Role of Adipokines in the Crosstalk Between Human Adipose Tissue and Skeletal Muscle Cells

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Zusammenfassung

Typ 2 Diabetes (T2D) hat weltweit eine Verbreitung epidemischen Ausmaßes erreicht. Die hohe Prävalenz sowie die erwartete Zunahme von T2D Fällen in naher Zukunft verdeutlichen den Bedarf an kostengünstigen und effizienten Therapien. Hierzu ist das Verständnis der zugrunde liegenden molekularen Mechanismen unentbehrlich. So ist bereits bekannt, dass die Entstehung von T2D mit einer vermehrten Fettgewebsmasse sowie einer verminderten Insulinsensitivität korreliert. Dieser negative Crosstalk zwischen Fettgewebe und Muskel wird durch eine verstärkte Freisetzung von bioaktiven Proteinen aus dem Fettgewebe, den so genannten Adipokinen, vermittelt. Die Wirkung einzelner Adipokine auf die generelle sowie die Muskel-spezifische Insulinsensitivität wurde bereits in verschiedenen Studien untersucht. Moderne Analysemethoden zeigen jedoch, dass das Adipozytensekretom ein hochkomplexes Gemisch aus hunderten verschiedener sekretierter Proteine und Peptide darstellt. Dadurch wird deutlich, dass die Analyse einzelner Adipokine nur einen limitierten Ausschnitt der komplexen physiologischen Situation liefern kann. Daher war es Ziel dieser Arbeit, weitere Einblicke in die zusammenhängende Wirkung des gesamten Adipozytensekretoms in Kombination mit physiologischen Konzentrationen von Fettsäuren auf den Metabolismus von Skelettmuskelzellen zu gewinnen. Des Weiteren wurde der Einfluss der Muskelkontraktion im Zusammenspiel mit der Wirkung von Adipokinen und Fettsäuren auf den Skelettmuskel untersucht.

Der Einfluss des gesamten Adipozytensekretoms wurde mittels Adipozyten-konditionierter Medien (CM) untersucht. Dabei konnte gezeigt werden, dass Adipokine die Abundanz des Fettsäuretransportproteins CD36 sowie die Fettsäureaufnahme in primären humanen Skelettmuskelzellen (SkMC) erhöhen. Während negative Effekte einzelner Adipokine, wie z.B. Chemerin, auf die Insulinsensitivität von SkMC gezeigt werden konnten, konnte kein isoliertes Adipokin die CM-vermittelte Wirkung auf den Lipidstoffwechsel nachahmen.

Obwohl T2D häufig mit erhöhten Plasmaspiegeln von freien Fettsäuren einher geht, ist wenig über die gemeinsame Wirkung von Adipokinen und Fettsäuren

bekannt. Im Rahmen dieser Arbeit konnte jedoch gezeigt werden, dass die gleichzeitige Behandlung von SkMC mit CM und Fettsäuren zu einer vermehrten Ansammlung von intramyozellulären Lipidtropfen sowie Triacylglycerol (TAG) führte, wobei unterschiedliche Effekte für Ölsäure (OA) und Palmitinsäure (PA) beobachtet werden konnten. So führte die Kombination von OA+CM zu einer vermehrten Ausbildung von intramyozellulären Lipidtropfen und einer deutlichen Ansammlung von TAG, während die Behandlung mit PA+CM eine weniger strukturierte Akkumulierung von Lipiden in der Zelle zur Folge hatte. Darüber hinaus konnte gezeigt werden, dass die Behandlung von SkMC mit CM und Fettsäuren zu einer gestörten Fettsäureoxidation führt, wobei die schwerwiegendsten Defekte durch die Kombination von PA+CM hervorgerufen wurden.

Da Lebensstilinterventionsstudien, unter anderem mittels vermehrter sportlicher Aktivität, eine Verbesserung der Insulinsensitivität zeigen konnten, und *in vitro* Studien eine dauerhafte Beeinträchtigung des Insulinsignals nahe legen, wurde die Reversibilität der CM-induzierten Insulinresistenz untersucht. Dabei konnte nur eine partielle Aufhebung der CM-vermittelten Defekte festgestellt werden. Um die zu zugrunde liegenden positiven Auswirkungen vermehrter körperlicher Aktivität genauer zu untersuchen, wurde ein *in vitro* Modell elektrisch-induzierter Muskelkontraktion heran gezogen, um den Einfluss der Kontraktion im Zusammenspiel mit Adipokinen und Fettsäuren auf den Skelettmuskel zu untersuchen. In diesem Zusammenhang konnte gezeigt werden, dass die elektrische-Puls-Stimulation (EPS) von SkMC die Beeinträchtigung der Fettsäureoxidation nach OA+CM-Behandlung verhindern konnte. Interessanterweise war dies jedoch nach PA+CM-Behandlung nicht zu beobachten.

Zusammenfassend deuten die Daten dieser Arbeit auf eine neue Rolle für Adipokine bei der Verstärkung der lipotoxischen Wirkung von Fettsäuren hin. Diese Wirkung konnte nicht durch einzeln eingesetzte Adipokine nachgeahmt werden. Darüber hinaus wurde festgestellt, dass die Behandlung mit CM und gesättigten Fettsäuren, im Gegensatz zu ungesättigten Fettsäuren, zu einer dauerhaften Schädigung von SkMC führt. Abschließend konnte gezeigt werden, dass die Behandlung mit EPS ein wertvolles *in vitro* Modell darstellt, mit dessen Hilfe der selektive Schutz vor Lipotoxizität-induzierten Defekten durch Muskelkontraktion realisiert werden konnte. Diese Ergebnisse geben neue Einblicke in das komplexe Wechselspiel von Adipokinen, Fettsäuren und Muskelkontraktion bei der Entstehung

von Adipositas und T2D, die zu der Entwicklung verbesserter oder neuer therapeutischer Ansätze bei der Behandlung von T2D und der einhergehenden Komplikationen beitragen können.

Summary

The worldwide prevalence of type 2 diabetes (T2D) has reached epidemic proportions and the expected increase of T2D cases emphasizes the urgency of understanding the molecular mechanisms underlying the etiology of this disease in order to develop cost-efficient and effective therapeutic strategies. Pathophysiology of T2D is closely associated with obesity and the induction of skeletal muscle insulin resistance. In this context, adipocyte-secreted factors, referred to as adipokines, have been implicated in the negative crosstalk between adipose tissue and skeletal muscle. A number of studies have investigated the contribution of isolated adipokines to impaired whole body or skeletal muscle insulin sensitivity, however, highly sensitive state-of-the-art proteomic analyses have revealed the complex nature of the adipocyte secretome, comprising hundreds of different secreted peptides and proteins. Thus, studies of single adipokines are only able to yield a limited picture of the complex physiological condition. Therefore, this thesis aimed to obtain further insight into the interrelated and combined impact of the entire adipocyte secretome together with physiological concentrations of fatty acids (FA) on skeletal muscle metabolism. Additionally, the influence of muscle contraction on muscle cell metabolism in the interplay with adipokines and FA was to be assessed.

Adipocyte-conditioned medium (CM) was used to investigate the influence of the entire adipocyte secretome. In this context, adipokines were found to increase protein abundance of the fatty acid transporter CD36 as well as enhance fatty acid uptake in primary human skeletal muscle cells (SkMC). Although novel adipokines, such as chemerin, could be demonstrated to contribute to the negative crosstalk of adipocytes and muscle with regard to impaired skeletal muscle insulin sensitivity, no single adipokine could be identified to be able to mimic the CM-mediated influence on lipid metabolism.

Although T2D patients are frequently characterized by increased plasma free FA levels, data on the combined influence of adipokines and FA are scarce. Simultaneous exposure of primary human SkMC to CM and FA was found to

induce severe accumulation of intramyocellular lipid (IMCL) droplets and triacylglycerol (TAG), whereas distinct effects of oleic acid (OA) and palmitic acid (PA) could be identified. While combined treatment of OA+CM resulted in formation of a multitude if IMCL droplets and profound accumulation of TAG, incubation of SkMC with PA+CM yielded unstructured IMCL accumulation dispersed throughout myotubes. Additionally, SkMC FA oxidation was found to be impaired as a consequence of CM- and FA-treatment, with the most profound defects induced by combination of PA+CM.

In vivo data obtained from lifestyle intervention studies, involving enhanced physical activity, have indicated the potential of SkMC recovery from insulin resistance. In contrast to that, *in vitro* studies have demonstrated retained impairments of insulin signaling. Therefore, CM-treatment was used to investigate the reversibility of muscle insulin resistance, indicating only partial recovery of SkMC functions. To further unravel the beneficial influences of physical exercise, an *in vitro* model of electrically stimulated skeletal muscle contraction was applied to characterize the impact of muscle contraction in the interplay with adipokines and FA. In this context it was revealed that subjecting SkMC to electrical pulse stimulation (EPS) could prevent impairments of OA+CM-treatment on FA oxidation. Strikingly, this was not the case for PA+CM treatment.

Collectively, data obtained in this thesis indicate a novel role for adipokines in promoting lipotoxic effects of FA, which could not be mimicked by isolated adipokines. Furthermore, muscle cell functionality was found to be persistently impaired in response to saturated but not unsaturated FA. Finally, EPS-treatment was identified as a valuable *in vitro* exercise model, revealing the selective potential of muscle contraction to prevent lipotoxicity-induced cellular defects. These findings contribute to the understanding of the molecular interplay of adipokines, FA, and muscle contraction in the pathogenesis of obesity as well as the transition to T2D and may help to develop improved or novel therapeutic strategies to combat T2D and its associated complications.

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List of Abbreviations

ACC	Acetyl-CoA carboxylase
ADA	American Diabetes Association
ADRP	Adipocyte differentiation-related protein
AICAR	5-Aminoimidazole-4-carboxamide riboside
AMPK	AMP-activated protein kinase
AMP	Adenosine monophosphate
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
CVD	Cardiovascular disease
CM	Adipocyte-conditioned medium
CPT-1	Carnitine palmitoyltransferase-1
DAG	Diacylglycerol
EPS	Electrical pulse stimulation
EERα	Estrogen-related receptor α
ETC	Electron transport chain
FFA	Free fatty acids
FGF21	Fibroblast growth factor 21
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GSK-3	Glycogen synthase kinase 3
HDL	High-density lipoproteins
HSL	Hormone-sensitive lipase
IDL	Intermediate density lipoproteins
IKKβ	IκB kinase β
IL	Interleukin
IMCL	Intramyocellular lipid
IR	Insulin receptor
IRS	Insulin receptor substrate

JNK	c-Jun N-terminal kinase
LADA	Latent autoimmune diabetes of adults
LDL	Low-density lipoproteins
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MEF2	Myocyte enhancer factor-2
MODY	Maturity onset diabetes of the young
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa B
NRF	Nuclear respiratory factor
OA	Oleic acid
OGTT	Oral glucose tolerance test
PA	Palmitic acid
PAI-1	Plasminogen activator inhibitor-1
PDK-1	Phosphoinositide-dependent protein kinase
PEDF	Pigment epithelium-derived factor
PGC-1α	PPAR gamma coactivator 1-alpha
PI3K	Phosphatidylinositol 3-kinase
PIP	Phosphatidylinositol phosphate
PKB	Protein Kinase B
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PTEN	Phosphatase and tensin homolog
PTP1B	Protein-tyrosine phosphatase 1B
SH2	Src-homology-2
SHIP2	SH2-containing 5'-inositol phosphatase 2
SkMC	Skeletal muscle cells
SOCS	Suppressor of cytokine signaling
T2D	Type 2 diabetes mellitus
TAG	Triacylglycerols
TCA	Tricarboxylic acid
TNFα	Tumor necrosis factor-alpha
TZD	Thiazolidinediones
VLDL	Very-low-density lipoprotein
WHO	World Health Organization

CHAPTER 1

General Introduction

1.1 Type 2 diabetes and insulin signaling

1.1.1 Pathophysiology of type 2 diabetes

Diabetes is recognized as a group of heterogeneous disorders with the common elements of hyperglycemia and glucose intolerance resulting from defects in either insulin secretion, insulin action, or the combination. Depending on its etiology and clinical presentation diabetes can be mainly subdivided into type 1 and type 2 diabetes. Type 1 diabetes is also known as insulin-dependent, juvenile-onset, or immune-mediated diabetes, since it develops as a consequence of an autoimmune destruction of the insulin-producing β-cells of the pancreas[1]. While it accounts for 5-10 % of all diabetes cases today, the incidence of type 1 diabetes is increasing. The reasons for which are unclear but are likely to be mainly due to changes in environmental risk factors such as increased weight development, increased maternal age at delivery, and possibly also aspects of diet and exposure to certain viral infections that may initiate autoimmunity or accelerate an already ongoing β-cell destruction[2].

Type 2 diabetes (T2D), accounting for >90 % of diabetes cases, is characterized by a combination of resistance to insulin action[3] and an insufficient response of pancreatic β-cell insulin production[4]. Commonly it takes years to decades before the vicious cycle of these two defects culminates in the manifestation of the disease.

However, prior to its manifestation, impaired fasting glucose as well as impaired glucose tolerance have been found to identify individuals at high risk for progression to diabetes[5]. In these individuals, hyperglycemia at levels below the current diagnostic thresholds for diabetes is now recognized to be sufficient to cause functional and pathological changes in various tissues, especially the heart and circulatory system[5-7]. These changes often go undetected since they occur without clinical symptoms. In contrast to type 1 diabetes, patients with T2D do not necessarily depend on insulin supplementation and at least in the beginning plasma glucose levels may be lowered by lifestyle modifications and oral anti-diabetic drugs[8].

The American Diabetes Association (ADA) and the World Health Organisation (WHO) describe several diagnostic criteria for diabetes. These include a fasting plasma glucose (FPG) of ≥ 126 mg/dl, or hyperglycaemia and a plasma glucose of ≥ 200 mg/dl, or plasma glucose levels of ≥ 200 mg/dl two hours after an oral glucose load[9,10]. Today it is recognized that T2D commonly coincides with a range of associated metabolic disorders like obesity, hypertension, and dyslipidemia. These metabolic conditions constitute risk factors for the development of each other and are collectively described as the metabolic syndrome (also known as syndrome X). The WHO defines five criteria, three of which are sufficient to diagnose a patient with the metabolic syndrome. These comprise abdominal obesity defined by a waist circumference > 88 cm in women and > 102 cm in men as well as elevated serum triglycerides (≥ 150 mg/dl) and reduced high density lipoprotein cholesterol levels ≤ 50 mg/dl in women and ≤ 40 mg/dl in men. Further criteria are elevated fasting blood glucose levels of ≥ 110 mg/dl as well as hypertension ≥ 135/88 mmHg. Although T2D patients commonly exhibit a combination of obesity, dyslipidemia, hypertension, and insulin resistance, especially the body mass index (BMI) has been identified to increase the risk of developing T2D in a dose-dependent manner[11-13]. Accordingly, it has been shown that the prevalence of T2D in obese adults is 3-7 times higher compared to normal-weight controls and that morbidly obese patients (BMI ≥35) have a 20 times increased risk to develop T2D than normal-weight controls (BMI between 18.5 and 24.9)[11,14,15]. Furthermore, it has been shown that weight gain during adulthood directly correlates with an increased risk of T2D[12,16-18]. Interestingly, obesity-associated health risk is greater when excess fat is deposited in the abdominal region, indicating that this phenotype is a stronger predictor of cardiovascular disease and T2D than overall BMI[19-22].

While formerly diagnosis of T2D commonly occurred above the age of 45 years[23], recent development provoked an increase of the number of younger cases, especially due to increased incidence of obesity in children. In this context, several studies have demonstrated an increased prevalence of overweight and obesity in children and adolescents, associated with impaired glucose regulation and T2D[24-27]. Interestingly, the risk for children to develop overweight or obesity was found to be related to the socioeconomic status, migration background, or an increased weight of the mothers[26,28]. Furthermore, the incidence of T2D in prospering developing countries is increasing above average. According to the *Diabetes Atlas 2007* of the International Diabetes Federation, 80 % of the 246 million adults (5.9 % of adult population), that have been diagnosed with diabetes (especially T2D) worldwide in 2007, lived in developing countries. While estimates for developed countries only expect a 20 % increase, it has been estimated that the incidence of diabetes in developing countries will increase from 2010 to 2030 by 69 %, leading to an estimated 439 million cases of diabetes worldwide in 2030[29]. While diabetes prevalence is known to be high in Europe as well as North and Central America (e.g. Germany 11.8%, Switzerland 11.2 %, United States 9.2 %, Puerto Rico 12.8 %), it even represents the most common chronic disease in adults in Arabian countries (United Arab Emirates 19.5 %, Saudi Arabia 16.7 % prevalence, respectively).

Poor glycaemic control in the course of T2D as well as its associated comorbidities like hypertension and dyslipidemia can introduce major alterations regarding the vascular system, leading to severe disease complications in later stages including cardiovascular disease, nephropathy, amputation, or neuropathy. These may ultimately result in death due to myocardial infarction, stroke, or renal failure[30]. Hence, adequate glycaemic control is of utmost importance in disease management[31]. While these clinical symptoms develop in the advanced state of the disease, insulin resistance of peripheral tissues like liver, adipose tissue, pancreas, and skeletal muscle is a characteristic hallmark early in the progression of T2D. Since insulin resistance is a fundamental contributor to the development of T2D, much effort has been made in the attempt to resolve the molecular cause of the disease[32]. Especially insulin resistance of skeletal muscle represents a critical determinant, since skeletal muscle constitutes one of the major insulin-sensitive organs responsible for postprandial glucose clearance[33]. Therefore, understanding the multiple mechanisms involved in the disturbance of proper insulin action and hence

the development of skeletal muscle insulin resistance will provide the basis to develop new strategies to improve insulin sensitivity or prevent insulin resistance.

1.1.2 Insulin signaling in skeletal muscle

The healthy human body carefully maintains plasma glucose levels within a limited range. In poorly controlled diabetic patients however, plasma glucose levels are chronically elevated, leading to adverse effects on tissues and organs. Adequate plasma glucose maintenance can only be achieved by precisely balancing glucose absorption, production, uptake, and metabolism. In this context, insulin operates as the key regulatory factor of plasma glucose levels by stimulating glucose uptake in skeletal muscle cells and adipose tissue and inhibiting hepatic gluconeogenesis[34]. Instead, insulin promotes the storage of energy by enhancing glycogen synthesis in liver and skeletal muscle as well as lipogenesis in adipose tissue, while inhibiting lipolysis, glycogenolysis, and protein breakdown. Furthermore, insulin is also known to regulate cell growth and differentiation.

Synthesis of insulin by pancreatic β-cells involves several processing steps[35] before mature insulin, composed of an α-chain and a β-chain, is ultimately secreted by clathrin-coated secretory vesicles. Secretion is achieved by several stimuli, including intestinal hormones and increased plasma glucose levels after a meal. Subsequently, insulin is transported to its target tissues via the circulation. As one of its target tissues, skeletal muscle strongly depends on insulin to enable glucose uptake and ensure sufficient nutrient supply for the cell as well as to clear excess glucose from the circulation after a meal. To this end, insulin stimulates the translocation of glucose transporter (GLUT)4 containing vesicles to the plasma membrane via the phosphatidylinositol 3 kinase (PI3K)/Akt pathway. Translocation of GLUT4-containing vesicles is an essential step of the insulin signaling cascade, as in unstimulated muscle cells over 90 % of GLUT4 is located intracellularly and only 3–10 % reside at the plasma membrane[36]. Additionally, insulin is able to trigger the mitogen activated protein kinase (MAPK) pathway. In order for insulin to induce its effects, a complex signaling network needs to be activated, initiated by binding of insulin to the insulin receptor (IR). The IR belongs to the receptor tyrosine kinase

family and consists of two disulfide-linked heterodimers, each of them containing an α- and a β-subunit, with the β-subunit harboring a tyrosine kinase domain and the α-subunit inhibiting the tyrosine kinase activity under unstimulated conditions. Binding of insulin to the α-subunit initiates a conformational change of the receptor molecule, which results in subsequent abrogation of the allosteric inhibition and activation of the tyrosine kinase. Upon activation, specific tyrosine residues within the catalytic β-subunit are autophosphorylated, resulting in conformational changes that further increase kinase activity. This allows for tyrosine phosphorylation of several downstream proteins including insulin receptor substrate (IRS) proteins 1-4 (Fig. 1.1). Among IRS proteins, IRS1 and IRS2 are the most important regarding insulin-stimulated GLUT4-mediated glucose uptake[37].

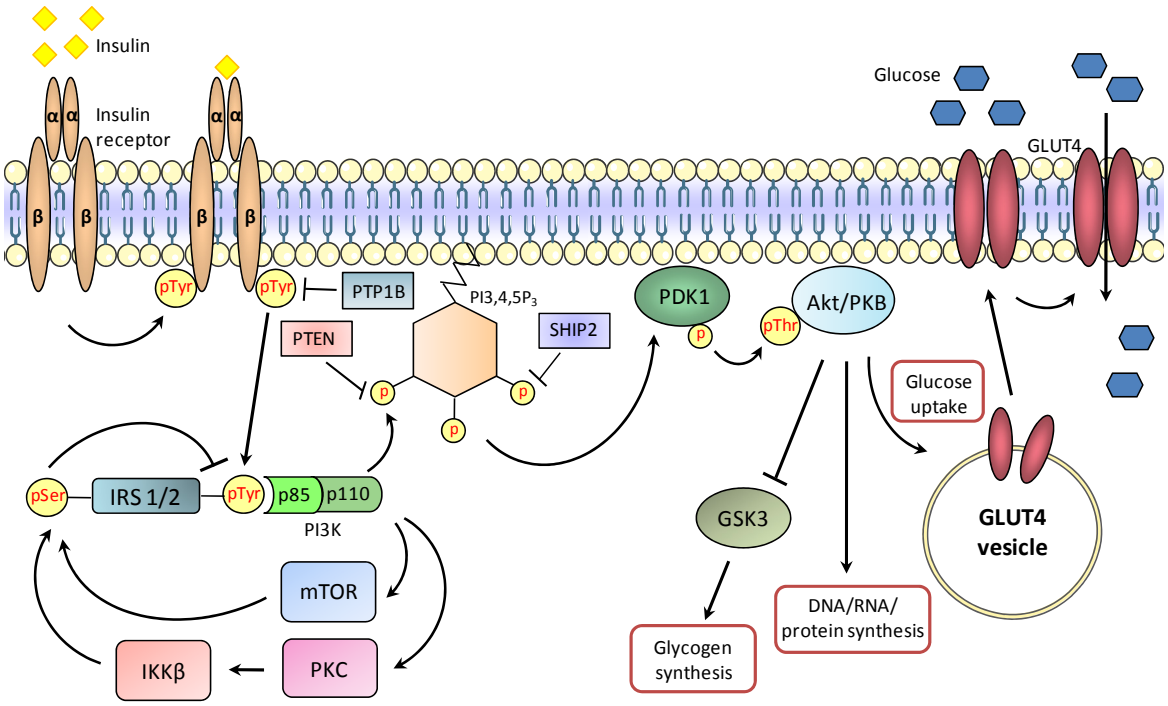

Fig. 1.1 **Simplified overview of the insulin signaling cascade**. Binding of insulin to the insulin receptor initiates a complex signaling cascade. This involves phosphorylation and therefore activation of the insulin receptor, insulin receptor substrate (IRS) proteins, PI3 kinase (PI3K), phosphatidylinositol phosphates (PIP), PI3K-dependent kinase 1 (PDK1), and Akt. Activated Akt subsequently mediates GLUT4 translocation and glucose uptake, glycogen synthesis, as well as DNA/RNA/protein synthesis processes. In a negative feedback loop, mTOR, protein kinase C (PKC) isoforms, and IKKβ mediate serine phosphorylation of IRS, thereby inhibiting insulin signaling.

The phosphorylated tyrosines in the substrate proteins provide binding sites for proteins containing Src-homology-2 (SH2) domains. One such protein is the p85 regulatory subunit of PI3K. PI3K consists of the p85 regulatory subunit, which interacts with the tyrosine-phosphorylated motifs in IRS proteins, and a catalytic p110 subunit. Activated PI3K catalyses the phosphorylation of phosphatidylinositol (4,5) bisphosphate (PI(4,5)P2) leading to the formation of PI(3,4,5)P3. These phospholipids are important second messengers in forwarding the insulin signal as they directly activate PI3K-dependent serine/threonine kinase 1 (PDK1). Since PIP-proteins are restricted to the plasma membrane, this results in recruitment of Akt and PDK1 to the plasma membrane. Translocation to the plasma membrane as well as colocalization with activated PDK1 leads to conformational changes and subsequent phosphorylation of Akt on Thr308[38], resulting in partial activation of Akt. Full activation of Akt, additionally requiring phosphorylation of Ser473 by the rictor-mTOR complex[39], then regulates the activity of many downstream targets, like glycogen synthase kinase 3 (GSK3). Phosphorylation of GSK3 leads to its inactivation and thus initiates glycogen synthesis[35]. However, a key action of insulin is to stimulate glucose uptake into cells by inducing translocation of GLUT4-containing vesicles from intracellular stores to the plasma membrane along the actin cytoskeleton as the final step of the insulin signaling cascade.

As with any adaptive system in physiologically healthy conditions, there also have to be negative feedback mechanisms terminating insulin signaling in order to precisely meet the body's demands. One such mechanism features phosphatases like phosphatase and tensin homologue (PTEN), Src-homology-2 containing inositol 5'-phosphatase 1 and 2 (SHIP1/2), and protein-tyrosine phosphatase 1B (PTP1B), which dephosphorylate critical members of the insulin signaling cascade. While PTP1B directly dephosphorlyates the IR, PTEN and SHIP degrade PI(3,4,5)P3 to PI(4,5)P2 or PI(3,4)P2, respectively. Furthermore, termination of insulin signaling may also be achieved by internalization of the insulin-IR complex into endosomes with subsequent degradation of insulin by insulin degrading enzyme[40]. Additionally, it is becoming apparent that Ser/Thr phosphorylation of IRS proteins has a dual function in serving either as a positive or a negative modulator of insulin signaling, depending on the specific site of phosphorylation[41]. For this purpose phosphorylation sites have been identified that protect activated IRS proteins from phosphatase activity[42], while phosphorylation of other Ser/Thr sites may either induce the dissociation of IRS

proteins from the IR[43,44], inhibit Tyr-phosphorylation sites[45], release the IRS proteins from intracellular complexes that maintain them in close proximity to the IR[46,47], induce degradation of IRS proteins[48,49], or turn IRS proteins into inhibitors of the kinase activity of the IR[50]. As part of the negative-feedback control mechanism resulting in termination of insulin signaling, phosphorylation of these sites can be mediated by insulin-activated proteins like mTOR and PKCζ either directly or through activation of downstream effectors such as IκB kinase β (IKKβ).

The appropriate balance of this delicately orchestrated signaling cascade is crucial for the maintenance of glucose homeostasis and the regulation of carbohydrate, lipid, and protein metabolism. In T2D patients however this tightly regulated balance is disturbed resulting in insulin resistance. Insulin resistance is a defect observed early in the pathogenesis of T2D and describes the inability of insulin to correctly stimulate insulin signaling. Great effort has been made in numerous *in vivo* and *in vitro* studies to identify the underlying mechanisms and causative agents engaged in the development of insulin resistance. Various studies indicate the inter-organ crosstalk between skeletal muscle and the increased adipose tissue mass often associated with T2D to be involved. Furthermore, several studies have suggested a role for lipid-derived factors in the pathophysiology of insulin resistance. Understanding the molecular mechanisms of insulin resistance as one of the fundamental defects in T2D would help to prevent, reverse, or treat T2D.

1.2 Disturbing influences leading to skeletal muscle insulin resistance

1.2.1 Skeletal muscle insulin resistance – a critical determinant of type 2 diabetes

The term insulin resistance describes a condition in which insulin becomes increasingly ineffective in lowering blood glucose levels due to a failure of insulin target tissues to adequately respond to the circulating insulin levels. In an attempt to

compensate for this ineffectiveness, pancreatic β-cells secrete increasing amounts of insulin up to a point where β-cells cannot cope with this increased demand anymore and perish. Insulin resistance in muscle and fat cells reduces glucose uptake, whereas insulin resistance in liver cells results in reduced glycogen synthesis and storage and a failure to suppress glucose production and release into the blood. As a consequence blood glucose levels rise above the normal range, leading to the development of T2D. Insulin resistance of skeletal muscle is especially critical, since this is the tissue mainly responsible for postprandial glucose clearance from the circulation[33]. However, impairments in this insulin-mediated postprandial glucose uptake and utilization are central defects in the majority of T2D patients[51-53], which are often present even years before T2D is diagnosed.

In an effort to improve whole-body glucose homeostasis, intense interest has been focused on understanding the molecular basis for the development of insulin resistance in skeletal muscle[54]. After several studies have demonstrated an impaired action of insulin on skeletal muscle glucose transport in T2D patients[55-58], various studies were aimed to unravel whether this reduction results from alterations in the insulin signal transduction pathway[58-64] or from alterations in either traffic or translocation of GLUT4 to the plasma membrane[65,66]. In this context, several studies have demonstrated reduced IR-kinase activity in skeletal muscle from non-obese T2D subjects[62,67,68], as well as defects in PI3K activity and insulin-stimulated IRS-1 tyrosine phosphorylation[60,64,69] in lean to moderately obese T2D subjects, that were however not related to changes in IRS-1 protein content[60,70]. Studies investigating the involvement of Akt as an intermediate step of the insulin signaling cascade have however yielded conflicting data. While some studies demonstrated reduced insulin-stimulated Akt phosphorylation[58,71], others observed no differences in phosphorylation or enzymatic activity of Akt between controls and T2D subjects[69,72]. These diverging results may be attributed to isoform-specific functions of the 3 different Akt isoforms. While it has been reported that the activities of Akt2 and Akt3 are decreased in skeletal muscle biopsies from insulin-resistant morbidly obese subjects, this was not the case for Akt1[73]. Furthermore, isoform-specific alterations of Akt phosphorylation in response to insulin have been found in myotubes of T2D patients, with decreased Ser473 phosphorylation on Akt2 and decreased Thr308 phosphorylation on Akt1[74]. Additionally, impaired activation of atypical PKC isoforms

have been shown to also contribute to skeletal muscle insulin resistance in subjects with impaired glucose tolerance and T2D[75,76].

As the final biological event of the insulin signaling cascade, proper translocation of GLUT4 containing vesicles to the plasma membrane is essential for adequate insulin action. However, defects in the GLUT4 translocation machinery could also contribute to impaired insulin-stimulated, whole-body glucose uptake[65,66,77]. Indeed, GLUT4 protein expression was found to be reduced in skeletal muscle of morbidly obese subjects[78], while in lean T2D patients total GLUT4 protein expression was not altered[79,80]. In these cases reduced glucose uptake may be explained by impaired function or distribution of GLUT4 or failure of GLUT4 vesicles to translocate, dock, or correctly fuse with the plasma membrane. In this context, abnormal subcellular localization of GLUT4 in skeletal muscle from insulin-resistant subjects without or with overt T2D[65] as well as reduced insulin-stimulated GLUT4 translocation in muscle from T2D patients[77] have been demonstrated. Thus, it has been shown that in insulin-resistant muscles the insulin signaling cascade may display multiple defects at different steps of the cascade ranging from early and intermediate steps to final GLUT4 trafficking. However, the chronological course and causative interrelation of events as well as possible external influences inducing these defects are only incompletely understood and are still frequently subject of investigation.

1.2.2 Impact of obesity on insulin resistance

Development of adipose tissue is a life-long process, constantly influenced by several factors like nutrient intake, gender, or hormones. Traditionally, the function of adipose tissue was thought to be restricted to store energy in the form of triglycerides during states of excess energy, and provide the body with energy in the form of free fatty acids (FFA) in periods of energy shortage[81]. However, recently it has become evident that adipose tissue also has to be regarded as a secretory organ capable of producing various cytokines, collectively referred to as adipokines, which may act locally as well as systemically[81-84]. A successive lifestyle change in our today's society, comprising a persistent oversupply of energy accompanied by a decreased routine demand for physical activity, has promoted the development of obesity. Consequently, the prevalence of obesity has reached epidemic proportions in both

developed and developing countries. This is especially critical since obesity has been recognized as a worldwide serious threat to public health[85] by substantially increasing the risk for morbidity from numerous chronic disorders, such as T2D[86,87], hypertension[88,89], and cardiovascular disease[90,91].

The strong correlation of obesity with the development of T2D has been demonstrated in many studies conducted in humans and various animal models[92-94]. Specifically the link between obesity and insulin resistance is now well recognized[95]. Besides mere expansion of adipose tissue, it has also been shown that the different adipose tissue depots as well as adipocyte biology play a critical role. In this context, especially adipose tissue of the visceral region seems to be associated with insulin resistance and also constitutes a risk factor for cardiovascular disease[96,97]. A study conducted in rats showed that removal of visceral fat but not subcutaneous fat improved insulin sensitivity[98]. Similarly, a human study investigating the influence of liposuction in women demonstrated that removing only subcutaneous adipose tissue, did not ameliorate insulin sensitivity[99]. While the importance of especially the visceral fat depot as a critical determinant has already been acknowledged by the fact that one of the five WHO criteria for metabolic syndrome is the waist circumference, recent studies even propose waist circumference as a more precise risk indicator for T2D and cardiovascular disease than general obesity[85,100-102]. As men commonly display adipose tissue expansion predominantly in the abdominal and women in the gluteal and femoral regions[103], these gender-associated differences of fat depot expansion predispose men to dyslipidemia, hyperglycemia, and hyperinsulinemia[104]. As these gender-specific differences seem to be at least partially mediated by sex steroids, fat distribution patterns shift towards a more male-like pattern in post-menopausal women. As a result, the differences are compensated and these women display more metabolic similarities to fat mass-matched men, which further demonstrates fat depot-specific contribution to metabolic dysfunction[105]. Furthermore, it has been shown that T2D patients display a significant higher percentage of visceral adipose tissue than carefully matched non-diabetic controls[106]. While the critical role of visceral adipose tissue in T2D and especially insulin resistance is now undisputed, the exact molecular mechanisms leading to impaired insulin sensitivity are still only incompletely understood.

Besides localization, abnormal development of adipose tissue has also been demonstrated to play a crucial role in the progression of insulin resistance. An excess

of energy, resulting from an imbalance between energy intake and expenditure, leads to enlargement of adipose tissue. If this state of excess energy persists, it poses an increased burden on the lipid storage and processing capacities of adipocytes that may finally exceed the capacities and induce adipocyte dysfunction including impaired mitochondrial function, increased fatty acid release, altered adipokine secretion, or signaling[107,108]. These defects may in turn contribute to the development of insulin resistance through multiple routes.

On the one hand these alterations may contribute to a chronic state of low grade inflammation[109,110] comprising an increased release of pro-inflammatory cytokines like tumor necrosis factor α (TNFα) and monocyte chemotactic protein-1 (MCP-1), which promote the recruitment of immune cells such as macrophages into the adipose tissue. Macrophages in turn are able to secrete numerous paracrine and autocrine factors and they have been shown to contribute extensively to adipose tissue remodeling[111,112]. Furthermore, increased abundance of pro-inflammatory cytokines like TNFα has been shown to directly interfere with insulin signaling, culminating in the degradation of IRS[113,114]. Additionally, inflammatory signaling including c-Jun N-terminal kinase (JNK) and IKKβ pathways may be activated[115,116], resulting in negative regulation of insulin signaling via serine phosphorylation of IRS-1[117,118].

Besides its contribution to the pro-inflammatory state and the associated disturbances of insulin signaling, the altered adipocyte secretion pattern also comprises several adipokines that have been shown to be regulated by obesity and are known to influence insulin action in peripheral tissues like muscle and liver, as well as in adipose tissue itself. Most of the adipokines are significantly increased in obesity, like leptin[119], plasminogen activator inhibitor-1 (PAI-1)[120], and resistin[121], each disturbing proper insulin action, while secretion of a small number of adipokines with beneficial influences, like adiponectin[122], is decreased in obesity. Although a number of adipokines have been identified and their disturbing mechanisms thoroughly studied for years or decades, modern and highly sensitive proteomic analyses have recently demonstrated the complexity of the adipocyte secretome comprising hundreds of different peptides and are identifying an ever growing number of adipokines[123-126].

The correlation between adipocyte biology and T2D is also further underlined by the fact that the thiazolidinediones (TZD), a large class of (former) anti-diabetic drugs

like troglitazone and rosiglitazone, are agonists of the transcription factor peroxisome proliferator-activated receptor (PPAR) γ, possibly the most important regulator of adipogenesis[127] that is also involved in glucose and lipid homeostasis. Activation of PPARγ has been shown to result in adipose tissue remodeling involving the production of more adipocytes of smaller average size[128,129], while obesity is commonly characterized by bigger adipocytes. Smaller adipocytes are generally believed to be more insulin sensitive and to have lower rates of lipolysis, while bigger adipocytes, especially those in visceral depots[104], are highly lipolytic and thereby contribute to FFA release into the circulation. Additionally, it has been shown that large adipocytes secrete less adiponectin and higher amounts of pro-inflammatory cytokines such as TNFα[130].

Furthermore, obesity-associated adipocyte dysfunction and adipocyte insulin resistance have been shown to lead to an increased release of fatty acids into the circulation. Under normal conditions, fatty acids are taken up by adipose tissue from the blood in the postprandial phase after hydrolysis of triacylglycerols (TAG) by lipoprotein lipase (LPL) and stored as TAG in intracellular depots for times when energy supply is limited. In order to mobilize this energy reserve, hormone sensitive lipase (HSL) as well as adipose triglyceride lipase (ATGL) are necessary for proper hydrolysis of TAG[131]. In this context, insulin plays a central role as regulator of adipocyte fat content, as it promotes fatty acid uptake by activating LPL and suppresses lipolysis by inhibiting HSL[81]. However, in obesity-associated adipocyte insulin resistance these actions of insulin are impaired and fatty acids are released into the circulation by adipose tissue even in the fed state[132].

Taken together, an increased adipose tissue mass is able to contribute to the development of insulin resistance by promoting a constant state of low grade inflammation via secretion of pro-inflammatory cytokines as well as other adverse adipokines, both ultimately resulting in disturbances of the insulin signaling cascade. Additionally, insulin-resistant adipose tissue releases increased amounts of fatty acids into the circulation, further exacerbating an already adverse situation in obesity, as lipids and lipid derivatives have also been shown to impair insulin action in peripheral tissues.

1.2.3 Role of free fatty acids and lipid-derived factors in skeletal muscle insulin resistance

Fatty acids are essential to provide and store energy for the body. As energy-dense molecules they provide nine calories per gram, while proteins and carbohydrates only contain four calories per gram. Since their metabolism yields large quantities of ATP, many tissues, particularly heart and skeletal muscle, rely on either fatty acids or glucose as their source for energy. Besides metabolic fuel, the body further needs fatty acids for membrane components, precursors of lipid mediators such as hormones or intracellular messengers, regulators of ion channels, modulators of gene expression, and various post-translational modifications of proteins (e.g. palmitoylation)[133-135].

Most fatty acids are ingested in the form of TAG. In an average diet, approximately 95 % of dietary lipids are TAG, mainly composed of long-chain fatty acids with an aliphatic tail of more than 12 carbons, while the remaining dietary lipids are phospholipids (4.5 %) and sterols[133]. As TAG are not able to cross cellular membranes, they have to be hydrolyzed by pancreatic lipase in the small intestine to form glycerol and free fatty acids (FFA) in order to be absorbed by the intestinal enterocytes. These cells then reassemble the TAG and combine them with cholesterol, phospholipids, and apolipoproteins to form chylomicrons[108]. Packaged like this, the poorly water-soluble lipids are released into the intercellular space, drained by the lymph system, and travel via circulation to the liver, adipose tissue, or cardiac and skeletal muscle. Besides chylomicrons there are four other species of lipoproteins, which enable lipids to move within the hydrophilic environment of the blood. Very-low-density lipoproteins (VLDL) are formed predominantly by the liver to transport nutrient- or endogenously derived TAG, cholesterol, and cholesteryl esters to extra-hepatic tissues. Special apolipoproteins in the sheath of the chylomicrons and VLDL are able to bind to and activate membrane-bound LPL, primarily at adipose tissue and muscle, where TAG are once again degraded to FFA and glycerol in order to be taken up by these cells. Short chain fatty acids may passively pass through the cell membrane, while an active transport involving different fatty acid transport proteins is suggested for long chain fatty acids[136]. Intracellularly, TAG may be resynthesized and stored in lipid droplets or they may be oxidized to provide energy.

As the lipid load of VLDL is progressively hydrolyzed, these molecules get smaller and denser, turning into intermediate-density lipoproteins (IDL) and further into low-density lipoproteins (LDL). Instead of delivering lipids to peripheral tissues, the primary function of high-density lipoproteins (HDL) is to acquire cholesterol from peripheral tissues and transport it back to the liver to recycle it or convert it into bile salts. Besides in the form of TAG and packaged in lipoproteins, FFA may also occur in the bloodstream merely coupled to albumin. Assessing the amounts of serum FFA, LDL, and HDL is an important tool providing information on the physiological state of a patient. Increased levels of FFA as well as LDL and decreased levels of HDL, as in dyslipidemia, have been described to be associated with a number of pathologies like cardiovascular disease, hypertension, and T2D.

Fatty acids can be divided into different subgroups, e.g. saturated and unsaturated, short-, medium-, and long-chain, as well as essential or nonessential fatty acids. Owing to their different chemical structures and physical properties, each subclass may have different implications for the body. The fatty acid subclass predominately found in Western diets is saturated fatty acid, like stearic acid and palmitic acid. Foods that contain large amounts of saturated fatty acids are dairy products, fatty meats, palm oil, butter, lard, coconut oil, and some processed foods[108]. Consuming a diet rich in saturated fat has been linked to an increased risk of cardiovascular disease (CVD)[137], and this effect is thought to be mediated primarily by an increase in low density lipoprotein (LDL) cholesterol concentrations[138].

Unsaturated fatty acids, like oleic acid or myristoleic acid, are predominantly found in vegetable fats like olive oil or canola oil. As a subgroup of the unsaturated fatty acids, certain polyunsaturated fatty acids like alpha-linolenic acid (an ω-3 fatty acid) and linoleic acid (an ω-6 fatty acid) are known as essential fatty acids, since they cannot be synthesized by the human body and need to be taken up from foods like fish, soya oil, canola oil, pumpkin seeds, sunflower seeds, or walnuts. In the body, essential fatty acids serve multiple functions and play a role in a variety of signaling pathways, especially those involving inflammation or the central nervous system. It is suggested that a high ω-3: ω-6 ratio is most favorable[139]. Clinical data have shown that substituting dietary saturated fatty acids with polyunsaturated fatty acids results in a reduced incidence of CVD[138,140,141]. The basis for this well-documented relationship between dietary fat quality and CVD may be found in differential capacity of saturated, mono- and polyunsaturated fatty acids to modify

plasma LDL-cholesterol, blood pressure, cardiac function, endothelial function, and vascular reactivity as well as different influences on platelet aggregation and inflammation[142,143].

Besides the correlation with CVD, clinical studies have also suggested a link between dietary fat quality and insulin sensitivity[143]. In this context, epidemiological studies report a direct association of saturated fatty acids and impaired insulin sensitivity[144]. In contrast to that, higher intake of monounsaturated and ω-3 polyunsaturated fatty acids clearly improve insulin action[145]. Furthermore, intervention studies in healthy subjects[146] or diabetic patients and obese/non-obese people[147] have revealed that moderate substitution of saturated fat with unsaturated fat is able to significantly improve insulin sensitivity[144]. As insulin resistance is one of the most important pathophysiological traits leading to T2D, it is clinically relevant to investigate whether the quality of dietary fat also influences the risk for T2D. A number of prospective studies have indeed demonstrated a positive association between the consumption of saturated/animal fat and incidence of T2D, while an inverse association has been found for unsaturated/vegetable fats[144,148-150]. Several potential mechanisms how dietary fat quality may influence insulin resistance and development of T2D are being discussed. Fatty acids may differentially affect inflammatory pathways[151] that are tightly related to impaired insulin sensitivity, such as toll-like receptor 4 signaling, or they might change the composition of lipids in the cell membrane. In this context, fatty acids can modify membrane function by changing overall membrane fluidity, affecting membrane thickness/volume, modifying lipid phase properties, inducing changes in the membrane environment, or by interactions of specific lipid components with membrane proteins[143,152,153]. Furthermore, a diet generally rich in lipids with concomitant low energy expenditure increases serum lipid levels, which may contribute to impaired insulin sensitivity by eliciting secondary adverse effects.

Together with hyperglycemia, elevated plasma triglycerides are a criterion for the metabolic syndrome, suggesting a close association. Indeed, already more than 50 years ago Biermann *et al* observed elevated plasma FFA levels in patients with uncontrolled T2D[154], an observation that has subsequently been extended to non-diabetic but insulin-resistant humans, such as obese patients and first-degree relatives of T2D patients[155,156]. Generally plasma FFA concentrations vary in over-night fasted humans between 0.1 and 0.5 mmol/l, primarily due to different rates of

lipolysis, however concentrations may increase in overt T2D patients to 0.7-0.9 mmol/l[156]. Increased plasma FFA levels in T2D patients may result from an imbalance between nutrient intake and energy expenditure resulting in excess amounts of available lipids. Initially adipose tissue undergoes hyperplasia to increase storage capacity of intracellular lipid droplets[108], however ultimately the lipid storage and processing capacities of adipocytes may be exhausted, resulting in adipocyte dysfunction. Besides the production and release of altered adipokines as well as contribution to chronic low grade inflammation by cell death and release of pro-inflammatory cytokines, adipocyte dysfunction also comprises decreased TAG synthesis and increased lipolysis, consequently resulting in systemic release of FFA[108]. Additionally, insulin resistance of adipose tissue results in impaired uptake and untimely lipolysis of TAG by adipocytes in the postprandial state, thereby further contributing to elevated circulating FFA levels. When adipose tissue lipid stores are exhausted, excess FFA are redistributed to non-adipose tissues such as liver, pancreas, cardiac and skeletal muscle, accumulating as ectopic lipid stores[157]. However, these non-adipose tissues are not designed for long-term lipid storage and many studies have demonstrated a variety of cellular impairments associated with ectopic lipid accumulation[158]. In this context, intrahepatocellular lipid accumulation has been positively correlated with BMI, percent body fat, and central obesity[159], while a negative correlation has been demonstrated for intrahepatocellular lipid content and whole body as well as hepatic insulin sensitivity, the latter resulting in decreased hepatic glycogen synthesis and impaired suppression of endogenous gluconeogenesis[160,161]. Furthermore, ectopic lipid stores in cardiac muscle cells and pancreatic β-cells have been correlated with impaired cardiac performance[162,163] as well as cardiomyocyte[164] and β-cell[165] insulin resistance, respectively. However, the impact of ectopic lipid accumulation on skeletal muscle function is especially relevant when considering the essential function of skeletal muscle as an insulin-responsive organ for whole body glucose homeostasis.

While several studies have demonstrated that the amount of intramyocellular lipids (IMCL) is positively correlated with BMI, waist-to-hip ratio, central adiposity, and percent body fat[166-169], others have shown a direct association between IMCL accumulation and skeletal muscle insulin resistance[170,171]. While Randle *et al* have suggested a direct inhibitory effect of fatty acids on cellular glucose consumption already almost 50 years ago[172], subsequent lipid infusion studies have suggested that

not the fatty acids themselves elicit insulin resistance. Instead IMCL may be metabolized to lipid intermediates like diacylglycerol (DAG) and ceramide, whose intracellular levels have been positively correlated with high IMCL accumulation in obese insulin-resistant subjects[168,173,174]. DAG and ceramide have been shown to activate PKCα, -θ, and -ε isoforms, IKKβ as well as JNK. When these kinases are activated, they are subsequently able to phosphorylate inhibitory serine/threonine residues of the IR and of IRS-1[175], thereby preventing insulin-mediated phosphorylation of tyrosine residues[176] (Fig. 1.2). Additionally, PKC isoforms may

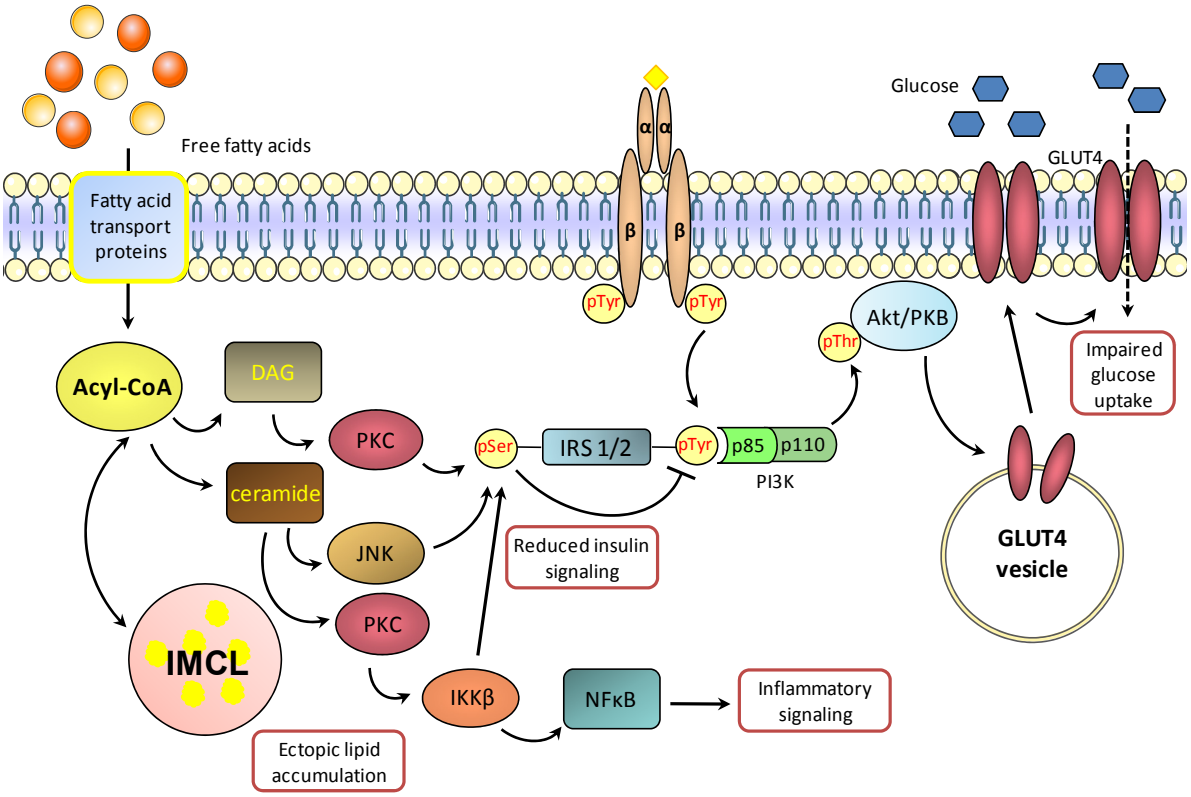

Fig. 1.2 **Schematic summary of lipid-mediated impairments of insulin signaling**. Increased availability and uptake of fatty acids elevates intracellular acyl-CoA levels and elicits either storage of lipids in intramyocellular lipid (IMCL) droplets or metabolization to diacylglycerol (DAG) or ceramides. These then activate c-Jun N-terminal kinase (JNK) and protein kinase C (PKC) isoforms, resulting in activation of pro-inflammatory signaling via activation of nuclear factor κB (NFκB) and increased serine phosphorylation of IRS. This in turn reduces insulin-mediated tyrosine phosphorylation of IRS and ultimately impairs insulin-stimulated glucose uptake.

prevent proper insulin signal transduction by decreasing phosphorylation of PKB/Akt[177,178]. As a consequence, insulin-mediated activation of the IR, IRS-1 (tyrosine phosphorylation), and PKB/Akt is attenuated, impairing insulin signaling and impeding translocation of GLUT4-containing vesicles to the plasma membrane. Hence, insulin mediated glucose uptake may be reduced or even abrogated. Furthermore, IKKβ is able to activate nuclear factor-κB (NFκB), which in turn regulates the activation of proinflammatory pathways as well as production of proinflammatory cytokines such as TNFα and IL-6[179].

Even though this association of obesity, IMCL accumulation, DAG/ceramide levels, and disruption of insulin signaling has been well established, studies conducted in trained individuals have demonstrated functional insulin signaling despite IMCL accumulation[180]. These observations, known as the athlete's paradox, suggest that additional mechanisms associated with physical activity seem to play a pivotal role in this complex cascade of interrelated events. Consequently, additional attention should be focused on physical activity and muscle contraction, not only in the context of energy expenditure but also with regard to subcellular modifications.

1.3 Beneficial influences of physical exercise on skeletal muscle insulin sensitivity

Until the last century, physical activity belonged to and was necessary for daily routine. However, technological advances have introduced modernization of e.g. transportation, communication, and food provision, simplifying everyday life and reducing the daily demand of physical labor. Besides decreased physical activity, these changes have also introduced effortless access to high-energy and processed foods. Today this often leads to a disadvantageous lifestyle of imbalanced nutrient intake and energy expenditure, frequently resulting in obesity, insulin resistance, and T2D, as described in the previous sections. In addition to T2D[181], it is well established that physical inactivity also increases the risk for other diseases like CVD[182], colon cancer[183], and postmenopausal breast cancer[184]. Furthermore, recent evidence suggests that physical exercise also has an impact on cognitive performance[185]. In

this context, studies in middle aged and older adults have demonstrated that physical activity appears to protect against dementia or cognitive decline [185-187].

In newly diagnosed T2D patients, the first therapeutic strategy commonly involves a lifestyle intervention, comprising weight management, dietary modification, and an increase of physical activity. Several studies have demonstrated lifestyle modifications to be an effective way to nearly normalize impaired glucose metabolism[181,188-190]. Additionally, these studies identified a protective effect of increased physical activity by delaying or even preventing the development of T2D. This protective effect was found to be strongest for individuals at high risk of developing T2D[181,189,191]. Further studies have demonstrated a positive association between physical activity and insulin sensitivity without accompanying dietary modifications. While insulin-mediated glucose uptake was shown to be increased in the immediate post-exercise period in rodents[192], strong reduction of physical activity in healthy men has been revealed to reduce peripheral insulin sensitivity within two weeks[193,194].

Physical activity may exert its positive effects involving different mechanisms. One the one hand, acute exercise is a associated with increased glucose uptake in skeletal muscle[192]. It has been revealed that this contraction-mediated increased glucose disposal is mediated via an insulin-independent pathway. In this pathway, exercise-associated muscle contraction activates AMP-activated kinase (AMPK)[195,196] and increases cytoplasmic calcium concentrations[197,198], which have both been shown to lead to insulin-independent increases in glucose transport. AMPK is a heterotrimeric enzyme complex frequently regarded as an intracellular fuel sensor and a metabolic master switch[199]. Two isoforms have been described for the catalytic α-subunits and the regulatory β-subunits, respectively, while three isoforms have been identified for the γ-subunits, which comprise binding sites for AMP[200]. Due to these biochemical properties, AMPK is able to sense enhancement in the cellular AMP:ATP ratio, which may either evolve by increased ATP consumption, like in muscle contraction, or by impaired ATP production like in hypoxia, glucose deprivation, or ischemia[201]. In response to this deprivation of cellular energy, AMPK is able to regulate catabolic and anabolic processes, like fatty acid oxidation, lipogenesis, protein synthesis, or gluconeogenesis[202,203] in order to maintain sufficient ATP concentrations. AMPK-mediated activation of these catabolic processes results in increased utilization of glucose and fatty acids, thereby

equalizing or even favorably influencing whole body energy balance. Thus, physical exercise may also mediate its positive effects by arresting adipose tissue expansion or even reducing adipose tissue mass. As increased adipose tissue mass has been correlated with an unfavorably altered adipokine profile, improved exercise-associated insulin sensitivity may also be partially attributed to a reduction in adverse adipokines[204-206].

Skeletal muscle displays a high capacity to change its plasticity in response to nutrient availability and contractile activity. Hence it has been reported that physical activity does not only increase energy expenditure, thereby positively influencing energy balance, chronic physical activity also elicits an array of cellular adaptation processes. In this context, PGC-1α is an important player and a frequently studied target as it is regarded as a crucial activator of mitochondrial biogenesis. By binding and coactivating transcription factors, it is able to regulate expression of nuclear genes, encoding mitochondrial proteins and mitochondrial transcription factor A, which regulates mitochondrial DNA transcription[207]. This way, PGC-1α is able to increase the mitochondrial content of skeletal myotubes and consequently also the oxidative capacity of entire skeletal muscles. While several studies have demonstrated a reduced expression of PGC-1α in obesity and T2D[208,209], endurance trained athletes display high PGC-1α levels and concomitantly enhanced fatty acid oxidation capacities. These adaptive changes are suggested to be responsible for the athlete's paradox. This term describes the observation that endurance trained athletes display enhanced accumulation of IMCL, which is usually associated with impaired insulin sensitivity as discussed in the previous section. However, in parallel to IMCL accumulation, endurance trained individuals show increased insulin sensitivity. It is suggested that activation of PGC-1α, stimulating mitochondrial biogenesis and concomitantly augmenting oxidative capacity, has induced fundamental adaptations in response to endurance training. As a consequence, IMCL stores in endurance trained athletes may more efficiently be metabolized to yield energy rather than serving as a source for potentially detrimental lipid metabolites.

Furthermore, a role of physical activity in whole body inflammatory processes has been suggested[210]. In this context it has been discussed that physical inactivity promotes accumulation of especially visceral rather than subcutaneous adipose tissue, thereby exacerbating the detrimental effects associated with visceral fat[211]. Additionally, physical inactivity has also been shown to correlate with chronic

systemic inflammation independent of obesity[212]. Besides atherosclerosis, neurodegeneration, and tumor growth, chronic inflammation also promotes development of insulin resistance[210,211]. Therefore, physical inactivity may also promote progression of these diseases by contributing to a pro-inflammatory milieu.

Additionally, skeletal muscle has been proposed to be considered as an endocrine organ, as it is able to produce and secrete a number of cytokines and other peptides that may exert paracrine and endocrine effects[213]. These so-called myokines include IL-6, IL-8, IL-15[214], and brain derived neurotrophic factor (BDNF)[215], as well as fibroblast growth factor-21 (FGF21)[216] and follistatin-like-1[217]. Due to its substantial (up to 100-fold) increase in circulation during physical exercise, IL-6 has received considerable attention[213]. While chronically elevated IL-6 levels have been associated with obesity and reduced insulin action[213,218], short-term contraction-associated increases in IL-6 levels have been shown to have highly beneficial effects. In this context a number of studies have identified IL-6 as a potent insulin sensitizer and regulator of fat metabolism, able to increasing glucose uptake, activate AMPK, augment lipolysis and enhance fat oxidation[210,211,213,219]. Additionally, IL-6 has also been suggested to exhibit anti-inflammatory properties as it has been demonstrated to stimulate the production of classical anti-inflammatory cytokines such as IL-1ra and IL-10[220]. Besides IL-6, BDNF has also been revealed to increase phosphorylation of AMPK and acetyl-CoA carboxylase (ACC)[210,211], thereby enhancing fat oxidation. Interestingly, BDNF levels were found to be low in individuals with obesity and T2D[221], while BDNF mRNA and protein expression are increased in response to exercise[222]. Furthermore, IL-15 has also been suggested to play a role in lipid metabolism[223], as IL-15 overexpression resulted in a decrease of visceral but not subcutaneous fat mass[224].

In general, increased physical activity represents an efficient way to ameliorate or prevent insulin resistance and T2D, as it is associated with increased glucose uptake in the immediate post-exercise period, while chronic physical activity induces substantial cellular remodeling, rendering the muscle generally more insulin sensitive. Additionally, myokines, secreted upon muscle contraction, may affect muscle signaling pathways involved in glucose uptake and fat oxidation as well as create a systemic anti-inflammatory environment, exerting specific effects on visceral fat. These systemic alterations indentify physical exercise to be a potent regulator of muscle and whole body insulin sensitivity. Therefore, understanding the

precise underlying molecular mechanisms mediating the beneficial effects of muscle contraction would be helpful to prevent or treat T2D.

1.4 Objectives

In light of the persistently increasing number of T2D patients worldwide and considering the expected increase in T2D cases in developing countries in the near future, it is of utmost importance to find ways of how to efficiently combat T2D. As described in the previous sections, profound evidence has established the negative crosstalk between expanded adipose tissue mass and skeletal muscle insulin resistance. Additionally, accumulating data have demonstrated a pivotal role for lipids and lipid-derived mediators in the pathophysiological network of obesity, dyslipidemia, and T2D. However, the underlying mechanisms are only incompletely understood. Comprehending the molecular link between obesity-associated adipokines and elevated plasma FFA levels with the induction of insulin resistance and the progression to T2D is a prerequisite to develop effective therapeutic and prevention strategies. Although physical activity has long been known to be an effective, safe, and commonly cost-efficient measure to ameliorate multiple defects associated with T2D, only recently an increasing number of studies have begun to decipher the biochemical basis for the observed beneficial effects. However, a number of questions has still remained unanswered and studies investigating the combined effects of adipokines, lipids, and physical exercise are scarce. Therefore, the starting point of the present work was to more closely investigate the individual influence of adipokines, fatty acids, and contraction on skeletal muscle cell metabolism and subsequently determine the impact of the combination of these influences.

- A number of studies have investigated the effects of single adipokines such as TNFα, MCP-1, angiotensinogen, and resistin on muscle glucose metabolism. However, recent studies applying highly sensitive proteomic technologies have revealed the adipocyte secretome to be comprised of hundreds of different factors. Therefore, an *in vitro*

model applying adipocyte-conditioned medium (CM) was used to investigate the impact of the entire adipocyte secretome on skeletal muscle lipid metabolism. Additionally, the role of novel adipokines, such as chemerin and PEDF, in the negative crosstalk between adipose tissue and muscle glucose and lipid metabolism was to be assessed.

- Dyslipidemia has been positively correlated with the pathophysiology of chronic diseases such as CVD, and T2D. In this context, elevated plasma FFA levels have been linked to the accumulation of IMCL and development of insulin resistance. In obesity, FFA coincide with an altered adipokine profile, enabling synergistic influences. However, so far only very few studies have analyzed the combined impact of fatty acids and adipokines. To obtain further insight into the combined impact of fatty acids and adipokines, primary human skeletal muscle cells were exposed to a combination of adipocyte-conditioned medium (CM) as well as physiologically healthy concentrations of fatty acids to analyze their combined impact on muscle lipid metabolism.

- On the one hand, lifestyle intervention studies have demonstrated potential recovery from insulin resistance, while on the other hand *in vitro* studies have indicated retained cellular defects. Therefore, the extent and underlying molecular mechanisms of reversibility of insulin resistance were investigated. Although physical exercise has been implicated as a cornerstone of lifestyle intervention therapies, the molecular basis of its beneficial effects have remained incompletely characterized. Hence, an *in vitro* model of primary skeletal muscle cell contraction was established to obtain further insight into the implication of contraction in the interplay with adipokines and fatty acids with regard to lipotoxicity-induced impairments of muscle cell functionality.

CHAPTER 2

Study 1

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

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Running title: Reversibility of muscle insulin resistance

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

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

Abstract

Insulin resistance in skeletal muscle is an early event in the development of diabetes, with obesity being one of the major contributing factors. In vitro, conditioned medium (CM) from differentiated human adipocytes impairs insulin signaling in human skeletal muscle cells, but it is not known if insulin resistance is reversible and which mechanisms may underlie this process. CM induced insulin resistance in human myotubes at the level of insulin-stimulated Akt and GSK3 phosphorylation. In addition, insulin-resistant skeletal muscle cells exhibit enhanced production of reactive oxygen species and ceramide as well as a downregulation of myogenic transcription factors such as myogenin and MyoD. However, insulin resistance was not paralleled by increased apoptosis. Regeneration of myotubes for 24 or 48 h after induction of insulin resistance restored normal insulin signaling. However, the expression level of myogenin could not be reestablished. In addition to decreasing myogenin expression, CM also decreased the release of IL-6 and IL-8 and increased monocyte chemotactic protein-1 (MCP-1) secretion from skeletal muscle cells. Although regeneration of myotubes reestablished normal secretion of IL-6, the release of IL-8 and MCP-1 remained impaired over 48 h after withdrawal of CM. In conclusion, our data show that insulin resistance in skeletal muscle cells is only partially reversible. Although some characteristic features of insulin-resistant myotubes normalize in parallel to insulin signaling after withdrawal of CM, others such as IL-8 and MCP-1 secretion and myogenin expression remain impaired over a longer period. Thus, we propose that the induction of insulin resistance may cause irreversible changes of protein expression and secretion in skeletal muscle cells.

Introduction

Obesity is one of the major risk factors contributing to the development of insulin resistance and type 2 diabetes[1]. In this context, the negative crosstalk between adipose tissue and skeletal muscle is involved in early metabolic disturbances leading to insulin resistance[2,3]. Adipocytes from obese patients have a different secretion pattern as compared to lean donors with the release of pro-inflammatory factors and adipokines being increased[4]. In fact, these adipose-derived molecules might be key contributors to the development of insulin resistance and other diseases such as endothelial dysfunction and atherosclerosis[5]. In vitro, we were able to show that adipocyte-conditioned medium (CM) containing various adipokines induces insulin resistance in skeletal muscle cells[6,7].

The development of insulin resistance is a reversible process. Reduction of adipose tissue mass by weight loss is a validated approach to reverse insulin resistance[8,9]. In parallel to improved insulin sensitivity, weight reduction also normalizes adipokine blood level which has been demonstrated for IL-6[10], high molecular weight adiponectin[11], monocyte chemotactic protein-1 (MCP-1)[12] and TNFα[13]. It could be shown that insulin resistance disappears in cultured skeletal muscle biopsies from obese patients[14,15] demonstrating that insulin resistance might be a reversible feature that can be acquired with obesity. However, other studies in muscle biopsies from obese and diabetic patients demonstrated that insulin resistance is retained in culture[14,16,17]. This study was aimed at analyzing reversibility of adipocyte-induced insulin resistance in skeletal muscle cells and underlying mechanisms.

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 by Sigma (München, Germany). Polyclonal antibodies anti-phospho GSK-3α/β ($Ser^{21/9}$), anti-phospho-Akt (Ser^{473}), and anti-glucose transporter 4 (GLUT4) were supplied by Cell Signaling Technology (Frankfurt, Germany) and anti-tubulin by Calbiochem (Darmstadt, Germany). Antibodies for myogenin came from Acris (Hiddenhausen, Germany), for MyoD from Imgenex (San

Diego, CA), and the one for myosin heavy chain (MHC) from Upstate (San Diego, CA). Horse radish peroxidase (HRP)-conjugated goat-anti-rabbit and goat-anti-mouse IgG antibodies were purchased from Promega (Mannheim, Germany). Collagenase CLS type 1 was obtained from Worthington (Freehold, NJ), and culture media were obtained from Gibco (Berlin, Germany). Primary human skeletal muscle cells and supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

Culture of human skeletal muscle cells. Primary human skeletal muscle cells of four healthy Caucasian donors (2 males, 9 and 47 y; 2 females, 10 and 48 y) were supplied as proliferating myoblasts (5 x 10^5 cells) and cultured as described previously[6]. 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 and used for experiments.

Adipocyte isolation and culture. Adipose tissue samples were obtained from the mammary fat of normal or moderately overweight women (BMI 24.5 ± 0.9, aged between 23 and 41 yr) undergoing surgical mammary reduction. The procedure to obtain adipose tissue was approved by the ethical committee of Heinrich-Heine-University, Duesseldorf, Germany. All subjects were healthy, free of medication, and had no evidence of diabetes according to routine laboratory tests. Adipose tissue samples were dissected from other tissues and minced in pieces of ~10 mg in weight. Preadipocytes were isolated by collagenase digestion as previously described[18]. Isolated cell pellets were resuspended in Dulbecco's modified Eagle's/Ham's F-12 medium supplemented with 10% FBS, seeded on membrane inserts (3.5 x 10^5/4.3 cm^2) or in a six-well culture dish, and kept in culture for 16 h. After washing, culture was continued in an adipocyte differentiation medium (DMEM-F-12, 33 µM biotin, 17 µM D-pantothenic acid, 66 nM insulin, 1 nM triiodo-L-thyronin, 100 nM cortisol, 10 µg/ml apo-transferrin, 50 µg/µl gentamycin, 15 mM HEPES, 14 mM $NaHCO_3$, pH 7.4). After 15 days, 60-80 % of seeded preadipocytes developed to differentiated adipose cells, as defined by cytoplasm completely filled with small or large lipid

droplets. These cells were then used for generation of CM, as previously described by us[19]. Briefly, after *in vitro* differentiation, adipocytes were incubated for 48 h in skeletal muscle cell differentiation medium. This CM was then harvested, centrifuged to remove any cell debris, and immediately frozen in aliquots for future use. CM from 350.000 adipocytes was used to stimulate one cavity in a six-well plate of skeletal muscle cells. In control experiments, skeletal muscle cell differentiation medium was incubated for 48 h without adipocytes and tested upon its effect on skeletal muscle. No difference in insulin signaling could be found using this medium compared to fresh skeletal muscle cell differentiation medium (data not shown).

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

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

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

dichlorodihydrofluorescein diacetate incubation. For measurement of nitric oxide (NO), skeletal muscle cells were incubated with 10 µM 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF) (Molecular Probes) solved in phenol red-free DMEM for 30 min. As a positive control for NO production, cells were also treated with 500 µM SNAP (Calbiochem, Darmstadt, Germany) for 30 min in parallel with to DAF. Afterwards, cells were lysed in the above-mentioned lysis buffer and fluorescence measured using an excitation wavelength of 595 nm on a Fluostar-P (SLT, Salzburg, Austria).

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

Measurement of apoptosis. Apoptosis was monitored by assessment of caspase-3 activity and nuclear fragmentation in skeletal muscle cells treated with CM. The DEVD-cleaving activity of the caspase-3 class of cystein proteases was determined in cell lysates using Ac-DEVD-AMC (BD Biosciences, Heidelberg, Germany) as fluorogenic substrate according to the manufacturer's protocol. The ability of cell lysates to cleave the specific caspase-3 substrate was quantified by spectrofluorometry using an excitation wavelength of 390 nm and an emission wavelength of 460 nm with a microplate reader. For detection of nuclear fragmentation, the cells were double-stained with Hoechst 33342 and propidium iodide. Skeletal muscle cells were washed twice with PBS and were stained with 10 µg/ml Hoechst 33342 and 1 µg/ml propidium iodide at 37°C for 15 min. Fluorescence was observed under a Leica DM IRB fluorescence microscope. At least 400 cells were counted for each experiment. Cells with condensed or fragmented nuclei were

defined to be apoptotic and cells with normally shaped nuclei were supposed to be viable.

Quantitative evaluation of ceramide. Lipids from skeletal muscle cells were extracted in chloroform/methanol/water (2:1:0.1, vol/vol/vol) for 24 h at 48°C. Lipid extracts were applied to thin layer Silica Gel 60 plates (Merck, Darmstadt, Germany) as described earlier[21]. Ceramides were resolved twice using chloroform-methanol-acetic acid (190:9:1, vol/vol/vol) as developing system. Following development, plates were air-dried, sprayed with 8% (wt/vol) H_3PO_4 containing 10% (wt/vol) $CuSO_4$, and charred at 180°C for 10 min. Lipids were identified by their Rf value, using authentic lipid samples as references. Individual lipid bands obtained by thin-layer chromatography (TLC) were evaluated by photodensitometry (Shimadzu, Kyoto, Japan). Assuming constant cholesterol amounts in all samples, densitometric data obtained for ceramide were normalized to cholesterol.

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

Results

CM-induced insulin resistance of insulin signaling in skeletal muscle cells is a reversible process. CM of differentiated human adipocytes impairs insulin signaling at the level of Akt in human skeletal muscle cells (Fig. 2.1*A*). Insulin-stimulated GSK-3α/β phosphorylation is only slightly decreased by CM treatment, whereas basal phosphorylation is significantly increased, leading to an insignificant insulin effect (Fig. 2.1*B*). Withdrawal of CM for 24 or 48 h reestablishes normal insulin signaling in skeletal muscle cells, with Akt and GSK3α phosphorylation being similar to control and GSK3β phosphorylation being even higher than in the control situation.

Insulin resistance is accompanied by reduced expression of myogenic transcription factors in skeletal muscle cells and an irreversible downregulation of

myogenin. During differentiation, skeletal muscle cells display an increased expression of myogenin, MHC, and myoD, all markers of myogenesis (Fig. 2.2, *A*-*C*). Analysis of myogenic transcription factors revealed that CM-treated skeletal muscle cells have significantly reduced expression of myogenin, MHC, and MyoD (Fig. 2.3, *A*-*C*). Skeletal muscle cells display an increasing GLUT4 level (Figure 2.4*A*, *top*). However, CM-treatment did not affect GLUT4 expression in differentiated myotubes (Fig. 2.4*A*, *bottom*), and the cells exhibited an unaltered morphology as compared to control cells (Fig. 2.4*B*). Withdrawal of CM for 24 or 48 h reverses the downregulation of MHC and MyoD, whereas the expression of myogenin remains decreased over the whole period as compared to control (Fig. 2.3). Thus, in spite of reestablished insulin signaling, skeletal muscle cells do not normalize myogenin expression after CM-treatment and withdrawal.

CM-treated skeletal muscle cells are characterized by a partially irreversible secretory dysfunction. Skeletal muscle cells secrete various myokines, including IL-6, IL-8, and MCP-1. As compared to adipocytes that secrete ~500 pg*ml^{-1}*24 h^{-1} IL-6, skeletal muscle cells exhibit lower secretion of this cytokines with 23 ± 1 pg*ml^{-1}*24 h^{-1} (*n* = 5). Treatment with CM leads to a significantly lower IL-6 secretion during the first 24 h of regeneration of myotubes (Fig. 2.5*A*). Forty-eight hours after CM withdrawal, however, IL-6 secretion is comparable to control cells.
IL-8 secretion is also lower in skeletal muscle cells (94 ± 12 pg*ml^{-1}*24 h^{-1}; *n* = 5) compared to adipocytes (~ 500 pg*ml^{-1}*24 h^{-1}). CM-treated skeletal muscle cells display significantly impaired IL-8 secretion over the whole regeneration period of 48 h when compared to control. This suggests that IL-8 secretion might be irreversibly disturbed in insulin-resistant myocytes (Fig. 2.5*B*).
MCP-1 is a cytokine robustly released from human adipocytes (~3 ng*ml^{-1}*24 h^{-1}) but also secreted at low levels from myotubes (37 ± 11 pg*ml^{-1}*24 h^{-1}; *n* = 5). Induction of insulin resistance in skeletal muscle cells significantly stimulates MCP-1 secretion after 24 h of regeneration with an additional increase after 48 h (Fig. 2.5*C*).

Insulin-resistant skeletal muscle cells exhibit increased oxidative stress and decreased mitochondrial capacity but no apoptosis. ROS and NO are both potential players in the induction of insulin resistance. As presented in Fig. 2.6, a significant increase in both ROS and NO production was observed in skeletal muscle cells

treated with CM. SDH activity was measured in whole cell lysates of skeletal muscle cells to assess oxidative capacity. CM treatment slightly but significantly reduced SDH activity in whole cell lysates after 24 h (Fig. 2.7). Longer incubation with CM over 96 h further reduced the level of SDH activity. The parallel induction of insulin resistance and oxidative stress cannot, however, be assigned to apoptosis in skeletal muscle cells. Measurement of caspase-3 activity revealed no increase in CM-treated cells as compared to controls [1.08 ± 0.13 vs. 1.06 ± 0.17 arbitrary units, significantly elevated positive control (campthotecin for 5 h) 1.95 ± 0.03 arbitrary units; *n* = 3-4]. Furthermore, nuclear fragmentation was not elevated in CM-treated cells compared to controls (2.6 ± 0.1 % vs. 2.2 ± 0.2 % apoptotic cells, significantly elevated positive control (campthotecin for 5 h) 5.0 ± 1.0 % apoptotic cells; *n* = 3-4)

Insulin-resistant skeletal muscle cells contain higher ceramide levels. Ceramide constitutes a well-known player in insulin resistance. Fatty acids and ceramide can induce insulin resistance in skeletal muscle cells[22,23]. Analysis of lipid extracts by thin layer chromatography revealed a nearly three-fold increase of ceramide content in insulin-resistant skeletal muscle cells as compared to controls (Fig. 2.8).

Discussion

Adipose tissue expansion and increased release of adipokines have been shown to play a crucial role in the induction of insulin resistance[24]. We could demonstrate in several studies that adipocyte-derived factors can induce insulin resistance in skeletal muscle cells *in vitro*[6,7,25]. The data presented here now demonstrate that CM-treated skeletal muscle cells are not only characterized by impaired insulin signaling but also by various other defects. Insulin-resistant skeletal muscle cells downregulate the expression of myogenin and display oxidative stress, lower mitochondrial capacity, and higher ceramide content. Furthermore, insulin-resistant myotubes have disturbed secretion of the myokines IL-6, IL-8, and MCP-1.

In vitro differentiated skeletal muscle cells are characterized by a high abundance of the myogenic transcription factors such as myogenin and MyoD. We demonstrate here for the first time that adipocyte-derived factors lead to a marked downregulation of myogenin in skeletal muscle cells. It is known from the literature that TNFα suppresses the differentiation process in C_2C_{12} myoblasts[26], but nothing is

known about its effect on differentiated cells. However, CM contains very low doses of TNFα (less than 0.02 pmol/l[7]), making it probable that another adipokine with higher concentration in CM might be the culprit for downregulation of myogenin. The loss of myogenin in insulin-resistant skeletal muscle cells is, however, associated with a conservation of skeletal muscle phenotype, as myotubes display normal morphology and GLUT4 expression. However, it cannot be completely ruled out that the downregulation of multiple markers, including MyoD, MHC, and SDH, points to a dedifferentiation of skeletal muscle cells, and it is impossible so far to speculate on the meaning of this finding for the situation in skeletal muscle *in vivo*.

IL-6, IL-8, and MCP-1 are known secretory products from skeletal muscle with different roles in myogenesis, exercise, inflammation, and insulin sensitivity. Increased IL-6 levels are associated with insulin resistance *in vivo*[27], but short-term treatment of skeletal muscle cells with IL-6 can increase insulin sensitivity[28]. The reported increase of IL-6 during exercise[29] makes it likely that IL-6 has completely different acute and chronic effects. As for myogenesis, IL-6 is a promyogenic factor[30] explaining the parallel decrease of myogenic markers and IL-6 secretion in the myotubes. IL-8 and MCP-1 are proinflammatory chemokines that are increased in serum of obese and diabetic patients[31-33]. MCP-1 is a potent inducer of insulin resistance in skeletal muscle cells[25] and plays a role in myopathies[34]. TNFα and INFγ have been described to induce MCP-1 transcription in myoblasts[34]. Although IL-8 secretion is almost completely inhibited in CM-treated skeletal muscle cells, MCP-1 release increases, pointing to an inflammatory effect of CM.

SDH activity is known to be slightly but significantly reduced in skeletal muscle lysates from diabetic patients as compared to controls[35]. We also observe a reduction in SDH activity in CM-treated skeletal muscle cells, indicating a possible role of decreased oxidative capacity in the initiation of skeletal muscle cell insulin resistance. Notably, in diabetic patients, reduced oxidative capacity in parallel with increased glycolytic activity is due to a significant alteration of skeletal muscle fiber composition.

Oxidative stress is a result of increased ROS or NO production and can lead to oxidation and damage of DNA, protein, and lipids[36]. Increasing ROS production as observed in our model could cause damage to mitochondria and so-called mitoptosis and explain the loss of mitochondria observed in states with increased oxidative stress such as insulin resistance and diabetes. Thus, increased ROS or NO levels could

also explain decreased SDH activity in insulin-resistant skeletal muscle cells. Other work in L6 muscle cells shows that palmitate-induced insulin resistance is also characterized by higher levels of ROS and NO[37]. However, it should be noted that fatty acids are barely detectable in CM when an HPLC approach is used (data not shown). Therefore, we conclude that adipocyte-derived factors produce an increase in ROS and NO similar to that produced by fatty acids.

NO and inducible NO synthase (iNOS) are known to be increased in the diabetic state and are linked to chronic inflammation[38]. However, it is not known how NO induces or exacerbates insulin resistance. In C2C12 skeletal muscle cells, the NO donor SNAP inhibits Akt activity, making it possible that an intracellular increase in skeletal muscle cell NO might contribute to insulin resistance[39]. Furthermore, diabetic patients are characterized by higher blood levels of nitrates and nitrites as well as higher expression of iNOS in skeletal muscle[40]. In our primary myotubes we also observed an increase in NO production after treatment with CM, which might, together with ROS, contribute to the development of insulin resistance. It should be noted in this context that CM-treated skeletal muscle cells are not apoptotic, as shown by unaltered percentage of cells with nuclear fragmentation and similar caspase-3 activity, compared to controls, so that NO and ROS elevation cannot be attributed to apoptosis.

The sphingolipid ceramide is described to be a possible link between obesity and diabetes. Fatty acids and resulting higher levels of ceramide can induce insulin resistance in skeletal muscle cells[22,23]. In this study, insulin resistant skeletal muscle cells are also characterized by increased ceramide levels, which may contribute to adipokine-induced insulin resistance and illustrate disturbed lipid metabolism.

In this study, we were able to show that adipocyte-induced insulin resistance is a reversible process in skeletal muscle cells, at least at the level of insulin signaling. However, some alterations are not fully reversible and may illustrate longer lasting damage to the myotubes by one-time treatment with CM. Skeletal muscle cells display long-lasting myogenin downregulation and secretory defects of IL-8 and MCP-1. Differentiation of skeletal muscle involves a group of transcription factors, including myogenin and MyoD, that activate muscle-specific gene expression and have each a distinct function during myogenesis[41]. In our model, we observe a loss of myogenin expression with preservation of muscle phenotype. At this point, we cannot evaluate the physiological impact of the loss of myogenin. Our data clearly

shows that the loss of myogenin is unrelated to early steps in insulin signaling, myotube morphology, and GLUT4 expression. Certainly, our model of *in vitro*-differentiated skeletal muscle cells has limitations as to how our findings on downregulation of myogenic markers underlie obesity-related insulin resistance *in vivo*.

Future work should be aimed to relate our findings to the *in vivo* situation in diabetic and obese patients in this respect. In summary, we could demonstrate that adipocyte-derived insulin resistance in skeletal muscle cells impacts on various aspects of skeletal muscle cell physiology. The analysis of mechanisms involved in skeletal muscle insulin resistance and its reversibility might lead to a better understanding of this process and a possible discovery of muscular targets for the treatment of type 2 diabetes.

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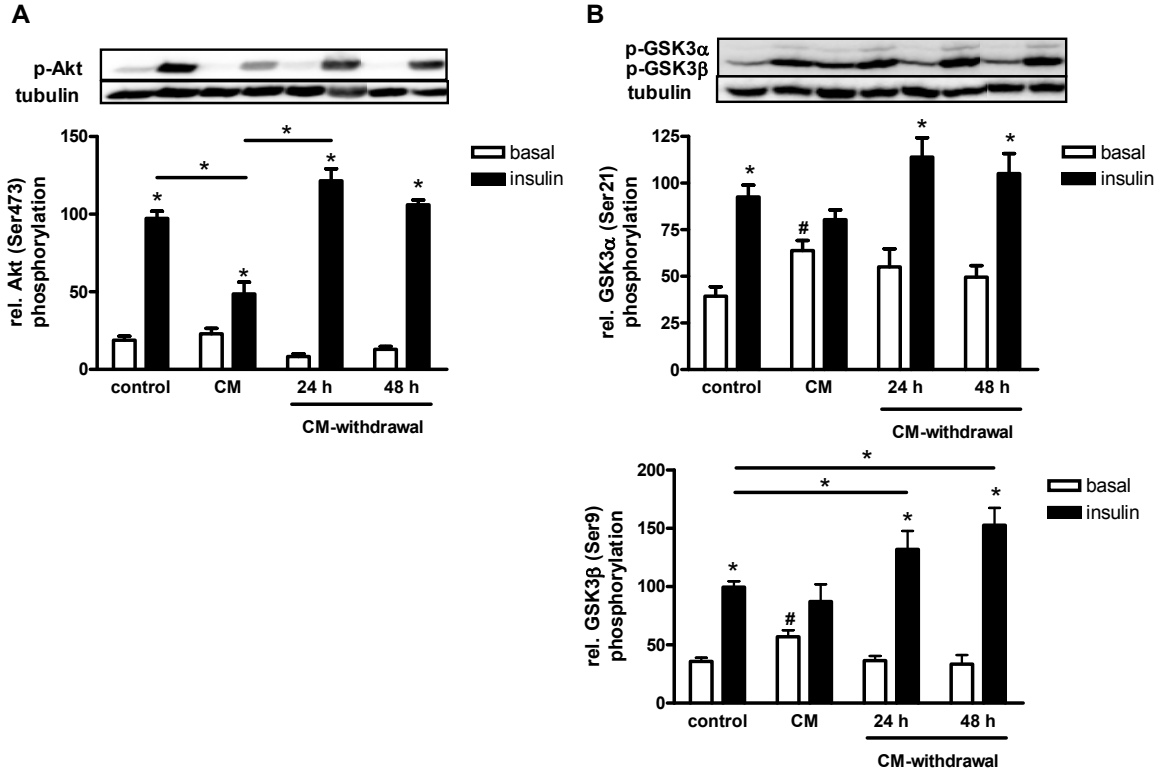

Fig. 2.1 Effect of adipocyte-conditioned medium (CM) on insulin signaling in skeletal muscle cells. Differentiated skeletal muscle cells from 2–3 donors were treated with CM for 24 h and stimulated with insulin (100 nM, 10 min) directly or after regeneration for 24 or 48 h. Five micrograms of total lysates were resolved by SDS-PAGE and blotted to polyvinylidene difluoride membranes. Membranes were blocked with 5% milk in Tris-buffered saline containing 0.1% Tween-20 and incubated overnight with phosphorylated (p)Akt (*A*) or pGSK-3 (*B*) antibodies. After incubation with the appropriate horseradish peroxidase-coupled secondary antibody, the signal was detected by enhanced chemiluminescene. Signals were analyzed on a LUMI Imager work station. Data are tubulin-normalized means ± SE (n=5–6). *Significant insulin stimulation or significantly different from designated insulin-stimulated value. #Significantly different from basal control.

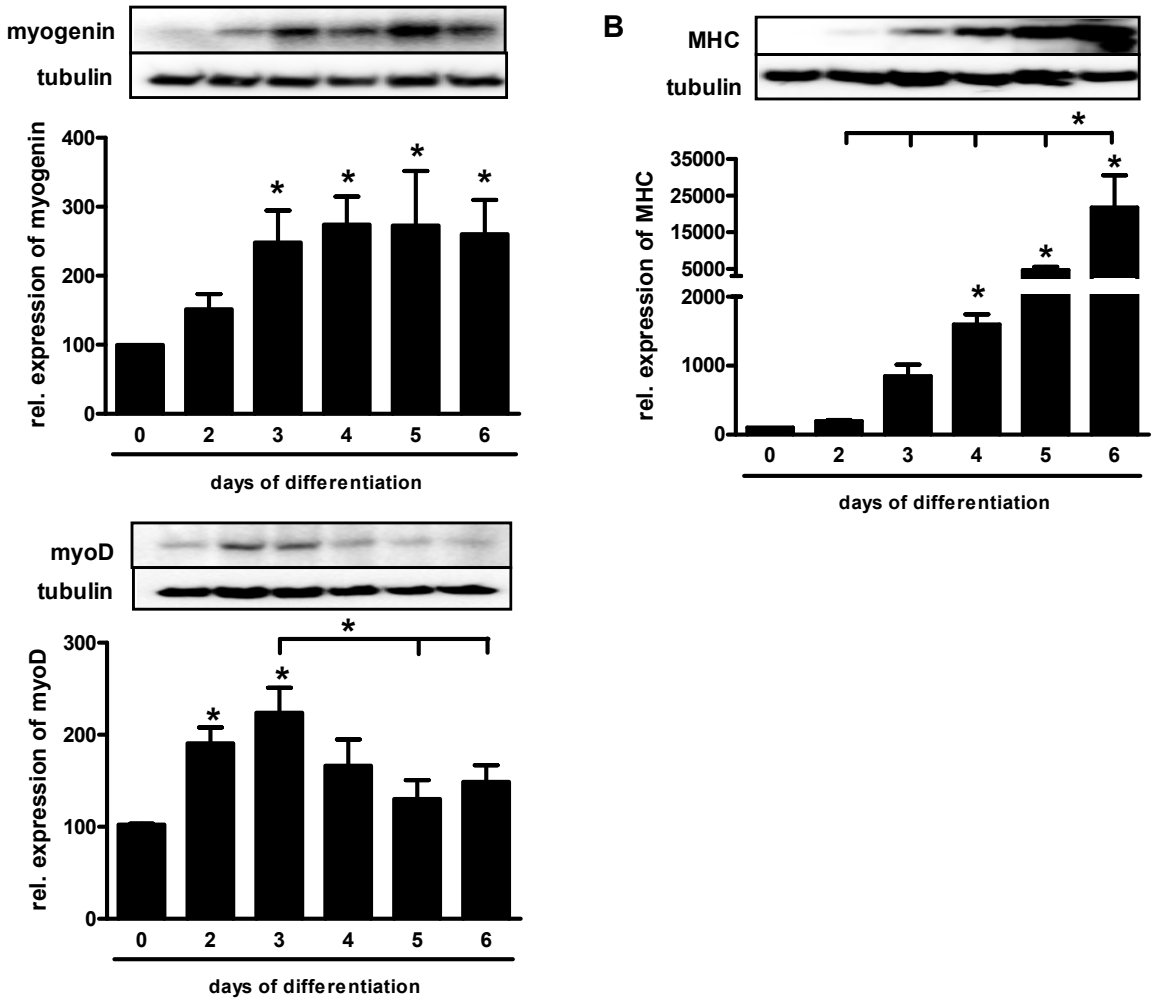

Fig. 2.2 Expression of differentiation markers in skeletal muscle cells. Myoblasts or skeletal muscle cells differentiated for 2-6 days from 4 donors were lysed and used for Western blots as described in Fig. 2.1. Blots were incubated overnight with myogenin (*A*), MHC (*B*) and MyoD (*C*) antibodies. Data are tubulin normalized mean values ± SEM (n = 4). * significantly different from myoblasts or from designated values.

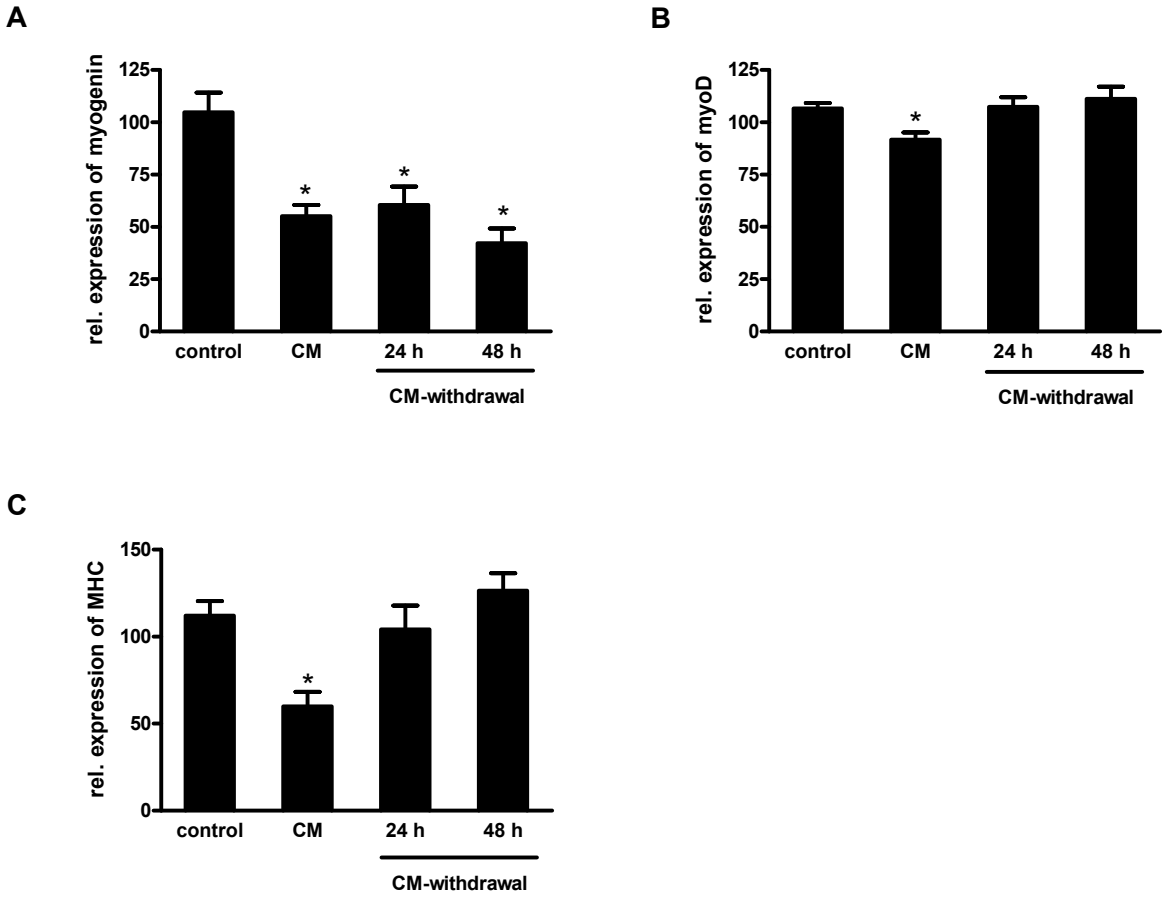

Fig. 2.3. Effect of CM-treatment and CM-withdrawal on myogenic markers. Differentiated skeletal muscle cells from 2-3 donors were treated with CM for 24 h and lysed directly or after regeneration for 24 or 48 h. Lysates were used for Western blot as described in Fig. 2.1 and detected with myogenin (*A*), MHC (*B*) and MyoD (*C*) antibodies. Data are tubulin normalized mean values ± SEM (n = 6-12). * significantly different from control.

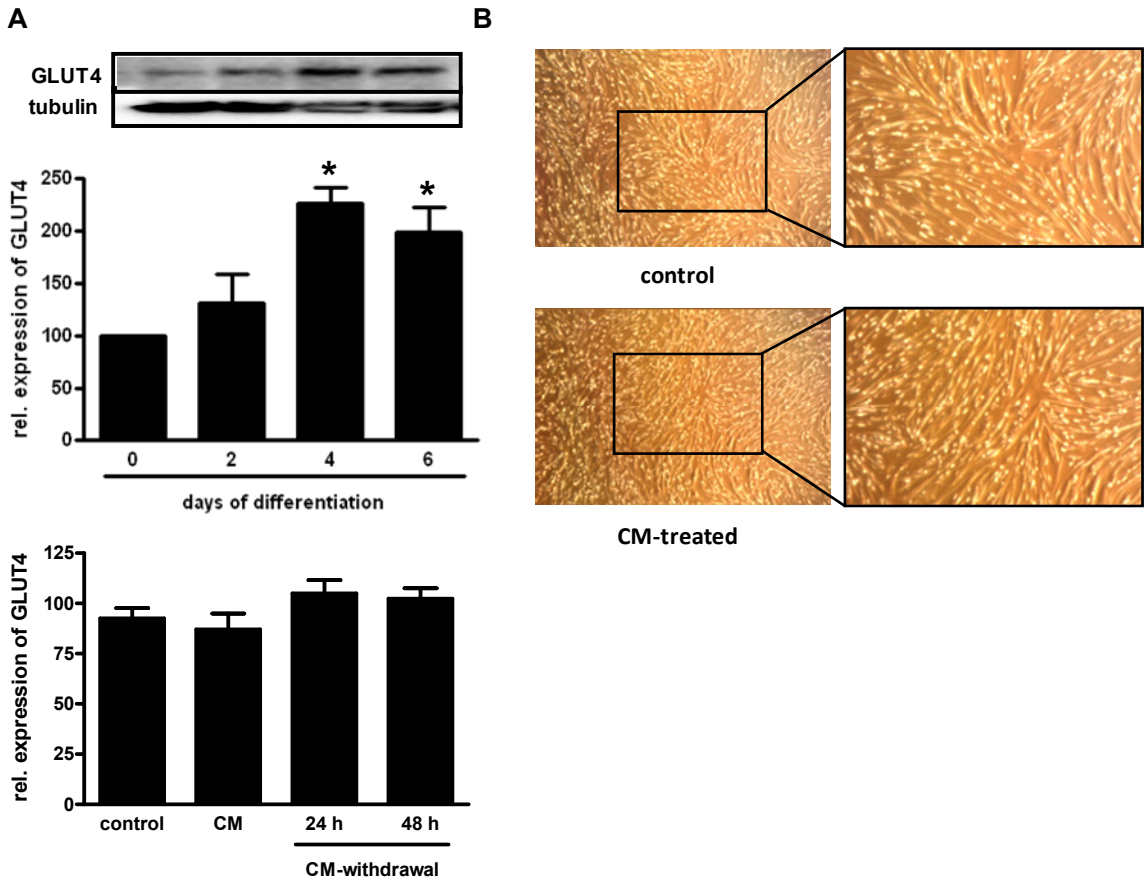

Fig. 2.4. GLUT4 expression and morphology of insulin-resistant skeletal myotubes. *A*: Myoblasts and differentiated skeletal muscle cells from 3 donors were analyzed for GLUT4 expression during differentiation (upper panel) and differentiated skeletal muscle cells from 2-3 donors were treated with CM for 24 h (lower panel) and lysed. Lysates were used for Western blot as described in Fig. 2.1 and detected with a GLUT4 antibody. Data are tubulin normalized mean values ± SEM (n = 3 during differentiation and n = 6-12 for CM-treatment). * significantly different from day 0 of differentiation. *B*: Myotubes were treated with CM for 24 h and a representative micrograph showing unaltered myotube morphology in insulin-resistant skeletal muscle cells is presented. Magnification 4x.

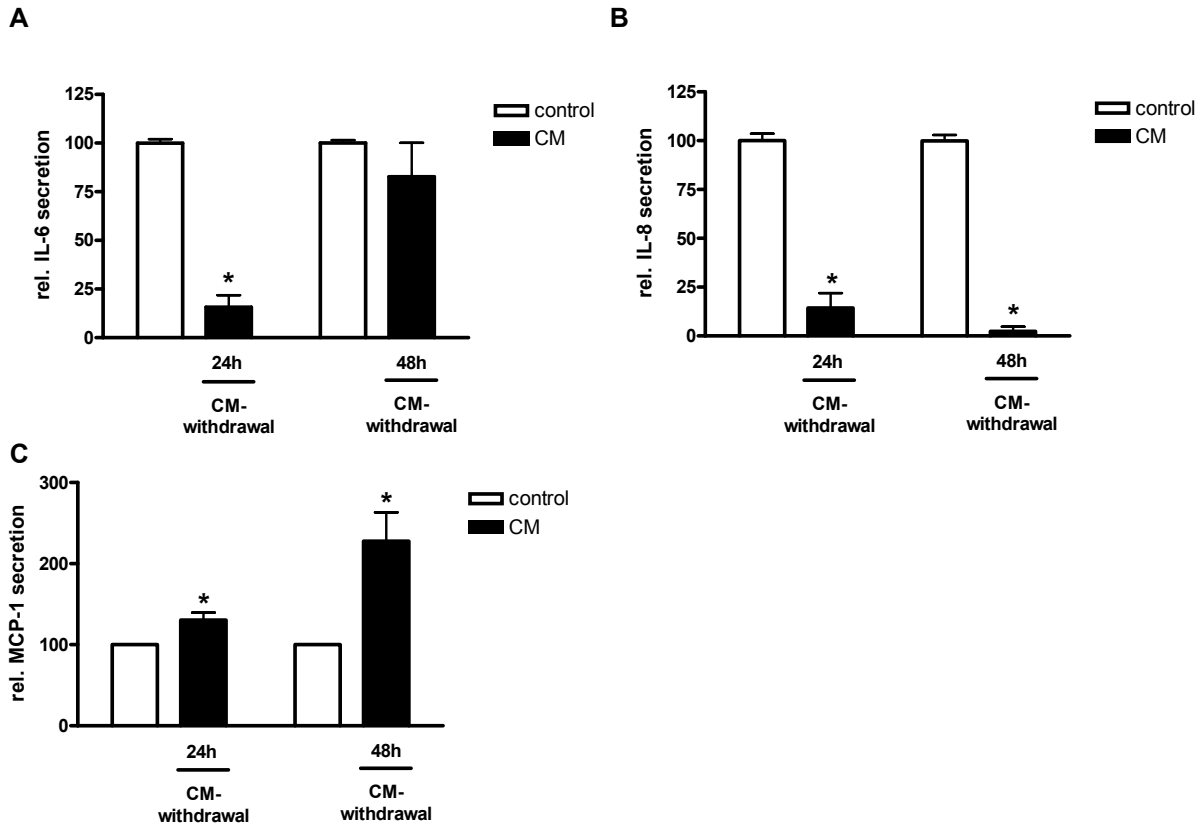

Fig. 2.5. Effect of CM-treatment on skeletal muscle cell secretion. Differentiated skeletal muscle cells from 2-3 donors were treated with CM for 24 h. After 2-times washing with PBS, cells were given fresh differentiation medium for 24 h followed by medium collection. Differentiation medium was then added again for 24 h and collected for the 48 h time point. IL-6 (*A*), IL-8 (*B*), and MCP-1 (*C*) secretion from the myotubes were analyzed by ELISA. Data are mean values ± SEM (n = 3-4). * significantly different from control.

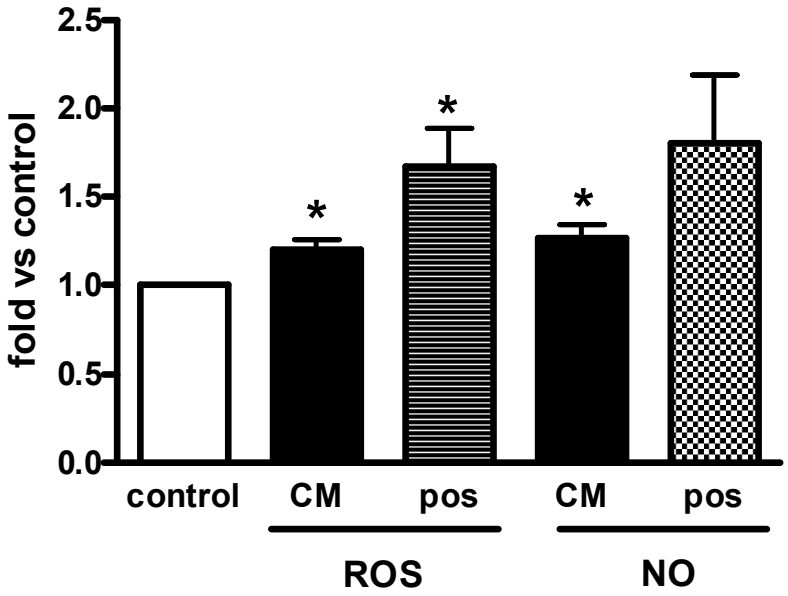

Fig. 2.6. Effect of CM-treatment on skeletal muscle ROS and NO production. Differentiated skeletal muscle cells from two donors were treated with CM for 24 h and subsequently analyzed for their capacity to produce ROS and NO as described in Materials. As a positive control, cells were treated 30 min prior to the beginning of the experiment with H_2O_2 and SNAP, respectively. Data are mean values ± SEM (n = 3-4). *significantly different from control.

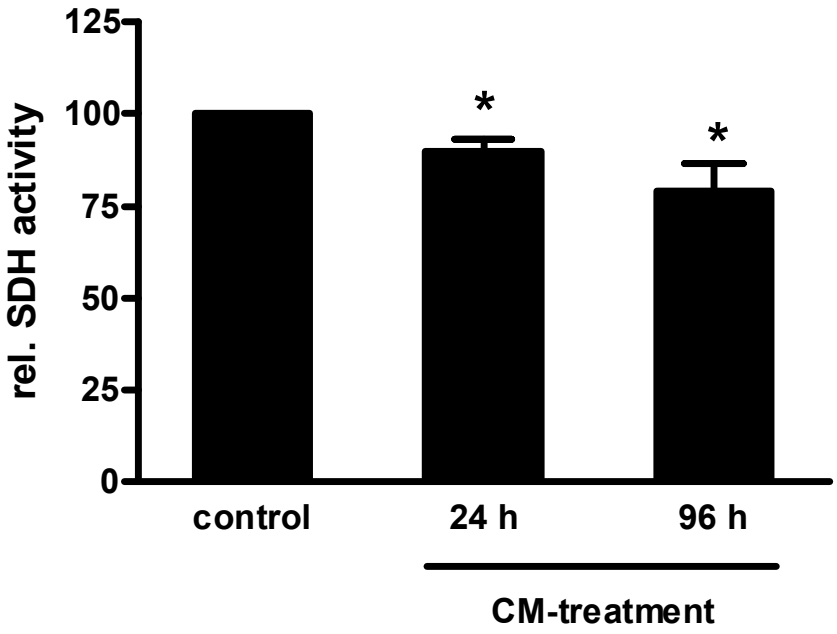

Fig. 2.7. Effect of CM-treatment on skeletal muscle SDH activity. Skeletal muscle cells from two different donors were treated with CM for 24 h or 96 h. Total cell lysates were analyzed for SDH activity as described in Materials. Data are mean ± SEM (n = 4). *significantly different from control.

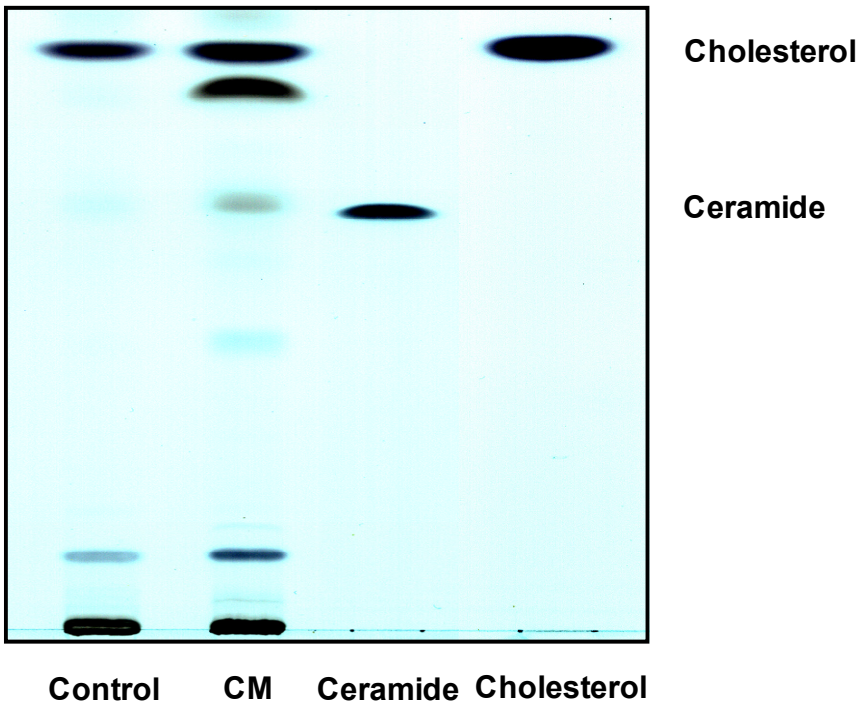

Fig. 2.8. Effect of adipocyte-conditioned medium on ceramide content in skeletal muscle cells. Cells were incubated overnight in control media or CM. Then cells were harvested and lipids extracted, separated by thin layer chromatography, and quantitatively evaluated as described in Material and Methods. Similar results were obtained in 3 different experiments.

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CHAPTER 3

Study 2

Chemerin Is a Novel Adipocyte-Derived Factor Inducing Insulin Resistance in Primary Human Skeletal Muscle Cells†

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Running title: Chemerin and muscle insulin resistance

Abbreviations: CMKLR1, chemokine-like repetor 1; ERK, extracellular signal-regulated kinase; GSK3, glycogen synthase kinase 3; IL, interleukin; IRS-1, insulin

receptor substrate-1; MCP-1, monocyte chemotactic protein-1; NFκB, nuclear factor-κB; p38 MAP kinase, phospho-38 mitogen-activated protein kinase; SkM, skeletal muscle cell; TNFα, tumor necrosis factor α

Abstract

Objective: Chemerin is an adipokine that affects adipogenesis and glucose homeostasis in adipocytes and increases with BMI in humans. This study aimed at investigating the regulation of chemerin release and its effects on glucose metabolism in skeletal muscle cells.

Research Design and Methods: Human skeletal muscle cells were treated with chemerin to study insulin signaling, glucose uptake, and activation of stress kinases. The release of chemerin was analyzed from *in vitro* differentiated human adipocytes and adipose tissue explants from 27 lean and 26 obese patients.

Results: Human adipocytes express chemerin and chemokine-like receptor 1 (CMKLR1) differentiation dependently and secrete chemerin (15 ng/ml from 10^6 cells). This process is slightly but significantly increased by TNFα and markedly inhibited by >80 % by peroxisome proliferator-activated receptor-γ activation. Adipose tissue explants from obese patients are characterized by significantly higher chemerin secretion as compared to lean controls (21 and 8 ng from 10^7 cells, respectively). Chemerin release is correlated with BMI, waist-hip-ratio, and adipocyte volume. Furthermore, higher chemerin release is associated with insulin resistance at the level of lipogenesis and insulin-induced antilipolysis in adipocytes. Chemerin induces insulin resistance in human skeletal muscle cells at the level of insulin receptor substrate 1, Akt and glycogen synthase kinase 3 phosphorylation, and glucose uptake. Furthermore, chemerin activates p38 mitogen-activated protein kinase, nuclear factor-κB, and extracellular signal-regulated kinase (ERK)-1/2. Inhibition of ERK prevents chemerin-induced insulin resistance, pointing to participation of this pathway in chemerin action.

Conclusions: Adipocyte-derived secretion of chemerin may be involved in the negative crosstalk between adipose tissue and skeletal muscle contributing to the negative relationship between obesity and insulin sensitivity.

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], and increased adipose tissue mass, especially in the visceral compartment, is now described as 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 proinflammatory adipokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, angiotensinogen, and resistin[7,8]. It is likely that some of these secreted molecules may be factors underlying the association of increased body fat to insulin resistance in peripheral organs, such as skeletal muscle. We previously demonstrated that skeletal muscle cells treated with conditioned medium from adipocytes or the adipokine monocyte chemotactic protein (MCP)-1 are characterized by an impairment of insulin signaling and glucose uptake[9,10] and could thereby define the mechanism of a negative crosstalk between adipose tissue and skeletal muscle.

Recently, the rapidly growing adipokine family was expanded by chemerin, a secreted chemoattractant protein. Initially discovered in body fluids associated with inflammatory processes[11], chemerin and its receptor, chemokine-like receptor 1 (CMKLR1, or ChemR23) are also highly expressed in adipose tissue[12,13]. In adipocytes, chemerin and CMKLR1 are necessary for adipogenesis[13]. *In vivo* data revealed that chemerin is elevated in adipose tissue of diabetic *Psammomys obesus* compared with control subjects[12]. However, no difference in chemerin levels between diabetic and control patients could be observed, despite a correlation of chemerin levels with BMI, blood triglycerides, and blood pressure[12]. Because skeletal muscle is the major postprandial glucose-uptaking organ, the current study was meant to describe effects of the novel adipokine chemerin on skeletal muscle insulin sensitivity in the context of the negative cross talk between adipose tissue and skeletal muscle.

Materials and Methods

BSA (fraction V, fatty acid free) was obtained from Roth (Karlsruhe, Germany). Reagents for SDS-PAGE were supplied by GE Healthcare (Munich, Germany) and by Sigma (Munich, Germany). Polyclonal antibodies anti-phospho-glycogen synthase kinase (phospho-GSK)3α/β (Ser21/9), anti-phospho-Akt (Ser473), anti-phospho-nuclear factor-κB (NFκB [p65, Ser536]), anti-phospho-extracellular signal-regulated kinase (phospho-ERK)-1/2 (Thr202/Tyr204), and anti–phospho-p38 mitogen-activated protein (phosphop-38 MAP) kinase (Thr180/Tyr182) were supplied by Cell Signaling Technology (Frankfurt, Germany) and anti-tubulin from Calbiochem (Merck Biosciences, Schwalbach, Germany). Chemerin, CMKLR1, actin, and adiponectin antibodies were purchased from Abcam (Cambridge, U.K.) and an antibody against myosin heavy chain (MHC) from Upstate (San Diego, CA). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG antibodies were from Promega (Mannheim, Germany). Collagenase NB4 standard grade was obtained from Serva (Heidelberg, Germany) and culture media from Life Technologies/Gibco (Berlin, Germany). Recombinant human chemerin (*Escherichia coli* derived, molecular mass 16 kDa) was supplied by R&D Systems (Wiesbaden-Nordenstadt, Germany). TNFα was purchased from Sigma and adiponectin from Biovendor (Heidelberg, Germany). 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 GE Healthcare. The enzyme-linked immunosorbent assay (ELISA) kit for phospho-insulin receptor substrate-1 (phospho-IRS-1 [Ser307]; coated antibody against mouse IRS-1 and detection antibody against rabbit phosphor-IRS-1 [Ser307]) was purchased from Cell Signaling Technology. 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 rectus abdominis muscle by enzymatic digestion with trypsin followed by a purification step with fibroblast-specific magnetic beads to prevent contamination with fibroblasts. Primary human skeletal muscle cells from three different healthy Caucasian donors were supplied as proliferating myoblasts (5 x 10^5 cells) and cultured as described in our earlier study[14]. For an individual experiment, myoblasts were seeded in six-well culture dishes 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 before they were used in experiments. The expression of the myogenin markers myogenin, MyoD, and MHC reached a plateau at 4 days until at least 6 days of culture. Experiments were repeated with the same donor but always with a different cryo-conserved aliquot of cells. The cells were always used in the 4th passage.

Adipocyte isolation and culture. Adipose tissue samples were obtained from the mammary fat of normal or moderately overweight women undergoing surgical mammary reduction. The procedure to obtain adipose tissue was approved by the ethical committee of Heinrich-Heine-University Duesseldorf (Duesseldorf, Germany). All subjects were healthy, free of medication, and had no evidence of diabetes according to routine laboratory tests. Adipose tissue samples were dissected from other tissues and minced in pieces of ~10 mg in weight. Preadipocytes were isolated by collagenase digestion as we have previously described[14]. Isolated cell pellets were resuspended in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium supplemented with 10% FCS and kept in culture for 16 h. After washing, culture was continued in an adipocyte differentiation medium (DMEM/F12, 33 µmol/l biotin, 17 µmol/l D-pantothenic acid, 66 nmol/l insulin, 1 nmol/l triiodo-L-thyronin, 100 nmol/l cortisol, 10 µg/ml apo-transferrin, 50 µg/µl gentamicin, 15 mmol/l HEPES, and 14 mmol/l $NaHCO_3$, pH 7.4) supplemented with 5 µmol/l troglitazone for 3 initial days. After 15 days, 70-90% 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 experiments or generation of adipocyte-conditioned medium, as we have recently described[15]. Briefly, after *in vitro* differentiation, adipocytes were washed with skeletal muscle cell differentiation medium, and conditioned medium was then generated by culturing adipocytes for 48 h in the same medium followed by collection of the medium.

We used subcutaneous adipose tissue from both abdominal and mammary areas (see subsection "Clinical studies"). There was no evidence of apparent differences in chemerin effects using preadipocytes from the two sources.

Clinical studies of chemerin release from adipose tissue. A total of 26 obese (BMI ≥30 kg/m2) otherwise healthy and 27 lean (BMI <25 kg/m^2) healthy women were investigated in the morning after an overnight fast. They were investigated ~1

week prior to the expected start of their menstruation period. Their age (mean ± SD) was 36 ± 7 and 35 ± 8 years, respectively. A venous blood sample was obtained for analysis of glucose and insulin to be used as an estimation of insulin sensitivity *in vivo*, using homeostasis model assessment (HOMA) index as previously described[16]. Thereafter, an abdominal subcutaneous adipose tissue biopsy was obtained by needle aspiration as previously described[17]. One part of the tissue was used for measurements of chemerin release as described elsewhere[18]. In brief, ~300 mg of tissue was incubated at 37°C for 2 h in an albumin buffer (pH 7.4), and chemerin in the medium was determined and related to the number of fat cells incubated or to the adipose tissue wet weight. Methodological experiments revealed that chemerin release was linear with time for at least 3 h, suggesting no important cell damage. Another part of the tissue was subjected to collagenase administration, and mean adipocyte volume and weight were determined as previously described[19]. Isolated fat cells were incubated for 2 h at 37°C in an albumin concentration buffer without or with increasing concentrations of insulin. Lipolysis or lipogenesis (incorporation of ^{14}C glucose into lipids) was determined as previously described[19]. From the insulin concentration response curves, the following parameters were determined as previously described[19]: *1*) half-maximum effective concentration, which was transformed to a negative logarithm value (pD2); *2*) lipolysis or lipogenesis at maximum effective insulin concentration expressed either per number of fat cells or per lipid weight of the incubated cells; and *3*) basal lipolysis or lipogenesis. The study was explained in detail to each woman, and written informed consent was obtained. The investigation was approved by the ethics committee at Karolinska Institutet.

Measurement of chemerin in conditioned medium and supernatant from adipose tissue explants. Chemerin ELISA kits were purchased from Biovendor and used according to the manufacturer. The intra- and interassay coefficients of variation for the chemerin ELISA were 6.1 and 7.6%, respectively.

Immunoblotting. Muscle cells and adipocytes were treated as indicated and lysed in a buffer containing 50 mmol/l HEPES (pH 7.4), 1% (vol/vol) Triton-X, PhosStop, and Complete protease inhibitor cocktail from Roche. After incubation for 2 h at 4°C, the suspension was centrifuged at 10,000g for 15 min. Thereafter, 5 µg of lysates were separated by SDS-PAGE using 10 or 15% horizontal gels and transferred

to polyvinylidene fluoride filters in a semidry blotting apparatus[20]. Detection filters were blocked with Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk and subsequently incubated overnight with a dilution of appropriate antibodies in Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk or 5% BSA. After washing, filters were incubated with secondary horseradish peroxidase-coupled antibody and processed for enhanced chemiluminescence detection using Uptilight (Interchim, France) or Immobilon Western detection reagents (Millipore, Schwalbach, Germany). Signals were visualized and evaluated on a LUMI Imager work station (Boehringer Mannheim, Mannheim, Germany).

Glucose uptake in skeletal muscle cells. At 4 days after start of differentiation, skeletal muscle cells were used for glucose uptake experiments. Uptake of 2-desoxy-glucose was measured for 2 h after an acute 30-min insulin stimulus (10^{-7} mol/l insulin) as described before[21].

Presentation of data and statistics. Data are the means ± SEM. Unpaired two-tailed Student *t* test or one-way ANOVA (post hoc test: Newman-Keuls multiple comparison test) were used to determine statistical significance. All statistical analyses were performed using Prism (GraphPad, LA Jolla, CA) considering a *P* value of <0.05 as statistically significant. Corresponding significance levels are indicated in the figures. Additional patient characteristics with appropriate statistics are provided as supplementary material in the online appendix, available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-0277/DC1.

Results

Chemerin and CMKLR1 expression in primary human adipocytes and skeletal muscle cells. In adipocytes, we could detect chemerin protein throughout differentiation (Fig. 3.1A). Chemerin levels increased with differentiation but decreased at the end of differentiation to levels similar to that of preadipocytes at day 0. In comparison to chemerin, adiponectin expression was only detectable after 3 days of differentiation and remained high until day 13 of differentiation. CMKLR1 levels also increased during differentiation and decreased again with full

differentiation after 13 days (Fig. 3.1A), very similar to chemerin but reaching the maximal expression already at day 1 of differentiation.

Skeletal muscle cells did not express chemerin but they did express CMKLR1 (Fig. 3.1B). The expression of CMKLR1 was highest in undifferentiated myoblasts and decreased during differentiation. After 6-8 days of differentiation, when myotube formation was completed and fused skeletal muscle cells were characterized by high expression of the myogenic marker MHC, the levels of CMKLR1 in the myotubes was 60% lower compared with myoblasts.

Regulation of chemerin expression and secretion from adipocytes. Chemerin expression in human adipocytes was upregulated by TNFα but not influenced by either adiponectin or troglitazone administration (Fig. 3.2A). In the same cells, adiponectin was upregulated by troglitazone administration, in accordance with the literature[22,23]. CMKLR1 expression was not regulated by any of the treatments.

Human *in vitro* differentiated adipocytes secreted measurable amounts of chemerin during 48 h (Fig. 3.2B). The secretion of chemerin increased constantly during 48 h to 15 ng/ml from 10^6 cells. The secretion of chemerin was slightly but significantly upregulated by TNFα in accordance with increased chemerin expression in this situation (Fig. 3.2A and C). Adiponectin did not influence chemerin secretion from adipocytes, whereas troglitazone administration led to a marked decrease by >80% in secretion of this adipokine. It can be concluded that at least in this setting, peroxisome proliferator-activated receptor (PPAR)-γ activation led to decreased chemerin release with unchanged intracellular chemerin levels (Fig. 3.2A and C).

We also tested whether macrophages isolated from human adipose tissue, using a method described by Curat et al.[24], secrete chemerin. Chemerin could not be detected in culture media from these macrophages and in media from skeletal muscle cells (data not shown).

Chemerin secretion from adipose tissue explants increased with obesity. Measurement of chemerin release over the course of 2 h from adipose tissue biopsies obtained from lean and obese female subjects revealed a significantly higher release of chemerin in obesity (Fig. 3.3). Chemerin secretion was significantly higher when expressed as nanograms of release per gram adipose tissue explant and also when expressed in relation to fat cell number.

Chemerin release correlated with BMI, waist-to-hip ratio, and fat cell volume (Fig. 3.4), whereas no correlation could be found for clinical parameters such as insulin, HOMA, blood lipids, and blood pressure (data not shown). However, a significant relationship could be found between secretion of chemerin and insulin sensitivity of adipocyte lipogenesis and insulin-stimulated antilipolysis. At the same time, chemerin did not correlate with basal or maximal insulin-inhibited lipolysis or lipogenesis (data not shown).

Chemerin impaired insulin signaling and glucose uptake in skeletal muscle cells. After overnight incubation with chemerin, skeletal muscle cells showed a marked decrease in insulin-stimulated Akt phosphorylation at Ser473, GSK3β phosphorylation at Ser21, and GSK3α phosphorylation at Ser9 (Fig. 3.5A and B). This effect was dose dependent because administration with 250 ng/ml of chemerin showed a trend toward induction of insulin resistance, whereas 1 µg/ml of chemerin provoked a significant decrease in insulin-stimulated phosphorylation of Akt and GSK3α/β. Expression of Akt and GSK3α/β remained unchanged by administration of chemerin (data not shown). Upstream of Akt, chemerin increased basal serine phosphorylation of IRS-1 and further increased insulin-stimulated phosphorylation of this substrate (Fig. 3.5C). This IRS-1 serine site is targeted by several kinases and is known to negatively modulate insulin action.

To test whether the observed effect on insulin signaling also translates into changes of glucose homeostasis in the skeletal muscle cell, we measured glucose uptake. Administration of chemerin significantly decreased insulin-stimulated glucose uptake with a slight but not significant parallel impairment of basal glucose uptake (Fig. 3.5D). Overall, the insulin-stimulated increase of glucose uptake over the respective basal level was significantly lower in skeletal muscle cells treated with chemerin (910 ± 110 vs. 620 ± 80 cpm/3.5 x 10^5 cells, respectively; n = 3).

Chemerin levels were lower in conditioned medium than the concentrations used to stimulate skeletal muscle cells. Therefore, we also used a combination of conditioned medium containing different adipokines besides chemerin and added chemerin in high concentrations to analyze whether higher chemerin levels can add to the effect of conditioned medium to induce insulin resistance, as described in earlier work[10,15]. Conditioned medium and chemerin induced insulin resistance to a similar degree, whereas addition of chemerin to conditioned medium further

decreased insulin-stimulated Akt phosphorylation (Fig. 3.6). This additive effect of conditioned medium containing low chemerin concentrations and added chemerin in a high concentration might indicate the stimulation of different pathways by adipokines present in conditioned medium and chemerin.

Chemerin activated the NFκB pathway and MAP kinases in skeletal muscle cells. Different pathways were analyzed to identify mechanisms that might mediate the effect of chemerin. Chemerin rapidly activated p38 MAP kinase and ERK-1/2 in a dose-dependent manner (Fig. 3.7A and B). The activation of these kinases reached its maximum after 30 min, and the phosphorylation of both kinases slowly decreased. The NFκB pathway was activated in a more prolonged fashion, reaching a maximal activation of p65 by chemerin after 60 min. After 24 h of chemerin administration, there was no significant increase in p38 MAP kinase phosphorylation but still significant activation of ERK and p65 (Fig. 3.7C). The expression of p38 MAP kinase, ERK-1/2, and p65 remained unchanged by administration with chemerin (data not shown).

ERK inhibition partially restored insulin sensitivity in chemerin-treated skeletal muscle cells. To analyze the role of ERK in the impairment of insulin signaling by chemerin, we preincubated skeletal muscle cells with the specific ERK inhibitor PD 98059. In short-term experiments (Fig. 3.8A), preincubation with the ERK inhibitor completely blocked ERK activation by TNFα and chemerin. After overnight incubation, the ERK inhibitor had no effect on insulin signaling itself but could partially prevent the impairment of insulin signaling by chemerin (Fig. 3.8B). Similarly, inhibition of ERK could prevent a chemerin-induced decrease in glucose uptake (Fig. 3.8C). We concluded that ERK activation is necessary for the specific effect of chemerin on insulin signaling in skeletal muscle cells but that other pathways could also be involved in this process because ERK inhibition is not able to completely reverse chemerin-induced reduction in insulin-stimulated Akt phosphorylation.

Discussion

Adipokines such as TNFα and interleukin (IL)-6 are significantly increased in obesity and are good predictors for the development of type 2 diabetes[25,26]. TNFα , IL-6, and MCP-1 have been identified to contribute *in vitro* to insulin resistance[27,28] and might be regulators of insulin sensitivity. Increased concentrations of the chemokines MCP-1, IL-8, and IP-10 (interferon-γ–inducible protein-10) have been described to be associated with incident type 2 diabetes[29,30]. Their role as important immune mediators in physiological and pathological processes might also translate into increased macrophage infiltration into adipose tissue as it has been observed in obesity[31-33]. Therefore, chemoattractant proteins represent an ideal link between obesity-associated changes in adipose tissue and an increased risk for metabolic diseases such as type 2 diabetes.

Chemerin is a novel chemokine and adipokine with a described role in host survival defense, including complement fibronolysis and coagulation. Although first described as a chemokine occurring in fluids during inflammatory processes such as cancer and rheumatoid arthritis[11], chemerin is also expressed in adipose tissue. We can demonstrate that *in vitro* differentiated adipocytes release measurable amounts of chemerin and express CMKLR1. Several studies describe similarly that chemerin mRNA expression increases with adipogenesis in 3T3 L1 adipocytes, but two of the studies comprise completely divergent data on the regulation of CMKLR1[12,13,34]. In human adipocytes chemerin and CMKLR1 mRNA expression increase with differentiation[13], the effect being more pronounced for CMKLR1. We can also find increased expression of chemerin and CMKLR1 at the protein level during differentiation that does not, however, last until the end of differentiation. The use of freshly isolated subcutaneous adipocytes and a distinct differentiation protocol might explain this difference. In adipose tissue, chemerin can also be found in the stroma-vascular fraction, pointing to a contribution of different adipose tissue cell types to chemerin production. Unable to detect chemerin release from macrophages isolated from adipose tissue, we can demonstrate that this cell type, which is so critical for adipose tissue inflammation, does not contribute to chemerin release. However, it has been described that macrophages express CMKLR1 and are chemerin responsive[35]. Chemerin is expressed similarly in human preadipocytes and

adipocytes, making it possible that adipocytes and preadipocytes are the main cells secreting this adipokine within adipose tissue.

The current knowledge of chemerin is more complicated, including more targets than chemerin and CMKLR1. Chemerin is synthesized as prochemerin with low affinity to CMKLR1[11]. Prochemerin is rapidly converted into chemerin by proteolytic cleavage of a COOH-terminal peptide, involving serine proteases of the coagulation and inflammation cascades[11]. COOH-terminal peptides derived from chemerin by cysteine protease cleavage bind to CMKLR1 with much higher affinity than chemerin itself and exert potent anti-inflammatory effects on activated macrophages[36,37]. This divergent effect of chemerin and chemerin-derived peptides can be explained by their binding to other receptors recently identified, such as GPR1 (G-protein-coupled receptor 1) and orphan serpentine receptor chemokine (CC motif) receptor-like 2 (CCRL2)[36,38]. In detail, chemerin binds with its COOH terminus to CMKLR1, directly activating cells, but chemerin can also bind CCRL2 on its NH_2-terminal domain and present the COOH terminus to CMKLR1 on neighboring cells. Differently, chemerin-derived peptides can only bind to CMKLR1 and inhibit an inflammatory response, a process that is similarly known for other chemokines, such as MCP-1 or RANTES (regulated on activation, normal T expressed and secreted)[39,40]. In this study, we observed a proinflammatory action of chemerin on skeletal muscle cells. The role of the novel chemerin receptors and chemerin-derived peptides in the context of obesity and type 2 diabetes is not known. Further work is also necessary to assess the effects of chemerin-derived peptides in this respect.

Chemerin expression is not increased in adipose tissue of genetically obese mice[13] or even lower in *db/db* mice[41], but it is higher in obese, insulin-resistant *P. obesus*[12]. The initial study on chemerin in humans revealed a correlation of chemerin blood levels with BMI, independent of glucose tolerance[12]. Very recent publications could also demonstrate an association of chemerin with markers of the metabolic syndrome and inflammation[42-44]. We can show that the release of chemerin is clearly increased from adipose tissue explants of obese patients compared with lean control subjects. Furthermore, chemerin release from adipose tissue correlated with waist-to-hip ratio and fat cell volume, whereas no correlation could be found with blood pressure and HOMA. Interestingly, chemerin secretion was negatively correlated with insulin sensitivity of the adipocytes because higher chemerin release was associated with lower insulin sensitivity of lipogenesis and lower insulin-stimulated

antilipolysis. In conclusion, we could show that chemerin was released by human adipose tissue, and in obesity its amounts were increased. Chemerin correlated with insulin sensitivity of the fat cell, potentially leading to local insulin resistance in obesity. Thus, chemerin might, clinically, serve as a marker for body composition. Its possible role as marker for metabolic control and homeostasis needs to be explored further.

We demonstrated in this study that chemerin secretion by adipocytes is regulated *in vitro* and that the release of chemerin from adipose tissue explants correlates with various features of the metabolic syndrome. It would be interesting to also study the *in vivo* effect of chemerin. This is unfortunately not possible because chemerin is not registered for therapeutic use in humans. Chemerin is secreted at concentrations of ~15 ng/ml from 10^6 cells, which is relatively low compared with the concentrations used on skeletal muscle cells of 250 ng/ml to 1 µg/ml. The latter reflect serum concentrations of this chemokine in lean (249 ± 71 ng/ml measured in 142 patients)[12] and obese patients (measured chemerin levels in 4 morbidly obese women of 674 ± 37 ng/ml, BMI 48–55 kg/m^2, unpublished data). The contribution of liver, lung, and other chemerin-producing organs to chemerin blood levels have to be taken into account.

In accordance with chemerin secretion being elevated from adipose tissue of obese patients and published data on chemerin showing it to be related to obesity[12], the expression and secretion of this chemokine by adipocytes was upregulated by TNFα . Interestingly, the PPAR-γ agonist troglitazone strongly suppressed the release of chemerin while having no effect on its expression. This observation might add to the wide area of action of these compounds[45]. A very recent publication revealed that chemerin secretion is increased by insulin in adipose tissue explants, whereas metformin is able to reduce chemerin secretion[46]. Metformin also reduces chemerin blood levels without decreasing BMI in women with polycystic ovary syndrome.

A role for chemerin was not only described in chemoattraction and macrophage infiltration into adipose tissue, but also in adipogenesis and adipocyte metabolism[13,34,41]. Chemerin is necessary for normal adipogenesis, but, as we show here, also induces insulin resistance in peripheral tissues such as skeletal muscle. We observed a clear inhibitory effect of chemerin on skeletal muscle cell glucose uptake, but in 3T3 adipocytes chemerin has the opposite effect[41], namely increasing insulin-stimulated glucose uptake. Thus, chemerin may exert different action in endocrine

and paracrine/autocrine ways. Other chemokines such as MCP-1 are also known to induce insulin resistance in skeletal muscle cells also involving activation of ERK-1/2 similarly to chemerin[9]. However, there are differences in their mode of action because MCP-1 does not activate the NFκB pathway, whereas chemerin does.

In summary, our data show that skeletal muscle is a target tissue for chemerin, which might be involved in the negative cross talk between skeletal muscle and adipose tissue. The possible role of chemerin as a link between obesity and diabetes needs, however, 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. Other adipokines already tested for their ability to disturb insulin-stimulated glucose homeostasis in skeletal muscle are also involved in inflammation, and it must be emphasized that there is most likely not a single adipokine that is fully responsible for obesity-associated metabolic complications, including insulin resistance in skeletal muscle. It is apparent that the role of adipokines in obesity and its associated metabolic complications is complex, involving numerous proteins that may act independently or in consonance. A rather complicated interplay between a huge number of adipokines and their overlapping physiological effects adds to other environmental or genetic factors to decide the development of type 2 diabetes.

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No potential conflicts of interest relevant to this article were reported.

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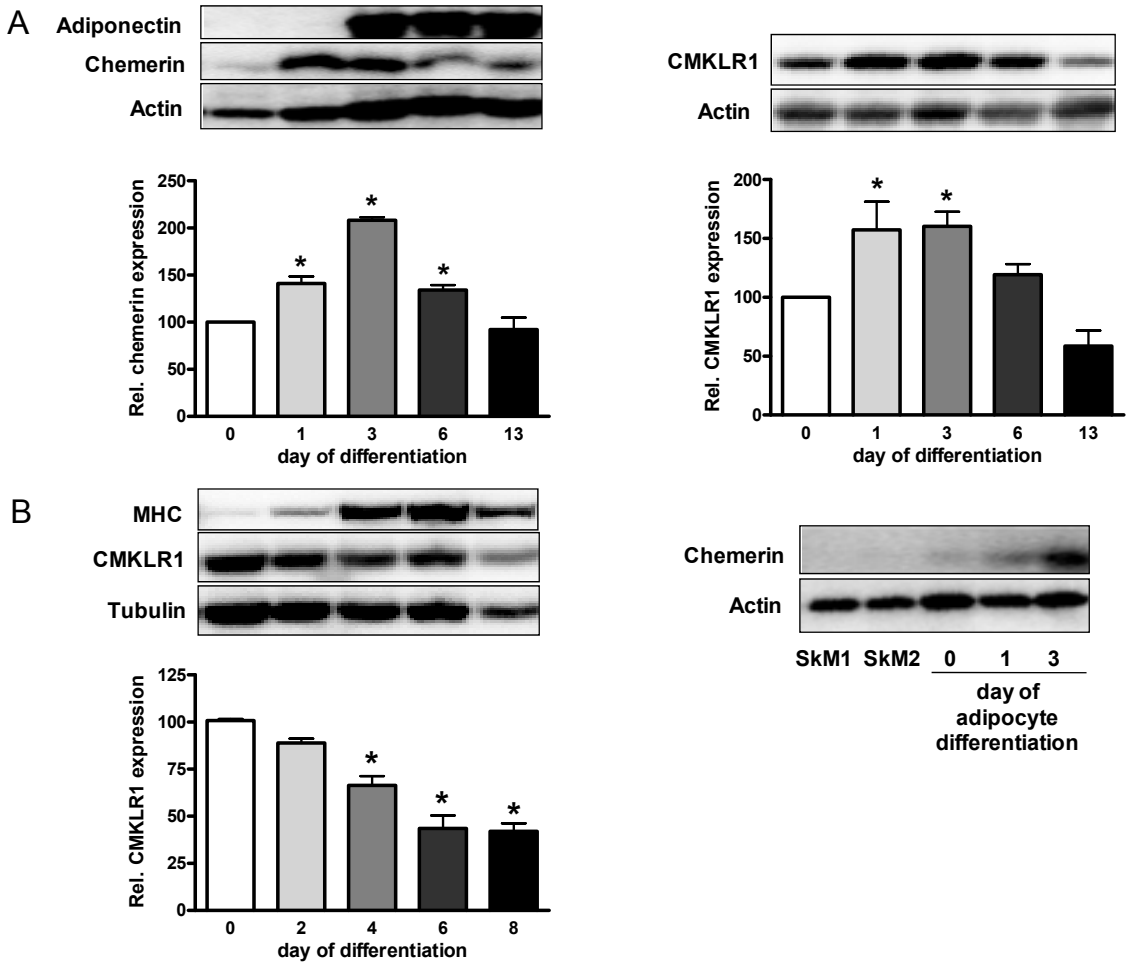

Fig. 3.1 Expression of chemerin and CMKLR1 in human skeletal muscle cells and adipocytes. A: Human adipocytes from different donors were differentiated for the indicated time, and total cell lysates were resolved by SDS-PAGE. Western blots for chemerin, adiponectin, and CMKLR1 as well as normalization for actin are shown. Data are the means ± SEM of three to four independent experiments. All data were normalized to the level of actin expression and are expressed relative to the expression level at day 0. *Significantly different from day 0. B: Skeletal muscle cells from different donors were differentiated for the indicated time, and total cell lysates were resolved by SDS-PAGE. Western blots for CMKLR1 and MHC as well as normalization for tubulin are shown. Data are the means ± SEM of three independent experiments. All data were normalized to the level of tubulin expression and are expressed relative to the expression level at day 0. The right panel shows that skeletal muscle cells from two different donors (SkM1 and SkM2) have no expression of chemerin compared with adipocytes harvested at day 0, 1, and 3 of differentiation. *Significantly different from day 0.

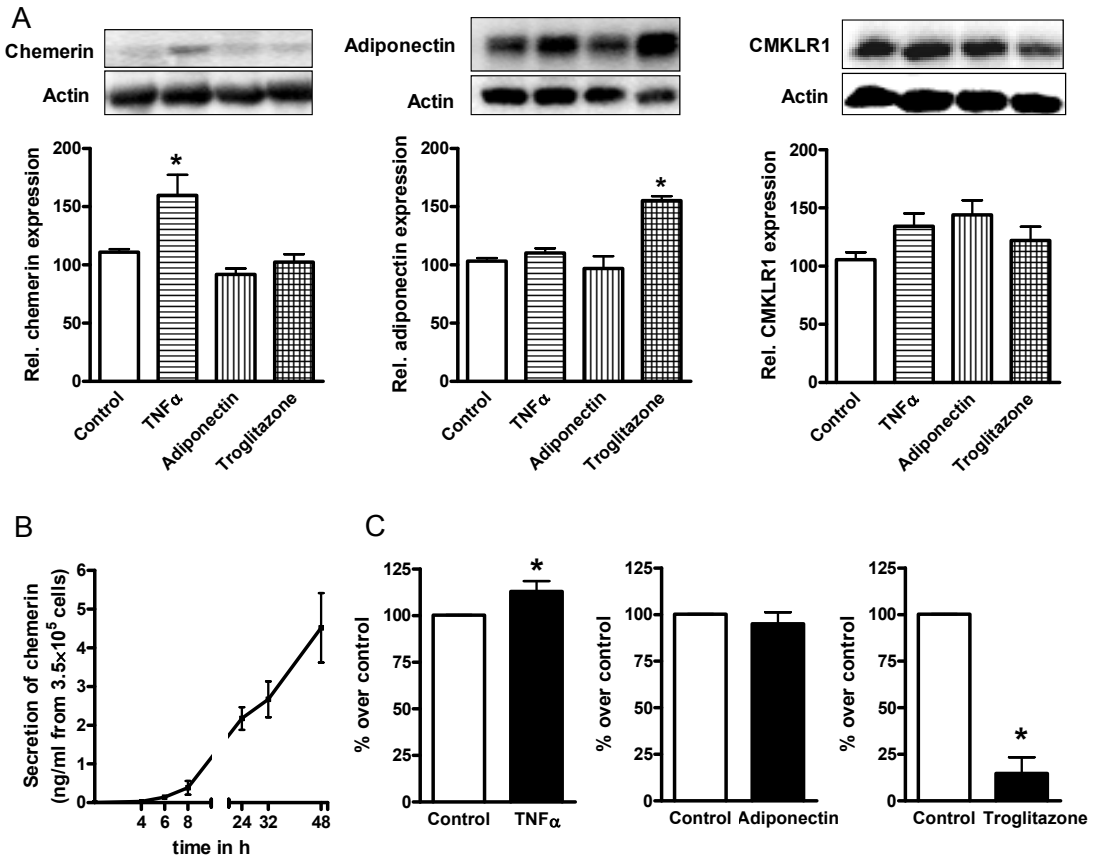

Fig. 3.2 Regulation of chemerin and CMKLR1 expression and chemerin secretion in human adipocytes. A: Human adipocytes from different donors were differentiated and incubated with either 2.5 nmol/l TNFα , 5 nmol/l adiponectin, or 5 µmol/l troglitazone overnight. Total cell lysates were resolved by SDS-PAGE. Western blots for chemerin, adiponectin, and CMKLR1 as well as normalization for actin are shown. Data are the means ± SEM of three to four independent experiments. All data were normalized to the level of actin expression and are expressed relative to the unstimulated control. *Significantly different from control. B: Human adipocytes from different donors were differentiated, and conditioned medium was collected after different periods of incubation. The release of chemerin was analyzed using a chemerin ELISA. Data are the means ± SEM of three independent experiments. C: Human adipocytes from different donors were treated with 2.5 nmol/l TNFα , 5 nmol/l adiponectin, or 5 µmol/l troglitazone overnight, and the conditioned medium was collected for chemerin measurement. Data are the means ± SEM of three independent experiments. All data are expressed relative to the unstimulated control. *Significantly different from control.

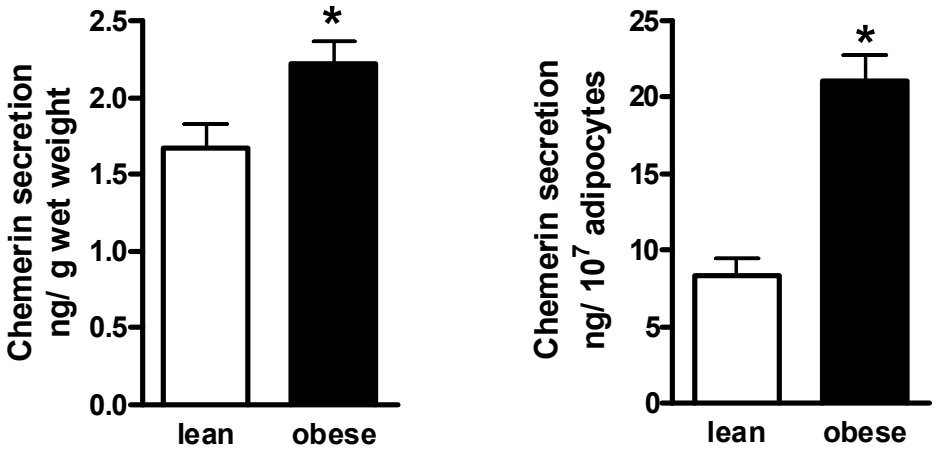

Fig. 3.3. Secretion of chemerin from adipose tissue explants derived from lean and obese female subjects. Adipose tissue explants were treated as detailed in the RESEARCH DESIGN AND METHODS section, and the release of chemerin was measured by ELISA. Data are the means ± SEM of tissue explants from 53 individuals. *Significantly different from lean control subjects.

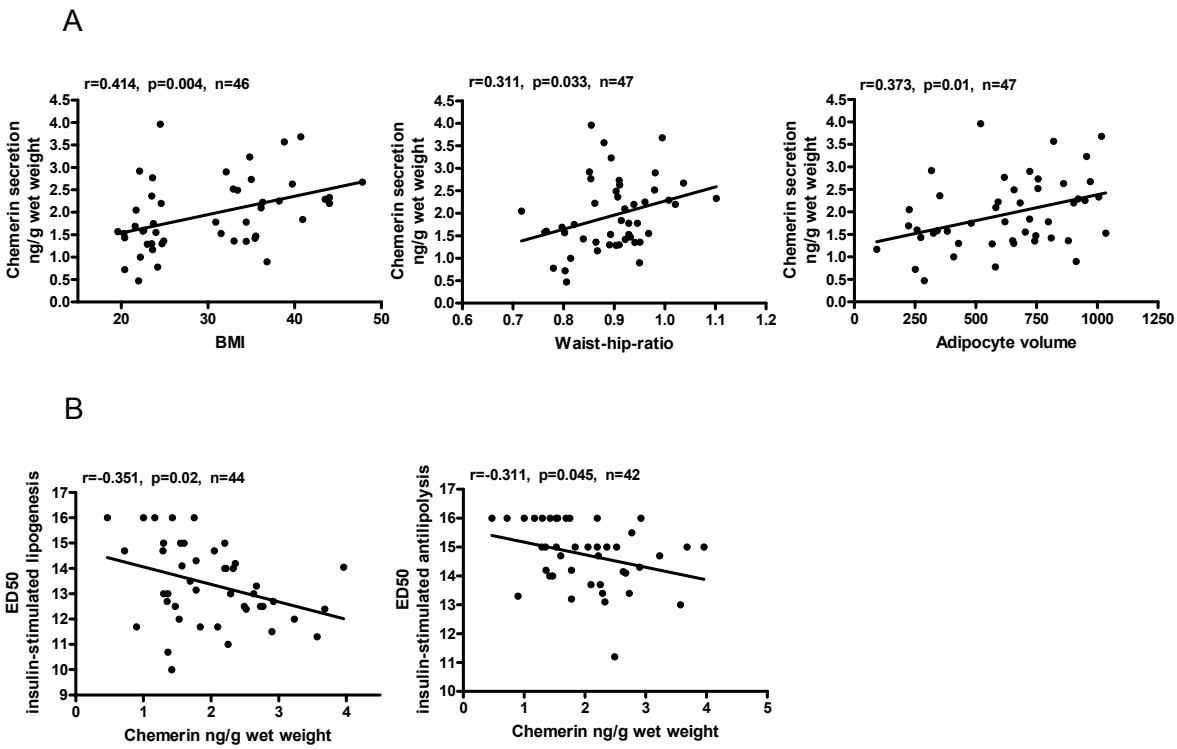

Fig. 3.4. Correlation of chemerin release from adipose tissue explants with different parameters. A: Adipose tissue explants were treated as detailed in the RESEARCH DESIGN AND METHODS section, and the release of chemerin was measured by ELISA. Chemerin release was correlated with BMI, waist-to-hip ratio, and adipocyte volume. B: Lipogenesis and insulin-stimulated antilipolysis was measured as described in the RESEARCH DESIGN AND METHODS section. Values for antilipolysis are not normally distributed, but the correlation remains significant, using a nonparametric test (Spearman rank test).

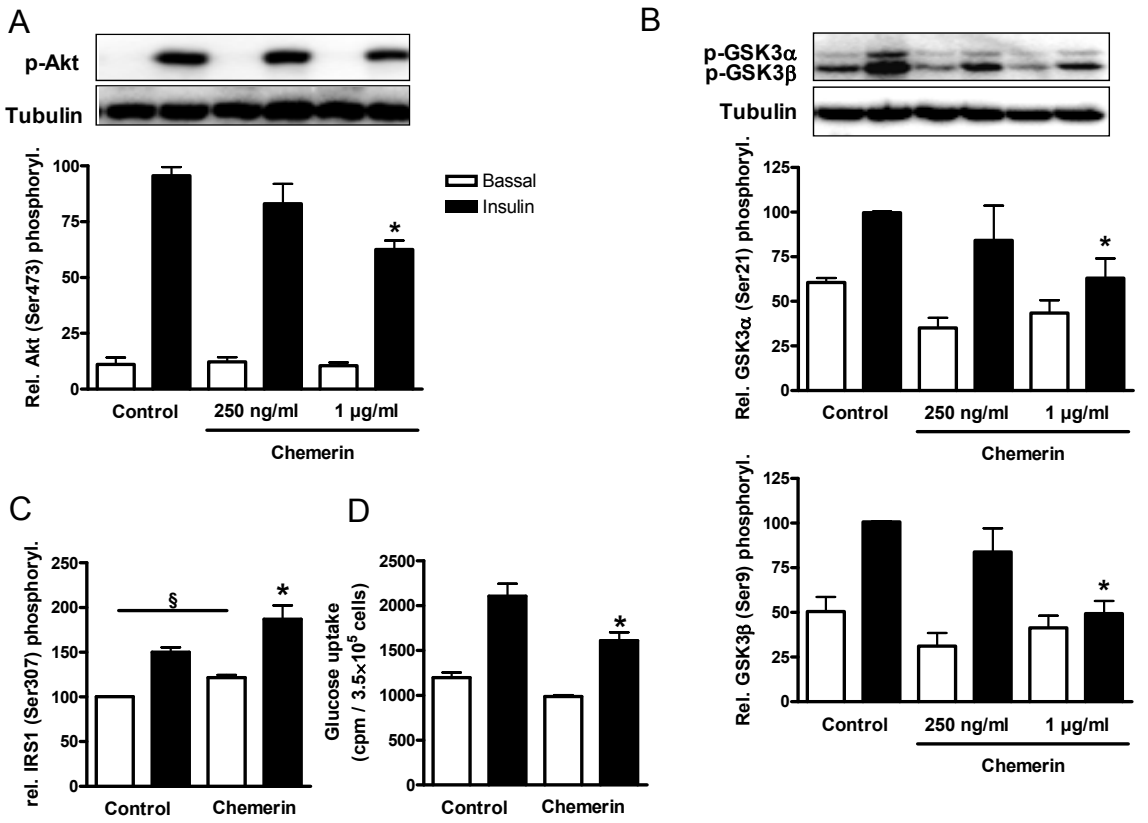

Fig. 3.5. Effect of chemerin on insulin signaling and glucose uptake in human skeletal muscle cells. A: Myocytes from different donors were cultured with increasing concentrations of chemerin (250 ng/ml and 1 µg/ml) for 24 h. After acute stimulation with insulin, total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt antibody and tubulin antibody. Data are the means ± SEM of five independent experiments. All data were normalized to the level of tubulin expression and are expressed relative to the insulin-stimulated control value. B: Myocytes from 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 the means ± SEM of four independent experiments. All data were normalized to the level of tubulin expression and are expressed relative to the insulin-stimulated control value. C and D: Skeletal muscle cells were cultured for 24 h in absence or presence of chemerin (1 µg/ml). IRS-1 phosphorylation and glucose uptake was assessed after acute stimulation with insulin, as outlined in the RESEARCH DESIGN AND METHODS section. Data are the means ± SEM of three independent experiments. *Significantly different from insulin-stimulated control; §significantly different from respective insulin-stimulated control. black bars, insulin; white bars, basal.

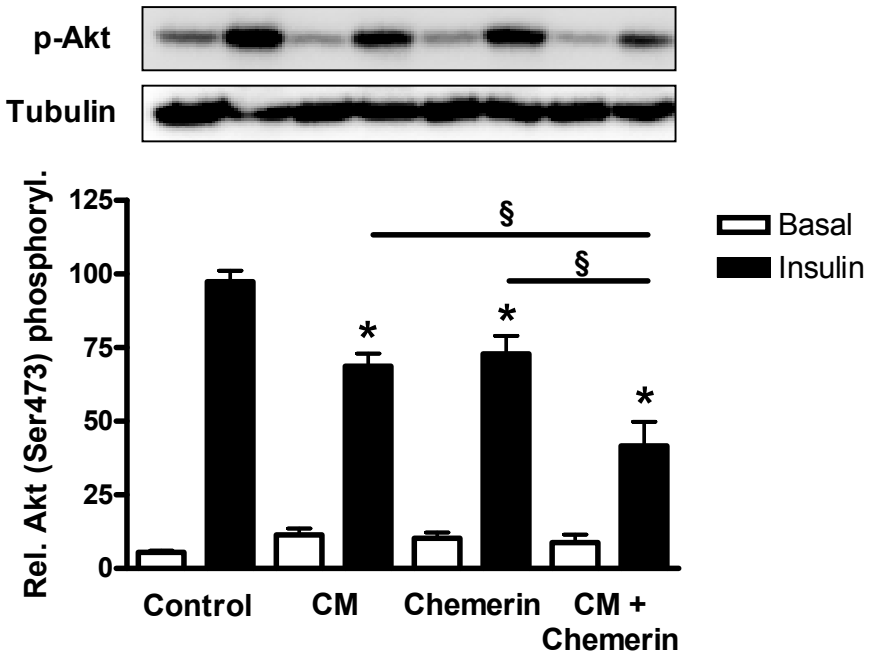

Fig. 3.6. Additive effect of chemerin and conditioned medium (CM) on insulin signaling in skeletal muscle cells. Skeletal muscle cells from different donors were incubated with chemerin, conditioned medium, or a combination of both overnight. After insulin stimulation, total cell lysates were resolved by SDS-PAGE and immunoblotted with a phosphospecific antibody for Akt and tubulin for loading control. Representative blots are shown. Data are the means ± SEM of three to four independent experiments. *Significantly different from respective basal; §significantly different from respective insulin-stimulated control.

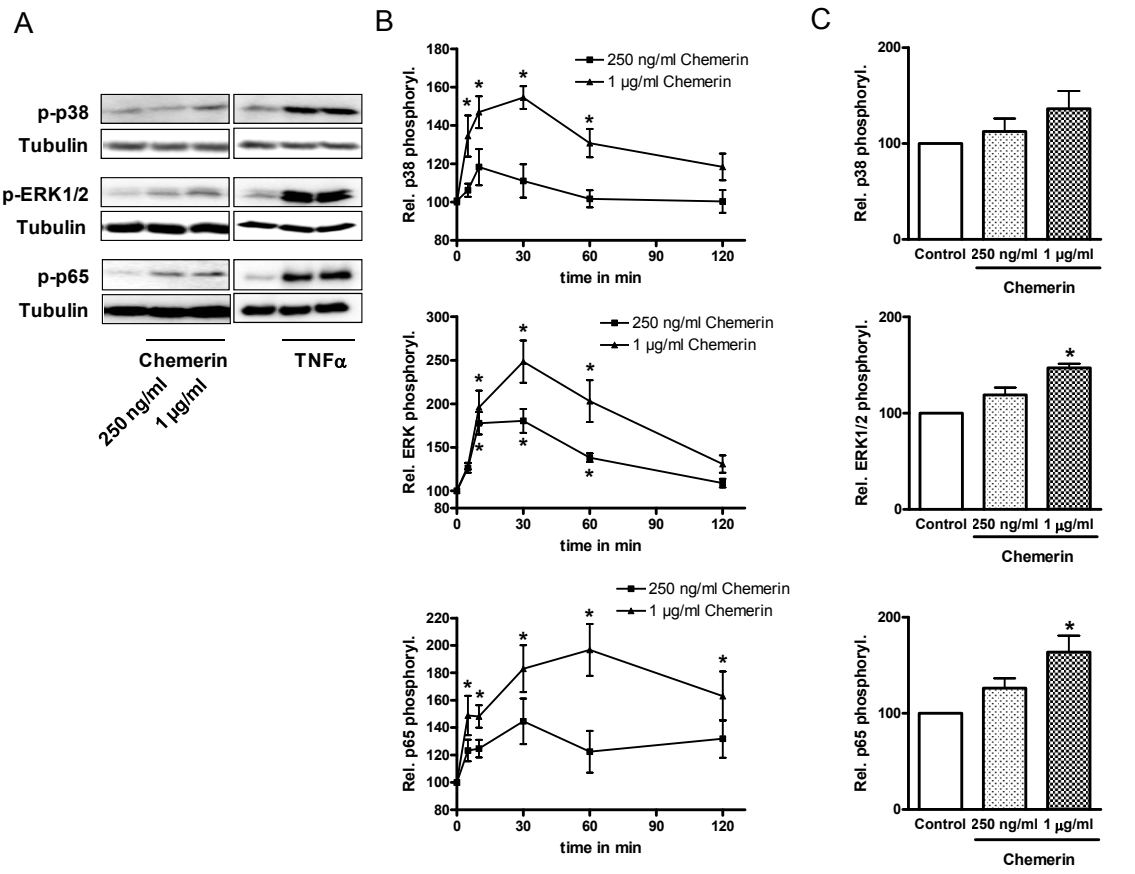

Fig. 3.7. Chemerin signaling in skeletal muscle cells. A: Skeletal muscle cells from different donors were cultured with chemerin for 30 min and as a control with 2.5 nmol/l TNFα for 10 min. Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific antibodies for p38 MAP kinase, the p65 subunit of NFκB (p65), and ERK-1/2 and tubulin for loading control. Representative blots are shown. B: Skeletal muscle cells from different donors were cultured with chemerin for 10-120 min. Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific antibodies for p38 MAP kinase, the p65 subunit of NFκB (p65), and ERK-1/2 and tubulin for loading control. Data are the means ± SEM of four to five independent experiments. *Significantly different from unstimulated control. C: Skeletal muscle cells from different donors were cultured with different concentrations of chemerin for 24 h. Total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific antibodies for p38 MAP kinase, the p65 subunit of NFκB (p65), and ERK-1/2 and tubulin for loading control. Data are the means ± SEM of four to five independent experiments. *Significantly different from unstimulated control.

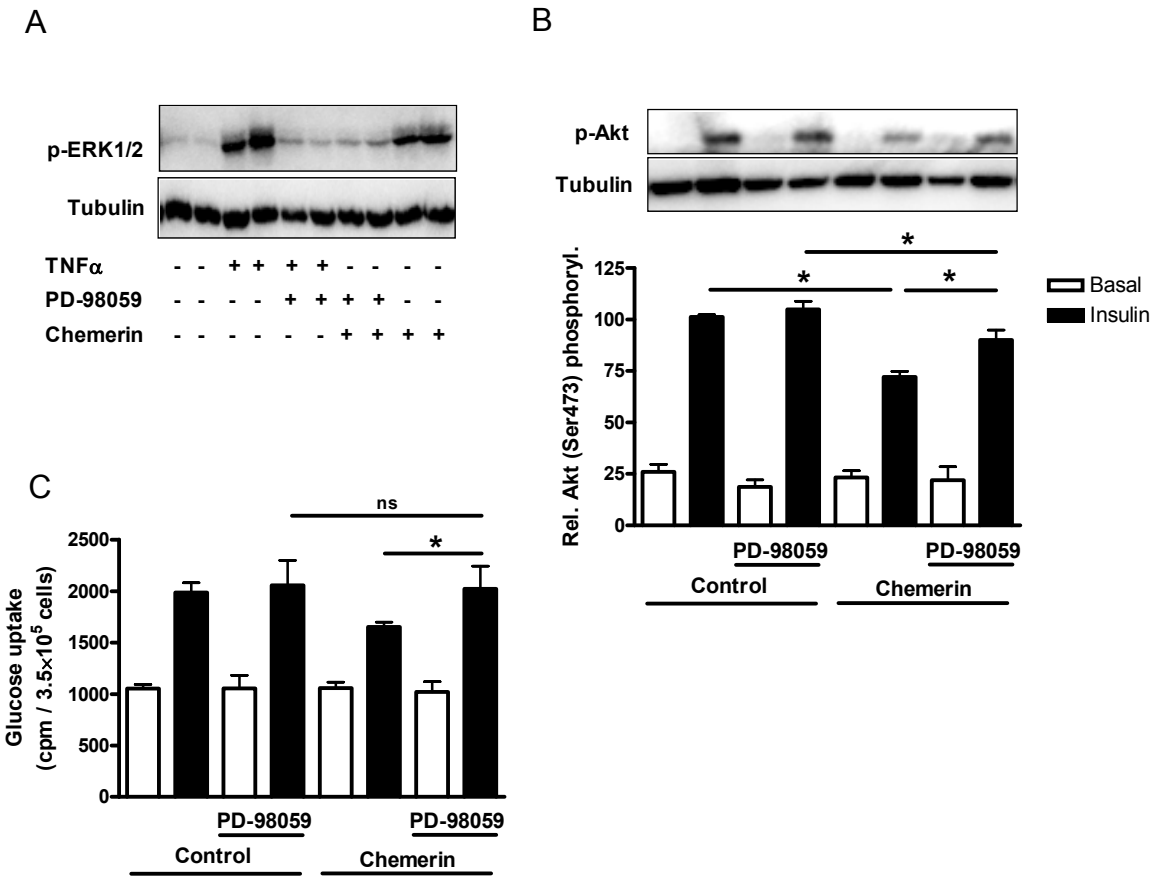

Fig. 3.8. Prevention of chemerin-induced insulin resistance by ERK inhibition. A: Skeletal muscle cells from different donors were precultured with or without 50 µmol/l of the specific ERK inhibitor PD 98059 for 15 min before starting the administration with chemerin or TNFα . The cells were then treated with chemerin for 30 min and as a control with 2.5 nmol/l TNFα for 10 min. Total cell lysates were resolved by SDS-PAGE and immunoblotted with a phosphospecific antibody for ERK-1/2 and tubulin for loading control. Representative blots are shown. B and C: After pretreatment for 15 min with PD 98059 (50 µmol/l), skeletal muscle cells from different donors were treated with chemerin overnight. After insulin stimulation, total cell lysates were resolved by SDS-PAGE and immunoblotted with a phosphospecific antibody for Akt and tubulin for loading control. Representative blots are shown. Data are the means ± SEM of four independent experiments. Glucose uptake was measured as outlined in the RESEARCH DESIGN AND METHODS section. Data are the means ± SEM of three independent experiments. *Significantly different from respective insulin-stimulated control. black bars, insulin; white bars, basal.

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CHAPTER 4

Study 3

Role of lipid-derived mediators in skeletal muscle insulin resistance†

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Abbreviations: 2-AG, 2-arachidonoyl-ethanolamide; ACC, acetyl-CoA carboxylase; AEA, anandamide; AMPK, 5´-AMP-activated protein kinase; CB1R, type 1 cannabinoid receptor; CM, adipocyte-conditioned medium; DAG, diacylglycerol; EC, endocannabinoids; EMCL, extramyocellular lipids; ETC, electron transport chain; FAox, fatty acid oxidation; FFA, free fatty acids; GLUT4, glucose transporter 4; IMCL, intramyocellular lipids; IRS-1, insulin receptor substrate-1; MCD, malonyl-CoA decarboxylase; PGC1α, peroxisome proliferator activated receptor γ coactivator 1α; ROS, reactive oxygen species; SkM, skeletal muscle; T2DM, type 2 diabetes mellitus; TCA, tricarboxylic acid; TNFα, tumor necrosis factor α; UCP3, uncoupling protein 3

Abstract

Imbalance between nutritional intake and energy expenditure has been described to culminate in obesity, which predisposes to insulin resistance and type 2 diabetes mellitus (T2DM). In such states of energy oversupply, excess amounts of lipids are available in tissues and circulation. Over the past years, an increasingly important role in development of skeletal muscle (SkM) insulin resistance has been attributed to lipids and impaired fatty acid metabolism. In this review we reflect the current state of knowledge about the effects of various lipid-derived mediators on SkM insulin sensitivity. Furthermore, potential mechanisms underlying the biogenesis of intramyocellular ectopic lipid stores are discussed. Previously, a pivotal role was attributed to mitochondrial dysfunction, however results of recent studies have suggested an important role for exercise deficiency, accompanied by decreased expression levels of peroxisome proliferator activated receptor γ coactivator 1α (PGC1α) and subsequent incomplete β-oxidation. Additionally, we summarize the implications of increased levels of lipid-derived endocannabinoids (EC) for metabolic control in peripheral tissue and highlight the benefits of targeting the EC system.

Introduction

Obesity has become a growing threat to global health by reaching epidemic proportions. It is closely associated with other diseases like hypertension, dyslipidemia, and hyperglycemia. However most importantly, obesity is a major risk factor for the development of type 2 diabetes mellitus (T2DM). A complication found in > 90% of type 2 diabetic patients is an unresponsiveness of skeletal muscle (SkM) cells to the insulin stimulus, known as insulin resistance[8]. This condition is marked by hyperinsulinemia, enhanced hepatic gluconeogenesis, and impaired insulin-stimulated glucose uptake into SkM cells[88]. Furthermore, insulin resistance is considered as an early sign for the development of T2DM[105]. Since SkM is a key metabolic tissue, accounting for ~80% of total glucose disposal under insulin-stimulated conditions[17,65], defects of insulin action in this tissue are central to the pathogenesis of T2DM.

Multiple pathways may lead to the condition of insulin resistance. Well-known and often described contributors are adipose tissue-derived cytokines. These

so-called adipocytokines exert a variety of effects on peripheral tissues, especially on SkM, and are thereby able to impair insulin-responsiveness (reviewed in [96]). Furthermore, macrophage infiltration of adipose tissue accompanied by a constant low grade inflammation has been described. As a consequence of adipocytokines as well as inflammatory mediators such as tumor necrosis factor α (TNFα), general systemic as well as adipose tissue lipolysis is altered, leading to elevated levels of free fatty acids (FFA). Enhanced availability of liberated as well as dietary FFA has been shown to result in increased amounts of ectopic lipid stores in non-adipose tissues. These lipids together with their metabolites are able to contribute to the development of insulin resistance (see Fig. 4.1). However the precise mechanisms by which ectopic lipid stores develop and how they affect insulin signaling are still part of ongoing debate and will be discussed in this review.

Besides FFA, endocannabinoids (EC) have also been described as another class of lipid-derived mediators, which are able to contribute to the pathogenesis of obesity and insulin resistance. The EC system is an important modulator of energy homeostasis and has been shown to be dysregulated in obesity and T2DM[29].

In this review we will discuss how increased ectopic lipid stores in SkM affect insulin signaling, leading to insulin resistance. Additionally, we will review the concepts of mitochondrial dysfunction and incomplete β-oxidation as possible mechanisms underlying biogenesis of ectopic lipid stores in SkM. Finally, we will summarize the implications of increased levels of EC for metabolic control in peripheral tissue and the benefits of targeting the EC system.

Ectopic lipid stores impair organ function

Obesity, which results from an imbalance between nutritional intake and energy expenditure, is characterized among others by increased amounts of available lipids. Additionally, lipid levels are further elevated by adipose tissue insulin resistance, which is frequently observed in obese patients. Under normal conditions the level of FFA increases during fasting, while in the fed state lipolysis in adipose tissue is suppressed by insulin. However, obesity is characterized by an inadequate insulin action in the fed state that resembles conditions of a normal fasted state, resulting in the release of FFAs into the circulation. In such states of lipid oversupply,

storage of available FFA cannot be by accomplished by adipose tissue anymore. Instead FFA are also stored in other non-adipose tissues not intended for long-term lipid storage like SkM, liver, heart, or pancreas[115]. As a consequence, increased amounts of ectopic lipid stores are found in obese patients.

Several studies performed in obese individuals have demonstrated a correlation between the amount of ectopic lipid stores found inside SkM cells, referred to as intramyocellular lipids (IMCL), and parameters of lipid oversupply like body-mass-index (BMI), waist-to-hip ratio, central adiposity, and percent body fat[32,66,73,101]. Whereas small amounts of intracellular triglycerides represent an important energy source especially for skeletal and cardiac muscle in periods of low glucose supply, increasing amounts of ectopic lipid depositions have been demonstrated to be able to impair organ function, known as lipotoxicity. Consequently, in the studies mentioned above an association between the amount of IMCL and impairment of SkM function marked by insulin resistance has been described. This association is further supported by additional studies performed in non-obese, non-diabetic humans[63] as well as in lean offspring of type 2 diabetic patients, a model of *in vivo* insulin resistance, where the amount of IMCL was identified as a main predictor of muscle as well as whole body insulin resistance[49,79]. Accordingly, subsequent studies have shown that reduction of IMCL content results in improved insulin sensitivity[75,91,98].

Although the majority of studies have reported a correlation between improved insulin sensitivity and reduced IMCL content, some studies have also described improved insulin sensitivity without a reduction of IMCL. Restriction of dietary glycemic index has been shown to improve insulin sensitivity without changes in IMCL content in healthy volunteers[33]. Exercise intervention studies in obese Zucker rats[110] and type 2 diabetic patients[15] demonstrated improved SkM insulin sensitivity without corresponding decrease in long chain acyl-CoA and diacylglycerol (DAG). Furthermore, a study investigating the potential effects of weight loss and physical activity on IMCL content in obese previously sedentary subjects described a significant increase in insulin sensitivity in response to weight loss and exercise without a significant change in IMCL content[41]. Instead they found a significant decrease in lipid droplet size. The authors have proposed that the reduced lipid droplet size may coincide with increased oxidative enzyme capacity, resulting in improved insulin sensitivity. The results of this study suggest that there might not be

a straightforward connection between the amount of IMCL and insulin resistance, but that other factors, like lipid droplet size may also play a role.

An additional source for lipid-derived mediators besides IMCL are lipid stores outside SkM in adipocytes interspersed between muscle fibers, called extramyocellular lipids (EMCL). Similar to IMCL, a significant association between EMCL content and obesity, percent body fat, and central adiposity has been observed[101]. Analysis of EMCL content in obese and/or type 2 diabetic subjects has demonstrated an association between EMCL content and insulin resistance[37]. However, no such correlation was found in a study conducted in lean insulin resistant offspring of type 2 diabetic patients[49,101]. Due to these divergent results, it may be speculated that the influence of EMCL content on insulin sensitivity may not be a direct, but rather a secondary effect depending on other parameters of obesity. Further studies are needed to clarify the contributing effects of EMCL to SkM insulin resistance.

Besides SkM, ectopic lipids stores may also develop in other peripheral tissues, where they are able to impair tissue functionality (reviewed in [107]). Accordingly, several studies have described a correlation between obesity and liver steatosis[39,87] as well as between intrahepatocellular lipids and BMI, percent body fat, and central obesity[109]. Increased intrahepatocellular lipids content has been shown to be negatively correlated with whole body and hepatic insulin sensitivity, as demonstrated by impaired suppression of endogenous glucose production and decreased hepatic glycogen synthesis during hyperinsulinemic clamps[3,62].

Furthermore, correlations have been described between obesity and ectopic lipid stores in cardiac muscle[50,97,106]. Like in SkM and liver, intramyocardial lipids have adverse effects on organ function. Intramyocardial lipids have been shown to correlate with concentric left ventricular hypertrophy, nonischemic heart failure, and decreased regional systolic performance[97,106]. Furthermore, there is evidence for the development of cardiomyocyte insulin resistance[23].

Lipid-derived metabolites impair SkM insulin signaling

The observation that insulin resistance correlates with IMCL content is especially relevant when considering the essential function of SkM as an insulin-

responsive organ for whole body glucose homeostasis. However, the complex mechanisms by which IMCL impair insulin signaling are not yet fully unraveled. While the lipid droplets themselves may not be harmful, they provide a source of substrate to fuel high rates of fatty acid metabolism and concomitantly to generate fatty acid metabolites which may interfere with insulin signaling. Derivatives like DAG or ceramide are of special interest and it has been shown that IMCL accumulation is associated with elevated levels of these metabolites[85,117]. They are able to activate PKCα, -θ, and -ε isoforms as well as IκB kinase (IKKβ) and c-Jun N-terminal kinases (JNK). These in turn are then able to phosphorylate serine/threonine residues of the insulin receptor and of insulin receptor substrate-1 (IRS-1) and decrease phosphorylation of PKB/Akt[90,100]. As a consequence, activation of insulin receptor, IRS-1 (tyrosine phosphorylation), and PKB/Akt are attenuated, impairing insulin signaling and impeding glucose transporter 4 (GLUT4) translocation to the plasma membrane compartment. Hence, insulin-mediated glucose uptake may be reduced or even abrogated. Furthermore, IKKβ is able to activate NFκB, which in turn regulates the production of pro-inflammatory cytokines such as TNFα and IL-6[99].

Recently, an increase of fatty acylcarnitine ester level has been described in muscle and plasma of an obese/insulin-resistant animal model[59,60]. Accumulation of these intermediates may be explained by a lipid-induced upregulation of ß-oxidation rates while downstream metabolic pathways such as the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC) are not upregulated accordingly[74], leading to incomplete ß-oxidation. Indeed, it has been shown that high-fat feeding decreases levels of organic acid metabolites of the TCA cycle[60]. The model of incomplete ß-oxidation is further supported by studies which have either used siRNA-mediated silencing of malonyl-CoA decarboxylase (MCD) in human primary myotubes[13] or knockout of MCD in mice[60] to restrict fatty acid uptake into mitochondria. The reduction of MCD causes an increase of malonyl-CoA, which in turn inhibits carnitine palmitoyl transferase 1, thereby suppressing fatty acid uptake[70,84]. This manipulation enhances glucose uptake and glucose oxidation in primary human myotubes[13] and protects $MCD^{-/-}$ mice from diet-induced glucose intolerance despite a high level of IMCL[60]. Recently, a study in type 2 diabetic african-american women described increased concentrations of fatty acylcarnitines, which were positively correlated with fasting blood HbA1c[1]. Additionally, it was shown that medium-chain acylcarnitines are able to activate NFκB in a monocytic cell line, thus

providing a potential mechanism of how acylcarnitines may interfere with insulin signaling.

Is IMCL Accumulation a Consequence of Mitochondrial Dysfunction?

Although it has been demonstrated that obesity is significantly associated with increased levels of IMCL, the precise mechanism that causes IMCL to accumulate is still subject of ongoing discussion[44]. A concept that has been proposed states that mitochondrial dysfunction results in insufficient oxidation of FFA leading to accumulation of IMCL. However, studies investigating SkM mitochondrial oxidative capacity in obese and/or insulin-resistant subjects have revealed conflicting results. On the one hand, several studies have described reduced SkM fatty acid oxidation (FAox) in obesity[40,48,51,53,57] and T2DM[55,82]. On the other hand, studies have reported unchanged FAox rates in obese humans[11,46] and type 2 diabetic patients[12] or even increased rates in different animal models[24,45,114]. An explanation for this discrepancy may be found when a closer look at study details is taken. Besides differences in study subjects, like diet-induced vs genetic obesity or obese insulin-resistant vs genetically insulin-resistant subjects, divergent methods of analysis also have to be considered. When whole muscle FAox was analyzed, frequently a reduction in FAox capacity was found[40], whereas examination of isolated mitochondria revealed enhanced FAox capacity[24,45,114].

When trying to interpret these results, one has to consider that SkM of insulin-resistant obese individuals generally shows some alterations compared to healthy controls. It has been described that SkM of obese individuals exhibit ~30% less mitochondria and a generally higher content of type II muscle fibers, which are characterized by a decreased oxidative capacity. Both features are consequences of sedentary lifestyle, since studies have shown that exercise can normalize muscle mitochondrial content in type 2 diabetic patients[111] and evoke fiber type switching. Furthermore, several studies in obese humans[11] and rats[40,45] have reported increased amounts of fatty acid transporter CD36 at the plasma membrane of SkM cells, accompanied by an enhanced transport of FFA into SkM[11,42,113]. Additionally, a

reduced expression of peroxisome proliferator activated receptor γ coactivator 1α (PGC1α), a coactivator that plays a key role in regulating mitochondrial biogenesis has been described[78]. Another observation in SkM of obese individuals is that there is a failure in substrate switching during the transition from fed to fasted state[52,54,74].

These alterations might be the reason why FAox in whole muscle was found to be reduced, while oxidative capacity of isolated mitochondria was reported to be intact or even increased. In light of these findings, it seems unlikely that mitochondrial dysfunction is the major cause of IMCL accumulation and that rather an increased delivery and/or uptake of FFA exceeding SkM energy requirements may underlie its accumulation[44]. Nevertheless, it has to be considered that depending on the subject's exact physical constitution other mechanisms may apply. Hence it has been described that in aging and severe obesity pathological changes in mitochondrial structure, morphology, and function can be observed[38,47,81], still providing a possible role for mitochondrial dysfunction in the pathogenesis of insulin resistance under these conditions.

Role of PGC1α and Oxidative Stress

IMCL themselves are unlikely to be deleterious for SkM insulin signaling, they rather serve as a source pool for a variety of metabolites, which in turn have detrimental effects on the insulin signaling cascade. Therefore the question remains why lipids are not completely oxidized to CO_2, despite reportedly unchanged or increased mitochondrial oxidative capacity. The concept of incomplete β-oxidation provided by Koves *et al*[59] and Muoio and Koves[74] might supply an answer. As mentioned above, high rates of mitochondrial β-oxidation may not be accompanied by equivalently enhanced activity of downstream metabolic pathways such as the TCA cycle or the ETC. PGC-1α has been described as an important player in coordinating interaction between these metabolic cycles[59]. Therefore, it is interesting to note that several studies have shown a reduced expression of PGC-1α in obesity and T2DM[72,78] and as a consequence of high fat feeding[59,102]. Again exercise deficiency of sedentary obese individuals may contribute to this situation because it has been described that content of PGC-1α in red muscle fibers (type I) is greater than in white muscle fibers (type II)[59], which however are more commonly found in

sedentary individuals. Furthermore, studies have shown that exercise is able to increase muscle PGC-1α content. On average, the induced changes of protein expression in rodent muscle ranged from 1.5- to 2.5-fold[10]. Since PGC-1α has been shown to I) stimulate mitochondrial biogenesis, II) regulate genes involved in oxidative phosphorylation, and III) coordinate the induction of β-oxidation with downstream metabolic pathways[59], overexpression models to further analyze its role under high fat conditions have been generated. Surprisingly, muscle-specific overexpression of PGC-1α in transgenic mice has lead to augmentation of diet-induced insulin resistance[18]. A reason for this unexpected finding may be that massive overexpression of PGC-1α was associated with increased expression of CD36 and acetyl-CoA carboxylase (ACC)2. Consequently, decreased SkM insulin sensitivity may result from increased CD36-mediated FFA uptake, which exceeds FAox capacity. On the other hand, modest overexpression of PGC-1α in muscle leads to increased GLUT4 expression and insulin-stimulated glucose uptake[5], pointing to the importance of balanced changes within physiological limits in order to allow improvements of insulin sensitivity.

However, if β-oxidation and TCA cycle do not function in a coordinated fashion, rising NADH/NAD and ATP/ADP ratios as well as depletion of free CoA, carnitine, and organic acids may ensue. This high-energy redox state affects TCA cycle and the ETC, resulting in accumulation of acyl-CoAs, acylcarnitines, and finally the production of reactive oxygen species (ROS)[74]. If ROS formation exceeds detoxification mechanisms, oxidative stress may lead to further deleterious effects. TCA cycle enzymes may be inhibited, stress-induced serine kinases may be activated, and fatty acids in the matrix or in the mitochondrial inner membranes might become subject to lipid peroxidation[92]. Indeed, it has been shown that lipid peroxides are increased in the obese state and they pose a potential risk of further peroxide-induced oxidative damage to mitochondrial matrix, comprised of DNA, RNA, and enzymes. Hence, a protective mechanism, limiting the import of FFA in conditions of high FFA availability as well as the production of ROS has been discussed.

Based on the literature available on the regulation of uncoupling proteins (UCP), it has been speculated that SkM UCP3 may play a crucial role in preventing such lipid-induced oxidative damage[19,92]. Indeed, it has been shown that in conditions of high FFA availability, UCP3 activity and expression are upregulated[94]. On the other hand, UCP3 is down-regulated again when FAox capacity is high, e.g. in

endurance training or due to weight loss. It has been speculated that a lack of SkM UCP3 would result in lipid-induced oxidative damage to the mitochondria. Indeed, UCP3 ablated mice are characterized by increased levels of muscular lipid peroxidation and oxidative damage to proteins and DNA[14]. Despite an anticipated increase of lipid-induced oxidative stress in type 2 diabetic patients, reduced muscle UCP3 contents have been demonstrated in prediabetic and type 2 diabetic patients[61,93,95]. This would suggest that, due to impaired availability of protective effects of UCP3, ROS and lipid peroxides may introduce additional oxidative damage further impairing mitochondrial integrity.

The scenario of general exercise deficiency as a central component of lipid-induced insulin resistance is supported by the endurance trained paradox. This concept comprises the observation that increased levels of IMCL may not only be found in sedentary individuals but also in endurance trained athletes. However, in contrast to obese individuals, where an increased level of IMCL content correlates with insulin resistance, endurance-trained athletes remain highly insulin sensitive[86]. Explanations for this might be that, besides their increased exercise-induced mitochondrial biogenesis[47], their enhanced content of highly oxidative type I muscle fibers, and their preserved ability of substrate switching, exercise-stimulated expression of PGC1α[59] also enables complete oxidation of FFA, severely reducing the amounts of deleterious lipid-derived metabolites as well as ROS. Hence, IMCL in SkM of endurance trained individuals are completely metabolized and exclusively serve as a source for the increased amount of energy required during training. Nevertheless, this model demonstrates that IMCL themselves are not deleterious and that exercise can protect against lipid-induced insulin resistance. The effects of exercise on whole body insulin sensitivity are numerous and their detailed description would go beyond the scope of this review.

The Peripheral EC System and Its Dysregulation in Obesity

EC are a family of lipid ligands derived from cell membrane phospholipids that play an important role in the control of energy homeostasis mainly through binding and subsequent activation of type 1 cannabinoid receptors (CB1R). CB1R have been shown to be expressed in central[26,43,83,112] and peripheral tissues like adipose tissue,

liver, and SkM[4,16,20,28,76,77]. The on-demand synthesis of EC in neurons and their involvement in regulation of neurotransmitter release has been known for some time[83]. However, it has been described recently that the components of EC synthesis and degradation are also present in nonneuronal tissues like liver[76] or pancreas[7,104]. Additionally, also adipose tissue has been recognized as a source for EC production as shown by the expression of the required enzymes for synthesis and degradation as well as by the detection of EC within the tissue[77,103]. We have recently demonstrated that *in vitro* differentiated human adipocytes derived from subcutaneous fat produce and secrete EC including anandamide (AEA) and 2-arachidonoyl-ethanolamide (2-AG)[28], thereby confirming the result of an earlier study[36].

In obesity the EC system becomes dysregulated as reflected by increased circulating levels of AEA and 2-AG as well as elevated levels of 2-AG in visceral adipose tissue[9,30,68] of obese patients. Genetically and diet-induced obese animal models show elevated levels of EC in hypothalamus, adipose tissue, liver, and endocrine pancreas[27,68,76]. The biochemical mechanisms underlying aberrant EC levels are not completely understood but the increased bioavailability in obesity may be the result of both increased synthesis and decreased degradation[29]. Several studies revealed a reduction of mRNA expression and/or activity of the AEA-degrading enzyme fatty acid amide hydrolase[9,30,56,76]. Furthermore, downregulation of CB1R expression in visceral and subcutaneous adipose tissue of obese patients compared with lean controls have been described[9,30,56]. TNFα, which is known to be elevated in obesity, has been identified as one factor directly involved in decreasing mRNA expression of fatty acid amide hydrolase[56]. Another study conducted in 3T3-L1 adipocytes revealed a role for insulin in regulating EC levels by rapidly decreasing mRNA expression of synthesizing enzymes as well as by increasing mRNA expression of degrading enzymes upon stimulation[22]. However, in insulin-resistant adipocytes insulin failed to reduce intracellular EC levels. Together with TNFα-mediated disturbances of EC degradation, absence of insulin effects may contribute to increased concentrations of EC associated with obesity.

Another aspect that may influence peripheral EC levels could be a certain dietary fatty acid composition, since the availability of biosynthetic precursor may be affected. A study conducted in 3T3-F442A adipocytes showed that incubation of the cells with arachidonic acid (AA) increased the amount of 2-AG and elevated the level

of AA esterified in triglycerides or in sn-2 but not in sn-1 position of phospholipids. Treatment with docosahexaenoic acid (DHA) decreased 2-AG and AEA concentration as well as the amount of AA esterified in both positions of phospholipids[67]. Similar results have been obtained by an earlier study which has analyzed the influence of dietary AA and docosahexaenoic acid on EC concentrations in mouse brain[6,116]. Further feeding studies support the idea that dietary fatty acids are able to influence the levels of ECs[2,69,104].

Effect of Overactivated EC System at Tissue Level

Several studies have investigated the tissue-specific contribution of EC to metabolic regulation and the consequences of overactivating vs. antagonizing CB1R. In adipose tissue EC signaling participates in the differentiation process of adipocytes and it has been described that chronic activation of CB1R leads to accelerated differentiation[68]. Blocking CB1R provides indirect evidence for a potential role in the determination of adipocyte number in adipose tissue as antagonizing the receptor inhibits preadipocyte proliferation[34]. In mature adipocytes stimulation of CB1R causes activation of lipoprotein lipase[20], inhibition of 5´-AMP-activated protein kinase (AMPK) with subsequent inhibition of FAox[58], and enhanced basal as well as insulin-stimulated glucose uptake[35,77]. Under normal conditions these mechanisms are subject to regulation (e.g. by insulin as outlined above), which limits EC levels to avoid excessive lipid accumulation. However, in states of obesity and insulin resistance this regulation is disturbed. The action of EC on adipose tissue results in routing excess energy to the adipocytes and enhancing the storage of fat, thereby increasing fat depots. Additionally, stimulation of CB1R has been shown to cause inhibition of adiponectin production and release[68,80].

In liver stimulation of CB1R leads to increased *de novo* fatty acid synthesis as well as increased lipogenesis by inducing expression of lipogenic transcription factor sterol regulator element-binding protein-1c and its targets ACC1 and fatty acid synthase[76]. Similar to adipose tissue, activation of AMPK is also inhibited in liver. This observation may be implicated in increasing ectopic lipid stores in liver, as AMPK activity has been shown to inhibit lipogenesis and increase lipid oxidation[58]. Thereby,

elevated levels of EC associated with obesity contribute to the development of ectopic lipid stores in liver and associated insulin resistance.

However, tissue-specific contribution of the EC system to metabolic regulation in SkM is investigated less extensively. Very recently we have published a study examining the involvement of EC in the negative crosstalk between adipose tissue and SkM[28]. We used conditioned media (CM) generated from *in vitro* differentiated human adipocytes to induce insulin resistance in human SkM cells and were able to partially prevent this effect by treating the cells with the selective CB1R antagonist rimonabant. Concerning the very low levels of ECs detected in conditioned media, we assume that EC play a role in inducing insulin resistance in a complex interplay with other adipocyte-released factors, which may enhance CB1R signaling. Nevertheless, we could demonstrate that stimulation of CB1R in SkM cells with high doses of AEA leads to impaired insulin-stimulated PkB/Akt (Ser^{473}) phosphorylation and reduced insulin-stimulated glucose uptake. Furthermore, we were able to show that activation of extracellular regulated kinase 1/2 and p38 mitogen-activated protein kinase as well as enhanced IRS-1 (Ser^{307}) phosphorylation is involved in mediating the effects of AEA, potentially underlying the development of insulin resistance. The results of our study add EC to the growing list of adipocyte-derived factors that mediate SkM insulin resistance.

In summary, EC system overactivity leads to enhanced fatty acid synthesis and lipogenesis in adipose and non-adipose tissue and thereby contributes to increased triglyceride storage, hepatic steatosis, and SkM insulin resistance, while adiponectin levels are decreased. Numerous studies investigating the effects of blocking CB1R with selective antagonists like rimonabant have collectively demonstrated an overall increase of metabolic parameters like improvement of dyslipidemia, hyperinsulinemia, insulin sensitivity, as well as reduction of inflammation and hepatic steatosis (reviewed in [25] and [89]). These effects are partially mediated by central mechanisms like reduction of food intake. However, it has been shown that food-intake independent effects of CB1R antagonism play an important role as well. Rimonabant has been described to increase expression of adiponectin in adipocytes, which may partially mediate several of the anti-obesity effects of rimonabant, like reduction of hyperinsulinemia[4] and improvement of insulin sensitivity[71]. Additionally, it has been shown that rimonabant is able to I) reduce triglyceride levels in plasma and SkM[21], II) increase glucose uptake in SkM cells[31], and III) promote mitochondrial

biogenesis in adipocytes[108]. These data indicate that potential CB1R antagonists, which exclusively act on peripheral systems, should have beneficial effects on treating metabolic dysfunctions associated with obesity and T2DM. Thereby adverse effects observed during clinical application of rimonabant, which are caused by affecting the central nervous system, may be circumvented[64].

Concluding remarks

Due to the central role of SkM insulin resistance in the pathophysiology of T2DM, it has become increasingly more important to understand cellular mechanisms underlying the development of SkM insulin malfunction. In this review we have reported an important role for lipid-derived metabolites such as DAG, ceramide, acylcarnitine, and EC, in addition to the more classically described adipocytokines. The amount of available conflicting data on the role of IMCL and mitochondrial function indicates the presumed complex nature of the etiology of insulin resistance. Although clear evidence for the contributive effects of lipid-derived metabolites exists, many steps of the route leading to SkM insulin resistance have yet remained unraveled. In order to be able to combat insulin resistance and therefore also T2DM, further studies investigating possible yet unknown contributing factors and interactions are needed. Understanding these mechanisms might help to find potential new targets for anti-diabetic therapy.

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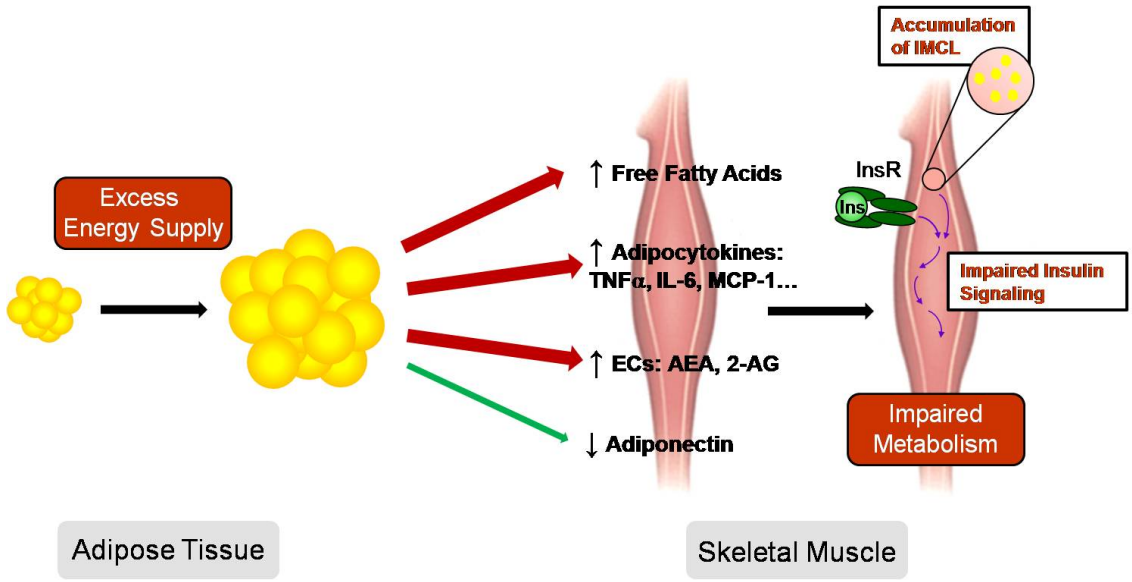

Fig. 4.1 Consequences of increased adipose tissue mass on skeletal muscle (SkM) metabolism. Energy overload due to high caloric intake and reduced physical activity leads to an increase of adipose tissue mass, enhanced release of free fatty acids, and changes in adipocyte secretion profile. Adipocyte-derived factors like free fatty acids, TNFα, IL-6, monocyte chemoattractant protein-1 (MCP-1), and endocannabinoids (ECs), among others, disturb the metabolism of SkM cells. As a consequence, intramyocellular lipids (IMCL) accumulate, insulin signaling is disturbed, and glucose uptake is impaired. AEA, anandamide; 2-AG, 2-arachidonoyl-ethanolamide; Ins, insulin; InsR, insulin receptor.

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CHAPTER 5

Study 4

Obesity-associated insulin resistance in skeletal muscle: role of lipid accumulation and physical inactivity †

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Key words: obesity, skeletal muscle, IMCL, insulin resistance, PGC-1α, exercise

Abbreviations: ACC, acetyl-CoA carboxylase; ATGL, adipose triglyceride lipase; BDNF, brain-derived neurotrophic factor; CPT-1, carnitine palmitoyl transferase 1; DAG, diacylglycerol; ETC, electron transport chain; FAox, fatty acid oxidation; fATPase, flux through ATP synthase; FFA, free fatty acids; GRx, glutathione peroxidase; IMCL, intramyocellular lipids; LIF, leukemia inhibitory factor; MCD, malonyl-CoA decarboxylase; PCr, phosphocreatine; PGC1α, peroxisome proliferator activated receptor γ coactivator 1α; ROS, reactive oxygen species; SOD, superoxide dismutase; T2D, type 2 diabetes mellitus; TCA, tricarboxylic acid;

Abstract

An alarming increase in the prevalence of obesity, type 2 diabetes mellitus, and associated diseases can be observed world-wide during the past 20 years. In obesity, profound alterations in the secretion profile of adipokines and inflammatory markers as well as increased lipolysis occur, leading besides other events to elevated levels of free fatty acids, which in turn are distributed to nonadipose tissue such as skeletal muscle. While the amount of intramyocellular lipids can be used as a marker of insulin resistance in physical inactive individuals, these neutral triglycerides themselves are not thought to be harmful. However, they provide a source for the generation of harmful lipid metabolites such as diacylglycerol and ceramide, which are implicated in insulin resistance by perturbing insulin signaling pathways. In this review, we will discuss the role of lipid metabolites in insulin resistance and potential mechanism involved in accumulation of intramyocellular lipids. Furthermore, we will highlight the key role of PGC-1α, which is a master regulator of mitochondrial biogenesis and coordinates the activation of genes involved in oxidative energy production as well as genes involved in fiber type transformation. Finally, the role of exercise in stimulating PGC-1α activity and expression as well as the release of contraction-induced myokines is discussed.

Introduction

Changes in lifestyle such as increased consumption of high-caloric diets combined with reduced physical activity are known to play an important role in the worldwide dramatic increase of obesity. According to the World Health Organization, an estimate of ~1.6 billion adults worldwide were overweight (body mass index [BMI] ≥ 25 kg/m²) and at least 400 million were obese (BMI ≥ 30 kg/m²) in 2005. By 2015, these numbers are expected to reach 2.3 billion and 700 million, respectively. Obesity is closely associated with metabolic disturbances like hypertension, dyslipidemia, and hyperglycemia. Furthermore, epidemiological studies indicate that central obesity is an important risk factor for type 2 diabetes mellitus (T2D), cardiovascular disease, certain types of cancer, and premature death[1]. An early defect occurring in the pathogenesis of T2D is insulin resistance of insulin-responsive tissues. Its consequences are an impaired ability of insulin to suppress hepatic

glucose production and to stimulate peripheral glucose clearance as well as compensatory hyperinsulinaemia. Since skeletal muscle is a key metabolic tissue, accounting for ~70-90% of total glucose disposal under postprandial conditions[2-5], defects of insulin action in this tissue are central to the pathogenesis of T2D.

Adipose tissue is an active secretory organ whose secretion profile is dramatically changed in overweight and obesity. While adiponectin levels are strongly reduced in obesity[6], the circulating concentrations of other adipokines like leptin[7], or resistin[8] are increased. Furthermore, adipose tissue is infiltrated by macrophages which are the main source of inflammatory cytokines such as TNFα. An increase in circulating levels of these adipocyte- and macrophage-derived factors in obesity leads to a chronic low-grade inflammatory state that has been linked to the development of insulin resistance and T2D[9]. In addition, bacterial lipopolysaccharide (LPS) is recognized as mediator of inflammatory responses by activating toll-like receptor-4 (TLR4) thereby inducing proinflammatory pathways and expression/secretion of cytokines in a variety of cell types. As circulating LPS levels are increased upon high-fat diet (HFD), e.g. due to increased gut permeability, endotoxins potentially provide a molecular link between HFD and obesity-associated inflammation[10,11]. Modified secretion of adipokines and inflammatory mediators partially contributes to altered systemic as well as adipose tissue lipolysis, leading to elevated levels of circulating free fatty acids (FFA). Enhanced availability of FFA has been shown to increase the amounts of ectopic lipid stores in nonadipose tissues like skeletal muscle, liver, heart, or pancreas[12]. In particular, metabolites of these lipids are thought to be involved in the development of skeletal muscle insulin resistance.

In this review we will discuss the impact of lipid-derived metabolites on insulin signaling and current concepts concerning the development of ectopic fat accumulation in skeletal muscle. In addition, we will outline the effects of physical exercise and its importance as a strategy to boost oxidative capacity and fat metabolism in order to prevent and treat obesity and insulin resistance.

Obesity and intramyocellular lipids

Obesity results from an imbalance between nutritional intake and energy expenditure and is characterized by increased amounts of available FFA in addition to other defects. Moreover, FFA levels are further elevated by insulin resistance of

adipose tissue, which is frequently observed in obese patients. Under normal conditions, the level of FFA increases during fasting, while in the fed state lipolysis is suppressed in adipose tissue by insulin. However, in obesity insulin action is impaired and even in the fed state FFA are released into the circulation[13]. In such states of lipid oversupply, fat is redistributed to non-adipose tissues such as skeletal muscle and liver[12]. As a consequence, increased amounts of ectopic lipid stores are found in obese patients.

Several studies have reported an association between the amount of intramyocellular lipids (IMCL) and parameters of overnutrition like BMI, waist-to-hip ratio, central adiposity, and percent body fat[14-16]. Small amounts of intracellular triglycerides represent an important energy source, especially for skeletal and cardiac muscle in periods of low glucose supply. However, the studies mentioned above have also demonstrated elevated amounts of IMCL to be associated with insulin resistance. Further evidence supporting this notion comes from studies conducted in non-obese, non-diabetic humans[17], as well as in lean offspring of T2D patients, where the amount of IMCL was identified as a main predictor of muscle as well as whole body insulin resistance[18,19].

Differing results have been reported regarding changes in IMCL content after intervention by diet-induced weight loss or physical exercise training both leading to improved insulin sensitivity. While diet-induced reduction in body weight resulted in declined IMCL content[20-22], exercise training has been reported to even increase IMCL content[23-25]. Furthermore, a study investigating potential effects of combined weight loss and physical activity on IMCL content in obese, previously sedentary subjects described an increase in insulin sensitivity without a change in IMCL content[26]. Instead, a significant decrease in lipid droplet size was found and it has been proposed that the reduced lipid droplet size may coincide with increased oxidative enzyme capacity, resulting in improved insulin sensitivity. In addition, mice lacking adipose triglyceride lipase (ATGL) display a ~3fold increased triglyceride content in skeletal muscle but exhibit increased glucose tolerance and increased insulin sensitivity compared to wild-typ mice[27]. The results of these studies suggest that there might not be a straightforward connection between the amount of IMCL and insulin resistance, but that other factors, such as lipid droplet size or training status, may also play a role.

Lipid-derived metabolites impair skeletal muscle insulin signaling

Results from different studies mentioned above indicate that IMCL content correlates with insulin resistance only in untrained individuals. Individuals performing regular physical exercise display normal insulin sensitivity and high oxidative capacity, despite IMCL content similar or even higher than that found in obese and T2D patients[24]. This observation, often referred to as the athlete's paradox, leads to the assumption that lipid droplets per se may not be harmful. While IMCL represent an important substrate source to fuel fatty acid metabolism, these storage depots are also presumed to generate bioactive lipid metabolites such as diacylglycerol (DAG) and ceramide, that may interfere with insulin signaling (Fig. 5.1). Several studies revealed an association between high IMCL accumulation and elevated levels of DAG and/or ceramide in obese insulin-resistant subjects[14,28,29]. Abnormal accumulation of these metabolites are thought to be involved in activation of protein kinase C (PKC) α, θ, and ε isoforms as well as IκB kinase (IKKβ) and c-Jun N-terminal kinases (JNK). These are then able to phosphorylate serine/threonine sites of the insulin receptor (IR) and of insulin receptor substrate-1 (IRS-1), as well as to decrease phosphorylation of protein kinase B (PKB)/Akt[30,31]. As a consequence, activation of IR, IRS-1 (tyrosine phosphorylation), and PKB/Akt is attenuated, impairing insulin signaling and impeding GLUT4 translocation to the plasma membrane compartment. Hence, insulin-mediated glucose uptake may be reduced or even abrogated. Furthermore, IKKβ is able to activate nuclear factor (NF)-κB, which in turn regulates the production of pro-inflammatory cytokines such as IL-6[32].

Recently, an increase of fatty acid-derived acylcarnitine intermediates have been reported in muscle of obese rodents[33,34], which may accumulate due to incomplete β-oxidation. The concept of incomplete β-oxidation describes a lipid-induced upregulation of ß-oxidation rates, while downstream metabolic pathways such as the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC) are not upregulated accordingly. Indeed, it has been shown that HFD feeding decreases levels of organic acid metabolites of the TCA cycle[34]. Furthermore, human studies have also reported increased plasma acylcarnitine levels in association with insulin resistance and T2D[35-37]. The model of incomplete ß-oxidation is further supported by knockout models of malonyl-CoA decarboxylase (MCD)[34,38], which leads to restriction

of fatty acid uptake into mitochondria via inhibition of carnitine palmitoyl transferase 1 (CPT-1) due to increased intracellular malonyl-CoA levels[39]. This manipulation enhances glucose uptake and glucose oxidation in primary human myotubes[38] and protects MCD-/- mice from diet-induced glucose intolerance despite a high level of IMCL[34]. Furthermore, treatment of L6 myotubes with the CPT-1 inhibitor etomoxir has been shown to prevent lipid-induced insulin resistance[34].

In contrast to studies supporting the concept of incomplete ß-oxidation, other groups have found that increasing fatty acid flux into mitochondria can also exert insulin-sensitizing effects. Prolonged inhibition of CPT-1 with etomoxir increased IMCL accumulation and promoted insulin resistance in rats fed a HFD[40], while overexpression of CPT-1 protects myotubes against lipid-induced insulin resistance[41,42]. In addition, muscle-specific overexpression of CPT-1 was shown to improve insulin action in HFD fed insulin-resistant rats[43]. In this study, CPT-1 was overexpressed in one hind limb of a rat while the other hind limb served as sham-treated control, which means that the analyzed muscles were subjected to the same circulating factors. Increase of muscle CPT-1 expression (~40%) and activity (~20%) was sufficient to increase fatty acid oxidation (FAox) without major changes in markers of downstream capacity of the TCA cycle or increases of acylcarnitine species. The insulin-sensitizing effects of CPT-1 overexpression were associated with a reduction of IMCL content, membrane DAG and ceramide content, PKCθ activity, and HFD-induced phosphorylation of JNK and IRS-1 at Ser307[43].

Based on current knowledge, it does not seem possible to finally answer the question whether or not an increase of FFA entry into mitochondria may be of advantage for improving insulin action in muscle since convincing data for both scenarios exist. Future studies will be needed to clarify the precise molecular mechanisms and the role of CPT-1 in order to interpret the observed discrepancies in recent studies.

Which mechanisms cause accumulation of IMCL?

While it has been demonstrated that obesity is significantly associated with increased levels of IMCL, the precise mechanism leading to increased IMCL accumulation is still subject of ongoing discussion. Excessive IMCL accumulation could result from reduced FAox and/or an increased rate of fatty acid uptake across

the plasma membrane. Studies in obese and/or insulin-resistant subjects investigating skeletal muscle mitochondrial function, which were based on markers of mitochondrial metabolism such as mitochondrial enzyme activities, have yielded inconsistent results. Several studies have described reduced skeletal muscle FAox in obesity[44,45] and T2D[46,47], while other studies have reported unchanged FAox rates in obese humans[48,49] and T2D patients[50] or even increased rates in different animal models[51,52]. An explanation for this discrepancy may be a different methodology as earlier studies used to analyze whole muscle FAox[44,45], while in later studies mitochondrial function has either been examined using isolated mitochondria or normalized to mitochondrial content[49-51].

New technologies using magnetic resonance spectroscopy (MRS) has allowed assessment of *in vivo* mitochondrial function by determining the rate of unidirectional flux through ATP synthase (fATPase) as well as phosphocreatine (PCr) kinetics during recovery from submaximal exercise. In addition, flux through the TCA cycle can be assessed by carbon-13 (^{13}C) MRS. Using these techniques, several groups reported compromised mitochondrial function in insulin-resistant subjects. This was evident by e.g. lower rates of skeletal muscle ATP synthesis and reduction in TCA cycle flux in lean, but insulin-resistant offspring of T2D patients compared to healthy subjects[53,54], and prolonged half-times of PCr resynthesis after moderate exercise in the obese T2D patients vs. BMI-matched controls[55]. In addition, a decrease of fATPase as well as failure of insulin-stimulated increase of fATPase was reported in non-obese metabolically well controlled T2D patients compared to young, healthy controls[56]. Interestingly, IMCL content was similar in the subjects of the last two mentioned studies.

In general, skeletal muscle of insulin-resistant obese individuals displays certain alterations compared to healthy controls. It has been shown to contain ~30% less mitochondria and a generally higher content of type IIB fibers, which are characterized by a lower number of mitochondria and lower oxidative capacity. Furthermore, decreased expression of peroxisome proliferator activated receptor γ coactivator 1α (PGC-1α), a master regulator of mitochondrial biogenesis, has been reported in obesity[57]. These features are related to physical inactivity and have been shown to be normalized by exercise[58]. In addition, studies in obese humans[16,48] and rats[59,60] revealed increased amounts of fatty acid transporter CD36 at the plasma membrane of skeletal muscle cells as well as an enhanced transport of FFA into

skeletal muscle [48,60]. Further support for the functional implication of CD36 in skeletal muscle IMCL accumulation is provided by a recent study demonstrating a dramatic reduction in IMCL content after specific inhibition of CD36 in myotubes obtained from obese patients[16]. Another observation in skeletal muscle of obese individuals is a failure in substrate switching during the transition from fed to fasted state referred to as metabolic inflexibility[61,62] (Fig. 5.2).

Conclusively, these data suggest that in obesity reduction of FAox observed at the whole muscle level may be attributable to a decrease in mitochondrial content as well as further alterations, while the ability of mitochondria to oxidize fat itself is not altered. In light of these findings, it seems unlikely that reduced mitochondrial function is the major cause of IMCL accumulation. Instead, increased delivery and/or uptake of FFA, exceeding the rates of intracellular FAox, may rather underlie its accumulation[63]. However, skeletal muscle mitochondrial aberrations in T2D can be detected, but it is so far not known if these aberrations are causally related to the development of the disease. As increased plasma FFA concentrations reduce insulin-stimulated skeletal muscle fATPase[64], and are negatively related to fasting skeletal muscle ATP synthesis[56], it cannot be excluded that a reduced mitochondrial function may be the consequence of the diabetic state[65].

Role of PGC-1α

Skeletal muscle cells of obese subjects contain a reduced number of mitochondria. PGC-1α is an important activator of mitochondrial biogenesis and several studies have revealed a reduced expression of PGC-1α in obesity and T2D[57,66]. Furthermore, acute elevation of plasma FFA levels was shown to reduce PGC-1α expression[67,68]. PGC-1α engages in the whole process of mitochondrial biogenesis and respiration by docking on and co-activating transcription factors regulating expression of nuclear genes. These in turn encode mitochondrial proteins and induce expression of mitochondrial transcription factor A, which regulates mitochondrial DNA transcription, thus leading to coordinated expression of mitochondrial proteins[69].

To further analyze the role of PGC-1α under high fat conditions, overexpression models have been generated. Surprisingly, muscle-specific overexpression of PGC-1α has lead to augmentation of diet-induced insulin

resistance[70]. This finding may be explained by the observed increased expression of CD36 and acetyl-CoA carboxylase 2 (ACC-2) which may ultimately result in increased CD36-mediated FFA uptake exceeding FAox capacity. Consequently, increased IMCL accumulation, an increase in membrane DAG content, activation of PKCθ, decreased insulin-stimulated IRS-1 tyrosine phosphorylation, and skeletal muscle insulin resistance was observed in these animals. On the other hand, modest overexpression of PGC-1α in muscle leads to increased GLUT4 expression and insulin-stimulated glucose uptake[71], pointing to the importance of balanced changes within physiological limits in order to allow improvements in insulin sensitivity.

The reduced level of PGC-1α expression associated with obesity and T2D may be connected to exercise deficiency, since these patients frequently have a sedentary lifestyle. PGC-1α is preferentially expressed in muscle enriched with type I fibers[72], while muscle of sedentary individuals commonly contain more type IIB fibers. Furthermore, studies have shown that exercise is able to increase muscle PGC-1α content and to promote an adaptive muscle fiber transformation, thereby increasing the content of type I and type IIA fibers in trained muscle. When PGC-1α is overexpressed in muscle enriched with type IIB fibers, expression of proteins characteristic for type I fibers, such as troponin I (slow) and myoglobin, is induced in addition to activation of genes involved in oxidative energy production[72]. Exercise-induced activation of calcium signaling pathways appears to play a major role in the stimulation of PGC-1α transcription through calcineurin and calcium-dependent protein kinases, which culminate in the activation of several transcription factors, such as CREB and MEF2, leading to increased PGC-1α expression. In addition, PGC-1α activity is stimulated due to phosphorylation by p38 MAPK and AMPK as well as via deacetylation by SIRT1. Importantly, AMPK itself is activated by exercise through contraction-induced increased energy demand and enhances SIRT1 activity by increasing intracellular NAD+ levels[73]. These data implicate that PGC-1α, acting downstream of calcium signaling pathways, coordinates the expression of both metabolic and contractile properties of muscle fibers.

Very recently, microRNA miR-696 was identified to regulate PGC-1α protein content in skeletal muscle of mice depending on physical exercise[74]. While muscle inactivity resulted in upregulation of miR-696 leading to decreased PGC-1α protein level along with reduced expression of mRNAs of its downstream genes, exercise led to miR-696 decrease resulting in increased PGC-1α protein level.

Exercise and role of contraction-induced myokines

Exercise evokes a complex set of physiological responses. Stimulation of PGC-1α leading to mitochondrial biogenesis and concomitant augmentation of oxidative capacity represent major adaptations observed in endurance exercise training. In general, it is associated with increased insulin-stimulated glucose uptake in the immediate postexercise period, while chronic physical activity enhances insulin sensitivity. In addition, it is known that reactive oxygen species (ROS) are generated during exercise and induce expression of PGC-1α and downstream targets such as superoxide dismutase (SOD) and glutathione peroxidase (GRx) which are involved in antioxidant defense[75,76]. A study by Ristow et al. demonstrated the beneficial effect of exercise-induced oxidative stress on insulin sensitivity by showing that preventing the formation of ROS due to antioxidants, like vitamin C and E, abolished observed changes in gene expression of SOD and GRx and the increase in insulin sensitivity following exercise[77].

Skeletal muscle is also known to express a variety of cytokines and other peptides referred to as myokines, which play a role in mediating metabolic changes associated with exercise as well as the following training adaptation. The endocrine function of muscle is stimulated through contraction, leading to release of e.g. IL-6[78], IL-8[79], brain-derived neurotrophic factor (BDNF)[80,81], and leukemia inhibitory factor (LIF)[82]. However, obesity is often accompanied by physical inactivity, which has been shown to increase the risk of T2D[83]. Interestingly, a strong reduction of physical activity in healthy young men has been shown to reduce peripheral insulin sensitivity within two weeks[84,85].

IL-6 is a cytokine with a dual role as it is implicated both as a mediator of impaired insulin action in obesity and also as a facilitator of increased fuel metabolism during exercise. In states of obesity and insulin resistance, plasma levels of IL-6 are significantly higher compared to lean controls and display a strong relationship with obesity and insulin resistance[86]. Several studies have demonstrated that palmitate, which is the most abundant saturated FFA in plasma, induces the expression of IL-6 in myotubes via activation of NFκB[87,88]. Chronic exposure of skeletal muscle to IL-6 has been shown to result in impaired insulin-stimulated IRS-1/Akt signaling and glucose uptake and involves activation of JNK1/2, expression of SOCS3, and activation of PTP1B[89]. However, it has been observed that during physical

exercise expression of IL-6 and its receptor is enhanced. Additionally, an up to 100-fold increase of IL-6 in the circulation has been shown during exercise[90]. Acute treatment with IL-6 increases insulin-stimulated glucose disposal and FAox in skeletal muscle via activation of LKB1 and AMPK[91,92]. Furthermore, IL-6 possesses anti-inflammatory effects due to stimulation of IL-1ra and IL-10 as well as inhibition of TNFα and IL-1 production[78]. A negative association between the amount of regular physical activity and basal plasma IL-6 levels has been observed in several epidemiological studies[93-95]. Furthermore, results from intervention studies indicate that regular exercise may be able to reduce basal IL-6 levels[96,97]. In severely obese subjects, combination of a hypocaloric diet and regular physical activity for 15 weeks led to reduction of plasma IL-6 as well as IL-6 mRNA content in adipose tissue and skeletal muscle [98]. Another effect observed in response to regular exercise training is upregulation of IL-6 receptor mRNA in skeletal muscle, pointing towards a sensitization to IL-6 at rest[99].

IL-8 is another myokine which is stimulated by contraction. Its plasma concentration has been reported to be increased in response to exhaustive exercise such as running involving eccentric muscle contractions[100-102], while concentric exercise such as bicycle ergometry[103] or rowing[104] of moderate intensity did not increase plasma IL-8 concentration. In muscle biopsies obtained during and after concentric exercise, an increase of IL-8 mRNA has been observed as well as an increase of IL-8 protein within muscle fibers, which peaked 3-6 h postexercise[79]. However, the low release of IL-8 from muscle did not result in an increase in systemic plasma concentration of IL-8, suggesting that muscle-derived IL-8 may play a local role and exerts its effect in an autocrine or paracrine fashion. The majority of systemic IL-8 increase as seen during eccentric exercise is most likely due to an inflammatory response[78]. The physiological function of IL-8 in skeletal muscle is not yet well established but studies indicate that it may stimulate angiogenesis through CXCR2 receptor signaling[105] and play a role in neutrophil chemotaxis[106].

Very recently, expression of BDNF has been described in human skeletal muscle[80]. This factor has been reported before to play a role in metabolism by reducing food intake and improving blood glucose and lipid metabolism in genetically modified *(db/db)* obese mice[107]. Recent studies have demonstrated that BDNF plasma levels of T2D patients are decreased independently of obesity[108] and that exercise can increase circulating BDNF levels in healthy humans[109-112]. Matthews *et*

al. proved that BDNF expression in human skeletal muscle and C2C12 cells were stimulated by contraction, hereby adding BDNF to the list of contraction-induced myokines[80]. Effects of BDNF in skeletal muscle involve phosphorylation of AMPK and ACCβ, leading to enhanced FAox. However, while contraction led to increased BDNF protein abundance, it could not be detected in the media from cells that underwent contraction. Whether BDNF plays an important role for skeletal muscle *in vivo* remains to be established.

LIF, a member of the IL-6 cytokine superfamily, is constitutively expressed at a low level primarily in type I fibers[113] and has been reported to be involved in skeletal muscle growth and regeneration by increasing myoblast proliferation and survival as well as increasing the number and size of myotubes in regenerating skeletal muscle[114,115]. A recent study reported that acute exercise induced expression of LIF mRNA in human skeletal muscle, however this was not accompanied by an increase of LIF protein[82]. Additionally, the results also suggest that Ca^{2+} may be involved in regulation of LIF, since treatment of primary skeletal muscle cells with ionomycin increased both LIF mRNA and protein. It may be concluded that different training stimuli or repetitive bouts of exercise seem to be necessary to induce an increase in skeletal muscle LIF protein content. While it has been shown that LIF can decrease fat mass in animal models[116,117], its physiological function in skeletal muscle requires further investigations.

Summary and concluding remarks

Despite intense research, current understanding of molecular mechanisms underlying lipid-induced insulin resistance remains incomplete. However, links between IMCL accumulation, generation of lipid-derived metabolites, and insulin resistance are in the focus. In addition, the role of microRNAs in regulation of key proteins involved in these processes will become increasingly important. Recently, the microRNA pattern of T2D patients has been analyzed indicating that a third of microRNAs in muscle were altered, many changes occurring even prior to the onset of clinical diabetes[118]. Thus, investigation of microRNAs and their regulation in the context of insulin resistance will open a novel field of research.

Recognition of skeletal muscle as an endocrine organ, whose function is stimulated by contraction, highlights the importance of physical exercise in order to

prevent and treat obesity, insulin resistance, T2D, and associated diseases. Physical exercise does not only increase energy expenditure, thereby positively influencing energy balance, but also stimulates adaptations such as mitochondrial biogenesis, fiber type conversion, improvement of insulin sensitivity, and release of myokines, which may impact on metabolism of skeletal muscle as well as other tissues. Understanding molecular mechanisms behind exercise-related adaptations and their positive impact on metabolism has become increasingly important due to the augmented prevalence of the metabolic syndrome.

Identification of potential pharmacological targets and development of safe and effective therapeutics to treat obesity, insulin resistance, and associated diseases is complicated due to the pathophysiological complexities of metabolic dysfunction. Though combination of diet and exercise has been proven to be the most effective therapy, to many patients it is not much appealing.

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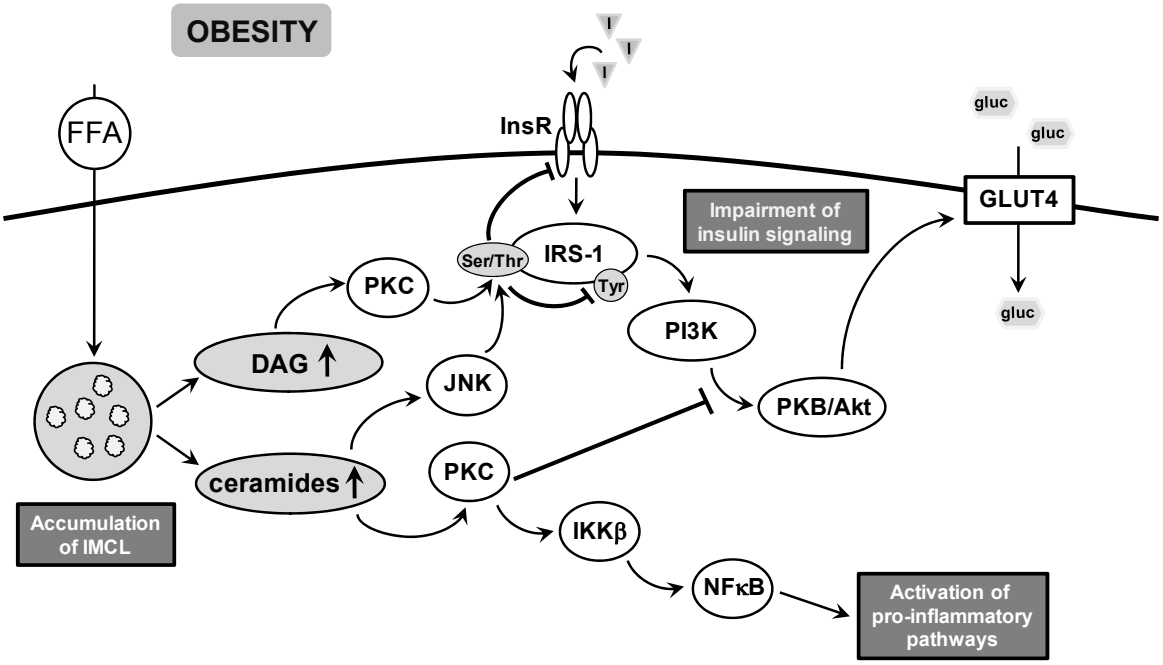

Fig. 5.1 Simplified overview of intracellular signaling cascades involved in skeletal muscle insulin resistance by lipid metabolites. In obesity lipid oversupply leads to accumulation of IMCL which provide a source for lipid metabolites such as DAG and ceramides. Increased level of these mediators are involved in activation of different PKC isoforms, JNK, and IKKβ, which in turn impair insulin-stimulated signaling cascades leading to e.g. disturbed glucose uptake. In addition, pro-inflammatory pathways involving NFκB are activated. I, insulin; gluc, glucose; PI3K, phosphatidylinositol 3-kinase

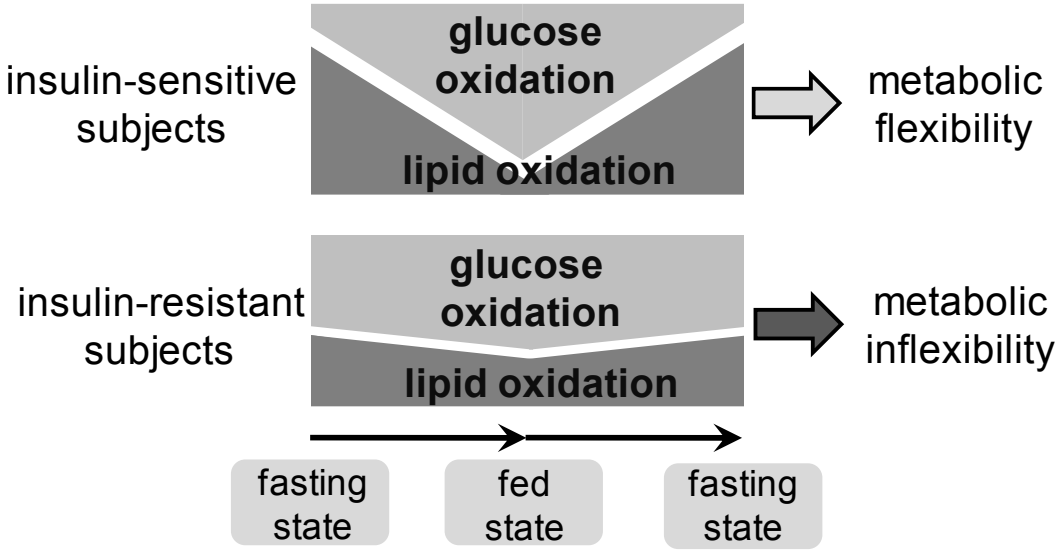

Fig. 5.2 Concept of metabolic inflexibility. In insulin-sensitive subjects lipid uptake and oxidation in skeletal muscle is high during fasted state while glucose oxidation is low. In fed state, lipid oxidation is suppressed while glucose uptake, oxidation and storage is increased. Transition to fasting state is accompanied again by increasing rate of lipid oxidation and decreasing rate of glucose oxidation. This substrate switch is impaired in insulin-resistant subjects, and no modulation in the relative reliance of lipid and glucose oxidation can be observed. Insulin-resistant subjects exhibit higher rates of glucose oxidation in fasted state compared to insulin-sensitive subjects, while in the fed state higher rates of lipid oxidation occur due to insufficient suppression of lipid oxidation by insulin

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CHAPTER 6

Study 5

Electrical Pulse Stimulation of Human Skeletal Muscle Cells Mimics Exercise and Prevents Insulin Resistance Induced by Adipocyte-conditioned Medium†

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Abbreviations: αMEM, α-modified Eagle's medium; AMPK, AMP activated protein kinase; ACC, acetyl-CoA carboxylase; AICAR, aminoimidazol carboxamid ribonukleotide; CM, adipocyte-conditioned medium; ELISA, enzyme-linked immunosorbent assay; EPS, electrical pulse stimulation; FA, fatty acid; IKK, IκB kinase; I229 , selective inhibitor of IκB kinase; IL-6, interleukin-6; MCP-1, monocyte chemotactic protein-1; NFκB, nuclear factor 'kappa-light-chain-enhancer' of activated

B-cells; OA, oleic acid; PA, palmitic acid; TBS, tris buffered saline; TNFα, tumor necrosis factor α

Abstract

Objective: Obesity is closely associated with muscle insulin resistance and a major risk factor for the pathogenesis of type 2 diabetes. Regular physical activity does not only prevent obesity, but also considerably improves insulin sensitivity and skeletal muscle metabolism. The aim of our project was to establish and characterize an *in vitro* model of human skeletal muscle contraction to study signalling pathways and mechanisms, which are involved in beneficial effects of muscle activity.

Research Design and Methods: Contracting human skeletal muscle cells were generated by applying electrical pulse stimulation. To induce insulin resistance, skeletal muscle cells were incubated with human adipocyte-derived conditioned medium, MCP-1, and chemerin.

Results: Electrical pulse stimulation induced contractile activity combined with formation of sarcomeres, activation of AMPK, and increased IL-6 secretion, similar to *in vivo* exercising skeletal muscle. Insulin-stimulated glucose uptake was substantially elevated in contracting cells compared to control. Incubation of skeletal muscle cells with adipocyte-derived conditioned media, chemerin, and MCP-1 significantly reduced the insulin-stimulated phosphorylation of Akt. This effect was abrogated by concomitant pulse stimulation of the cells. Additionally, pro-inflammatory signalling by adipocyte-derived factors was completely prevented by electrical pulse stimulation of the myotubes.

Conclusion: Our results provide direct evidence that muscle contractile activity improves insulin action and eliminates insulin resistance by blocking pro-inflammatory signalling pathways. This novel model provides a unique tool to investigate the molecular mechanisms which mediate the beneficial effects of muscle contraction.

Introduction

Obesity in combination with a lack of exercise is a strong risk factor to develop type 2 diabetes (T2D). It is well established that physical inactivity causes accumulation of visceral fat and that the health consequences of both are related to systemic low-grade inflammation[1,2]. Importantly, the visceral fat compartment is a major secretory and endocrine active tissue producing a wide variety of cytokines that regulate energy metabolism and insulin sensitivity[3-5]. Adipocytes from obese subjects are characterized by altered endocrine function leading to an increased secretion of pro-inflammatory adipokines, such as tumor necrosis factor (TNF)α, angiotensinogen, chemerin, monocyte chemotactic protein (MCP)-1, and resistin[6-9]. Activation of inflammatory pathways leads to the development of insulin resistance in peripheral tissues such as skeletal muscle and adipose tissue, which is an early defect in the pathogenesis of T2D. Insulin resistant and type 2 diabetic patients are characterized by impaired insulin action on whole-body glucose uptake, in part due to impaired insulin-stimulated glucose uptake in skeletal muscle.

It is well accepted that physical activity exerts major beneficial effects on the prevention of chronic diseases like T2D, cardiovascular disease, dementia, and depression[10,11]. Regular physical activity does not only prevent obesity and reduce adipose tissue mass, it is known to increase insulin-stimulated glucose uptake in the immediate post-exercise period[12], while chronic physical activity enhances insulin sensitivity in human skeletal muscle[13,14]. Acute exercise increases glucose uptake in skeletal muscle by an insulin-independent mechanism that bypasses insulin signalling defects associated with the pathophysiological conditions[15]. Exercise activates AMP activated protein kinase (AMPK), which phosphorylates acetyl CoA carboxylase (ACC), resulting in inhibition of ACC activity, which causes a reduced malonyl CoA content, resulting in an enhanced fatty acid oxidation[16].

At the present stage, the molecular mechanisms mediating the health-promoting effects of physical activity are not entirely understood. In the last decade it became evident that the muscle is an endocrine organ that produces and releases myokines in response to contraction[17]. Most likely, specific myokines mediate these health-promoting effects of physical activity. Myokines, like interleukin (IL)-6 and brain derived neurotrophic factor, are produced and released by skeletal muscle cells

after exercise. These cytokines lead to enhanced fatty acid oxidation in an AMPK-dependent fashion[18,19].

We previously showed that skeletal muscle cells incubated with adipocyte-conditioned medium (CM) from primary human adipocytes or treated with adipokines like MCP-1 and chemerin are characterized by an impairment of insulin signalling and glucose uptake[7,8,20]. The aim of our current project was to establish and characterize an *in vitro* contraction model of human skeletal muscle cells that mimics exercise, with the overall goal to analyze signalling pathways and mechanisms, which are involved in the beneficial effects of muscle activity. Furthermore, in the present study we combined this contraction model with the insulin resistance model to analyze the cross-talk between adipocytes and contracting human skeletal muscle cells. We show here that contractile activity of skeletal muscle cells exerts an anti-inflammatory action, which prevents the induction of insulin resistance.

Research Design and Methods

Materials. Reagents for SDS-PAGE were supplied by GE Healthcare (Munich, Germany) and by Sigma (Munich, Germany), rotiphorese was supplied by Carl Roth (Karlsruhe, Germany). The following antibodies were used: anti-phospho glycogen synthase kinase (phospho-GSK) 3α/β (Ser21/9), anti-phospho Akt (Ser473, Thr308), anti-Akt, anti-phospho nuclear factor-κB (NFκB) (Ser536), anti-NFκB, anti-IKKα/ß, anti-IκBα, anti-phospho AMPKα (Thr172), anti-AMPKα supplied by Cell Signalling Technology (Frankfurt, Germany), anti-tubulin obtained from Calbiochem (Merck Biosciences, Schmalbach, Germany), sarcomeric α-actinin purchased from Sigma, anti-myosin heavy chain (MHC) supplied by Upstate (San Diego, CA). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG were purchased from Promega (Mannheim, Germany). Collagenase NB4 standard grade was obtained from Serva (Heidelberg, Germany) and culture media from Gibco (Berlin, Germany). Recombinant human chemerin (*Escherichia coli* derived) was supplied by R&D Systems (Wiesbaden-Nordenstadt, Germany), MCP-1 by PeproTech (Hamburg, Germany), and TNFα from Sigma. Primary human skeletal muscle cells and supplement pack for growth medium were obtained from PromoCell (Heidelberg,

Germany). Horse serum for the differentiation medium was provided by Gibco (Berlin, Germany). All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

Culture of human skeletal muscle cells. Primary human skeletal muscle cells isolated from rectus abdominis muscle of five healthy Caucasian donors (3 males, 16, 21 and 47 of age; 2 females, 33 and 37 of age) were supplied as proliferating myoblasts and cultured as described in our earlier study[21]. For an individual experiment, myoblasts were seeded in six-well culture dishes at a density of 10^5 cells/well and were cultured in α-modified Eagle's (αMEM)/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 αMEM containing 2% horse serum until day 5 of differentiation followed by overnight starvation in αMEM without serum. The differentiated cells were incubated as indicated with CM, 2 µg/ml chemerin, 2 ng/ml MCP-1 and 50 pg/ml TNFα, respectively. Afterwards, cells were stimulated with 100 nmol/l insulin for 10min.

Electrical Pulse Stimulation (EPS). EPS was applied to fully differentiated human myotubes in six-well dishes using a C-Dish in combination with a C-Pace pulse generator (C-Pace 100, IonOptix, Milton MA). The instrument emits bipolar stimuli to the carbon electrodes of the C-dish which are placed in the cell culture media. The myotubes were stimulated with a frequency of 1Hz, pulse duration of 2ms and intensity of 11.5V for 2 to 24h. The medium was changed directly before the stimulation.

Adipocyte isolation and culture. Adipose tissue samples were obtained from subcutaneous fat of normal or moderately overweight women (BMI 27.9±0.9 kg/m^2, aged between 26 and 44 years). The procedure to obtain adipose tissue was approved by the ethical committee of Heinrich-Heine-University Duesseldorf (Germany). All subjects were healthy, free of medication, and had no evidence of diabetes according to routine laboratory tests. Adipose tissue samples were dissected from other tissues and minced in pieces of about 10mg in weight. Preadipocytes were isolated by collagenase digestion and differentiated as previously described[8,22]. After 15 days, 70-90% of seeded preadipocytes developed to

differentiated adipocytes, as defined by accumulation of lipid droplets. These mature adipocytes were then used to generate CM by incubation with αMEM for 48h followed by collection of the medium, as previously described[23].

Immunofluorescence staining. Human skeletal muscle cells were seeded on glass cover slips, differentiated and stimulated by electrical pulse stimulation. After 8 and 24h cells were fixed with 2% paraformaldehyd dissolved in PBS for 15min at room temperature. The cells were washed twice and permeabilized with 0.2% TritonX in buffer containing 20 mmol/l HEPES, 300 mmol/l saccharose, 50 mmol/l NaCl, 3 mmol/l $MgCl_2$ for 5min on ice. After blocking with 5% nonfat dry milk in PBS, the myotubes were incubated with anti-sarcomeric α-actinin, washed, and incubated with a secondary rhodamin-conjucated antibody. Myotubes were washed with 2xSSC buffer (0.3 mol/l NaCl, 0.03 mol/l sodium citrate, pH 7.0) and incubated with 100 µg/ml RNAse in 2xSSC buffer for 20min at 37°C. After washing with 2x SSC buffer, nuclei were stained with 5 µmol/l Syto13green for 5min at room temperature.

Electron Microscopy. Embedding of skeletal muscle cells in Epon 812 was performed as described by Luft[24] and modified by Reale[25]. In brief, cells were fixed in 2.5% glutaraldehyde/190 mmol/l cacodylat buffer, pH 7,4 and postfixed in 1% osmium tetroxide. 1% uranyl acetate and lead citrate[26] were used for staining ultrathin sections. Sections were investigated using a TEM 920A (Zeiss, Oberkochem, Germany).

Measurement of IL-6 and VEGF. Supernatants were collected after indicated time points and IL-6 concentration was determined by IL-6 ELISA (Biovendor) and VEGF ELISA (Biovendor), respectively, both assays used according to the manufacturer.

Cell Viability Assays. Human skeletal muscle cells were differentiated and electrically stimulated for 2 to 24h. *ATP Assay.* Changes of relative ATP levels were analyzed using ApoSENSOR ATP Cell Viability Assay Kit (BioVision) according to the instructions. *Lactate Assay.* L-(+)-lactate was detected in the supernatant applying Lactate Assay Kit II (BioVision). As positive control, lysis reagent supplied with the kit was used. *MTT assay*. The NADH content in the cells was determined using MTT (3-

(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) purchased from Sigma. Myotubes were incubated with 1.2 mmol/l MTT solution in medium for 2h at 37°C. Afterwards, cells were washed with PBS and lysed with 500 µl DMSO. Absorption at 540nm was determined using a plate reader (InfiniteM200, Tecan, Maennedorf, Switzerland). *Cytotoxicity assay.* Cell death was quantified by measuring lactate dehydrogenase (LDH) release into cultured media. Supernatant was collected after 8 and 24h and LDH measurements were performed with Cytotoxicity Detection Kit Plus (Roche Applied Science, Mannheim, Germany) and used according to the manufacturer's protocol.

Marker of mitochondrial function. Skeletal muscle cells were incubated with 1 µmol/l JC-1 for 30min under culture conditions after indicated time points. Afterwards, skeletal muscle cells were washed and analyzed using a plate reader (InfiniteM200, Tecan). JC-1 monomers were assessed using excitation/emission wavelengths of 485/530nm, while J-aggregates were measured using 560/595 nm.

Glucose uptake. Skeletal muscle cells were electrically stimulated for 7h on day 6 of differentiation. Uptake of 2-desoxy-glucose was measured for 2h after an acute 30min insulin stimulus (100nmol/l) as described before[8,27].

FA oxidation. Skeletal muscle cells were seeded on 10mm cover slips in six-well culture dishes and electrically stimulated for 24h as described above. Subsequently, cover slips were transferred to 48-well culture dishes and 11.1 kBq/well of ^{14}C-OA or ^{14}C-PA supplemented with 1 µmol/l L-carnitine were added to skeletal muscle cells. Culture dishes were incubated for 4h in an oxidation chamber, which allows gas exchange between two neighbouring wells. Filter papers soaked with NaOH were placed in the empty neighbouring wells. Oxidation was stopped and CO_2 was liberated via acidification of culture media by injecting 1 mol/l HCl and trapped in filter paper. Radioactivity was counted in a liquid scintillation counter (Beckman).

Immunoblotting. Human skeletal muscle cells were treated as indicated and lysed in a buffer containing 50 mmol/l HEPES (pH7.4), 1% TritonX, PhosStop, and Complete Protease Inhibitor cocktail from Roche. After sonification for 10min at 4°C,

the suspension was centrifuged at 15,000rpm for 15min. Thereafter, 5 µg of total cell lysates were separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane. The membranes were blocked with Tris-buffered saline (TBS) containing 0.1% Tween-20 and 5% nonfat dry milk or 5% BSA, respectively, and incubated overnight with the appropriate antibodies. After repeated washing, membranes were incubated with secondary horseradish peroxidase-coupled antibody and processed for enhanced chemiluminescence detection using Immobilion Western detection reagents (Millipore, Schwalbach, Germany). Signals were visualized and evaluated on a VersaDoc 4000 MP (BioRad, Munich, Germany) work station and analyzed by quantity one analysis software (Version 4.6.7).

Presentation of data and statistics. Data are presented as means ± SEM. Unpaired two-tailed Student's t test or one-way ANOVA (post hoc test: Bonferroni multiple comparison test) were used to determine statistical significance. All statistical analyses were performed using Prism5 (GraphPad, LA Jolla, CA) considering a P value of <0.05 as statistically significant. Corresponding significance levels are indicated in the figures.

Results

EPS induces de novo sarcomere structure assembly in human skeletal myotubes. After six days of differentiation, most of the human skeletal muscle cells fused and formed typical multinucleated myotubes. As reported in our earlier study[20], differentiating skeletal muscle cells display an increased expression of myogenin, MyoD, and MHC, which are typical markers of myogenesis, but cells did not show spontaneous contractile activity. However, after 2 to 3h of continuous EPS with 1Hz, 2ms, and 11.5V, a subset of myotubes showed a noticeable, vigorous contraction, and after 8h EPS most of the myotubes contracted. Immunofluorescence staining of sarcomeric α-actinin showed a uniform distribution of the protein in the cytoplasm of unstimulated cells. After up to 24h of EPS, a reorganization of the cytoskeleton and *de novo* formation of sarcomeric structures was observed (Fig. 6.1A-C). These findings could be confirmed by electron microscopy (Fig. 6.1D-F). After 8h of EPS, sarcomeres became visible with a typical

striated pattern. The stimulated cells had a highly ordered periodic structure composed of thin filaments attached to the Z-line and thick filaments located in the middle of the sarcomere. The typical Z-lines appeared as a series of dark lines as described for cross-striated muscles. The sarcomeric structures were still visible after 24h of EPS.

EPS increases IL-6 and VEGF secretion as well as AMPK activation. EPS did not influence the protein abundance of motor proteins like MHC and sarcomeric α-actinin. In addition, protein level of glucose transporters GLUT1 and GLUT4 was not altered (Fig. 6.2A). Contraction induced by EPS upregulated secretion of IL-6 and VEGF, two known myokines. Secretion of IL-6 increased in non-stimulated cells during the first 4h to 14±4 pg/ml, but did not change afterwards. EPS significantly upregulated IL-6 secretion after 8 and 24h compared to controls, reaching a concentration of 35±8 pg/ml after 24h (Fig. 6.2B). Secretion of VEGF increased in control cells from 32 ng/ml after 4h to 55±30 pg/ml after 24h. EPS immediately upregulated VEGF secretion. VEGF concentration in the supernatant is increased after 2h of EPS, reaching a maximum after 24h with 274±40 pg/ml (5fold increase compared to control, Fig. 6.2C).

ATP content in skeletal muscle cells significantly increased during the first 2h of EPS (1.5fold). Afterwards, the ATP level in skeletal muscle cells decreased (Fig. 6.2D). After 8h of EPS, ATP content was slightly, but not significantly, lower than in controls and this decrease became significant after 14 and 24h. AMPK is not phosphorylated in non-stimulated human skeletal muscle cells after 8 and 24h. EPS of skeletal muscle cells induced a 3.8fold increase in AMPK(Thr172) phosphorylation after 8h (Fig. 6.2E). This effect was diminished but still significant after 24h.

EPS exerts no cell damage on myotubes. Decreased levels of ATP and increased levels of ADP are recognized in apoptotic cells. To exclude that EPS exerts any cell damage, several cell based assays were performed. Cytotoxicity, as assessed by LDH release, did not differ significantly between cells with or without 8 or 24h of EPS (Fig. 6.3A). The NADH level in the cells, as assessed by MTT assay, was not changed after 8 and 24h EPS compared to control cells (Fig. 6.3B). JC-1 is a common tool to assess the polarization status of mitochondrial membranes, as this fluorescent dye forms so-called J-aggregates in intact negatively charged

mitochondria. When the mitochondrial membrane potential collapses, JC-1 is dispersed throughout the cell in a monomeric form. However, a change in ratio of JC-1 aggregates to monomers could not be observed during culture time neither in control cells nor in electrically stimulated cells after 8 and 24h (Fig. 6.3C). Concentration of lactate in the medium significantly increased from 8 to 24h culture time (Fig. 6.3D) from 9±0.4 nmol/ml to 17±0.5 nmol/ml. EPS induced significantly higher lactate concentrations in the medium compared to controls. Based on these results we conclude that EPS induces contraction of human skeletal myotubes without damaging the cells.

EPS augments insulin-stimulated glucose uptake, but does not change FA oxidation. To test whether EPS induces changes of glucose homeostasis in skeletal muscle cells, we measured glucose uptake. Insulin-stimulated glucose uptake was significantly increased over basal level (2.6fold, Fig. 6.4A), as reported in our earlier studies[20,27]. Contraction of skeletal muscle cells led to a significant 2.4fold increase compared to basal control. Importantly, skeletal muscle cells showed a marked increase in insulin-stimulated glucose uptake after EPS compared to basal control (5.0fold, n=5). The difference between insulin-stimulated and basal glucose uptake, designated as increment of insulin-stimulated glucose uptake, is profoundly augmented by contractile activity of myotubes (Fig. 6.4B). Fatty acid oxidation of oleic acid and palmitic acid was assessed by $^{14}CO_2$ production. However, oxidation of both fatty acids was not changed after contraction induced by EPS (Fig. 6.4C).

EPS protects skeletal muscle cells from impaired insulin signalling induced by CM, MCP-1, and chemerin. Under control conditions, insulin induced a significant increase of Akt phosphorylation (Ser473) in human skeletal muscle cells, with a significant ($p<0.01$) reduction of this response after incubation with CM (Fig. 6.5A). When cells were electrically stimulated during incubation with CM, this effect on insulin-stimulated Akt phosphorylation was abrogated. Comparable effects were observed at the level of GSK3α (Ser21) phosphorylation, with a very prominent inhibition by CM, which was completely prevented by EPS. Chemerin and MCP-1 induced a significant reduction of insulin-stimulated Akt phosphorylation at Ser473 and Thr308 sites, respectively, as reported in our earlier studies[7,8,21]. By applying EPS during treatment with chemerin and MCP-1, respectively, normalized insulin

signalling in skeletal muscle cells was observed, with insulin-stimulated Akt phosphorylation levels being similar to control (Fig. 6.5B).

EPS prevents activation of NFκB and p42/44 MAPK in skeletal muscle cells. NFκB and IκB kinase (IKK) β protein abundances were significantly reduced in EPS-treated cells, whereas IKKα and IκBα protein abundances were not affected (Fig. 6.6A). Incubation of cells with TNFα led to an activation of NFκB, which is specifically mediated by IκB kinase downstream signalling, as shown by application of the IKK inhibitor I229 (Fig. 6.6B). Incubation of skeletal muscle cells with TNFα induces proinflammatory signalling reaching a maximal activation of NFκB after 10min (3.2fold, Fig. 6.6C). NFκB activation is diminished after EPS, because of a reduced NFκB protein abundance. Most importantly, maximal NFκB activation in response to TNFα is substantially reduced after 8h (1.7fold after 10min). Thus contractile activity exerts an anti-inflammatory action and interferes with TNFα-induced NFκB signalling.

Incubation of skeletal muscle cells with TNFα led to a significant decrease of 59% after 20min in IKKβ protein level (Fig. 6.6D). In contrast, IKKβ protein level was significantly reduced after 8h EPS compared to controls (Fig. 6.6A) and was not altered after TNFα treatment (Fig. 6.6D). Incubation of myotubes with TNFα diminished IκBα protein abundance by more than 60% after 20min, while TNFα had no effect on IκBα protein level of contracting cells (Fig. 6.6D). CM and chemerin induced activation of NFκB (1.8fold and 1.5fold, Fig. 6.7A), while MCP-1 induced significant activation of p44/p42 MAPK (1.5fold, Fig. 6.7C). Both effects on NFκB and p44/p42 MAPK activation were diminished to control level after EPS (Fig. 6.7B and C).

Discussion

Exercise has been shown to have a positive impact on a number of diseases in humans, including obesity and T2D[1,2,10]. The pathogenesis of T2D has intensively been studied and is characterized by chronic hyperglycaemia resulting in defects in insulin secretion, insulin action, or both. Research in the last 5 to 10 years highlighted the positive impact of exercise in the context of obesity[1,10,28]. However, knowledge of the interplay between different molecular signalling pathways during exercise is still incomplete and experimentally adequate model systems for exercise remain elusive.

In this study we established and validated a model system of contracting human skeletal muscle cells and used this system to further analyze the beneficial effect of exercise in the context of insulin resistance.

It is well known that human skeletal muscle cells display increased expression of typical myogenesis markers like myogenin, MyoD, MHC, and GLUT4 during differentiation *in vitro*[20]. One limitation of these models is the lack of contraction, one important chief characteristic of muscle cells. Therefore, we stimulated skeletal muscle cells with electrical pulses, resulting in vigorous contraction and in formation of striation pattern of sarcomeric structures, visualized by immunofluorescence staining of α-actinin and electron microscopy. Accordingly, Fujita et al observed *de novo* formation of sarcomers after EPS in murine C2C12 cells, whereas calcium channel blockers like verapamil and BAPTA-AM suppressed sarcomere structure development[29]. Thus, it can be speculated that manipulated Ca^{2+} transients by applying an appropriate EPS to differentiated human skeletal muscle cells are primarily necessary to accelerate *de novo* sarcomer assembly and to rapidly develop contractile activity.

In skeletal muscle three energy systems function to replenish ATP in muscle, namely creatine kinase, glycolysis, and mitochondrial respiration. All energy systems contribute to different degrees to replenishment of ATP based on an interaction between the intensity and duration of exercise[30]. Using 1Hz frequency, 2ms pulse duration, and 11.5V intensity, we could observe an enhanced glucose uptake after 8h and an increased lactate concentration after 8h and 24h of EPS compared to unstimulated control. During high performance sports glucose is catabolised and pyruvate is substantially generated. When the mitochondrial capacity is exceeded, pyruvate is reduced to lactate resulting in oxidation of $NADH/H^+$ to NAD^+. Since we could not observe an increased fat oxidation after 24h EPS, it is likely that cells mainly perform glycolysis for ATP regeneration under the applied conditions.

C2C12 cells derived from mouse skeletal muscle are mostly used to investigate muscle cell differentiation and development, including sarcomere development and myotube contraction[29,31]. However, cells do not express sufficient levels of GLUT4 proteins, and their insulin responsiveness is reportedly minimal even after differentiation[32]. In our model, GLUT4 expression was not altered in cells during EPS, but human cells exhibited a profound insulin-stimulated glucose uptake (2.6fold), compared to C2C12 cells (1.4fold)[31]. Most importantly, we observed higher

insulin-stimulated glucose uptake after EPS. Human studies using the one-legged exercise model followed by a euglycemic hyperinsulinemic clamp showed increased insulin sensitivity after exercise[33]. There is considerable amount of data supporting the hypothesis that AMPK plays a role in stimulation of glucose uptake[34-36]. First evidence came from a study showing that treatment with AICAR, an activator of AMPK, in perfused rat hindlimb muscles had the dual effect of enhancing fatty acid oxidation and increasing basal glucose uptake to provide energy for muscle contraction[36]. Thus, increased AMPK activity after contraction may play a role in increasing uptake of glucose to meet increased energy requirements, but might not be the only regulation of contraction-induced muscle glucose uptake. Therefore, pathways which are involved in regulating contraction-stimulated glucose uptake would be an attractive target for pharmacological treatment of T2D.

One of the major advancements in obesity research is the recognition that adipose tissue is a major endocrine organ, which secretes a tremendous variety of adipokines. Approximately one hundred separate adipokines have now been identified. Secretion of adipokines is changed dramatically in the obese state, leading to a wide range of physiological functions and to insulin resistance in skeletal muscle among others[6,9,37]. A main finding of the present study was that contracting skeletal muscle cells exhibited improved insulin sensitivity in conditions of insulin resistance. As previously shown by our group, adipocyte conditioned medium, which contains the whole secretory output of mature adipocytes including several adipokines like chemerin, PEDF, and MCP-1, induces insulin resistance in skeletal muscle cells at the level of Akt and GSK3 as well as reduces insulin-stimulated glucose uptake by activating inflammatory signalling pathways[7,8,21,38]. Incubation of human skeletal muscle cells with CM or adipokines is a suitable model to dissect different mechanisms leading to muscle insulin resistance. In this study, we could show that EPS completely prevented insulin resistance in skeletal muscle cells at the level of Akt and GSK3 during incubation with CM, MCP-1, or chemerin.

A potential reason for this observation might be the blocking effect of EPS on NFκB activation by CM and several adipokines. Some adipokines that are involved in the development of insulin resistance are known to activate inflammatory signalling pathways by activating IKK and its downstream effector NFκB[39]. These proteins belong to a family of transcription factors that controls the production of pro-inflammatory proteins. In our model CM, chemerin, and TNFα induced an activation

of NFκB, which is in agreement with elevated NFκB activity in muscle of insulin-resistant subjects in comparison to lean control subjects under basal conditions[40]. EPS, mimicking contraction of an active muscle, diminished NFκB activity and prevented activation of NFκB by CM and chemerin. Additionally, the increment of NFκB activation by TNFα treatment after 8h of EPS was profoundly diminished. Thus, contraction seems to be an inhibitor for the activation of NFκB. Other studies showed that silencing of IKK via siRNA prevents TNFα-induced insulin resistance[41] and *in vivo* experiments carried out in humans have shown that salicylates, which function as inhibitors of NFκB, improve insulin sensitivity in T2D[42]. Thus, our model provides evidence that the anti-inflammatory potential of contraction might mediate the beneficial effect of muscle contraction.

With this contraction model we could observe that the risk of insulin resistance is diminished by contraction, since insulin signalling was not disturbed after incubation with adipocyte-conditioned medium and inflammatory signalling was not activated. This model provides a unique tool to investigate mechanisms and underlying signalling pathways, which mediate the beneficial effects of muscle contraction and will help to further clarify the potential of exercise in order to combat insulin resistance.

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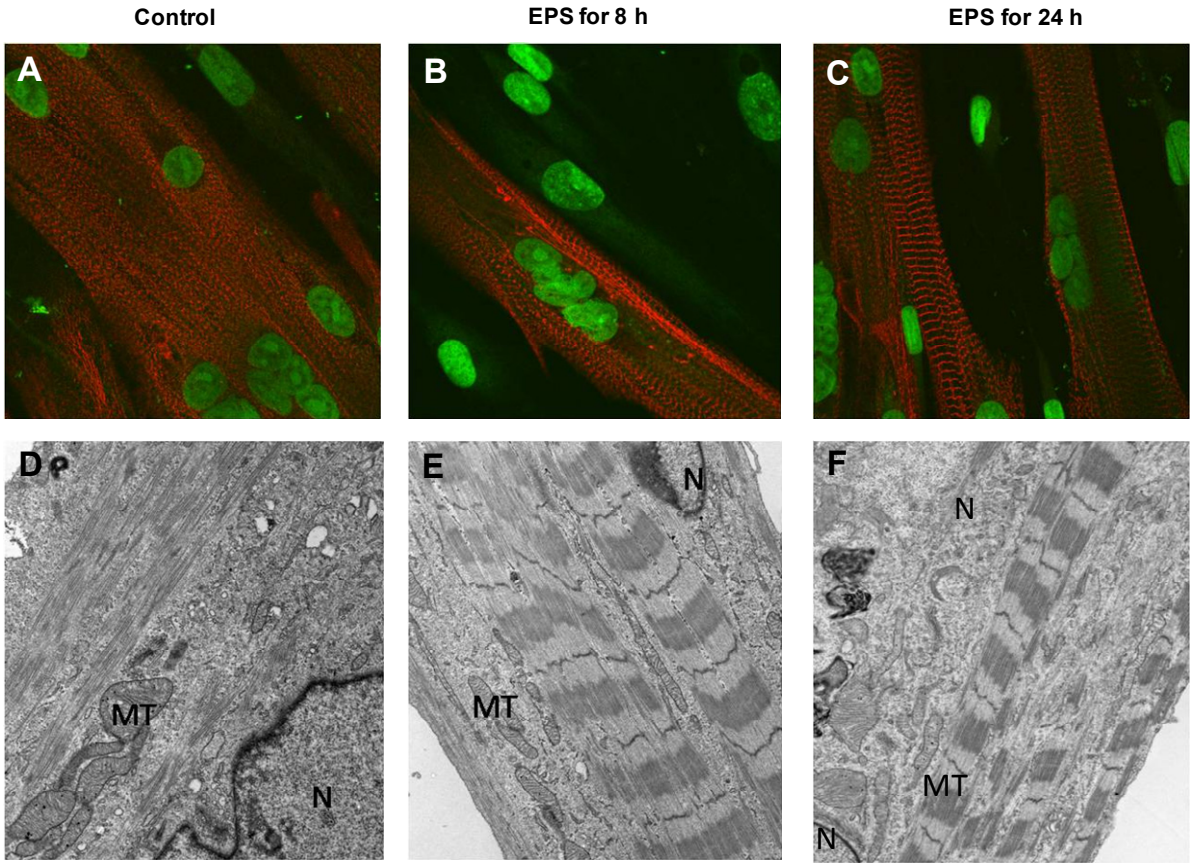

Fig. 6.1. Effect of EPS on sarcomere structure assembly in human skeletal myotubes. A-C: The cells were fixed and analyzed for the localization of sarcomeric α-actinin after 8 and 24h EPS (1Hz, 2ms, 11.5V) by immunofluorescence staining, as described in Research Designs and Methods. Red, sarcomeric α-actinin; green, sytogreen; 100x magnification. Sarcomeric α-actinin is evenly distributed in the cytoplasm without EPS (A). Sarcomers become rapidly visible after EPS and Z-lines appear as a series of red lines (B, C). These findings could be confirmed by electron microscopy (D-E). MT = mitochondria, N = nucleus

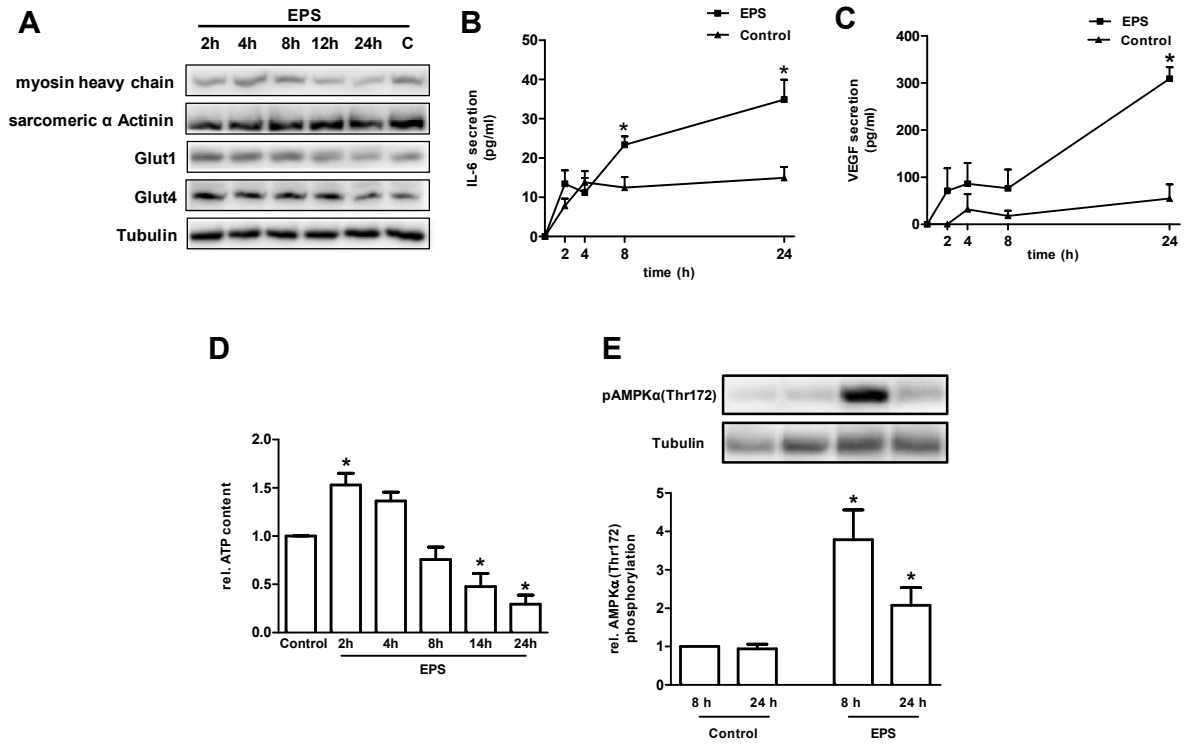

Fig. 6.2. Effects of EPS on the expression of muscle proteins, secretion of IL-6 and VEGF, and AMPK activation. A: Human skeletal muscle cells were differentiated and electrically stimulated for 2, 4, 8, 12h and 24h with 1Hz, 2ms, and 11.5V. Total cell lysates were resolved by SDS-PAGE and representative blots for MHC, sarcomeric α-actinin, GLUT1, and GLUT4 are shown. B and C: IL-6 and VEGF secretion of human myotubes with and without EPS was measured after indicated time points by ELISA, respectively ($n \geq 3$, $p < 0.05$). D: Relative ATP concentration of myotubes was measured after indicated time points of EPS and in control, as described in Research Design and Methods. E: Total cell lysates were obtained and analyzed by SDS-PAGE and Western blot using phospho-specific AMPKα antibody. Data are means ± SEM, $n \geq 3$ and are normalized to tubulin, * $P \leq 0.01$ vs. corresponding basal level.

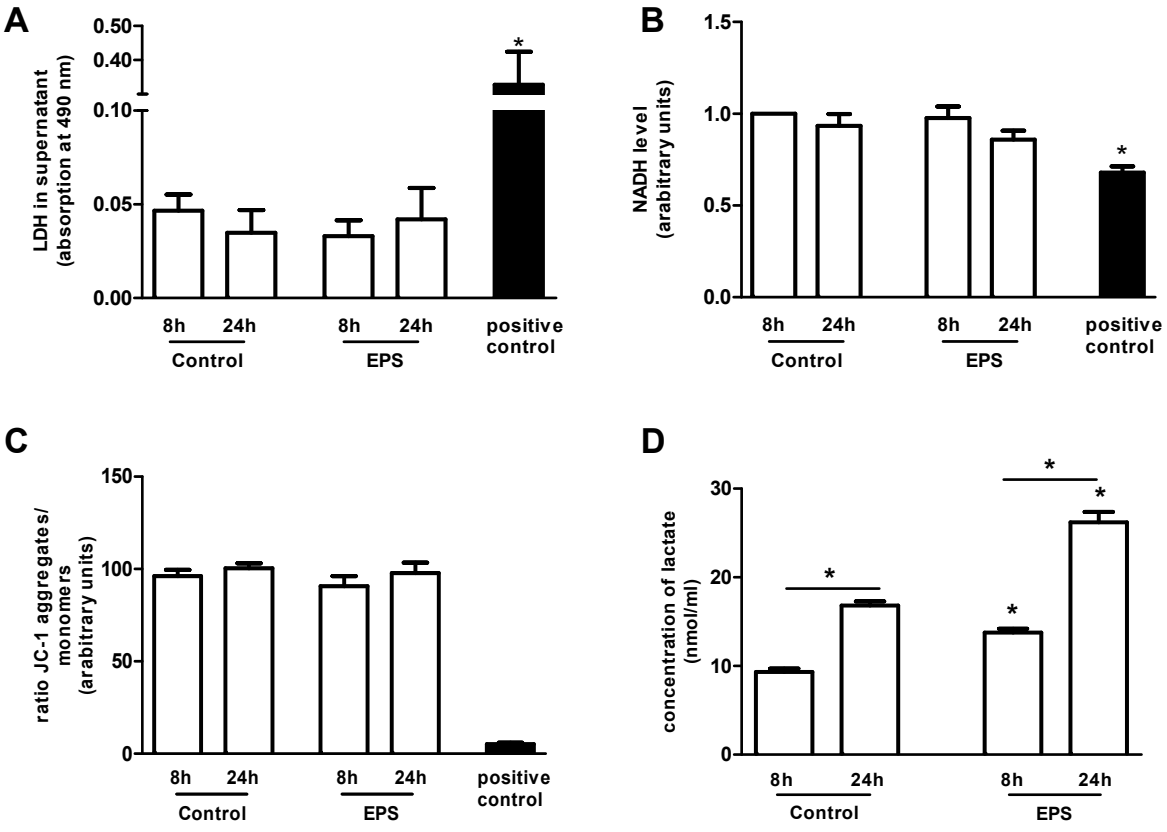

Fig. 6.3. Determination of metabolic activity of skeletal muscle cells after EPS. Human skeletal muscle cells were differentiated and stimulated for 8 and 24h with 1Hz, 2ms, 11.5V. A: Lactate dehydrogenase abundance in the medium was measured as described in Research Designs and Methods ($n \geq 5$). B: The quantity of NADH in the cells was measured using a MTT assay as described in Research Designs and Methods ($n \geq 3$). C: Ratio of JC-1 aggregates to monomers was determined. As positive control 100 µmol/l CCCP for 45 min was used prior to JC-1 staining. D: Concentration of lactate in the supernatant of electrically stimulated and untreated cells was determined as described in Research Designs and Methods ($n \geq 3$, * $P < 0.001$).

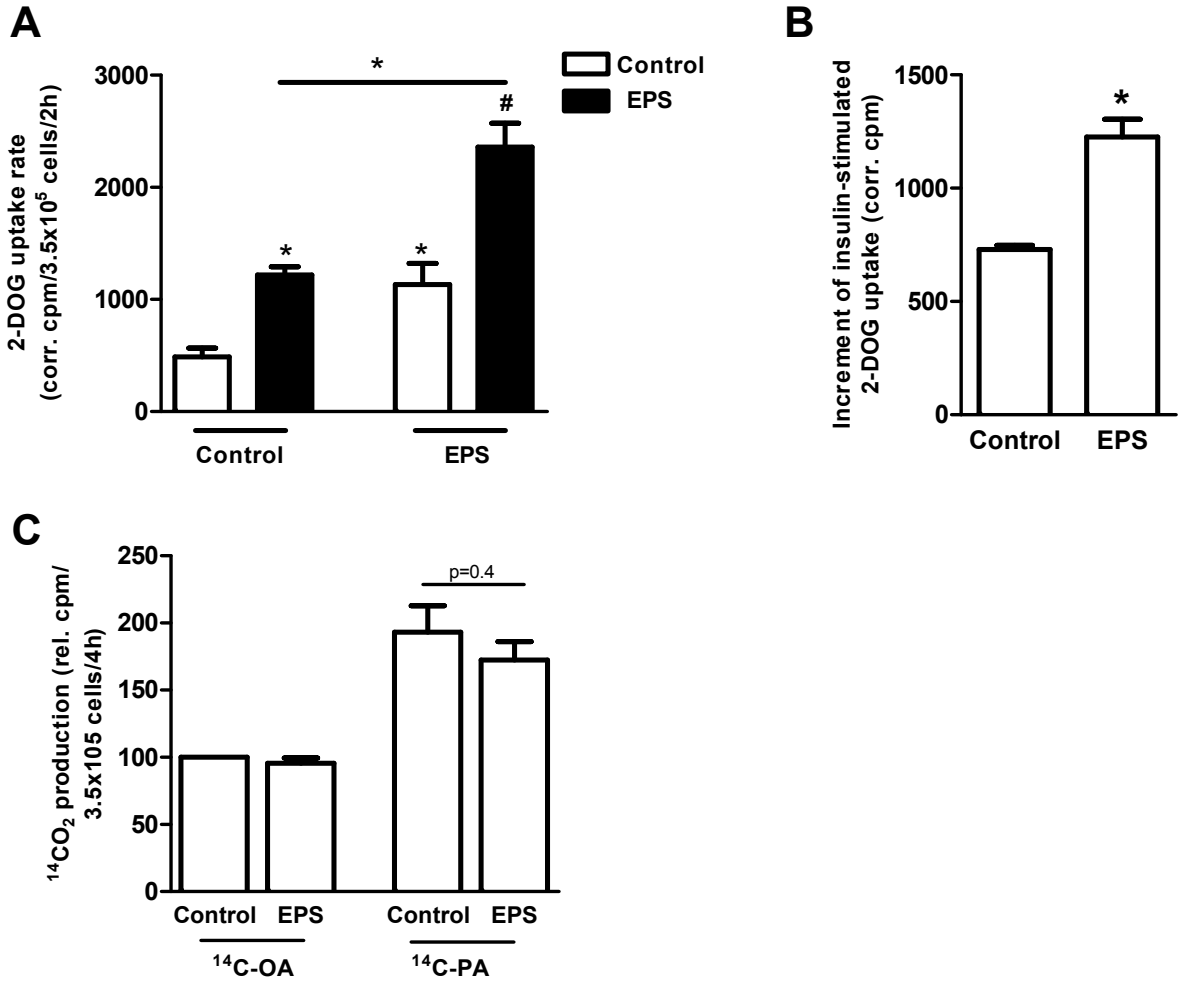

Fig. 6.4. Effect of EPS on glucose uptake and fat oxidation in human skeletal muscle cells. A: Glucose uptake was assessed for 2h after acute stimulation with 100 nmol/l insulin after 8h EPS (1Hz, 2ms, 11.5V) (n = 5, *p < 0.05 vs basal control, # p < 0.05 vs basal control and EPS control). B: Increment of insulin-stimulated glucose uptake was calculated as the difference between insulin stimulated glucose uptake and basal control (n = 5, *p< 0.05). C: Fat oxidation was assessed as outlined in Research Design and Methods section.

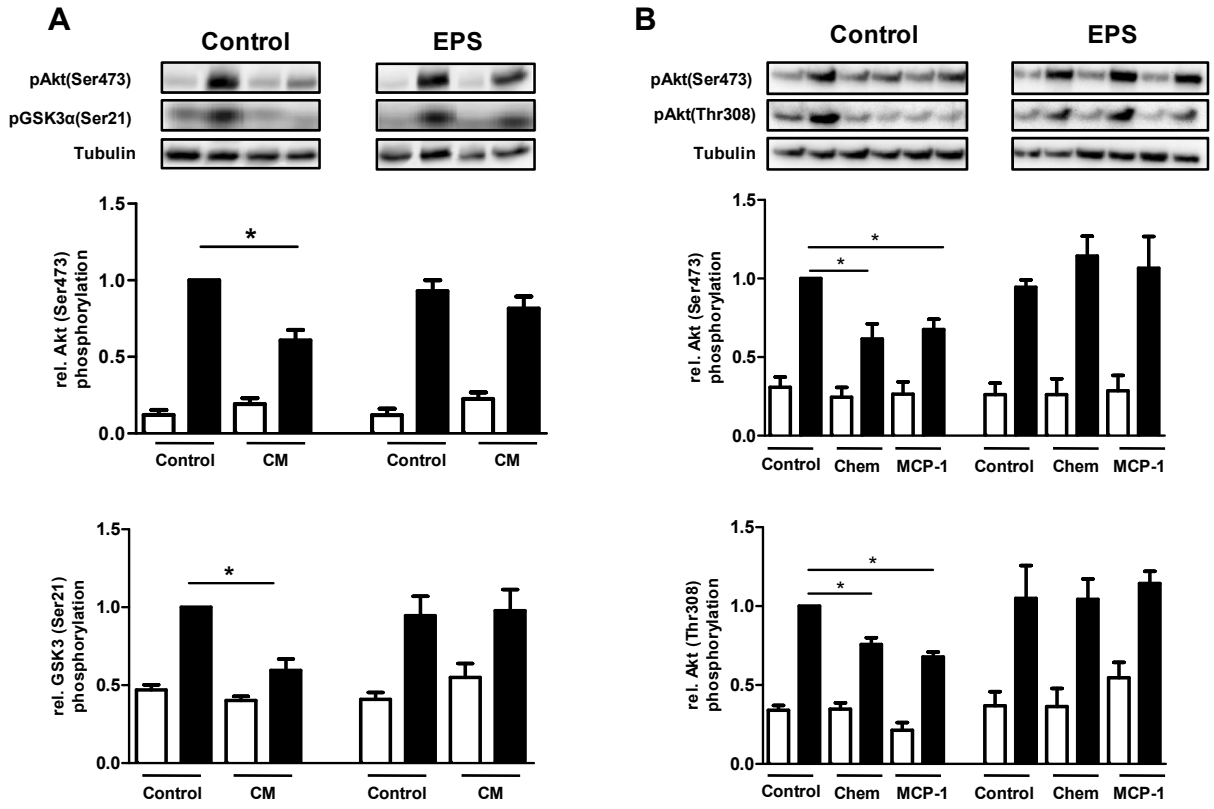

Fig. 6.5. Effect of CM, chemerin, and MCP-1 on insulin signalling in control and electrically stimulated human skeletal muscle cells. A. Myotubes were simultaneously incubated with CM for 8h and electrically stimulated. After acute stimulation with insulin, total cell lysates were obtained, resolved by SDS-PAGE and immunoblotted with phospho-specific Akt and GSK3 antibodies. All data were normalized to the level of tubulin and are expressed relative to insulin-stimulated control value. Data are means ± SEM, n ≥ 5, * P < 0.05. B. Myotubes were incubated with 2 µg/ml chemerin and 2 ng/ml MCP-1, respectively, and simultaneously stimulated electrically. After acute stimulation with insulin, total cell lysates were obtained, resolved by SDS-PAGE and immunoblotted with phospho-specific Akt antibodies. All data were normalized to the level of tubulin and are expressed relative to the insulin-stimulated control value. Data are presented as means ± SEM, n ≥ 4, * P < 0.05 vs. insulin-stimulated control. White bars: basal, black bars: 100 nmol/l insulin.

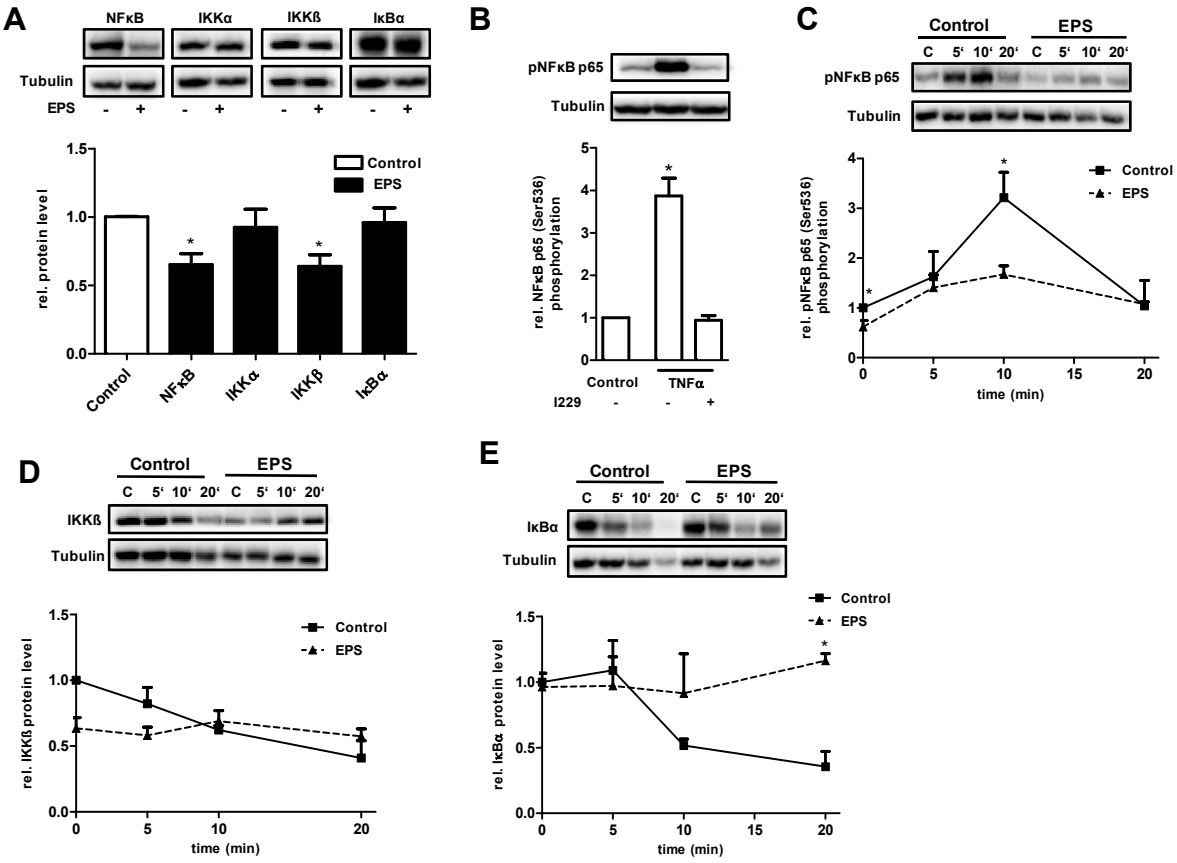

Fig. 6.6. Prevention of TNFα-induced inflammatory signalling by EPS. A. Skeletal muscle cells were stimulated by EPS for 8h, total cell lysates were resolved by SDS-PAGE and protein levels of NFκB, IKKα, IKKβ, and IκBα were determined by Western Blotting. B. Skeletal muscle cells were pre-incubated with 10 µmol/l specific IKK inhibitor I229 and afterwards stimulated with 50 pg/ml TNFα for 10 min. C-E. Cells were treated with 50 pg/ml TNFα for 5, 10, and 20 min after 8h EPS. Total cell lysates were resolved by SDS-PAGE and immunoblotted with phospho-specific antibody for NFκB (C), specific antibody for IKKß (D) and IκBα (E). All data were normalized to the level of tubulin. Data are presented as means ± SEM, n ≥ 3, * P < 0.05 vs. basal level.

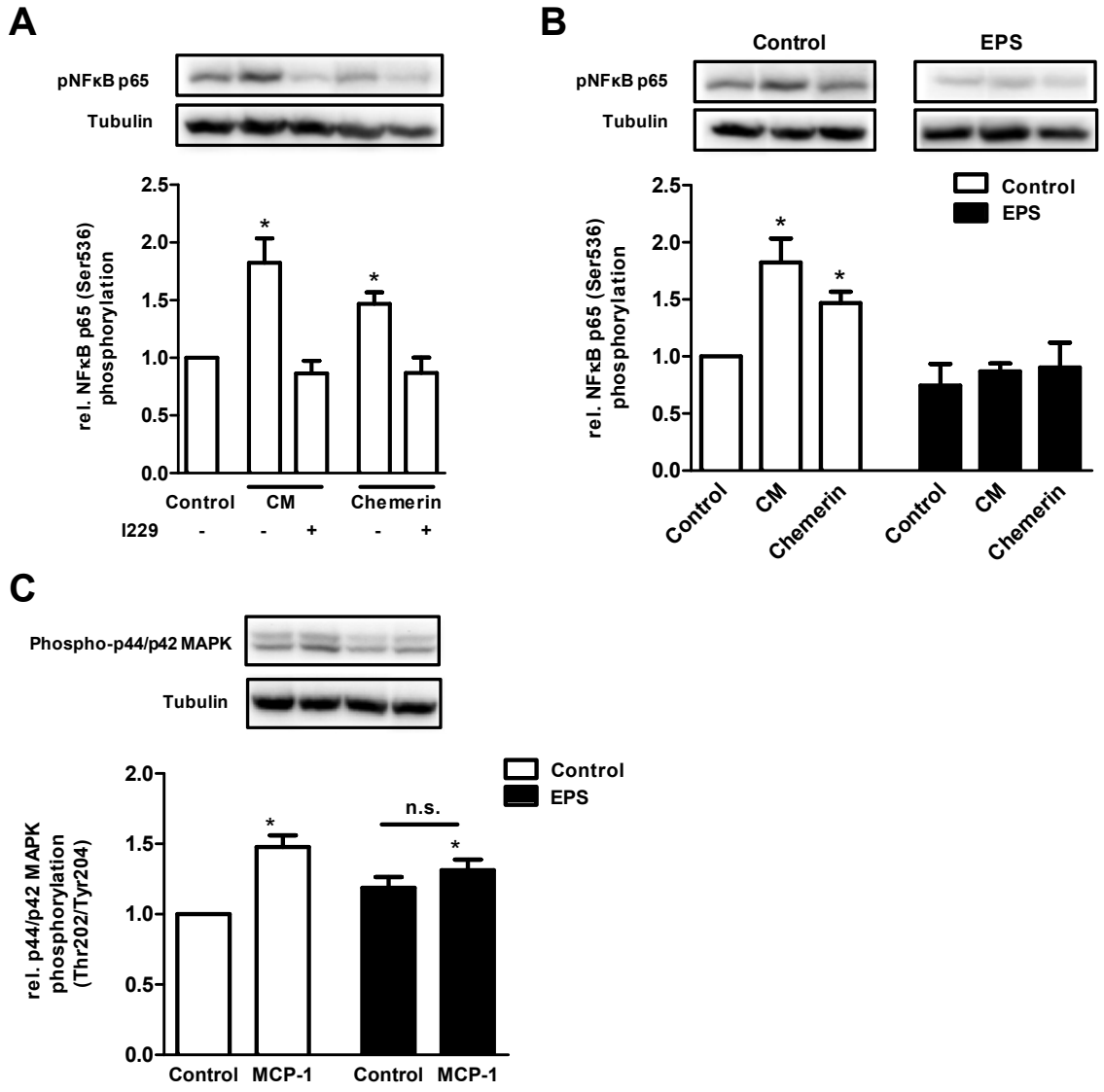

Fig. 6.7. Prevention of CM-, MCP-1-, and chemerin-induced inflammatory signalling by EPS. A: Skeletal muscle cells were pre-incubated with or without 10 µmol/l of the specific NFκB inhibitor I229. Afterwards, cells were treated with CM and chemerin for 30 min. Total cell lysates were resolved by SDS-PAGE and immunoblotted with a phospho-specific antibody for NFκB. B+C: Skeletal muscle cells were electrically stimulated for 8h with 1Hz, 2ms, and 11.5V. Cells were treated with CM and chemerin (B) with 2 ng/ml MCP-1 (C) or for the last 30 min of stimulation. Total cell lysates were resolved by SDS-PAGE and immunoblotted with a phospho-specific antibody for NFκB. All data were normalized to the level of tubulin. Representative Blots are shown. Data are the means ± SEM, n ≥ 4, * $P < 0.05$ vs. basal level.

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CHAPTER 7

Study 6

Adipokines Promote Lipotoxicity of Fatty Acids in Human Skeletal Muscle Cells†

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Abbreviations: ADRP, adipocyte differentiation-related protein; ^{14}C-BrPA, ^{14}C-bromo palmitic acid; CM, adipocyte-conditioned medium; EPS, electrical pulse stimulation; FA, fatty acids; IMCL, intramyocellular lipids; MCP-1, monocyte chemotactic protein-1; OA, oleic acid; PA, palmitic acid; PEDF, pigment epithelium-derived factor; SkMC, skeletal muscle cells; VEGF; vascular endothelial growth factor

Abstract

Isolated adipokines as well as increased plasma free fatty acid (FA) levels have been independently associated with the development of type 2 diabetes (T2D). In order to analyze the more physiological combination of the entire adipocyte secretome with FA, this study investigated the lipotoxic potential of adipocyte-conditioned medium (CM) combined with physiological concentrations of oleic (OA) and palmitic acid (PA) on human skeletal muscle cell (SkMC) metabolism. Since regular physical activity has been demonstrated to induce beneficial effects on muscle insulin sensitivity and metabolic performance, the potential of electrical pulse stimulation (EPS) to counteract adipokine- and FA-induced effects was investigated. Combination of OA+CM led to massive accumulation of intramyocellular lipid (IMCL) droplets. Treatment of SkMC with CM increased CD36 protein abundance and FA uptake. Incubation of SkMC with OA+CM and PA+CM profoundly reduced FA oxidation. Although EPS-treatment could not attenuate IMCL accumulation, CM-induced increases in CD36 protein abundance and FA transport were prevented by EPS. Importantly, EPS was able to counteract impairments of FA oxidation induced by OA+CM but not PA+CM. In conclusion, the results of our study suggest (I) a novel role for adipokines in promoting lipotoxicity of relatively low FA concentrations, (II) severe impairments of cellular functionality in response to saturated but not unsaturated FA-treatment and (III) the potential of contractile activity to selectively prevent these lipotoxicity-mediated impairments. Unraveling the physiological interplay between adipokines, FA, and physical exercise will help to find efficient therapeutic strategies to prevent or reverse adverse effects leading to T2D.

Introduction

Obesity is one of the major risk factors contributing to the development of type 2 diabetes (T2D)[1]. Enlargement of adipose tissue, especially in the visceral region, is characterized by an altered adipokine secretion pattern[2-4]. As part of the negative crosstalk between adipose tissue and skeletal muscle, these obesity-associated adipokines promote skeletal muscle insulin resistance[5-8], an early impairment and central defect in the pathogenesis of T2D[9,10]. Considering the essential role of skeletal muscle in postprandial glucose disposal[11], muscle insulin resistance is especially critical in the pathogenesis of T2D. Several studies have demonstrated deleterious effects of isolated adipokines like leptin[12], adiponectin[13], resistin[14], TNFα[15], and IL-6[16] on muscle FA uptake, oxidation, lipolysis, or insulin response[12-17]. However, recent studies applying highly sensitive proteomic approaches have revealed the complex nature of the human adipose tissue secretome, comprising hundreds of different factors[18-21]. Thus, application of single adipokines yields a limited picture of the *in vivo* situation. To more closely mimic the physiological complexity, we have previously established an *in vitro* crosstalk model using adipocyte-conditioned medium (CM) derived from human differentiated adipocytes to induce skeletal muscle cell insulin resistance and impaired glucose uptake[22,23].

In addition to skeletal muscle insulin resistance, T2D patients frequently exhibit increased plasma lipid levels. On the one hand, this may be a consequence of an obesity-associated lifestyle, comprising imbalanced nutrient intake and energy expenditure. On the other hand, adipose tissue dysfunction and resistance to the lipolysis-suppressing effect of insulin may contribute to these increased plasma free fatty acid (FA) levels[24]. As a consequence, FA concentrations may rise from physiologically healthy levels (~0.1 mmol/l) to pathological levels of 0.6 mmol/l to 0.9 mmol/l in obesity and T2D[25]. However, high circulating plasma FA concentrations have been demonstrated to promote the development of skeletal muscle insulin resistance[26-29], most likely via accumulation of lipids inside the muscle cell[30-32]. Impaired skeletal muscle mitochondrial function including a decreased capacity to oxidize fat, as it is often observed in T2D patients, has been suggests to play a central role in this context[33-35]. Lifestyle intervention therapies involving nutrition

modifications and increased physical activity have been shown to effectively improve insulin sensitivity and delay or prevent the onset of T2D[36,37]. Especially, enhanced physical exercise has been demonstrated to induce muscle adaptation processes leading to augmented insulin sensitivity and improved mitochondrial performance[38]. While a number of studies have investigated the impact of isolated adipokines and various FA concentrations, respectively, the role of adipokines in the interplay with FA and the combined impact on skeletal muscle metabolism however remain poorly understood. Therefore, the aim of this study was to investigate the combined effects of the entire adipocyte secretome and physiological concentrations of FA on human skeletal muscle metabolism. In this context, saturated and unsaturated FA were studied to identify possibly diverging implications. Additionally, a recently established innovative *in vitro* model of muscle contraction was applied to assess the potential influence of physical activity on skeletal muscle metabolism in the interaction with adipokines and FA.

Materials and Methods

Materials. Reagents for SDS-PAGE were purchased from GE Healthcare (Uppsala, Sweden). Oleic acid [1-^{14}C] (^{14}C-OA) and palmitic acid [1-^{14}C] (^{14}C-PA) were purchased from Perkin Elmer (Waltham, MA, USA), while 2-bromopalmitic acid [1-^{14}C] (^{14}C-BrPA) was obtained from Hartmann Analytic GmbH (Braunschweig, Germany). Liquid scintillation Aqua safe 300 plus was provided by Zinsser Analytic (Frankfurt, Germany). VEGF and PEDF were purchased from Millipore (Billerica, MA, USA), MCP-1 from Peprotec (Hamburg, Germany), and chemerin from R&D Systems (Minneapolis, MN, USA). All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

*Generation and analysis of adipocyte-conditioned media (CM)***.** CM was generated as described previously[22,23]. Briefly, human preadipocytes were isolated from adipose tissue samples obtained from subcutaneous fat of normal or moderately overweight women. Preadipocytes were cultured and differentiated for 15 days. Mature adipocytes were used to generate CM by incubation with α-

modified Eagle's medium (αMEM) for 48h followed by collection of the medium. Several components of the CM have been analyzed in previous studies[39]. Myostatin content was quantified using an ELISA (Hoelzel Diagnostika GmbH, Cologne, Germany).

Culture of human SkMC. Primary human skeletal muscle cells (SkMC) of 6 healthy Caucasian donors were supplied as proliferating myoblasts (PromoCell, Heidelberg, Germany; Lonza, Basle, Switzerland) and cultured as described previously[40]. For individual experiments, myoblasts were cultured in αMEM/Ham's F-12 medium (Gibco, Berlin, Germany) containing SkMC growth medium supplement (PromoCell) up to near confluence. Subsequently, SkMC were differentiated and fused by culture in αMEM containing 2% horse serum for 5 days. On day 5 the medium was changed to αMEM without serum. Differentiated SkMC were then incubated with CM, VEGF (250 pg/ml), PEDF (10 nmol/l), MCP-1 (200 pg/ml), chemerin (1 µg/ml), heat inactivated CM (95°C, 10 min), oleic acid (OA; 100 µM) or palmitic acid (PA; 100 µM) (OA and PA bound to FA free BSA at a ratio of 2.5:1 dissolved in αMEM), as indicated in figure legends. Furthermore, SkMC were incubated with corresponding amounts of BSA as controls.

Electrical Pulse Stimulation (EPS). Differentiated human myotubes were subjected to EPS-treatment in 6-well dishes using a C-Dish in combination with a C-Pace pulse generator (C-Pace 100, IonOptix, Milton MA, USA). The instrument emits bipolar stimuli to the carbon electrodes of the C-dish, which are placed in the cell culture media. SkMC were stimulated with a frequency of 1 Hz, a pulse duration of 2 ms, and an intensity of 11.5 V for 24h. Electrical stimulation was performed in parallel to CM-treatment for 24h. Culture medium was changed before the start of the stimulation.

Nile Red staining. After incubation with CM and FA, SkMC were fixed and stained for 20 min with 100 µg/ml Nile Red (Biomol, Hamburg, Germany) dissolved in DMSO. SkMC were viewed with a 20x plan objective lense using a Zeiss Axiovert 200M microscope and Zeiss LSM 5 PASCAL software (Zeiss, Jena, Germany). Simultaneous excitation with wavelengths of 488 nm and 543 nm was applied to visualize red as well as green/gold fluorescence.

*Immunoblotting***.** For analysis of protein levels of CD36 (kind gift from J.F. Glatz, Maastricht), β-actin (Abcam, Cambridge, UK), tubulin (Calbiochem, Darmstadt, Germany), and FA transport protein 4 (FATP4, Abnova, Taipei City, Taiwan), SkMC lysates were prepared and analyzed using SDS-PAGE and Western Blot as described previously[41]. Western Blotting studies on ADRP protein abundance were performed with whole-cell lysates in NP40-lysis buffer (62.5 mmol/l TRIS-HCL, 12.5% glycerol, 2%NP40, 1.25 mmol/l EDTA, and protease inhibitors) mixed 1:1 with Laemmli sample buffer (Bio-Rad, Mississauga, Ontario, Canada), and incubated with anti-ADRP antibody (Progen, Heidelberg, Germany).

*Quantification of intracellular triglyceride content***.** A commercial triglyceride quantification kit (BioCat, Heidelberg, Germany) was used to assess triglyceride content. SkMC were treated as indicated and lysed in a 5 % Triton-X100 solution. Lipids were dissolved by heating the lysates to 95°C for 5 minutes followed by slow cooling of samples to room temperature. This was preformed twice before the lysate was cleared by centrifugation (13000 rpm, 5 min). The supernatant was used for the triglyceride assay according to the manufacturer´s instruction.

FA uptake and bromo-palmitic acid transport assay. FA uptake after 24h was determined by incubation of pretreated SkMC with 37 kBq/ml ^{14}C-OA or ^{14}C-PA (adjusted to 100 µmol/l with unlabeled FA bound to BSA), respectively. Subsequently, SkMC were lysed using 1 mol/l NaOH. In order to determine ^{14}C-BrPA transport, SkMC were preincubated as indicated, before ^{14}C-BrPA (3.7 kBq/ml per well) was added. After 30 min of incubation at 37°C, SkMC were lysed with 1 mol/l NaOH. Radioactivity of all cell lysates was counted in a liquid scintillation counter (Beckman, Munich, Germany). Kinetics for fatty acid uptake were obtained via a scintillation proximity assay as described previously[42]. Briefly, SkMC were cultured in 96-well Cytostar-T plates (GE Healthcare Bio-Sciences), preincubated with CM and treated with 37 kBq/ml ^{14}C-OA (adjusted to 100 µmol/l with unlabeled FA). Cell associated radioactivity was measured at indicated time points using a Wallac 1450 Microbeta Plus Liquid Scintillation Counter (Perkin Elmer).

FA oxidation. In order to determine FA oxidation, SkMC were seeded in every other row of 48-well culture dishes and cultured as described above. For EPS-

treatment experiments, SkMC were instead seeded on 10mm cover slips in 6-well culture dishes, treated and electrically stimulated as described above. Subsequently, cover slips were carefully transferred to 48-well culture dishes. After treatment with CM, FA, and EPS as indicated, culture medium was exchanged and SkMC were cultured in FA free media for additional 24h in order to prevent dilution of radioactively labeled FA. Afterwards, 11.1 kBq/well of ^{14}C-OA or ^{14}C-PA supplemented with 1 µmol/l L-carnitine were added to SkMC, respectively, corresponding to the FA during preincubation. Culture dishes were incubated for 4h in an oxidation chamber, which allows gas exchange between two neighboring wells. Filter papers soaked with NaOH were placed in the empty neighboring wells. Oxidation was stopped and CO_2 was liberated via acidification of culture media by injecting 1 mol/l HCl and trapped in filter paper. Radioactivity was counted in a liquid scintillation counter (Beckman).

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

Results

Characterization of adipocyte-conditioned medium. Collecting the culture media from *in vitro* differentiated adipocytes provided us with conditioned media (CM) containing all secretory products of the adipocytes. Several components of the CM could be identified and quantified, like myostatin, PEDF, and chemerin, while other factors like leptin, TNFα, and especially FA are not detectable in our CM (Table 1)[39]. Novel and very sensitive proteomic analyses of the CM performed in our laboratory have further revealed the complex nature of the CM with several hundred secreted proteins (manuscript submitted), corresponding to previous studies demonstrating a similar complexity of the adipocyte secretome[18-21]. Hence, by applying this CM to skeletal muscle cells instead of isolated adipokines, we obtain a unique model to simulate the crosstalk between adipose tissue and skeletal muscle in its full physiological complexity. Furthermore, absence of FA in the CM enables us

to determine potentially synergistic effects of adipokines at exactly defined FA concentrations under controlled *in vitro* conditions.

Detection of lipid droplets, lipid droplet coating protein ADRP, and FA uptake. Nile Red staining of SkMC results in a red background staining of polar lipids in cell membranes and a yellow/green staining of neutral lipids like triglycerides in lipid droplets. BSA-treatment was used as control, after it was verified that BSA-treated SkMC did not show differences compared to completely untreated SkMC (data not shown). While BSA-treated SkMC displayed no lipid droplets, only sporadic lipid droplets were visible in very few myotubes after incubation with CM, OA, or PA (Fig. 7.1A). In contrast to that, numerous clearly defined lipid droplets were found in the majority of SkMC after co-application of OA+CM. Incubation with PA+CM however showed a more diffuse staining covering entire myotubes. The massive accumulation of lipid droplets after incubation with OA+CM coincided with a significantly increased protein abundance of the lipid droplet coating protein ADRP (Fig. 7.1B). FA-treatment alone showed a tendency to increase ADRP protein levels, which however failed to reach statistical significance.

This apparent lipid accumulation in the combination situation observed with Nile Red staining also correlates with increased fatty acid uptake of SkMC. Tracing FA uptake for up to 8h revealed an increasingly enhanced uptake of ^{14}C-OA into cells that were preincubated with CM (Fig. 7.1C). Similarly, uptake of ^{14}C-OA as well as ^{14}C-PA after 24h showed an 1.7 and 1.5-fold increase, respectively, in CM-treated SkMC compared to their respective controls (Fig. 7.1D).

Effect of CM and single adipokines on FA transport and FA transporter expression. While the protein level of FATP4 remained unaltered upon CM-treatment (Fig. 7.2B), incubation of SkMC with CM increased the protein abundance of the FA transporter CD36 more than 2-fold (Fig. 7.2A). When analyzing isolated known components of the CM like VEGF, PEDF, and MCP-1 for their potential to influence CD36 protein abundance, neither was able to reproduce the CM-induced effect (Fig. 7.2C). Incubation of SkMC with chemerin even evoked a significant reduction of CD36 abundance. Importantly, heat inactivation of CM resulted in loss of the CM-induced increase of CD36 protein level, demonstrating a protein-mediated effect (Fig. 7.2C). Increased CD36 protein abundance translates into increased capacity of SkMC to

take up FA. Application of ^{14}C-BrPA, which cannot be metabolized by the cell, revealed that FA transport was significantly increased in SkMC after incubation with CM (Fig. 7.2D).

Impact of CM and FA-treatment on SkMC FA oxidation. Since IMCL accumulation has been linked to skeletal muscle oxidation capacity, oxidation of ^{14}C-labelled OA and PA was analyzed. ^{14}C-OA oxidation was reduced in SkMC treated with CM (~34%), OA (~23%), or both (~50%), after incubation with 100 µmol/l FA (Fig. 3A). While oxidation of ^{14}C-PA was not significantly altered in SkMC incubated with CM or PA alone, combination of PA+CM severely decreased ^{14}C-PA oxidation by more than 70% compared to PA treatment alone. FA oxidation experiments were also performed in SkMC pretreated with 300 µmol/l FA (Fig. 7.3B). Similar to the experimental setting using 100 µmol/l PA, here the most profound reduction (> 90%) could be observed after incubation with PA+CM, while neither CM nor PA alone significantly reduced ^{14}C-PA oxidation. Generally, oxidation of ^{14}C-PA was found to be approximately twice as high as oxidation of ^{14}C-OA when comparing control situations (Fig. 7.3A).

Influence of EPS on lipid accumulation in SkMC. We have recently established and validated an *in vitro* model of skeletal muscle contraction by subjecting cells to electrical pulse stimulation (EPS). By using this model we are able to analyze the effects of contractile activity under precisely controlled conditions. This novel EPS-technique closely mimics the effects of physical exercise, as it activates AMPK, induces secretion of characteristic exercise-stimulated myokines like IL-6 and VEGF as well as improves insulin-stimulated glucose uptake (data not shown). Further details on this model will be reported elsewhere. In order to assess the influence of contraction on the degree of intramyocellular lipid (IMCL) droplet accumulation in SkMC, Nile Red staining was performed. Electrically stimulating SkMC in parallel to CM- and FA-treatment did not induce apparent changes in the amount of lipid droplet content compared to the respective controls without contraction (Fig. 7.4A). While SkMC incubated with CM, OA, or PA only displayed sporadic lipid droplets in singular cells both with and without contraction, massive lipid droplet accumulation was observed after incubation with OA+CM. However, this was the case for contracted and control cells. Instead of clearly defined lipid droplets, SkMC treated

with PA+CM resulted in a more diffuse staining of IMCL loosely scattered throughout myotubes. Subjecting SkMC to 24h EPS did not induce apparent changes of this result.

In order to determine the degree of lipid accumulation more precisely, the amount of triglycerides was quantified. The data obtained largely resembled the results from Nile Red staining. While incubation with CM or PA did not induce significant changes compared to control, treating SkMC with OA elevated the intracellular triglyceride content more than 3-fold (Fig. 7.4B). Combination of OA+CM even increased the amount of triglycerides more than 7-fold, while treatment of PA+CM still elicited an ~5-fold increase of SkMC triglycerides. This finding supports the observation obtained from Nile Red staining that lipids seem to accumulate after incubation with PA+CM, even though no distinct lipid droplets are formed. Applying EPS-stimulation to SkMC did not change the intracellular triglyceride content in control cells. Similarly, no alterations were obtained for CM-, OA-, and PA-treatment. In contrast to that, electrical stimulation was found to reduce the triglyceride content by ~15% and ~20% after incubation with OA+CM and PA+CM, respectively.

Effect of electrical stimulation on CD36 protein abundance and FA transport. Compared to unstimulated control cells, SkMC subjected to EPS treatment displayed a ~30% increase in CD36 protein abundance (Fig. 7.5A). While CM-treatment significantly increases CD36 protein levels by ~2-fold in unstimulated SkMC, simultaneous electrical pulse stimulation attenuated this increase. Furthermore, results obtained using ^{14}C-BrPA suggest that EPS-treatment also seems to have an impact on FA transport. While incubation of unstimulated cells with CM elicited a ~40% increase in ^{14}C-BrPA transport compared to control cells (Fig. 7.5B), this CM-mediated significant increase was abrogated by EPS-treatment. ^{14}C-BrPA transport in electrically stimulated SkMC incubated with CM was even found to be significantly lower compared to CM-treated cells without EPS.

Impact of EPS-treatment on SkMC FA oxidation. Electrical pulse stimulation was found to selectively influence muscle cell FA oxidation. While pre-treatment of SkMC with CM or OA reduced the cells' capacity to oxidize ^{14}C-OA, data obtained with simultaneous electrical stimulation indicate a trend for improved ^{14}C-OA oxidation, which however did not reach statistical significance (Fig. 7.6A). A profound

~50% reduction of ^{14}C-OA oxidation was observed in response to treatment with OA+CM in unstimulated cells. Subjecting these SkMC to EPS treatment robustly increased ^{14}C-OA oxidation to the control level, thus preventing the reductive effect of OA+CM. Oxidation of ^{14}C-PA was not altered in response to CM or PA pre-treatment with or without EPS (Fig. 7.6B). Pre-incubating SkMC with PA+CM however severely impaired ^{14}C-PA oxidation by more than 70%. In contrast to OA-treatment, electrical pulse stimulation in parallel to PA+CM incubation was not able to improve SkMC oxidative activity. Instead, ^{14}C-PA oxidation remained at the substantially reduced level.

Discussion

Numerous studies have demonstrated the correlation of FA as well as adipokines with impaired muscle metabolism, however commonly single adipokines and high FA concentrations have been investigated as isolated factors. To more closely simulate their physiological interplay under controlled *in vitro* conditions, this study used a unique model of incubating skeletal muscle cells with CM in combination with FA concentrations found in physiologically healthy subjects. In this context, the synergistic impact of adipokines and FA on skeletal muscle metabolism could be investigated. By using CM we are able to mimic the physiological complexity of the adipocyte secretome, while the absence of FA from CM comprises the advantage of precisely assessing the effects of FA.

Nile Red staining revealed massive accumulation of lipid droplets exclusively in SkMC that were incubated with OA+CM, coinciding with increased protein abundance of ADRP. On the one hand this supports previous reports on differential storage and metabolizing processes for saturated and unsaturated FA with unsaturated FA rather accumulating as lipid droplets[43,44], while saturated FA like palmitate rather accumulate as DAG and ceramide[43,45]. On the other hand, it supports a previously proposed role for ADRP in attenuating lipotoxic effects due to the sequestration of lipids in lipid droplets[46]. An explanation why this massive lipid droplet accumulation is exclusively observed after incubation with OA+CM may lie in an coincidence of two events: (I) CM-treatment leads to increased CD36-mediated uptake of FA, however in the situation where SkMC were treated with CM alone, no

FA were present in the culture media to be taken up and (II) adipokines seem to be able to amplify the previously described potential of various long-chain FA to stimulate ADRP protein levels[47]. However, our data suggest that there has to be a different mechanism for PA, as there are no clearly defined lipid droplets in SkMC after incubation with PA or PA+CM.

In order to determine the underlying mechanism accounting for this prominent lipid accumulation, several steps of FA uptake were analyzed. We describe here that the protein level of the FA transport protein CD36 is significantly increased after incubation with CM, an effect that could not be mimicked by a variety of adipokines when added alone. Since CM is a very complex mixture, we presume that most likely a combination of several yet undefined factors may be responsible for the CM-induced effects. Additionally, inability of heat-inactivated CM to increase CD36 protein abundance indicates that possible causative agents are protein factors. Concomitantly to increased CD36 protein levels, we observe enhanced FA transport and uptake in SkMC incubated with CM. In this study we analyzed CD36 protein in whole cell lysates and did not differentiate between intracellular and plasma membrane CD36 pools. However, increased FA transport and uptake in absence of increased protein levels of another important FA transporter in SkMC (FATP4) indicate that increased amounts of functional CD36 have to be available at the plasma membrane after incubation with CM to mediate this FA transport. Our data suggest that this CM-induced increase in CD36-mediated FA uptake might play an important role in the accumulation of lipid droplets in SkMC after incubation with OA+CM. It has been discussed previously that CD36 may contribute to intracellular lipid accumulation[48,49] and might resume a role as a mediator of lipotoxicity[50]. Thus it has been shown in animal models that ablation of CD36-mediated lipid uptake in muscle or liver prevents lipotoxicity[51-53], while specific induction of CD36 in liver contributed to steatosis[54]. Although there is only a modest increase in FA transport, there is severe accumulation of intracellular lipid droplets in SkMC after incubation with OA+CM, however additional cellular processes like reduced FA oxidation are likely to contribute to further lipid accumulation.

As a number of studies have demonstrated an association of intramyocellular lipid (IMCL) accumulation and impaired muscle function, we aimed to investigate the consequences of this lipid droplet accumulation. While insulin signaling at the level of Akt and insulin-stimulated glucose uptake were not changed in response to the OA

and PA concentrations used in this study (data not shown), FA metabolism was found to be altered. Incubating SkMC with CM and OA both decreased ^{14}C-OA oxidation. However, reduction is even more pronounced after incubation with OA+CM. Importantly, in case of ^{14}C-PA only the combination of PA+CM caused a profound reduction of oxidation, while CM or PA alone do not significantly affect ^{14}C-PA oxidation. Notably, this was also the case when higher PA concentrations were used, even yielding a more substantial reduction of ^{14}C-PA oxidation after combined incubation with CM and 300 µmol/l PA. This increased effect, nearly abolishing FA oxidation, further emphasizes the lipotoxic potential of this fatty acid.

Physical exercise has been demonstrated to be a major regulator of skeletal muscle metabolism, potently influencing mitochondrial function[38]. In this context, several studies have shown that increased physical activity is capable to elicit a complex set of biological responses, resulting in increased oxidative capacity of the skeletal muscle[55,56]. Therefore, we asked the question, whether electrically induced contraction of SkMC could counteract the defects in FA oxidation induced by combined treatment of FA and CM.

Subjecting SkMC to EPS-treatment for 24h in parallel with CM and FA-treatment did not markedly alter IMCL accumulation assessed by Nile Red staining. Furthermore, triglyceride quantification revealed that electrical stimulation only altered triglyceride content when applied in parallel to the combination of OA+CM or PA+CM. An explanation for this observation might be found when analyzing the data obtained for ^{14}C-BrPA transport. In these experiments, EPS-treatment was demonstrated to reduce the amount of ^{14}C-BrPA transported into the cell in response to CM-treatment. In light of these findings it may be speculated that FA uptake and triglyceride accumulation in response to FA-treatment is strongly promoted by adipokines, as discussed above, while electrically stimulated contraction predominantly seems to exert its effects by preventing adipokine-associated increased FA transport.

Regulating CD36 protein abundance might be a way in which EPS-treatment could influence FA transport. It has been previously demonstrated that exercise training potently elicits a series of adaptation processes in skeletal muscle cells, many of which involving critical components of FA metabolism[38,57]. In this context, PPAR gamma coactivator 1-alpha (PGC-1α), a key regulator of the expression of mitochondrial FA oxidation enzymes, has been demonstrated to be increased in

response to physical exercise[58,59]. Therefore, it could be speculated that the increased CD36 protein abundance observed after EPS-treatment could represent another step in this adaptation process, contributing to ensure the cell's increased demand for energy during exercise. On the other hand electrical stimulation prevented the CM-induced increase in CD36 protein abundance. It may be discussed that moderately increased CD36 protein levels, potentially necessary to meet the increased energy demands during exercise, might be beneficial, while disproportional CD36 protein abundance in response to adipokines, exceeding the cells requirements and capacities, may be detrimental for the cell. Thus, regulating FA transport proteins could be another way in which physical exercise might mediate its protective effects. Despite this potential mechanism, lipid droplet accumulation was not found to be altered after EPS-treatment. A reason for this could be that adipokines might employ more direct cellular mechanisms mediating their potential effects than electrical stimulation, requiring an earlier onset or longer duration of EPS-treatment to adequately counteract the CM-induced effects. Furthermore, since ^{14}C-BrPA uptake did not precisely resemble CD36 protein abundance, it may be speculated that additional mechanisms in FA transport or CD36 trafficking might be involved.

Several studies have demonstrated that T2D subjects are characterized by a decreased capacity to oxidize fat[33,34]. Since oxidative capacity, intracellular lipid accumulation, metabolic flexibility and ultimately also insulin resistance are closely related [60], appropriate FA oxidation is essential for maintaining insulin sensitivity. Whether IMCL accumulation is cause or consequence of impaired mitochondrial function is still subject of ongoing discussion[60,61], however, accumulating evidence suggests that not IMCL themselves but rather lipid metabolites like diacylglycerol (DAG) or ceramide interfere with skeletal muscle cell metabolism[62,63]. In this context, several studies have revealed that skeletal muscle cells of endurance trained athletes are characterized by enhanced insulin sensitivity despite increased IMCL accumulation[64,65]. Adaptation of skeletal muscle to exercise stimuli comprising increased mitochondrial oxidative capacity has been suggested to be the underlying mechanism of this phenomenon. In this study we demonstrate that impairment of ^{14}C-OA oxidation and lipid droplet accumulation are both more severe in response to the combination of OA+CM than to OA or CM alone. However, subjecting these SkMC to electrical pulse stimulation prevented the adverse effect on ^{14}C-OA

oxidation. Thus it may be speculated, that although EPS could not prevent the accumulation of IMCL, the cells retained their capacity to adapt to contraction-induced stimuli, enhance their oxidative capacity, and successfully switch to OA as metabolic substrate.

In case of PA, the results obtained are considerably different. While incubation of SkMC with PA or CM alone did not alter IMCL accumulation or ^{14}C-PA oxidation, respectively, PA+CM-treatment caused diffuse lipid accumulation and profoundly diminished ^{14}C-PA oxidation. Furthermore, electrical pulse stimulation was not able to prevent this severe reduction of ^{14}C-PA oxidation, in contrast to OA+CM-treatment. Thus it may be speculated that diffuse distribution and possibly further metabolism of saturated FA like PA induces profound cellular impairments, which are not preventable by contraction stimuli. In contrast to that, unsaturated FA like OA seem to be properly stored and possibly sequestered, involving lipid droplet coating proteins, thereby preserving the cells' potential to respond to contraction, despite an initially impaired FA oxidation. These findings support previous studies demonstrating improved metabolic switching in response to exercise training[66] as well as reports indicating differential storage and metabolism of saturated[43,45] and unsaturated FA[67] with more detrimental results induced by saturated FA.

In summary, we demonstrate here for the first time a novel role for adipokines in the pathogenesis of T2D by increasing the lipotoxic potential of FA, notably of relatively low concentrations. This would imply an increased lipotoxic risk already at an early stage of weight gain, when lipolysis has not yet contributed to increased plasma FFA levels. Furthermore, we could clearly observe differential effects of OA and PA, supporting the notion that saturated FA are more detrimental than unsaturated. Additionally, the results of our study demonstrate that contractile activity is able to counteract the lipotoxicity-mediated impairments of FA oxidation. However, this was only the case for OA- but not for PA-treatment. These data indicate novel mechanisms involved in the pathogenesis of obesity, transition to T2D, and response to exercise training. Understanding the underlying molecular mechanisms of these events will help to find efficient therapeutic strategies to prevent or reverse the adverse developments leading to T2D.

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Disclosures

The authors have no conflict of interest to disclose.

Table 1 Concentrations of various factors in CM from human adipocytes

Adipocyte-secreted factor	Concentration in CM (ng ml^{-1})
Adiponectin	**6.545 ± 1.46**
Chemerin	**2.175 ± 0.294**
DPP4	**2.19 ± 1.37**
FFA	**n.d.**
IL-6	**0.025 ± 0.002**
IL-8	**0.055 ± 0.013**
Leptin	**n.d.**
MCP-1	**0.54 ± 0.08**
Myostatin	**12.64 ± 4.44**
PEDF	**45.7 ± 0.82**
Resistin	**n.d.**
TNFα	**n.d.**
VEGF	**0.329 ± 0.09**

Abbreviations: CM, conditioned media; DPP, dipeptidyl peptidase; FFA, free fatty acids; IL, interleukin; MCP-1, monocyte chemotactic protein-1; PEDF, pigment epithelium-derived factor; TNF, tumor necrosis factor; VGEF, vascular endothelial growth factor; n.d., not detectable. Concentrations of adipocyte-secreted factors of CM were obtained by enzyme-linked immunosorbent assay. Data are means ± SEM, n = 4-20

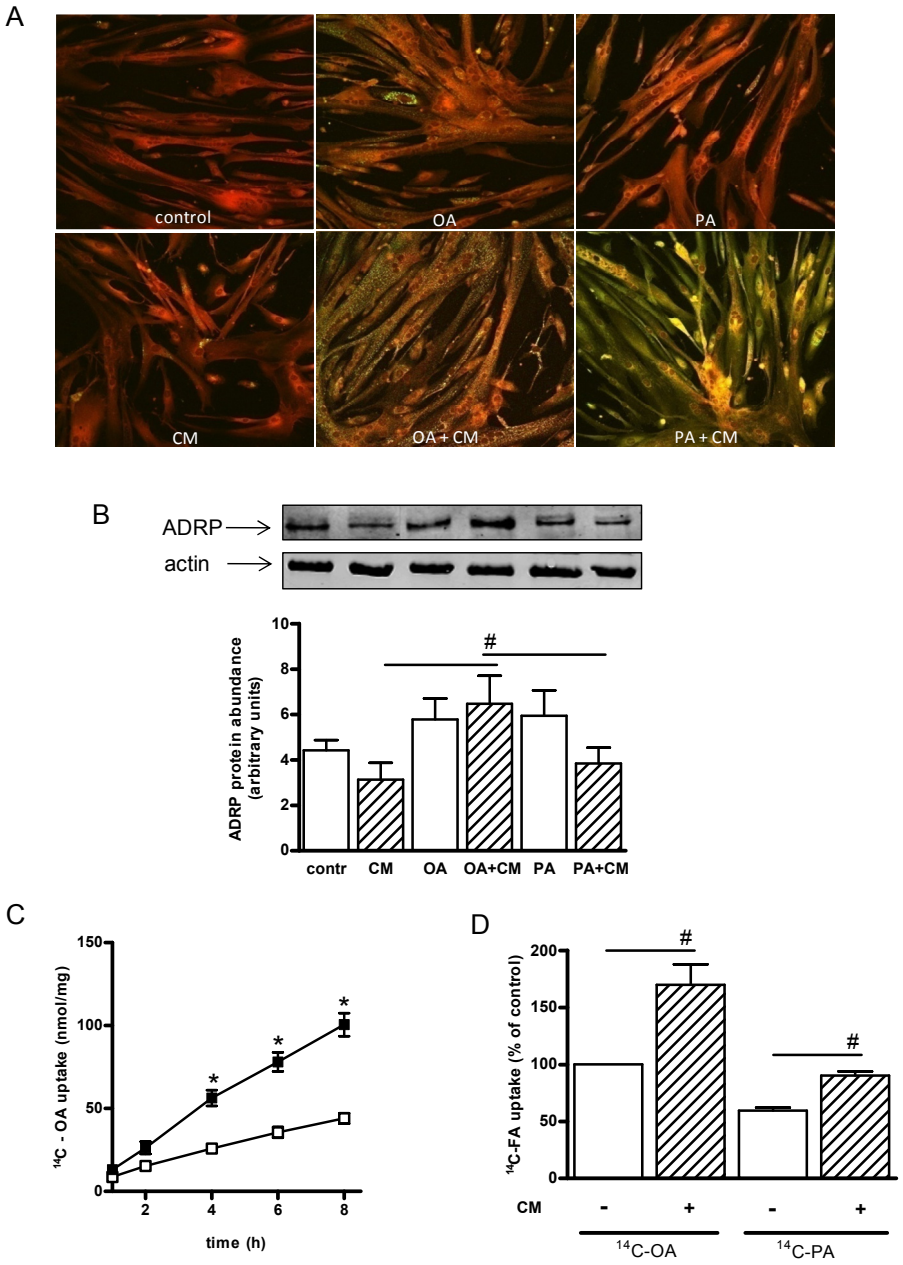

Fig. 7.1. Influence of CM and FA on lipid droplet formation, ADRP protein abundance, and FA uptake. A: SkMC were pre-incubated with CM for 6h, OA and PA (100 µmol/l) were then added over-night. Subsequently, SkMC were fixed using picric acid and lipid droplets were stained using Nile Red. Representative images of treated cells are shown. B: SkMC were incubated as described under A. Subsequently, SkMC were lysed and analyzed by SDS-PAGE and Western Blot. Values were normalized to the protein level of actin. C: SkMC were incubated with CM for 24h. Subsequently, SkMC were incubated with ^{14}C-OA and radioactivity was assessed at the designated time points. Values were corrected for non-specific uptake

measured immediately after start of experiment. Open boxes: control; black boxes: CM. D: SkMC were incubated with CM for 24h. Subsequently, SkMC were incubated for 24h with ^{14}C-OA or ^{14}C-PA. Values were corrected for non-specific uptake measured immediately after start of experiment. Data are mean values ± SEM of ≥ 3 independent experiments. * $p < 0.05$ vs control; # $p < 0.05$ vs designated data.

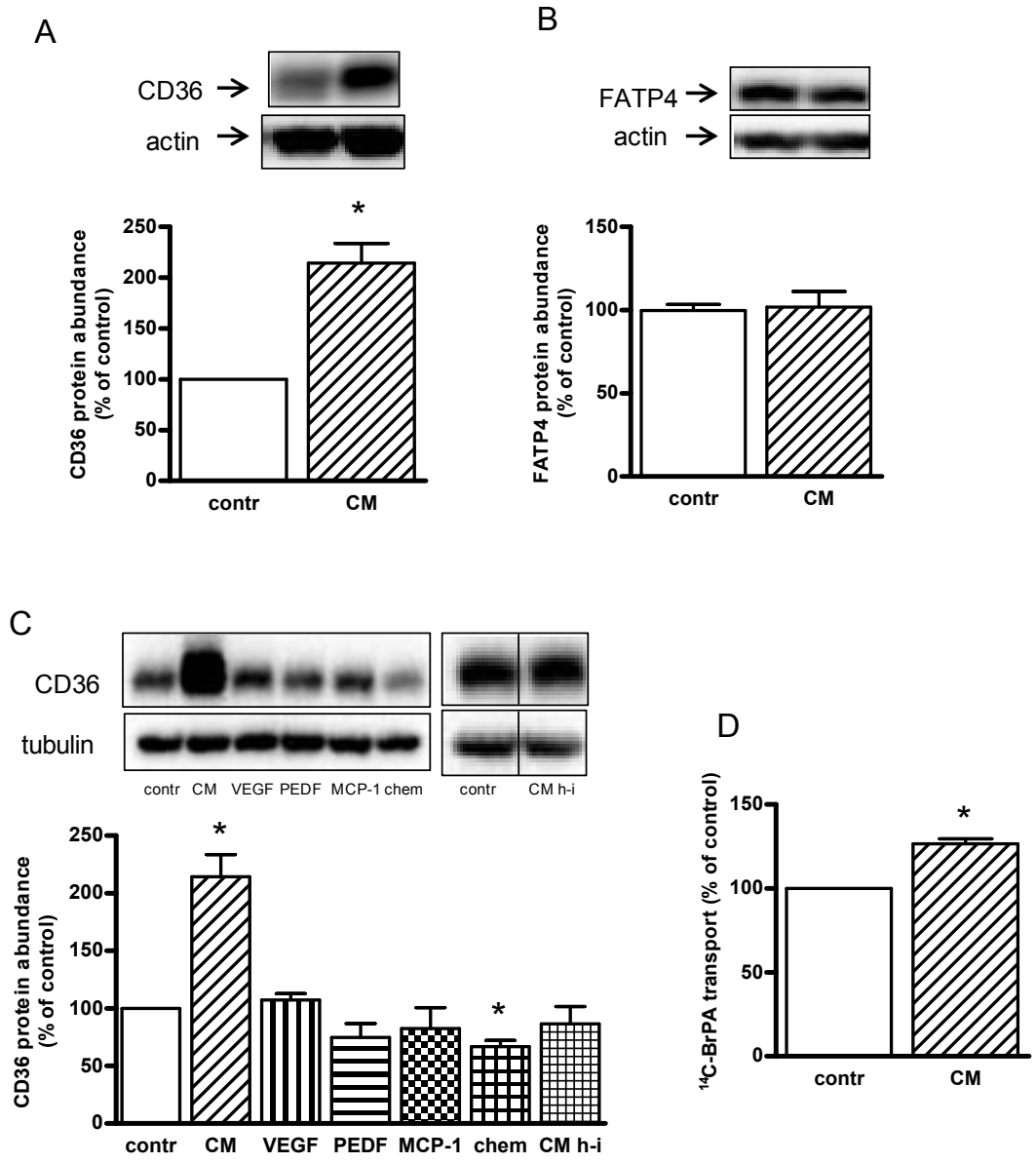

Fig. 7.2. Effect of CM and isolated adipokines on FA transport proteins and FA transport. A, B: SkMC were incubated with CM for 24h. Subsequently, SkMC were lysed and analyzed by SDS-PAGE and Western Blot. Representative images of

Western Blots for CD36 (A) and FATP4 (B) are shown. All data were normalized to the protein level of β-actin and are expressed relative to the protein levels of control cells. C: SkMC were incubated with CM, VEGF, PEDF, MCP-1, chemerin, or heat inactivated CM for 24h. Subsequently, SkMC were lysed and analyzed by SDS-PAGE and Western Blot. Representative images of Western Blots for CD36 and tubulin are shown. All data were normalized to the protein level of tubulin and are expressed relative to the protein levels of control cells. D: SkMC were incubated with CM for 24h. Subsequently, SkMC were incubated with ^{14}C-BrPA for 30 min and lysed. For quantification, control cells were used as reference and set to 100%. All data are mean values ± SEM of ≥ 3 independent experiments. * $p < 0.05$ vs control.

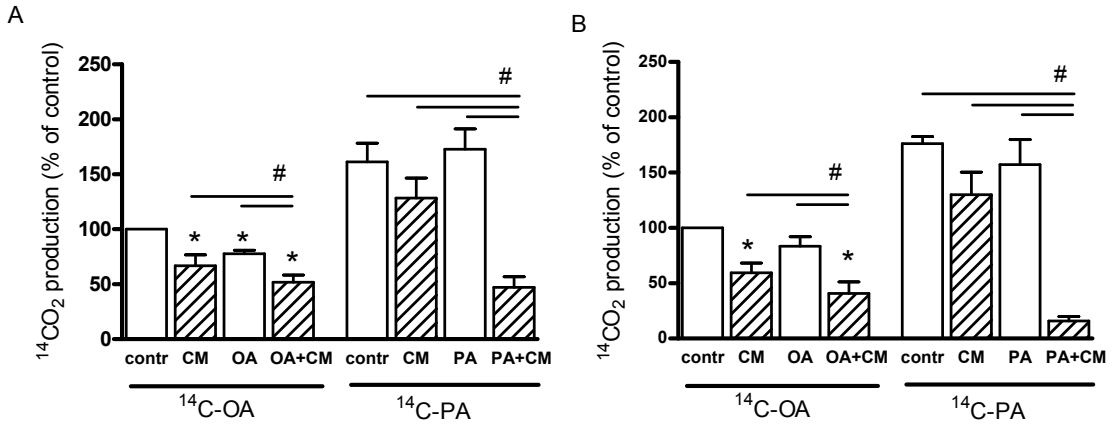

Fig. 7.3. FA oxidation after incubation with CM and FA. A: SkMC were pre-incubated with CM for 6h, OA and PA (A: 100 µmol/l; B: 300 µmol/l) were then added over-night. Subsequently, SkMC were incubated with ^{14}C-OA or ^{14}C-PA in an oxidation chamber for 4h. Liberated $^{14}CO_2$ was trapped and radioactivity was assessed. Values were corrected for non-specific oxidation obtained by immediate media acidification after addition of radioactivity. All data are mean values ± SEM of ≥ 3 independent experiments. * $p < 0.05$ vs control; # $p < 0.05$ vs designated data.

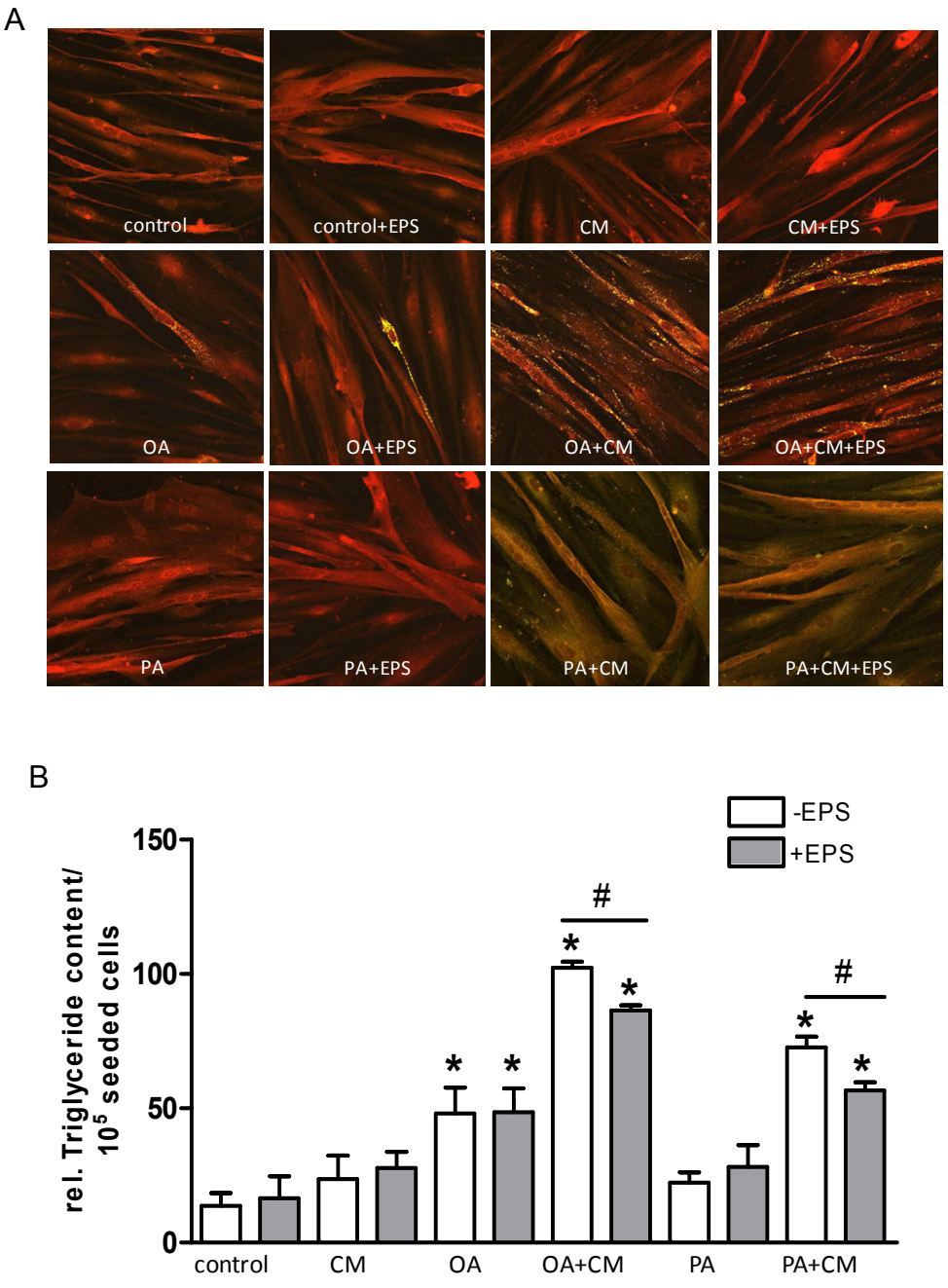

Fig. 7.4. Influence of EPS on lipid accumulation in SkMC incubated with CM and FA. A: Differentiated SkMC were incubated with CM and subjected to EPS treatment as indicated. After 6h of pre-treatment, OA and PA (100 µmol/l) were added over-night, respectively. Subsequently, SkMC were fixed using picric acid and lipid droplets were stained using Nile Red. Representative images of treated cells are shown. B: SkMC were treated as in A and subsequently lysed and subjected to triglyceride quantification as described in Materials and Methods.

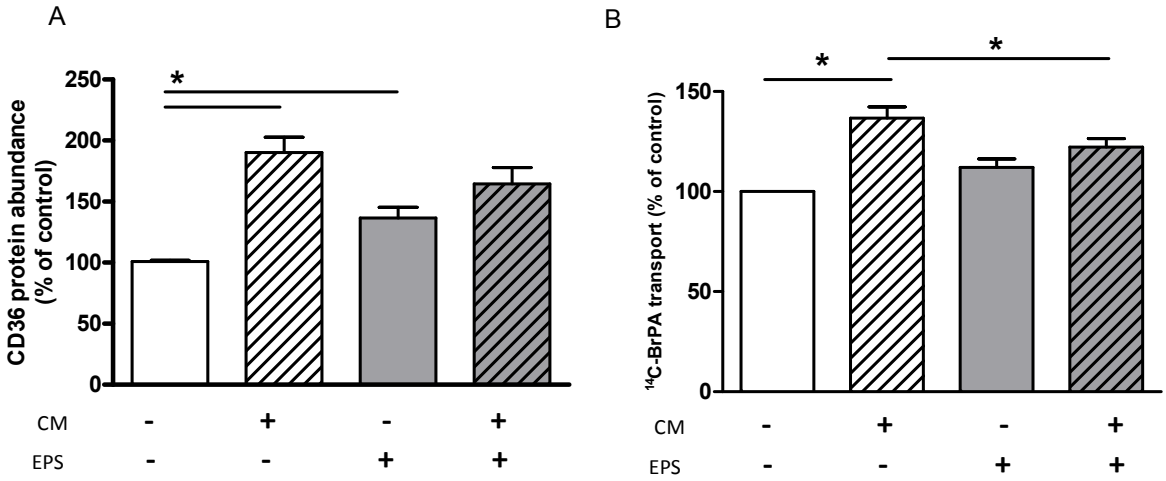

Fig. 7.5. CD36 protein abundance and FA transport after treatment with CM, FA, and EPS. A: Differentiated SkMC were incubated with CM for 24h and simultaneously subjected to EPS-treatment as indicated. Subsequently, SkMC were lysed and analyzed by SDS-PAGE and Western Blot. Representative images of Western Blots for CD36 and tubulin are shown. All data were normalized to the protein level of tubulin and are expressed relative to the protein levels of control cells. B: SkMC were treated as described in A. Subsequently, SkMC were incubated with ^{14}C-BrPA for 30 min and lysed. For quantification, control cells were used as reference and set to 100%. All data are mean values ± SEM of ≥ 3 independent experiments. * $p < 0.05$ vs designated data.

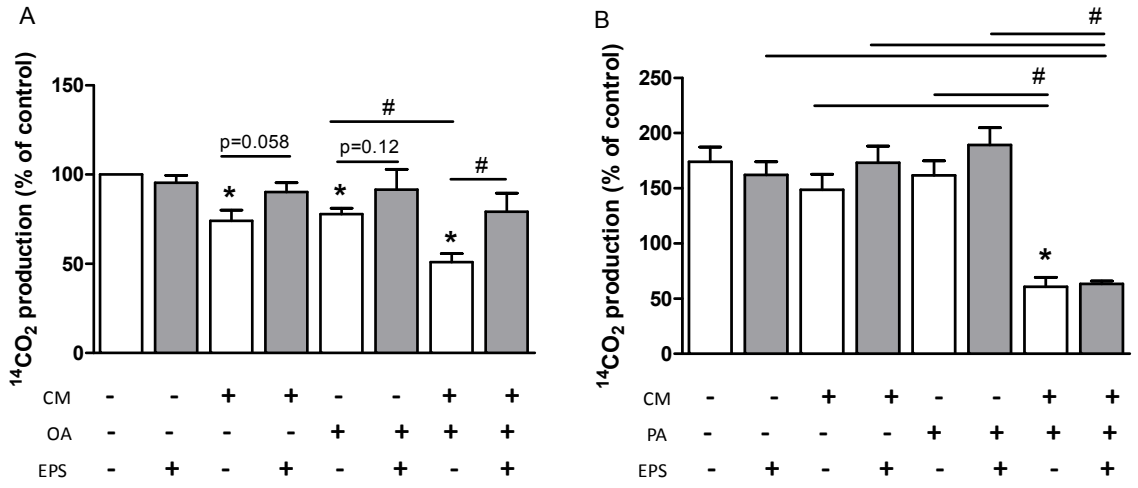

Fig. 7.6. FA oxidation of SkMC after treatment with CM, FA, and EPS. Differentiated SkMC were incubated with CM and subjected to EPS treatment as indicated. After 6h of pre-treatment, OA and PA (100 µmol/l) were added over-night. Subsequently, SkMC were incubated with ^{14}C-OA (A) or ^{14}C-PA (B) in an oxidation chamber for 4h. Liberated $^{14}CO_2$ was trapped and radioactivity was assessed. Values were corrected for non-specific oxidation as described in Fig. 3. All data are mean values ± SEM of ≥ 3 independent experiments. * $p < 0.05$ vs control; # $p < 0.05$ vs designated data.

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CHAPTER 8

General Discussion

8.1 Adipokines as players in the crosstalk between adipose tissue and skeletal muscle

8.1.1 Impact on glucose metabolism

Obesity is a hallmark of the metabolic syndrome and represents a major global health problem associated with the development of dyslipidemia, hypertension, and chronic diseases like T2D and CVD[225,226]. Especially the development of insulin resistance, a critical defect observed early in the pathogenesis of T2D, has been correlated with obesity-associated expansion of adipose tissue, especially of the visceral region. Accordingly, numerous studies have demonstrated an association between the degree of skeletal muscle or whole body insulin resistance and markers of obesity like BMI, waist-to-hip ratio, physical inactivity, and dyslipidemia. In this context, a complex inter-organ crosstalk between adipose tissue, liver, and muscle has been implemented in the etiology and progression of T2D. Since more than a decade ago, accumulating data have indicated that adipose tissue is capable of secreting a variety of bioactive proteins and peptides, in addition to the well-known secretion of FFA during fasting. Discovery of

this additional role of adipose tissue as a global regulator of whole body energy homeostasis has opened an entire new field of research aimed to unravel the molecular mechanisms underlying obesity-associated diseases[227].

The concept of adipose tissue as a major secretory organ is now commonly accepted. In order to dissect the precise effects of the adipocyte secretome, early studies have investigated the effects of single adipokines. In this context, the complex network of autocrine, paracrine, and endocrine signals, including regulatory feedback loops able to elicit molecular vicious cycles, were revealed. Together with factors like leptin, IL-6, MCP-1, angiotensinogen, or PAI-1, TNFα is one of the most frequently described and well-characterized adipokines associated with obesity. Primarily, TNFα is involved in systemic inflammation and regulation of immune cells. Chronic inflammation is thought to contribute to the development of atherosclerosis, tumor growth, neurodegeneration, and insulin resistance[228]. Indeed, low-grade systemic inflammation, defined as a two- to fourfold elevation in circulating levels of pro-inflammatory and anti-inflammatory cytokines as well as acute phase proteins[211], is frequently observed in obesity and T2D[229,230]. In this context, TNFα has been suggested to play a central role in directly inhibiting insulin signaling. Thus, it has been demonstrated that TNFα infusion into healthy humans impaired insulin signaling and whole-body glucose uptake[231]. Functionally, TNFα has been suggested to impair insulin signaling by increasing IRS-1 serine phosphorylation via activation of JNK and IKKβ[232]. Furthermore, TNFα has been demonstrated to increase lipolysis in human and 3T3 adipocytes, resulting in increased release of FFA, thereby further contributing to the already elevated plasma FFA levels in obesity[211].

Besides the above mentioned well-studied adipokines, an increasing number of novel adipokines are being discovered. Chemerin belongs to the continuously growing list of novel adipokines and its role in the context of negative crosstalk between adipose tissue and skeletal muscle was investigated in the course of this thesis. Chemerin was initially described as a chemokine occurring in fluids during inflammatory processes such as cancer and rheumatoid arthritis[233]. However, expression of chemerin as well as its receptor, chemokine-like receptor 1 (CMKLR1, or ChemR23) has also been demonstrated in adipose tissue, where it is necessary for adipogenesis[234,235]. Furthermore, several human studies have revealed a correlation of serum chemerin levels with BMI[234] as well as markers of the metabolic syndrome and inflammation[236-238]. This sparked the question as to which role chemerin plays in

the crosstalk between adipose tissue and skeletal muscle. After establishing that chemerin is secreted in measurable amounts by *in vitro* differentiated primary human adipocytes, which is even exacerbated by obesity, chemerin was found to promote skeletal muscle insulin resistance (chapter 3). We could demonstrate that SkMC express the chemerin receptor CMKLR1 in a differentiation dependent manner and that exposure of SkMC to chemerin resulted in decreased insulin-stimulated Akt and GSK3 phosphorylation as well as increased basal IRS-1 serine phosphorylation. As a physiological consequence insulin-stimulated glucose uptake was found to be significantly decreased. Thus, chemerin could be established as a potential player contributing to the negative crosstalk between adipose tissue and skeletal muscle by inducing skeletal muscle insulin resistance.

Pigment epithelium-derived factor (PEDF) is another protein recently suggested to be a potential player in the fat-muscle crosstalk. While PEDF was first identified in conditioned media of human retinal epithelial cells and described as a potent regulator of neuronal differentiation[239], later studies also demonstrated that PEDF is secreted by 3T3-L1 adipocytes[240]. A recent study conducted in our laboratory also identified PEDF as one of the most abundant proteins secreted by primary human adipocytes[241]. Furthermore, studies in humans[242,243] and mice[244] revealed a positive correlation between PEDF, obesity, and insulin resistance. Recently, we were also able to demonstrate that *in vitro* administration of PEDF induces insulin resistance in adipocytes, skeletal and smooth muscle cells at the level of insulin-stimulated Akt phosphorylation[241]. Although these findings suggest a role for PEDF in influencing glucose metabolism, the precise signaling pathways of PEDF are only incompletely understood.

As outlined above, a number of studies have investigated the individual contribution of a variety of adipokines to the negative crosstalk between adipose tissue and peripheral insulin responsive organs. In this context, the individual capacity of adipokines like MCP-1[245,246], chemerin (chapter 3), and PEDF[241] to negatively influence skeletal muscle insulin sensitivity could be demonstrated. However, recent studies applying highly sensitive state-of-the-art proteomic technology have revealed the adipocyte secretome to be comprised of hundreds of different secreted factors[123,125]. A recent study of our laboratory has confirmed these findings with regard to our adipocyte-conditioned medium (manuscript submitted). Additionally, in all studies mentioned, a number of novel adipokines have been

identified, suggesting that the adipocyte secretome remains incompletely characterized. In light of the previously described complex signaling network and considering the potential synergistic influences of a multitude of adipokines, it becomes apparent that most likely not a single adipokine is fully responsible for obesity-associated complications, such as insulin resistance in skeletal muscle. Thus, investigation of the entire adipocyte secretome is required to comprehend the full molecular impact of the complex physiological condition. Understanding the multi-level signaling networks established by the adipocyte secretome is a prerequisite for the development of efficient treatment strategies of T2D and obesity-related diseases[247].

To tackle such challenge, co-culture models have generally been accepted to investigate paracrine interactions between two cell types[248,249]. We have previously been able to establish a co-culture model involving primary human adipocytes as well as primary human skeletal muscle cells, enabling us to closely study the direct interactions between these two cell types via the shared culture media. Co-culture has been shown to decrease insulin-stimulated phosphorylaton of IRS-1, Akt, and GSK3, and markedly reduced GLUT4-translocation to the plasma membrane in skeletal muscle cells[250,251]. Thus, our co-culture model has provided direct evidence for a negative crosstalk between adipocytes and skeletal muscle. As a variation of this co-culture model, generation of adipocyte-conditioned medium (CM) has been established. To this end, culture media from differentiated adipocytes is collected after 48 h of cultivation, thus containing all adipokines secreted by adipocytes during that period of time. As shown previously[245,250,252] and also confirmed in several studies of this thesis (chapter 2 and 6), CM is able to successfully mimic the co-culture approach, causing similar reduction of insulin-stimulated IRS-1 and Akt phosphorylation.

8.1.2 Impact on fatty acid uptake

The *in vitro* crosstalk model utilizing CM has also been applied here to investigate further details of the adipocyte-myocyte axis. Interestingly, in the course of this thesis, CM was found to not only influence skeletal muscle glucose

metabolism but also affect fatty acid metabolism (chapter 7). Thus, we could show that incubation of skeletal muscle cells with CM increases fatty acid transport and uptake. In this regard, this increased fatty acid uptake could have been mediated by an increased protein abundance of the fatty acid transport protein CD36 in response to CM-treatment.

CD36 is a membrane glycoprotein present on platelets, mononuclear phagocytes, adipocytes, hepatocytes, myocytes, and some epithelia[253]. This diverse expression pattern reflects the multiple cellular functions of CD36. On microvascular endothelial cells, it is a receptor for thrombospondin-1[254,255] and several other proteins containing peptide domains known as thrombospondin type I repeats (TSRs)[256]. In this context, it has been shown to play a role as an endogenous negative regulator of angiogenesis[257], and therefore is involved in tumor growth, inflammation, wound healing, and other pathological processes requiring neovascularization. On phagocytes, it functions as a pattern recognition receptor, recognizing specific oxidized phospholipids and lipoproteins presented by pathogens or by pathogen-infected cells[258]. In this context, CD36 participates in internalization of apoptotic cells and elimination of foreign agents such as bacteria, parasites, and viruses[259,260]. However, CD36 also recognizes endogenously derived ligands, including oxidatively modified low-density lipoproteins[258,261]. Thus, CD36 was attributed a role in foam cell formation, fatty streak development, and hence atherosclerotic diseases. Furthermore, on adipocytes, muscle cells, enterocytes, and hepatocytes, CD36 functions as a facilitator of long-chain fatty acid transport[262-266], thus participating in muscle lipid utilization, adipose energy storage, and gut fat absorption.

In its function as a fatty acid transporter, CD36 has been suggested to contribute to intracellular lipid accumulation[267,268], thereby promoting lipotoxicity and possibly contributing to the pathogenesis of metabolic disorders, such as T2D and obesity[253]. Accordingly, it has been shown in animal models that ablation of CD36-mediated lipid uptake in muscle or liver prevents lipotoxicity[269-271], while specific induction of CD36 in liver contributed to steatosis[263]. Furthermore, several studies have demonstrated that skeletal muscle in obesity and T2D is characterized by increased rates of fatty acid transport, however, no alteration of muscle CD36 protein content was observed[267,272]. These studies sparked the notion that the degree of CD36-mediated fatty acid uptake may more critically depend on the localization of CD36 than on total protein abundance. Indeed, accumulating evidence

indicates that in skeletal muscle CD36 may not only be present at the plasma membrane, but also in intracellular vesicles[136]. Accordingly, a number of studies have now identified certain conditions in which CD36 location is altered. In this context, muscle denervated for 1 week showed reduced rates of fatty acid transport, attributed to reduced plasmalemmal CD36 content, while CD36 protein expression was not altered[273]. Similarly, increased CD36 plasma membrane content has been associated with conditions such as obesity and T2D[267,272]. Collectively, these studies indicate that, similar to GLUT4, CD36 may alter its subcellular distribution and that certain stimuli permit CD36 to traffic between intracellular storage depots and the plasma membrane.

When attempting to unravel factors regulating CD36, several studies revealed that CD36 location as well as expression and protein abundance may be altered in response to chronic and acute stimuli. Thus, it has been shown that on the one hand exposure of cardiac myocytes and perfused hearts to insulin or AICAR for 90-120 min increased the rate of fatty acid uptake as a result of enhanced expression and plasmalemmal content of CD36. Furthermore, chronic stimulation of muscle contraction (24 h/d for 7 d) also increased fatty acid transport as a consequence of upregulated CD36 protein expression and plasmalemmal content[273,274]. While chronic exposure to insulin, AMPK-activating AICAR, and muscle contraction have been shown to increase CD36 expression, prolonged leptin treatment (2 week leptin infusion) has been shown to downregulate CD36 protein expression, reduce plasmalemmal content, and hence diminish the rate of fatty acid transport into muscle[275]. On the other hand, acute stimulation of muscle with contraction[276], AICAR[277,278], oligomycin[279], insulin[280], or leptin[136] has been shown to induce the translocation of CD36 from its intracellular depot to the plasma membrane within minutes. Collectively, these data indicate that acute influences on skeletal muscle promote translocation of CD36, while different chronic stimuli alter CD36 protein abundance. Permanent relocation of CD36 to the plasma membrane observed in obesity and T2D has been linked to increased lipid accumulation in insulin resistant muscle in animals and humans[136].

Alteration of CD36 protein abundance in response to several stimuli is one key finding of this thesis. Incubation of primary human skeletal muscle cells (SkMC) with the adipokine mixture of CM was revealed to increase CD36 protein abundance (chapter 7). It is noteworthy that whole cell lysates were analyzed, which did not

allow for assessment of subcellular CD36 localization. However, increased fatty acid transport in the absence of augmented protein abundance of fatty acid transport protein 4, suggests that increased amounts of CD36 protein must have been present at the plasma membrane, mediating the observed increase in fatty acid transport. In an attempt to identify the causative agent of CM responsible for this increase, several known adipokines of CM were individually assessed for their capacity to increase CD36 protein abundance.

One of these factors studied was PEDF. This adipokine has been described as a multifunctional protein with different functions on diverse cell types. In this context, multiple receptors and ligands have been suggested to be involved in PEDF signaling. Thus, patatin-like phospholipase domain containing protein-2, also known as ATGL, has been described as one specific receptor for PEDF[281]. This finding suggests a role for PEDF also in lipid metabolism. Indeed, there is upcoming evidence that PEDF affects lipolysis, thereby influencing lipid homeostasis of adipose tissue[244,281,282]. In this context, PEDF was suggested to be involved in accumulation of hepatic lipids and induction of lipid mediators. Analyzing the effect of PEDF on SkMC CD36 protein level in the course of this thesis however revealed that PEDF did not seem to have a direct influence on CD36 protein abundance (chapter 7). Thus, it might be speculated that, with regard to lipid metabolism, PEDF either predominantly acts on adipocytes and hepatocytes, as suggested by literature as well as an earlier study of our laboratory[241], or it may exert its effects on SkMC without involving CD36.

In addition to PEDF, VEGF was also studied for its potential to individually influence CD36 protein abundance. The vascular endothelial growth factors are major angiogenic regulators and are involved in several aspects of endothelial cell physiology[283]. While VEGF-A is required for vasculogenesis and angiogenesis[284,285], a recent report has suggested a novel role for VEGF-B in controlling fatty acid uptake in endothelial cells via transcriptional regulation of vascular fatty acid transport proteins[286]. Since we have previously shown that VEGF is part of the adipokine repertoire of CM[287], the influence of VEGF on skeletal muscle CD36 expression was assessed here. However, incubation of SkMC with VEGF did not alter CD36 protein abundance (chapter 7). Hence, similar to PEDF, the regulatory properties of VEGF on fatty acid transport protein expression may be restricted to certain cell types, such as endothelial cells.

Besides the above mentioned role of chemerin in inducing skeletal muscle insulin resistance, it has been attributed to a variety of other effects. Thus, chemerin has been described to play a role in chemoattraction and macrophage infiltration into adipose tissue, in adipogenesis and adipocyte metabolism as well as in insulin-stimulated glucose uptake in 3T3 adipocytes[235,288,289]. Additionally we have shown here that chemerin also contributes to inflammatory signaling in skeletal muscle via activation of the NFκB pathway (chapter 3). Hence, various endocrine and paracrine/autocrine pathways have been described for chemerin. To further characterize its potential biochemical repertoire, the influence of chemerin on CD36 expression was assessed. We found that incubation of SkMC with chemerin moderately downregulated CD36 protein abundance (chapter 7). As this finding was contrary to the effect observed for CM, additional studies are needed to unravel the underlying molecular processes. Nevertheless, this is the first indication that chemerin may also influence skeletal muscle lipid metabolism, besides inflammatory signaling and glucose metabolism.

Although neither of the investigated adipokines was able to elicit the same reaction as CM, the causative agent of this effect is still suggested to be a protein factor, since heat inactivation of CM resulted in loss of the CM-mediated increased CD36 protein abundance. Considering the highly complex nature of CM and the possible interrelated and synergistic effects of multiple adipokines in CM, it is well possible that the increase of CD36 requires the interplay of several factors of CM. Although increased CD36 protein abundance and concomitantly enhanced fatty acid uptake have been linked to increased IMCL accumulation in literature as discussed above, no significant accumulation of IMCL was observed in response to increased CM-mediated CD36 protein abundance in this thesis (chapter 7). Only combined treatment of CM and fatty acids elicited severe accumulation of IMCL and TAG. As a reason for this it has been speculated that treatment of SkMC with CM increased the ability of SkMC to take up fatty acids, namely increased CD36 protein abundance, however, since CM is devoid of measurable amounts of fatty acids, there was no substrate present in the culture media to be taken up. In contrast to that, in the combined treatment situation, CM enhanced CD36 protein abundance and the simultaneous addition of fatty acids provided the substrates for increased CD36-mediated fatty acid uptake, resulting in IMCL accumulation.

Besides treatment with CM, subjecting SkMC to electrical pulse stimulation, simulating physical activity, has also been demonstrated to moderately increase CD36 protein abundance in this thesis (chapter 7). This is in accordance with literature, where chronic muscle contraction was shown to upregulate CD36 protein abundance[273,274]. However, while chronic contraction persisted for 7 days in these studies, increased CD36 protein abundance was already observed after 24 h EPS-treatment in this thesis. Additional studies will be needed to assess whether the degree of increased CD36 protein abundance correlates with the duration of contraction stimuli. Furthermore, EPS-treatment was shown to prevent CM-mediated prominent increase in CD36 protein abundance. As discussed in the context of that study, it may be speculated that a moderate increase of CD36 protein levels in response to contraction stimuli with concomitantly increased mitochondrial function is beneficial in meeting the increased energy demand implied by contraction activity, while disproportional upregulation of CD36 in response to CM-treatment may be detrimental in promoting IMCL accumulation. Further influences of contraction on mitochondrial activity as well as IMCL accumulation will be discussed in the following.

8.1.3 Role of adipocyte size

As previously outlined, composition of the adipocyte secretome has been demonstrated to be influenced by a number of factors. An increasing body of evidence has indicated that adipocyte size relates to an altered adipokine profile. In this context, adipocyte hypoxia associated with adipose tissue enlargement has also been shown to be able to modify adipocyte secretome.

Adipose tissue expansion in obesity-associated weight gain is mainly characterized by hypertrophy of individual adipocytes[290,291]. *De novo* differentiation of new adipocytes from preadipocytes has been discussed to contribute only marginally to adipose tissue enlargement and to rather balance adipocyte death due to apoptosis, maintaining a rather stable adipocyte cell number[292,293]. Nevertheless, it has previously been demonstrated that enlargement of adipocytes is associated with substantial changes in metabolic functions, including lipid metabolism[294,295]. In this context, smaller adipocytes are generally believed to be more insulin sensitive

and to have lower rates of lipolysis, whereas obesity-associated bigger adipocytes, especially those in visceral depots, are highly lipolytic, thereby contributing to FFA output into circulation. This way, these alterations may contribute to the increased health risks associated with obesity[130]. Indeed, a recent study has identified adipocyte size in the subcutaneous abdominal depot to be a significant predictor for the future development of T2D[296]. Furthermore, composition of the adipocyte secretome has been demonstrated to be influenced by adipocyte size. A positive correlation has been demonstrated between adipocyte size and secretion of a variety of adipokines including TNFα, leptin, IL-6, IL-8, MCP-1, and granulocyte colony stimulating factor[130], thereby shifting the secretome profile to a more pro-inflammatory composition. Thus, obesity-associated adipocyte hypertrophy has been suggested to promote an inflammatory state in human adipose tissue.

As a consequence of adipocyte hypertrophy, adipose tissue may be characterized by hypoxia, another critical determinant of adipocyte metabolic functions. When adipocytes continuously increase in size to meet the enhanced demand for lipid storage capacity in obesity, they may reach a size of ~150–200 µm in diameter[297]. This pushes normal O_2 diffusion distance of 100–200 µm[298] to its limits. Although adipose tissue expansion has been demonstrated to require angiogenesis[299], white adipose tissue is generally considered to be poorly vascularized[300]. Thus, combination of an insufficient blood supply and severe adipocyte hypertrophy may result in adipose tissue hypoxia. Indeed, adipose tissue was found to be in a hypoxic state in mouse models of genetic and diet-induced obesity[301,302]. Accumulating evidence suggests a role for adipose tissue hypoxia in the induction of insulin resistance. In this context, hypoxia has been identified as a potent stimulus for activation of ER stress, macrophage recruitment, and inhibition of preadipocyte differentiation as well as adipocyte apoptosis. Additionally, hypoxia reduces adiponectin and increases leptin expression, promotes AMPK-mediated activation of adipocyte lipolysis, inhibits mitochondrial respiration and biogenesis, and activates the IKKβ/NFκB inflammatory pathway[303]. Collectively, these data further underline the complexity involved in obesity-associated adipocyte dysfunction and the resulting implications for the development of insulin resistance and T2D.

8.2 Fatty acids influence skeletal muscle cells in multiple ways

8.2.1 Impact on glucose metabolism

Although FFA are an essential oxidative fuel for skeletal muscle cells, chronically elevated FFA levels have been linked to the development of skeletal muscle insulin resistance. In this context, several studies have demonstrated that acute elevation of plasma FFA, via intravenous lipid infusion, is able to decrease insulin-stimulated glucose uptake in diabetic and non-diabetic subjects in a dose-dependent manner[304-307]. Several mechanisms have been proposed to explain the underlying processes of these FFA-induced defects. The glucose fatty-acid cycle as postulated by Randle, couples an increased lipid availability and fat oxidation to a decreased glucose uptake and oxidation[172]. While the physiological principle of the Randle cycle is still valid, accumulating evidence now argues against a role of the glucose fatty-acid cycle in explaining fatty acid-induced insulin resistance in skeletal muscle[53,304,308,309]. Instead, it is suggested that fatty acids primarily inhibit glucose transport and not glycolysis[310]. A delay (2-4 h) between the rise in plasma FFA and the appearance of insulin resistance gave rise to the notion that FFA must be re-esterified inside muscle cells before they may cause insulin resistance[307]. This hypothesis was supported by studies conducted in animals and humans, demonstrating a close relationship between muscle fat content and insulin resistance[145,311-315]. Collectively, these studies demonstrated that muscle insulin resistance was related to the content of intramyocellular lipids (IMCL), however accumulating data suggest that not IMCL themselves cause insulin resistance, but rather indicate a role for other lipid metabolites like diacylglycerol (DAG), ceramide, and fatty acylcarnitines[177,316-318]. In this context, several studies have shown that accumulation of these lipid metabolites is positively correlated with impaired insulin signaling via activation of PKC isoforms and serine phosphorylation of the insulin receptor or IRS-1[307,316].

As outlined above, obesity, elevated plasma FFA levels, insulin resistance, and T2D are closely related. Numerous studies have investigated the implication of

dietary lipid composition for the development of chronic diseases like T2D or CVD, as discussed in chapter 1. Thus, it is generally accepted that saturated fatty acids induce insulin resistance[146,148,319], whereas monounsaturated fatty acids increase insulin sensitivity in T2D patients[320,321]. Furthermore, palmitic acid (PA), as a saturated fatty acid, as well as oleic acid, as an unsaturated fatty acid, have frequently been subject of *in vitro* studies. In this context, it could be demonstrated that the impact of each fatty acid on skeletal muscle metabolism depends on the channeling to distinct cellular metabolic fates. Exposure of skeletal muscle cells to PA has been shown to increase DAG levels[322-324], resulting in activation of the PKCθ-NFκB pathway including subsequent impairment of insulin action[325], enhanced secretion of IL-6, as well as downregulation of PGC1-α and diacylglycerol acyltransferase 2 (DGAT2)[326]. In contrast to that, exposure of skeletal muscle cells to OA has not been associated with accumulation of DAG. Supplementation of OA to PA-treated skeletal muscle cells was even shown to prevent PA-mediated accumulation of DAG and subsequent activation of the PKCθ-NFκB pathway[326]. A central role in this scenario has been attributed to DGAT2, as it is suggested to channel PA to TAG instead of DAG. In contrast to DAG, accumulation of TAG has not been associated with lipotoxic complications. Instead, TAG accumulation has been described as a physiological adaptive event in obese and T2D muscle fibers[324], reducing the formation of other more deleterious lipid species, thus possibly serving to prevent lipotoxic events[327]. Thus, the impact of fatty acid exposure on skeletal muscle insulin signaling has been suggested to critically depend on the intracellular channeling of different fatty acids.

Due to this distinct cellular impact, PA has been frequently applied to induce insulin resistance, reduce glucose uptake, and impair insulin signaling in a variety of cell types. In this context, PA has been applied in a large concentration interval, ranging from 50 µM to 2 mM. However, depending on the cell type, very high PA concentrations may not only elicit lipotoxic but also cytotoxic effects. In the course of this thesis, skeletal muscle cells were exposed to rather low fatty acid concentrations (100 µM), since one primary aim was to evaluate the combined effects of fatty acids and adipokines in the pathophysiology of obesity and T2D (chapter 7). Thus, it was desired (i) to not elicit maximal effects already due to high fatty acid concentrations, (ii) to simulate the physiological situation in the course of pathogenesis of these diseases, and (iii) to eliminate cytotoxic complications. The fatty acid concentrations

used here were found to not influence glucose metabolism, however, combined with adipokines severe implications for lipid metabolism were found.

8.2.2 Combined impact of fatty acids and adipokines on fatty acid metabolism

Although T2D patients are commonly characterized by dyslipidemia and enhanced adipose tissue mass, including an obesity-associated altered adipocyte secretory profile, data on the impact of the physiological coincidence of fatty acids and adipokines on skeletal muscle metabolism are scarce. Only few studies have investigated the combination of single adipokines with selected fatty acids. In this context, a study conducted in C2C12 myotubes combined PA-treatment with co-culture of myotubes with J774A.1 macrophages. The authors could show that while PA-treatment alone increased mRNA and protein levels of protein tyrosine phosphatase 1 B (PTP1B), additional co-culture with macrophages resulted in an even augmented PTP1B expression[328]. This effect was attributed to increased concentrations of the cytokines TNFα and IL-6 present in the culture media. Thus, synergistic effects of PA and classical adipocyte-associated cytokines could be demonstrated.

However, as discussed above, considering the complexity of the adipocyte secretome and the possible interrelated and synergistic effects of multiple adipokines in the CM, analysis of single adipokines can only provide limited insight into the physiological condition. Therefore, one aim of this thesis was to analyze the effects of combining CM and fatty acids on skeletal muscle metabolism, to obtain novel insights into the pathogenesis of obesity and transition to T2D. As described above, CD36 protein abundance and FA uptake were found to be increased in response to CM-treatment. In the presence of OA or PA this led to severely increased concentrations of TAG (chapter 7). This finding has been attributed to a CM-mediated increased capacity of SkMC to take up fatty acids, while supplementation of OA or PA was required to yield the necessary substrate for fatty acid transport, as regular culture media and CM are devoid of fatty acids.

Furthermore, in case of OA+CM, massive accumulation of IMCL droplets was detected, whereas this was not the case for PA+CM. While formerly lipid droplets were considered inert storage sites of energy dense fats, today lipid droplets are increasingly considered as dynamic functional organelles involved in many intracellular processes like lipid metabolism, vesicle trafficking, and cell signaling[329-331]. Lipid droplets are coated by a monolayer of phospholipids interspersed with a variety of proteins, termed PAT proteins, which regulate the delicate balance between lipid droplet synthesis, growth, and degradation in close interaction with lipases like HSL and ATGL[331]. Expression of PAT proteins, including perilipin, adipocyte differentiation-related protein (ADRP), and tail-interacting protein of 47 kDa (TIP47) as well as the recently discovered S3-12 and OXPAT, has been described to vary between tissues. In skeletal muscle, ADRP is the predominant lipid droplet-associated protein[332]. Furthermore, ADRP protein abundance has been found to be upregulated in circumstances of improved glucose tolerance, such as weight loss in obese, non-diabetic subjects or treatment of T2D subjects with insulin sensitizers[332]. Therefore, it has been hypothesized that ADRP might be able to sequester fatty acids as TAG in discrete lipid droplets, thereby protecting muscle from the detrimental effects of insulin-desensitizing intermediates on insulin action and glucose tolerance[331-333]. Thus, it may be speculated that OA+CM-treated cells appropriately store and potentially sequester lipids, leading to the accumulation of numerous lipid droplets observed in the course of this thesis, while PA+CM-treated SkMC lack this ability, possibly contributing to the adverse cellular effects observed in this situation.

Accumulation of IMCL has been the subject of ongoing debate, as it is discussed whether lipids accumulate in response to reduced mitochondrial activity or mitochondrial activity is reduced as a consequence of IMCL accumulation[310,334]. This controversy has been fuelled by conflicting data on mitochondrial function in metabolic states associated with IMCL accumulation such as obesity and insulin resistance. While some studies have reported a reduction in SkMC fatty acid oxidation in obesity[335-339] and T2D[340,341], others have described unchanged fatty acid oxidation rates in obese humans[267,342] and T2D patients[343] or even increased rates in different animal models[344-346]. Evaluation of this situation is also impeded by the finding that muscles of T2D patients exhibit less mitochondria compared to controls[347], further complicating objective comparison. As mitochondria are the power plants of a cell, providing energy in the form of ATP for cellular processes

including skeletal muscle contraction, their proper function is essential for maintaining cellular functionality. Turnover of ATP strongly depends on a variety of factors such as skeletal muscle fiber type, duration and intensity of the contraction stimuli, supply of energy fuel, and overall constitution of the organism. Dysfunction of any of these processes may result in defective mitochondrial performance. Thus, recent studies have demonstrated that an uncoordinated upregulation of mitochondrial β-oxidation and downstream metabolic pathways such as the TCA cycle and the electron transport chain results in incomplete β-oxidation and accumulation of detrimental lipid metabolites, like acyl-carnitines[348,349]. This further underlines the general understanding that IMCL themselves are not lipotoxic but rather protect from deleterious lipid metabolites by sequestering and properly storing excess intracellular lipids. Furthermore, additional factors influencing IMCL accumulation and IMCL droplet biogenesis, such as availability of different fatty acid subtypes (OA vs PA) or lipid droplet coating proteins, as discussed above, have been described. Nevertheless, many questions remain unanswered concerning the correlation between IMCL accumulation, formation of detrimental lipid intermediates, and mitochondrial fatty acid oxidation capacity. Further well-controlled studies will be needed to unravel, whether the hypothesized impaired mitochondrial function and reduced mitochondrial number represent the primary starting point in the pathogenesis of insulin resistance, or whether they constitute another feature of the insulin resistant state.

In our study, fatty acid oxidation was found to be profoundly reduced in the same situations that displayed increased amounts of IMCL (chapter 7). Thus, we could confirm the previously described association between IMCL accumulation and impaired fatty acid oxidation. However, additional studies are needed to clarify whether IMCL are cause or consequence of impaired mitochondrial performance. Nevertheless, we could show that stimulating contraction, via electrical pulse stimulation (EPS), in parallel to CM- and fatty acid-treatment was able to restore the severely reduced ^{14}C-OA oxidation in SkMC treated with OA+CM. Interestingly, this was not the case for PA+CM. On the one hand, these data might indicate that mitochondrial oxidative capacity was not permanently disturbed in SkMC treated with OA+CM, while this could be the case for SkMC incubated with PA+CM. On the other hand, it may be speculated that SkMC treated with PA+CM or OA+CM retained a different capacity to respond to the contraction stimulus. Skeletal muscle

contraction is able to elicit a wide variety of cellular responses (discussed in chapters 1.3 and 8.3), but eliciting a modification of fuel oxidation in response to changes in nutrient availability, termed metabolic switching, is one of the most essential functions. Metabolic flexibility is positively correlated with insulin sensitivity[350]. Furthermore, anti-diabetic treatment with TZDs[351] as well as a program of weight-loss and improved aerobic fitness training[352] are able to improve metabolic flexibility. The ability to change substrate oxidation in response to the nutritional status strongly depends on the cellular equipment of oxidation and storage capacities, thus intact balance and proper function of e.g. cellular fuel sensors, oxidative enzymes, mitochondrial biogenesis, and lipid droplet coating proteins is required. A dysbalance of these processes will impair metabolic switching and result in accretion of fuel substrates. Indeed, a state of metabolic inflexibility has been suggested to contribute to IMCL accumulation and thus contribute to the development of insulin resistance and T2D[310]. However, considering the open questions concerning the etiology of IMCL accumulation, it could also be a reflection of the insulin resistant state, with both carbohydrate and fat oxidation being less sensitive to the normal physiological response of insulin. In summary, one could hypothesize that the improved ^{14}C-OA oxidation in SkMC treated with OA+CM in response to electrical pulse stimulation observed here (chapter 7) might indicate functional metabolic switching, while absence of this EPS-stimulated effect in PA+CM-treated SkMC may indicate a potential metabolic inflexibility. Although EPS treatment showed a tendency to increase ^{14}C-OA oxidation in CM- and OA-treated SkMC, it is noteworthy that significant improvement of ^{14}C-OA oxidation was only observed in states of severely impaired oxidation, as observed after incubation with OA+CM. It may be speculated that ^{14}C-OA oxidation had potentially reached its maximum in the remaining situations.

The data obtained in this part of the thesis indicate that (i) adipokines influence cellular fatty acid uptake, thereby promoting lipotoxicity, (ii) OA and PA are differentially stored and metabolized by SkMC, demonstrating the capacity and necessity of lipid droplet coating proteins to properly store and thereby potentially sequester intracellular lipids, and (iii) fatty acid oxidation may be restored in response to contraction stimuli in the presence of IMCL droplets, while inadequate lipid handling in response to PA+CM-treatment seems to persistently impair the cells' capacity to respond to contraction stimuli (Fig. 8.1).

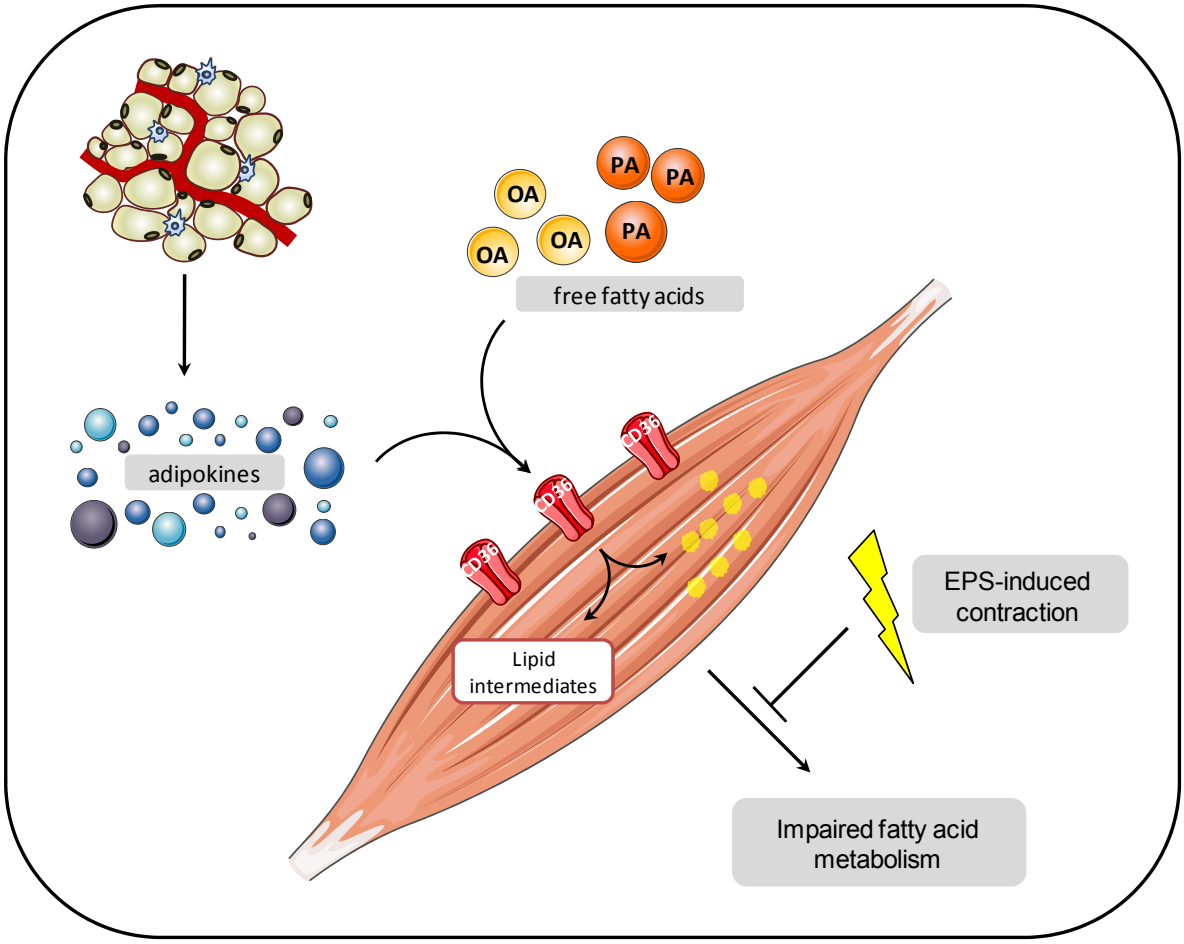

Fig. 8.1 **Schematic description of important results.** Combined exposure of skeletal muscle cells (SkMC) with adipokines and oleic acid (OA) or palmitic acid (PA) increases fatty acid uptake and leads to accumulation of intramyocellular lipid droplets or possibly lipid intermediates, respectively. As a result, fatty acid metabolism is impaired, which can selectively be prevented by electrical pulse stimulation (EPS).

8.2.3 Synergistic impact of fatty acids and adipokines on myogenic markers

As outlined in the previous section, adipokines and fatty acids are able to induce multi-facetted and sometimes persistent alterations of skeletal muscle metabolism. Interestingly, we also found several myogenic markers to be affected by

CM-treatment. Analyses of SkMC at successive days of differentiation have revealed that *in vitro* differentiating skeletal muscle cells display increasing protein levels of myogenic transcription factors such as myogenin, MHC, and MyoD, and that differentiated skeletal muscle cells are still characterized by a high protein abundance of myogenin and MHC (chapter 2). Treatment of SkMC with CM could be demonstrated to reduce the protein abundance of myogenin, MyoD, and MHC. In order to gain further insight into the complex cellular modifications introduced by adipokines and fatty acids, protein abundance of these myogenic markers was analyzed in SkMC after combined incubation with CM and fatty acids.

In this context, it has been discovered that exposure of SkMC to CM and fatty acids introduces severe alterations of myogenin (Fig. 8.2A). Protein abundance of the transcription factor myogenin was found to be significantly decreased in SkMC after incubation with CM (~50%), while treatment with OA increased myogenin protein levels by ~50% compared to control cells. Combination of OA+CM eliminated this upregulation. Incubation of SkMC with PA did not significantly alter myogenin protein levels, whereas combination of PA+CM caused a severe reduction of protein abundance by ~80% compared to control cells. In addition to myogenin, protein levels of GLUT4 and MyoD, two other markers of differentiated myotubes, were analyzed. GLUT4 protein abundance remained constant in all investigated situations (Fig. 8.2B), and also MyoD protein levels were not significantly altered (Fig. 8.2C).

The cellular implications of this severe manipulation of myogenin in differentiated skeletal muscle cells however remain incompletely understood. While myogenin has received considerable attention as a key regulatory molecule involved in differentiation of satellite cells and myoblasts with a variety of regulating factors, its role in differentiated myotubes has been less well studied. So far a study conducted in transgenic mice suggested a role for myogenin as a putative messenger in the pathway linking nerve-evoked action potential activity to muscle gene expression[353]. Furthermore, a recent study in myogenic cells demonstrated that diversification towards expression of fast or slow skeletal muscle fiber types can be determined by the activity and dose of myogenin[354].

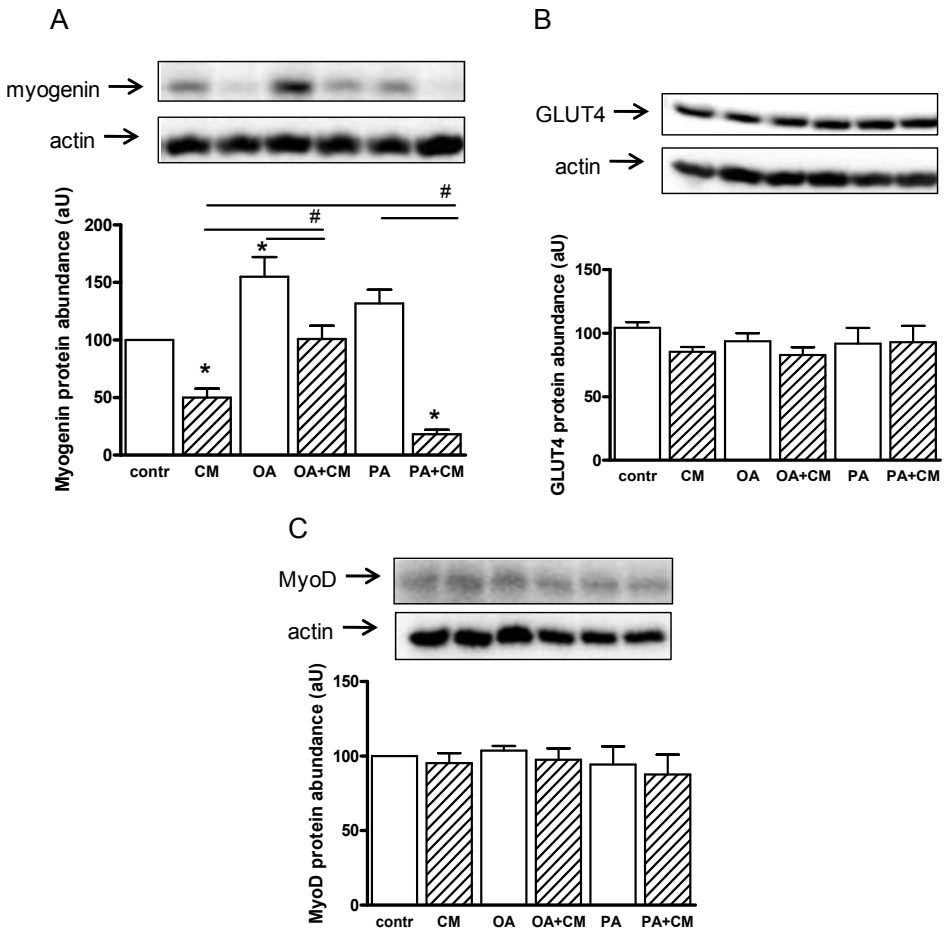

Fig. 8.2 **Influence of CM- and fatty acid-treatment on myogenic markers.** Protein abundance of myogenin, GLUT4, and MyoD was analyzed in SkMC after incubation with adipocyte-conditioned medium (CM) and fatty acids. While treatment conditions were found to severely alter myogenin protein abundance, GLUT4 and MyoD protein levels were not changed.

Generally, muscle fibers are classified as slow-twitch fibers, also referred to as type I or red fibers, as well as fast-twitch fibers, also known as type II or white muscle fibers[355,356]. Each fiber type is associated with a specific set of proteins, enzymes and transcription factors influencing metabolic and contractile properties of the muscle fibers. Thus, slow-twitch fibers are characterized by e.g. a high capillary and mitochondrial density, enabling the muscle fibers to preferentially perform oxidative metabolism, while fast-twitch fibers display increased enzyme activities of e.g. glycogen phosphates, resulting in a high glycolytic potential[356]. These metabolic properties have been demonstrated to determine the contractile performance of the skeletal muscle. Thus, it has been revealed that endurance trained athletes display a

higher content of slow-twitch than fast-twitch fibers, while sprinters have been shown to exhibit muscles predominantly composed of fast-twitch fibers[357-359]. Additionally, is has been demonstrated that insulin sensitivity correlates with the proportion of slow-twitch oxidative fibers[360], resulting in increased insulin-stimulated glucose transport in skeletal muscle enriched with slow-twitch muscle fibers[361-363], thus priming slow-twitch muscle for accelerated glucose uptake and metabolism[356]. Muscle fiber type formation has been demonstrated to be influenced by several stimuli, with exercise being the most well-known determinant of fiber type formation. Signaling pathways involved in the regulation of fiber type formation include Ras/mitogen-activated protein kinase (MAPK)[364], calcineurin[365,366], calcium/calmodulin-dependent protein kinase IV[367], and PPARγ coactivator-1α (PGC-1α)[368]. It has been proposed that these pathways represent the basis of a signaling network that controls skeletal muscle fiber type formation and metabolic profiles that protect against insulin resistance and obesity[356].

As mentioned above, previously published data on myogenin suggest that it might also be involved in muscle fiber type formation. Thus, data presented in this thesis allow for speculation that adipokines and fatty acids might also be involved in the regulation of fiber type formation or differentiation status via influencing myogenin protein abundance. However, since cell morphology was observed to be normal and protein levels of other myogenic differentiation markers GLUT4 and MyoD remained unaltered, it may be speculated that severe loss of myogenin in response to PA+CM might indicate a subtle partial dedifferentiation possibly only involving certain aspects of SkMC performance. Indeed, fatty acid oxidation, a core competence of skeletal muscle cells, was found to be severely and persistently disturbed in SkMC after incubation with PA+CM (chapter 7).

These data support the previously proposed role for adipokines in promoting lipotoxic effects of fatty acids, as the strongest influence on myogenin protein abundance was observed after incubation with PA+CM (Fig. 8.1). Additionally, previously reported significant differences between OA and PA could be confirmed here. Most interestingly, these data suggest a possible impact of adipokines and fatty acids on the cellular framework of skeletal muscle, involving fundamental properties such as mitochondrial function associated with fiber type specificity. Further studies will need to unravel, whether skeletal muscle function is a target of adipokine and fatty acid action.

8.3 Exercise as important regulator of skeletal muscle function

8.3.1 Exercise as a therapeutic strategy

In light of the epidemic prevalence and estimated increasing rates of T2D cases worldwide, there is an urgent need for cost efficient and effective therapeutic strategies to combat the development of this disease and its associated complications. Weight reduction has been highlighted as a primary therapeutic strategy in all obese individuals with T2D or at risk of developing T2D[11]. Although an increasing number of pharmacological agents and surgical measures are available today to induce or assist weight reduction, these therapies are generally only applicable or available for a limited number of patients. Thus, exercise has been recognized as a cornerstone in the treatment and prevention of T2D[369-371]. Current guidelines recommend diet and/or exercise-induced weight loss as the principal therapy for overweight and obese individuals in the prevention and management of T2D[11]. In this context, 30-45 min of moderate intensity aerobic physical activity (40–60% of maximal oxygen uptake, or 50–70% of maximum heart rate) 3–5 d/wk initially with a gradual increase in the duration and frequency of the activity have been recommended[11]. A number of clinical studies have demonstrated such lifestyle intervention therapies, comprising weight management, dietary modification, and an increase of physical activity, to be effective strategies to delay or prevent the onset of T2D[181,188-190].

Exercise-induced reduction of adipose tissue mass is suggested to exert positive influences by attenuating previously discussed obesity-associated alterations, such as adipokine secretion, adipocyte size, and hypoxia. Thus, weight loss has been indicated to be effective in normalizing adipokine levels such as IL-6[205], MCP-1[204], and TNFα[206]. However, recent studies have also revealed beneficial effects of exercise even without weight loss[372,373]. In this context, moderate-intensity exercise has been demonstrated to be able to significantly reduce total and abdominal fat in both obesity and T2D, indicating that changes in fat distribution in response to exercise favor reduction of visceral fat[369]. Considering the pivotal role of

visceral adipose tissue in obesity-associated insulin sensitivity[374], as discussed above, reduction of visceral fat may beneficially influence whole body glucose homeostasis even without changes in BMI.

Additionally, acute physical activity has also been associated with improved insulin sensitivity[375], enhanced insulin-stimulated glucose uptake in the immediate post-exercise period[192], as well as insulin-independent activation of glucose transport via activation of AMPK[195,196] without accompanying dietary modifications. Accordingly, several of these characteristic features of physical exercise could be demonstrated in the *in vitro* model of electrically stimulated muscle contraction. In this context, EPS-treatment was revealed to activate AMPK and increase basal as well as insulin-stimulated glucose uptake (chapter 6). Furthermore, electrically stimulated muscle contraction was also able to prevent impairments of insulin signaling induced by incubation with CM, chemerin, or MCP-1. Hence, this *in vitro* model proved to be a valuable tool to simulate muscle contraction and, in accordance with literature, indicated protection of physical exercise against adipokine-induced insulin resistance.

Generally, results from lifestyle intervention studies have suggested that defects associated with obesity, such as insulin resistance, adverse adipokine secretion, or adipose tissue expansion are reversible. However, *in vitro* studies have yielded conflicting results. While some studies have revealed no differences of insulin-stimulated signaling events like autophosphorylation of IR and Akt phosphorylation as well as insulin-stimulated glucose transport in cultured human SkMC obtained from obese non-diabetic patients with impaired insulin action compared to non-obese controls[376], others have demonstrated defective insulin-stimulated glucose transport displayed by cultured human SkMC obtained from obese T2D patients[377,378]. The former studies suggest that insulin resistance is an acquired feature of obesity, whereas the latter results indicate retained insulin resistance in culture. It may be discussed that the capacity to recover from insulin resistance may depend on the duration of exposure of SkMC to insulin resistance-promoting conditions like elevation of adipokines or FFA levels. Nevertheless, the precise time course involved as well as the extent of reversibility remain unclear.

In order to obtain further insight into the processes involved in reversing insulin resistance, the CM model of our laboratory was applied. Besides previously reported defects of insulin signaling and action in response to CM-treatment[245,250,252],

we demonstrated additional defects in SkMC such as downregulation of myogenin, MHC and MyoD, reduction of IL-6 and IL-8 secretion, and increased MCP-1 secretion (chapter 2). Furthermore, ROS and NO production were elevated and SDH activity was reduced upon treatment with CM, pointing to increased oxidative stress and reduced mitochondrial capacity. Withdrawal of CM normalized insulin signaling, IL-6 secretion, and expression of MyoD and MHC. However, myogenin levels as well as disturbed secretion of IL-8 and MCP-1 were retained over the 48 h period of CM withdrawal. In summary, insulin signaling was observed to recover after CM withdrawal, while defects of selected myogenic markers and secreted factors were retained even in absence of CM, illustrating longer-lasting damages. Therefore, it may be speculated that although lifestyle intervention strategies involving weight reduction and enhanced physical activity indicate improvement of insulin sensitivity, some features of insulin resistance might be retained, possibly depending on the duration of exposure to insulin-resistance inducing factors. Additional studies are needed to further unravel the mechanisms involved in reversibility of insulin resistance.

8.3.2 Impact of exercise on muscle cell function

Besides exercise-associated weight reduction and contraction-stimulated acute improvements of glucose uptake, prolonged physical activity has been suggested to also mediate its positive effects by eliciting a complex set of physiological responses. Skeletal muscle has been demonstrated to display an enormous capacity to adapt to external stimuli such as nutrient availability or contractile activity. Persistent alterations and sustained remodeling of skeletal muscle physiology have been demonstrated to be the consequence. As outlined in the introduction, PGC1α has been identified as key regulator of muscle adaptation processes[207,379]. In this context, PGC1α has been revealed to stimulate mitochondrial biogenesis through coactivation of nuclear respiratory factor (NRF)[380] and to regulate genes involved in oxidative phosphorylation through interactions with the NRFs as well as estrogen-related receptor α (ERRα)[381]. Additionally, PGC1α regulates genes involved in fatty acid catabolism through coactivation of PPARs and EERα[381-384], and

induces expression of GLUT4 by activating myocyte enhancer factor-2 (MEF2)[385]. Interestingly, PGC1α was recently shown to regulate a broad program of antioxidant defense, thereby limiting potential damage due to increased mitochondrial activity[386]. Furthermore, PGC1α has been attributed a role in muscle fiber type formation. In this context, PGC1α mRNA levels have been revealed to be more abundant in slow-twitch oxidative fibers[349], PGC1α gene expression and protein abundance have been shown to increase in response to exercise[387-390], and PGC1α was demonstrated to potently drive transcription of myofibrillar genes typical of oxidative muscle fibers by coactivating MEF2C and MEF2D[368]. Consequently, physical exercise is able to evoke switch from quickly fatigued fast-twitch fibers to oxidative slow-twitch fibers via activation of PGC1α. This way, PGC1α procures enhanced mitochondrial oxidative capacity associated with type 1 slow-twitch fibers and mediates coordinated upregulation of the enzymatic machinery of β-oxidation and downstream metabolic pathways, such as the tricarboxylic acid (TCA) cycle and electron transport chain[349]. As a consequence, lipids are metabolized more efficiently, conferring protection against accumulation of IMCL-associated lipid metabolites, such as fatty acylcarnitine esters, DAG, or ceramide, and ultimately protect against induction of insulin resistance. These molecular processes are the underlying principle of the athlete's paradox. Furthermore, in the course of this thesis we have observed severe accumulation of IMCL droplets after incubation with OA+CM, however exercise was revealed to be able to increase previously diminished ^{14}C-OA oxidation (chapter 7). In light of the previously described pivotal role for PGC1α, it may be hypothesized that electrical pulse stimulation improved ^{14}C-OA oxidation via activation of PGC1α. Future studies will need to investigate the involvement of PGC1α in EPS-induced changes observed in our *in vitro* model.

Besides PGC1α, an important role has also been attributed to carnitine palmitoyltransferase-1 (CPT-1)[391], an important rate-limiting step in mitochondrial fatty acid uptake and subsequent oxidation. Cross-sectional studies have reported reduced CPT-1 activity in the skeletal muscle of obese individuals[339], whereas CPT-1 activity was demonstrated to be increased in the muscle of trained individuals compared with untrained individuals[392,393]. Furthermore, endurance training of obese individuals has recently been shown to elevate muscle CPT-1 activity associated with an increased mitochondrial FA oxidation[394]. Additionally, CD36 has been shown to be present at the mitochondrial membrane, increase long-chain fatty acid oxidation,

and colocalize with CPT-1[391,395,396]. Increased colocalization in response to endurance exercise training has been demonstrated to highly correlate with whole-body fat oxidation[396]. In light of this fairly new role for CD36, it may be speculated that our observation of EPS-induced increase of CD36 protein abundance (chapter 7) might represent an additional feature of skeletal muscle adaptation in response to exercise stimuli, thereby potentially contributing to the EPS-induced increase in fatty acid oxidation observed in that study. However, additional analyses are needed to unravel the subcellular localization of CD36 protein in our model system.

Conclusively, physical exercise has been demonstrated to be a potent regulator of skeletal muscle physiology as it is able to elicit fiber type switch and increase mitochondrial oxidative capacity, thereby ameliorating or even preventing skeletal muscle insulin resistance. With our *in vitro* models of CM-induced SkMC insulin resistance and EPS-induced SkMC contraction, we have valuable tools at hand to further investigate the underlying mechanisms of exercise-mediated improvements of insulin sensitivity.

8.3.3 Muscle as a secretory organ

Recently, skeletal muscle has been proposed to be considered as an endocrine organ, as it is able to produce and secrete a number of cytokines and other peptides that may exert paracrine and endocrine effects[185,210,211,213]. Frequently described myokines include IL-6, IL-8, IL-15[214], brain derived neurotrophic factor (BDNF)[215], as well as fibroblast growth factor-21 (FGF21)[216] and follistatin-like-1[217]. These factors have been discussed to contribute to the beneficial effects of physical activity.

IL-6 is the first identified and most studied myokine, however its role in insulin resistance is highly controversial[213]. On the one hand it is a well-known detrimental adipokine and chronically elevated IL-6 levels have been associated with obesity and reduced insulin action[213,218]. On the other hand short-term contraction-associated augmentation of IL-6 levels has been demonstrated to have highly beneficial effects. In this regard, IL-6 has been described as potent insulin sensitizer and regulator of fat metabolism, able to increase glucose uptake, activate AMPK, augment lipolysis and

enhance fat oxidation[210,211,213,219]. Furthermore, IL-6 has also been suggested to exhibit anti-inflammatory properties as it has been demonstrated to stimulate the production of classical anti-inflammatory cytokines such as IL-1ra and IL-10[220]. In this regard, data obtained in this thesis nicely correspond to these previous findings. We indeed observe increased secretion of IL-6 in response to electrically induced muscle contraction (chapter 6). Additionally, EPS-treatment is able to prevent activation of inflammatory signaling mediated by incubation with CM, chemerin, or MCP-1. These findings support previously reported anti-inflammatory effects of exercise on the one hand, and emphasize the potential of our *in vitro* model to accurately mimic physical activity on the other hand.

In conclusion, physical exercise may engage 3 different possible mechanisms to mediate its beneficial effects: (i) reduction of adipose tissue mass, (ii) induction of adaption processes in skeletal muscles, resulting in enhanced insulin sensitivity as well as improved mitochondrial oxidative capacity, and (iii) secretion of contraction-induced myokines. The multitude of changes and adaptation processes induced by muscle contraction highlight the important role of physical activity in preventing, reversing, and managing obesity and T2D and its essential contribution as an element of lifestyle therapeutic strategies. Unfortunately, regular physical activity sustained for a persistent duration of time has been proven to not be much appealing to the majority of T2D patients.

8.4 Perspectives

Data obtained in the course of this thesis met several objectives proposed in the introduction. Although novel adipokines, like chemerin, could undoubtedly be revealed to contribute to the negative crosstalk between adipose tissue and skeletal muscle, only application of CM, comprising the entire adipocyte secretome, could be shown to profoundly influence skeletal muscle lipid metabolism. These alterations might substantially contribute to the development of muscle insulin resistance and transition from obesity to type 2 diabetes (T2D). Therefore, additional studies directed to further dissect the composition of the adipocyte secretome and elaborate studies characterizing the potential effects of defined combinations of adipokines will be helpful in identifying possible causative agents as potential systemic markers or valuable therapeutic targets.

Results obtained from the exposure of primary human SkMC to the combination of CM and fatty acids revealed a novel role for adipokines in promoting the lipotoxic potential of physiologically healthy concentrations of fatty acids. It may be speculated that these data indicate an increased lipotoxic risk already early in the pathogenesis of obesity and T2D. Future studies will have to unravel the contribution of adipokine-promoted lipotoxicity in the progression of impaired insulin sensitivity to T2D. Furthermore, protein levels of myogenic differentiation markers were found to be affected by treatment with CM, fatty acids, or their combinations. As muscle cell insulin sensitivity has been shown to closely correlate with fiber type-associated mitochondrial oxidative capacity, alterations of muscle cell physiology might substantially contribute to impaired fatty acid oxidation, increased accumulation of IMCL-associated detrimental lipid metabolites, and ultimately attenuated muscle insulin sensitivity. Although sedentary T2D patients are frequently characterized by altered muscle plasticity compared to physiologically active individuals, we are only beginning to understand the underlying molecular mechanisms. Potential regulation of muscle fiber type formation by adipokines and fatty acids represents an interesting and novel aspect that warrants further investigation.

Combining CM-, fatty acid-, and EPS-treatment has revealed that *in vitro* muscle contraction is able to prevent impairments of fatty acid oxidation induced by incubating SkMC with OA+CM, while this was not the case for PA+CM. Although differential effects of saturated and unsaturated fatty acids have been previously described, these data indicate a substantial and persistent impairment of SkMC to respond to contraction stimuli after PA+CM-treatment, while the ability to adapt to electrically stimulated contraction was retained in SkMC treated with OA+CM. These data might contribute to the understanding of metabolic inflexibility frequently observed in T2D, however the complexity of skeletal muscle adaption processes elicited by physical exercise demands further investigation of the precise molecular processes. *In vitro* models of the adipocyte-myocyte crosstalk as well as muscle contraction, applied in this thesis, constitute valuable tools enabling precise investigation of the processes involved under tightly controlled conditions, and the absence of detectable amounts fatty acids from CM allows detailed analysis of the contributing impact of defined concentrations of fatty acids.

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Contribution to chapter 2-7

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

Chapter 2

- Cultivation of SkMC and preparation of SkMC lysates at different days of differentiation
- Analysis of differentiation-dependent protein levels of myogenin and GLUT4
- Preparation of microscopic images of SkMC

Chapter 3

- Cultivation of SkMC and preparation of SkMC lysates at different days of differentiation
- Establishment and optimization of the glucose uptake protocol for SkMC
- Analysis of glucose uptake

Chapter 4

- Preparation of manuscript together with Kristin Eckardt and Prof. Jürgen Eckel
- Literature research on various topics including ectopic lipids, IMCL accumulation, and PGC-1α
- Preparation of schematic figure together with Kristin Eckardt

Chapter 5

- Preparation of manuscript together with Kristin Eckardt and Prof. Jürgen Eckel
- Literature research together with Kristin Eckardt

Chapter 6

- Staining of SkMC with JC-1
- Measurement of SkMC glucose uptake together with Silja Lambernd
- Assessment of fatty acid oxidation

Chapter 7

- Preparation of manuscript together with Prof. Jürgen Eckel
- Cultivation of SkMC together with Birgit Platzbecker and Annette Schober
- Design and execution of CM-, fatty acid-, and EPS-treatment protocols together with Silja Lambernd
- Nile Red staining of SkMC and generation of microscopic images
- Preparation of SkMC lysates for ADRP protein analyses
- Assessment of fatty acid uptake
- Measurement of fatty acid transport
- Analysis of protein abundance of CD36 and FATP4
- Staining of SkMC with JC-1
- Assessment of fatty acid oxidation experiments
- Performance of triglyceride quantification

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Annika Taube