is highly expressed in adipose tissue-located macrophages than in adipocytes [209]. Its release from adipocytes but not from macrophages is decreased by atrial natriuretic peptide that also stimulates lipolysis. OPG is a member of the TNF family and serves as a soluble receptor for osteoclast differentiation factor. High OPG levels inhibit bone formation. Interestingly, leptin can regulate OPG expression and may therefore represent a link between adipose tissue and bone mass [210]. A recent study on plasma OPG levels in aging men revealed that it is associated with lower triglycerides, fasting glucose, and higher adiponectin, while lower levels of this adipokine could be found in patients with the metabolic syndrome [211]. However,

in our studies OPG secretion was lower from adipocytes treated with adiponectin so that its role in insulin resistance, if any, needs further analysis.

Different organs such as brain, liver, and skeletal muscle have been demonstrated to be affected by adipokines when it comes to glucose and lipid metabolism [212]. Leptin, TNFα, IL-6, and resistin exert anorexigenic effects in the brain. Adiponectin increases energy expenditure when injected intracerebroventricularly. As for the liver, leptin and adiponectin activate AMP-activated protein kinase (AMPK) and thereby enhance insulin sensitivity and β-oxidation. TNFα and IL-6 are related to hepatic dysfunction. In skeletal muscle, leptin and adiponectin activate insulin sensitivity via AMPK but TNFα clearly induces insulin resistance. The role of IL-6 for skeletal muscle insulin signaling is still controversial. The *in vitro* crosstalk between adipocytes and skeletal muscle cells corroborates several of the above-mentioned effects observed *in vivo*. The entire secretory output from adipocytes in form of CM induces insulin resistance at the level of IRS-1, Akt, and GSK3. In this work, it was also demonstrated for the first time that CM impairs insulin-stimulated GLUT4 translocation in skeletal muscle cells. TNFα is not secreted by *in vitro* differentiated adipocytes but induces insulin resistance in myocytes at high doses.

Adipose tissue and adipocytes are also secretory active from a metabolic point of view by releasing FFA into circulation. *In vitro*, the release of FFA by differentiated adipocytes is very low and barely detectable by high performance liquid chromatography (unpublished data) so that it can be presumed that FFA do not play an important role in the co-culture model. However, for human physiology, FFA released from adipose tissue play an important role. In obesity, the regulation of fat storage and energy supply by adipose tissue is impaired so that plasma FFA levels become elevated and excessive metabolism of FFA including storage of triglycerides and high levels of FFA metabolites occurs in nonadipose tissue such as liver, pancreas, and skeletal muscle [213]. These so-called ectopic fat stores have lipotoxic effects on skeletal muscle and other peripheral tissues because these organs are not designed to store so large amounts of lipids. As for the abnormal lipid storage in adipose tissue, it has been proposed that adipose tissue insulin resistance occurs before muscle insulin resistance [214]. In adipose tissue, insulin resistance is characterized by inadequate insulin action in the fed state that resembles conditions in the normal fasting state: spilling of FFA in the circulation to deliver energy to skeletal muscle. This inadequate insulin response creates a situation where postprandial FFA are directly transported to skeletal muscle and liver where energy is not needed. But

FFA and their metabolites are not only substrates in metabolism but also signaling molecules interacting with the insulin signaling pathway [215]. It was proposed in 1963 that the simultaneous metabolism of glucose and FFA in skeletal muscle is regulated by a series of events that integrate both metabolic pathways [216]. The observed substrate competition between glucose and FFA is referred to as "Randle cycle". Several studies demonstrated that effects of FFA on glucose homeostasis are normally characterized by prominent direct effects on glucose transport rather than glucose metabolism [213]. This makes the "Randle cycle" describing substrate competition, which presumes that increased fatty acid oxidation causes a commensurate decrease in glucose oxidation, untenable for the diabetic state. It is now known that FFA interfere with insulin signaling in skeletal muscle at the level of IRS-1 serine phosphorylation involving activation of PKC θ, inhibitor κB kinase (IKK), and JNK [217].

While some scientists favor the idea that FFA are most important in the molecular understanding of obesity related insulin resistance, a second group of researchers believe that insulin resistance is completely based on adipose tissue adipokine production and release. Paradoxically, the total lack of adipokines observed in lipodystrophic patients leads to diabetes [218]. However, lipodystrophy is characterized by ectopic fat in liver and skeletal muscle but also by a lack of fat in normal fat stores. In fact, intramyocellular lipid stores are observed in both obese and lipodystrophic patients and their abundance strongly correlates with insulin resistance [219]. Furthermore, intramyocellular fat is better associated with insulin resistance than FFA in serum. A mouse model without any ectopic fat caused by ablation of the fatty acid transporter CD36 is highly insulin sensitive despite very high FFA plasma levels [220]. Therefore, reducing ectopic fat in skeletal muscle of diabetic patients may be a good strategy for diabetes therapy and might be part of TZD action [221]. The second important issue in understanding the link between lipodystrophy and insulin resistance is that there is clearly a deficiency in adipocyte secretion, especially in leptin and adiponectin, which are exclusively produced by adipocytes. Finally, lipodystrophy proves that the functions of adipose tissue are crucial in maintaining lipid and glucose homeostasis as the absence of adipocytes is similarly detrimental as hypertrophy of adipose tissue.

6.2 Adiponectin as a positive adipokine

Some adipokines have been shown to directly or indirectly affect insulin signaling or molecules involved in glucose and lipid homeostasis. Of these adipose-derived factors, adiponectin has attracted attention due to its anti-diabetic and also anti-atherogenic action which clearly distinguish it from all other known adipokines. Adiponectin is an attractive therapeutic target for the treatment of type 2 diabetes and several other components of the metabolic syndrome. Besides leptin, adiponectin is the only known "real" adipokine as it is only expressed and secreted from adipocytes in white and brown adipose tissue. Hypoadiponectinemia is not only associated with obesity and diabetes but also with dyslipidemia, cardiovascular disease, hypertension, oxidative stress, and deregulated sexual hormones (androgen and testosterone) [222]. Thus, it must be presumed that circulating adiponectin or its lack affects various cell types that underlie these pathologies. In liver, adiponectin stimulates AMPK improving glucose and lipid homeostasis [223]. In isolated hepatocytes, adiponectin decreases gluconeogenesis leading to reduced hepatic glucose output [224]. Furthermore, adiponectin positively influences the release of different lipoproteins and may therefore improve dyslipidemia [225]. Adiponectin has also beneficial effects on the cardiovascular system [226]. *In vitro*, this adipokine stimulates endogeneous nitric oxide synthase activity and expression in endothelial cells thus being vasoprotective [201, 227]. Smooth muscle cell proliferation and migration are inhibited by adiponectin so that this adipokine can be seen as a positive regulator of vascular remodeling [227].

Several aspects of adiponectin action have also been studied in animal models. Transgenic mice overexpressing adiponectin due to a mutation in the collageneous domain are more insulin sensitive and display higher AMPK activation in liver [228]. Higher adiponectin levels also impair adipogenesis and fat accumulation explaining higher insulin sensitivity [229]. Furthermore, transgenic mice are protected from the adverse effects of HF diet [230]. On the other hand, adiponectin ablation in mice causes severe insulin resistance and increases the cardiovascular risk [231, 232].

Despite good insight into the regulation of adiponectin expression in adipocytes, little is known about the effect of adiponectin on fat cells. We were the first to report on adiponectin acting as an autocrine regulator of adipocytokine secretion of the human fat cell. Another group working on adiponectin reported at the same time that adiponectin acts as an autocrine regulator of adipocyte differentiation and insulin sensitivity [233]. In the co-culture of adipocytes and skeletal muscle cells, the pres-

ence of adiponectin prevents the impairment of insulin signaling in muscle cells. CM generated in the presence of adiponectin does not impair insulin signaling in skeletal muscle cells, while concomitant treatment with adiponectin and CM impairs insulin signaling in skeletal muscle cells similar to CM alone. In this regard, a depressed release of several adipocytokines is the main effect of adiponectin on adipocytes. This regulatory effect of adiponectin also appears in human adipocytes *in vitro* per se. A few CM (approximately 10%) were unable to induce insulin resistance in skeletal muscle cells, an effect that is due to higher adiponectin levels and lower levels of cytokines compared to active CM. The question as to why certain CM are characterized by higher adiponectin levels and no insulin resistance inducing capacity is still open as there is no correlation with BMI or age of the fat donors. Interestingly, the use of different differentiation protocols for adipocytes results in different levels of adiponectin secretion in differentiated cells with higher adiponectin levels always predicting less insulin resistance inducing capacity. However, since our *in vitro* differentiated adipocytes are predominantly characterized by a low adiponectin secretion accompanied by a high release of pro-inflammatory adipocytokines, they therefore represent an ideal model to study the negative crosstalk between skeletal muscle and adipose tissue in obesity. In conclusion, adiponectin acts as an autocrine/paracrine regulator of adipocyte secretion suggesting that adiponectin-regulated cytokines may represent a molecular link between adipose tissue and insulin resistance in skeletal muscle cells.

The adiponectin receptors, AdipoR1 and AdipoR2, were discovered in 2003 [203]. We could demonstrate that both AdipoRs are expressed in human adipocytes and skeletal muscle cells but we have no information on the regulation of these receptors in insulin resistance. While both adipocytes and skeletal muscle cells express adiponectin receptors, adiponectin could be shown to act primarily on adipocytes in our system. In humans, the expression of AdipoRs is not regulated by insulin and seems not to correlate with insulin sensitivity [234]. Furthermore, the expression of AdipoRs in isolated myotubes is not regulated by PPARγ or PPARα agonists [235, 236]. AdipoR1 is highly expressed in adipose tissue and might there contribute to an autocrine/paracrine action of adiponectin [237]. In adipose tissue, AdipoR1 expression correlates with BMI as it is decreased in obesity and increased during weight loss in humans. This negative correlation of AdipoR expression together with reduced levels of adiponectin in obese patients might have additive effects on reduced biological action of adiponectin in adipose tissue. In rodents, AdipoR ex-

pression correlates with insulin sensitivity and is regulated by both PPARγ and PPARα agonists [238] pointing to different regulation of AdipoRs in humans and rodents. The importance of AdipoRs in rodents was confirmed by the very recent generation of mice with AdipoR ablation as loss of adiponectin binding is accompanied by insulin resistance and increased adipose tissue inflammation and oxidative stress [239]. In addition to AdipoRs, T-cadherin was described to bind adiponectin on skeletal muscle cells [240]. The fact that T-cadherin is an extracellular glycosylphosphatidylinositol-anchored protein raises the possibility that it may be an adiponectin-binding protein or co-receptor, although there is a lack of information about its effect in adiponectin cellular signaling.

Adiponectin resistance is a term that appeared in recent years and represents similarity to what is discussed for leptin resistance. This idea came from the fact that increased leptin levels could not counterbalance the development of obesity thereby pointing to peripheral and/or central leptin resistance. While the paradoxical decrease in adiponectin plasma levels may not immediately indicate a peripheral adiponectin resistance, this notion is supported by several studies [241–243]. In genetically obese mice, globular adiponectin has a strongly impaired effect on AMPK in skeletal muscle compared to controls. In parallel, these mice exhibit reduced expression of AdipoR1 in muscle. As for obese patients, there is also a defect in adiponectin stimulated glucose transport and fatty acid oxidation. Very recent data extend the idea of adiponectin resistance to HF diet-induced obesity in rats that display defects in adiponectin-stimulated fatty acid oxidation in muscle independently from glucose transport which remains normal. During pregnancy, higher adiponectin but decreased insulin sensitivity may also be explained by adiponectin resistance [244].

6.3 MCP-1 as an adipocytokine linking obesity and skeletal muscle insulin resistance

After the identification of multiple adipokines in CM and their regulation by adiponectin, it was necessary to find out which of these adipokines is capable of inducing insulin resistance in skeletal muscle cells. Initial studies with resistin and IL-6 revealed that these adipokines can be ruled out as being responsible for the insulin resistance-inducing effect of CM [121]. IL-8 and MIP-1β have to be applied at very high concentrations, usually not found in the circulation, not even in diabetic or obese patients. The chemokine MCP-1 turned out to be a promising candidate as it is the only adipokine tested so far to induce insulin resistance at physiological doses and even at supraphysiological ones.

MCP-1 is a chemokine and a member of the small inducible cytokine family which plays a role in the recruitment of monocytes and T lymphocytes to sites of injury and infection [133]. It is expressed by a number of cell types including smooth muscle and endothelial cells when exposed to inflammatory stimuli [131, 245]. We could show that MCP-1 is also secreted from adipocytes, thus confirming earlier observations [132]. MCP-1's main receptor is CCR2 that is expressed in various cell types including adipocytes and macrophages [132]. We could demonstrate for the first time that CCR2 and other chemokine receptors are expressed in skeletal muscle cells. Interestingly, CCR2 is present in skeletal muscle cells at levels that are significantly higher than in adipocytes. This could explain why skeletal muscle cells are highly sensitive to MCP-1.

MCP-1 was first described to play a role in atherosclerosis as a secretory product of monocytes and endothelial cells [246]. MCP-1, acting through its receptor CCR2, is now thought to play a central role in the recruitment of monocytes to atherosclerotic lesions and in the development of intimal hyperplasia after arterial injury. These crucial roles in monocyte recruitment in vascular and nonvascular diseases made MCP-1 and CCR2 important therapeutic targets in cardiovascular research. Furthermore, MCP-1 plays a role in inflammation in liver and muscle [247, 248]. Finally, it was only recently that a role in the pathophysiology of obesity has been attributed to MCP-1 [249].

First evidence that MCP-1 is linked to obesity was provided by the observation that MCP-1 is overexpressed in obese rodents [129, 130]. It also attains significantly higher plasma levels in obese [249] and diabetic patients [250]. Different depots of

adipose tissue show increased expression of MCP-1 in obese patients such as visceral, subcutaneous, and epicardial adipose tissue [251–253]. Expression of MCP-1 has been found to be higher in visceral adipose tissue than in subcutaneous tissue and is closely related to the number of residing macrophages [251]. This differential expression of MCP-1 could especially contribute to the high risk for the metabolic syndrome associated with visceral obesity. On the other hand, overexpression of MCP-1 especially in epicardial adipose tissue might be crucial for arterial inflammation [134]. Conversely, obese patients that lose weight show decreased levels of MCP-1 and display decreased macrophage infiltration in adipose tissue [249, 254]. These studies demonstrate that the beneficial effect of weight loss is mediated at least partially by reducing the inflammatory status of adipose tissue. MCP-1 may not only describe inflammation in fat but may also actively be involved in adipose tissue expansion and remodeling. MCP-1 has been shown to have angiogenic effects in endothelial cells [135], thus enabling neovascularization in growing fat. The ability of MCP-1 to accelerate wound healing, a process that involves vessel formation, also highlights an angiogenic effect related to this chemokine [255].

MCP-1 is secreted from adipocytes in rodents [129] and humans as also demonstrated in this work. Large adipocytes release higher amounts of MCP-1 together with other pro-inflammatory cytokines [99]. *In vitro*, MCP-1 expression and secretion is highly regulated in adipocytes. In 3T3-L1 adipocytes, MCP-1 expression is increased by insulin, TNFα, GH, and IL-6 [256], all of which are increased in obese patients. Conversely, treatment of 3T3-L1 adipocytes with MCP-1 was found to impair glucose uptake indicating that this cytokine may contribute to the pathogenesis of insulin resistance [129]. In fact, this was the first study to demonstrate that MCP-1 might be a direct inflammatory link between obesity and insulin resistance. We could extend this effect of MCP-1 to skeletal muscle further demonstrating the importance of MCP-1 in this context.

In fact, many chemokines have been shown to possess biological activity beyond the recruitment of immune cells. This applies to MCP-1, for which insulin resistance-inducing capacity was first postulated in adipocytes [129]. Clinical data provide good evidence for a relationship between MCP-1 serum levels and insulin resistance, and type 2 diabetes as diabetic patients display elevated MCP-1 levels [257, 258]. Conversely, diabetes treatments such as exercise [259], rosiglitazone [260], and weight loss [249], all of which improve insulin sensitivity in obese patients, reduce MCP-1 plasma concentrations. In contrast, metformin treatment, which re-

duces insulin resistance, has no effect on MCP-1 serum levels, perhaps due to the fact that this treatment is not able to reduce the macrophage content of adipose tissue [261]. Besides traditional anti-diabetic and anti-obesity treatments, lower MCP-1 levels due to a MCP-1 G-2518 gene variant have been shown to protect from the development of diabetes [258]. The A-2518G polymorphism in MCP-1 leads to decreased MCP-1 plasma concentrations and is related to a reduced prevalence of insulin resistance and diabetes.

Our *in vitro* studies on MCP-1 treatment of skeletal muscle cells have demonstrated specific insulin resistance-inducing effects of MCP-1, which constitute evidence for this chemokine being a link between inflamed adipose tissue and insulin resistance. In adipocytes where MCP-1 exerts the same insulin resistance-inducing capacity, it could be demonstrated that MCP-1 is an insulin-responsive protein, since insulin stimulates its expression and secretion [129]. In insulin-resistant adipocytes and *ob/ob* mice, MCP-1 is significantly increased but still insulin-responsive. Thus, hyperinsulinemia accompanying insulin-resistant states might explain the altered expression of MCP-1. White adipose tissue is the main source of MCP-1 where it induces insulin resistance and dedifferentiation of adipocytes, thereby contributing to the development of type 2 diabetes. In addition to these important initial findings, it has been shown that MCP-1 expression is upregulated by insulin resistance-inducing cytokines such as GH and IL-6 in adipocytes. This may represent a molecular link between insulin resistance and obesity as already proposed by the above-mentioned clinical studies. Furthermore, MCP-1 secretion is also stimulated by isoproterenol [262] demonstrating that increased sympathetic stimulation as observed in insulin resistance and obesity might lead to increased levels of this adipokine. Resistin overexpression in adipocytes increases the release of MCP-1, as well as other inflammatory cytokines [263]. We could demonstrate, in addition to all shown treatments that upregulate MCP-1, that secretion of this chemokine from adipocytes can also be downregulated by stimuli that prevent insulin resistance, namely adiponectin, AICAR, and troglitazone.

MCP-1 causes insulin resistance in an autocrine/paracrine fashion by reducing glucose uptake in adipocytes and concomitantly increasing the release of pro-inflammatory adipokines. MCP-1 affects insulin resistance in skeletal muscle and survival of β-cells in an endocrine way. Our findings on MCP-1 inducing insulin resistance in primary skeletal muscle cells have been confirmed in a skeletal muscle cell line [264]. The fact that very low levels of MCP-1 are able to induce insulin

resistance in skeletal muscle suggests that even a slight elevation of MCP-1 due to only moderate weight increase might affect skeletal muscle insulin sensitivity. MCP-1 might therefore underlie early stages of insulin resistance - an assumption that still needs to be verified. Furthermore, MCP-1 might also be related to β-cell survival and type 1 diabetes. It is known that CD4-positive cells migrate into the pancreas and contribute to the development of diabetes. Th1 cells, but not Th2, are specifically involved in the destruction of β-cells in autoimmune diabetes, at least in an *in vitro* model [265]. Th1 cells differ from Th2 cells by producing MCP-1 and other inflammatory cytokines and this marked difference in cytokine secretion might be important in the induction of diabetes. In addition, human β-cells also express and secrete MCP-1, even without the presence of immune infiltrates [266]. MCP-1 expression is regulated by IL-1β, TNFα, and lipopolysaccharides (LPS) but not by glucose. Nevertheless, insulin secretion is not affected by MCP-1 treatment. On the other hand, MCP-1 expression in β-cells predicts the outcome of islet transplantation [267]. Islets that are not functional after transplantation have increased MCP-1 secretion supporting the notion that this chemokine may contribute to graft rejection.

Very recently, generation of a mouse model where MCP-1 is specifically overexpressed in adipose tissue has provided exciting new insights into the role of MCP-1 in the pathogenesis of insulin resistance and adipose tissue inflammation [268]. Overexpression of MCP-1 in adipose tissue alone mimics effects of diet-induced obesity such as insulin resistance, macrophage infiltration into adipose tissue, and liver steatosis, which occurs in the absence of any increase in body weight. Thus, MCP-1-induced increase in adipose tissue inflammation is sufficient to induce several features of the metabolic syndrome, at least in rodents. It has also been shown by the same investigators that in contrast to MCP-1 overexpression, MCP-1 deficiency in diet-induced obese mice or inhibition of MCP-1 expression in *db/db* mice ameliorates insulin resistance and reduces macrophage content in adipose tissue. A second study involving mice with adipose tissue overexpression of MCP-1 has demonstrated that MCP-1 can reduce insulin sensitivity in an endocrine way in skeletal muscle [264]. Thus, our *in vitro* results with MCP-1 are indicatory for the situation *in vivo*.

6.4 Pahtways involved in skeletal muscle cell insulin resistance

The co-culture model of adipocytes and skeletal muscle cells also serves to study pathways underlying the development of insulin resistance. For skeletal muscle cells, several pathways are already identified to contribute to insulin resistance such as systemic inflammation-mediated JNK, IKK and NF-κB activation. Furthermore, defects in oxidative phosphorylation and therefore oxidative stress are crucial components of the development of insulin resistance in muscle. By studying the effect of MCP-1 on skeletal muscle cells, we could identify extracellular signal-regulated kinase (ERK) to be a key mediator in its induction of insulin resistance. ERKs are members of the mitogen-activated protein (MAP) kinase family that plays an important role in cell proliferation, differentiation, motility, and survival [269]. In cancer therapy, ERK inhibitors are upcoming drugs [270]. Particularly in skeletal muscle, ERKs are emerging as important signaling molecules in the regulation of metabolism and gene expression. It was recently shown *in vivo* that ERK activation is increased in skeletal muscle samples from insulin-resistant patients [56]. Acute induction of insulin resistance in C2C12 myotubes by TNFα was shown to decrease insulin-stimulated protein synthesis involving ERK [271]. The selective ERK inhibitor PD-98059 could partly prevent this effect. A role of ERK has also been demonstrated for palmitate-induced insulin resistance [272]. In primary human skeletal muscle cells, ERK plays an essential role in MCP-1-induced insulin resistance which could, in contrast to C2C12 myotubes, be completely reversed by ERK inhibition.

Obesity and insulin resistance are associated with markers of systemic inflammation. JNKs are mitogen-activated protein kinases with an emerging role in inflammation that regulate transcription by phosphorylation of activator complex proteins such as c-Jun [273]. It was confirmed by various studies that JNKs constitute a link between inflammation and metabolic diseases [274, 275]. JNKs integrate metabolic and immune pathways. In the context of obesity, JNKs are activated by pro-inflammatory cytokines such as TNFα and have been demonstrated to play a role in the negative regulation of insulin signaling [276]. JNK1 KO leads to protection from HF feeding-induced obesity and insulin resistance in lean mice [275]. However, JNK2 KO does not influence insulin sensitivity making it probable that JNK1 is more important in this context. Furthermore, JNK1 suppression by a dom-

inant negative form positively influences insulin sensitivity [277]. Taken together, JNKs might be key mediators in the progression of insulin resistance *in vivo*. *In vitro*, JNKs seem to play a minor role in the induction of skeletal muscle cells insulin resistance by CM since it is not activated after overnight incubation [278]. However, JNK inhibitors are able to partly restore insulin sensitivity in skeletal muscle cells treated with CM pointing to a possible participation of JNKs in the induction of insulin resistance at earlier time points in a transient way. In skeletal muscle, basal JNK phosphorylation is increased in samples from obese and insulin-resistant patients compared to lean controls [56]. In 3T3 adipocytes, JNKs were shown to be involved in FFA-induced insulin resistance upstream of TNFα release [279] but data on the role of JNKs in primary adipocytes is so far not available.

There is increasing evidence that inflammatory processes also referred to as low grade systemic inflammation result from underlying metabolic diseases including insulin resistance and diabetes. This subject is more closely treated later in section 6.7 with special emphasis on inflammation in adipose tissue but inflammatory pathways also play a role in skeletal muscle. IKK is a central coordinator of inflammatory responses by activating NF-κB, the primary transcription factor involved in inflammatory processes [280] and a critical pathway underlying the link between chronic inflammation, obesity, and insulin resistance. It seems that both JNKs and IKK are essential for TNFα-induced insulin resistance in cells. Suppression of IKK can increase insulin sensitivity. In fact, non steroidal anti-inflammatory drugs such as aspirin increase insulin sensitivity in adipocytes through inhibition of IKK and JNKs [281]. In the same context, NF-κB inhibitors downregulate the secretion of pro-inflammatory cytokines from human adipose tissue and skeletal muscle [282]. Furthermore, it has been shown that IKK is involved in the induction of insulin resistance in skeletal muscle cells by co-culture with adipocytes [121]. In our model, inhibition of IKK reverses insulin resistance that is characterized by NF-κB activation.

Defective oxidative phosphorylation is another mechanism that seems to be involved in insulin resistance in both skeletal muscle [283] and adipose tissue [284]. Muscle oxidative capacity correlates with defense against oxidative stress in diabetic patients [285] and is already decreased in insulin-resistant relatives of diabetic patients which are at high risk of becoming diabetic later in life [286]. This loss of mitochondrial oxidative capacity can be explained by the fact that insulin resistance is accompanied by fewer muscle mitochondria. Accordingly, skeletal muscle

from obese patients also contains a smaller number of mitochondria due to a lower ratio of type 1 to type 2 muscle fibers. Furthermore, a deficit in muscle oxidative phosphorylation could also be due to defects in mitochondrial function. In fact, analysis of gene expression in skeletal muscle of diabetic patients revealed the lower expression of a cluster of oxidative genes related to PPARγ coactivator 1 [283] and mitochondrial dysfunction [287]. Whether intramyocellular lipid accumulation is the cause or an effect of decreased mitochondrial oxidative capacity is unclear [288].

6.5 Pathways involved in the regulation of adipokine secretion

In chapter 4 AMPK was described to be a regulator of adipokine release. Generally, AMPK is described as a regulator of cellular and systemic energy homeostasis. In this respect, AMPK is an evolutionary well-conserved serine/threonine kinase that senses cellular energy status [289]. Activation of AMPK occurs by any form of stress that depletes cellular ATP levels. In adipose tissue, the kind of stress can be manifold and might be related to oxidative capacity, hypoxia, or inflammation. Leptin and adiponectin both activate AMPK but this happens in an endocrine way in skeletal muscle, liver, and heart also involving the hypothalamo-sympathetic axis. Only adiponectin was shown to directly act on glucose uptake in rat adipocytes by activating AMPK [290]. In adipose tissue and skeletal muscle, adipokines such as leptin and adiponectin exert their metabolic function via activation of AMPK leading to increased β-oxidation in skeletal muscle. AMPK can be activated by adipokines thus enabling skeletal muscle to cope with increased FFA supply, a process that seems not to be functioning in the obese state where adiponectin is diminished. On the other hand AMPK also functions as a regulator of cytokine release. The AMPK activator AICAR results in an attenuated secretion of pro-inflammatory cytokines from human adipose tissue *in vitro* [291]. Furthermore, AMPK appears to play a role in adipose tissue secretion through a positive feedback loop related to adiponectin. It is also believed that AMPK activation is part of the anti-diabetic action of PPARγ agonists, in this case also related to increased adiponectin expression and secretion [289]. We could demonstrate that AICAR, adiponectin, and the PPARγ agonist troglitazone activate AMPK in human adipocytes and that all these treatments share a common change of adipokine release decreasing the secretion of pro-inflammatory adipokines while increasing the release of adiponectin. AMPK might therefore play a crucial role in the crosstalk between adipose tissue and skeletal muscle where it integrates metabolic needs and secretory function.

Some evidence exists that hypoxia in adipose tissue could be involved in adipokine dysregulation in obesity. In fact, expansion of adipose tissue is not followed by increased vascularization making it impossible to maintain normoxia [292]. Larger adipocytes with diameters of up to 150 µm are then partially deprived of oxygen which can diffuse only over 100 µm [293]. The response to hypoxia might be the release of inflammatory adipokines which serve to increase blood flow and induce

angiogenesis. In fact, some adipokines such as leptin, vascular endothelial growth factor, and PAI-1 are angiogenic factors. Furthermore, adipocytes express a key transcription factor involved in cellular response to hypoxia, hypoxia-inducible factor (HIF)-1, which is increased in obesity and regulated by TNFα [254]. *In vitro*, hypoxia induces HIF-1 in adipocytes leading to downregulation of adiponectin which is in complete agreement with the hypothesis that hypoxia is an integral component of inflammation in enlarged adipose tissue [293]. Very recent data demonstrates that measured hypoxia in adipose tissue of obese mice correlates with increased expression levels of pro-inflammatory adipokines and decreased adiponectin mRNA stability [294]. This publication also links hypoxia with another mechanism that could be involved in the regulation of adipose tissue secretion, endoplasmic reticulum (ER) stress.

ER stress is described by hyperactivation of JNK and subsequent inhibitory phosphorylation of IRS-1 which can be observed in adipose tissue and liver of *ob/ob* obese mice and HF diet-induced obese mice [295]. The "unfolded protein response" and ER chaperones are part of ER stress and seem to be altered in diabetes [296]. However, a clear role of ER stress in adipose tissue secretion needs to be further demonstrated.

6.6 Adipose tissue inflammation

There are two major scientific evidences that obesity can be seen as a state of chronic inflammation. First, there is the above-described increase in pro-inflammatory adipokines in obese patients. Second, macrophage infiltration was recently described to be characteristic of expanding adipose tissue [297]. The first description of inflammation in adipose tissue goes back to 1993 when constitutive production of TNFα from adipose tissue was demonstrated [112]. Commonly, lymphoid organs and the liver are most important for the expression and release of inflammatory factors but advances in adipose tissue biology provided evidence for a prominent role of adipose tissue in this respect too.

Electron microscopic studies revealed an important new aspect of adipose tissue biology as numerous cell types other than adipocytes were recognized [254]. These cell types comprise pericytes, endothelial cells, monocytes, macrophages, preadipocytes, and other pluripotent stem cells, which altogether are referred to as the stroma-vascular fraction. There are some studies that provide evidence for a higher contribution of nonadipose cells to adipose tissue secretion [298, 299] making it possible that adipocytes are neither the only nor the main contributor to insulin resistance *in vivo*. In this context, it was also proposed that adipocytes and macrophages share some characteristics such as secretion of TNFα, MCP-1, and IL-6, regulation of differentiation and maturation by PPARγ activation, and presence in the same tissue [300]. This may implicate a much closer interaction between these two cell types than previously thought. Therefore, it can be assumed that the complete secretory activity of adipose tissue cannot be attributed merely to adipocytes.

The infiltration of macrophages increases with BMI and adipocyte hypertrophy but is however not associated with increased macrophage content in other insulin-sensitive tissues such as muscle or liver [297, 301]. The origin of fat-infiltrating macrophages is still under investigation but there is some evidence that they are essentially bone marrow-derived similar to preadipocytes differentiating into new adipocytes [84, 297]. Completely surrounding adipocytes, macrophages arrange in crown-like structures similar to macrophage arrangements in other local inflammatory states. The mechanisms by which macrophages are attracted to adipose tissue and finally infiltrating fat are multifactorial. Paracrine and endocrine signals as well as adipocyte hypertrophy and hyperplasia might contribute to this phenomenon. *In vitro*, leptin is a potent factor for adherence and transmigration-inducing factor for bone marrow-derived macrophages in co-culture with endothelial cells [302].

Adiponectin inhibits this process [303]. Thus, adiponectin might not only have a paracrine effect on adipocytes but also on adipose tissue-located macrophages. Very recent data provides further insight into the nature of adipose tissue-located macrophages. Not only the number of macrophages increases in obesity but also the activation status of these cells is altered [304]. Macrophages in adipose tissue of lean mice express markers of M2 macrophages (alternatively activated macrophages) while obese mice have more M1 macrophages (classically activated) that have higher expression of TNFα and inducible nitric oxide synthase. MCP-1 and CCR2 are also critical actors involved in inflammation in adipose tissue. CCR2 KO mice have reduced macrophage content [305]. Furthermore, mice lacking CCR2 on HF diet are protected from infiltration of M1 macrophages [304]. Data on CCR2 regulation in adipose tissue of obese and diabetic patients and therefore evidence for a role of CCR2 in humans is not yet available.

MCP-1 and its receptor both seem to highly contribute to macrophage accumulation in adipose tissue in obesity but knowledge on the MCP-1/CCR2 axis in skeletal muscle is very limited. Skeletal muscle cells also express CCR2 and other chemokine receptors. We demonstrated for the first time that CCRs are regulated in human skeletal muscle cells by insulin resistance-inducing treatments. We observed an increased expression of CCR2 in insulin-resistant myotubes after treatment with glucose/insulin and TNFα. This increase can also be observed in monocytes from diabetic patients [306]. In contrast, expression of CCR4 and CCR10 tend to decrease in insulin-resistant human myotubes but specific functions of these receptors are unknown. Despite CCR2 upregulation by insulin resistance-inducing treatments, we are not able to prove a general correlation between the level of insulin resistance and CCR2 expression in myotubes. We hypothesize that the observed increase in CCR2 expression in insulin-resistant myotubes might rather illustrate dedifferentiation of myotubes as described in the literature and in accordance with our own data on CCR2 expression decreasing with differentiation of myotubes [307]. Clinical evaluation of CCR2 expression in different patient groups would help to further understand the role of CCR2 in skeletal muscle.

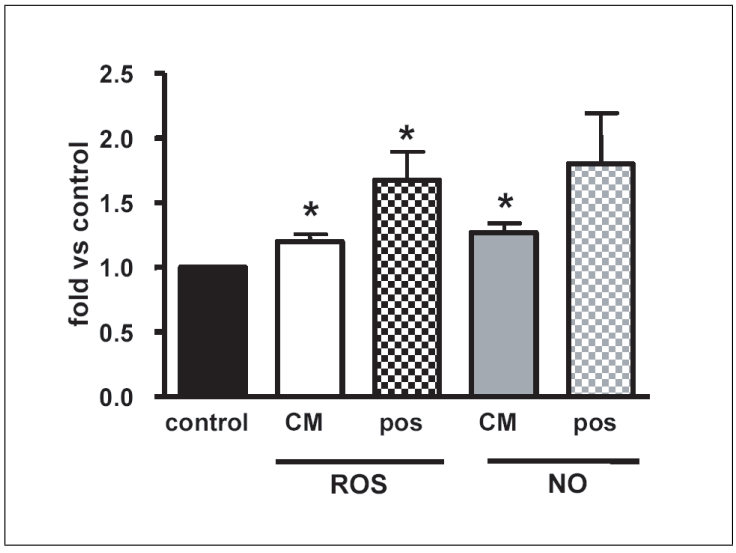
As for the role of macrophages in adipose tissue, two different major functions are described in the literature. First, macrophages accumulate around dead, stressed, or aged cells [308]. In fact, adipocytes that are surrounded by macrophages express lipofuscin in cytoplasmic granules which is a good indicator of cellular stress [254]. This role of macrophages includes the above-mentioned concomitant

increased release of pro-inflammatory cytokines, a deleterious systemic effect. Second, macrophages might play an important role in neovascularization of expanding fat [309]. Macrophages may therefore counterbalance hypoxia in adipocytes of expanding adipose tissue, a beneficial local effect on adipose tissue function. Thus, the role of macrophages in adipose tissue inflammation seems to be dual and must be differentiated.

6.7 Perspectives

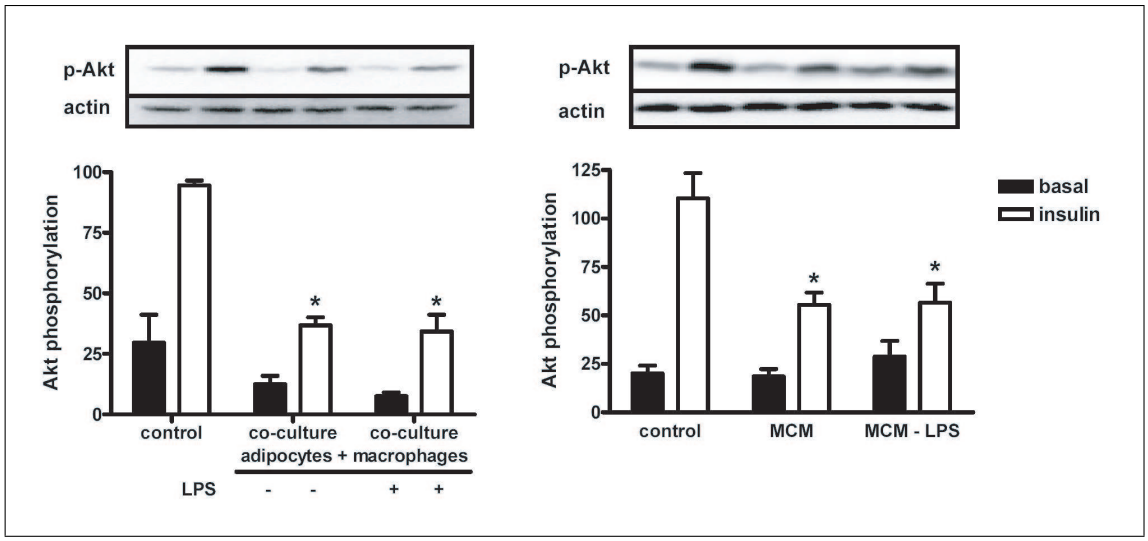
The present work met several of the objectives put forward in the introduction. We were able to elucidate the role of adiponectin in the negative crosstalk between human adipocytes and skeletal muscle cells. Adiponectin acts on the secretory function of adipocytes and prevents insulin resistance. In the second study, we could demonstrate that an isolated secretory factor from adipocytes, namely MCP-1, can mimick CM-induced insulin resistance in skeletal muscle cells. Thus, we could show that adipokines are a real link between obesity and insulin resistance. From a mechanistic point of view, ERK was shown to be involved in MCP-1-induced insulin resistance. Another study showed that IKK is involved in the induction of insulin resistance by CM [121]. Very recent preliminary data (Figure 1) indicates that oxidative stress is also an underlying mechanism of insulin resistance in our system as reactive oxygen species (ROS) and nitric oxide (NO) levels are significantly higher in insulin-resistant skeletal muscle cells. Furthermore, we observed a dedifferentiation of skeletal muscle cells that were treated with CM (data not shown). Future work should be aimed to elaborate these notions in our system and also to analyse insulin resistant skeletal muscle cells concerning their metabolic and mitochondrial capacity.

Figure 6.1: Oxidative stress in insulin-resistant skeletal muscle cells



In human adipocytes, we studied their secretory function and the regulation of adipokine release by different stimuli. We could demonstrate that adipocytes secrete

Figure 6.2: Macrophages induce insulin resistance in human adipocytes



various pro-inflammatory adipokines that are downregulated by adiponectin and anti-diabetic drugs such as PPARγ agonists. By identifying these pro-inflammatory factors, we could prove that the concept of adipose tissue inflammation can be studied in our system. The concept of inflammation in adipose tissue as an underlying mechanism of insulin resistance led us to study the crosstalk between adipocytes and macrophages. In fact, the crosstalk between adipocytes and macrophages has recently been shown to have an impact on the development of insulin resistance through the secretion of pro-inflammatory cytokines by adipose tissue [310, 311]. In preliminary experiments, we could show that co-culture of human adipocytes and macrophages induces insulin resistance in adipocytes (Figure 2). Macrophage-conditioned medium (MCM) is also able to induce insulin resistance at the level of insulin-stimulated Akt phosphorylation. Interestingly, LPS activation of macrophages has no additional impact on insulin signaling in the adipocytes suggesting that basal macrophage secretion is sufficient to create an inflammatory environment.

Finally, it may be considered to extend this co-culture to skeletal muscle cells because it is reasonable to assume that an interaction between macrophages and adipocytes in adipose tissue might also be relevant to the crosstalk with other tissues such as muscle and liver.

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Contribution to chapter 2-5

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

Chapter 2

- Analysis of GLUT4 translocation
- Analysis of adiponectin receptor expression by RT-PCR
- Effect of adiponectin in the co-culture of human skeletal muscle cells with adipocytes together with Daniela Dietze-Schroeder
- Effect of medium conditioning in the presence or absence of adiponectin on insulin signaling in skeletal muscle cells together with Daniela Dietze-Schroeder
- Generation of all CM together with Daniela Dietze-Schroeder
- Cytokine arrays of CM
- Quantification of cytokines in CM
- Preparation of manuscript together with Daniela Dietze-Schroeder and Jürgen Eckel

Chapter 3

- Analysis of insulin signaling in CM-treated skeletal muscle cells
- Cytokine arrays of CM
- Comparison of CCR2, 4 and 10 expression in skeletal muscle cells and adipocytes
- Dose-dependent effect of MCP-1 on insulin signaling in skeletal muscle cell

- Effect of MCP-1 on glucose uptake in skeletal muscle cells
- Analysis of ERK activation by MCP-1
- Effect of ERK inhibition on insulin signaling in skeletal muscle cells
- Effect of IL-8 and MIP-1β on insulin signaling in skeletal muscle cells
- Preparation of manuscript together with Jürgen Eckel

Chapter 4

- Preadipocyte isolation together with Daniela Dietze-Schroeder and Kristin Eckardt
- Cytokine arrays of CM
- Analysis of AMPK activation
- Preparation of manuscript together with Jürgen Eckel

Chapter 5

- Analysis of CCR2, 4 and 10 expression in different skeletal muscle cell donors
- Analysis of CCR2, 4 and 10 expression during differentiation of myotubes
- Analysis of CCR4 expression in insulin-resistant skeletal muscle cells
- Analysis of CCR2, 4 and 10 expression in skeletal muscle cells treated with MCP-1/TNFα and CM/TNFα
- Supervision of practical work done by Ulrike Kaiser
- Preparation of manuscript together with Jürgen Eckel

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