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The Role of Contractile Activity in the Crosstalk between Human Skeletal Muscle Cells and Adipocytes

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Summary

Nowadays, the prevalence of type 2 diabetes is increasing worldwide and reaches epidemic proportions already. Obesity and inactivity are closely associated with muscle insulin resistance, which is a major risk factor for the pathogenesis of type 2 diabetes. In this context, adipocyte-secreted factors, so-called adipokines, have been intensively studied and implicated in the negative crosstalk between adipose tissue and skeletal muscle. In contrast, regular physical activity does not only prevent obesity, but also considerably improves insulin sensitivity and skeletal muscle metabolism. While the detrimental effect of adipokines has been intensively studied, less is known about the underlying mechanisms of the beneficial effect of muscle acitivity, partly due to the lack of suitable models. Most likely, specific myokines released from contracting muscle mediate these health promoting effects.

Therefore, we thought to establish and characterise an *in vitro* model of human skeletal muscle contraction, with a view to directly studying the signalling pathways and mechanisms that are involved in the beneficial effects of muscle activity. In this study, contracting human skeletal myotubes were established by applying electrical pulse (EPS) stimulation. We showed that the effects of EPS on myotubes were similar to the effect of exercise on skeletal muscle *in vivo* in terms of *de novo* sarcomer formation and enhanced AMPK activation, glucose uptake, and mitochondrial biogenesis. Additionally, the secretion of myokines like IL-6 was enhanced. Most interestingly, muscle contractile activity eliminates insulin resistance, as insulin signalling was not disturbed after incubation with various adipokines and inflammatory signalling was not activated.

Using the contracting myotubes, we found 44 novel contraction-regulated myokines and 52 myokines, which have not been described as myokines before. Comparing the secretome of primary human adipocytes and myotubes revealed an extensive overlap. We termed cytokines released by both cell types 'adipo-myokines'. PEDF is a good example, since it is one of the most abundant adipokines secreted from primary human adipocytes. Nevertheless, PEDF secretion is increased by contractile activity of myotubes, while whole body PEDF serum levels of humans decrease after one acute bout of exercise. Beside PEDF, DPP4 and follistatin-like 1 have been validated as myokines within the scope of this study.

Just recently, irisin, a novel contraction-regulated myokine, has drawn attention, since it was reported that the beneficial effect of exercise can partly be ascribed to irisin. In mice, viral delivery of FNDC5, the gene encoding the precursor protein of irisin, diminished diet-induced weight gain and metabolic dysfunction, since it caused a browning of subcutaneous fat. Brown adipose tissue AT is primarily a thermogenic tissue that burns fat to generate heat by uncoupling the proton electrochemical gradient. Thus, this tissue is discussed as target for novel type 2 diabetes drug therapies. However, data generated here in primary human adipocytes using recombinant irisin revealed evidence against the beneficial effect of irisin in humans. Irisin was not contraction-regulated in human myotubes and most interestingly, it had no effect on the browning of primary human adipocytes.

The *in vitro* contraction model provided the starting point to investigate mechanisms and underlying signalling pathways that mediate the beneficial effects of muscle contraction. With this model, we found that contractile acitivity of skeletal muscle abrogates inflammatory signalling and insulin resistance. We identified 44 contraction-regulated myokines and validated PEDF, DDP4 and follistatin-like 1. In our model system irisin was not contraction-regulated and the beneficial effect could not be determined. Taken together, these findings will contribute to understand the molecular pathways of physical activity, which will help to further clarify the potential of exercise as a way of combating insulin resistance.

Zusammenfassung

Weltweit steigt die Anzahl der Typ 2 Diabetiker und erreicht mittlerweile epidemische Ausmaße. Einer der wichtigsten Risikofaktoren für die Pathogenese des Typ 2 Diabetes ist die Insulinresistenz des Skelettmuskels, die vor allem durch Adipositas und Bewegungsmangel ausgelöst wird. Die Forschung der letzten Jahre hat gezeigt, dass unter anderem die verstärkte Freisetzung von Zytokinen aus dem Fettgewebe, so genannter Adipokine, zu einer Insulinresistenz im Skelettmuskel führt. Im Gegensatz dazu hat regelmäßige körperliche Bewegung nicht nur einen vorbeugenden Effekt zur Vermeidung von Adipositas, sondern verbessert die Insulinsensitivität und den Metabolismus des Skelettmuskels. Während die negative Wirkung von Adipokinen auf die Insulinsensitivität des Skelettmuskels bereits in vielen Studien untersucht wurde, ist über die positiven Effekte der Muskelkontraktion auf das Insulinsignalling weniger bekannt, nicht zuletzt weil geeignete primäre Zellkulturmodelle fehlten. Aktuelle Forschung deutet darauf hin, dass kontraktionsregulierte Myokine positive Effekte auf die Gesundheit haben können.

Aus diesem Grunde war das Ziel dieser Arbeit, mittels elektrisch stimulierter Kontraktionen von primären humanen Skelettmuskelzellen, ein Zellkulturmodell zur Simulation von Muskelaktivität zu etablieren und zu charakterisieren. Durch dieses Modell können unter genau definierten Bedingungen Signalwege und Mechanismen untersucht werden, die für die positiven Effekte von Muskelaktivität auf das Insulinsignalling verantwortlich sind.

Die elektrische Stimulation von humanen Skelettmuskelzellen zeigte vergleichbare Effekte, wie sie schon für das Training in der Skelettmuskulatur in der Literatur beschrieben sind. Durch die Kontraktion konnten wir eine *de novo* Anordnung funktionell aktiver Sarkomere zeigen und weiterhin nachweisen, dass die Aktivität der AMPK, die Glukoseaufnahme und die Mitochondrienbiogenese erhöht sind. Durch dieses Modell konnten wir zeigen, dass allein die kontraktile Aktivität der Muskelzellen das Auslösen der Insulinresistenz durch Adipokine verhindert. Dies folgern wir aus der Beobachtung, dass das Insulinsignalling nach der Inkubation mit Adipokinen nicht verändert und inflammatorische Signalwege nicht aktiviert werden, wenn die Myotuben vorher elektrisch stimuliert worden waren.

Wir haben in dieser Arbeit 52 neue, noch nicht beschriebene Myokine und 44 kontraktions-regulierte Myokine identifiziert. Der Vergleich des Sekretoms von primären humanen Adipozyten und Myotuben zeigt eine große Übereinstimmung. Wir haben Zytokine, die von beiden Zelltypen sezerniert werden, Adipo-Myokine benannt. PEDF ist hierfür ein gutes Beispiel. Es wird vom Fettgewebe sezerniert und ist eines der höchst abundanten Adipokine. Zusätzlich wird es auch von human Myotuben sezerniert, ist durch die kontraktile Aktivität reguliert, während der PEDF Serumlevel nach einer akuten Belastung verringert ist. Neben PEDF wurden DPP4 und Follistatin-like 1 als Myokine in dieser Arbeit validiert.

Vor kurzem hat Irisin, ein neu beschriebenes kontraktionsreguliertes Myokin, die Aufmerksamkeit der Wissenschaft auf sich gezogen. Denn es wurde beschrieben, dass die positiven Effekte der körperlichen Bewegung teilweise auf Irisin zurückgeführt werden können. Virale Überexpression von FNDC5 in Mäusen, dem Gen, das das Vorläuferprotein von Irisin transkribiert, vermindert die nahrungsinduzierte Gewichtszunahme und metabolische Dysfunktion in Mäusen. Dieser Effekt wurde durch die vermehrte Bildung von braunem Fettgewebe erklärt, einem Gewebe, in dem der elektrochemische Protonengradient durch das Protein UCP1 entkoppelt wird und Energie in Wärme umgewandelt wird. Die Aktivierung des braunen Fettgewebes im Menschen ist zurzeit ein Forschungsansatz für die Entwicklung von neuen Therapien zur Behandlung des Typ 2 Diabetes. Daten aus dieser Arbeit mit primären humanen Adipozyten und rekombinantem FNDC5/Irisin deuten jedoch darauf hin, dass dieser positive Effekt von Irisin nicht auf humane Zellen übertragen werden kann. Irisin ist in humanen Myotuben nicht kontraktionsreguliert und vor allem hatte es keinen Effekt auf die Bildung von braunen Adipozyten.

Das in dieser Arbeit entwickelte Kontraktionsmodell war der Ausgangspunkt um Mechanismen und Signalwege der Muskelkontraktion, die für die positiven Effekte von körperlicher Bewegung verantwortlich sind, zu analysieren. Durch dieses Modell konnten wir zeigen, dass die kontraktile Aktivität allein das Auslösen inflammatorischer Signalwege durch Adipokine hemmt und somit das Auslösen einer Insulinresistenz verhindert. Wir haben 44 neue kontraktionsregulierte Myokine identifiziert und 3 Myokine validiert. In unserem Modell ist Irisin nicht kontraktions-reguliert und die positiven Effekte können nicht

nachgewiesen werden. Diese Ergebnisse geben neue Einblicke in die Mechanismen von körperlicher Bewegung auf molekularer Ebene, die zur Behandlung des Typ 2 Diabetes von Bedeutung sind.

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List of abbreviations

ACC	CoA carboxylase
ADP	Adenosine diphosphate
AICAR	5-Aminoimidazole-4-carboxamide riboside
AMP	Adenosine monophosphate
AMPK	AMP activated protein kinase
ATP	Adenosine triphosphate
aPKC	Atypical protein kinase C
AS160	Akt substrate of 160 kD
BDNF	Brain-derived neurotrophic factor
CaM	Calmodulin
CaMKKβ	Ca^{2+}/Calmodulin-dependent kinase kinase β
CBP	CREB (cyclic AMP response element–binding protein)-binding protein
CD36	Cluster of Differentiation 36
CoA	Acetyl-coenzyme A
DAG	Diacylglycerol
DPP4	Dipeptidyl peptidase 4
EPS	Electrical pulse stimulation
ERK	Extracellular signal-regulated kinase
ESI	Electrospray ionization
FAT	Fatty acid transporter
FFA	Free fatty acids
FNDC5	Fibronectin type III domain containing 5
Foxo	Forkhaead box O
Fstl1	Follistatin-like 1
GLUT	Glucose transporter
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase 3
IκB	Inhibitors of κB
IKK	IκB kinase
IL	Interleukin
IR	Insulin receptor
IRS	Insulin receptor substrate
LC	Liquid chromatography
LKB	Tumor suppressor kinase
MALDI	Matrix-associated laser desorption/ionization

MAPK	p38 mitogen activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MS	Mass spectrometry
mTOR	Mammalian target of rapamycin
NAD	Nicotinamide adenine dinukleotide
NFκB	Nuclear factor kappa B
NRF	Nuclear respiratory factor
OXPHOS	Oxidative phosphorylation
PAGE	polyacrylamid gel electrophoresis
PAI-1	Plasminogen activator inhibitor 1
PDK	Phosphoinositide-dependent protein kinase
PEDF	Pigment epithelium-derived factor
PGC1α	PPAR gamma coactivator 1-alpha
PI3K	Phosphatidylinositol 3-kinase
PIP	Phosphatidylinositol phosphate
PKC	Protein kinase C
RICTOR	Rapamycin insensitive companion of mTOR
ROS	Reactive oxygen species
SIRT1	Sirtuin-1
TBC1D1	(tre-2/USP6, BUB2, cdc16) domain family member 1
TNFα	Tumor necrosis factor-alpha

1 Introduction

It takes nothing more than 12000 steps for a healthier life. A study from 2012 showed that a reduction in steps of healthy active volunteers from habitual 12000 steps per day down to 5000 steps per day already markedly decreased insulin sensitivity (1).

The world is facing a huge challenge with the severe increased prevalence of type 2 diabetes throughout the world. Changes of nutritional behaviour and a sedentary lifestyle lead to an imbalance of energy uptake and energy expenditure. This results in obesity and an increasing number of type 2 diabetic patients worldwide. In this pathophysiological change three preserved, biological mechanisms are involved; regulation of glucose homeostasis, insulin sensitivity, and physical activity. These three mechanisms strongly interfere with each other and detrimental changes in glucose homeostasis and/or physical activity reduce insulin sensitivity leading to type 2 diabetes.

This thesis focused on the detrimental effects of inflammatory cytokines released by adipose tissue and the possibility to combat insulin resistance by contractile activity of skeletal muscle, all studied on the molecular level.

1.1 Glucose transport in skeletal muscle

Glucose is a fundamental source of energy for all eukaryotic cells and transported across the cell membrane by a family of glucose transporters (GLUT). The GLUT family comprises 14 proteins (2). These polytopic membrane proteins form an aqueous pore across the membrane through which the hydrophilic glucose can move by facilitated transport down a concentration gradient (3;4). Many mammalian tissues have been endowed with GLUTs that are constitutively targeted to the cell surface, like GLUT1-3 (3). In adipose tissue and skeletal muscle GLUT4 is the most prominent transporter (5;6). GLUT4 is located in the membrane of intracellular vesicles. In the basal state, GLUT4 vesicles cycle slowly to and from the cell surface, with the vast majority situated in the perinuclear compartment (5;7). Exposure to a distinct stimulus leads to an enhanced exocytic rate of GLUT4, which is increased by 10-40

fold within a few minutes (8), (Figure 1). GLUT4 vesicles are translocated to the sarcolemma, and consequently GLUT4 is redistributed through the plasma membrane (7;9;10). Simultaneously, endocytosis of the transporter is attenuated. Insulin and muscle contraction are the most important stimuli for GLUT4 mobilization to the plasma membrane and for glucose uptake into skeletal muscle (7). Once glucose is cleared from the bloodstream, GLUT4 is internalized and recycled back to intracellular compartments.

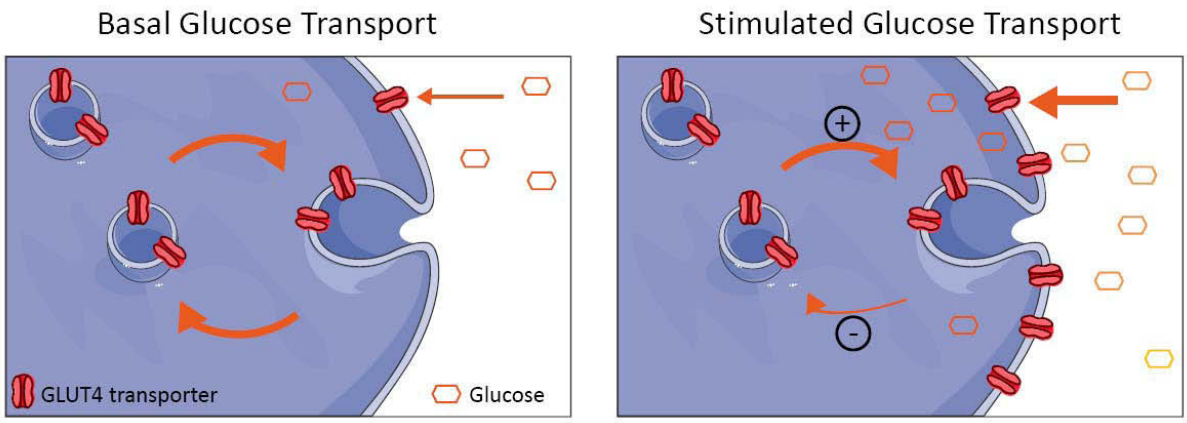

Figure 1: Glucose transport in skeletal muscle cells. Insulin and muscle contraction stimulate the translocation of GLUT4 vesicles from the cytoplasma to the membrane, allowing an increased glucose uptake and reduce GLUT4 endocytosis. GLUT, glucose transporter.

1.1.1 The classical insulin-dependent cascade

In skeletal muscle and adipose tissue insulin triggers an insulin-dependent signalling cascade. By binding to the insulin receptor (IR) at the cell membrane, it activates a signalling cascade resulting in GLUT4 vesicle transport to the membrane (11), (Figure 2).

Insulin itself is a polypeptide hormone secreted by pancreatic ß-cells. It is synthesized as preproinsulin and processed to proinsulin by cleavage of its signal peptide. This processing takes place during the insertion into the endoplasmatic reticulum. Proinsulin gains its appropriate tertiary structure by the formation of disulfide bonds. Through cleavage at both junction sites of the connecting segment peptide, proinsulin is processed to mature insulin and C-peptide. The mature insulin, consisting of α-chain and β-chain, is stored in vesicles.

Upon stimulation of the β-cells by a postprandial increase of plasma glucose level and by gut hormones like glucagon-like peptide-1, insulin is secreted to the plasma.

In insulin sensitive tissues, binding of insulin to the α-subunit of the IR activates the kinase activity of the ß-subunit by an autophosphorylation of tyrosine residues within the catalytic ß-subunit. This activation results in conformational changes of the receptor that further increases kinase activity. The activated IR allows binding and subsequenct phosphorylation of tyrosine residues of the insulin receptor substrate family (IRS 1 to 4) to initiate the so called 'classical insulin cascade' (12). IRS proteins interact with the regulatory subunit of phosphatidylinositol-3-kinase (PI3K) (13;14), which activates the catalytic subunit of this enzyme and stimulates the translocation of PI3K to the plasma membrane. This key component of the classical insulin cascade generates the lipid product phosphotidylinositol (PI) substrates to generate PI(3) phosphate, PI(3,4) diphosphate and PI(3,4,5) triphosphate. These phospholipids act as second messengers and forward the insulin signal by activating PI3K-dependent serine/threonine kinases, 3-phosphoinositide-dependent protein kinase (PDK).

Since PIP are restricted to the plasma membrane, this results in recruitment of PDK and protein kinase B (PKB or Akt) from the cytoplasm to the plasma membrane. PDK phosphorylates two proteins that have been shown to be essential key factors for insulin-stimulated glucose transport: Akt (15;16) and atypical protein kinase C (aPKC) (17;18). PDK leads to conformational changes and subsequent phosphorylation of Akt on Thr308, resulting in partial activation of Akt. Full activation of Akt requires the phosphorylation of Ser473 by an enzyme complex consisting of mTOR (mammalian target of rapamycin) and RICTOR (rapamycin insensitive companion of mTOR) (19). The activated protein Akt is a signalling protein for several insulin actions. It activates glycogen synthesis, protein synthesis, GLUT4 translocation, and thereby increases glucose transport. Glycogen synthesis is activated by a phosphorylation and inactivation of glycogen synthase kinase (GSK)-3. This results in a decrease of glycogen synthase (GS) phosphorylation, thus increasing its activity state (20).

The Akt substrate of 160 kD (AS160), which is a Rab-GAP protein, links the insulin signal with GLUT4 translocation from intracellular stores to the plasma membrane along the actin

cytoskeleton as the final step of the classical insulin signalling cascade (21-23). Upon insulin stimulation AS160 is phosphorylated, which leads to an inactivation of the Rab-GAP activity of AS160. Rab small G proteins on the GLUT4 storage vesicles turn to an active, GTP-bound state. Activation of Rab proteins promotes processes that lead to mobilization and fusion of GLUT4 at the plasma membrane (24;25).

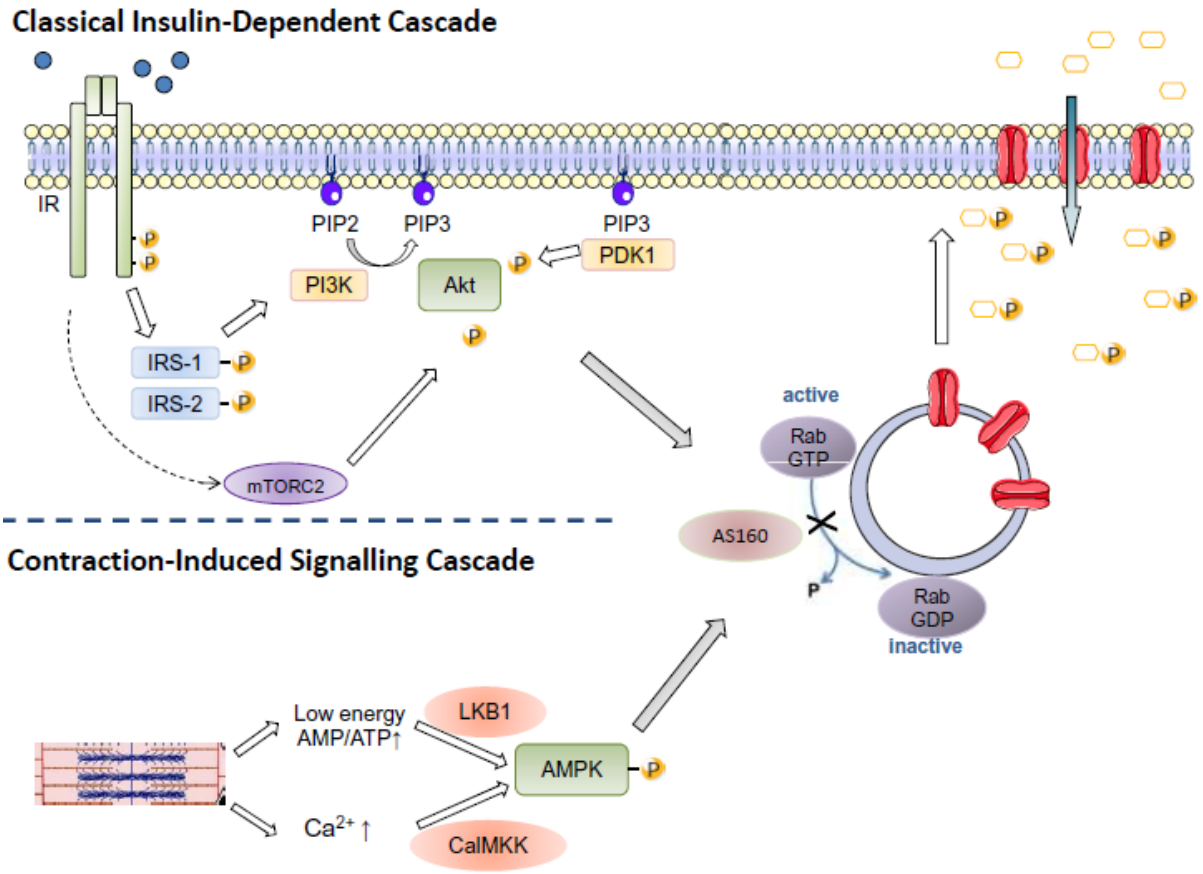

Figure 2: Currently proposed signalling pathways for insulin- and contraction-stimulated glucose transporter 4 (GLUT4) translocation in muscle. Insulin stimulates Akt through two different upstream mediators, phosphoinositide-dependent protein kinase-1 (PDK1) and mammalian target of rapamycin complex-2 (mTORC2). Activated Akt phosphorylates and inactivates AS160 promoting conversion of less active GDP-loaded Rab to more active GTP-loaded Rab. The more active GTP-loaded Rab then allows GLUT4 storage vesicles to move to and fuse with the plasma membrane. Contraction through both energy depletion (i.e., an elevated AMP/ATP ratio) and elevated intracellular [Ca2+] leads to activation of AMP-activated protein kinase (AMPK) via LKB1 and Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK), respectively. These regulators lead to AS160 phosphorylation as well.

The binding of insulin to its receptor also stimulates the extracellular signal-regulated kinase (ERK) and p38 mitogen activated protein kinase (MAPK) (26). This pathway has no direct impact on glucose transport (26), but results in the stimulation of different transcription factors for cell proliferation and differentiation (27).

1.1.2 The contraction-induced cascade

Similar to the effect of insulin an acute bout of physical exercise also causes GLUT4 translocation and thus glucose uptake in skeletal muscle (Figure 2).

Maximal effective insulin concentration in combination with contractile activity stimulates muscle glucose transport (28-30) and GLUT4 translocation (31) to a greater degree than either stimulus alone. Additionally, exercise or muscle contraction is insufficient to activate signalling molecules of the classical insulin signalling cascade like the insulin receptor, IRS1, PI3K, or Akt (32-34). Both observations led to the hypothesis that two distinct pathways exist for the stimulation of glucose transport in mammalian skeletal muscle.

An important step in understanding how muscle contraction affects glucose transport was the identification of adenosine monophosphate (AMP) as an activator of protein kinase, which resulted in the name AMPK (35). In the last years, tremendous research has been conducted to elucidate the role of AMPK in contraction-stimulated glucose transport *in vitro* (36;37), in *situ* (38) and *in vivo* (39;39;40).

Upstream Targets of AMPK

AMPK is a heterotrimeric protein consisting of one catalytic α-subunit and two regulatory β- and γ-subunits being highly specific for AMP (41;42) (Figure 3). The α-subunit has a kinase domain at the N-terminus that is only active after phosphorylation at Thr-172 (43). After several years of intensive research on the protein AMPK, Hardie and Carling described the AMPK as a protein which protects individual cells by acting as a 'fuel gauge' or perhaps more accurately a 'low-fuel warning system', being switched on by depletion of adenosine triphosphate (ATP) (44). AMP activates AMPK and ATP antagonizes this effect. Although

adenosine diphosphate (ADP) is the direct product of the hydrolysis of ATP during muscle contraction, it is rapidly converted to AMP via the adenylate kinase reaction. Thus AMP is a signal of low cellular energy status. Binding of AMP promotes the phosphorylation of the α-subunit at Thr-172. Up to now, two kinases were identified acting upstream of AMPK, the tumor suppressor kinase LKB1 (45) and calmodulin-dependent kinase kinase β (CaMKKβ) (46-48) (Figure 2).

Pharmacological activation of AMPK can be achieved by treating cells with 5-amino 4-imidazolecarboximide riboside (AICAR) (49). By developing a muscle specific LKB1 knockout mouse, it has been shown that the AMPK activation and glucose uptake induced by AICAR and by muscle contraction were diminished in LKB1 deficient muscle (50). Cytosolic accumulated AMP directly binds the γ subunit of AMPK (51) allowing, by allosteric modification, the accessibility to the threonine residue of the AMPK, which is targeted by the LKB1 complex. This leads to its phosphorylation and subsequently to AMPK activation (46;52). The LKB1 has a high basal activity and continuously phosphorylates Thr-172, but in the absence of AMP, AMPK is immediately dephosphorylated (53).

The CaMMK activates AMPK in response to a rise in cytosol Ca^{2+} in brain, endothelium and lymphocytes (47;54). In these tissues CaMMKβ seems to be the dominant isoform (47;54), while CaMMKα may be the most important isoform in muscle (55). A study using an inhibitor for CaMMKα indicates that CaMMK acts as an upstream AMPK kinase in mouse skeletal muscle regulating glucose uptake at the onset of mild tetanic contraction (56).

Downstream Targets of AMPK

Once activated by AMP, AMPK switches on catabolic pathways that generate ATP, while it inactivates anabolic pathways and other ATP-consuming processes (44) (Figure 3). This restores the energy balance within the cell.

Fatty acid oxidation, as a catabolic pathway, is activated by AMPK. Therefore, AMPK phosphorylates and inactivates acetyl-coenzyme A (CoA) carboxylase (ACC) (57-59) that produces malonyl-CoA in the mitochondrial intermembrane space. Since malonyl-CoA is an

inhibitor of fatty acid uptake into the mitochondria by the carnitin-palmitoyl-CoA tranferase-1 system and inhibition of ACC by AMPK has a stimulating effect on fatty acid oxidation (Figure 3).

Additionally, AMPK activates mitochondrial biogenesis in skeletal muscle (60) by up-regulating the expression of the transcriptional coactivator PPARγ coactivator-1α (PGC1α) (61), which stimulates both DNA replication and nuclear-encoded mitochondrial genes (Figure 3).

Consistent findings have established a convincing connection between AS160 and AMPK (Figure 3). Besides insulin, contraction and AICAR stimulate AS160 phosphorylation in isolated muscle as well (62). Using *in vivo*, *in vitro*, and *in situ* methods, insulin, contraction, and AICAR have been shown to increase AS160 phosphorylation in mouse skeletal muscle (63). Insulin-stimulated AS160 phosphorylation was fully blunted by wortmannin, a PI3K

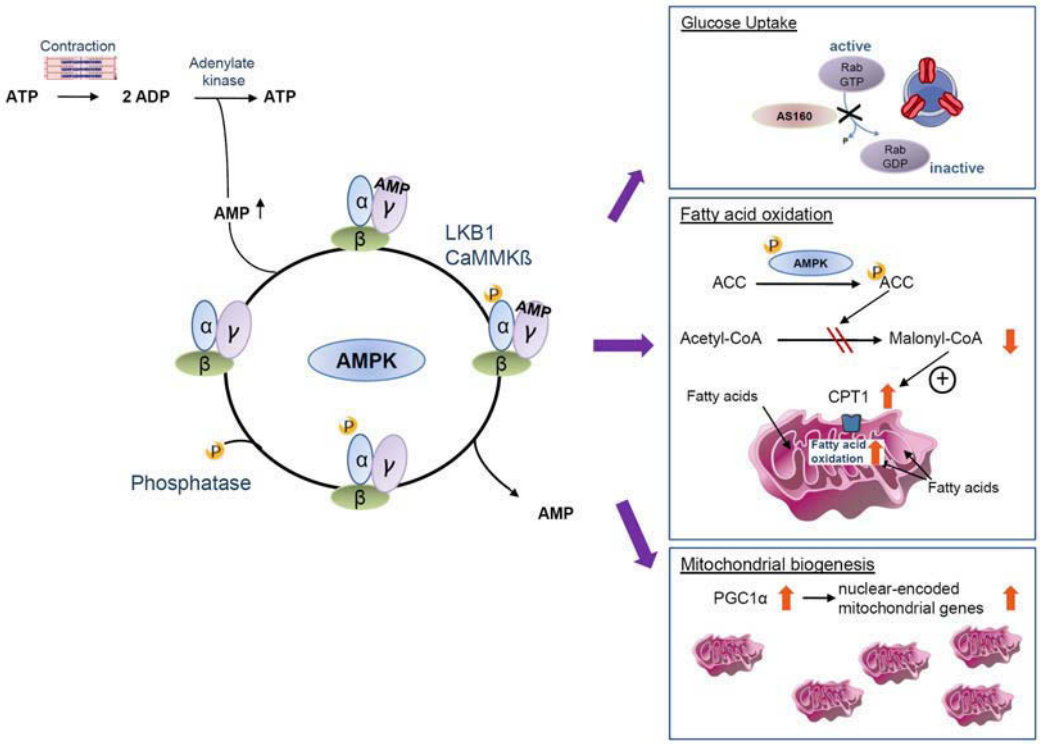

Figure 3: The AMPK signalling cascade. The AMP-activated protein kinase (AMPK) is a sensor of cellular energy status. It is activated, by a mechanism requiring the tumor suppressor LKB1 or CaMMKß, by metabolic stresses that increase cellular AMP:ATP ratios like contraction. Once activated, it switches on catabolic pathways that generate ATP like fatty acid oxidation. It affects glucose uptake and enhances mitochondrial biogenesis, while switching off biosynthetic pathways and cell-cycle progress.

inhibitor, *in vitro*, while contraction-stimulated AS160 phosphorylation was only partially decreased by wortmannin (63). A supporting human study has also observed that Akt phosphorylation was not changed by resistance training, while AS160 activation was enhanced (64). Additionally, mutations in AS160 that prevent it from being phosphorylated decrease muscle glucose uptake during contractions (58). These studies strongly suggest AS160 to be an important AMPK downstream regulator and a key element linking insulin- and contraction regulated glucose uptake.

Thus, contraction as well as insulin stimulates glucose transport by separate pathways and have been shown to be at least additive with respect to GLUT4 translocation (65-67).

1.1.3 The long-term effect of exercise on glucose transport

As described, the acute effect of muscle contractions on glucose transport is independent of insulin and reverses after termination of exercise within 2-4 hours. In addition to the acute effect on glucose transport, the sensitivity of muscle to insulin is markedly increased for more than 48 hours in human subjects (68;69).

Insulin sensitivity itself is defined as the insulin concentration needed to produce one-half of the maximal response (70), while insulin responsiveness is defined as the maximal response to insulin (70).

Richter at al. were the first to discover that exercise increases the sensitivity of the glucose transport process to insulin in skeletal muscle (70). Prior exercise lowered the concentration of insulin that half-maximally stimulated glucose utilization and modestly increased its maximum effect (70). A following study by Richter et al. demonstrated that glucose incorporation into glycogen and the uptake of glucose were strongly increased in the previously contracted muscles compared to non-stimulated control muscles (71). Subsequently, increased insulin sensitivity was shown in studies of human subjects (68;72-74).

There has been considerable progress over the past decade in understanding the molecular basis for clinically important effects of exercise on glucose uptake and insulin sensitivity in skeletal muscle. Up to now, the mechanisms for increased insulin sensitivity are not

completely understood. Long time it was hypothesized that the increase in insulin sensitivity after exercise is mediated by an amplification of the insulin signal. However, if rat muscles are treated with insulin 3-4 hours after exercise, IR tyrosine kinase activity, IRS1 tyrosine phosphorylation, and PI3K activity are not enhanced in skeletal muscle (75;76).

Nowadays, there is substantial evidence in animals (75-77) and humans (65;69;78;79) that long-term exercise training increases the expression of GLUT4 protein level in skeletal muscle. Additionally, translocation of GLUT4 to the cell surface is described to be increased (76;80). Current research tries to elucidate the role of distal signalling components in the insulin signalling pathway such as atypical protein kinase C, Rac1, AS160 and TBC1D1. These candidates are all affected by insulin and exercise and might regulate insulin sensitivity (reviewed in (80)).

Beside a direct effect of AMPK on glucose uptake, chronic activation of AMPK increases GLUT4 transporter protein level in skeletal muscle (81). Thus, acute activation of AMPK increases GLUT4 vesicle transport to the membrane, while chronic activation increases the number of GLUT4 transporters in skeletal muscle.

1.2 Pathophysiology of insulin resistance and type 2 diabetes

The prevalence of insulin resistance and type 2 diabetes is increased throughout the world. Epidemiological evidence strongly associates the development of type 2 diabetes with obesity due to physical inactivity and excess energy intake (82).

Type 2 diabetes, accounting for more than 90 % of diabetes cases, is characterized by hyperglycemia resulting from relative impaired insulin secretion of pancreatic ß-cells and impaired insulin action in peripheral tissues as liver, adipose tissue and skeletal muscle, or both (83). Commonly it takes years to decades before the adverse cycle of these two defects culminates in the manifestation of the disease. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels due to macrovasuclar and microvascular complications (83).

The World Health Organization published in 2012 that 347 million people worldwide suffer from diabetes (84). Between 2008 and 2030 diabetes deaths will probably increase by two thirds (84). About 1 billion people in the world are overweight or obese, compared to 850 million who are underweight (85). Although the understanding of the role of genetics in obesity and type 2 diabetes is increasing (86-88), roughly 60% of all cases of diabetes can be directly attributed to weight gain (89). The lack of success controlling the obesity epidemic in an aging population is exacerbating the problem.

1.2.1 Normal glucose homeostasis

Under normal conditions, plasma glucose is tightly regulated and remains in a narrow range between 4 and 7 mmol/l and fasting plasma glucose of <5.6 mmol/l (less than 100 mg/dl).

Post-prandial, especially after a carbohydrate rich meal, plasma insulin concentrations increase. This leads to the inhibition of lipolysis in adipocytes and stimulates glucose uptake in skeletal muscle. About 70–90% of insulin-stimulated glucose disposal is incorporated into muscle glyocogen (90). Thus, skeletal muscle is the pre-dominant site of insulin-mediated glucose uptake. Under physiologic conditions, approximately two-thirds of all glucose is converted to glycogen, and one third enters glycolysis (91). Glycogen content in skeletal muscle is normally about 100 mmol/kg in humans. A young man of about 70 kg can store about 500 g carbohydrate as muscle glycogen, while the liver contains about 100 g (69). Thus a major part of carbohydrates is stored as muscle glycogen.

Beside glucose, skeletal muscle utilizes free fatty acids (FFA) as fuel sources for energy production. During the fasting state, the plasma insulin concentration and glucose uptake is low, while plasma FFAs are elevated. In this state, glucose is exclusively utilized by the brain, while FFA serves as the main fuel source in skeletal muscle. The ability of skeletal muscle to switch from fat oxidation in the fasting state to glucose utilization during the post-prandial state has been referred to as metabolic flexibility.

During exercise and rising exercise intensity, glucose and intramuscular glycogen becomes an increasingly important energy substrate (reviewed in (92)). Glycogenolysis is regulated by

glycogen phosphorylase and the activity is increased by allosteric binding of AMP and competed by ATP or glucose. For whole body glucose homeostasis, glycogen stores have to be depleted during times of higher energy expenditure, so that post-prandial glycogen stores in muscle can be replenished. A large decrease in calorie expenditure due to a lack of physical activity is the cause for filled glycogen stores. In this state, excess plasma glucose is stored in adipose tissue.

In healthy lean subjects, plasma glucose levels are tightly regulated, because glycogen synthesis and depletion in skeletal muscle are in balance with the ratio of energy uptake and energy expenditure.

1.2.2 Disturbed glucose homeostasis leading to insulin resistance

In patients diagnosed with insulin resistance or type 2 diabetes glucose homeostasis is disturbed due to an imbalance between energy intake and energy expenditure.

To maintain energy balance, the American Diabetes Association recommends a minimum of 30 minutes moderate-intensity physical activity per day (93). The American Association of Diabetes Educators recommends to walk or to perform other moderate-intensity physical activity 3 or more days a week for a total of 150 minutes per week and engage in weight lifting or other muscle-strengthening resistance exercise 3 days a week (94). Furthermore, the American College of Sports Medicine recommends 12000 steps per day to maintain a healthy level of physical activity.

A study performed in the US showed that most residents do not fulfil these recommendations. Less than 5% of adults and only 8% of adolescents (aged 12-19 years) adhere to the recommendation of daily physical exercise (95), leading to low energy expenditure. In physical inactivity muscle glycogen is not depleted and excess energy is stored in form of triglycerides in adipose tissue, the major organ in mammals for energy storage.

Insulin Resistance is a characteristic feature of type 2 diabetes and is defined as impaired insulin-stimulated glucose disposal in target tissues, such as the skeletal muscle, liver and adipocytes. In 1939, Himsworth and Kerr were the first to demonstrate that tissue sensitivity

to insulin is diminished in type 2 diabetic patients (96). The dose response curve, relating insulin-stimulated glucose uptake and plasma insulin concentration, reveals a decrease in insulin sensitivity by an increased EC50 from 80-100 μU/ml in healthy subjects to 120-140μU/ml in subjects with type 2 diabetes. The onset of insulin action in skeletal muscle in type 2 diabetic subjects is markedly delayed (97). Additionally, the rate of insulin-stimulated muscle glycogen synthesis was decreased over 50% in patients with type 2 diabetes and this is a major factor responsible for insulin resistance under hyperinsulinemic-hyperglycemic clamp conditions (98)

A minor degree of hyperglycemia with a fasting glucose of 5.6-6.9 mmol/l (100-125 mg/dl) is sufficient to cause pathologic and functional changes in target tissues. At this stage insulin levels appear normal or elevated. In the process of insulin resistance manifestation, the higher blood glucose levels in these diabetic patients would be expected to result in even higher insulin values assuming normal ß-cell function. Thus, insulin secretion is defective in these patients and insufficient to compensate for insulin resistance. This results in a fasting plasma glucose level of more than 7 mmol/l (126 mg/dl) (99).

Stephens et al. showed that increased sedentary time combined with a positive energy balance lowered insulin-stimulated glucose disposal by 39%. When sedentary living occurred in energy balance, due to reduced energy intake, 19% reduction were measured for insulin-stimulated glucose disposal (100). Thus, at least half of the reduction in insulin sensitivity is due to a positive energy balance. Krogh-Madsen et al. showed that a two week reduction of ambulatory activity from about 10000 steps per day down to 1500 steps per day attenuates peripheral insulin sensitivity determined by hyperinsulinemic-euglycemic clamp (101). Furthermore, it has been shown that weight gain directly correlates with an increased risk to develop type 2 diabetes (102-104), not only in the adult population, but also amongst children (105-107).

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 type 2 diabetes than overall BMI (108-110). These data illustrate the tight relation

between a positive energy balance, obesity and insulin resistance and that obesity is the most common cause of insulin resistance.

1.2.3 Adipose tissue dysfunction and induction of insulin resistance

Positive energy balance leads to the storage of excess energy as triglycerides in adipocytes. Adipose tissue grows by two mechanisms: hyperplasia (cell number increase) and hypertrophy (cell size increase). Beyond a critical threshold of hypertrophy and hyperplasia excess adipose tissue is associated with several dysfunctions, including apoptotic signalling, disruption of mitochondrial function induced by oxidative stress of reactive oxygen species (111), endoplasmatic reticulum stress (112) and increased fatty acid release (113).

During the past 15 years, adipose tissue has been widely recognized to play a more important role than that of a fuel depot. Adipose tissue releases FFA by lipolysis and additionally a huge variety of proteins and cytokines (114;115), called adipokines. The comparison of seven proteomic profiling studies conducted with human material revealed more than 600 potentially secretory proteins (114). Clustering these adipose tissue derived proteins according to their postulated functions revealed that these adipokines fall into several groups, including adipokines contributing to the extracellular matrix, involved in inflammation, metabolism, oxidative stress, or the control of angiogenesis and blood pressure (116).

Obesity-associated tissue inflammation is now recognized as a major cause of decreased insulin sensitivity. Adipocyte hypertrophy in obesity alters adipokine secretion resulting in increased release of pro-inflammatory adipokines. The first studies that established the concept of obesity-induced adipose tissue inflammation were conducted by Hotamisligil et al., who found that TNFα was elevated in obese rodents and that neutralization of TNFα ameliorated insulin resistance (117). Although one has to be careful extrapolating from the rodent study to the human situation, since primary human adipocytes secrete only minor levels of TNFα (118), it is now well established that the level of inflammatory cytokines secreted from adipocytes are increased with obesity. Clinical studies have clearly linked e.g. interleukin (IL)-6 (119;120), IL-8 (120-122), chemerin (123;124), plasminogen activator

inhibitor 1 (PAI-1) (125), monocyte-chemotactic protein 1 (MCP-1) (126;127) with obesity. These pro-inflammatory cytokines lead to disturbances in skeletal muscle insulin signalling and finally to insulin resistance. *In vitro* studies using adipocyte-conditioned medium, containing the whole secretory output of mature adipocytes (128;129), as well as single adipokines such as chemerin (130), PEDF (131), MCP-1 (132), showed a reduction of insulin-stimulated glucose uptake in skeletal muscle cells. Subsequent analysis revealed that adipocyte-derived factors reduce insulin-stimulated phosphorylation of proteins of the insulin signalling cascade leading to reduced GLUT4 translocation to the membrane and thus to reduced glucose uptake.

More recently, dipeptidyl peptidase 4 (DPP4) was identified as a novel adipokine, which strongly correlates with adipocyte size. Potentially adipose tissue represents an important source of DPP4 serum levels in obesity (133). DPP4 had gained considerable interest as a therapeutic target before, since DPP4-inhibitors enhance glucose-dependent insulin secretion from pancreatic ß-cells. Lamers et al. showed that DPP4 impairs insulin sensitivity in skeletal muscle on the level of insulin-stimulated Akt phosphorylation, indicating a second role of DPP4 in humans (133).

Besides an augmented adipokine secretion, hypoxia and adipocyte cell death have been prosposed to be inititators of macrophage infiltration and therefore inflammation in adipose tissue. Cinti et al. showed that adipocyte cell death, resulting from hypertrophy, stimulated macrophage recruitment in individuals with obesity (134). Infiltrating macrophages formed crown-like structures around necrotic adipocytes, where they ultimately form multinucleate giant cells, a hallmark of chronic inflammation (134). The presence of pro-inflammatory M1 macrophages in adipose tissue correlates with insulin resistance and states of overnutrition (135). Obesity induces a phenotype switch from anti-inflammatory M2 macrophages to inflammatory M1 macrophages (135).

Additionally, current literature discusses adipocyte hypertrophy to lead to local adipose tissue hypoxia during early stages of expansion (136) and *in vitro* studies proved that a variety of pro-inflammatory adipokines are upregulated by hypoxia (137-140). Adipose tissue hypoxia is

controversial in humans because studies showed contradicting values for adipose tissue oxygenation depending on the technique used (141;142).

Taken together, inflammation within expanding adipose tissue is a characteristic of obesity leading to increased secretion of pro-inflammatory adipokines which can induce insulin resistance in peripheral tissues.

1.2.4 Insulin resistance on the molecular level

Impaired insulin-stimulated glucose uptake in skeletal muscle from severely obese subjects is accompanied by a deficiency in insulin receptor signalling. IR phosphorylation, IRS-1 phosphorylation, and PI3K activity are decreased in intact skeletal muscle strips from obese subjects (143). Additionally, insulin-stimulated phosphorylation of AS160, linking insulin signalling and GLUT4 trafficking, is impaired in skeletal muscle of type 2 diabetic subjects (144). However, the amount of GLUT4 expressed in skeletal muscle was unaffected by type 2 diabetes (145). Thus, the decreased glucose disposal in type 2 diabetes is not associated with a diminished number of insulin regulable glucose transporters, but rather an impaired insulin signalling induced by pro-inflammatory cytokines released from adipose tissue.

NFκB: a pivotal transcription factor in chronic inflammatory diseases

A mechanistic link between insulin resistance and inflammatory processes was established by showing that the signalling pathways leading to activation of inhibitor of κB kinase-β (IKKβ) and nuclear factor-κB (NFκB) are stimulated in obesity and insulin resistance (146;147).

The expression of genes encoding for many cytokines, enzymes, and adhesion molecules in chronic inflammatory diseases is increased by NFκB. NFκB represents a group of structurally related, evolutionary conserved proteins, which normally play a pivotal role in defence of the host against certain pathogens. Several different NFκB proteins have been characterized: Rel (c-Rel), RelA (p65), RelB, NFκB1 (p50) and NFκB2 (p52) (reviewed in (148)). In resting cells, NFκB-dimers (p50-p65) are bound to regulatory proteins called inhibitors of κB (IκB), which prevent the complex to enter the nuclei and is found in the cytoplasm (149-151).

In response to a variety of extracellular stimuli, such as inflammatory signals, IkB proteins are rapidly phosphorylated by the IkB kinase (IKK) complex (150), (Figure 4). The IKK complex consists of at least three subunits, including the kinases IKKα and β and the regulatory subunit IKKγ. IKK activation initiates IκBα phosphorylation, which targets these inhibitory proteins for rapid polyubiquitination and degradation through the proteasome (152;153). NF-κB dimers are liberated from the cytoplasmic NFκB-IκB complex. The removal of IκB activates protein kinase A (154), which phosphorylates the p65 subunit and allowing the NFκB complex to translocate to the nucleus (155). Additionally, the phosphorylation of p65 weakens the interaction of the p65 N-terminal region with the C-terminal region, unmasking the CREB-binding protein (CBP)/p300 -interaction domain in p65 (154). The transcriptional coactivator CBP/p300 associates with NF-kB p65 through two sites to further enhance the transcriptional activity of p65 (154), inducing transcription of pro-inflammatory genes (155). Activation of NFκB is typically transient. Shortly after stimulation of cells with inducers of NFκB, newly formed NFκB-IκB complexes begin to reaccumulate in the cytoplasm (150;151;153;156).

Yuan et al. revealed that the IKKβ pathway contributes to obesity and insulin resistance in type 2 diabetes by diminishing insulin signalling. Inflammatory signalling, mimicked by TNFα-treated murine 3T3 adipocytes, activated the IKKβ pathway and resulted in negative regulation of insulin signalling by decreased insulin-stimulated phosphorylation of the IR β-subunit and IRS-1. Reduced signalling through the IKKβ pathway, either by salicylate inhibition or decreased IKKβ expression, was accompanied by improved insulin sensitivity in rodents (147). Furthermore, it has been shown that inflammatory adipokines released by human adipocytes activate IKK signalling and induce insulin resistance in human skeletal muscle cells. Inhibition of IKK by a specific inhibitor reduces NFκB activation and reverses insulin resistance in skeletal muscle (128;157).

Thus, inflammation is an essential survival mechanism, but it can have adverse effects if it becomes converted to the chronic state, as it is in obesity and type 2 diabetes.

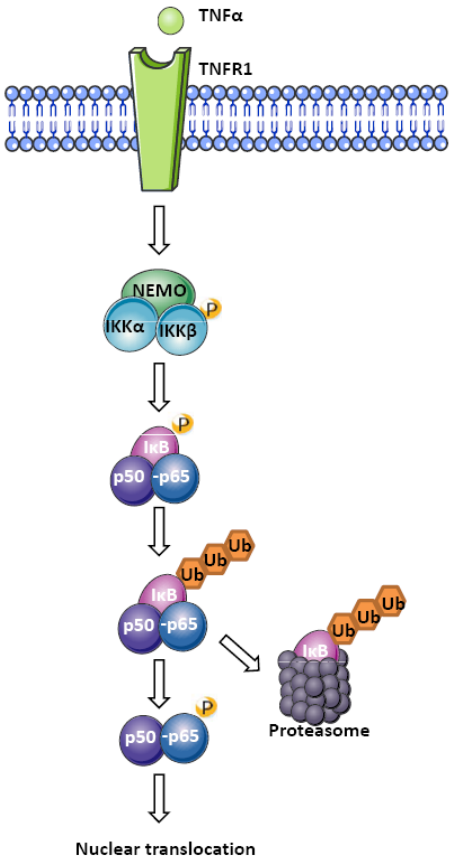

Figure 4: The classical NFκB activation cascade. NFkB dimers (p50-p65) are held in the inactive state by a family of inhibitors called IkB. Receptor signalling leads to activation of a multisubunit IkB kinase (IKK) complex which phosphorylates IkB marking it for degradation by the ubiquitin pathway. NFkB dimer is liberated to translocate to the nucleus, bind to the DNA and activate transcription of target genes.

1.2.5 Beneficial influence of physical activity in the context of insulin resistance

Once elevated blood glucose and insulin resistance or type 2 diabetes is diagnosed, the major aim of therapy is to archieve normoglycemia, with a fasting glucose <110 mg/dl. Thereby reducing incidence of diabetic micro- and macrovascular complications and the increased mortality associated with these complications. The first therapeutic strategy in newly diagnosed type 2 diabetic patients is to increase physical activity and to modify dietary habits with the major goal to induce weight loss in overweight patients.

Regular physical activity improves insulin function and glucose tolerance in healthy individuals (158), patients with obesity (159), insulin resistance (79), and diabetics (160;161). Sedentary individuals can markedly increase their endurance by regularly performed exercise. After a few weeks of endurance training, such as long distance running, individuals can often exercise comfortably for prolonged periods, even at exercise intensities that they could maintain for only a few minutes prior to training. For many years it was thought that this

increase in the capacity of endurance exercise was exclusively the result of the cardiovascular adaptations to endurance training. The main effect of exercise training was thought to be the increased capacity to deliver O_2 to the working muscles and the large increase in maximal O_2 uptake capacity (162).

However, it has long been appreciated that an acute bout of exercise is associated with changes in the metabolism of liver and adipose tissue that results in an increased provision of fuel for the contracting muscle. A large body of evidence from both humans and experimental animals has linked these changes temporally to increased levels of the hormone and neurotransmitter norepinephrine (also known as noradrenalin), which is released by the sympathetic nervous system. Norepinephrine occurs very early during moderate and intense exercise. It inhibits the insulin secretion from the pancreas, while it triggers glucagon secretion. It stimulates glycogenolysis in the liver and muscle, and stimulates glycolysis in muscle. In adipocytes the lipolysis is activated, releasing long chain nonesterified fatty acids into the circulation (for review see [51]).

Overall, exercise itself decreases blood glucose and acts against hyperglycemia. A single session of resistance- or endurance-type exercise substantially reduces the prevalence of hyperglycaemia during the subsequent 24 h period in individuals with impaired glucose tolerance (163). Both modes of exercise, endurance and strength training, similarly reduced the duration of excess glucose levels by more than 30% in patients with type 2 diabetes (163). A recent meta-analysis showed that structured aerobic, resistance or combined exercise training results in a decline in HbA1c in patients with type 2 diabetes, with exercise duration of 150 min per week being more efficient than duration of less than 150 min per week (164). Although physical activity advice is associated with lower HbA(1c) only when combined with dietary advice (164). While an acute bout of exercise reduces the risk of adverse effects of hyperglycemia, the long-term adherence to exercise might ultimately determine efficacy to improve glycaemia and, importantly, morbidity and mortality. Additionally, exercise training, even without weight loss, increases insulin sensitivity in previously sedentary adults (165).

The long-term anti-inflammatory effect of exercise may be mediated by a reduction in visceral fat mass, accompanied with reduced macrophage infiltration into adipose tissue of humans

with obesity and decreased release of pro-inflammatory adipokines (166;167). Moderate exercise training combined with a hypocaloric diet reduces inflammation more than diet alone (168), underlining the beneficial effect of exercise in this context.

Additionally, a new concept proposing the skeletal muscle as a major endocrine organ assumes that it releases peptides and cytokines which might be partly responsible for the beneficial effects of physical exercise.

1.3 Skeletal muscle as endocrine organ

In line with the acceptance of adipose tissue as an endocrine organ, path-breaking work during the last decade demonstrated that skeletal muscle is an active endocrine organ. Skeletal muscle releases a host of proteins that can communicate with cells in an autocrine/paracrine manner, locally within the muscles, or in an endocrine fashion to distant tissues. Cytokines and proteins that are expressed and released by muscle fibres were termed myokines.

Interleukin-6 – the best characterized myokine

Some of the first reports in this research field identified myostatin (169) and interleukin (IL)-6 as secreted proteins from skeletal muscle (170;170;171). The identification that contracting human skeletal muscle releases significant amounts of IL-6 into the circulation during prolonged single-limb exercise was a milestone in this research field and identified skeletal muscle as an endocrine organ (172). Up to now, IL-6 is the most prominent muscle-derived protein, which was demonstrated to be upregulated in plasma after exercise without muscle damage (170;173;174), (Figure 5). The level of circulating IL-6 increases in an exponential fashion in response to exercise (172;175;176), and declines in the post-exercise period (177). The magnitude by which plasma levels increase is related to exercise duration, intensity and the muscle mass involved in the mechanical work (172;175;176;178).

Before this observation, IL-6 was described to be overexpressed in human fat cells from insulin-resistant subjects (179), to be increased in the plasma of obese patients (180;181), and to decrease after bariatric surgery (182). IL-6 expression was known to be activated by pro-

inflammatory IKKß/NFκB signalling pathway which is thought to contribute to the development of obesity-induced insulin resistance (119;183). Additionally, *in vitro* IL-6 has been shown to induce insulin resistance in hepatocytes (184), adipocytes (179), and in skeletal muscle cells after treatment with high doses (128). Since exercise is thought to increase insulin sensitivity, the observation that IL-6 is also increased after exercise, seemed quite paradoxical. Carey et al. observed that increased circulating IL-6 concentrations in patients with type 2 diabetes are strongly related to fat mass and not insulin responsiveness (185). Additionally, the regulation of intracellular signalling mechanisms, mediating IL-6 expression, differs from the classical pro-inflammatory pathway. IL-6 expression in contracting muscle is regulated by c-Jun terminal kinase (JNK)/activator protein-1 (AP-1) (186), increases insulin-stimulated glucose disposal in humans and glucose uptake as well as fatty acid oxidation in rat myotubes *in vitro* (187).

1.3.1 Contraction-regulated myokines

Research of the last decade revealed that several myokines are regulated by contraction, like angiopoietin-like 4 (188), brain-derived neurotrphic factor (BDNF) (189), fibroblast growth factor 21 (190), IL-6 (170;171), IL-7 (191), IL-15 (192), irisin (193), leukemia inhibitory factor (194), myonectin (195), myostatin (196) and vascular endothelial growth factor (197;198). For some of these reported myokines, the description as a contraction-regulated myokine is based on RNA data of repeated skeletal muscle biopsies before and after exercise or mRNA/protein data of cell cultures. Repeated biopsy sampling from one muscle is necessary to investigate muscular adaptation to different forms of exercise. The adaptation is thought to be the result of cumulative effects of transient changes in gene expression in response to single exercise bouts. Nevertheless, it was shown that multiple fine needle biopsies obtained from the same muscle region can per se influence the expression of marker genes induced by an acute bout of resistance exercise (199). Additional results are needed to consolidate mRNA data obtained from biopsies. Not for all the above mentioned myokines an increase in plasma levels after

exercise or an increase in protein level in supernatants of myotubes was reported. For BDNF even basal secretion by skeletal muscle cells has not been proven before (189).

A second good example is the myokine IL-15. This cytokine was discussed as a contraction-regulated myokine in the literature which may play a role in muscle-fat crosstalk (200;201) mediating some of the beneficial effects of physical activity. However, long time there are conflicting data published whether physical activity affects IL-15 expression, protein level and secretion from skeletal muscle. Nielsen et al. reported that IL-15 mRNA content increased 24 hours following a single bout of resistance exercise, while this increase was not accompanied by an increase in muscular IL-15 protein level (202). Plasma IL-15 did not change after 10 weeks of resistance exercise training and was not associated with variability in muscle responses with training (203). Riechman et al. demonstrated only an approximately 5 % increase in plasma IL-15 immediately after the end of one resistance exercise bout (203). A third study in humans demonstrated no change in IL-15 mRNA level in vastus lateralis muscle biopsy samples, which were taken immediately after 2 h intensive resistance training (204). Yang et al. published that IL-15 mRNA expression in soleus and gastrocnemius muscle is increased after 8 weeks treadmill running training in mice, while plasma IL-15 level was not changed (205). Recently, it has been shown that 30-min treadmill running at 70% of maximum heart rate resulted in a significant increase in circulating IL-15 level in untrained healthy young men, measured 10 min after exercise (206).

IL-15 was higher expressed at the mRNA level in muscle biopsies taken from from marathon runners compared to other cytokines like IL-6, IL8 and TNFα (204). Most interestingly, it is higher expressed in skeletal muscle compared to adipocytes (207). In 3T3 adipocytes, administration of IL-15 inhibited lipid accumulation, and stimulated secretion of the adipocyte-specific hormone adiponectin. Additionally, IL-15 overexpression in mice promotes endurance, oxidative energy metabolism and enhances exercise-related transcription factors in muscle (208) and IL-15 treatment improves glucose homeostasis and insulin sensitivity in obese mice (209). Since both IL-15 and physical exercise have positive effects on body composition, IL-15 was discussed as a contraction-regulated myokine in the literature which may play a role in muscle-fat cross-talk (200;201) mediating some of the

beneficial effects of physical activity. However, it has to be proven by future research that the rather short increase of IL-15 plasma level is sufficient to exert systemic effects.

Irisin

Just recently, a novel identified myokine draw the attention. Boström et al. observed that overexpression of PGC1α in mice muscle as well as exercise induces the expression of the FNDC5 gene, encoding the membrane protein that is cleaved and secreted as novel messenger molecule called irisin (193). Viral delivery of FNDC5 caused a browning of the subcutaneous fat depot, stimulated oxygen consumption, and diminished diet-induced weight gain and metabolic dysfunction (193), (Figure 5).

However, Timmons et al. observed no effect on FNDC5 mRNA level neither after aerobic exercise in younger subjects nor in a resistance training study (210). Huh et al. observed only minor effects on irisin plasma levels after 1 week of exercise and no effect after prolonged training over 8 weeks (211). Thus, irisin is also controversially discussed to be regulated by contraction.

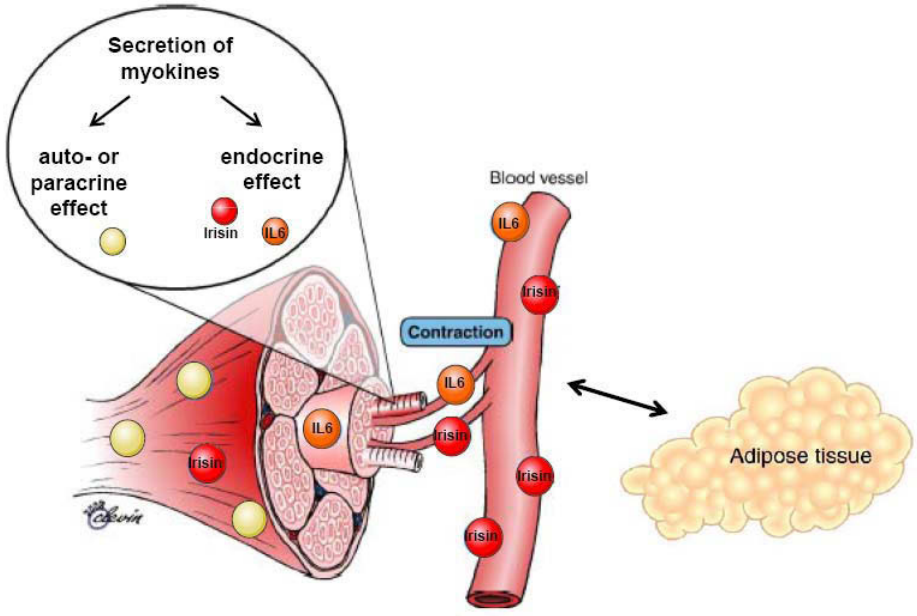

Figure 5: **Secretion of myokines.** Cytokines and proteins secreted from skeletal muscle are called myokines. Its secretion is proposed to have a positive impact either on skeletal muscle itself or on other tissues and organs in an endocrine fashion. For some myokines a contraction-reguated expression has been observed, raising myokine serum levels (e.g. IL-6 and irisin) and affecting adipose tissue metabolism (modified from (212)).

Secretome of muscle cells

To gain a broader view, recent efforts have focused on exploring the complete secretome of skeletal muscle by proteomic studies. While Chan et al. and Henningsen et al. have investigated altered regulation of secretome components at different time points of muscle differentiation of murine C2C12 cells by a quantitative proteomics approach (213-215), Yoon et al. have studied the regulation of protein secretion by rat skeletal muscle cells after insulin stimulation (216) and TNFα-treatment (217). Recently, Hittel et al. have explored the secretome from cultured myotubes derived from extremely obese compared with healthy non-obese women (218). All these studies found hundreds of secreted proteins from skeletal muscle, some regulated by insulin or TNFα. A drawback of all these studies is the use of non-contracting cells, although contraction is a major characteristic of skeletal muscle activating intracellular signalling pathways, metabolic adaption and change of its plasticity.

Nevertheless, all these studies show that skeletal muscle cells are, like adipocytes, major secretory cells. Research indicates that myokines might be part of a highly complex network which operates to regulate metabolic functions. The previous sections of this thesis and the current literature describe a negative crosstalk between excess body fat and skeletal muscle (219), while the data of IL-6 and irisin indicate a positive effect of muscle-secreted proteins on the adipose tissue. However, for other myokines the systemic effect of contraction-regulated myokines is still controversially discussed.

1.4 Objectives

Profound evidence has established the negative crosstalk between expanded adipose tissue of obese patients and skeletal muscle insulin sensitivity. As emphasized in the previous sections, the mechanisms involved in the beneficial effects of exercise attract increasing attention because of continuously increasing prevalence of type 2 diabetes. Understanding these mechanisms induced by contraction can potentially be used in drug development as well as in developing exercise interventions. Although physical activity has long been known to be an effective and commonly cost-efficient therapeutic strategy for the treatment of multiple complications associated with type 2 diabetes, molecular mechanisms in skeletal muscle itself are only incompletely understood.

Studies examining the effect of contraction on the molecular level in skeletal muscle cells mainly use rat or mice cells and chemical stimuli as AICAR and caffeine as exercise mimetics are used, which activate particular signalling pathways involved in skeletal muscle contraction. To not only activate one particular contraction-regulated pathway and to better model the complex network of signalling pathways involved in skeletal muscle contraction,

the first aim of this study was to establish and validate an *in vitro* contraction model of primary human skeletal muscle cells.

Type 2 diabetes is associated with an increase in inflammatory cytokines secreted by adipose tissue inducing insulin resistance in skeletal muscle. Our working group has previously shown that primary human skeletal muscle cells incubated with adipocyte-conditioned medium from primary human adipocytes or treated with adipokines like MCP-1 and chemerin are characterised by impaired insulin signalling and glucose uptake. In this study we combined the novel contraction model with the insulin resistance model to analyse the crosstalk between adipocytes and contracting hSkMC.

The second aim was to analyse the signalling pathways and mechanisms involved in the beneficial effects of muscle activity in the context of insulin resistance.

Research now indicates that skeletal muscle is also an endocrine organ secreting numerous peptides and proteins. Novel contraction-regulated myokines that have a positive impact on muscle or whole body physiology may display new pharmacological targets for the treatment of insulin resistance, low-grade systemic inflammation and type 2 diabetes. Although proteomic approaches based on rat and mice skeletal muscle cells emphasized the complexity of the whole skeletal muscle secretome, the secretome of contracting muscle remains incompletely characterized. In that perspective, the identification of novel contraction-regulated myokines could lead to the discovery of new pharmacological targets.

The third aim was to use the in vitro contraction model to identify novel contraction-regulated myokines.

2 Published Articles

2.1 Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways

Lambernd S, Taube A, Schober A, Platzbecker B, Görgens SW, Schlich R, Jeruschke K, Weiss J, Eckardt K, Eckel J (2012) Diabetologia 55: 1128-1139

2.2 Adipokines promote lipotoxicity in human skeletal muscle cells

Taube A, **Lambernd S**, van Echten-Deckert G, Eckardt K, Eckel J (2012) Arch.Physiol Biochem. 118: 92-101

2.3 Identification and validation of novel contraction-regulated myokines released from primary human skeletal muscle cells

Rascke S, Eckardt K, Bjørklund Holven K, Jensen J, Eckel J (2013) PLOS ONE, in revision

2.4 Regulation of Follistatin-like 1 expression in primary human skeletal muscle cells

Görgens SW*, **Raschke S***, *, Bjørklund Holven K, Jensen J, Eckardt K and Eckel J (2013) Arch.Physiol Biochem, accepted for publication

2.5 Evidence against a beneficial effect of irisin in humans

Raschke S, Elsen M, Gassenhuber H, Sommerfeld M, Schwahn U, Brockmann B, Romacho T, Eckardt K, Eckel J (2013) Diabetologia, submitted

2.6 Contribution Statement

Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways

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Abstract

Aims/hypothesis • Obesity is closely associated with muscle insulin resistance and is a major risk factor for the pathogenesis of type 2 diabetes. Regular physical activity not only prevents obesity, but also considerably improves insulin sensitivity and skeletal muscle metabolism. We sought to establish and characterise an in vitro model of human skeletal muscle contraction, with a view to directly studying the signalling pathways and mechanisms that are involved in the beneficial effects of muscle activity.

Methods • Contracting human skeletal muscle cell cultures were established by applying electrical pulse stimulation. To induce insulin resistance, skeletal muscle cells were incubated with human adipocyte-derived conditioned medium, monocyte chemotactic protein (MCP)-1 and chemerin.

Results • Similarly to in exercising skeletal muscle in vivo, electrical pulse stimulation induced contractile activity in human skeletal muscle cells, combined with the formation of sarcomeres, activation of AMP-activated protein kinase (AMPK) and increased IL-6 secretion. Insulin-stimulated glucose uptake was substantially elevated in contracting cells compared with control. The incubation of skeletal muscle cells with adipocyte-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, proinflammatory signalling by adipocyte-derived factors was completely prevented by electrical pulse stimulation of the myotubes.

Conclusions/interpretation • We showed that the effects of electrical pulse stimulation on skeletal muscle cells were similar to the effect of exercise

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Abbreviations

AMPK	AMP-activated protein kinase
CM	Adipocyte-conditioned medium
EPS	Electrical pulse stimulation
GSK	Glycogen synthase kinase
hSkMC	Human skeletal muscle cells
IKK	IκB kinase
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemotactic protein
αMEM	α-Modified Eagle's medium
MHC	Myosin heavy chain
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B cells
SSC	Saline-sodium citrate
VEGF	Vascular endothelial growth factor

on skeletal muscle in vivo in terms of enhanced AMPK activation and IL-6 secretion. In our model, muscle contractile activity eliminates insulin resistance by blocking pro-inflammatory signalling pathways. This novel model therefore provides a unique tool for investigating the molecular mechanisms that mediate the beneficial effects of muscle contraction.

Introduction

Obesity in combination with a lack of exercise is a strong risk factor for the development of type 2 diabetes. 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 numerous cytokines that regulate energy metabolism and insulin sensitivity [3–5]. Adipocytes from obese persons are characterised by altered endocrine function, leading to increased secretion of proinflammatory adipokines, such as TNFα, chemerin, monocyte chemotactic protein (MCP)-1 and resistin [6–9]. The activation of inflammatory pathways leads to insulin resistance [10] in peripheral tissues such as skeletal muscle and adipose tissue, constituting an early defect in the pathogenesis of type 2 diabetes [11]. Insulin-resistant and type 2 diabetic patients display impaired insulin action on wholebody glucose uptake, in part due to impaired insulinstimulated glucose uptake in skeletal muscle [11].

It is well accepted that physical activity exerts major beneficial effects on the prevention of chronic diseases like type 2 diabetes, cardiovascular disease, dementia and depression [12, 13]. Regular physical activity not only prevents obesity and reduces adipose tissue mass, but is also known to increase insulin-stimulated glucose uptake in the immediate post-exercise period [14], while chronic physical activity enhances insulin sensitivity in human skeletal muscle [15, 16]. Acute exercise increases glucose uptake in skeletal muscle by an insulin-independent mechanism that bypasses the insulin signalling defects associated with pathophysiological conditions [17]. Additionally, exercise activates AMP-activated protein kinase (AMPK), which phosphorylates and thereby inhibits acetyl-CoA carboxylase, resulting in reduced malonyl CoA content and hence enhanced fatty acid oxidation [18].

At present, the molecular mechanisms mediating the health-promoting effects of physical activity are not entirely understood. In the last decade, it became evident that skeletal muscle is an endocrine organ that produces and releases myokines in response to contraction [19]; these myokines probably mediate the health-promoting effects of physical activity. Myokines such as IL-6 and brain derived neurotrophic factor are released by skeletal muscle cells after exercise and lead to enhanced fatty acid oxidation in an AMPK-dependent fashion [20, 21]. We have previously shown that primary human skeletal muscle cells (hSkMC) incubated with adipocyte-conditioned medium (CM) from primary human adipocytes or treated with adipokines like MCP-1 and chemerin are characterised by impaired insulin signalling and glucose uptake [8, 9, 22]. Our aim here was to establish and characterise an in vitro contraction model of hSkMC, which mimics exercise; the overall goal was to analyse the signalling pathways and mechanisms involved in the beneficial effects of muscle activity. We also combined this contraction model with the insulin resistance model to analyse the cross-talk between adipocytes and contracting hSkMC. We show here that the contractile activity of hSkMC exerts an anti-inflammatory action, which prevents the induction of insulin resistance.

Methods

Materials • Reagents for SDS-PAGE were supplied by GE Healthcare (Munich, Germany) and Sigma (Munich, Germany), and rotiphorese was supplied by Carl Roth (Karlsruhe, Germany). The following antibodies were used: antiphospho glycogen synthase kinase (GSK) 3α/β (Ser21/9), anti-phospho Akt (Ser473, Thr308), anti-Akt, anti-phospho nuclear factor 'kappa-light-chain-enhancer' of activated B cells (NFκB) (Ser536), anti-NFκB, anti-IκB kinase (IKK)α/ß, anti-IκBα,

anti-phospho AMPKα (Thr172) and anti- AMPKα (Cell Signalling Technology, Frankfurt, Germany); anti-tubulin (Calbiochem, Merck Biosciences, Schmalbach, Germany); sarcomeric α-actinin (Sigma); anti-myosin heavy chain (MHC) (Upstate, San Diego, CA, USA); and mitochondria oxidative phosphorylation antibody cocktail and anti-GLUT12 antibody (Acris, Herford, Germany). 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 was supplied by R&D Systems (Wiesbaden-Nordenstadt, Germany) and MCP-1 by PeproTech (Hamburg, Germany). Primary hSkMC and the supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). Horse serum for the differentiation medium was provided by Gibco. All other chemicals were of the highest analytical grade commercially available and purchased from Sigma.

Culture of hSkMC • Primary hSkMC from five healthy white donors (three males, 16, 21 and 47 years old; two females, 33 and 37 years old) were supplied as proliferating myoblasts and cultured as described earlier [23]. For an individual experiment, myoblasts were seeded in six-well culture dishes at a density of 1×10^5 cells/well and cultured to near-confluence in α-modified Eagle's medium (αMEM)/Ham's F-12 medium containing skeletal muscle cell growth medium supplement. The cells were then differentiated in αMEM containing 2% (vol./vol.) horse serum until day 5 of differentiation, followed by overnight starvation in αMEM without serum. Differentiated cells were electrically stimulated and incubated as indicated with CM, chemerin, MCP-1 and TNFα, respectively. Afterwards, cells were stimulated with 100 nmol/l insulin for 10 min.

Electrical pulse stimulation • Electrical pulse stimulation (EPS) was applied to fully differentiated myotubes in sixwell dishes using a C-dish combined with a 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 medium. The myotubes were stimulated at 1 Hz, 2 ms

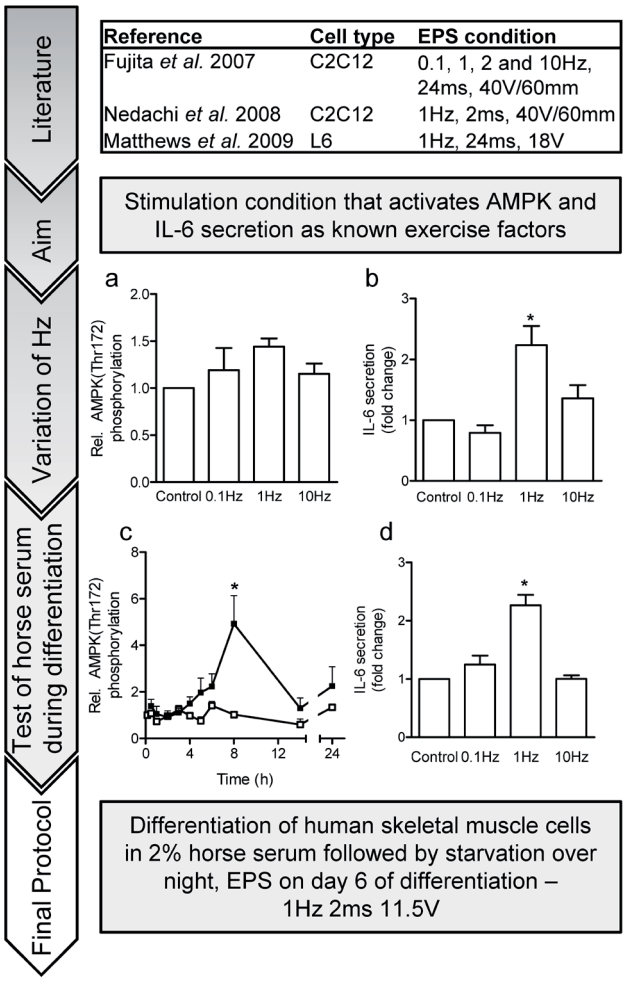

Figure 1. Process of selecting the conditions of EPS protocol. Based on previously published conditions of EPS, frequencies of 0.1, 1 and 10 Hz were tested [21, 30, 42]. The conditions were selected with regard to optimised AMPK activation and IL-6 secretion. Effects of variation of frequency **(a, b)** and test of differentiation with horse serum **(c, d)** were quantified. **a, b** Myotubes were differentiated in αMEM serum-free medium and subjected to EPS at 0.1, 1 and 10 Hz, 2 ms and 11.5 V. Total cell lysates **(a)** were obtained, resolved by SDS-PAGE and immunoblotted with phospho-specific (p) AMPK (Thr172) antibody; n=3. **b** IL-6 secretion was determined in supernatant fractions; n=4–5; **p<0.01 vs control. **c** Myotubes were differentiated in αMEM serum-free medium containing 2% (vol./vol.) horse serum during differentiation, in combination with overnight starvation. Total cell lysates were obtained and treated as above **(a)**; n≥3; *p<0.05. White symbols, control; black symbols, EPS. **d** IL-6 secretion was determined in supernatant fractions; n≥3; *p<0.001 vs control

and 11.5 V for 2 to 24 h. The medium was changed directly before stimulation. To document the contraction of the stimulated myotubes, a series of two images per s was taken using a microscope (DM RBE; Leica, Heidelberg, Germany) and camera (HV-C20; Hitachi, Tokyo, Japan). The images, shown at a rate of two images per s, can be viewed in the electronic supplementary material [ESM] Video 1.

Adipocyte isolation and culture • Adipose tissue samples were obtained from subcutaneous fat of normal or moderately overweight women (BMI 27.9±0.9 kg/m2 [mean±SEM], age 26–44 years). The procedure for obtaining adipose tissue was approved by the Ethics Committee of Heinrich-Heine-University, Duesseldorf, Germany. All tissue donors were healthy, free of medication and had no evidence of diabetes according to routine laboratory tests. Pre-adipocytes were isolated by collagenase digestion and differentiated as previously described [9, 24]. After 15 days, 70–90% of the seeded pre-adipocytes 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 48 h, as previously described [25].

Immunofluorescence staining • hSkMC were seeded on glass coverslips, differentiated and stimulated by EPS. Afterwards cells were fixed with 2% (wt/vol.) paraformaldehyde dissolved in PBS for 15 min at room temperature. Cells were washed twice and permeabilised on ice for 5 min with 0.2% (vol./vol.) Triton-X in buffer containing 20 mmol/l HEPES, 300 mmol/l saccharose, 50 mmol/l NaCl and 3 mmol/l $MgCl_2$. After blocking with 5% (wt/vol.) non-fat dry milk in PBS, myotubes were incubated with anti-sarcomeric α-actinin, washed and incubated with a secondary rhodamine-conjugated antibody. Myotubes were washed with 2× saline-sodium citrate (SSC) buffer (0.3 mol/l NaCl, 0.03 mol/l sodium citrate, pH 7.0) and incubated with 100 μg/ml RNAse in 2× SSC buffer for 20 min at 37°C. After washing with 2× SSC buffer, the nuclei were stained with 5 μmol/l Syto13 green for 5 min at room temperature.

Electron microscopy • Embedding of hSkMC in Epon 812 was performed as described by Luft [26] and modified by Reale [27]. In brief, cells were fixed in 2.5% (vol./vol.) glutaraldehyde/190 mmol/l cacodylate buffer, pH 7.4, and post-fixed in 1% (wt/vol.) osmium tetroxide. We used 1% (wt/vol.) uranyl acetate and lead citrate [28] to stain ultra-thin sections. Sections were investigated using a transmission electron microscope (TEM910; Zeiss, Oberkochem, Germany).

Measurement of IL-6 and vascular endothelial growth factor • The cytokine concentration in the supernatant fractions was determined by IL-6 and vascular endothelial growth factor (VEGF) ELISA (Biovendor, Heidelberg, Germany), respectively, both assays used according to the manufacturer's protocol.

Cell viability assays • hSkMC were differentiated and electrically stimulated for 2–24 h.

ATP assay • Changes of relative ATP level were analysed using an ATP cell viability assay kit (ApoSENSOR; Bio-Vision, Heidelberg, Germany) according to the instructions.

Lactate assay • L-(+)-lactate was detected in the supernatant fraction with a kit (Lactate Assay Kit II; BioVision).

MTT assay • The NADH content in the cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Myotubes were incubated for 2 h at 37°C with 1.2 mmol/l MTT solution in medium. Afterwards, cells were washed with PBS and lysed with 500 μl DMSO. Absorption at 540 nm was determined using a plate reader (InfiniteM200; Tecan, Maennedorf, Switzerland).

Cytotoxicity assay • The supernatant fraction was collected and lactate dehydrogenase release into the medium measured with a kit (Cytotoxicity Detection Kit Plus; Roche Applied Science, Mannheim, Germany) used according to the manufacturer's protocol.

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

RNA-isolation and quantitative real-time PCR • Total RNA was isolated and reverse-transcribed using kits (RNeasy Mini, Omniscript Reverse Transcription; Qiagen, Hilden, Germany) according to the manufacturer's instructions. Gene expression was determined by quantitative real-time PCR using QuantiTect primer assays and SYBR green reagents (Qiagen) with 0.016 to 20.00 ng cDNA on a cycler (Step One Plus; Applied Biosystems, Carlsbad, CA, USA). Expression of the genes investigated was normalised to actin. Gene expression was analysed via the $\Delta\Delta C_t$ method.

Glucose uptake • hSkMC were electrically stimulated for 7.5 h on day 6 of differentiation. During the last 30 min of EPS, cells were treated with insulin (100 nmol/l) and uptake of 2-deoxyglucose was measured for 2 h as described above [9, 29].

Fatty acid and glucose oxidation • hSkMC were seeded on 10 mm coverslips in six-well culture dishes and electrically stimulated as described above. Subsequently, coverslips were transferred to 48 well culture dishes, and 11.1 kBq/well of [^{14}C]oleic acid and [^{14}C]palmitic acid supplemented with 1 µmol/l L-carnitine, or 7.4 kBq/well [U-^{14}C]glucose supplemented with 0.35 mmol/l glucose were added to hSkMC. Culture dishes were incubated for 4 h 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, Munich, Germany).

Immunoblotting • hSkMC 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 (Roche). Western blot analysis was performed as described before [9]. Signals were visualised and evaluated on a work station (VersaDoc 4000 MP; BioRad, Munich, Germany) and analysed with an analysis software package (Quantity One, version 4.6.7, BioRad, Munich, Germany).

Presentation of data and statistics • Data are presented as means±SEM. Unpaired two-tailed Student's t test or oneway ANOVA (post hoc test Bonferroni's multiple comparison test) were used to determine statistical significance. All statistical analyses were performed using Prism5 (GraphPad, La Jolla, CA, USA), with a value of $p<0.05$ considered to be statistically significant. Corresponding significance levels are as indicated.

Results

EPS induces de novo sarcomere structure assembly in human skeletal myotubes • After 6 days of differentiation, most of the hSkMC fused and formed typical multinucleated myotubes. As reported in our earlier study [22], differentiating hSkMC display increased protein abundance of myogenin, myoblast determination protein and MHC, which are typical markers of myogenesis, but cells did not contract spontaneously. The conditions of 1 Hz frequency, 2 ms pulse duration and 11.5 V intensity were selected with regard to most marked AMPK activation and IL-6 secretion after differentiation with 2% horse serum (Fig. 1). Using this protocol, we observed that after a few hours of continuous EPS, a subset of myotubes showed noticeable, vigorous contraction, with most of the myotubes contracting after 8 h EPS (ESM Video 1). Immunofluorescence staining of sarcomeric α-actinin showed uniform distribution of the protein in the cytoplasm of unstimulated cells. After EPS, a reorganisation of the cytoskeleton and de novo formation of sarcomeric structures with their typical striated pattern were observed (Fig. 2a–c). These findings were confirmed by electron microscopy (Fig. 2d–f). The typical Z-lines appeared as a series of dark lines as described for cross-striated muscles.

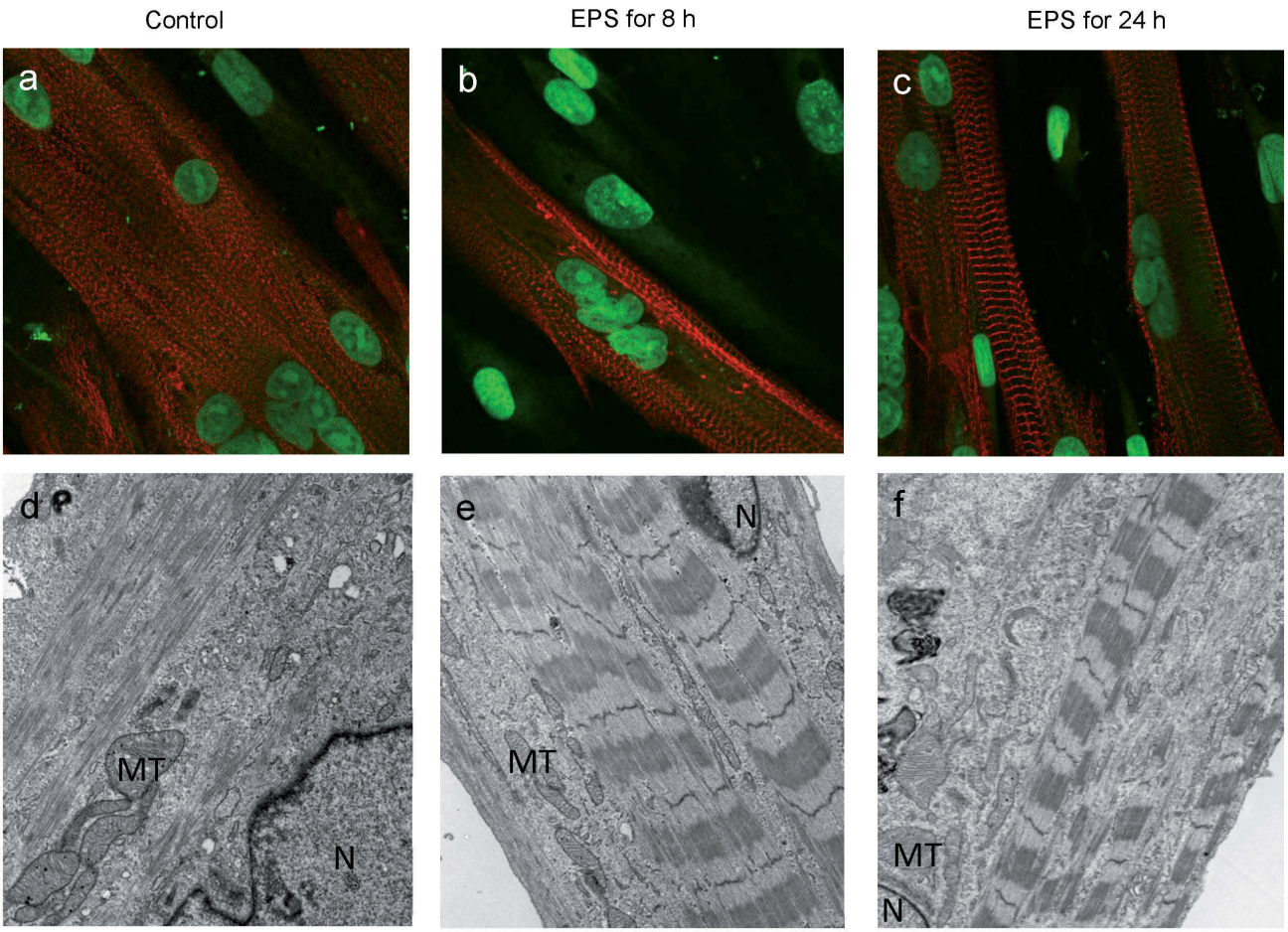

Figure 2. Effect of EPS on sarcomere structure assembly in human skeletal myotubes. **a** The cells were fixed and analysed for localisation of sarcomeric α-actinin without (control), and after 8 **(b)** and 24 h **(c)** of EPS (1 Hz, 2 ms, 11.5 V) by immunofluorescence staining, as described. Red, sarcomeric α-actinin; green, sytogreen. Magnification×100. Sarcomeric α-actinin was evenly distributed in the cytoplasm without EPS **(a)**. Sarcomeres became rapidly visible after EPS, with **(b, c)** Z-lines appearing as a series of red lines. These findings were confirmed by electron microscopy **(d–f)**. Magnification×10,000. MT, mitochondria; N, nucleus

EPS exerts no damaging effect on myotubes • Decreased levels of ATP and increased levels of ADP are recognised in apoptotic cells. To rule out the possibility that EPS may cause cell damage, several cell-based assays were performed. Cytotoxicity, as assessed by L-lactate dehydrogenase release, did not differ significantly between cells with or without EPS (Fig. 4a). The NADH level in cells, as assessed by MTT assay, was not changed by EPS compared with control cells (Fig. 4b). The concentration of lactate in the medium significantly increased from 8 to 24 h of culture (Fig. 4c) from 187±7 nmol/ml (mean±SEM) to 336±10 nmol/ml. EPS induced significant, higher lactate concentrations in the medium compared with controls. The polarisation of mitochondrial membranes was not disturbed, since the ratio of JC-1 aggregates to monomers was not changed during culture, either in unstimulated or in EPS-treated cells (Fig. 4d). Protein abundance of the complexes of the electron transport chain showed a significant increase for complexes II, IV and V (Fig. 4e). In addition UCP3, PGC1α (also known as PPARGC1A) and COX2 mRNA were significantly increased compared with non-stimulated cells (Fig. 4f).

EPS augments insulin-stimulated glucose uptake, but does not change fatty acid oxidation • To test whether EPS induces changes in glucose homeostasis in hSkMC, we measured glucose uptake. Insulin-stimulated glucose uptake was significantly increased over the basal level (2.6-fold; Fig. 5a), as

reported in our earlier studies [22, 29]. Contraction of hSkMC led to a significant 2.4-fold increase compared with basal control. Importantly, hSkMC showed a marked increase (fivefold) in insulin-stimulated glucose uptake after EPS compared with basal control. The incremental increase in insulin-stimulated glucose uptake, which reflects the efficiency of insulin action, was profoundly augmented by contractile activity of myotubes (Fig. 5b). However, levels of insulin-sensitive glucose transporters, namely GLUT4 and GLUT12, were not affected (Fig. 5c). Oxidation of the fatty acids, oleic acid and palmitic acid, and glucose oxidation were assessed by $^{14}CO_2$ production. Glucose oxidation was increased in response to insulin and EPS alone, while the combined effect was additive (Fig. 5d). However, oxidation of both fatty acids was not changed after EPS-induced contraction (Fig. 5e).

EPS protects hSkMC from impaired insulin signalling induced by CM, MCP-1 and chemerin • Under control conditions, insulin-induced a significant increase of Akt phosphorylation (Ser473) in hSkMC, with a significant reduction of this response after incubation with CM (Fig. 6a). When the cells were EPS-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 very marked inhibition by CM and complete prevention 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 [8, 9, 23]. The application of EPS during treatment with chemerin and MCP-1, respectively, restored insulin signalling in hSkMC (Fig. 6b).

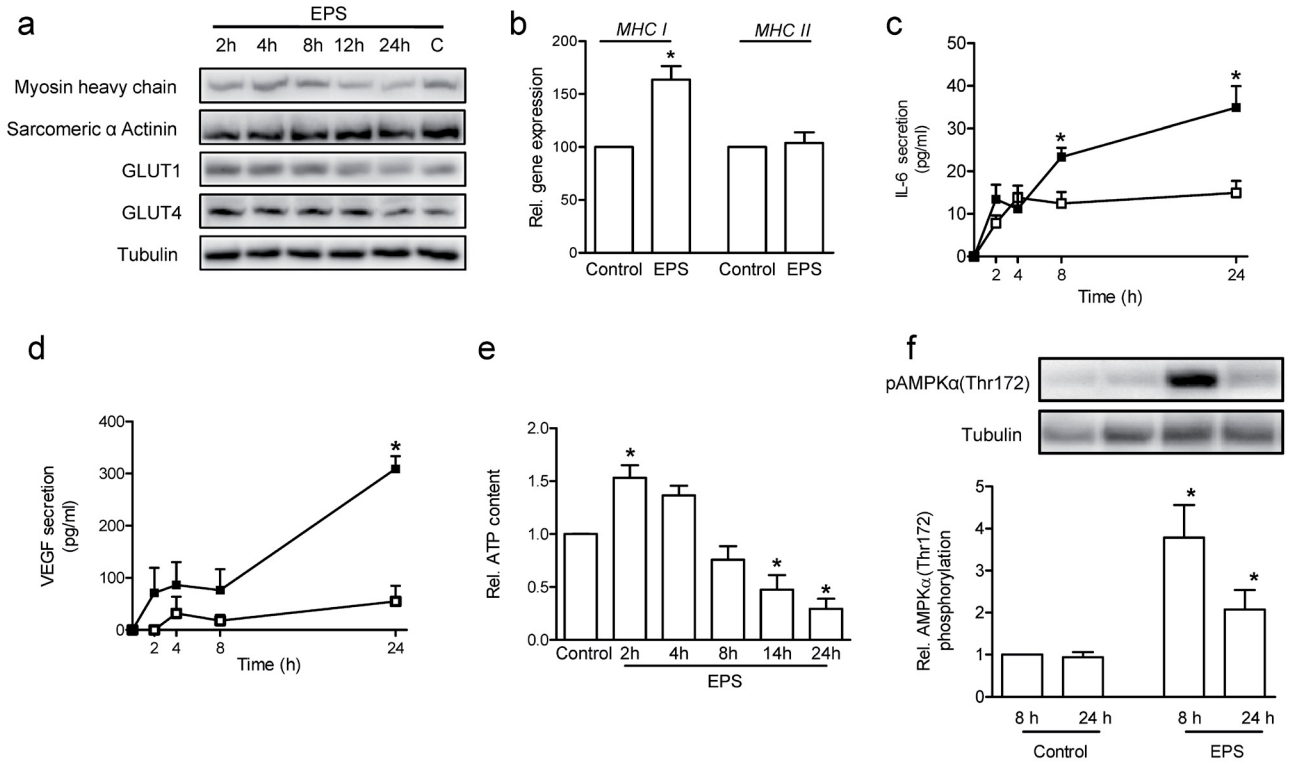

Figure 3. Effect of EPS on the abundance of muscle proteins, secretion of IL-6, and on VEGF and AMPK activation. hSkMC were differentiated and exposed to EPS for the indicated times at 1 Hz, 2 ms and 11.5 V. **a** Total cell lysates were resolved by SDS-PAGE; representative blots of proteins as labelled are shown. **b** Relative gene expression of MYH1 and MYH2 was measured by real-time PCR as described; n=4–5; *p<0.001. **c** IL-6 and (**d**) VEGF secretion of human myotubes with and without EPS was measured after indicated time points by ELISA; n≥3; *p<0.05. White symbols, control; black symbols, EPS. **e** Relative ATP concentration of myotubes was measured after indicated time points of EPS and in control, as described; n=8, *p<0.05. **f** Total cell lysates were obtained and analysed by SDS-PAGE and western blot using phospho-specific (p) AMPKα antibody. Quantified data were normalised to tubulin; n≥3; *p<0.01 vs corresponding basal level. Data (**b–f**) are mean±SEM

EPS prevents activation of NFκB and p42/44 mitogenactivated protein kinase in hSkMC • NFκB and IKKβ protein abundance was significantly reduced in EPS-treated cells, whereas IKKα and IκBα protein abundance were not affected (Fig. 7a). Incubation of cells with TNFα led to activation of NFκB, reaching a maximum after 10 min (3.2-fold; Fig. 7c). As shown by application of the selective inhibitor of IKK, I229 (Fig. 7b), this effect is specifically mediated by IKK downstream signalling, After EPS, NFκB activation was diminished due to reduced NFκB protein abundance. Most importantly, after EPS, maximal NFκB activation in response to TNFα as substantially reduced (1.7-fold after 10 min). Thus contractile activity exerts an anti-inflammatory effect and interferes with TNFα-induced NFκB signalling.

Incubation of hSkMC with TNFα led to a significant decrease (59% after 20 min) of IKKβ protein levels (Fig. 7d). In contrast, IKKβ protein abundance was significantlyr reduced after EPS, compared with quiescent controls (Fig. 7a), but was not altered after TNFα treatment (Fig. 7d). The incubation of myotubes with TNFα diminished IκBα protein abundance by more than 60% after 20 min, while TNFα had no effect on IκBα levels in contracting cells (Fig. 7e). CM and chemerin induced NFκB activation (1.8-fold and 1.5-fold; Fig. 8a), while MCP-1 induced significant activation of p44/p42 mitogen-activated protein kinase (MAPK) (1.5-fold; Fig. 8c). Both effects on NFκB and p44/p42 MAPK activation were diminished to control level after EPS (Fig. 8b, c).

Discussion

Exercise has been shown to have a positive impact on a number of diseases in humans, including obesity and type 2 diabetes [1, 2, 13]. The pathogenesis of type 2 diabetes has been intensively studied and is characterised by chronic hypergly-

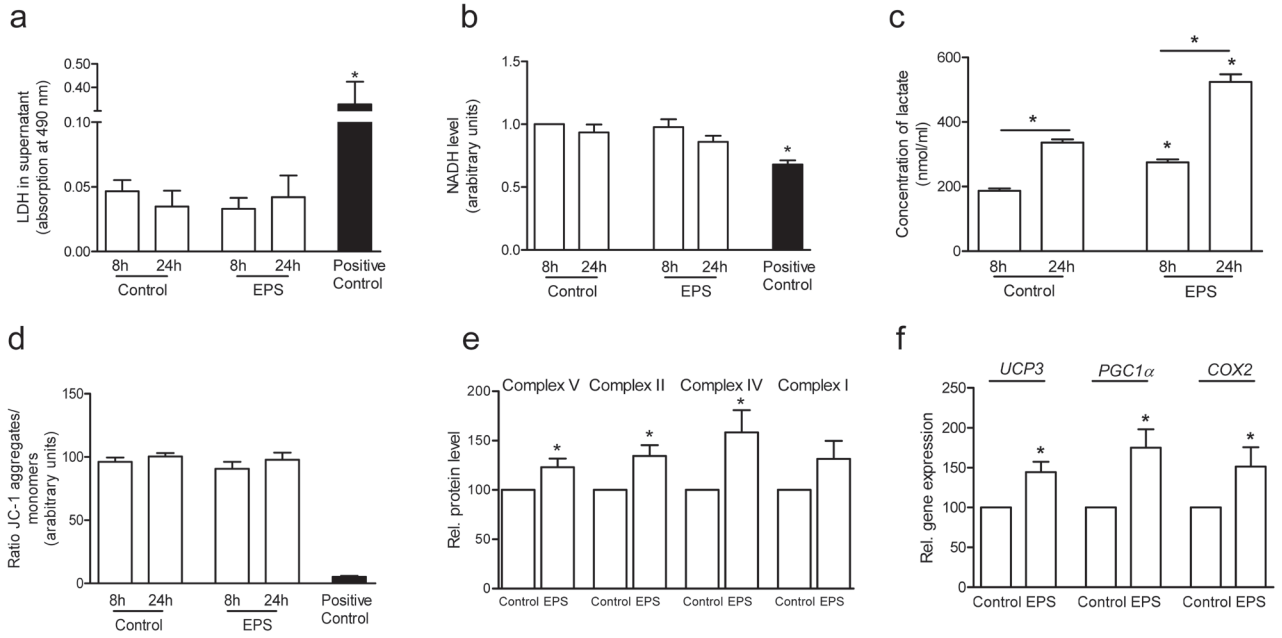

Figure 4. Determination of metabolic activity of skeletal muscle cells after EPS. hSkMC were differentiated and stimulated for 8 and 24 h at 1 Hz, 2 ms and 11.5 V. **a** Lactate dehydrogenase (LDH) abundance in the medium; n≥5, *p<0.001. **b** The quantity of NADH in the cells was measured using an MTT assay; n≥3, *p<0.01. **c** Lactate concentrations in the supernatant fractions of EPS-treated and untreated cells. As positive control, lysis reagent (supplied with the kit) was used. n≥3, *p<0.001. **d** The ratio of JC-1 aggregates to monomers. As a positive control, exposure to 100 μmol/l CCCP (carbonyl-cyanide mchlorophenyl hydrazone) for 45 min was used prior to JC-1 staining. **e** Total cell lysates were obtained and analysed by SDS-PAGE and western blot using an oxidative phosphorylation antibody cocktail. Data are normalised to tubulin; n=4, *p<0.05. **f** Relative gene expression of UCP3, PGC1α and COX2 as measured by real-time PCR; n04–5; *p<0.01 for UCP3 and PGC1α, *p>0.05 for COX2. Values **(a–f)** are mean±SEM

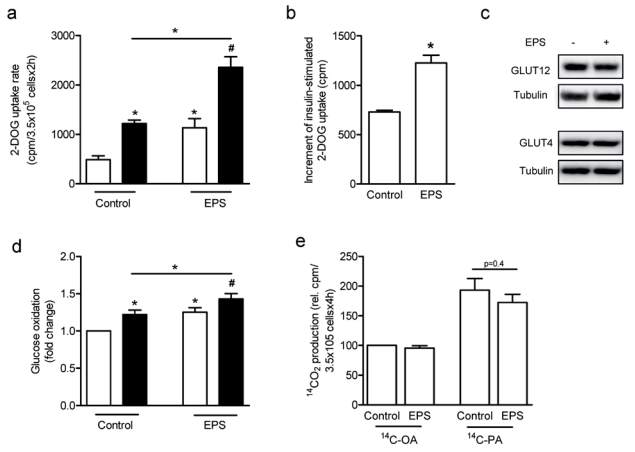

Figure 5. Effect of EPS on glucose uptake and fatty acid oxidation in hSkMC. **a** In the last 30 min of 7.5 h of EPS (1 Hz, 2 ms, 11.5 V) cells were stimulated with 100 nmol/l insulin. Glucose uptake was assessed for 2 h after acute insulin stimulation. n=5; *p<0.05 vs basal control; #p<0.05 vs basal control and EPS control. White bars, basal; black bars, insulin-stimulated. **b** The increment of insulin-stimulated glucose uptake was calculated as the difference between insulin-stimulated glucose uptake (2-deoxy-glucose, 2-DOG) and basal control; n=5; *p<0.05. **c** Total cell lysates were obtained and analysed by SDS-PAGE and western blot. Representative blots for proteins as indicated are shown. **d** Glucose oxidation. Cells were treated with 100 nmol/l insulin as indicated during incubation in the oxidation chamber; n=4–5; *p<0.05 vs basal control; #p<0.05 vs basal control and EPS control. White bars, basal; black bars, insulin-stimulated **e** Fatty acid oxidation as assessed after 24 h EPS; n≥3. Data **(a, b, d, e)** are means±SEM. OA, oleic acid; PA, palmitic acid

caemia, resulting in defects in insulin secretion, insulin action or both. However, knowledge of the interplay between different molecular signalling pathways during exercise is still incomplete and experimentally adequate models of exercise remain elusive. In this study, we established and validated a model of contracting hSkMC and used it to further analyse the beneficial effect of exercise in the context of insulin resistance.

It is well known that hSkMC display increased levels of typical myogenesis markers like myogenin, myoblast determination protein, MHC and GLUT4 during differentiation in vitro [20]. One limitation of the models used is the lack of contraction, a central characteristic of muscle cells. Therefore, we stimulated hSkMC with electrical pulses, resulting in vigorous contraction and formation of striation patterns of sarcomeric structures, as visualised by immunofluorescence staining of α-actinin and electron microscopy. In line with this, Fujita et al. observed de novo formation of sarcomeres after EPS in murine C2C12 cells, whereas calcium channel blockers like verapamil and BAPTA-AM suppressed sarcomere structure development [30]. Thus it can be speculated that manipulated Ca^{2+} transients, achieved by applying an appropriate EPS to differentiated hSkMC, are primarily necessary to accelerate de novo sarcomere assembly and to rapidly develop contractile activity. In response to contraction, we observed an increase of MYHI mRNA expression after EPS, in combination with an increase of mitochondrial marker proteins and mitochondrial mRNA expression. MYHI fibres are more efficient at using oxygen to generateATP for continuous, extended muscle contractions over a long time, reflecting the experimental setup used by us with its rather long EPS time of 24 h.

In skeletal muscle, three energy systems function to replenish ATP, namely creatine kinase, glycolysis and mitochondrial respiration. All systems contribute to different degrees to the replenishment of ATP on the basis of an interaction between the intensity and duration of exercise [31]. Using EPS, we observed enhanced glucose uptake and increased lactate concentrations. 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^+ [31]. As we did not observe increased fatty acid oxidation after 24 h of EPS, it is likely that cells mainly catabolise glucose for ATP regeneration under the applied conditions. However, after depletion of muscle glycogen by contraction, cells may replenish glycogen stores, as well.

Human studies using the one-legged exercise model followed by a euglycaemic–hyperinsulinaemic clamp have shown increased insulin sensitivity after exercise [32]. C2C12 cells derived from mouse skeletal muscle are mostly used to investigate muscle cell differentiation, sarcomere development, myotube contraction and glucose uptake [30, 33]. However, these cells do not pro-

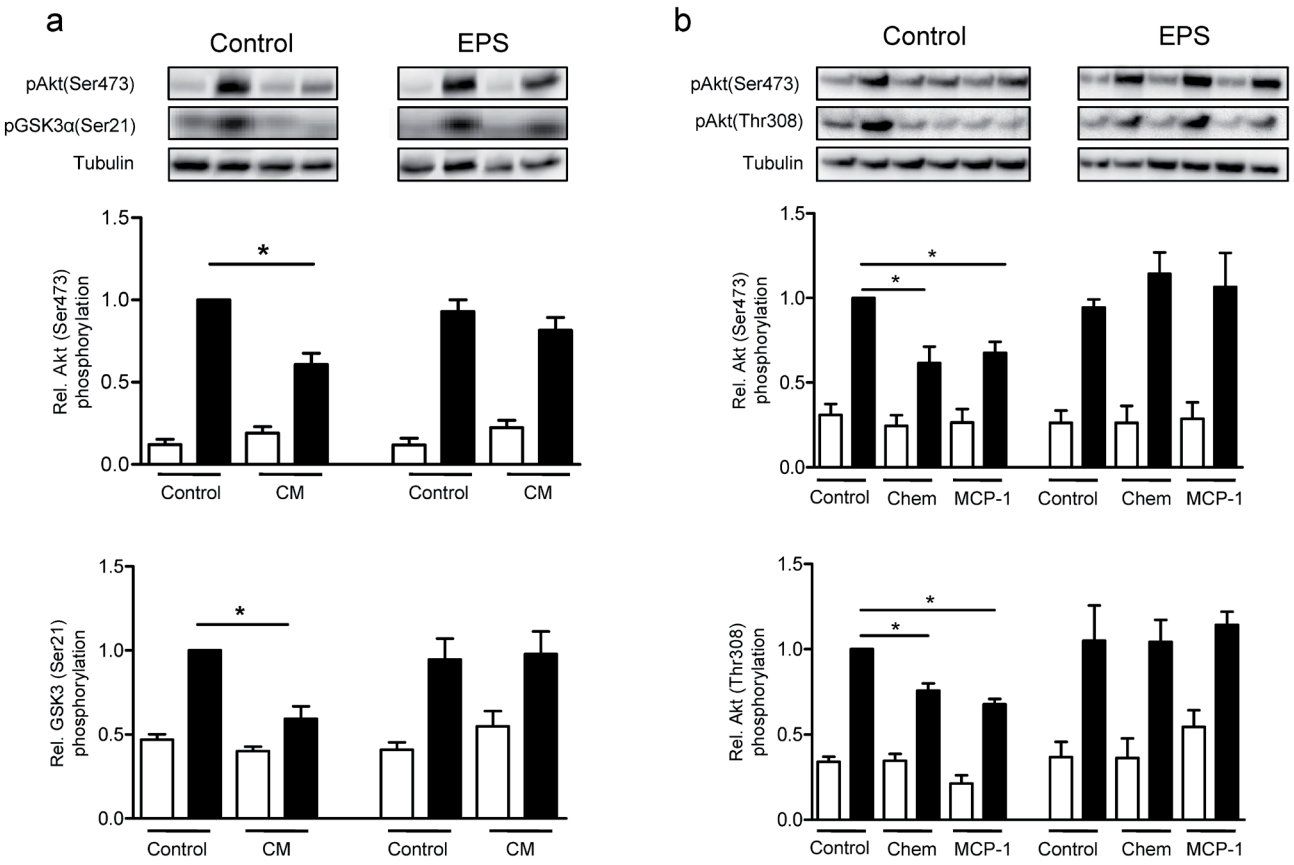

Figure 6. Effect of CM, chemerin and MCP-1 on insulin signalling in control and electrically stimulated hSkMC. **a** Myotubes were simultaneously incubated with CM for 8 h and exposed to EPS. After acute stimulation with insulin, total cell lysates were obtained, resolved by SDS-PAGE and immunoblotted with phospho-specific (p)Akt and GSK3 antibodies. All data were normalised to the level of tubulin and are expressed relative to insulin-stimulated control values. Data are means±SEM; n≥5; *p<0.05. **b** Myotubes were incubated with 2 μg/ml chemerin (Chem) and 2 ng/ml MCP-1, respectively, and simultaneously exposed to EPS. After acute stimulation with insulin, total cell lysates were obtained, resolved by SDS-PAGE and immunoblotted with phospho-specific Akt antibodies for Ser473 and Thr308. All data were normalised 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

duce sufficient levels of GLUT4 and their insulin responsiveness is reportedly minimal, even after differentiation [34]. In our model, GLUT12 and GLUT4 protein abundance was not altered in cells during EPS, but human cells exhibited a profound insulin-stimulated glucose uptake (2.6-fold greater), compared with C2C12 cells (1.4-fold) [33]. We observed increased insulin efficiency after EPS, resulting in substantially elevated glucose uptake in contracting myotubes. As insulin signalling, GLUT4 and GLUT12 protein abundance remained unaltered after EPS, it may be that contractile activity affects the GLUT4 trafficking machinery, mediating a more efficient mobilisation of the transporter by insulin. Future studies will be needed to address these issues.

One of the major achievements in obesity research is the finding that adipose tissue is a major endocrine organ, which secretes numerous adipokines. Indeed, several hundred adipokines have now been identified [35]. The secretion of adipokines is changed dramatically in the obese state, affecting a wide range of physiological functions and leading to insulin resistance in skeletal-muscle, among other tissues [6, 7, 36]. A main finding of the present study was the improved insulin sensitivity of contracting hSkMC in conditions of insulin resistance. As previously shown by our group, CM (which contains the whole secretory output of mature adipocytes, including adipokines like chemerin, pigment epithelium-derived factor and MCP-1) induced insulin re-

sistance in hSkMC at the level of Akt and GSK3, and reduced insulin-stimulated glucose uptake by activating inflammatory signalling pathways [8, 9, 23, 37]. Incubation of hSkMC with CM or adipokines is a suitable model to dissect different mechanisms leading to muscle insulin resistance. In this study, we showed that EPS completely prevented insulin resistance in hSkMC at the level of Akt and GSK3 during incubation with CM, MCP-1 or chemerin. To date, we have only analysed the effects of EPS with regard to insulin signalling. Future work will be required to assess its effects at effector systems located further downstream from insulin.

One potential reason for the observation described above might be the blocking effect of EPS on NFκB activation by CM and several adipokines. Some adipokines involved in the development of insulin resistance are known to activate inflammatory signalling pathways by activating IKK and its downstream effector NFκB [38]. These proteins belong to a family of transcription factors that controls production of pro-inflammatory proteins. In our model, CM, chemerin and TNFα induced activation of NFκB, which is consistent with the elevated NFκB activity observed in muscle of insulinresistant participants in comparison with lean control participants under basal conditions [39]. EPS, which mimics 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 EPS was profoundly diminished. Thus contractile activity of hSkMC appears to directly inhibit TNFα signalling and activation of NFκB. This may involve: (1) downregulation of pro-inflammatory signalling components like IKK and NFκB, as shown here; (2) Ca^{2+}-mediated activation of anti-inflammatory pathways; and (3) probably the release of myokines by contracting cells. The anti-inflammatory effect of exercise is well established [40] and muscular IL-6 is thought to play a key role in this process [41]. However, the effect of IL-6 is related to its profound increase in the circulation after exercise, as well as to the inhibition of TNFα and IL-1 receptor alpha production [19]. Thus additional myokines exerting an autocrine action may be involved in the anti-inflammatory effect of muscle contractile activity. Very recent work in our laboratory suggests that several hundred myokines are released from contracting hSkMC

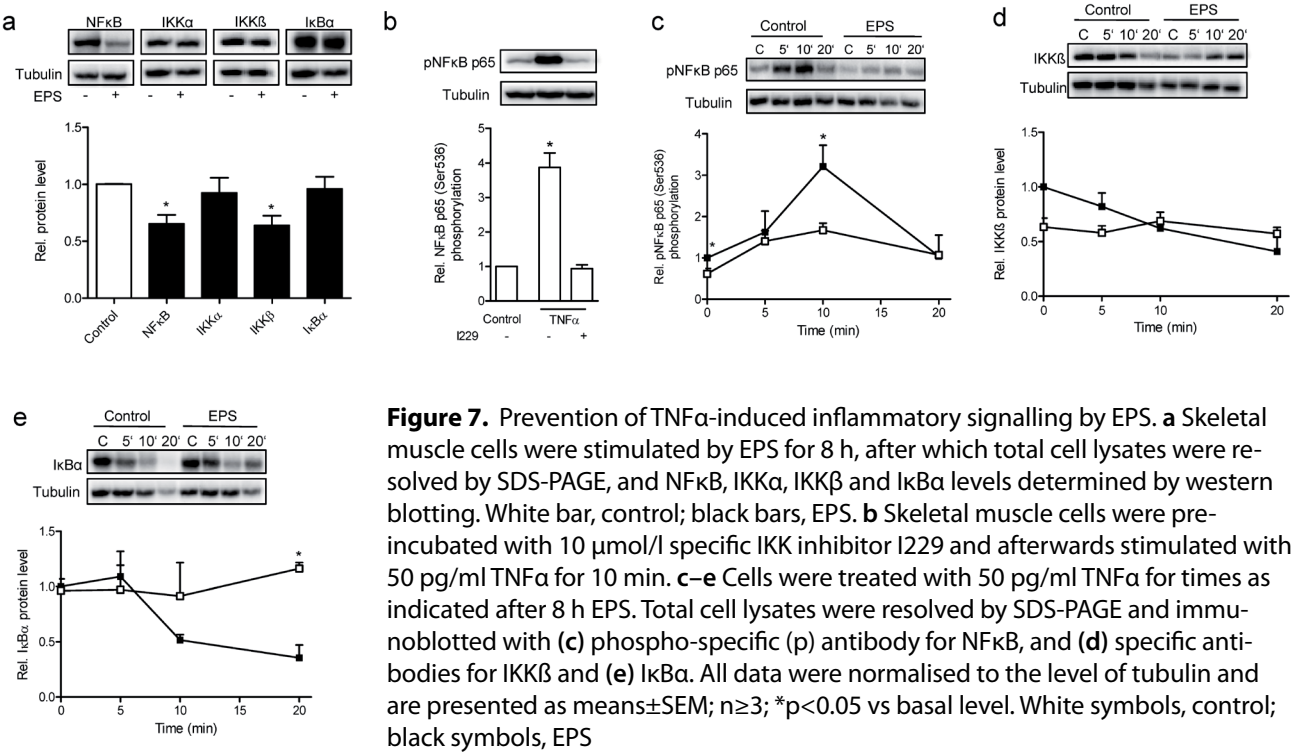

Figure 7. Prevention of TNFα-induced inflammatory signalling by EPS. **a** Skeletal muscle cells were stimulated by EPS for 8 h, after which total cell lysates were resolved by SDS-PAGE, and NFκB, IKKα, IKKβ and IκBα levels determined by western blotting. White bar, control; black bars, EPS. **b** Skeletal muscle cells were preincubated 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 times as indicated after 8 h EPS. Total cell lysates were resolved by SDS-PAGE and immunoblotted with **(c)** phospho-specific (p) antibody for NFκB, and **(d)** specific antibodies for IKKß and **(e)** IκBα. All data were normalised to the level of tubulin and are presented as means±SEM; n≥3; *p<0.05 vs basal level. White symbols, control; black symbols, EPS

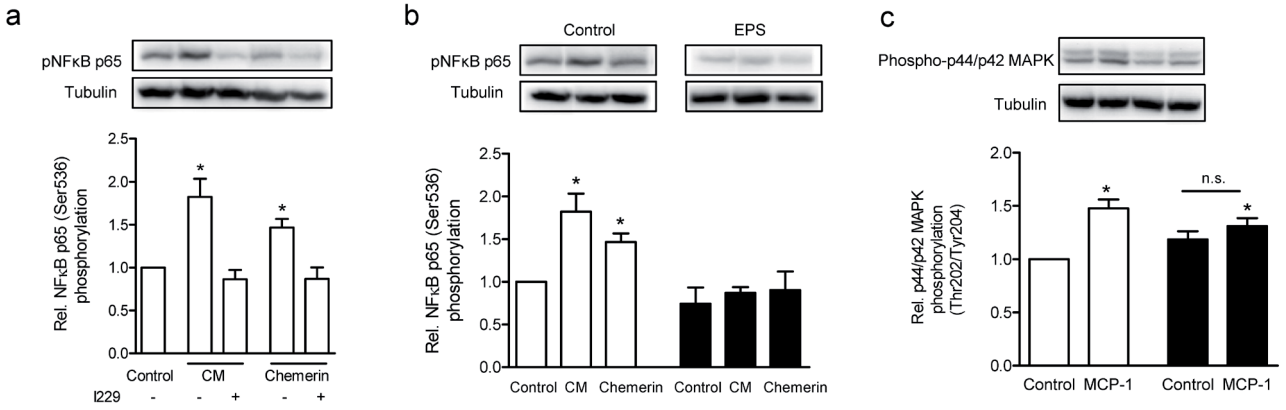

Figure 8. Prevention of CM-, MCP-1- and chemerin-induced inflammatory signalling by EPS. **a** Skeletal muscle cells were pre-incubated with or without 10 μmol/l of the specific IKK inhibitor I229. Afterwards, cells were treated with CM and 2 μg/ml chemerin for 30 min. Total cell lysates were resolved by SDS-PAGE and immunoblotted with a phospho-specific (p) antibody for NFκB. **b, c** Skeletal muscle cells were exposed to EPS for 8 h at 1 Hz, 2 ms and 11.5 V. Cells were treated with CM and 2 μg/ml chemerin **(b)** or with 2 ng/ml MCP-1 **(c)** for the last 30 min of stimulation. Total cell lysates were resolved by SDS-PAGE and immunoblotted with a phospho-specific antibody for NFκB **(b)** or MAPK **(c)**. Blots are representative. All data were normalised to the level of tubulin and are means±SEM; n≥4; *p< 0.05 vs basal level. **b, c** White bars, control (non-EPS); black bars, EPS

(S. Lambernd, unpublished observations). Identification of these factors will be instrumental to understanding the beneficial effects of muscle contraction.

In conclusion, using this model of contracting myotubes, we observed that the risk of insulin resistance is directly diminished, as insulin signalling was not disturbed after incubation with CM and inflammatory signalling was not activated. This model provides a unique tool for investigation of the mechanisms and underlying signalling pathways that mediate the beneficial effects of muscle contraction, and will help further clarify the potential of exercise as a way of combating insulin resistance.

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Contribution statement
SL contributed to the concept, acquired, analysed and interpreted data, wrote the manuscript and had the main responsibility together with JE. AT, AS, BP, SWG, RS, KJ and JW performed research and contributed to analysis and interpretation of data. KE and JE contributed to the concept, analysis of the data, and the discussion and revision of the manuscript. All authors approved the final version of the manuscript.

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

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Adipokines promote lipotoxicity in human skeletal muscle cells

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Abstract

Studies have shown the implication of specific adipokines or fatty acids (FA) in the pathogenesis of insulin resistance. However, the interplay of adipokines with FA remains poorly understood. This study aimed to investigate the combined effects of adipokines and low concentrations of palmitic acid (PA, 100 µmol/l) on skeletal muscle metabolism. Human skeletal muscle cells were incubated with adipocyte-conditioned medium (CM), PA or PA+CM, and FA transporter and FA metabolism were analysed. CM-incubation increased CD36 level (1.8 fold) and PA-uptake (1.4 fold). However, only co-application of PA+CM resulted in profound lipid accumulation (5.3 fold), 60% reduction of PA-oxidation and 3.5 fold increased diacylglycerol content. Our results support a novel role for adipokines in the pathogenesis of T2D by increasing the lipotoxic potential of PA, notably of low concentrations. This implies an increased lipotoxic risk already at an early stage of weight gain, when lipolysis has not yet contributed to increased plasma free FA levels.

Introduction

Obesity is one of the major risk factors contributing to the development of type 2 diabetes (T2D) (Felber et al., 2002). Enlargement of adipose tissue, especially in the visceral region, is characterized by an altered adipokine secretion pattern (Arner, 2001; Rajala et al., 2003; Trayhurn et al., 2001). As part of the negative crosstalk between adipose tissue and skeletal muscle, these obesity-associated adipokines promote skeletal muscle insulin resistance (Bloomgarden, 2000; Finegood, 2003; Sell et al., 2006a, 2006c), an early impairment and central defect in the pathogenesis of T2D (Bosello et al., 2000; Grundy et al., 2004). Considering the essential role of skeletal muscle in postprandial glucose disposal (DeFronzo et al., 1981), muscle insulin resistance is especially critical in the pathogenesis of T2D. Several studies have demonstrated deleterious effects of isolated adipokines such as leptin (Minokoshi et al., 2002), resistin (Junkin et al., 2009), TNFα (Hotamisligil, 1999), and IL-6 on muscle fatty acid uptake, oxidation, lipolysis, or insulin response (Hotamisligil, 1999; Junkin et al., 2009; Minokoshi et al., 2002; Nieto-Vazquez et al., 2008; Stefanyk et al., 2010). However, recent studies applying highly sensitive proteomic approaches have revealed the complex nature of the human adipose tissue secretome. A complementary protein profiling of supernatants obtained from human adipocytes by mass spectroscopy performed in our laboratory identified 347 proteins, 263 of which were predicted to be secreted (Lehr et al., 2011), corresponding to previous studies demonstrating a similar complexity of the adipocyte secretome (Alvarez-Llamas et al., 2007; Kim et al., 2010; Rosenow et al., 2010; Zhong et al., 2010). Thus, application of single adipokines yields a limited picture of the in

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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 impair glucose uptake (Sell et al., 2006b, 2008).

In addition to skeletal muscle insulin resistance, T2D patients requently 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 levels (Lelliott et al., 2004). As a consequence, fatty acid 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 (Roden, 2004). However, high circulating plasma fatty acid concentrations have been demonstrated to promote the development of skeletal muscle insulin resistance (Boden, 1997; Charles et al., 1997; Randle et al., 1963; Shulman, 2000), most likely via accumulation of lipids inside the muscle cell (Hegarty et al., 2003; Machann et al., 2004; Perseghin et al., 1999). Impaired skeletal muscle mitochondrial function including a decreased capacity to oxidize fat, as it is often observed in T2D patients, has been suggested to play a central role in this context (Blaak et al., 2000a, 2000b; Kelley et al., 1994). Lifestyle intervention therapies involving nutrition modifications and increased physical activity have been shown to effectively ameliorate insulin sensitivity and delay or prevent the onset of T2D (Knowler et al., 2002; Tuomilehto et al., 2001). Importantly, enhanced physical exercise has been demonstrated to induce muscle adaptation processes leading to augmented insulin sensitivity and improved mitochondrial performance (Hawley, 2009).

While a number of studies have investigated the impact of isolated adipokines and various fatty acid concentrations, respectively, the role of adipokines in the interplay with fatty acids and the combined impact on skeletal muscle metabolism remain poorly understood. Therefore, the aim of this study was to investigate the combined effects of the entire adipocyte secretome and the saturated fatty acid palmitic acid on human skeletal muscle metabolism. In this context, we used a relatively low concentration of 100 µmol/l palmitic acid compared with most other studies in order to closely mimic early stages in the pathogenesis of T2D where circulating levels of free fatty acids may not yet be elevated to pathophysiological levels. Additionally, a recently established innovative in vitro model of muscle contraction (Lambernd et al., 2012) was applied to assess the potential influence of physical activity on skeletal muscle metabolism in the interaction with adipokines and palmitic acid.

Methods

Materials • Reagents for SDS-PAGE were supplied by GE Healthcare Bio-Sciences (Uppsala, Sweden), Carl Roth (Karlsruhe, Germany) and Sigma (Munich, Germany). The phosphatase and protease inhibitor cocktail tablets were from Roche (Mannheim, Germany). The following antibodies were used: anti-CD36 (kind gift from J.F. Glatz, Maastricht), anti-fatty acid transport protein 4 (FATP4, Abnova, Taipei City, Taiwan), mitochondria OXPHOS antibody cocktail (Acris, Herford, Germany), anti-phospho-Akt (Ser473) and anti-Akt (Cell Signaling Technology, Danvers, MA, USA), anti-β-actin (Abcam, Cambridge, UK), and anti-tubulin (Calbiochem, Darmstadt, Germany). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG were purchased from Promega (Mannheim, Germany). Palmitic acid [1-14C] (14C-PA) was obtained from Perkin Elmer (Waltham, MA, USA) while liquid scintillation Aqua safe 300 plus was provided by Zinsser Analytic (Frankfurt, Germany). JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolylcarbocyanine iodide) was obtained from Calbiochem (Darmstadt, Germany). Primary human skeletal muscle cells (SkMC) were purchased from PromoCell (Heidelberg, Germany) and Lonza (Basle, Switzerland). Cell culture media was supplied by Gibco (Berlin, Germany) and SkMC supplement pack for growth medi-

um from PromoCell (Heidelberg, Germany). All other chemicals were of the highest analytical grade commercially available and were purchased from Sigma.

Generation of adipocyte-conditioned media (CM) • CM was generated as described previously (Sell et al., 2006b, 2008). Briefly, human pre-adipocytes were isolated from adipose tissue samples obtained from subcutaneous fat of normal or moderately overweight women. Pre-adipocytes were cultured and differentiated for 15 days. Mature adipocytes were used to generate CM by incubation with α-modified Eagle's medium (αMEM) for 48 h followed by collection of the medium.

Culture of human SkMC • Primary human SkMC of six healthy Caucasian donors were supplied as proliferating myoblasts and cultured as described previously (Dietze et al., 2002). For individual experiments, myoblasts were cultured in αMEM/ Ham's F-12 medium containing SkMC growth medium supplement 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 or palmitic acid (PA; 100 µM, bound to fatty-acid 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.

Nile Red staining • After incubation with CM and PA, SkMC were fixed with picric acid and stained for 20 min with 100 µg/ml Nile Red (Biomol, Hamburg, Germany) dissolved in DMSO. SkMC were viewed with a x20 plan objective lens 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.

Marker of mitochondrial function • 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. For this purpose, SkMC were preincubated as indicated and 1 µmol/l JC-1 was added for 30 min under culture conditions. Afterwards, SkMC were washed and analysed using an Infinite M200 plate reader (Tecan, Maennedorf, Switzerland). JC-1 monomers were assessed using excitation/emission wavelengths of 485/530 nm, while J-aggregates were measured using 560/595 nm. As a control, SkMC were incubated with 100 µmol/l CCCP (carbonyl-cyanide m-chlorophenyl hydrazone) for 45 min prior to JC-1 staining.

Immunoblotting • For analysis of protein levels of CD36, FATP4, and OXPHOS as well as for analysis of Akt (Ser473) phosphorylation SkMC whole cell lysates were prepared with icecold lysis buffer containing 50 mmol/l Hepes (pH 7.4), 1% (vol./ vol.) Triton X-100, phosphatase and protease inhibitor cocktail. Western blot analysis was performed as described before (Sell et al., 2009). The signals were visualized and evaluated on a VersaDoc 4000 MP work station (BioRad, Munich, Germany), and analysed by Quantity One analysis software (version 4.6.7).

Quantification of intracellular triglyceride content • A commercial triglyceride quantification kit (BioCat, Heidelberg, Germany) was used to assess triglyceride content. SkMC were incubated as indicated and lysed in a 5% Triton-X100 solution. Lipids were dissolved by heating the lysates to 95°C for 5 min 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.

TLC analysis • Lipids were evaluated as described earlier (Echten-Deckert, 2000). Briefly, lipids were extracted in chloroform/methanol/ water (2:1:0.1, v/v/v) for 48 h at 48°C and separated by thin layer chromatography (TLC) using Silica Gel 60 plates (Merck, Darmstadt, Germany). Diacylglycerols (DAG) were resolved using chloroform/me-

thanol/acetic acid (190:9:1, v/v/v) as developing system. Following development, plates were air-dried, sprayed with 8% (w/v) H_3PO_4 containing 10% (w/v) $CuSO_4$, and charred at 180°C for 10 min. Lipids were identified by their Rf value using authentic lipid samples as references. Individual lipid bands obtained by TLC were evaluated by photodensitometry (Shimadzu, Kyoto, Japan).

Electrical pulse stimulation (EPS) • Differentiated human SkMC were subjected to EPS treatment in six-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 24 h. Electrical stimulation was performed in parallel to CM-incubation for 24 h. Culture medium was changed before the start of the stimulation.

Palmitic acid uptake • PA uptake after 24 h was determined by incubation of pre-treated SkMC with 37 kBq/ml ^{14}C-PA (adjusted to 100 µmol/l with unlabelled PA bound to BSA). Subsequently, SkMC were lysed using 1 mol/l NaOH. Radioactivity of cell lysates was counted in a liquid scintillation counter (Beckman, Munich, Germany).

PA oxidation • In order to determine PA 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 10 mm cover slips in six-well culture dishes, treated and electrically stimulated as described above. Subsequently, cover slips were carefully transferred to 48-well culture dishes. After incubation with CM, PA, and EPS as indicated, culture medium was exchanged and SkMC were cultured in fatty acid-free media for additional 24 h in order to prevent dilution of radioactively labelled PA. Afterwards, 11.1 kBq/well of ^{14}C-PA supplemented with 1 µmol/l L-carnitine were added to SkMC. Culture dishes were incubated for 4 h 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).

Presentation of data and statistic • Data are presented as mean ± SEM. Statistical analysis was carried out by one-way ANOVA (post hoc test Tukey's multiple comparison test). All statistical analyses were performed using Prism5 (GraphPad, La Jolla, CA, USA). A p value of less than 0.05 was considered to be statistically significant. Corresponding significance levels are indicated in the figures.

Results

PA uptake and lipid accumulation in myotubes is enhanced by CM • Twenty-four hour incubation of SkMC with CM induced an increased uptake of ^{14}C-PA measured over a period of 24 h compared with the respective control (1.4 fold, Figure 1(a)). In order to determine the degree of lipid accumulation after incubation with CM, PA and the combination of both, the amount of triglycerides in cell lysates was quantified (Figure 1(b)). While incubation with CM or PA alone did not induce significant changes compared to control, incubating SkMC with the combination of PA and CM elicited a 5.3fold increase of triglycerides stored in the cells.

This apparent lipid accumulation after combined incubation correlated with increased Nile Red stainingof SkMC. Nile Red staining of cells 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-incubation was used as control, after it was verified that BSA-incubated SkMC did not show differences compared to completely untreated SkMC (data not shown). While BSA-incubated SkMC displayed no lipid droplets, only sporadic lipid droplets were visible in very few myotubes after incubation with CM and PA alone (as indicated by arrows in Figure 1(c)). In contrast

to that, co-incubation with PA and CM resulted in a diffuse yellow/green staining covering entire myotubes. However, no clearly defined lipid droplets were visible.

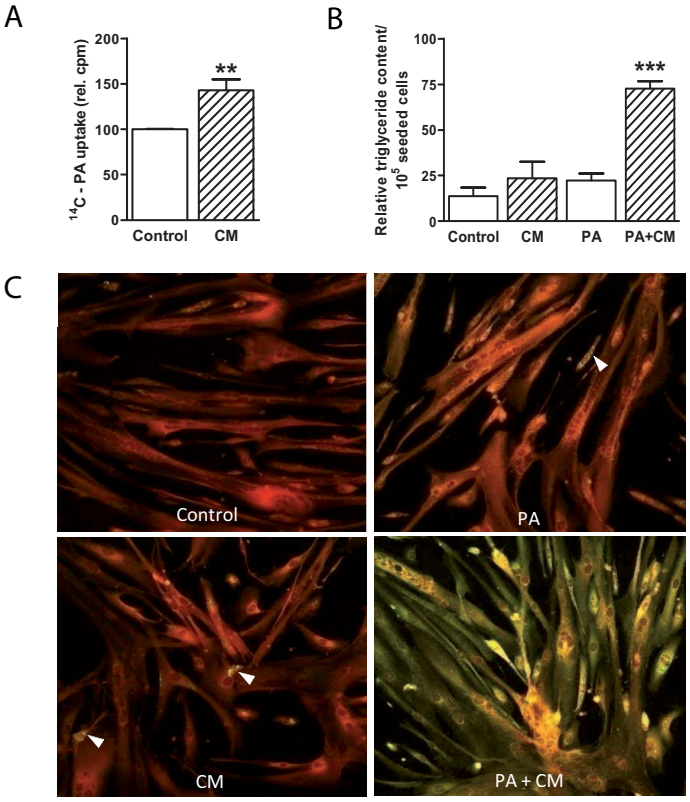


Figure 1. Effect of CM and PA on lipid content and PA uptake. (**a**) SkMC were incubated with CM for 24 h. Subsequently, SkMC were incubated for 24 h with ^{14}C-PA. Values were corrected for nonspecific uptake measured immediately after start of experiment; n = 4, **p < 0.01 vs control. (**b**) SkMC were pre-treated for 6 h with CM, and 100 µmol/l PA was added over night. Cells were lysed and subjected to triglyceride quantification as described in Materials and Methods; n = 3–4, ***p < 0.001 vs control. (**c**) SkMC were preincubated with CM for 6 h, and 100 µmol/l PA was added overnight. Cells were fixed and stained with Nile Red. Representative images are shown. All data are presented as mean ± SEM.

CM affects fatty acid transporter CD36 protein level • The protein level of FATP4 remained unaltered upon CM-incubation (Figure 2(a)). However, incubation of SkMC with CM increased the protein abundance of the fatty acid transporter CD36 more than 1.8 fold (Figure 2(b)). Importantly, heat inactivation of CM resulted in loss of the CM-induced increase of CD36 protein level, indicating a protein-mediated effect (Figure 2(b)). Incubation with PA alone and co-incubation with PA and CM increased CD36 protein level similar to CM alone (Figure 2(c)).

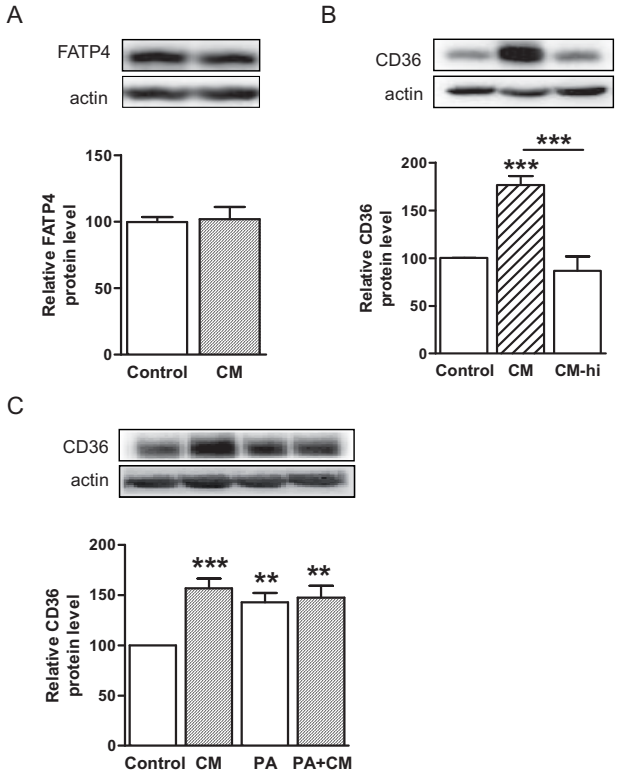


Figure 2. Influence of CM on fatty acid transport proteins. SkMC were incubated for 24 h as indicated. Subsequently, SkMC were lysed and analysed by SDS-PAGE and Western Blot. Representative images of Western Blots for FATP4 (**a**) and CD36 (**b, c**) are shown. All data were normalized to actin and are expressed relative to control cells. Data are presented as mean ± SEM, n = 4–6, ***p < 0.001 vs. control or as indicated, **p < 0.01 vs. control. CM-hi, heat-inactivated CM.

The combination of CM and PA has profound effects on PA oxidation, DAG content and mitochondrial membrane integrity • Since accumulation of intra-myocellular lipids (IMCL) has been linked to decreased skeletal muscle mitochondrial performance, oxidation of ^{14}C-labelled PA was analysed. Oxidation of ^{14}C-PA was not significantly altered in SkMC incubated with CM or low dose of PA alone, while the combination of PA and CM severely decreased ^{14}C-PA oxidation by more than 60% compared to PA and CM incubation

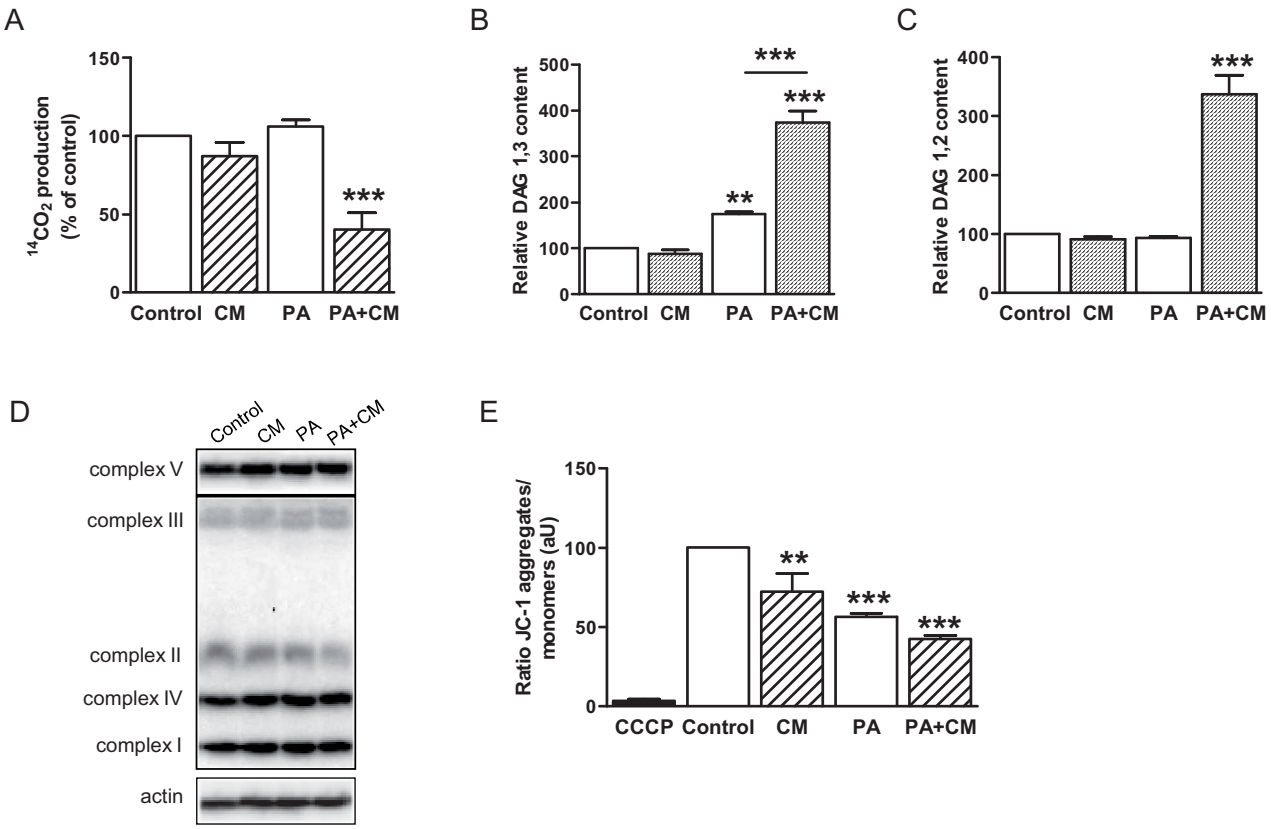


Figure 3. Effect of CM and PA treatment on PA oxidation, generation of DAG metabolites and markers of mitochondrial function. (**a**) SkMC were pre-incubated with CM for 6 h, then 100 µmol/l PA was added over night. Subsequently, SkMC were incubated with 14C-PA in an oxidation chamber for 4 h. Liberated 14CO2 was trapped and radioactivity was assessed. Values were corrected for non-specific oxidation obtained by immediate media acidification after addition of radioactivity; n = 7, ***p < 0.001 vs. control. (**b, c**) SkMC were pre-incubated with CM for 6 h, then 100 µmol/l PA was added over night. Afterwards, lipids were extracted and DAG 1,3 (**b**) or DAG 1,2 (**c**) content was analysed. For quantification control cells were used as reference and set to 100%; n = 6–7, ***p < 0.001 vs. control or as indicated, **p < 0.01 vs. control. (**d**) SkMC were pre-incubated as described before, lysed and analysed by Western Blot using an OXPHOS antibody cocktail. A representative blot of six independent experiments is shown. (**e**) Pretreated SkMC were stained using JC-1. As a positive control, SkMC were incubated with 100 µmol/l CCCP for 45 min prior to JC-1 staining. Shown is the ratio of J-aggregates to JC-1 monomers. For quantification control cells were used as reference and set to 100%. n ≥ 3, ***p < 0.001 vs. control; **p < 0.01 vs. control. All data are presented as mean ± SEM.

alone (Figure 3(a)). PA oxidation experiments were also performed in SkMC pre-incubated with 300 µmol/l PA (data not shown). Similar to the experimental setting using 100 µmol/l PA, here the most profound reduction (>90%) was observed after incubation with PA and CM, while neither CM nor PA alone significantly reduced ^{14}C-PA oxidation.

TLC analysis revealed a significant increase of lipid metabolite DAG 1,3 by ~70% in SkMC incubated with PA compared with control cells while CM had no effect on DAG 1,3 content (Figure 3(b)). SkMC incubated with a combination of PA and CM even showed a 3.7-fold increase. Similarly, content of DAG 1,2 was found to be increased 3.4-fold in SkMC co-incubated with PA and CM compared to CM-incubated or control cells (Figure 3(c)). However, PA alone did not affect DAG 1,2 content.

Next, the impact of CM, PA and the combined incubation of PA and CM on mitochondrial function was assessed. Analysing the protein levels of the electron transport chain proteins, complex I-V, showed no alterations by the incubation with CM, PA and the combination of PA and CM (Figure 3(d)). As a marker of mitochondrial integrity, the fluorescent dye JC-1 was used to assess the status of the mitochondrial membrane potential. Analysis revealed that incubation with CM alone reduced the ratio of JC-1 aggre-

gates to monomers by ~28% compared to control cells (Figure 3(e)) pointing towards a disturbed mitochondrial membrane integrity. Incubation of SkMC with PA even augmented the degree of mitochondrial damage (~44%), while the strongest effect on mitochondrial integrity could be observed after co-incubation with PA and CM (~58%) (Figure 3(e)).

Low doses of PA does not impact on insulin signalling • Under control conditions, insulin induced a significant increase of Akt phosphorylation (Ser473) in SkMC. This effect is significantly reduced by ~30% after 24 h incubation of myotubes with CM (Figure 4). Incubation of myotubes with 100 µmol/l PA had no effect on insulin-stimulated Akt phosphorylation while the effect of co-incubation of with PA and CM was not different from CM alone.

Negative effects of combined incubation with CM and PA cannot be reversed by contractile activity • We have recently established and validated a unique in vitro model of SkMC contraction by subjecting myotubes to electrical pulse

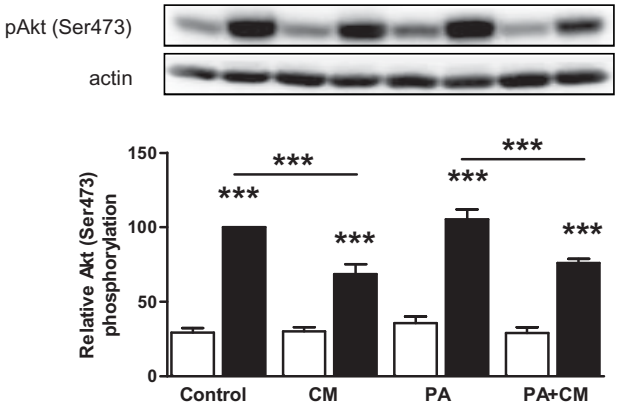
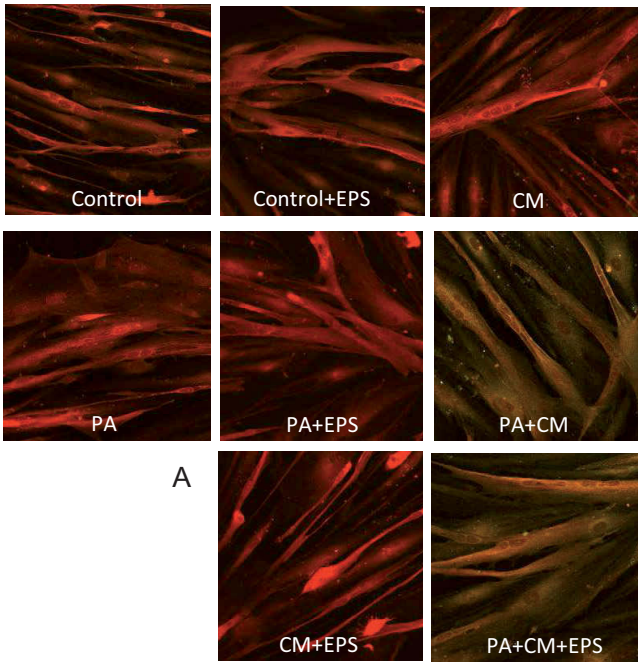
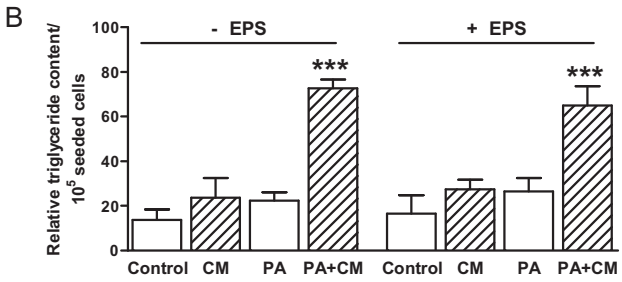


Figure 4. Effect of CM and PA on Akt phosphorylation. SkMC were incubated with CM and PA for 24 h, respectively or pre- incubated with CM for 6 h with subsequent addition of 100 µmol/l PA over night. After 10 min stimulation with 100 nmol/l insulin, total cell lysates were generated, resolved by SDS-Page and immunoblotted with phospho-specific Akt (Ser473) antibody. All data were normalized to the level of actin and are expressed relative to insulin-stimulated control values. Data are presented as mean ± SEM, n = 8, ***p < 0.001 vs. control and designated data, respectively.





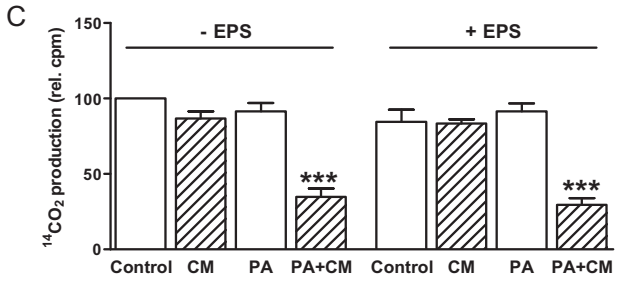


Figure 5. Effect of EPS on SkMC lipid accumulation and PA oxidation. SkMC were pre-incubated with CM for 6 h, and 100 µmol/l PA was added over night. Cells were simultaneously EPS-stimulated (1 Hz, 2 ms, 11.5 V). (**a**) SkMC were fixed using picric acid and stained using Nile Red. Representative images of treated cells are shown. (**b**) Cells were lysed and subjected to triglyceride quantification; n = 3–4, ***p < 0.001 vs control. (**c**) SkMC were incubated with ^{14}C-PA in an oxidation chamber for 4 h. 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; n ≥ 5, ***p < 0.001 vs. control. All data are presented as mean ± SEM.

stimulation (EPS). This novel EPS-technique closely mimics the effects of physical exercise as it activates AMPK, induces secretion of known exercise-stimulated myokines such as IL-6 and VEGF, and improves insulin-stimulated glucose uptake (Lambernd et al., 2012). In order to assess the influence of contraction on IMCL accumulation in SkMC, Nile Red staining was conducted. EPS-treatment of SkMC in parallel to CM- or PA-incubation did not lead to changes of the amount of lipid droplets compared to the respective controls without contraction (Figure 5(a)). Furthermore, the diffuse yellow/green staining covering entire myotubes after incubation of SkMC with PA and CM was not affected by simultaneous application of EPS. Similar results were obtained by analysing the intracellular triglyceride content. As shown in Figure 5(b), EPS stimulation of SkMC did not change the intracellular triglyceride content in control, CM- or PA-incubated and PA and CM co-incubated cells.

Next, we investigated the impact of EPS-induced contraction on PA oxidation. Contractile activity of myotubes did not alter PA oxidation in control, CM- and PA-incubated cells, respectively (Figure 5(c)). In addition, the severe impairment of PA oxidation in response to co-incubation with PA and CM by > 60% was not influenced by EPS.

Discussion

Numerous studies have demonstrated the correlation of fatty acids as well as adipokines with impaired muscle metabolism, however, commonly single adipokines and high fatty acid concentrations have been investigated as isolated factors (Chavez et al., 2003; Famulla et al., 2010; Jove et al., 2005; Sell et al., 2006b, 2009). To more closely simulate their physiological interplay under controlled in vitro conditions, in this study we used a model of incubating human skeletal muscle cells with CM derived from human adipocytes in combination with a low concentration of PA. In this context, the synergistic impact of adipokines and PA 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 fatty acids from CM (unpublished data) comprises the advantage of precisely assessing the effects of PA.

The incubation of SkMC with CM induced a 1.5 fold increased PA uptake indicating an enhanced ability of PA to enter the cells. The investigation of two fatty acid transport proteins, namely CD36 and FATP4, revealed a selective up-regulation of CD36 protein abundance by CM as well as PA. Heat-inactivation of CM prevented this effect thus indicating that possible causative agents are protein factors. Several factors were already described to up-regulate CD36 in different cell types such as interleukin-4 in monocytes (Yesner et al., 1996), and adiponectin in L6 myotubes (Fang et al., 2009). Another factor potentially involved in up-regulating CD36 is thrombospondin-1 (TSP1). Its over-expression in the carcinoma cell line A431 has been shown to increase CD36 abundance (Streit et al., 1999) and TSP1 was found among the 263 proteins we recently identified in CM (Lehr et al., 2011). Also, adiponectin is present in CM (Famulla et al., 2010) while IL-4 could not be detected (unpublished data). However, since CM is a very complex mixture, we presume that most likely a combination of several partially yet undefined factors may be responsible for the CM-induced effect on CD36. In this study we analysed CD36 protein in whole cell lysates and did not differentiate between intracellular and plasma membrane CD36 pools. However, increased PA uptake in absence of increased protein levels of another important fatty acid transporter in SkMC (FATP4) indicates that increased amounts of functional CD36 have to be available at the plasma membrane after incubation with CM to mediate the above described PA uptake. Our data suggest that this CM-induced increase in CD36-mediated PA uptake might play an important role in the accumulation of triglycerides in SkMC after co-incubation with PA and CM. It has been discussed previously that CD36 may contribute to intracellular lipid accumulation (Aguer et al., 2011; Bonen et al., 2004; Hegarty et al., 2002) and might resume a role as a mediator of lipotoxicity (Silverstein et al., 2009). Thus it has been shown in animal models that ablation of CD36-mediated lipid uptake in muscle or liver

prevents lipotoxicity (Koonen et al., 2007a; Koonen et al., 2007b; Yang et al., 2007), while specific induction of CD36 in liver contributed to steatosis (Zhou et al., 2008). Although there is only a modest increase in PA uptake, there is severe accumulation of triglycerides in SkMC co-incubation with PA and CM. However, additional cellular processes like reduced PA oxidation are likely to contribute to further lipid accumulation.

As a number of studies have demonstrated an association of IMCL accumulation and impaired muscle function, we aimed to investigate the consequences of the observed lipid accumulation after co-incubation with PA and CM. While CM alone impaired insulin-stimulated activation of Akt as observed in our earlier studies (Dietze-Schroeder et al., 2005; Sell et al., 2008), PA had no effect and did not augment the impairment of Akt phosphorylation in combination with CM indicating that the accumulation of lipids per se play only a minor role in the induction of insulin resistance at the level of Akt phosphorylation. Our data seem to be in contrast with several other studies showing an induction of insulin resistance by PA treatment as shown by enhanced IRS (Ser307) phosphorylation, decreased insulin-stimulated Akt phosphorylation and glucose uptake (Chavez et al., 2003; Coll et al., 2008; Hirabara et al., 2010; Jove et al., 2005). However, in these studies either C2C12 or L6 myotubes, higher PA concentrations (0.5–1 mmol/l) and/or longer incubation times were used, thus partly explaining the different results compared with our study. Notably, in our hands, incubation of human SkMC with PA concentrations above 0.5 mmol/l led to cytotoxic effects (unpublished observations). In spite of absent effects on insulin signalling, we found a profound decrease of PA oxidation after co-application of PA and CM, while both settings alone had no significant effects. Since muscle fatty acid oxidation rates strongly rely on functionally intact and active mitochondria, the observed reductions in PA oxidation could possibly be due to decreased mitochondrial function. While the protein abundance of OXPHOS complexes were not found to be affected by any incubation we observed alterations of the mitochondrial integrity, as measured with JC-1. However, as the JC-1 data not exactly match the PA oxidation pattern, it maybe discussed that there might be additional factors such as mitochondrial performance leading to the altered PA oxidation profile. Additionally, other factors essential for intact mitochondrial fatty acid oxidation like activity of proteins or enzymes involved in fatty acid transport or oxidation might be influenced, respectively. Observing a severely reduced $^{14}CO_2$ production might point to an impairment of complete fatty acid β-oxidation. The concept of incomplete β-oxidation has been described recently proposing an imbalance between up-regulation of lipid-induced β-oxidation rates and downstream metabolic pathways such as the tricarboxylic acid cycle and the electron transport chain (Koves et al., 2008; Muoio, 2010). Thus, increased levels of fatty acid-derived acylcarnitine intermediates have been reported in muscle of obese rodents (Koves et al., 2008) and in association with insulin resistance and T2D in humans (Adams et al., 2009; Huffman et al., 2009; Mihalik et al., 2010). However, impaired or insufficient mitochondrial uptake and oxidation of fatty acids results in their alternative use as substrates, generating harmful lipid signalling molecules (Petersen et al., 2006). Indeed, we observed lipid intermediates DAG 1,2 and DAG 1,3 to be increased drastically in SkMC after incubation with PA and CM.

Physical exercise has been demonstrated to be a major regulator of skeletal muscle metabolism, potently influencing mitochondrial function (Hawley, 2009). In this context, several studies have shown that increased physical activity is capable of eliciting a complex set of biological responses, resulting in increased oxidative capacity of the skeletal muscle (Hawley, 2002; Holloszy et al., 1977). Therefore, we asked the question, whether contraction of SkMC induced by EPS could counteract the defects in PA oxidation induced by combined application of PA and CM.

Subjecting SkMC to EPS for 24 h in parallel with PA and CM-incubation did not markedly alter lipid accumulation as assessed by Nile Red staining and triglyceride quantification. More importantly, EPS was not able to prevent the severe reduction of ^{14}C-PA oxidation in this situation. Although it is well described that adaptation of skeletal muscle to exercise stimuli comprises en-

hanced mitochondrial oxidative capacity, it has also been reported that saturated (Sabin et al., 2007; Schmitz-Peiffer et al., 1999) and unsaturated fatty acids (Lee et al., 2006) are differentially stored and metabolized with more detrimental results induced by saturated fatty acids. Thus it may be speculated that diffuse distribution and possibly further metabolism of saturated fatty acids like PA leading to generation of harmful lipid metabolites such as DAG induces profound cellular impairments, which are not preventable by contraction stimuli.

Conclusion

In summary, we demonstrate here for the first time a novel role for adipokines in the pathogenesis of T2D reflected by an increased lipotoxic potential of palmitic acid, 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 free fatty acid levels. Additionally, the results of our study demonstrate that contractile activity is not able to counteract the impairments of fatty acid oxidation induced by combination of adipokines and palmitic acid supporting the notion that saturated fatty acids are more detrimental than unsaturated. Our 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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Declaration of interest

The authors report no conflicts of interest.

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Identification and validation of novel contraction-regulated myokines released from primary human skeletal muscle cells

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Abstract

Proteins secreted by skeletal muscle, so called myokines, have been shown to affect muscle physiology and additionally exert systemic effects on other tissues and organs.

Although recent profiling studies have identified numerous myokines, the amount of overlap from these studies indicates that the secretome of skeletal muscle is still incompletely characterized. One limitation of the models used is the lack of contraction, a central characteristic of muscle cells. Here we aimed to characterize the secretome of primary human myotubes by cytokine antibody arrays and to identify myokines regulated by contraction, which was induced by electrical pulse stimulation (EPS). In this study, we validated the regulation and release of two selected myokines, namely pigment epithelium derived factor (PEDF) and dipeptidyl peptidase 4 (DPP4), which were recently described as adipokines. This study reveals that both factors, DPP4 and PEDF, are secreted by primary human myotubes. PEDF is a contraction-regulated myokine, although PEDF serum levels from healthy young men decrease after 60 min cycling at VO2max of 70 %. Most interestingly, we identified 52 novel myokines which have not been described before to be secreted by skeletal muscle cells. For 48 myokines we show that their release is regulated by contractile activity.

This profiling study of the human skeletal muscle secretome expands the number of myokines, identifies novel contraction-regulated myokines and underlines the overlap between proteins which are adipokines as well as myokines.

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Introduction

Skeletal muscle is the largest organ in the human body and path-breaking work during the last decade has demonstrated that skeletal muscle is an active endocrine organ releasing a host of so-called myokines [1].

Skeletal muscle exhibits profound adaptability in response to environmental influences. Exercise training enhances muscular endurance [2] and strength [3], expends calories, and leads to a decrease in adipose tissue mass. Additionally, it exerts major beneficial effects on the prevention of chronic diseases such as obesity and type 2 diabetes [4]. These systemic effects of

exercise cannot be explained exclusively by the expenditure of calories. It has been suggested that skeletal muscle communicates with other tissues such as liver, adipose tissue, heart, brain, and vasculature by secreting a variety of myokines. Some of these myokines are described to mainly affect muscle physiology in an autocrine fashion, while other myokines additionally exert systemic effects on other tissues and organs. Several myokines are described in the literature to be regulated by contraction, like angiopoietin-related protein 4 [5], fibroblast growth factor 21 [6], interleukin (IL)-6 [7,8], IL-7 [9], IL-15 [10], leukemia inhibitory factor [11], myonectin [12], myostatin [13] and vascular endothelial growth factor [14,15]. For some of the reported myokines, only increased mRNA or protein levels in cell or tissue lysates or biopsies are reported, while alterations in serum level after exercise or increased protein levels in supernatants of cultured myotubes after contraction were not reported. In this study, we define a myokine as a protein for which it is clearly proven to be released by skeletal muscle cells.

Boström et al. recently published that PGC1α over-expression in mouse muscle stimulates increased expression of FNDC5, a membrane protein that is cleaved at the N-terminus thereby releasing a soluble protein, called irisin. This protein acts on white adipose tissue-derived adipocytes in vitro and in vivo to stimulate a broad program of brown-fat-like development [16]. Up to now, IL-6 is the most prominent muscle-derived protein, which was demonstrated to be upregulated in plasma after exercise [8,17,18]. IL-6 increases insulin-stimulated glucose disposal in humans and glucose uptake as well as fatty acid oxidation in vitro in rat myotubes [19]. Infusion of IL-6 in healthy young men identified IL-6 as potent modulator of fat metabolism in humans, increasing fat oxidation and fatty acid (FA) reesterification [20]. Before this observation, IL-6 was described as a cytokine and adipokine. IL-6 is increased in the plasma of obese patients [21,22] and overexpressed in human fat cells from insulin-resistant subjects [23]. Additionally, IL-6 has been shown in vitro to induce insulin resistance in hepatocytes [24], adipocytes [23] and in skeletal muscle cells after treatment with high doses [25]. The current literature mainly describes a negative crosstalk between excess body fat and skeletal muscle [26], while the data of IL-6 and irisin indicate an additional crosstalk from the muscle to the adipose tissue.

Previously, we have published the secretome of primary human adipocytes and found 44 novel adipokines [27]. Among others, pigment epithelium derived factor (PEDF) [28] and dipeptidyl peptidase 4 (DPP4) [29] were described as novel adipokines. Both cytokines are associated with obesity and insulin resistance [28,29]. Interestingly, we also found these two proteins to be secreted by skeletal muscle cells and termed these proteins adipo-myokines.

To gain a broader view, recent efforts have focused on exploring the complete secretome of skeletal muscle by proteomic studies. Using this approach, Yoon et al. have studied the regulation of protein secretion by rat skeletal muscle cells after insulin stimulation [30], Chan et al. and Henningsen et al. have investigated altered regulation of secretome components during differentiation of murine C2C12 cells [31,32]. Hittel et al. have explored the secretome of cultured myotubes derived from extremely obese compared with healthy non-obese women [33]. Further, Norheim and colleagues have published the proteomic identification of secreted proteins from human skeletal muscle cells, but regulation of myokines by exercise was only assessed at the mRNA level [34]. A drawback of all these studies is the use of non-contracting cells, although contraction is a major characteristic of skeletal muscle activating intracellular signaling pathways and metabolic adaption.

Recently, we have established and characterized an *in vitro* model of human skeletal muscle cell contraction. Similarly to exercising skeletal muscle in vivo, electrical pulse stimulation (EPS) induced contractile activity in human myotubes, accompanied by formation of sarcomeres, activation of AMP-activated protein kinase and increased IL-6 secretion [35]. Thus, EPS application to human skeletal muscle cells represents an excellent tool to study the release of myokines induced by muscle contraction under in vitro conditions. Currently, no biochemical technique exists that

can efficiently separate and consistently detect the total protein composition of the cellular secretome, e.g. neither the rodent cell studies [30–32,36,37], nor the human cell studies [33,34] identified myokines belonging to the group of interleukins by a proteomic approach. In order to search for proteins undetected by the proteomics approaches, in this study, we have analyzed the secretome of contracting primary human myotubes by cytokine antibody arrays.

Experimental Procedures

Materials • Reagents for SDS-PAGE were provided by GE Healthcare (Munich, Germany) and by Sigma (Munich, Germany), rotiphorese was supplied by Carl Roth (Karlsruhe, Germany). The following antibodies were used: anti-DPP4 (Abnova, Heidelberg, Germany), anti-PEDF (Millipore, Darmstadt, Germany), anti-tubulin (Calbiochem/Merck Biosciences, Schmalbach, Germany). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG were purchased from Promega (Mannheim, 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 (PromoCell, Heidelberg, Germany) supplied as proliferating myoblasts were cultured as described in our earlier study [38]. Myoblasts of 5 different donors (three males 16, 21, and 47 years old [M16, M21, M47] and two females 33 and 37 years old [W33, W37]), were differentiated to myotubes and used for analysis to take biological variability into account. For an individual experiment, myoblasts were seeded in six-well culture dishes at a density of 10^5 cells/well and cultured in α-modified Eagle's (αMEM)/Ham's F-12 medium containing skeletal muscle cell growth medium supplement pack (PromoCell, Heidelberg, Germany) up to near-confluence. The cells were then differentiated in αMEM containing 2 % horse serum (Gibco, Berlin, Germany) until day 5 of differentiation followed by overnight starvation in αMEM without serum after washing with PBS twice.

Electrical pulse stimulation (EPS) • EPS was applied to fully differentiated myotubes in six-well culture dishes using a C-Dish in combination with a C-Pace pulse generator (C-Pace 100, IonOptix, Milton MA). Myotubes were stimulated with a frequency of 1 Hz, 2 ms pulse duration, an intensity of 11.5 V for 4 to 24 h. Cells were washed twice with PBS after overnight starvation and fresh medium was added directly before stimulation. Conditioned medium (CM) was collected after a period of 24 h.

Immunoblotting • Skeletal muscle cells were treated as indicated and lysed. The immunoblotting procedure was carried out as described before [39]. Signals were visualized on a VersaDOC 4000 MP (Bio-Rad Laboratories, Munich, Germany) and analyzed by Quantity One analysis software (version 4.6.7, Bio-Rad Laboratories).

RNA isolation and quantitative real-time PCR • Total RNA was isolated and reverse transcribed using the RNeasy Mini kit and Omniscript Reverse Transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Gene expression was determined by quantitative real-time PCR using QuantiTect primer assays and SYBR green reagents (Qiagen, Hilden, Germany) with 0.016-20 ng of cDNA on a Step One Plus Cycler (Applied Biosystems, Carlsbad, CA, USA). Expression levels of investigated genes were normalized to actin. Gene expression was analyzed via the $\Delta\Delta C_t$ method.

Cytokine antibody array and ELISA analysis • Primary human myotubes (4 different donors) were subjected to EPS or left unstimulated, and CM was collected over a period of 24 h to assure that differences of low abundant proteins were in the range of array and ELISA sensitivity. Undiluted CMs were then analyzed by RayBio human cytokine antibody arrays (array #5, #9, #10, Raybiotech, Norcross, GA, USA) according to the manufactures instructions and signals were visualized and evaluated on a LUMI Imager (Boehringer, Mannheim, Germany) work station. Only signals that were above the background and detected in the CM of at least three donors were included in the analysis. For further valida-

tion cytokine concentration in CM was determined using DPP4 (R&D Systems) and PEDF ELISA (Biovendor), respectively, both assays used according to the manufacturer's protocol.

Human Study • Eight well-trained healthy lean male volunteers (body mass index 23.1±0.6 kg/m^2, VO_2max 65.5±1.7 ml/min/kg) participated in the study. A baseline blood sample was taken before start of the exercise. Subjects cycled at 70 % VO2max for 60 min and then rested for 2 h. Blood was sampled immediately after the exercise session as well as 30 min and 120 min post exercise. Written informed consent was obtained from all the participants. The study was approved by the Regional Committee for Medical and Health Research Ethics, Region Sør-Øst-Norge, Norway (2011/927b).

Presentation of data and statistics • Data are the means ± SEM. Unpaired two-tailed Student t-test or one-way ANOVA (post hoc test Tukey's 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

Identification of the adipokines DPP4 and PEDF as myokines • In this study, we identified the two known adipokines DDP4 and PEDF to be secreted by skeletal muscle cells. The analyses of myocyte lysates confirmed the expression of PEDF and DPP4 in both human myoblasts and myotubes (Fig. 1A). Myocyte differentiation was confirmed by myosin heavy chain (MHC) expression (Fig. 1A).To study intracellular protein transport and to demonstrate weather the proteins are classically secreted, primary human myotubes were incubated with brefeldin A (BFA) for 24 h. BFA interferes with the transport from the endoplasmatic reticulum to the Golgi apparatus [40]. CM was analyzed for DPP4 by ELISA (Fig. 1B) and for PEDF by Western Blot (Fig. 1C), thereby validating the secretion of these factors from primary human myotubes. While DPP4 secretion was unaffected (Fig. 1B), since it is described to be released by shedding, BFA inhibited the secretion of PEDF (Fig. 1C).

The protein level of DPP4 was analyzed before (day 0 of differentiation) and up to 6 days of differentiation and increased significantly with myogenesis (Fig. 2A). Although secretion did vary between the donors (136.6±26.6 - 780±52.4 pg/ml on day 2 of differentiation and 592.6±176.2 -

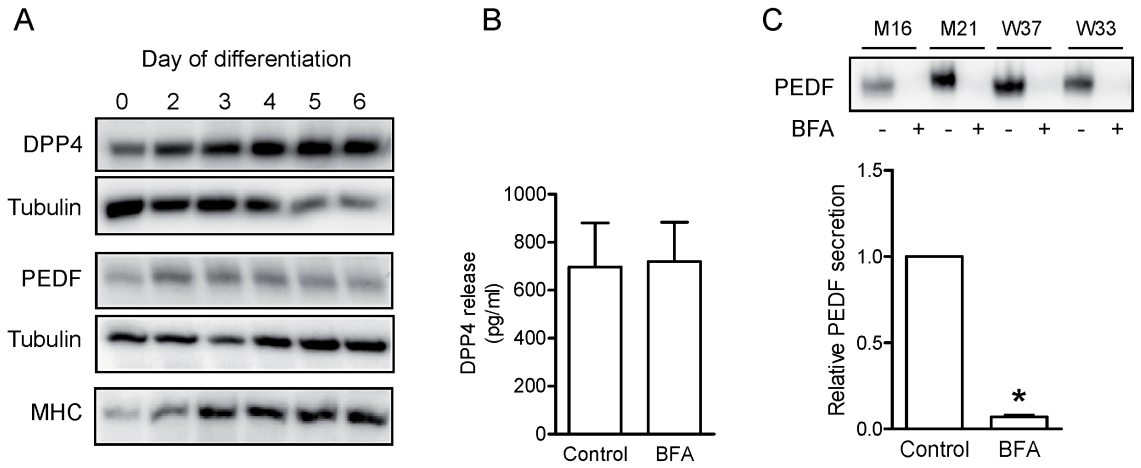

Figure 1. DPP4 and PEDF protein level during differentiation and release by human primary myotubes. A. Primary human myoblasts were differentiated for 0 to 6 days. Protein level of DPP4 and PEDF were analyzed in total cell lysates by SDS-PAGE and Western blot. MHC served as a positive control for differentiation. B+C. To analyze the secretion mechanism, myotubes were incubated with 1 µg/µl BFA for 24 h on day 5 of differentiation. The release of DDP4 was analyzed by ELISA (B, n=10) and the release of PEDF was analyzed by Western blot (C, n=6, *p<0.001).

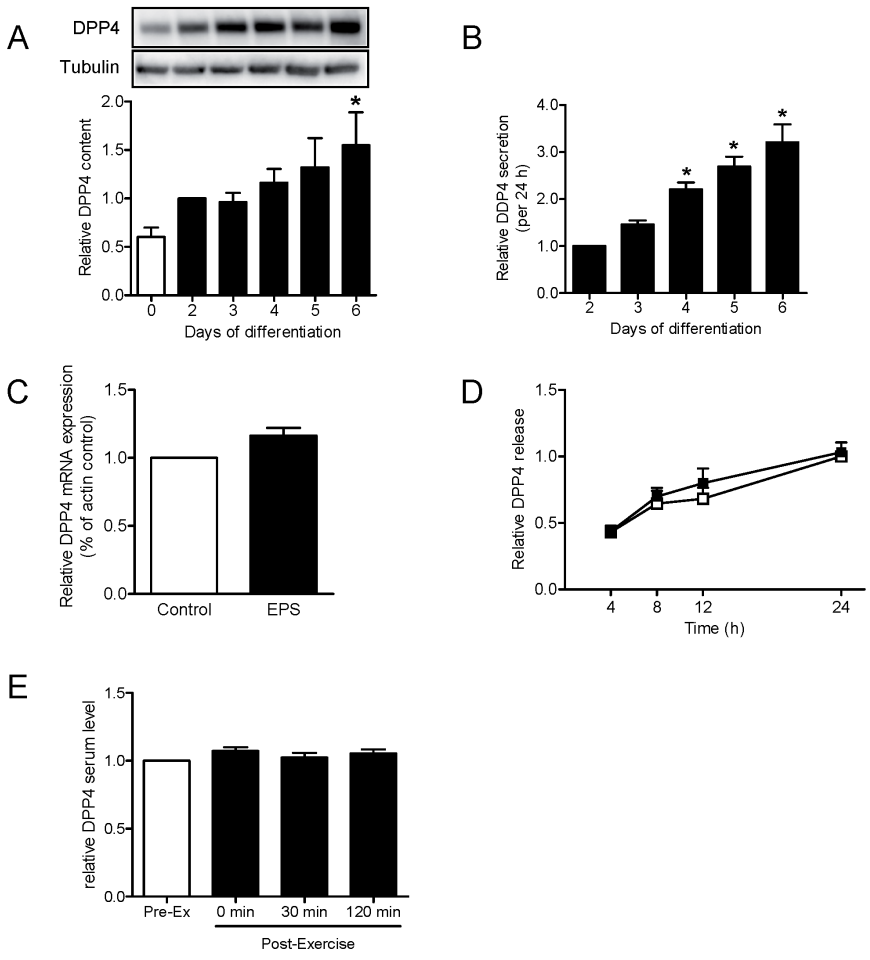

Figure 2. DPP4 protein level and release from myotubes and serum level after acute exercise. A. Primary human myotubes were differentiated and DPP4 protein level during differentiation was analyzed by SDS-PAGE and Western blot. Data were normalized to the protein level of tubulin and are expressed relative to day 2 of differentiation. n=4, *p<0.05 vs. day 2 of differentiation. B. Release of DPP4 during differentiation of myotubes was analyzed by ELISA. Data were normalized to day 2 of differentiation and are expressed relative to day 2 of differentiation. n=9-10, *p<0.001 vs. day 2 of differentiation. C. Relative gene expression of DPP4 after 24 h EPS (1 Hz, 2 ms, 11.5 V) was measured by real-time PCR as described, n=4. D. DPP4 released by human myotubes with and without EPS was measured after indicated time points using ELISA, n≥11. White symbols, control; black symbols, EPS. E. Serum samples were taken before and after 60 *min cycling (70 % VO2max) at indicated time points. Serum DPP4 was analyzed by ELISA, n=8. All data are mean values ± SEM.*

1850.8±112.5 pg/ml on day 6 of differentiation), relative secretion of DPP4 was significantly upregulated during differentiation (Fig. 2B). Contraction induced by EPS did neither increase DPP4 mRNA expression (Fig. 2C) nor DPP4 secretion (Fig. 2D). DPP4 serum levels from healthy young men were not changed after an acute bout of exercise (Fig. 2E).

PEDF mRNA expression and protein level were induced during the first days of myogenesis (Fig. 3A and B) reaching a maximum on day 3 and 4 of differentiation. Secretion of PEDF was not altered during differentiation from day 2 to day 6 (Fig. 3C). Relative mRNA expression was not changed after 24 h EPS (Fig. 3D). Although PEDF secretion from primary human myotubes was significantly enhanced after 8 and 24 h of EPS (Fig. 3E), measurement of PEDF serum level after 60 min cycling revealed a decrease 30 min post exercise (Fig. 3F) that was normalized after 120 min post exercise.

Identification of novel myokines • Using three different cytokine antibody arrays, we analyzed CM obtained from myotubes in order to identify novel proteins secreted by skeletal muscle cells. In total, 179 peptides were analyzed and for 116 of these a positive signal was detected after incubating the membranes with CM obtained from at least three different donors (Fig. 4A, Control). Among the identified peptides 52 factors are novel myokines such as brain-derived neurotrophic factor and somatotropin, for which a release by skeletal muscle cells has not been shown so far (Table 1). In addition, the list emphasizes that beside the known myokines IL-6 and IL-15, further interleukins are secreted such as IL-1α, IL-3, IL-16, IL-22, IL-28a, IL-29 and IL-31 (Table 1).

In the case of 40 factors, no signal was detected (Suppl. Table 1). Surprisingly, angiopoietin-related protein 4 was one of the cytokines which we could not detect in the CM of control and contracted myotubes. This protein was described be-

fore to be secreted by C2C12 cells after treatment with the PPARδ–specific activator GW501516 [41]. In addition, 19 cytokines were only detected in the CM obtained from one or two donors and were not included in the analyses.

Identification of contraction-regulated myokines • Human cytokine antibody array analysis was additionally used as a screening method to detect novel contraction-regulated myokines by directly comparing CM obtained from control and EPS-treated myotubes (Fig. 4A). IL-6, IL-7, IL-8, and VEGF were found to be increased in CM of EPS-treated cells compared to control (Fig. 4B). The release of these cytokines is known to be contraction-regulated and served as positive control. To validate the antibody array analysis, the increase of IL-6 and VEGF secretion was additionally determined by ELISA data and showed comparable results (Fig. 4C).

In addition to the four positive controls, 44 factors were significantly regulated by contraction of skeletal muscle cells (Table 2). Fifteen of these were upregulated by EPS more than 1.5 fold such as stromelysin-2 (2.12±0.35), growth-regulated alpha protein (1.88±0.91) and beta-2-microglobulin (1.81±0.54). Furthermore, 18 of the contraction-regulated myokines have not been described before to be secreted by skeletal muscle cells. Another 44 myokines were not regulated by

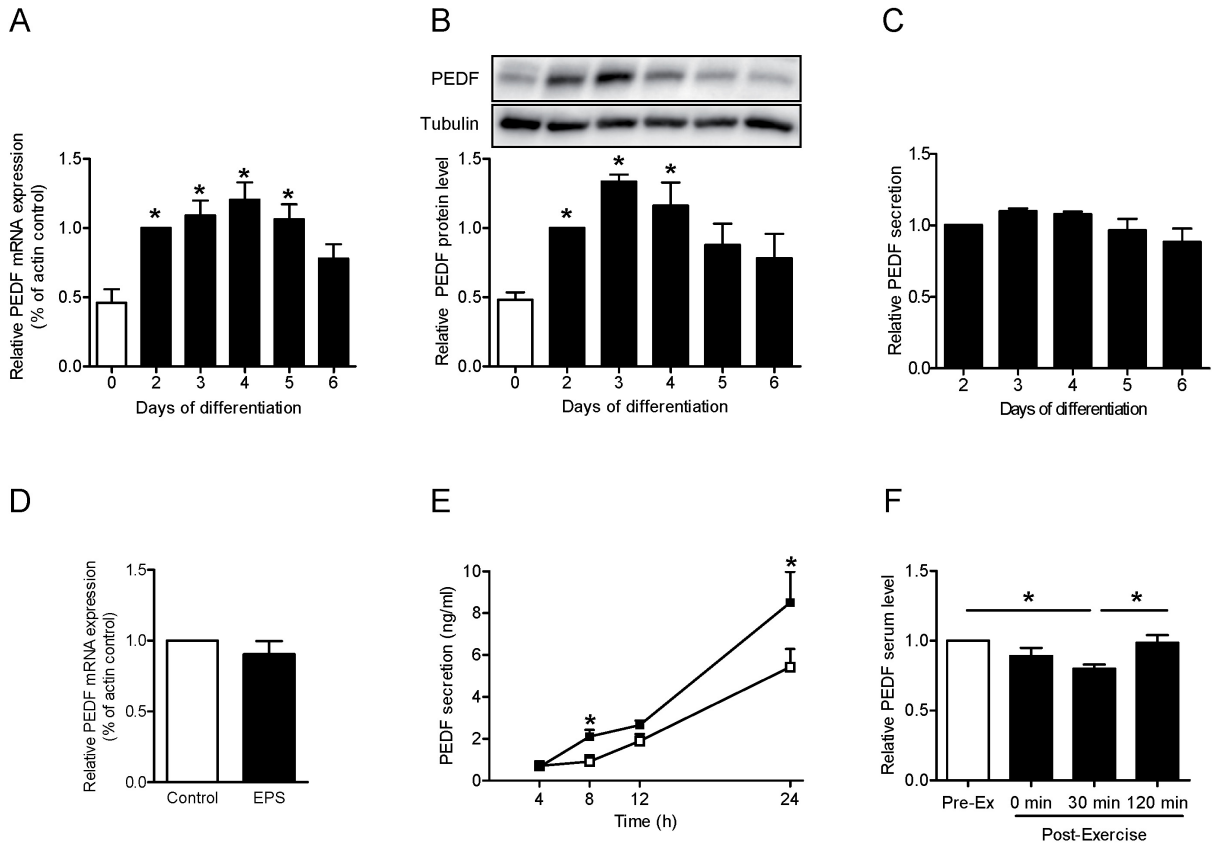

Figure 3. PEDF expression, protein level, and release in myotubes and serum concentration after acute exercise. A. Relative gene expression of PEDF during differentiation of myotubes was measured by real-time PCR as described and is expressed relative to day 2 of differentiation, n=3-4, *p<0.05 vs. day 0 of differentiation. B. PEDF protein level of primary human myotubes was analyzed during differentiation by SDS-PAGE and Western blot. Data were normalized to the protein level of tubulin and are expressed relative to day 2 of differentiation. n=5-6, *p<0.05 vs. day 0 of differentiation. C. Secretion of PEDF during differentiation of myotubes was analyzed by ELISA. Data are normalized to day 2 of differentiation, n=5. D. Relative gene expression of PEDF after 24 h EPS (1 Hz, 2 ms, 11.5V) was measured by real-time PCR as described, n=4. E. PEDF secretion of human myotubes with and without EPS was measured after indicated time points by ELISA, n=3-4, *p<0.05 vs. control. White symbols, control; black symbols, EPS. F. Serum samples were taken before and after 60 min cycling (70 % VO2max) at indicated time points and PEDF level was analyzed by ELISA, n=8, *p<0.05. All data are mean values ± SEM.

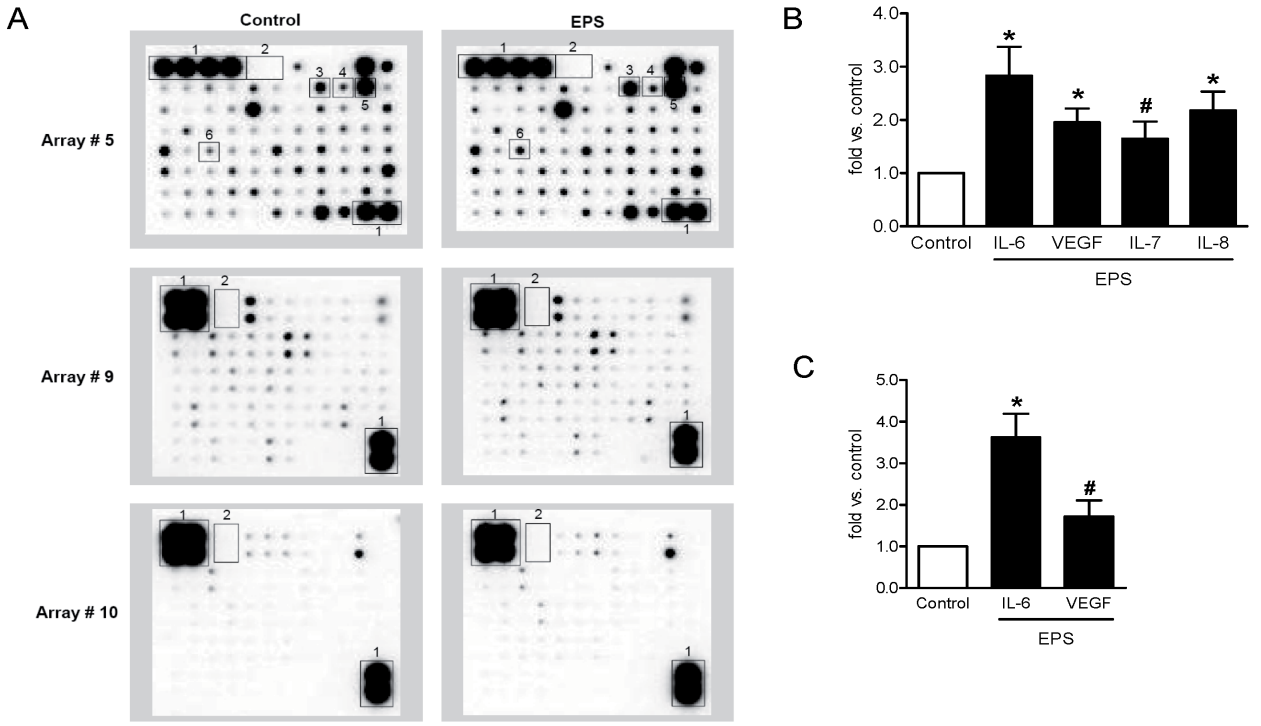

Figure 4. Assessment of contraction-regulated myokines by cytokine antibody arrays. CM of control and EPS-treated cells were collected after 24 h and analyzed as described in Material and Methods. A. Cytokine antibody array membranes #5, #9 and #10 after incubation with CM of control and EPS-treated cells are shown. The encircled areas reflect spots corresponding to the myokines shown in Fig.4B. 1=positive control; 2=negative control; 3=IL-6; 4=IL-7; 5=IL-8; 6=VEGF. B. Known contraction-regulated myokines were analyzed by cytokine antibody arrays and quantified as positive controls. IL, interleukin; VEGF, vascular endothelial growth factor, n=4, *p<0.01 vs. control, #p<0.05 vs. control. C. IL-6 and VEGF protein concentration were additionally analyzed by ELISA in CM of control and EPS-treated cells. IL-6, n=6, *p<0.01. VEGF, n=4, #p<0.05. White bars, control; black bars, EPS.

contraction (Suppl. Table 2). For three factors we observed a significant downregulation in the CM of contracted vs. non-contracted myotubes. Surprisingly, one of these proteins is leukemia inhibitory factor (LIF) which was described before to be upregulated after contraction. In our study we found a reduction of LIF by 14 %.

Discussion

Detailed characterization of the human skeletal muscle secretome is essential to understand the beneficial effect of exercise in the context of chronic diseases. Therefore, this study was focused on the identification of myokines released by contracting myotubes.

To consistently detect the total protein composition of the cellular secretome, it is important to combine various technical approaches, since no biochemical technique can efficiently detect all proteins [42]. Although hundreds of secreted proteins were already published by proteomics approaches [9,14,30–32,38,43–64], our cytokine array approach has identified 52 additional proteins, which we consider as novel myokines secreted by human skeletal myotubes. For several of these factors such as brain derived neurotrophic factor, C-C motif chemokine 4, IFNγ, neurotrophin-3 and -4, and VEGF-C, mRNA expression and/or protein abundance in skeletal muscle tissue or myotubes have been reported, however a clear prove of their release from skeletal muscle cells was not shown. In the case of oncostatin M, indirect evidence of its secretion by myotubes was provided by Hojman et al., however direct assessment of oncostatin M in the supernatant of myotubes is missing [65]. With the cytokine array approach, we could now clearly identify these proteins to be secreted from human skeletal muscle cells.

Table 1. Identification of novel myokines by cytokine antibody arrays. CM of myotubes was collected after 24 h and analysed as described using three different cytokine antibody arrays

Swissp rot accession	Protein name	Swiss prot accession	Protein name
Q15109	Advanced glycosylation end product-specific receptor	Q14005	Interleukin-16
P23560	Brain-derived neurotrophic factor	Q9GZX6	Interleukin-22
P22362	C-C motif chemokine 1	Q8IZJ0	Interleukin-28A
Q16663	C-C motif chemokine 15	Q8IU54	Interleukin-29
Q92583	C-C motif chemokine 17	P08700	Interleukin-3
P55774	C-C motif chemokine 18	Q6EBC2	Interleukin-31
O00585	C-C motif chemokine 21	P21781	Keratinocyte growth factor
P55773	C-C motif chemokine 23	P21583	Kit ligand
O00175	C-C motif chemokine 24	P01229	Lutropin subunit beta
Q9Y258	C-C motif chemokine 26	P01374	Lymphotoxin-alpha
P13236	C-C motif chemokine 4	P09237	Matrilysin
P01233	Choriogonadotropin subunit beta	Q29983	MHC class I polypeptide-related sequence A
P00746	Complement factor D	Q29980	MHC class I polypeptide-related sequence B
Q07325	C-X-C motif chemokine 9	P22894	Neutrophil collagenase
O43927	C-X-C motif chemokine 13	P20783	Neurotrophin-3
O75078	Disintegrin and metalloproteinase domain-containing protein 11	P34130	Neurotrophin-4
P78536	Disintegrin and metalloproteinase domain-containing protein 17	P13725	Oncostatin M
P08620	Fibroblast growth factor 4	Q99075	Proheparin-binding EGF-like growth factor (HB-EGF)
P10767	Fibroblast growth factor 6	Q9Y336	Sialic acid-binding Ig-like lectin 9
P31371	Fibroblast growth factor 9	P01241	Somatotropin
P49771	Fms-related tyrosine kinase 3 ligand	P40225	Thrombopoietin
P80162	Granulocyte chemotactic protein 2	P01222	Thyrotropin subunit beta
Q13651	Interleukin-10 receptor subunit alpha	O43557	Tumor necrosis factor ligand superfamily member 14
P08833	Insulin-like growth factor-binding protein 1	O14763	Tumor necrosis factor receptor superfamily member 10B
P01579	Interferon gamma	P28908	Tumor necrosis factor receptor superfamily member 8
P01583	Interleukin-1 alpha	P49767	Vascular endothelial growth factor C

Secretome analysis showed that the profile of proteins secreted by skeletal muscle changes in response to the treatment with insulin [30], TNFα [37] and during myogenesis [31,32,36]. Skeletal muscle is highly adaptable and physical activity exerts a highly complex physiological stimulus triggering multiple biochemical and biophysical aspects of cellular function. Additionally, we found that contraction is another stimulus changing the secretome of myotubes. Overall, our cytokine antibody array analysis revealed that the release of 45 myokines is regulated by contraction. Among these factors, 18 are described as myokines for the first time.

Norheim et al. described the proteomic identification of 236 proteins secreted from non-contracting human skeletal muscle cells and validated the expression of candidates in response to strength training [34]. Using computational analyses the authors distinguished between proteins targeted for export and proteins with specific intracellular retention signals by SignalP and SecretomeP and shortened the list of secreted proteins to 128. To validate expression and activity of secreted proteins on the gene level, Norheim et al. used two additional analyses, a comparison with Human Genome Expression Profile databases

Table 2. Identification of contraction-regulated myokines using cytokine antibody arrays. CM of control and EPS-treated myotubes were collected after 24 h and analysed as described. Novel myokines are in bold text. Data are presented as mean fold vs. control (± SEM), n=3-4, *p<0.01 vs. control, #p<0.05 vs. control (t-test).

Swissprot accession	Protein Name	Fold vs. Control
P05231	Interleukin-6	2.83 (±0.54)*
P10145	Interleukin-8	2.18(±0.35)*
P09328	Stromelysin-2	2.12 (±0.18)*
P15692	Vascular endothelial growth factor A	1.96 (±0.26)*
P09341	Growth-regulated alpha protein	1.88 (±0.45)#
P61769	Beta-2-microglobulin	1.81 (±0.28)*
P05121	Plasminogen activator inhibitor 1	1.73 (±0.38)#
Q9UBP4	Dickkopf-related protein 3	1.68 (±0.15)*
P13232	IL-7	1.65 (±0.32)*
Q29983	**MHC class I polypeptide-related sequence A**	**1.65 (±0.24)***
P05113	Interleukin-5	1.61 (±0.39)#
P01233	**Choriogonadotropin subunit beta**	**1.59 (±0.16)***
Q99075	**Heparin-binding EGF-like growth factor**	**1.56 (±0.16)***
P13500	C-C motif chemokine 2	1.55 (±0.33)#
O00585	**C-C motif chemokine 21**	1.53 (±0.20)*
P08253	72 kDa type IV collagenase	1.52 (±0.16)*
P09958	Furin	1.51 (±0.09)*
P01137	Transforming growth factor beta-1	1.50 (±0.18)*
Q13651	**Interleukin-10 receptor subunit alpha**	**1.39 (±0.06)***
P00746	**Complement factor D**	**1.38 (±0.17)#**
Q02297	Neuregulin-1	1.37 (±0.16)#
P07288	Prostate-specific antigen	1.36 (±0.21)#
Q29980	**MHC class I polypeptide-related sequence B**	**1.36 (±0.13)***
Q92823	Neuronal cell adhesion molecule	1.34 (±0.12)*
P01241	**Somatotropin**	**1.33 (±0.08)***
O14763	**Tumor necrosis factor receptor superfamily member 10B**	**1.33 (±0.13)***
P14543	Nidogen-1	1.32 (±0.15)#
P40225	**Thrombopoietin**	**1.31 (±0.17)#**
P50895	Basal cell adhesion molecule	1.30 (±0.06)*
P22894	**Neutrophil collagenase**	**1.29 (±0.13)#**
P60568	Interleukin-2	1.29 (±0.12)*
P01127	Platelet-derived growth factor subunit B	1.27 (±0.10)*
P02792, P02794	Ferritin (light chain, heavy chain)	1.24 (±0.09)#
P09237	**Matrilysin**	**1.24 (±0.09)#**
P28908	**Tumor necrosis factor receptor superfamily member 8**	**1.22 (±0.07)***
P17936	Insulin-like growth factor-binding protein 3	1.21 (±0.07)*
Q16663	**C-C motif chemokine 15**	**1.21 (±0.10)#**
P01229	**Lutropin subunit beta**	**1.20 (±0.03)***
P14210	Hepatocyte growth factor	1.20 (±0.06)*
P40933	Interleukin-15	1.19 (±0.08)*
P21781	**Keratinocyte growth factor**	**1.17 (±0.08)#**
P51671	Eotaxin	1.17 (±0.05)*
P22362	**C-C motif chemokine 1**	**1.16 (±0.07)***
P10767	**Fibroblast Growth Factor 6**	**1.14 (±0.04)***
Q6EBC2	**Interleukin-31**	**1.12 (±0.05)***
P15018	Leukemia inhibitory factor	0.86 (±0.06)*
P80162	**Granulocyte chemotactic protein 2**	**0.77 (±0.08)***
P34130	**Neurotrophin-4**	**0.76 (±0.06)***

together with a published mRNA-based reconstruction of the human skeletal muscle secretome [64]. By this approach, the number of secreted proteins was diminished to 18 classically secreted proteins. This strategy ensures that proteins are secreted by skeletal muscle cells and it narrows down the number of clearly, high abundant targets. However, this strategy might imply the loss of physiological important targets. The mRNA-based reconstruction of the muscle secretome included proteins putatively secreted based on the presence of a predicted signal peptide and the absence of predicted transmembrane regions [64]. This excludes all proteins which are secreted by shedding, the proteolysis of ectodomains of membrane proteins, like the novel myokine irisin [16] and DPP4 [66].

Validation of novel myokines • One myokine we have analyzed in this study is the exoprotease PEDF, which is a 50 kDa secreted glycoprotein that belongs to the non-inhibitory serpin group [67]. PEDF was initially purified from conditioned media of human pigment epithelial cells of the retina and identified as a differentiating factor for retinoblastoma cells [68,69]. PEDF serum levels are increased in subjects with metabolic syndrome [70] and in type 2 diabetic patients [71]. Insulin resistance is associated with elevated PEDF serum levels in morbidly obese patients [72], while serum PEDF was declined significantly after weight loss in individual patients [72,73]. All these data indicate that PEDF secreted from adipose tissue is associated with the metabolic syndrome. Despite these data, we found that an acute bout of exercise decreased PEDF serum levels, which was just recently described by Oberbach et al. as well [74]. Nevertheless, PEDF was detected as myokine by us and other secretome studies [32,34,64]. Most interestingly, we found that PEDF secretion of primary human myotubes is increased after contraction induced by EPS, although mRNA level was not changed. Norheim et al. showed that post-exercise to strength training transcription of muscle PEDF was increased in humans [34], indicating that the upregulation of PEDF in response to exercise is rather a long-term effect. A query using BioGPS, a gene annotation portal, indicates that PEDF is mainly expressed in adipose tissue, liver and retina [75]. PEDF is one of the most abundant proteins of primary adipocytes, and myotubes secrete PEDF at significantly lower concentrations compared to preadipocytes and adipocytes [28]. Thus, we assume that serum PEDF levels mainly originate from adipose tissue, and PEDF secreted from skeletal myotubes upon contraction acts rather in an autocrine/paracrine manner within the muscle.

The second validated myokine is DPP4, a cell surface type II membrane glycoprotein which is ubiquitously expressed. DPP4 cleaves N-terminal dipeptides of polypeptides like glucagon-like peptide 1 (GLP1) and glucose dependent insulinotropic polypeptide (GIP), two post-prandial activated incretins [76]. Insulin secretion is enhanced and glucose tolerance is improved in mice lacking DPP4 [77]. Therefore, DPP4 has gained considerable interest as a therapeutic target, and a variety of DPP4-inhibitors which enhance glucose-dependent insulin secretion from pancreatic ß-cells are now in clinical use as anti-diabetic drugs [78].

Substantial DPP4 activity is also found in plasma because of a soluble form of DPP4 lacking the cytoplasmic tail and the transmembrane region of this protein. BioGPS query revealed that DPP4 is predominantly expressed in the kidney, smooth muscle cells, cardiac myocytes, prostate and small intestine. However, the major source of circulating DPP4 and its regulation remain unknown. By using adipose tissue explants from lean and obese subjects, Lamers et al. observed an increase in DPP4 serum levels that strongly correlated with adipocyte volume and parameters of the metabolic syndrome, while DPP4 serum levels decreased to the lean level after weight reduction [29,66]. Since obesity is correlated with a low grade systemic whole body inflammation, we tested inflammatory cytokines to influence DPP4 secretion from myotubes. DPP4 secretion was not altered by inflammatory cytokines like TNFα, MCP-1, IFNγ and IL-6 as well as insulin (S. Raschke, unpublished observation). Additionally, contractile activity of myotubes did not change DPP4 secretion over 24 h. We could confirm that DPP4 is not classically secreted, since BFA did not

change soluble DPP4 concentration in the CM of myotubes. DPP4 function of the soluble form remains poorly understood, and it is unknown if the process of DPP4 release from cell membranes is regulated or not, and this should be addressed by future studies.

Contraction-regulated myokines • Most interestingly, we compared the CM of control and contracting myotubes by cytokine antibody array analysis. Among others, C-C motif chemokine 2 (MCP-1) and somatotropin were identified as contraction-regulated myokines.

MCP-1 resembles IL-6, which was longtime only recognized as inflammatory cytokine and then found to be an exercise factor with a positive impact on muscle physiology [79]. MCP-1 is a chemokine and member of the small inducible cytokine family and plays a crucial role in the recruitment of monocytes and T lymphocytes into tissues [80,81]. It is produced in primary adipocytes among other cell types and associated with obesity [82]. MCP-1-induced macrophage infiltration in adipose tissue leads to a chronic state of low-grade inflammation [83], which is linked to insulin resistance. Additionally, in vitro data demonstrate this factor has the ability to induce insulin resistance in adipocytes and skeletal muscle cells [84]. Here, we describe that MCP-1 is a contraction-regulated myokine. Increased MCP-1 mRNA expression in skeletal muscle was reported by Hubal et al. after a repeated eccentric exercise bout [85]. Garcia et al. showed an increase of circulating MCP-1 levels in healthy sedentary women after a single session of exercise [86]. In addition, a study using cultured human skeletal muscle cells revealed that mechanical strain enhanced the release of MCP-1 by these cells [45]. Future studies have to further characterize the effects of exercise on systemic and muscle MCP-1 and its potential autocrine/paracrine effects on skeletal muscle that may be related to adaptation processes.

Somatotropin is a pleiotropic peptide hormone with an important role in the regulation of metabolism via stimulation of lipid mobilization and oxidation [87]. It is well known that physical exercise increases somatotropin level in healthy subjects and there is also evidence that the acute somatotropin response to exercise is important in regulating fatty acid availability in the post exercise setting [88]. Several studies investigated the anabolic effects of somatotropin on skeletal muscle cells showing an increase of IGF-1 expression [89], protein synthesis [90], and the size of differentiated myotubes [91]. However, to the best of our knowledge we are the first to show that somatotropin is secreted by myotubes and regulated by contraction.

Adipo-Myokines • This study now revealed that there is a considerable overlap between the adipokines and myokines. A considerable number of secreted proteins from skeletal muscle are also secreted by adipocytes. We defined these proteins as adipo-myokines. Among others, DPP4 [29], PEDF [28] as well as MCP-1 [84], decorin [92] and PAI-1 [93] are described to be associated with conditions of obesity, insulin resistance and diabetes. Therefore, up to now, these cytokines were described as adipokines in the literature. Myokines itself might not only have autocrine and/or paracrine effects in the tissue of origin, several of them might also be distributed by the circulation and affect other tissues in an endocrine manner. Thereby, these adipo-myokines might participate in a bi-directional crosstalk between skeletal muscle and adipose tissue. The physiological role of adipo-myokines may vary depending whether the cytokines act in an autocrine and endocrine manner as well as in a chronic or acute manner. In healthy, normal weight subjects skeletal muscle is the largest tissue in the human body, accounting for 40-50 % of total human body mass, while body fat accounts for 20-35 %. In obese subjects the percentage of total body fat increases to 40-60 % resulting in an increased secretion of pro-inflammatory adipokines, while the percentage of skeletal muscle is decreased and so might the myokine serum level. The aim of future studies will be to identify adipo-myokines that are detectable in the circulation, to determine their origin and to analyze their role in the crosstalk between skeletal muscle and adipose tissue in the obese state and after exercise.

The present study contributes to the understanding of the endocrine function of human myotubes. Secretory proteins are part of a complex physiological network, and they exert different effects under various environmental conditions. Therefore, our current knowledge of contraction-regulated secretory proteins from skeletal muscle and their roles in distinct signaling pathways according to the organ has to be determined in future studies. For novel myokines presented in this study, additional basic research and clinical studies will provide a further understanding in exercise metabolism.

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Declaration of interest

The authors report no conflicts of interest.

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Suppl.Table 1. Cytokines that were not detected in CM of hSkMC using cytokine antibody arrays. CM of control and EPS-treated myotubes were collected after 24 h and analysed as described. This list contains all cytokines that did not produce a signal above the background.

Swissprot accession	Protein Name
Q9BY76	Angiopoietin-related protein 4
P00747	Angiostatin
Q16790	Carbonic anhydrase 9
P13688	Carcinoembryonic antigen-related cell adhesion molecule 1
Q16627	C-C motif chemokine 14a
P29965	CD40 Ligand
O94907	Dickkopf-related protein 1
Q9UBT3	Dickkopf-related protein 4
Q92838	Ectodysplasin-A
P16422	Epithelial cell adhesion molecule
P47929	Galectin-7
P09919	Granulocyte colony-stimulating factor
Q96D42	Hepatitis A virus cellular receptor 1
P01308	Insulin
P35225	Interleukin-13
Q9UHF5	Interleukin-17B
Q9P0M4	Interleukin-17C
Q96PD4	Interleukin-17F
Q96F46	Interleukin-17 receptor A
P31994	Low affinity immunoglobulin gamma Fc region receptor II-b
Q9Y5Y7	Lymphatic vessel endothelial hyaluronic acid receptor 1
Q14108	Lysosome membrane protein 2
P13591	Neural cell adhesion molecule 1
P41271	Neuroblastoma suppressor of tumorigenicity 1
Q07326	Phosphatidylinositol-glycan biosynthesis class F protein
P02776	Platelet Factor 4
P01258	Procalcitonin
P58294	Prokineticin-1
P04271	Protein S100-B
P04626	Receptor tyrosine-protein kinase erbB-2
Q9BQR3	Serine protease 27
Q15465	Sonic hedgehog protein N-product
P13385	Teratocarcinoma-derived growth factor 1
Q969D9	Thymic stromal lymphopoietin
P01266	Thyroglobulin
Q9NP99	Triggering receptor expressed on myeloid cells 1
Q02223	Tumor necrosis factor receptor superfamily member 17
P25942	Tumor necrosis factor receptor superfamily member 5
P62979	Ubiquitin-40S ribosomal protein S27a
P19320	Vascular cell adhesion protein 1

Suppl.Table 2. List of Myokines that were not regulated by contraction. CM of control and EPS-treated myotubes were collected after 24 h and analysed as described.

Swissprot accession	Protein Name	Swissprot accession	Protein Name
Q15109	Advanced glycosylation end product-specific receptor	P22301	Interleukin-10
P02771	Alpha Fetoprotein	P29459, P29460	Interleukin-12 p70 (subunit p35, subunit p40)
P03950	Angiogenin	Q14005	Interleukin-16
P23560	Brain-derived neurotrophic factor	P01583	Interleukin-1 alpha
Q99616	C-C motif chemokine 13	P01584	Interleukin-1 beta
Q92583	C-C motif chemokine 17	Q9GZX6	Interleukin-22
P55774	C-C motif chemokine 18	Q8IZJ0	Interleukin-28A
P55773	C-C motif chemokine 23	Q8IU54	Interleukin-29
O00175	C-C motif chemokine 24	P08700	Interleukin-3
Q9Y258	C-C motif chemokine 26	P05112	Interleukin-4
P13236	C-C motif chemokine 4	P21583	Kit ligand
P13501	C-C motif chemokine 5	P41159	Leptin
P80098	C-C motif chemokine 7	P01374	Lymphotoxin-alpha
P80075	C-C motif chemokine 8	P09603	Macrophage colony-stimulating factor 1
P78556	C-C motif chemokine 20	P14174	Macrophage migration inhibitory factor
Q07325	C-X-C motif chemokine 9	P01033	Metalloproteinase inhibitor 1
P02778	C-X-C motif chemokine 10	P16035	Metalloproteinase inhibitor 2
O43927	C-X-C motif chemokine 13	Q99733	Nucleosome assembly protein 1-like 4
P42830	C-X-C motif chemokine 5	P20783	Neurotrophin-3
O75078	Disintegrin and metalloproteinase domain-containing protein 11	P13725	Oncostatin M
P78536	Disintegrin and metalloproteinase domain-containing protein 17	P10451	Osteopontin
P08620	Fibroblast growth factor 4	P01133	Pro-epidermal growth factor
P31371	Fibroblast growth factor 9	Q9HD89	Resistin
P49771	Fms-related tyrosine kinase 3 ligand	Q9Y336	Sialic acid-binding Ig-like lectin 9
P19883	Follistatin	P48061	Stromal cell-derived factor 1
P78423	Fractalkine	P61812	Transforming growth factor beta-2
Q99988	Growth/differentiation factor 15	P10600	Transforming growth factor beta-3
P39905	Glial cell line-derived neurotrophic factor	Q15582	Transforming growth factor-beta-induced protein ig-h3
P04141	Granulocyte-macrophage colony-stimulating factor	P01375	Tumor necrosis factor
P08833	Insulin-like growth factor-binding protein 1	O43557	Tumor necrosis factor ligand superfamily member 14
P18065	Insulin-like growth factor-binding protein 2	O00300	Tumor necrosis factor receptor superfamily member 11B
P22692	Insulin-like growth factor-binding protein 4	Q9HAV5	Tumor necrosis factor receptor superfamily member 27
P05019	Insulin-like growth factor 1	P01222	Thyrotropin subunit beta
P01579	Interferon gamma	P49767	Vascular endothelial growth factor C

Regulation of follistatin-like protein 1 expression and secretion in primary human skeletal muscle cells

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*SWG and SR contributed equally to this study

Abstract

Follistatin-like protein 1 (Fstl1) is a secreted glycoprotein of the follistatin family. Fstl1 is secreted by C2C12 cells, and Akt1 over-expression in skeletal muscle leads to its induction in muscle and increased circulating levels. So far, secretion of Fstl1 by human myotubes and the effect of exercise on its circulating levels has not been investigated. Here, we examined both the regulation of Fstl1 expression and secretion in primary human skeletal muscle cells and the effect of acute exercise on Fstl1 serum concentrations in humans. We show that human myotubes express and secrete Fstl1 in a differentiation-dependent manner. Furthermore, IFNγ and IL-1ß significantly increase Fstl1 secretion. Electrical pulse stimulation (EPS)-induced contractile activity of myotubes did not regulate Fstl1. Interestingly, we observed that 60min cycling increased serum Fstl1 level by 22%. In conclusion, we demonstrate that Fstl1 is expressed and secreted by human myotubes and plasma Fstl1 levels are increased after exercise.

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Introduction

Skeletal muscle has been identified as an endocrine organ due to its capacity to produce and secrete a variety of cytokines (called myokines) and other proteins. Myokines affect muscle physiology and additionally exert systemic effects on other tissues and organs (Pedersen, 2011; Pedersen et al., 2012). It is well accepted that physical activity exerts major beneficial effects on the prevention of chronic diseases. Inactivity is accompanied by an increase in visceral adipose tissue mass, which is associated with a systemic low-grade inflammation in peripheral tissues like skeletal muscle, hence promoting the development of chronic diseases. For instance, regular physical activity induces changes in the secretion of the well-

known myokine interleukin-6 (IL-6), which induces hepatic glucose uptake and lipolysis in adipose tissue (Wolsk et al., 2010). But not only myokines are up-regulated during and after exercise, also other organs and tissues react to physical activity e.g. by an increased cytokine release. It has been shown that follistatin, which is a member of the TGF-ß super-family, is upregulated in plasma during exercise and most likely produced and secreted from the liver (Hansen et al., 2011). To date, myokines have primarily been studied in response to exercise. However, numerous observations suggest that skeletal muscle may also release myokines in response to stress exposure. Stress signals such as TNFα or IL-1ß, which serve as indicators of whole body stress or injury in other cells, act on skeletal muscle and affect the secretion of myokines. For example, Yoon et al. identified 28 TNF-α modulated myokines released from L6 skeletal muscle cells (Yoon et al., 2011).

Follistatin-like 1 (Fstl1), also known as TSC-36, is a secreted glycoprotein and belongs to the follistatin family of proteins (Sumitomo et al., 2000). Follistatin family members bind to TGF-ß super-family proteins and inhibit their functions (Shi et al., 2003). Oshima et al. have shown Fstl1 to be a cardiokine which is upregulated in ischemic injured and hypertrophic hearts of mice (Oshima et al., 2008). In a recent study they have shown that Fstl1 can prevent myocardial injury by inhibiting apoptosis and inflammatory responses through modulation of AMPK- and BMP-4-dependent mechanisms (Ogura et al., 2012). Therefore, they suggested Fstl1 as a novel therapeutic target for acute coronary syndrome.

In this study, we focused on the regulation and characterisation of Fstl1 in primary human skeletal muscle cells. We show here that Fstl1 is expressed and secreted by human skeletal muscle cells. Furthermore, we have investigated the effect of contraction and physical activity on Fstl1 expression and secretion. Although Fstl1 was not regulated by contractile activity of skeletal muscle cells in vitro, plasma Fstl1 levels were found to be increased post-exercise, making it likely that this myokine is involved in the crosstalk between muscle and other organs.

Material and Methods

Materials • Monocyte chemotactic protein-1 (MCP1) and tumor necrosis factor α (TNFα) were provided by Sigma-Aldrich (Munich, Germany). Interleukin 1-ß (IL-1ß) and interferon gamma (IFNγ) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Different recombinant Fstl1 was supplied by Creative BioMart (NY, USA), AdipoBioscience (Santa Clara, USA) and GenWay (San Diego, USA). 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). Anti-phospho Akt (Ser473) was supplied by Cell Signalling Technology (Frankfurt, 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 five healthy Caucasian donors (3 males, 16, 21 and 41 of age; 2 females, 33 and 37 of age) were supplied as proliferating myoblasts and cultured as described in our earlier study (8). For an individual experiment, myoblasts were seeded in six-well culture dishes at a density of 100,000 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 in αMEM containing 2% horse serum until day 5 of differentiation followed by overnight starvation in αMEM without serum. Differentiated cells were incubated as indicated with different cytokines for 24 h and recombinant Fstl1. Afterwards, cells were stimulated with 100 nmol/l insulin for 10 min.

Electrical pulse stimulation (EPS) • EPS was applied to fully differentiated myotubes in six-well dishes using a C-Dish in combination with a C-Pace pulse generator (C-Pace 100, IonOptix, Milton MA), as recently described by us (9). 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 1 Hz, pulse duration of 2ms and intensity of 11.5V for 2 to 24h. Medium was changed directly before stimulation.

Immunoblotting • Skeletal muscle cells were treated as indicated and lysed. The immunoblotting procedure was carried out as described before (Sell et al., 2008). Signals were visualized on a VersaDOC 4000 MP (Bio-Rad Laboratories, Munich, Germany) and analyzed by Quantity One analysis software (version 4.6.7, Bio-Rad Laboratories).

Measurement of Fstl1 • Supernatants from contracting and non-contracting hSkMC were collected after indicated time points and protein concentration was determined by Fstl1 DuoSet-ELISA (R&D systems). For measurement of Fstl1 serum concentration we used Fstl1 ELISA obtained by Uscn Life Science Inc.. Both ELISA kits were used according to the manufacturers protocol.

Cytokine treatment • Fully differentiated skeletal muscle cells were incubated with different cytokines (IFNγ, TNFα, MCP1 and IL-1ß) for 24 h. Fstl1 Secretion was assessed by ELISA according the manufacturer protocol.

RNA isolation and RT-PCR • Total RNA was isolated using the TriPure Isolation Reagent (Roche) according to the manufacturer's instructions. RNA concentration and purity was measured with a NanoDrop 2000 (Thermo Scientific). Two microgram of RNA was reverse transcribed using an Omniscript RT Kit (QIAGEN). Fstl1 mRNA expression was measured using pre-designed primers (Quantitect Primer Assay, Qiagen) in a SYBR Green-based real-time PCR. Amplification was done using SYBR Green technology (Applied Biosystems). The following conditions were used: 10 min by 95 °C for initial activation, 94 °C 15s, 55 °C 30 s, 72 °C 30 s (40 cycles). Samples were run in triplicate in 10 µl reaction volumes using StepOne plus sequence detection system for real-time PCR (Applied Biosystems). Melting curve analysis of the PCR products were performed to verify their specificity and identity. Target mRNA levels were normalize relative to actin.

Human Study • Eight well-trained healthy lean male volunteers (body mass index 23.1±0.6 kg/m2, VO2max 65.5±1.7 ml/min/kg) participated in the study. A baseline blood sample was taken before start of the exercise. Subjects cycled at 70 % VO2max for 60 min and then rested for 2 h. Blood were sampled immediately after the exercise session as well as 30 min and 120 min post exercise. The study was approved by the Regional Committee for Medical and Health Research Ethics, Region Sør-Øst-Norge, Norway (2011/927b).

Presentation of data and statistics • Data are the means ± SEM. Unpaired two-tailed Student 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

Differentiation-dependent Fstl1 expression and secretion • Fstl1 mRNA expression is regulated by skeletal muscle differentiation and shows a significant increase from undifferentiated cells (D0: 33±5 %) to day 2 of differentiation (70±10 %) (Tab. 1). The expression of Fstl1 remained at this level until the end of differentiation on day 6 (D2: 70±10% vs. D6: 100%). In addition, Fstl1 secretion was also constant from day 2 to day 6. In the supernatants we measured a mean Fstl1 concentration of 1.89±0.12 ng/ml (Tab. 1).

Fstl1 expression and secretion is regulated by inflammatory cytokines • Fstl1 mRNA expression was not regulated by contractile activity of skeletal muscle cells in vitro after 24 h of EPS (Fig. 1a). Also, Fstl1 secretion remained unchanged after 24 h EPS treatment (Fig. 1b). Fstl1 concentration in the supernatant of non-contracted cells was 1.9±0.8 ng/ml and 2.1±0.7 ng/ml after 24 h EPS treatment (Fig. 1b). However, we observed a significant 2fold upregulation of Fstl1 secretion after

Table 1. Differentiation-dependent secretion and expression of Fstl1 by human skeletal muscle cells. Cells were differentiated for the indicated time points and Fstl1 mRNA expression was analyzed by real time PCR. Data are normalized to expression of β-actin. Fstl1 protein secretion was analyzed in supernatants collected after 24 h by ELISA. Data are mean values ± SEM, n= 10, * p<0.05 vs. day 6 for Fstl1 mRNA expression.

Day of differentiation	Expression (Relative Fstl1 mRNA expression)	Secretion (ng/ml)
0	33 % ± 5 %	not determined
2	70 % ± 10 % *	1.82 ± 0.38
4	85 % ± 11 % *	1.85 ± 0.27
6	100 % *	1.91 ± 0.29

* p<0.05 vs. day 0 for Fstl1 mRNA expression.

24 h IFNγ treatment (Fig. 1c). The concentration of Fstl1 in the supernatant increased from 1.2±0.2 ng/ml to 2.1±0.4 ng/ml. In addition, we found a 2.7fold upregulation of Fstl1 secretion after treatment with IL-1ß. The concentration in the supernatant increased from 1.2±0.2 ng/ml to 3.2±0.5 ng/ml after 24 h (Fig. 1c). Interestingly, we did not observe a regulation of Fstl1 secretion after 24 h incubation with TNFα or MCP1 (Fig. 1c).

To investigate whether Fstl1 is classically secreted, we treated myotubes with brefeldin A (BFA). As shown in Fig. 1d, BFA is able to completely abrogate Fstl1 secretion by human skeletal muscle cells.

Fstl1 serum concentrations are affected by acute exercise • We analyzed the impact of acute exercise on Fstl1 serum concentrations in eight human male subjects. We observed a significant 1.2fold increase of Fstl1 serum levels after 60 min cycling (Fig. 2a). The concentration increased from 16.9±3.5 ng/ml pre-exercise to 20.1±3.1 ng/ml immediately after exercise (post-exercise 0 min). 30 min after completing the exercise the concentration was 21.9±2.9 ng/ml (Fig. 2b). The elevated Fstl1 serum concentrations were significantly reduced after a rest period of 120 min compared to post-exercise 0 min and 30 min and normalized at the basal level (0.99fold; 17.7±1.9 ng/ml, Fig. 2a/b).

Fstl1 has no effect on insulin-stimulated Akt phosphorylation • In order to analyze potential autocrine effects of Fstl1 on primary human skeletal muscle cells we stimulated myotubes with 100 ng/ml or 500 ng/ml recombinant Fstl1 for 24 h followed by 10 min insulin stimulation. Insulin-stimulated phosphorylation of Akt (Ser473) was assessed by Western blot analysis. Treatment of human skeletal muscle cells with insulin increased Akt activation 3fold compared to basal level (Fig. 3). Neither basal nor insulin-stimulated Akt phosphorylation was affected by Fstl1 treatment (Fig. 3). As shown in Fig. 3, treatment of myotubes with troglitazone, which was used as a positive control, resulted in a significantly increased insulin-stimulated Akt phosphorylation compared to insulin-stimulated control (2fold).

Discussion

The main finding of this study is that acute exercise induces increased levels of Fstl1 protein in the circulation. The kinetics revealed a significant increase of circulating Fstl1 during exercise and its normalization to basal level after a 120 min resting period. Thus, one hour of cycling resulted in a 1.2fold elevated serum concentration of Fstl1. We further demonstrate that Fstl1 is expressed and secreted by primary human skeletal muscle cells and that Fstl1 mRNA expression is upregulated during differentiation from myoblasts to myotubes. We also show an increase of Fstl1 secretion after treatment with pro-inflammatory cytokines such as IL-1ß and IFNγ while TNFα and MCP1 revealed no effects. Using our in vitro model of human skeletal muscle cells we could not detect effects of Fstl1 on Akt signaling. Our data suggest that Fstl1 is a myokine regulated by pro-inflammatory cytokines, and physical activity is able to increase circulating levels of this protein.

Prior studies have shown that Fstl1 is expressed and secreted by the murine skeletal muscle cell line C2C12. In addition, transgenic over-expression of Akt1 in mouse skeletal muscle leads to elevated circulating levels of Fstl1 (Ouchi et al., 2008). In this study, we show that Fstl1 is expressed and secreted by primary human skel-

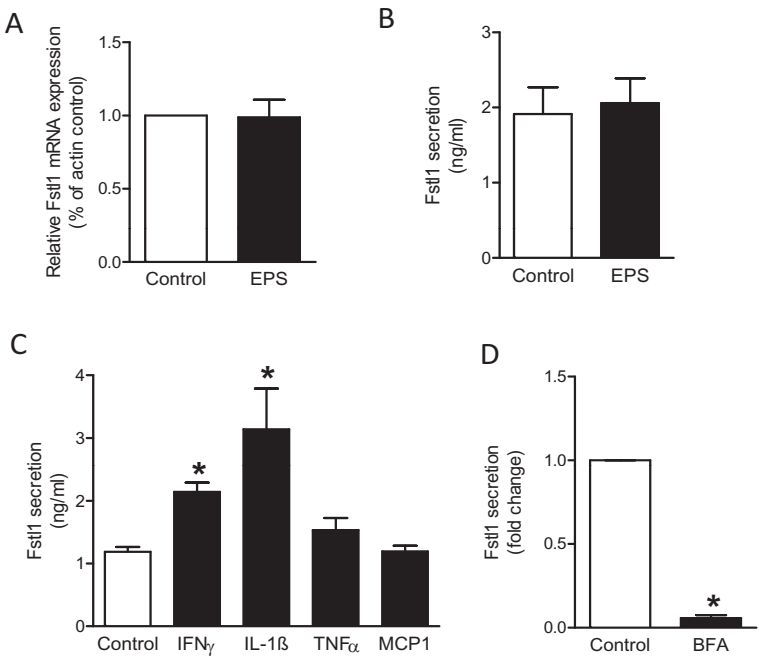

Figure 1. Comparison of Fstl1 secretion with and without EPS and regulation of Fstl1 secretion by different cytokines. (A) Comparison of Fstl1 mRNA expression in non-contracted skeletal muscle cells (control) and after 24 h EPS (1 Hz, 2 ms, 11.5 V). Fstl1 mRNA expression was analyzed by real time PCR and data are normalized to β-actin. (B) Supernatants of non-contracted (control) and contracted myotubes were analyzed by ELISA. (C) Differentiated human skeletal muscle cells were treated with IFNγ (10 ng/ml), TNFα (50 pg/ml), MCP1 (1 ng/ml) or IL-1ß (10 ng/ml) for 24 h and Fstl1 secretion was assessed by ELISA. Data are mean values ± SEM, n=8-10, * $p<0.05$ vs. control.

etal muscle cells. Furthermore, Fstl1 mRNA expression is significantly increased between myoblasts (day 0) and cells differentiated for two days. Rosenberg et al. showed that MyoD, a typical marker of the early state of myogenesis, inhibits Fstl1 expression due to activation of microRNA-206 transcription leading to a degradation of Fstl1 mRNA (Rosenberg et al., 2006). This may explain why Fstl1 mRNA expression is significantly lower in myoblasts compared to myotubes.

Recently, we have shown that stimulation of human skeletal muscle cells with EPS closely mimics the effect of exercise on skeletal muscle in vivo regarding enhanced AMPK activation, VEGF and IL-6 secretion (Lambernd et al., 2012). In this study, Fstl1 secretion was not regulated by contractile activity of skeletal muscle cells after 24 h EPS when we used our standard protocol (1 Hz, 2 ms, 11.5 V, 24 h). In addition, we analyzed the regulation of Fstl1 by different inflammatory factors and found an increased Fstl1 secretion after treatment with pro-inflammatory cytokines such as IFNγ and IL-1ß. Fstl1 was originally described as a TGF-β-inducible gene derived from the mouse MC3T3 osteoblast cell line. The induction of Fstl1 expression potentially involves the SMAD signaling pathway (Shibanuma et al., 1993). In addition, induction of Fstl1 by IL-1ß and LPS via an NFκB-dependent pathway has been reported (Clutter et al., 2009). We have observed that IL-1ß treatment doubled Fstl1 secretion in primary human skeletal muscle cells. These data suggest that Fstl1 is induced through NFκB signaling in addition to SMAD. Since it is known that TNFα and MCP1 also activate the NFκB pathway, we expected that these cytokines may trigger Fstl1 secretion, too. However, no effect of TNFα and MCP1 on Fstl1 secretion was observed. Wilson et al. have shown that IL-1ß, TNFα and IL-6 treatment resulted in increased Fstl1 secretion by mouse MC3T3 osteoblast cells (Wilson et al., 2010). Interestingly, TNFα, IL-1ß and IL-6 treatment had no effect on Fstl1 secretion by mouse 3T3L1 adipocytes, while IL-1ß and TNFα but not IL-6 increased Fstl1 secretion by fibroblast-like cells (Wilson et al., 2010). These and our data indicate that in different cell types distinct signaling pathways may be involved in the regulation of Fstl1 expression and secretion. Furthermore, we could show that IFNγ also increased Fstl1 secretion in primary human skeletal muscle cells. In addition to the well-known activation of the Stat1 pathway, a further mechanism may be involved in IFNγ-mediated induction of Fstl1 secretion. Further studies are needed to investigate in detail the mechanism underlying the processes by which Fstl1 expression and secretion is controlled in primary human skeletal muscle cells.

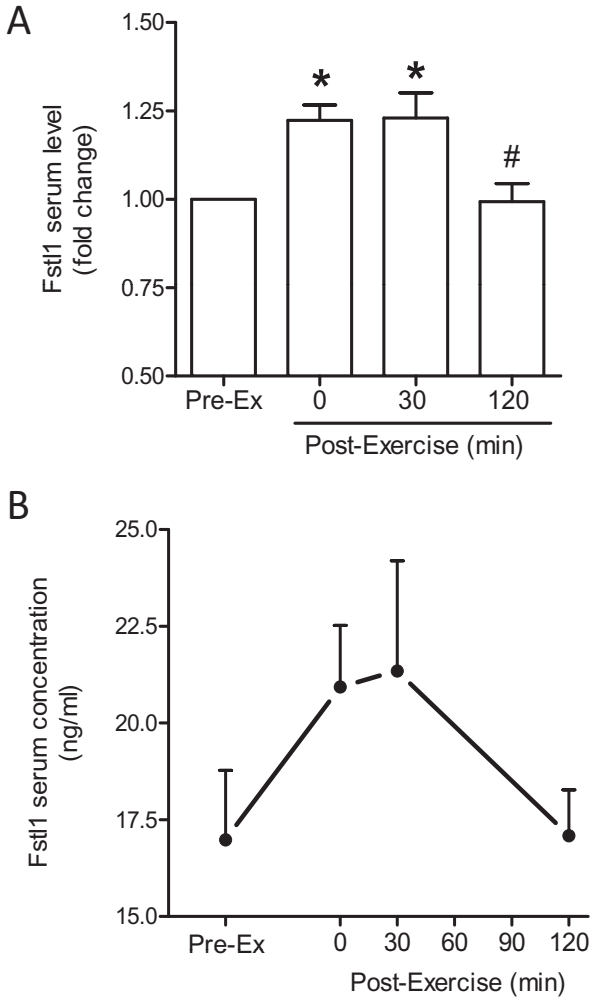

Figure 2. Fstl1 serum concentration is up-regulated after exercise. A baseline blood sample was taken before start of the exercise (Pre-Ex). Eight healthy lean male subjects cycled at 70 % VO2max for 60 min and then rested for 2 h. Blood was sampled immediately after the exercise session (Post-0 min) as well as 30 min and 120 min post exercise. (A) Shown is the fold change of Fstl1 serum level. (B) Absolute values of Fstl1 serum concentration. Data are mean values ± SEM, n=8, * p<0.05 vs. Pre-Ex, # p<0.05 vs. Post-0min and Post-30min.

Exercise is known to be an important regulator of myokine secretion. Up to now, only a few myokines have been identified to be regulated by exercise such as IL-6, IL-15, leukemia inhibitory factor and brain-derived neurotrophic factor (Broholm et al., 2011; Matthews et al., 2009; Nielsen et al., 2007; Pedersen, 2012). An acute increase of IL-6 after exercise, for example, increases insulin-stimulated glucose uptake in vitro and may enhance fatty acid oxidation (Carey et al., 2006). In the present study, we show that Fstl1 serum concentrations are increased after an acute bout of exercise and return back to basal levels after a resting period of 120 min. This observation indicates that Fstl1 circulation levels are regulated by short term exercise. Since muscle biopsie samples were not available for this study, we can not correlate the increase in serum level with elevated Fstl1 mRNA or protein level in skeletal muscle. Therefore, the elevation of Fstl1 serum concentration may be related to other organs and tissues that are influenced by exercise such as the heart. In a study by Oshima et al. secretion of Fstl1 by the myocardium has been reported (Oshima et al., 2008). However, it was recently shown that Fstl1 mRNA expression is regulated by strength training in human skeletal muscle. Muscle biopsies from m. vastrus and m. trapezius were taken from healthy male volunteers before and after 11 weeks of strength training and elevated Fstl1 mRNA expression was found in both muscles depots after exercise (Norheim et al., 2011). Taken together, these and our data provide evidences that exercise could be an important regulator of Fstl1 expression and secretion in humans. Interestingly, our in vitro standard EPS protocol had

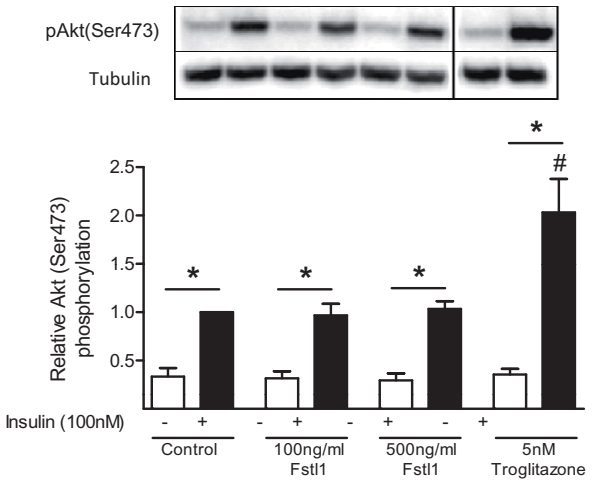

Figure 3. Effects of Fstl1 on Akt signaling in human skeletal muscle cells. Myotubes from different donors were cultured with two concentrations of Fstl1 (100 ng/ml and 500 ng/ml) or with troglitazone (5 nM) as positive control for 24 h. After acute stimulation with 100 nM insulin, total cell lysates were resolved by SDS-PAGE and immunoblotted with phosphospecific Akt (Ser473) as well as tubulin antibody. Data are the mean ± SEM of six experiments, were normalized to the level of tubulin and are expressed relative to the insulin-stimulated control value. *p<0.05 vs. basal. #p<0.05 vs. insulin-stimulated control.

no influence on Fstl1 mRNA expression in human skeletal muscle cells and release of Fstl1 into the supernatant. It might be speculated that a different EPS protocol, e.g. with higher intensity, may affect the regulation of Fstl1 in vitro. Future studies will help to analyze and understand the impact of contraction on the regulation of Fstl1 expression and secretion by human skeletal muscle cells.

Ouchi et al. (2008) have shown that basal Akt phosphorylation was increased after adenoviral Fstl1 transduction in human endothelial cells. We therefore expected an effect of Fstl1 on Akt signaling in skeletal muscle. It should be noted that the cells were treated with recombinant Fstl1 protein obtained from three different companies. However, treatment with Fstl1 had no effect on basal or insulin-stimulated Akt phosphorylation. It may be speculated that muscle-derived Fstl1 has no autocrine effect with regard to Akt signalling, but may be involved in the crosstalk between muscle and other organs acting in a rather endocrine manner. Shimano et al. (2011) have shown a time- and dose-dependent AMPK phosphorylation in neonatal rat ventricular myocytes using recombinant Fstl1. In addition, Ogura et al. (2012) reported that Fstl1 can prevent myocardial injury by inhibiting apoptosis and inflammatory responses through modulation of AMPK- and BMP-4-dependent mechanisms in the myocardium. We therefore suggest that an elevated Fstl1 serum concentration after exercise could be involved in the beneficial effects of physical activity.

Acknowledgements

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Declaration of interest

The authors report no conflicts of interest.

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Evidence against a beneficial effect of irisin in humans

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Abstract

Aims/hypothesis • Brown adipose tissue (AT) has gained interest as a potential target to treat obesity and metabolic diseases like type 2 diabetes. Irisin is a newly identified hormone, secreted from skeletal muscle cells, that regulates the browning of white fat cells which improves systemic metabolism by increasing energy expenditure in mice. The discovery of irisin raised expectations of its therapeutic potential to combat obesity and metabolic diseases; however the effect of irisin in humans is unclear.

Methods • Murine C2C12 cells and primary human myotubes were electrically pulse stimulated to induce contraction. Irisin serum levels were measured in mice after 4 weeks free wheel running. During differentiation of preadipocytes isolated from human subcutaneous AT, cells were treated with recombinant BMP7, FNDC5 and irisin.

Results • Our analyses of genomic DNA, mRNA and expressed sequence tags revealed that the human FNDC5 gene, the gene coding for the precursor of irisin, carries a point mutation in the start codon, which leads to a less effective translation of the protein in humans compared to mice. FNDC5 mRNA expression is contraction-regulated in murine C2C12 cells, but not in primary human skeletal muscle cells. Free wheel running of mice did not change irisin serum levels. BMP7 shows an effect on the browning of human adipocytes, but neither recombinant FNDC5 nor irisin.

Conclusions/interpretation • Targeting irisin might be a potential strategy to increase energy expenditure and to combat obesity by activating the browning of white AT. However, our findings make it unlikely that the beneficial effect of irisin observed in mice can be translated to humans.

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Introduction

Obesity and the involved risk of developing metabolic diseases is a major global public health challenge. In obese patients glucose homeostasis is disturbed due to an imbalance between energy intake and energy expenditure. Although the understanding of the role of genetics in obesity and type 2 diabetes is increasing [1-3], roughly 60% of all cases of diabetes can be directly attributed to weight gain [4].

Nowadays, brown adipose tissue (AT) has drawn the attention as novel therapeutic target

to treat obesity and metabolic diseases like type 2 diabetes. Brown AT is specialized in energy expenditure, while white AT is the primary site of triglyceride storage. In order to maintain body temperature in cold environment, brown AT is primarily a thermogenic tissue that oxidizes fat to generate heat [5] through the mitochondrial uncoupling protein 1 (UCP1). This protein uncouples the proton electrochemical gradient generated by respiration leading to the dissipation of heat. UCP1 knock-out mice are cold sensitive and tend to develop obesity, even when fed a control diet [6] whereas experimental approaches capable of increasing the amount of brown AT reduce the development of obesity [7].

In humans, it was thought that active brown AT exists only in newborns [8] and was lost later in life. In 2009, five independent research groups published studies using PET-CT (positron emission tomography-computed tomography) imaging to consistently prove that adult humans have metabolically active brown fat [9-13]. Brown AT is found in anatomically discrete depots, while the most common location in adults is the cervical-supraclavicular depot. Virtanen et al. were additionally able to confirm the presence of brown AT in this region by detecting UCP1 at the mRNA and protein level in biopsies [12].

Both white and brown AT derive from mesenchymal stem cells, although they have not the same lineage. Intrascapular brown adipocytes and skeletal muscle, but not white adipocytes, arise from progenitors expressing myf5 [14]. Additionally, brown AT differentiation is specifically underlying transcriptional regulators like PRDM16 [15], PGC1α [16], RIP140 [17], and others [18-21]. Rat epididymal white AT chronically stimulated with rosiglitazone, a PPARγ agonist, reveals a thermogenic competent population of UCP1-containing adipocytes [22]. These cells do not represent brown adipocytes of the same lineage as those found in classical brown AT depots, since they do not express the typical brown AT transcription factor PRDM16. These cells appear to be a particular type of adipocytes; called 'brite' (brown-in-white) adipocytes. Brite adipocytes manifest several classical brown adipocyte characteristics, but they do not derive from the same lineage as brown adipocytes. Thus, the possibility to switch from white AT to brown AT and to identify mechanisms that can activate brown adipocyte differentiation in response to pharmacological compounds is highly attractive in the context of obesity treatment.

De Matteis et al. observed browning of the visceral fat in rats after treadmill running by a supposed white-to-brown transdifferentiation [23]. Recently, Boström et al. published a promising mechanism for the induction of brown adipocytes in mice white AT depots after exercise. Overexpression of PGC1α in mice skeletal muscle, as well as exercise, induces the expression of the FNDC5 gene [24], a gene that was rarely studied before. Teufel et al. described FNDC5 as a protein containing a signal peptide, fibronectin type III repeats, and a hydrophobic region that is likely to encode a transmembrane domain [25]. However, in adult mouse tissues FNDC5 is highly expressed in heart and brain, less in skeletal muscle [25]. Boström et al. showed that the membrane protein is cleaved and the extracellular protein part is secreted as novel signalling molecule called irisin [24]. Viral delivery of FNDC5 caused a browning of subcutaneous fat, stimulated oxygen consumption, and diminished diet-induced weight gain and metabolic dysfunction in mice [24]. Thus, irisin induced a thermogenic mechanism in white AT, which improved whole body energy balance in mice. Although, the initial description of irisin was focused on mice, the highly conserved amino acid sequence among species raised the hope that exogenous administered irisin could have a therapeutic potential in the treatment of obesity and diabetes in humans. In the present paper, we demonstrate that great caution is needed when extrapolating from rodent data regarding FNDC5/irisin to the human situation.

Methods

Sequence Alignment • ClustalW was used for multiple alignments. Blast searches were done using NCBI-BLAST interface. FNDC5 exon 1 sequences were obtained from ENSEMBL. Single nucleotide polymorphism data were obtained from NCBI and

ENSEMBL. UNIPROT was searched for annotated non-canonical start sides in human proteins. The search for hairpin structures close to the start codon was done with the public tool AUG_hairpin (http://wwwmgs.bionet.nsc.ru/mgs/programs/aug_hairpin/).

Culture of primary human skeletal muscle cells • Human skeletal muscle cells from five healthy donors (three males, 16, 21 and 47 years old; two females, 33 and 37 years old) were supplied as proliferating myoblasts (PromoCell, Lonza and Tebu). For an individual experiment, myoblasts were seeded in six-well culture dishes at a density of 1×10^5 cells/well and cultured to near-confluence in α-modified Eagle's medium (αMEM)/Ham's F-12 medium containing skeletal muscle cell growth medium supplement (PromoCell). The cells were then differentiated in αMEM containing 2% (vol./vol.) horse serum (Gibco) until day 5 of differentiation.

Culture of C2C12 cells • C2C12 myoblasts were seeded in six-well culture dishes at a density of 1×10^5 cells/well and cultured to near-confluence in DMEM, high glucose containing 10% (vol./vol.) fetal calf serum. The cells were then differentiated in DMEM containing 2% (vol./vol.) horse serum until day 5 of differentiation, either followed by overnight starvation in DMEM without serum or followed by further cultivation in DMEM containing 2% (vol./vol.) horse serum.

Electrical pulse stimulation • Electrical pulse stimulation (EPS) was applied to fully differentiated myotubes in six-well dishes using a C-Dish combined with a pulse generator (C-Pace 100; IonOptix). The instrument emits bipolar stimuli to the carbon electrodes of the C-dish, which are placed in the cell culture medium. The human skeletal muscle cells and C2C12 cells were stimulated (1 Hz, 2 ms, 11.5 V) for 24 h after overnight starvation in medium without serum [26]. A protocol described by Burch et al., was used to induce a higher PGC1α expression in murine C2C12 cells [27]. Myotubes were stimulated in DMEM containing 2% (vol./vol.) horse serum using 1 s trains (50 Hz, 1 ms, 14 V) with 1 s pauses between the trains. RNA was isolated after 3 h rest. The medium was changed directly before stimulation.

Adipocyte culture • Subcutaneous AT was obtained from healthy lean or moderately overweight women (n = 11, body mass index 28.8 ± 3.8, age 32.5 ± 10.4 years) undergoing plastic surgery. The procedure was approved by the ethical committee of the Heinrich-Heine-University, Düsseldorf, Germany. Preadipocytes were isolated by collagenase digestion of AT as previously described by our group [28]. Isolated cell pellets were resuspended in basal medium (DMEM/F12 medium supplemented with 14 nmol/l NaHCO3, 33 mmol/l biotin, 17 mmol/l D-panthothenic-acid and 10% (vol./vol.) FCS, pH 7.4), seeded in six-well plates and maintained at 37°C with 5% CO_2. After cells were grown until confluence, cultures were washed and further incubated in an adipocyte differentiation medium (basal medium supplemented with 66 nM insulin, 1 nM triiodo-L-thyronine, 100 nM cortisol, 10 mg/ml apo-transferrin, 50 mg/ml gentamycin) for 14 days. Medium was changed every 2–3 days with addition of 5 mM troglitazone for the first three days. Adipocytes were treated with 50 ng/ml BMP7 (R&D systems), 200 ng/ml FNDC5 (Abnova and Phoenix), and irisin (Phoenix). To generate supernatants from the adipocytes differentiated with different treatments, adipocytes were incubated with αMEM for 24 h.

Animal experiments • All animal experiments were conducted according to German Animal Protection Law and approved by the Sanofi-Aventis Deutschland GmbH institutional animal care and use committee. The institution is AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) accredited. Endurance exercise was measured using in-cage voluntary running wheels, with electronic monitoring (FMI GmbH) as previously described [24]. Twenty-eight-week-old C57/BL6N mice were housed individually versus matched sedentary controls at 23°C and on a 12 h light-dark cycle. Mice were fed ad libitum with Ssniff standard diet. Plasma samples were taken before and after two or four weeks of endurance training, immediately frozen, and stored at -80°C, after adding a protease inhi-

bitor cocktail. Four weeks after initiation of voluntary running, mice were sacrificed and muscle and other tissues were removed for PCR analysis.

RNA-isolation and quantitative real-time PCR • Cells were lysed by Tripure (Roche Applied Science), RNA was isolated and reverse-transcribed using kits (RNeasy Mini, Omniscript Reverse Transcription, Qiagen) according to the manufacturer's instructions. Gene expression was determined by quantitative real-time PCR using primer as described in Suppl. Table 1 and GoTaq qPCR Master Mix (Promega) with 0.016 to 20.00 ng cDNA on a cycler (Step One Plus; Applied Biosystems). Expression of the investigated genes was normalised to actin or GAPDH and analysed via the ΔΔCt method.

Microfluidic card TaqMan gene expression assay • RNA integrity was tested on an Agilent 2100 Bioanalyzer using Agilent RNA Nano chips. Only RNAs with a RIN score of 7.5 or higher were used for analysis. Synthesis of cDNA was done from 0.5 µg of each total RNA preparation in a volume of 20µl with the Quantitect Reverse Transcription Kit from Qiagen according to the manufacturer's instructions. Thermal cycling of the PCR reactions was done in microfluidic cards on a ViiA7 Real Time PCR 384 well cycler and fluorescence plate reader from Applied Biosystems (see Suppl. Table 2 for details on used gene specific TaqMan assays).

Oil red staining • Adipocytes were washed with ice-cold PBS and fixed with a solution containing 71% picric acid, 24% acetic acid and 5% formaldehyde for 2 h. Afterwards, cells were washed with PBS, further incubated with 0.3% Oil Red O solution for 10 min and washed again with PBS. Subsequently, samples were photographed with a Canon EOS digital camera. Oil Red O staining was eluted in isopropanol and absorption was measured at 500 nm using a plate reader (InfiniteM200; Tecan, Maennedorf).

Detection of irisin • Supernatants of primary human skeletal and C2C12 myotubes were collected for 24 h, centrifuged at 1,100 rpm for 5 min and afterwards concentrated using centrifugal filter devices with a cut off of 3 kDa (Millipore). Irisin protein levels in concentrated supernatants were measured using EIA kit from Phoenix Pharmaceuticals according to the manufacturer´s instructions. Irisin levels in mice plasma were measured using ELISA kits from Phoenix Pharmaceuticals and Aviscera Bioscience as described by the manufacturer.

Adiponectin and leptin ELISA • Concentration of adiponectin and leptin in supernatants collected after 24 h of adipocytes were analyzed using ELISA kits (Leptin, R&D systems; Adiponectin, BioVendor). Samples were measured according to the manufacturer's instructions.

Statistics • Data are expressed as mean ± SEM. One-way ANOVA (post-hoc test: Tukey's multiple comparison test) and unpaired student's t-test were used to determine statistical significance. For animal study data were analyzed by using one-way ANOVA (post-hoc test: Dunnett's) or student's t-test. All statistical analyses were done using GraphPad Prism 5 considering a p-value of less than 0.05 as statistically significant.

Results

Start codon of FNDC5 gene is mutated in humans • As a general analysis of the FNDC5 protein level, we compared the irisin level secreted by differentiated primary human skeletal muscle cells compared to murine C2C12 cells. We observed a substantially lower irisin protein level in the supernatant of human cells compared to murine cells (Fig. 1a).

A multi-species sequence alignment of the FNDC5 exon 1 demonstrated that all analyzed FNDC5 genes display a conserved ATG translation start site, except for the human sequence (Fig. 1b). In contrast, at the position of the start codon the human sequence shows an ATA, encoding isoleucin, instead of the conserved ATG, encoding methionine. Using 5´-RACE-PCR of human brain and skeletal muscle samples, we could confirm experimentally the mutation within the start

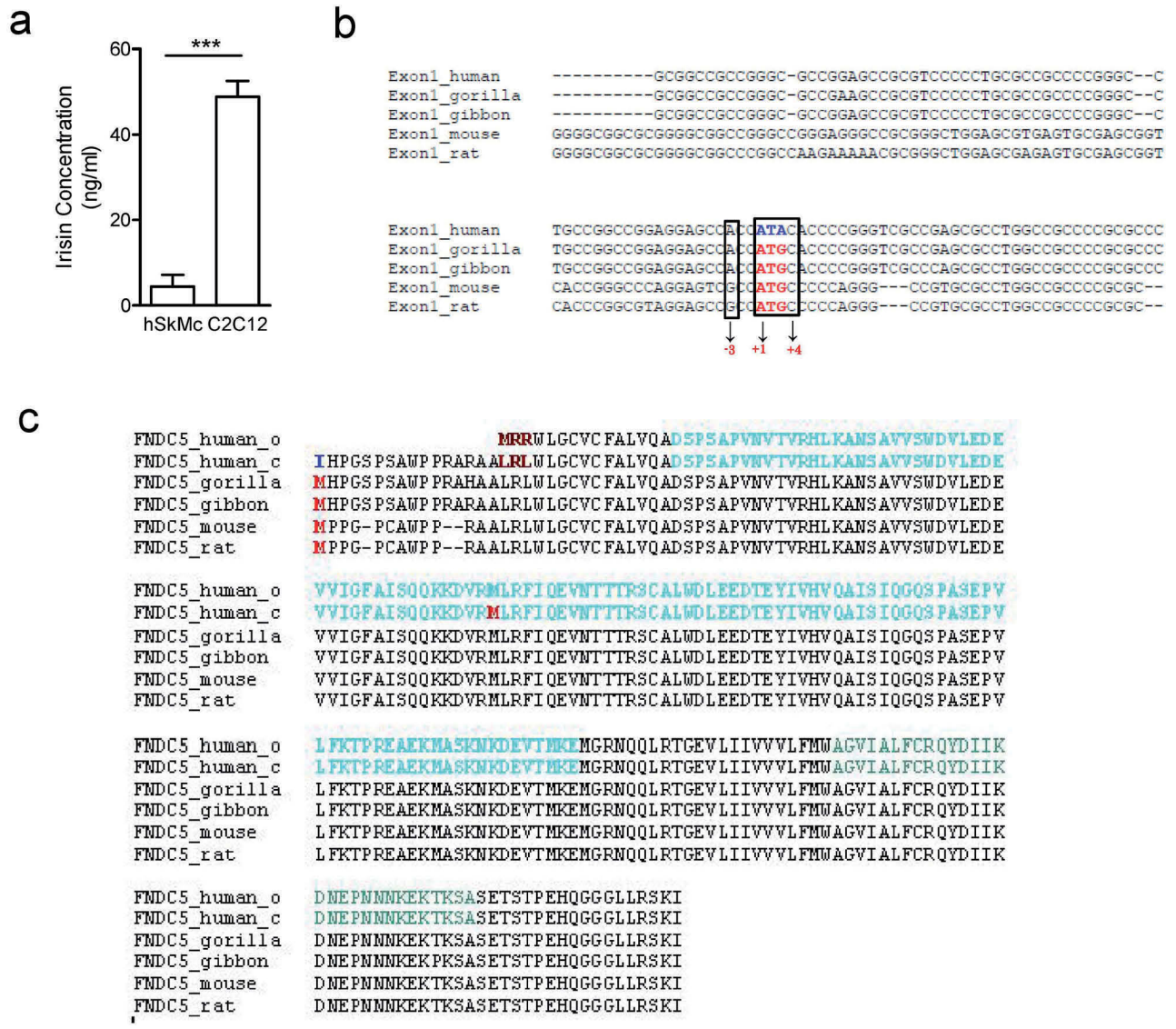

Figure 1. Irisin secretion of skeletal muscle cells is decreased in humans due to a point mutation in the start codon. **a** Primary human skeletal muscle cells and murine C2C12 cells were differentiated for 6 days. Medium was collected for the last 24 h of differentiation, centrifuged and concentrated by centrifugal filter devices (60times). Irisin protein levels in concentrated supernatants were determined by EIA kit. The medium control showed no signal, n = 5, *** $p < 0.001$. Data are presented as means±SEM. **b** Multiple alignment of the exon 1 sequences: The start codon is coloured. In the human sequence, the mutated ATG to ATA is bold and blue. There is no other ATG present in exon 1. The -3(A/G) and +4 (G) position, that define the Kozak sequence, and the position of the start codon is marked. As the +4 position is a C instead of a G, this start sequence posses a partial Kozak sequence. **c** Multiple sequence alignment of FNDC5 proteins of different species including two human versions. FNDC5_human_o: Sequence published in Boström et al.; FNDC5_human_c: current version in Uniprot; M = Start methionines including the potential downstream human start site; Light Blue = irisin sequence; I = mutated start site claimed to be a non canonical start side; LRL = sequence shown in UniProt (Q8NAU1_old) as MRR. Green sequence peptide used for the generation of the Abcam FNDC5 antibody

codon as ATA (Suppl. Fig. 1).

The current version of UNIPROT Q8NAU1 (Fig. 1c, FNDC5_human_c) annotates this ATA as a non-canonical start. However, Kozak et al. showed that ATA even in a perfect context (GCCRCCATG, R=A or G, Fig. 1b), is highly unlikely to serve as a translation site and results in low translation efficiency [29]. The next in-frame downstream ATG is a non-Kozak ATG and located within the sequence that was annotated as irisin (Fig 1c). Therefore an N-terminal truncated FNDC5 (represented by NP_ 715637/NM_153756) and irisin would be generated. In addition there are 3 upstream partial Kozak ATGs in this mRNA that are not in frame with the FNDC5 open reading frame (ORF) and would therefo-

re almost completely block translation from this new start site (Suppl. Fig. 3). It has been experimentally shown that the translation efficiency of non-canonical sides can be increased, if a hairpin slows down the scanning ribosome [30], as described for FGF2 (Suppl. Fig. 4a). Based on this observation, an ATG hairpin program predicts if there are stem-loop structures in appropriate distance to the ATA [31], which would increase the translation efficiency. However, for FNDC5 no eligible hairpin structures were found (Suppl. Fig. 4b).

Comparison of the published full-length human FNDC5 protein sequence by Boström et al. (Fig. 1c, FNDC5_human_o, [24]) with mRNA, expressed sequence tags (EST), genomic DNA and single-nucleotide polymorphism (SNP) data revealed that the first three amino acids (MRR, Fig. 1c) do not match human genomic DNA. We excluded that this is due to differential splicing, as 20 expressed sequence tags (EST) and two Ref_Seq cDNAs that cover this region perfectly matched the annotated exon 1 region (Suppl. Fig. 2). We looked up public SNP data, but could not find any hint that this codon might be altered.

FNDC5 gene is activated by contraction in murine, but not in human myotubes • PGC1α gene expression is induced in muscle by exercise and FNDC5 gene expression was reported to be PGC1α-dependent in mice [24]. To study contraction-regulated gene expression, we previously developed an in vitro contraction model using EPS of primary human skeletal muscle cells [26]. Using this EPS model, PGC1α mRNA expression was enhanced after 24 h of EPS in primary human skeletal muscle cells (1.7fold). However, FNDC5

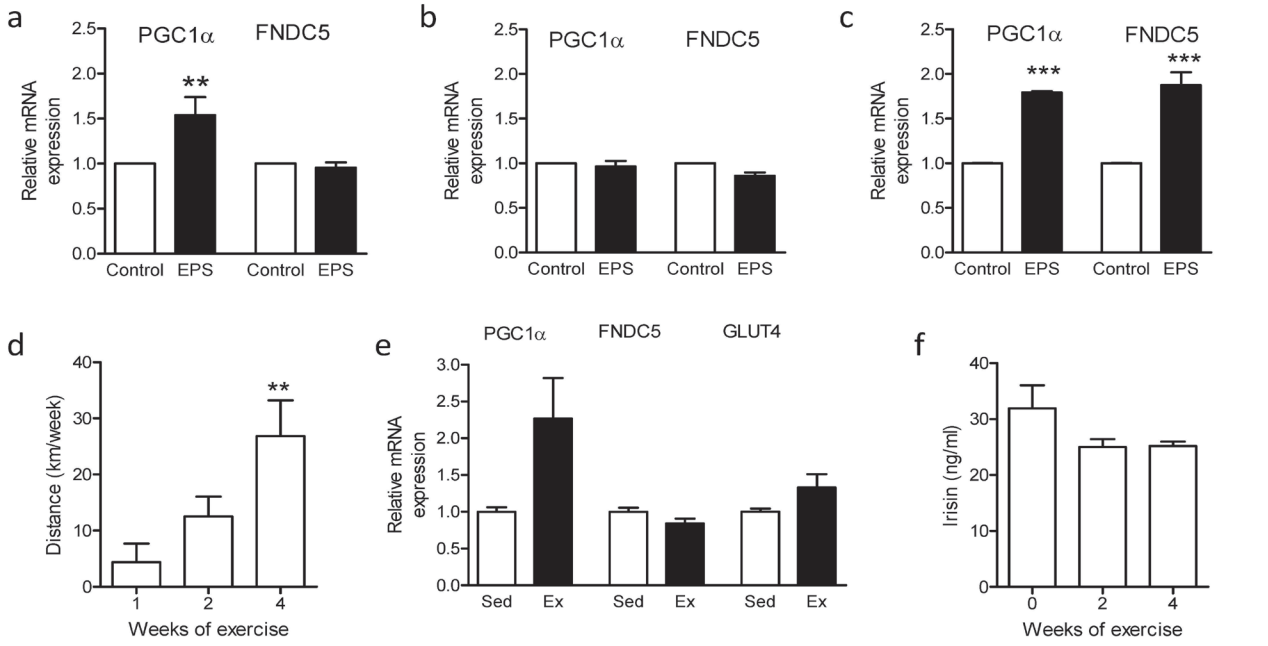

Figure 2. Differences in FNDC5 gene expression in mice and human contraction models. Primary human skeletal muscle cells (**a**) and murine C2C12 cells (**b**) were differentiated in αMEM containing 2% (vol./vol.) horse serum. Followed by overnight starvation, and electrical pulse stimulated for 24 h in serum-free medium (1 Hz, 2 ms, 11.5 V). **c** Murine C2C12 cells were differentiated and electrical pulse stimulated for 24 h in αMEM containing 2% (vol./vol.) horse serum (1 s trains [50 Hz, 1 ms, 14 V] with 1 s pauses between the trains), followed by 3 h rest. **a-c** Relative gene expression of PGC1α and FNDC5 was measured by RT-PCR; n=4-5 per group; **p < 0.01, ***p < 0.001. White bars, control (non-EPS); black bars, EPS. **d-f** Twenty-eight week old C57/BL6N mice were placed individually in cages equipped with electronically monitored running wheels. The mice were then allowed to use the wheels ad libitum. **d** Running distance per day of mice was measured using in-cage voluntary running wheels with electronic monitoring, n = 9. **p < 0.01 vs week 1. **e** After 4 weeks of free-wheel running, RNA of m. soleus was isolated and expression of PGC1α, FNDC5 and GLUT4 was measured by RT-PCR; n ≥ 4 per group. White bars, Sed, sedentary animals; black bars, Ex, exercise. **f** Before exercise study and after 2 and 4 weeks of free-wheel running, plasma samples were taken. Irisin level were determined using EIA kit provided by Phoenix, n = 11. Data are presented as means±SEM.

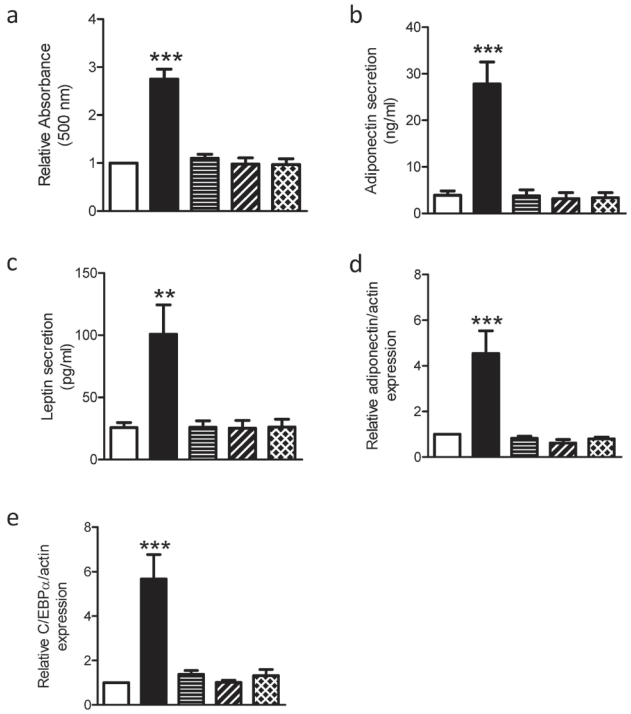

Figure 3. BMP7 promotes adipogenesis in human adipocytes, but not FNDC5 and irisin. Isolated preadipocytes from human subcutaneous AT of different donors were differentiated in the presence of 50 ng/ml BMP7, 200 ng/ml FNDC5 (obtained from Abnova), 200 ng/ml FNDC5 (obtained from Phoenix) and 60 ng/ml irisin (obtained from Phoenix). **a** Lipid droplet accumulation was analyzed at day 12-14 of differentiation by Oil Red O staining. The dye was eluted and quantified at 500 nm; n≥3; ***p < 0.001. **b+c** Protein concentration of adiponectin (b) and leptin (**c**) were measured in supernatants of mature adipocytes; n≥5; **p < 0.01 for leptin secretion, ***p < 0.001 for adiponectin secretion. **d+e** Relative gene expression of adiponectin and C/EBPα was measured by RT-PCR after 12-14 days of differentiation; n≥4; ***p < 0.001. White bars, control; black bars, BMP7; horizontally hatched bar, FNDC5 (obtained from Abnova), diagonally hatched bar, FNDC5 (obtained from Phoenix); crossed bar, irisin. Data are presented as means±SEM.

mRNA expression was not altered (Fig. 2a). Using the same protocol conditions for murine C2C12 cells we did neither observe enhanced mRNA expression of PGC1α nor of FNDC5 (Fig. 2b). We adjusted the EPS protocol for C2C12 cells according to Burch et al. [27], as stated in the Methods section, and observed an increased PGC1α gene (1.8fold) expression accompanied by increased FNDC5 gene expression (2.1fold) (Fig. 2c), while human myotubes started to detach after using this EPS protocol.

To validate the regulation of FNDC5 mRNA in mice skeletal muscle in response to exercise, we used an established model of voluntary endurance training [24]. After an adaptation period, the C57/BL6N mice increased the running distance per day and ran an equivalent of 3.8 km per day (Fig. 2d). After four weeks of free exercise running, PGC-1α expression tended to be about 2.3fold enhanced in the soleus muscle compared to sedentary controls (Fig. 2e). In contrast, FNDC5 mRNA level was not changed (Fig. 2e). Before the intervention and after two and four weeks of free-wheel running blood samples were taken and irisin plasma levels were measured. However, the irisin plasma levels in mice were not changed during four weeks of exercise (Fig. 2f). This result was confirmed by using a second commercially available kit provided by Aviscera BioScience (Suppl. Figure 5).

Recombinant FNDC5 and irisin have no effect on the browning of human preadipocytes • We isolated preadipocytes from primary human subcutaneous AT and differentiated these cells to mature adipocytes in the presence of recombinant FNDC5, irisin or BMP7 as positive control. FNDC5 was obtained from Abnova, which was also used by Boström et al. [24] and Wu et al. [32], and additionally from Phoenix.

Further analysis confirmed that BMP7 potently induced a brown-fat-like gene program in cultured adipocytes. Triglyceride storage assessed by Oil Red O staining was increased by BMP7 treatment (Fig. 3a) as well as adiponectin and leptin secretion (Fig. 3b+c). BMP7 treatment during differentiation induced an increased expression of general differentiation markers for adipogenesis like adiponectin (4.5fold, Fig. 3d), C/EBPα (5.7fold, Fig. 3e), and PPARγ (3.6fold) (Fig. 4a). Notably, UCP1, known as a brown/brite marker, was even stronger enhanced (6.4 fold, Fig. 4b), with the strongest effect in CD137 high expressing cells, a novel, recently described marker of preadipocytes which are susceptible to browning (Fig. 4c-e). The present study includes adipocytes of more than ten different donors. Analyzing the CD137 expression on day 0 revealed that the donors can be clustered in a CD137 low expressing

and a CD137 high expressing group (Fig. 4c). The CD137 high expressing adipocytes were more sensitive to the browning effect induced by BMP7, indicated by a higher UCP1 induction compared to the expression of the general differentiation marker PPARγ (Fig. 4d). In marked contrast to the gene activation by BMP7, FNDC5 and irisin showed no effect on classical and brite AT markers (Fig. 3d,e; Fig. 4a,b). CD137 expression level had no impact on the FNDC5 response of the adipocytes (Fig. 4e).

Additionally, the mRNA expression of TCF21, which is a marker for white AT, was significantly reduced after BMP7 treatment (Fig. 4f). ZIC1, a marker for classical brown AT with myogenic origin in mice [22], was not altered on mRNA level after BMP7 treatment of human adipocytes (Raschke, unpublished observation). Neither recombinant FNDC5 nor recombinant irisin had an effect on TCF21 (Fig. 4f) or ZIC1 mRNA expression.

In order to assess the potential upregulation of genes by FNDC5 and irisin of genes different than the previously analyzed (Fig. 3-4), we additionally performed a microfluidic card TaqMan gene expression assay including 40 genes associated with adipocyte differentiation or browning. A number of genes was upregulated by the treatment of BMP7 over differentiation including adiponectin, C/EBPα, FABPB4, leptin (LEP), perilipins (PLIN1, 2,4 and 5), and several others (Fig. 5, upper two panels). None of the gene expressi-

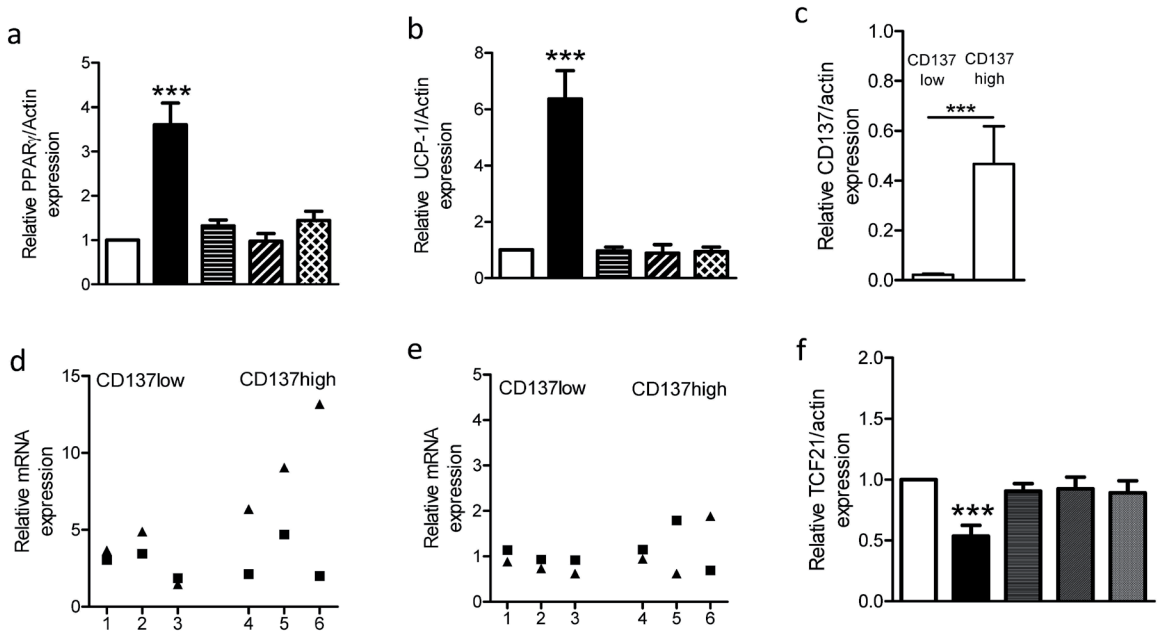

Figure 4. BMP7 activates the beige fat gene program in human adipocytes, but not FNDC5 and irisin. Isolated preadipocytes from human subcutaneous AT of different donors were differentiated in the presence of 50 ng/ml BMP7, 200 ng/ml FNDC5 (obtained from Abnova), 200 ng/ml FNDC5 (obtained from Phoenix) and 60 ng/ml irisin (obtained from Phoenix). **a+b** Relative gene expression of PPARγ and UCP1 was measured by RT-PCR after 12-14 days of differentiation; n≥4; ***p < 0.001. White bars, control; black bars, BMP7; horizontally hatched bar, FNDC5 (obtained from Abnova), diagonally hatched bar, FNDC5 (obtained from Phoenix); crossed bar, irisin. **c** Relative gene expression of CD137 was measured by RT-PCR on day 0 of differentiation without treatment with recombinant proteins; n=12; ***p < 0.001. **d+e** The increase in UCP1 and PPARγ expression of six individual donors was compared after BMP7 treatment (d) and FNDC5 (Abnova) treatment (**e**). Preadipocytes with a high CD137 expression showed a more robust activation of UCP1 compared to PPARγ after BMP7 treatment. Values are expressed relative to control in single experiments. Square, relative PPARγ expression after BMP7 (**d**) or FNDC5 (**e**) treatment over differentiation; triangle, relative UCP1 mRNA expression after BMP7(**d**) or FNDC5 (**e**) treatment over differentiation. Data are presented as means±SEM. (f) Relative gene expression of TCF21 was measured by RT-PCR after 12-14 days of differentiation; n≥4; ***p < 0.001. White bars, control; black bars, BMP7; horizontally hatched bar, FNDC5 (obtained from Abnova), diagonally hatched bar, FNDC5 (obtained from Phoenix); crossed bar, irisin. Data are presented as means±SEM.

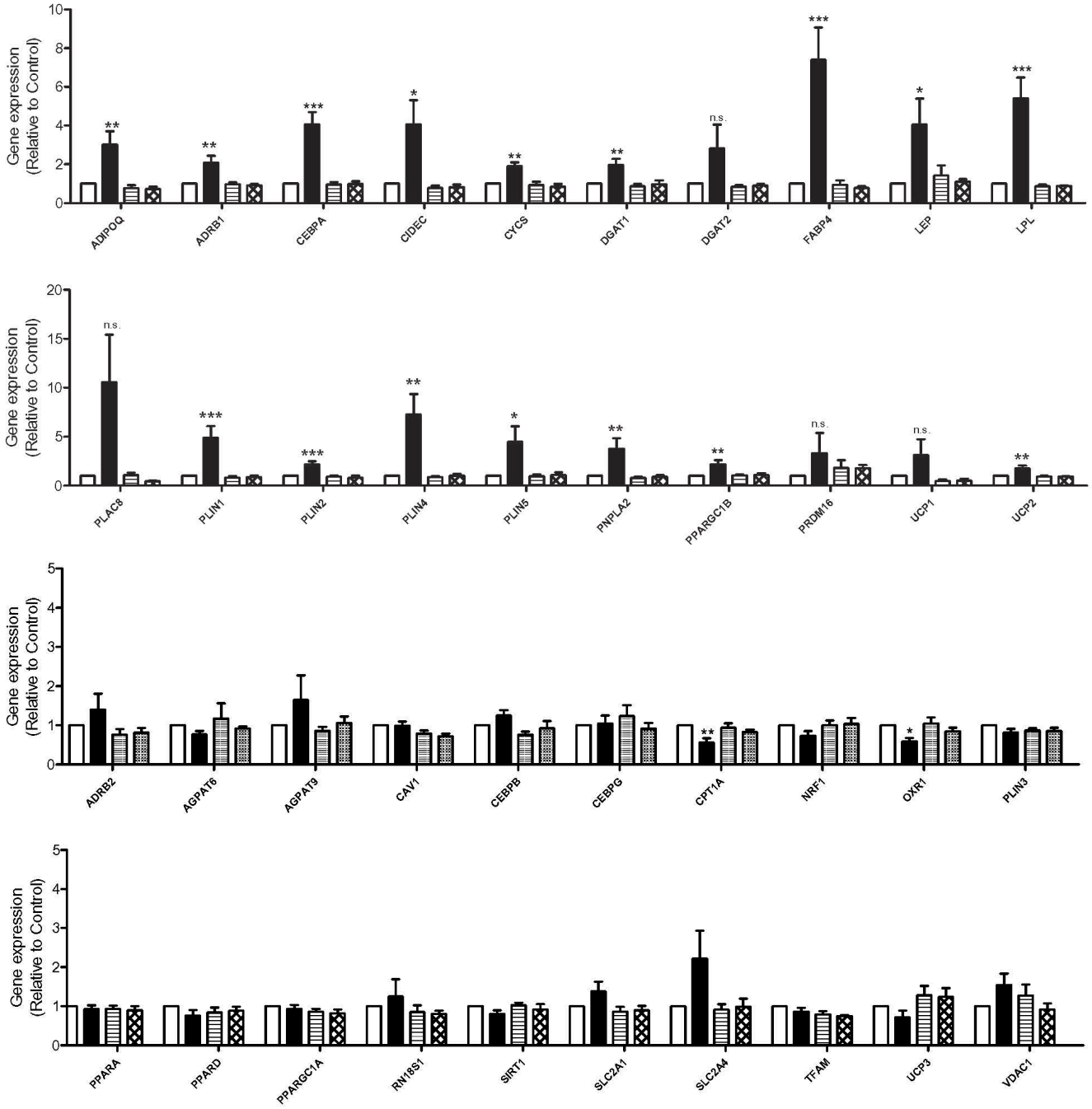

Figure 5. Gene expression analysis of human adipocytes after treatment with BMP7, FNDC5 and Irisin. Isolated preadipocytes from human subcutaneous AT of different donors were differentiated in the presence of 50 ng/ml BMP7, 200 ng/ml FNDC5 (obtained from Abnova), and 60 ng/ml irisin (obtained from Phoenix). Gene expression of 40 genes, related to adipocyte differentiation and browning, were assessed by using microfluidic card TaqMan gene expression assay, n ≥ 4, *p < 0.05, **p < 0.01, ***p < 0.001 vs control; n.s., not significant. White bars, control; black bars, BMP7; horizontally hatched bar, FNDC5; crossed bar, irisin. Data are presented as means±SEM.

ons was enhanced by FNDC5 or irisin. Genes that were not upregulated by BMP7, FNDC5 and irisin are presented in figure 5, lower two panels.

Since Boström et al. observed that viral delivery of FNDC5 potently improved diet-induced insulin resistance by an improved glucose tolerance test, we analyzed acute effects of recombinant FNDC5 on primary human skeletal muscle cells and adipocytes. Insulin-stimulated Akt phosphorylation was not changed by FNDC5 treatment neither in adipocytes (Suppl Figure 6a) nor in skeletal muscle cells (Suppl. Figure 6b).

Discussion

Targeting irisin and its downstream signalling pathways might be an interesting strategy to increase energy expenditure in humans and to combat obesity by inducing the browning of white adipose tissue. Due to the high identity between the mouse and human sequence, it was speculated that the translation from the mouse model to a human therapeutic approach would be less complex. Boström et al. stated that the cleaved and secreted portion of FNDC5, the hormone irisin, is highly conserved and 100% identical between all mammalian species sequenced [24]. Indeed, the FNDC5 gene is well conserved between organisms with one exception reported here, namely a mutation in the start codon of the human gene. Examining the human genomic sequence revealed that the start from UniProt entry FNDC5/Q8NAU1 (full-length protein as described by Boström et al. [24]) is not matched by an ATG and that the upstream conserved ATG of other species is mutated to an ATA codon in humans.

Ivanov et al. performed an algorithm based analysis of the 5'-UTRs of human GenBank RefSeq mRNAs to find non-ATG start codons in humans [33]. They have used sequences 5´ of the annotated start-codon and compared these to other vertebrate sequences. In this in silico study FNDC5 ranks high in their list, as the 5´ human amino acid sequence is almost identical to that in mouse. However, Kozak et al. showed that ATA results in low translation efficiency [29], which can be increased if a hairpin slows down the scanning ribosome [30]. However, ATG hairpin program predicts no eligible stem-loop structure or hairpin, respectively, for human FNDC5. We therefore suggest that the human FNDC5 gene might be a transcribed pseudogene that has substantially lost the ability to be translated into the FNDC5 protein and thus cannot be further processed to irisin. Thus, the mutation in the start codon of the human FNDC5 gene leads to low translation efficiency and might explain the decreased release of irisin observed from primary human myotubes compared to murine myotubes. The possibility that a non-canonical start site is used as annotated in the current version of Q8NAU1 cannot be ruled out completely. Further biochemical research is needed to prove this.

Physical activity promotes a more oxidative phenotype in skeletal muscle and is characterized by an increased expression of PGC1α in skeletal muscle [34], which stimulates enhanced expression of FNDC5 [24]. Inducing contractile activity in our in vitro model enhanced PGC1α expression in murine and human myotubes to a similar extent. Nevertheless, only in the murine cells the increased PGC1α expression resulted in a significantly enhanced FNDC5 expression. Boström et al. observed enhanced FNDC5 mRNA levels (2fold) in a cohort of older, obese subjects after a 10-week protocol of endurance exercise [24]. However, using gene-chip probe sets Timmons et al. demonstrated that FNDC5 induction in muscle occurred only in highly active elderly subjects compared to sedentary controls (1.3fold), which were a minority of analyzed subjects. They failed to confirm FNDC5 gene expression by aerobic exercise in younger subjects [35]. Additionally, another study showed that circulating irisin levels were only slightly increased (about 1.2fold) after 2 or 3 sets of double sprints at first week and not after 8 weeks of exercise [36]. This is the only study which proves circulating irisin levels in human plasma by using a commercially available ELISA kit. Boström et al. [24] and Sharma et al. [37] used Western Blot analysis to detect irisin in human and mouse serum. The antibody stated by the authors was provided by Abcam, which specifically detects the C-terminal region of the FNDC5 protein (peptide used for immunization/antibody synthesis was sequenced and is highlighted in Fig. 1c). FNDC5 is described as a transmembrane protein with the C-terminal tail located in the cytoplasm, while the extracellular N-terminal part is supposed to be cleaved and secreted. Thus, an antibody binding to the C-terminal region of the FNDC5 protein is unlikely to detect irisin in plasma samples.

A study with heart failure patients determined higher expression of both PGC1α and FNDC5 in subjects with higher aerobic performance, while no correlation was found in patients with low aerobic performance [38]. In the mouse exercise study performed in this project, PGC1α activation showed a trend, but was not signifi-

cantly upregulated and did not result in enhanced FNDC5 expression and plasma irisin level. Nevertheless, muscle-specific overexpression of PGC1α in transgenic mice showed a significant increase in FNDC5 mRNA level [24], which might indicate that a profound induction of PGC1α is necessary to activate the downstream target FNDC5. Up to now, there is only one study showing a robust activation of FNDC5 after exercise in humans measured by RT-PCR in muscle biopsies [24], however limited to a very small number of subjects.

Exercise enhanced putative brown adipocyte progenitor cells in brown AT [39] and was described as a new physiological stimulus for browning of the visceral fat in mice after controlled treadmill running [23] and free wheel running [24]. It has been shown that certain white AT depots have the capacity to shift in brown fat areas by cold exposure or stimulation with β3-adrenergic agonists [40, 41]. Increased UCP1 expression in white adipose tissue in response to external stimuli is described as a brite phenotype [22]. Several lines of evidence have suggested that bone morphogenetic proteins induce adipose cell fate determination in mammalian cells (reviewed in [42]). BMP-7 specifically triggers commitment of the multipotent mesenchymal cells into the brown adipocytes lineage, inducing the expression of brown fat-specific markers such as PRDM16 and UCP1 [7]. Embryos of BMP-7 knockout mice show a marked paucity of brown AT and nearly complete absence of UCP1, while adenoviral-mediated expression of BMP-7 in mice results in a significant increase in brown, but not in white AT and leads to an increase in energy expenditure [7]. Primary human adipocytes differentiated in vitro have a low basal level of UCP1 gene expression, as described for white adipocytes [32]. However, treatment of primary human preadipocytes with BMP7 during differentiation leads to an increase in PPARγ expression and an even more pronounced increase in UCP1 expression. In the present study, the induced cells are not brown adipocytes of the classic type. ZIC1, a marker for classical brown adipocytes [22], was not altered by BMP7 treatment and PRDM16 was barely detectable. The white adipocytes, emerging independently from the brown adipocytes, are molecularly identifiable through expression of TCF21 [22]. Primary human adipocytes isolated from subcutaneous AT and stimulated by BMP7 display enhanced UCP1 expression and decreased TCF21 expression. Cannon and Nedergaard raised the question how certain white-like adipocytes, which in general possess very few mitochondria, suddenly enhance their mitochondrial complement during the britening process and where these adipocytes originate from [43]. These important questions should be addressed by future studies.

Wu et al. isolated progenitor cells from subcutaneous white AT of mice, immortalized these cells and described the unique gene expression signature of adipocytes that could express UCP1 given an adequate stimulus. Genes expressed in a beige-selective manner include a developmental transcription factor (Tbx1), a component of lipid metabolism pathways (Slc27a1), as well as molecules known to be important in immune und inflammatory response pathways (CD40 and CD137) [32]. Thus, murine beige cells have a gene expression pattern distinct from either white or brown AT [32]. CD137 was used to define primary beige-fat-cell precursors and the CD137-high expressing group showed substantially elevated expression of UCP1 after treatment with irisin-Fc and recombinant FNDC5 compared to the CD137-low expressing cells [32]. In our study we could observe a 'britening' effect of human adipocytes after the treatment with BMP7, with the strongest effect in CD137-high expressing cells. However, neither recombinant FNDC5 nor the cleaved protein irisin had an effect on the britening of adipocytes in CD137-high expressing cells. Wu et al. examined the gene expression profile of brown fat from 11 adult humans and unexpectedly found that the profile was closer to that of mouse brite cells than to that of mouse classical brown cells [32], making adipocyte research from the energy-storing white fat cell to the energy-burning brown fat cell even more complex. Additionally, this study indicates that results obtained in the mouse model cannot always be extrapolated to humans.

In conclusion, human FNDC5 gene might be a transcribed pseudogene that has lost the ability to be effectively translated into a FNDC5 protein that than can be further processed to irisin.

Using an in vitro model to mimic contractile activity showed PGC1α-dependent activation of the FNDC5 gene, but only in mice cells. Serum levels of irisin were not increased in mice after four weeks of free wheel running, which might indicate that high-intensity exercise and strong activation of PGC1α is necessary to activate FNDC5. We observed no effect of recombinant FNDC5 and irisin on the britening of primary human adipocytes. Therefore, we conclude that the potential beneficial effects of irisin may not be relevant in humans.

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Duality of interest

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

Contribution Statement

S.R., M.E. and J.E. planned the majority of experiments, S.R. and J.E. wrote the paper. S.R., M.E., T.R. executed the adipocyte experiments. S.R. and M.S. analyzed the secretion of irisin. K.E. executed the EPS experiments. M.S. was responsible for the mice study. H.G. and B.B. performed the in silico analysis. U.S. performed RNA profiling. All authors contributed to the analysis and interpretation of the data and approved the final version of the manuscript.

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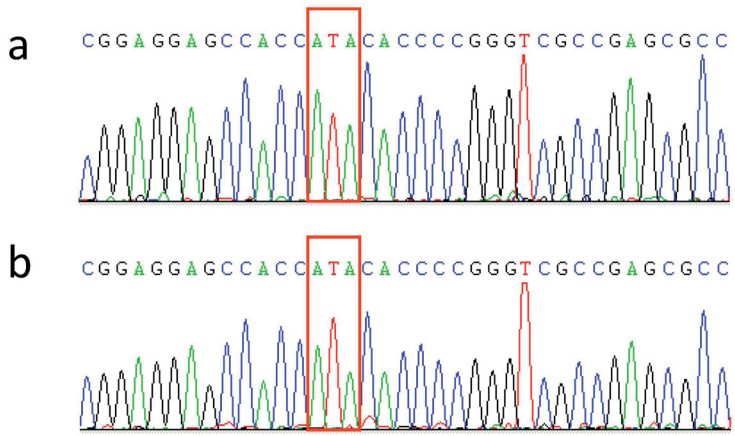

Suppl. Fig. 1. Genotyping of human FNDC5 exon 1 sequence. Source of mRNA for 5´-RACE was (a) human skeletal muscle and (b) human cerebellum. Identified sequences with bp 55-91 of human FNDC5 variant 2 (NM_153756) and variant 3 (NM_001171940).

```
NM_001171940.1  GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
NM_153756.2     GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
BF221649        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
AA908225        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCC-GCGCCCGCGC-GCGCTCC
BE467868        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
AA931673        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGC-CCCG-GCCG-GCTCC
BE502835        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
BF433165        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
BF108485        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
AI798367        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
AA913809        GAGCCACCATACACCCCGGGTCGCCGAG-GCCTGGCCGCCCCGCGCCCG-GCCG-GCTCC
BF445046        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
AI971851        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
BE468219        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
BE218308        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
BE551417        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
AI391724        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
AI937294        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
AW612671        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
AI458319        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
AI694150        GAGCCACCATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
DA685889        --------ATACACCCCGGGTCGCCGAGCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
DA070643        --------------------------GCGCCTGGCCGCCCCGCGCCCGCGCCGCGCTCC
```

Suppl. Fig. 2. Alignment of two Ref_Seq cDNAs (NM 001171940.1 and NM_153756.2) and 20 EST sequences covering the mutated start ATG to ATA codon and/or the CTG codon that should be the start ATG, if the FNDC5 protein sequence published by Böstrom et al. [24] is forced to match the exon1 sequence.

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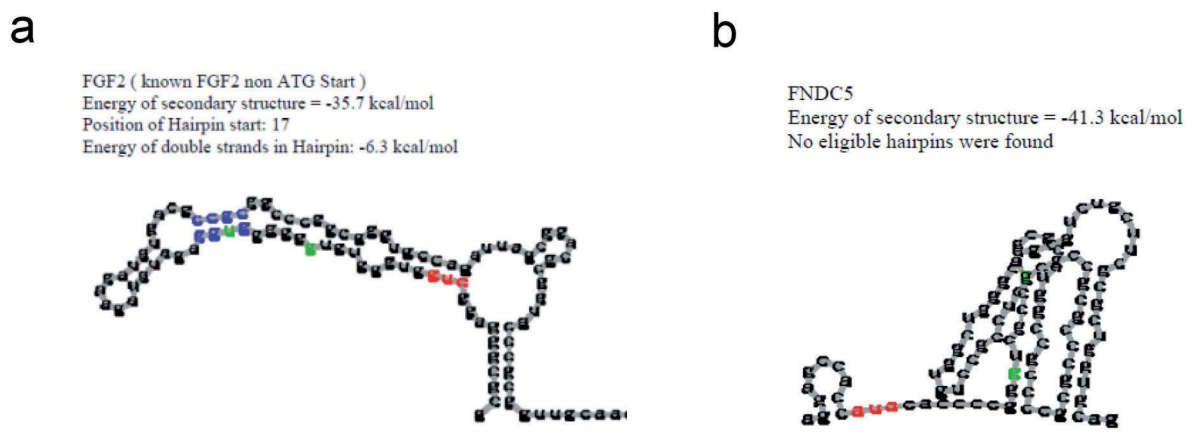
GAACGTCACCGTCAGGCACCTCAAGGCCAACTCTGCAGTGGTGAGCTGGGATGTTCTGGA
............................................................

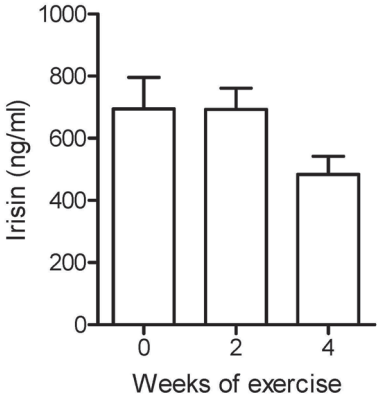
GGATGAGGTTGTCATCGGATTTGCCATCTCCCAGCAGAAGAAGGATGTGCGGATGCTGCG
.....................................................-M--L--R

CTTCATCCAGGAGGTGAACACCACCACCCGCTCATGTGCCCTCTGGGACCTGGAGGAGGA
--F--I--Q--E--V--N--T--T--T--R--S--C--A--L--W--D--L--E--E--D
```

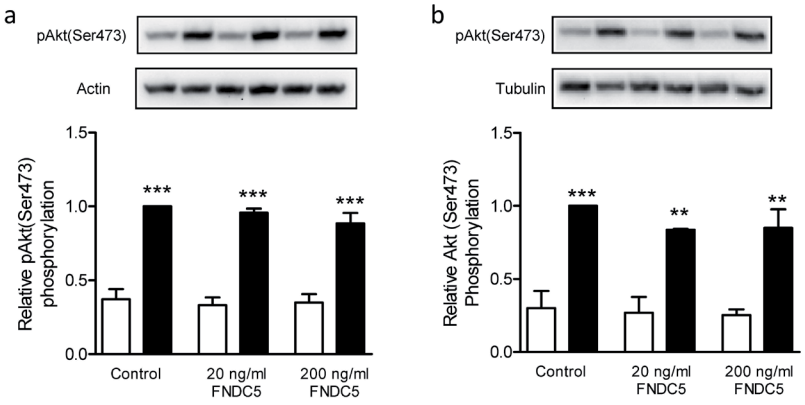
Suppl. Fig. 3. cDNA sequence showing the non-Kozak start ATG of NP_ 715637/NM_153756. Additionally, the 3 partial Kozak ATGs and the common stop codon for these 3 uORFs are boxed in red.



Suppl. Fig. 4. Using a program for prediction of a downstream hairpin which potentially increases initiation of translation at start AUG codon in a suboptimal context showed a positive result for FGF2 (a) and no result for FNDC5 (b).



Suppl. Fig. 5. Exercise training has no effect on irisin plasma levels in mice. 5 Twenty-eight week old C57/BL6N mice were placed individually in cages equipped with electronically monitored running wheels. The mice were then allowed to use the wheels ad libitum. Before exercise study and after 2 and 4 weeks of free-wheel running, plasma samples were taken. Irisin level were determined using EIA kit provided by Phoenix, n = 10-12. Data are presented as means±SEM.



Suppl. Fig. 6. Effect of FNDC5 on insulin signalling in human muscle cells and adipocytes. a+b Isolated preadipocytes from human subcutaneous AT (a) and human skeletal muscle cells (b) of different donors were differentiated and stimulated with 20 and 200 ng/ml for 24 hours at the end of differentiation followed by acute stimulation with insulin. Total cell lysates were obtained in a buffer containing 50 mmol/l HEPES (pH7.4), 1% (vol./vol.) Triton-X, PhosStop and compete protease inhibitor cocktail (Roche). Western Blot analysis was performed as described before [28]. Lysates were resolved by SDS-PAGE and immunoblotted with phospho-specific (p)-Akt antibody (Cell Signaling Technology). All data were normalised to the level of actin (abcam) (a) and tubulin (Calbiochem) (b) and are expressed relative to insulin-stimulated control values. Signals were visualised and evaluated on a work station (VersaDoc 4000 MP; BioRad) and analysed with an analysis software package (Quantity One, version 4.6.7, BioRad). Data are means±SEM; (a) n=6-7; ***p<0.001 vs. corresponding control; (b) n=3; ***p<0.001 and **p<0.01 vs corresponding control. White bars, basal; black bars, 100 nmol/l insulin. Data are presented as means±SEM.

Suppl. Table 1. Overview of used primers.

Gene	Supplier: Identifier	Forward Primer Sequenz (5'→3')	Reverse Primer Sequenz (5'→3')
Actin (human)	Qiagen: Hs_ACTB_2_SG	Sequence not provided	
Actin (mouse)	Qiagen: Mm_Actb_2_SG	Sequence not provided	
Adiponectin (human)	Qiagen: Hs_ADIPOQ_1 SG	Sequence not provided	
C/EBPα (human)	Qiagen: Hs_CEBPA_1_SG	Sequence not provided	
CD137 (human)	Eurofins MWG Operon	AGCTGTTACAACATA TAGCCAC	TCCTGCAATGATC TTGTCCTCT
FNDC5 (human)	Qiagen: Hs_FNDC5_1_SG	Sequence not provided	
FNDC5 (mouse)	Eurofins MWG Operon	ATGAAGGAGATGGG GAGGAA	GCGGCAGAAGAGAGC TATAACA
PGC1α (human)	Qiagen: Hs_PPARGC1A_1 SG	Sequence not provided	
PGC1α (mouse)	Eurofins MWG Operon	CCCTGCCATTGTTAA GACC	TGCTGCTGTTCCTGTTTTC
PPARγ (human)	Qiagen: Hs_PPARG_1 SG	Sequence not provided	
TCF21 (human)	Qiagen: Hs_TCF21_2_SG	Sequence not provided	
UCP1 (human)	Qiagen: Hs_UCP1_3_SG	Sequence not provided	
ZIC1 (human)	Qiagen: Hs_ZIC1_1_SG	Sequence not provided	

Suppl. Table 2. Gene symbols and corresponding TaqMan assay IDs provided by Applied Biosystems used for microfluidic card real-time PCR analysis.

Gene Symbol	Assay ID
ADIPOQ	Hs00605917_m1
ADRB1	Hs02330048_s1
ADRB2	Hs00240532_s1
CAV1	Hs00971716_m1
CEBPA	Hs00269972_s1
CEBPB	Hs00270923_s1
CEBPG	Hs01922818_s1
CIDEC	Hs01032998_m1
CPT1A	Hs00912671_m1
CYCS	Hs01588974_g1
DGAT1	Hs00201385_m1
DGAT2	Hs01045913_m1
FABP4	Hs01086177_m1
LEP	Hs00174877_m1
LPL	Hs00173425_m1
NRF1	Hs00192316_m1
OXR1	Hs00250562_m1
PLIN1	Hs00160173_m1
PLIN2	Hs00605340_m1
PLIN3	Hs00998421_m1
PLIN4	Hs00287411_m1
PLIN5	Hs00965990_m1
PNPLA2	Hs00386101_m1
PPARA	Hs00947539_m1
PPARD	Hs04187066_g1
PPARGC1A	Hs01016719_m1
PPARGC1B	Hs00991677_m1
PRDM16	Hs00922674_m1
RN18S1	Hs03928985_g1
SIRT1	Hs01009005_m1
SLC2A1	Hs00892681_m1
SLC2A4	Hs00168966_m1
TFAM	Hs00273372_s1
UCP1	Hs00222453_m1
UCP2	Hs01075227_m1
UCP3	Hs01106052_m1
VDAC1	Hs01631624_gH

2.6 Contribution Statement

Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways

Lambernd S, Taube A, Schober A, Platzbecker B, Görgens SW, Schlich R, Jeruschke K, Weiss J, Eckardt K, Eckel J (2012) Diabetologia 55: 1128-1139
Impact Factor: 6.8

SL contributed to the concept, acquired, analysed and interpreted data, wrote the manuscript and had the main responsibility together with JE. Unless otherwise stated, SL performed experiments and analyzed the data. SL and AT performed analysis of OXPHOS protein level and fatty acid oxidation. AT performed JC-1 staining. AS and BP supported cell culutre cultivation. SWG and SL analyzed mitochondrial markers by RT-PCR. RS and SL analyzed VEGF secretion. KJ and JW were responsible for the analysis by electron microscopy. KE and JE contributed to the concept, analysis of the data, and the discussion and revision of the manuscript.

Adipokines promote lipotoxicity in human skeletal muscle cells

Taube A, **Lambernd S**, van Echten-Deckert G, Eckardt K, Eckel J (2012) Arch.Physiol Biochem. 118: 92-101
Impact Factor: to be released
Cites per document (2y), computed by SCImago Journal Rankings using the same formula as for journal impact factor: 3.3

AT had the main responsibility together with KE and JE. Unless otherwise stated, AT performed experiments and analyzed the data. GED performed analysis of DAGs. SL and AT designed and executed EPS-treatment protocols. SL and AT performed analysis of OXPHOS and CD36 protein level. SL contributed to the concept of the manuscript.

Identification and validation of novel contraction-regulated myokines released from primary human skeletal muscle cells

Rascke S, Eckardt K, Bjørklund Holven K, Jensen J, Eckel J (2013) PLOS ONE, in revision
Impact Factor: 4.1

SR contributed to the concept, acquired, analysed and interpreted data, wrote the manuscript and had the main responsibility together with JE. Unless otherwise stated, SL performed experiments and analyzed the data. KBH and JJ obtained plasma samples. KE was responsible for antibody cytokine array analysis.

Regulation of follistatin-like protein 1 expression and secretion in primary human skeletal muscle cells

Görgens SW*, **Raschke S***, *, Bjørklund Holven K, Jensen J, Eckardt K and Eckel J (2013) Arch.Physiol Biochem, accepted for publication
* Both authors contributed equally to this work.
Impact Factor: to be released
Cites per document (2y), computed by SCImago Journal Rankings using the same formula as for journal impact factor: 3.3

SR contributed to the concept and had the main responsibility together with JE. SWG and SR performed experiments to characterize Fstl1 in skeletal myotubes. SR performed insulin signalling data. KBH and JJ obtained plasma samples. KE analyzed Fstl1 plasma levels. SR and SWG wrote the manuscript. KE and JE contributed to the discussion and revision of the manuscript.

Evidence against a beneficial effect of irisin in humans

Raschke S, Elsen M, Gassenhuber H, Sommerfeld M, Schwahn U, Brockmann B, Romacho T, Eckardt K, Eckel J (2013) Diabetologia, submitted

Impact Factor: 6.8

S.R., M.E. and J.E. planned the majority of experiments, S.R. and J.E. wrote the paper. S.R., M.E., T.R. executed the adipocyte experiments. S.R. analyzed the secretion of irisin. K.E. executed the EPS experiments. M.S. was responsible for the mice study. H.G. and B.B. performed the in silico analysis. U.S. performed RNA profiling. All authors contributed to the analysis and interpretation of the data.

3 Discussion

3.1 A novel model to study skeletal muscle contraction

It has been proven that exercise mediates beneficial systemic effects in subjects suffering from insulin resistance or type 2 diabetes. Physical activity may engage three different possible mechanisms to mediate its beneficial effects: (i) reduction of adipose tissue mass and subsequent reduction of circulating inflammatory cytokines, (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 with a beneficial impact on skeletal muscle or whole body metabolism.

Exercise training modulates the actions of enzymes, transcription factors, transporters and chaperones in skeletal muscle including AMPK, MAPK, PGC1α, NFκB, GLUT4, CD36, CaCMMKβ, and myokines. This highlights the complexity of the metabolism during contraction. However, knowledge of the interplay between different molecular signalling pathways during exercise is still incomplete and experimentally adequate models of exercise remain elusive. Long-time exercise-mimetics, such as AICAR, GW1516, and caffeine were used to study the molecular effects of contraction on skeletal muscle cells. These components activate single pathways involved in metabolic homeostasis and do not activate all enzymes, transcription factors, transporters, and chaperones which contribute to contraction metabolism. The major limitation of these models is the lack of contraction, a central characteristic of muscle cells. Thus, the first aim of this study was to establish an *in vitro* contraction model for comprehensive characterization of exercise signalling pathways and their manipulation. This study represents the first approach using EPS of primary human skeletal myotubes (220). EPS results in vigorous contraction of the myotubes and formation of sarcomeric structures, as visualised by immunofluorescence staining of α-actinin and electron microscopy. The contractile activity of myotubes activates AMPK, increases glucose transport and CD36 protein level, alters NFκB signalling, and changes the secretion pattern of myokines (220-222) (Table 1). Consequently, our approach influences not only a single pathway; instead EPS of human myotubes modulates the whole cell physiology.

Table 1: Alteration of signalling pathways in primary human skeletal myotubes after electrical pulse stimulation (EPS). Activity of signalling pathway is increased (⇧), decreased (⇩) and was not changed (⇨) by EPS.

	Alteration by EPS	*Published in*
Phosphorylation of AMPK	⇧	(220)
Glucose uptake	⇧	(220)
GLUT4 protein level	⇨	(220)
Insulin signalling	⇨	(220)
Induction of insulin resistance	blocked	(220)
CD36 protein level	⇧	(221)
Fatty acid oxidation	⇨	(221)
Glucose oxidation	⇧	(220)
Mitochondrial protein content	⇧	(220)
NFκB signalling	⇩	(220)
MAPK phosphorylation	⇨	(220)
Secretion of VEGF and IL-6	⇧	(220)
Secretion of novel myokines	⇧ ⇨ ⇩	(222)

Using the protocol of 1 Hz, 2 ms, and 11.5 V for 8 to 24 hours resembles the effect of an acute bout of exercise, rather than the effects of endurance exercise. Contraction as well as insulin stimulates glucose transport by separate pathways (65-67). Since insulin signalling and GLUT4 protein levels are not altered, the increase in contraction-regulated glucose uptake is due to an activation of AMPK, which is in turn activated by the contractile activity and the increase in AMP (220).

In skeletal muscle, three energy systems restore ATP, namely creatine kinase, glycolysis and mitochondrial respiration (223). The contractile activity of human myotubes induced by EPS results in an enhanced glucose uptake and glucose oxidation and increases lactate concentration (220). During high-performance exercise, 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^+ (223). As lipid accumulation and fatty

acid oxidation in human myotubes were not altered by EPS, it is likely that cells mainly use glucose as energy source for ATP regeneration under the applied conditions.

Since we could prove that EPS resembles some major effects of contraction, our strategy was to validate the beneficial effects of contractile activity on insulin resistance in primary human myotubes.

3.2 Anti-inflammatory effect of contraction

One breakthrough in diabetes research was the finding that adipose tissue is a major endocrine organ, which secretes hundreds of different adipokines (114;115). The secretion of adipokines is changed dramatically with obesity, affecting a wide range of physiological functions and leading to insulin resistance in skeletal muscle, among other tissues (142;157). A main finding of this study was the improved insulin sensitivity of contracting human myotubes in conditions of insulin resistance.

Contractile activity inhibits insulin resistance

Cell culture models provide easily accessible systems for detailed analysis of mechanisms potentially underlying the pathogenesis of insulin resistance. As previously shown by our group, adipocyte-conditioned medium induces insulin resistance in human skeletal myotubes. Adipocyte-conditioned medium, which contains the whole secretory output of mature adipocytes with more than twohundred identified adipokines (115), reduced insulin-stimulated Akt and GSK-3 phosphorylation, and diminished insulin-stimulated glucose uptake (118). Similar results have been shown treating human myotubes with single adipokines e.g. MCP-1 (132), PEDF (131), chemerin (130), and DDP4 (133). This is a valuable *in vitro* model to study and to dissect mechanisms leading to insulin resistance on molecular level.

We combined this insulin resistance model with the novel contraction model to analyse the cross-talk between adipocytes and contracting myotubes, with the overall goal to analyze the signalling pathways and mechanisms involved in the beneficial effects of muscle activity. In

this study, we showed that EPS completely prevented insulin resistance on the level of Akt and GSK3 during incubation with adipocyte-conditioned medium, MCP-1 and chemerin (220). Up to now, we have only analyzed the phosphorylation of the classical insulin signalling cascade. Future studies will address downstream targets like GLUT4 translocation and glucose uptake.

Contractile activity diminished inflammatory signalling

Depending on the type of exercise, human and rodent studies report divergent results for the activation status of NFκB after exercise. In rodents, endurance exercise like acute treadmill running increases NFκB activity in muscle (224;225), while human studies revealed that resistance exercise reduced NFκB activity measured immediately and in the post-exercise period (226;227). The discrepancy between these findings is likely related to the different time points and different modes of exercise (endurance vs resistance exercise) that were studied. These two modes of exercise activate different signalling pathways in skeletal muscle and have different effects on oxidative stress, a well known activator of this pathway. Acute muscle contraction generates reactive oxygen species (ROS), while chronic exercise reduces ROS (228). However, in our *in vitro* model EPS diminished the NFκB protein level and thus the level of activated NFκB.

Contractile activity diminished adipokine-induced inflammatory signalling

Chronic low-grade systemic inflammation is often associated with insulin resistance and type 2 diabetes. In this context, NFκB has been proposed as a critical bridge between insulin signalling and chronic inflammation. IκB protein abundance, the inhibitory protein of NFκB, is reduced in muscle of type 2 diabetic subjects (229). Thus, basal NFκB activity is significantly elevated in obese and type 2 diabetic subjects (230). Enhanced NFκB signalling is thought to inhibit insulin signalling by phosphorylating serine residues in IRS-1 (231). This impairs the ability of IRS-1 to activate downstream targets of the classical insulin-stimulated signalling cascade.

We could show that an acute incubation of myotubes with TNFα induces a strong inflammatory response in human skeletal myotubes. IκBα is degraded and NFκB significantly phosphorylated (220). After EPS, the increment of NFκB activation by TNFα treatment was profoundly diminished (220). In this model, the inhibitory protein of NFκB, IκBα, is not phosphorylated and degraded by TNFα treatment, when the cells were electrically stimulated to contract before. Additionally, the ratio of IκBα to NFκB protein level increased, since NFκB protein level was decreased after EPS. Thus, contractile activity of human myotubes seems to directly inhibit TNFα signalling. It might be speculated that the activation of NFκB is inhibited by blocking the degradation of IκBα in contracting myotubes (Figure 6).

A second link between the contractile activity and blocked inflammatory signalling might be the predominant intracellular receptor for Ca^{2+} signals; calmodulin (CaM). Hughes et al. published that CaM is essential for the activation of NFκB and CaM inhibitors prevent NFκB activation due to the prevention of inducible IκBα phosphorylation (232).

Emerging results indicate a third link between contractile activity and inflammatory signalling. AMPK signalling can inhibit the inflammatory response induced by the NFκB system (reviewed in (233)). Steinberg et al. observed that TNFα signalling through TNF receptor (TNFR) 1 suppresses AMPK activity via transcriptional upregulation of protein phosphatase 2C, an inhibitor of AMPK signalling (234). This in turn reduces ACC phosphorylation, suppresses fatty-acid oxidation, increases intramuscular diacylglycerol (DAG) accumulation, and causes insulin resistance in skeletal muscle. The suppressive effects of TNFα on AMPK signalling are reversed in TNFR1 and 2 null mice (234). *In vitro* experiments demonstrated that lipopolysaccheride-induced inflammatory response can be inhibited by activation of AMPK with AICAR (235-237). The anti-inflammatory effect of metformin, also an AMPK activator, was abolished by the knockdown of α1AMPK in endothelial cells (238), while constitutively active α1AMPK suppressed NFκB signalling in macrophages (239).

The NFκB subunits are not direct phosphorylation targets of AMPK, but the inhibition of NFκB signalling is mediated by several downstream targets of AMPK. For sirtuin-1 (SIRT1) an activation by AMPK has been shown (240), and SIRT1 can stimulate the activity of LKB1, which subsequently activates AMPK (241). This represents a positive feedback loop to

enhance cellular survival during energy deficiency. Additionally, SIRT1 was shown to interact with the RelA/p65 subunit of the NFκB complex. It deacetylates p65, which inhibits NFκB signalling (242).

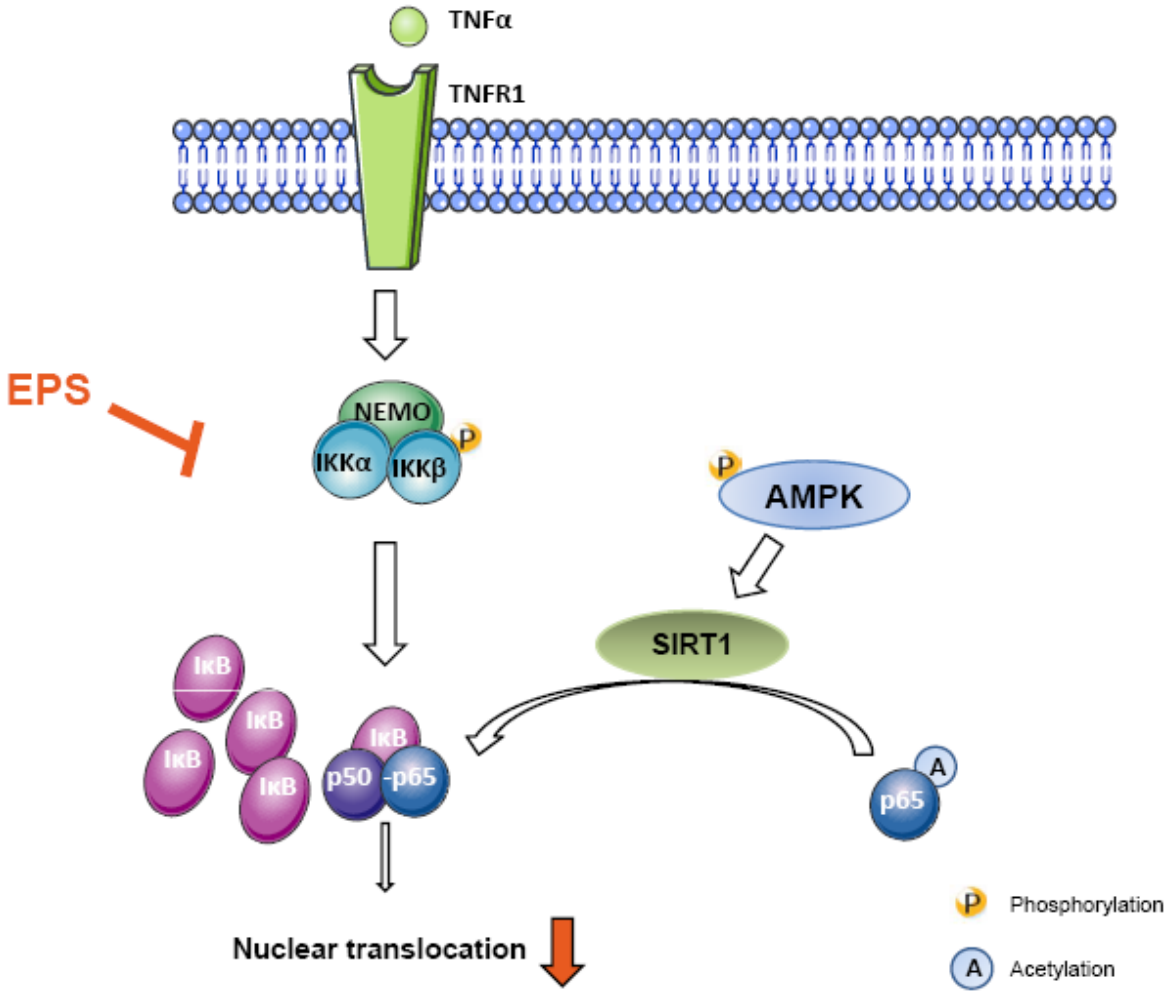


Figure 6: Contractile activity of myotubes influences classical NFκB signalling cascade. NFκB dimers are held in the inactive state by IkB. While receptor signalling leads to activation of a multisubunit IkB kinase (IKK) complex which phosphorylates IkB marking it for degradation by the ubiquitin pathway, electrical pulse stimulation (EPS) blocks the phosphorylation and degradation of IκB. The ratio of IκBα to NFκB protein level increases, since only NFκB protein level was decreased after EPS. Additionally, AMPK activates SIRT1, which deacetylates p65 and subsequently inhibits NFκB signalling. Taken together, after contraction NF-kB dimers translocate less to the nucleus.

NFκB has been proposed as the major link between insulin signalling and chronic inflammation. Thus, NFκB might be an interesting target for new types of anti-inflammatory, pharmacological treatment of obesity and type 2 diabetes. Salicylate prevents fat-induced insulin resistance in rat skeletal muscle (243). Aspirin and sodium salicylate inhibit the activation of NFκB, although only in relatively high concentrations (244). Additionally, AMPK was already in the focus of intensive drug discovery projects due to its beneficial effect on glucose uptake in the context of insulin resistance. This novel link between AMPK and NFκB signalling emphasizes that AMPK activators, e.g. AICAR, metformin, statins, and thiazolidinediones, have the capacity to repress a pro-inflammatory phenotype.

On the other hand, inactivation of NFκB for longer periods might have adverse side effects, since this pathway is a critical factor in the immune response and other defense mechanisms. Infusion with antioxidant N-acetylcysteine attenuates early positive adaptive responses to exercise in human skeletal muscle (245). Future studies will highlight, if the NFκB signalling pathway is an adequate target for the treatment of insulin resistance (245). Lifestyle modifications, combining dietary intervention and general exercise, remain the most important approach to combat obesity and insulin resistance, although the long-term success rate is diminished compared to pharmacotherapy and bariatric surgery (reviewed in (246)). No single agent will probably ever mimic the broad range of exercise-related health benefits. Targeting single aspects of the exercise response, like activation of AMPK, blocking NFκB signalling, enhancing insulin sensitivity, is likely to yield health benefits by an increase in energy expenditure, although it will most likely come along with adverse side effects.

3.3 Contractile activity does not influence lipotoxicity in human skeletal myotubes

In addition to increased inflammatory cytokine levels, type 2 diabetic patients frequently exhibit increased plasma lipid levels.

Under normal conditions, FFA plasma levels increase during fasting and decrease after a carbohydrate-rich meal since insulin suppresses lipolysis in adipose tissue. Plasma FFA concentrations may rise in the overnight fasted state from physiological normal level of 0.1-0.5 mmol/l to pathological levels of 0.6-0.9 mmol/l in obesity and type 2 diabetes (247). It has been shown that hyperglycemia, skeletal muscle insulin resistance, and the risk to develop type 2 diabetes correlate with the degree of plasma FFA (247).

Whereas small amounts of intracellular triglycerides represent an important energy source for skeletal muscle in periods of low glucose levels, excessive accumulation of FFA and lipid metabolites by dietary fat and altered lipolysis have been demonstrated to impair cell metabolism, known as lipotoxicity.

Raising plasma fatty acids in both rodents (248) and humans (249) abolishes insulin receptor tyrosine phosphorylation of IRS-1 activated by insulin (250). An imbalance of fatty acid uptake and ß-oxidation contributes to the accumulation of lipid metabolites such as long chain acyl CoAs, diacylglycerols (DAG), ceramide and triglycerols (251;252). Increased lipid metabolites in skeletal muscle are described to activate a number of different serine/threonine kinases such as protein kinase C (PKC) that negatively modulate insulin action (252-255). Impaired skeletal muscle mitochondrial function, including decreased capacity to oxidize fat, is observed in type 2 diabetic patients and has been suggested to play a central role in this context (255;256).

Taken together, beside an adipokine-induced insulin resistance a mechanism of fat-induced insulin resistance in skeletal muscle has been elucidated.

Adipokines promote lipotoxicity of palmitic acid

As a number of *in vitro* studies examined lipid accumulation or inflammatory signalling of adipokines as single factors, we aimed to investigate the consequences of the combined effect of FFA and adipocyte-conditioned medium to closely mimic the physiological complexity. Therefore, we studied lipid accumulation after co-incubation of skeletal muscle cells with relatively low concentrations of 100 µmol/ml palmitic acid and adipocyte-conditioned medium (221). Most interestingly, this study demonstrated a novel role for adipokines in the pathogenesis of type 2 diabetes. The lipotoxic effect of palmitic acid on primary human skeletal myotubes was tremendously increased by co-incubating fatty acid with adipokines. Co-incubation resulted in massive lipid accumulation (5.3fold) and 60% reduction of palmitic oxidation.

In view of their hydrophobic nature, fatty acids could cross the plasma membrane by simple diffusion. Passive flip-flop of the un-ionized form of fatty acids can occur rapidly and in a protein-independent manner across the lipid bilayer phase (reviewed in (235)). Alternatively, transmembrane protein CD36, acts as acceptor for fatty acids to increase their concentration at the cell surface and thus enhance the number of fatty acid diffusion events or facilitate the transport of fatty acids across the phospholipid bilayer (uptake by facilitated diffusion, reviewed in (235)). From experiments with cardiomyocytes from wildtype and CD36 null mice, it is known that the contribution of CD36 to fatty acid uptake is about 70% (257).

Beside palmitic acid, adipocyte-conditioned medium strongly increased CD36 protein levels in human myotubes. The investigation of a second fatty acid transporter, namely FATP4, revealed a selective upregulation of CD36 protein abundance by CM. Heat-inactivation of adipocyte-conditioned medium prevents this effect indicating that possible causative agents are protein factors. Several factors were already described to upregulate CD36 in different cell types like interleukin-4 in monocytes (258), adiponectin in L6 myotubes (259) and thrombospondin-1 in the carcinoma cell line A431 (260). Adiponectin (131) and thrombospondin-1 (115) are components of the adipocyte-conditioned medium and might be the responsible factors. However, since adipocytes secrete several hundred adipokines (115),

we presume that most likely a combination of several yet undefined factors might upregulate CD36 protein level in myotubes.

CD36 is stored in intracellular compartments, similar to GLUT4, and physiological stimuli induce the translocation of CD36 vesicles from intracellular membrane compartments to the sarcolemma (261). Permanent relocation of CD36 to the sarcolemma and increased fatty acid uptake were strongly linked in rodent models for insulin resistance (262-264) and obese humans (265;266). High fat-fed rats show an enhanced efficiency of fatty acid uptake which contributes to lipid accumulation in skeletal muscle. The accumulation of intramuscular lipid could be directly linked to increased CD36 mRNA expression (267). A human study showed that the triacylglycerol accumulation in human obesity and type 2 diabetes is associated with increased rates of skeletal muscle fatty acid transport and increased sarcolemmal CD36 (265). In accordance with these findings, it has been shown in animal models that ablation of CD36-mediated lipid uptake in muscle or liver prevents lipotoxicity (268).

Complete fatty acid oxidation strongly relies on intact and active mitochondria. The protein abundance of OXPHOS complexes in human myotubes were not affected by one of the treatments, however the co-incubation of palmitic acid and adipocyte-conditioned medium leads to diminished mitochondrial integrity. Impaired or insufficient oxidation of fatty acids results in generation of harmful lipid signalling molecules. Indeed, we observed DAG1,2 and DAG1,3 to be increased drastically in myotubes after incubation with palmitic acid and adipocyte-conditioned medium (221).

To summarize, in insulin resistance and type 2 diabetes CD36 protein level might be enhanced by increased levels of adipokines and FFA serum levels, CD36 permanently relocates to the sarcolemma in skeletal muscle, leading to increased fatty acid uptake and intracellular lipid accumulation, thereby promoting lipotoxicity (Figure 7). This study revealed a new role for adipokines in the pathogenesis of insulin resistance, thus adipokines promote lipotoxicity of palmitic acid in human myotubes, notably of low concentrations. This implies an increased lipotoxic risk already at an early stage of the pathologic development of insulin resistance.

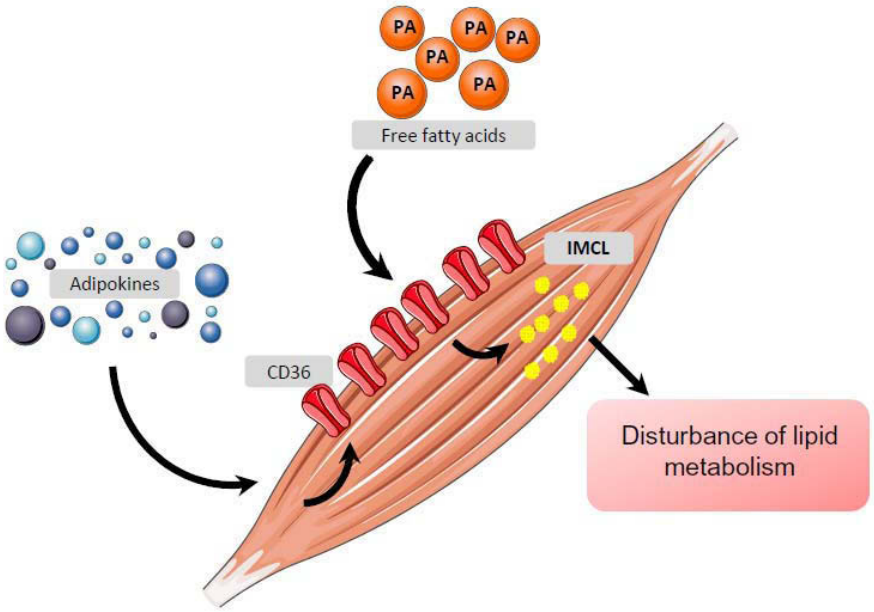


Figure 7: Adipokines promote lipotoxicity in skeletal myotubes. Combined exposure of skeletal muscle cells with adipokines and palmitic acid (PA) increases fatty acid uptake and leads to accumulation of (IMCL) intramyocellular lipid droplets or possibly lipid intermediates, respectively. As a result, fatty acid metabolism is impaired.

Effect of contractile activity on lipotoxicity

Physical exercise is a major regulator of skeletal muscle metabolism and potently influences mitochondrial biogenesis and function. Exercise stimulates mitochondrial biogenesis through PGC1α activation, which leads to an increase in the target genes, including nuclear respiratory factor (NRF)-1. NRF-1 is a transcription factor stimulating many nuclear-encoded mitochondrial genes such as OXPHOS genes. Using the established EPS model, PGC1α activation and protein levels of OXPHOS proteins were increased (220). Therefore, we asked the question, whether contraction of human myotubes induced by EPS could counteract the defects in palmitic acid oxidation induced by combined application of palmitic acid with adipocyte-conditioned medium.

However, contractile acitivity of human myotubes in parallel with palmitic acid and adipocyte-conditioned medium did not alter excessive lipid accumulation and severe reduction of palmitic acid oxidation. The used conditions for the induction of contraction in skeletal mytobues mainly activated glucose metabolism (220). We observed enhanced glucose uptake and increased lactate concentrations after EPS, but no activation of fatty acid oxidation (220). Although, enhanced availability of palmitic acid in the medium leads to increased lipid uptake. Randle et al. postulated a mechanism describing the competition between FFA and

glucose for mitochondrial oxidation. This leads to decreased glucose utilization in the presence of increased lipid concentrations (269). The rather low lipid concentrations used in these experiments of 100 µmol/l and high glucose concentration in the medium of 5.5 mmol/l did not lead to an increase in lipid oxidation. Future experiments should address how lower glucose concentrations could affect metabolic flexibility.

It has been reported that saturated and unsaturated fatty acids are differentially stored and metabolized with more detrimental effects induced by saturated fatty acids like palmitic acid (270;271). While unsaturated fatty acids are accumulating as lipid droplets (272), saturated fatty acid, as shown in our work for palmitic acid, leads to a more diffuse staining of fatty acids covering the entire myotubes. It may be speculated that diffuse distribution combined with further adverse metabolism of saturated palmitic acid leads to generation of harmful lipid metabolites such as DAG. These metabolites induce profound cellular impairments, which cannot be prevented by the used protocol for contractile activity of the myotubes.

3.4 Identification of novel myokines

It is well accepted that physical activity exerts multiple beneficial effects on the prevention of chronic diseases, both due to an improved energy balance and due to effects independent of obesity. A relatively new research concept proposes that the skeletal muscle is a major endocrine organ releasing myokines, which might in part be responsible for the beneficial effect of exercise (273;273). It is assumed that myokines play a pivotal role in the communication between muscle and other tissues such as pancreatic cells (274). One key step in getting a more comprehensive understanding of the beneficial effect of exercise in the context of chronic diseases is the detailed characterization of the human skeletal muscle secretome of contracting cells. For this aim to be achieved, first we focused on the identification of myokines released by contracting myotubes, followed by the second step; the differential analysis of contracting and non-contracting muscle cells for the identification of contraction-regulated myokines and finally, the validation of selected myokines.

Comprehensive profiling of skeletal muscle secretome

For the first part, supernatants of contracting primary human skeletal muscle cells were analyzed. Currently no biochemical technique exists that can efficiently separate and consistently detects the total protein composition of the cellular secretome. Hence, it is important to combine various technical approaches (275). Within this study, we combined two complementary, orthogonal proteomic profiling techniques, namely two-dimensional polyacrylamide gel electrophoresis/matrix-associated laser desorption/ionization mass-spectrometry (2D-PAGE/MALDI-MS) and one-dimensional SDS-PAGE/liquid chroma-tography-electrospray ionization (1D-PAGE/LC-ESI-MS/MS). Skeletal muscle proteomics attempts to establish the global identification and biochemical characterisation of all members of the secreted muscle proteins induced by contraction. We supplemented the study by cytokine antibody array technology for the analysis of as many different classes of secreted muscle proteins as possible.

We found by the combined MS profiling approach, followed by computer-based prediction of protein secretion approaches, 276 secreted proteins from primary human skeletal myotubes. A drawback of this approach is the need of relatively high amounts of starting material. A total volume of 800 ml conditioned medium from myotubes was collected, concentrated to a final volume of 200 µl. Although skeletal muscle secretes hundreds of proteins, the amount of secreted proteins is relatively low e.g. compared to primary human adipocytes (Table 2).

Additionally to the proteomics approach, cytokine antibody array analysis detected 121 proteins (222), which were mainly not found by the used MS approaches. Less than 5% of the identified proteins could be detected with both approaches. Proteins of the interleukin and fibroblast growth factor family were only found by cytokine antibody analysis. These results illustrate the importance to combine different techniques to provide a comprehensive analysis to identify as many myokines as possible.

Table 2: Concentrations of various factors in conditioned medium from primary human adipocytes and primary human myotubes. DPP, dipeptidyl peptidase; IL, interleukin; PEDF, pigment epithelium-derived factor; VEGF, vascular endothelial growth factor. Concentrations of secreted factors were obtained by enzyme-linked immunosorbent assay. Data are means ± SEM, n ≥ 3.

Secreted Factor	*Concentration in Adipocyte-conditioned medium (ng/ml)*	*Concentration in skeletal muscle-conditioned medium (ng/ml)*
Chemerin	2.175 ± 0.294	0.006±0.001
DDP4	2.19 ±1.37	0.69 ± 0.18
IL-6	0.025 ± 0.002	0.015 ±0.003
IL-8	0.055 ± 0.013	0.193 ± 0.086
Myostatin	12.64 ± 4.44	3.44 ± 1.638
PEDF	45.7 ± 0.82	5.4 ± 0.86
VEGF	0.329 ± 0.09	0.054 ± 0.03

The list of 276 myokines predicted to be secreted and found by both MS-analyses was compared with published secretome analyses of murine or rat myotube cell lines and of primary human myotubes (213;215-217;276-278). Our proteomics study uncovered 62 potential novel myokines, which have not been reported in previous approaches. For secreted myokines identified by antibody array analyses, existing data in the literature were carefully screened in order to identify candidates that have been described before to be secreted by skeletal muscle cells. To the best of our knowledge, we consider another 52 proteins as novel myokines (222).

Adipo-Myokines

Recently, our research group published the secretome of primary human adipocytes (115). Comparing the results of the proteomic analysis of the secretome of primary human adipocytes (115) (http://diabesityprot.org/) with the secretome of primary human myotubes revealed an extensive overlap of the identified secreted proteins. While 120 proteins seem to be exclusively secreted by adipocytes and 132 proteins by myotubes, about one half of the detected myokines (143 proteins) are secreted by primary human adipocytes as well as by primary human myotubes. We termed these proteins **adipo-myokines**.

The most intensively studied adipo-myokines in the current literature are IL-6, IL-7, IL-8, IL-15 and BDNF and are discussed in several reviews (279-283), (Table 3).

Table 3: Overview of selected adipo-myokines cited in reviews. IL, interleukin; BDNF, brain derived neurotrophic factor; n.d., not described; ✓, association has been shown in indicated publications; ✓, contradictory data published; **x**, association has not been shown.

Adipo-Myokine	*Associated with obesity*	*Associated with insulin resistance/T2D*	*Contraction-Regulated*	*Associated with improved glucose metabolism*
IL-6	✓ Plasma IL-6 is positively related to fat mass (185), elevated in type 2 diabetics (284;285)	✓ IL-6 promotes insulin resistance (286-288)	✓ High intensity exercise in humans increased plasma levels (171)	✓ Insulin-sensitizing effect in skeletal muscle (289;290), increases whole body fat oxidation (291)
IL-7	✓ Increased mRNA level in omental adipose tissue (292), although mice overexpressing IL-7 have reduced adipose tissue mass (293)	n.d.	✓ mRNA level in-creased in human muscle after strength training (294)	n.d.
IL-8	✓ Higher expression in visceral adipose tissue in type 2 diabetics and insulin resistant subjects (286;295)	✓ IL-8 plasma levels correlate with measures of insulin resistance (183;296)	✓ mRNA levels and plasma levels increased after exhaustive exercise (297-299) and after resistance training (227), but not after concentric exercise (300;301)	n.d.
IL-15	x Negative association between plasma IL-15 and total fat mass (302)	x IL-15 improves glucose homeostasis and insulin sensitivity (209)	✓ Increased in human serum after treadmill running (206) before contradictory data (203;205;303)	✓ IL-15 stimulates adiponectin secretion (207), improves glucose homeostasis and insulin sensitivity (209)
BDNF	x Plasma BDNF levels decraease in type 2 diabetics independently of obesity (304)	x Serum BDNF is not associated with metabolic syndrome (305), decraease in type 2 diabetics (304)	✓ EPS of C2C12 cells increased BDNF mRNA and protein level (189), EPS of human cells did not increase secretion (222)	✓ BDNF improves blood glucose levels in diabetic mice (306-308)

Nevertheless, the myokines IL-6, IL-7 and IL-8 are also known as adipokines and are described to be associated with obesity, insulin resistance and type 2 diabetes in contrast to IL-15 and BNDF (Table 3). IL-15 is most likely contraction-regulated (203;205;206;303) and is described to improve glucose homeostasis and insulin sensitivity (209).

Interleukin-6 was one of the first detected myokines and is up to now the most intensively studied myokine.

IL-6 – the prototype adipo-myokine

IL-6 is described as a myokine, produced in the working muscle during exercise. The level of circulating IL-6 increases after an acute bout of exercise in an exponential fashion in response to exercise (172;175;176), and declines in the post-exercise period (177). However, during one year of training intervention plasma levels of IL-6 remained unchanged (309). Additionally, the quantitative release from adipose tissue correlates positively with increasing body fat content resulting in systemic elevation of IL-6 plasma levels (284), while IL-6 mRNA expression decreased in subcutaneous and visceral adipose tissue 12 month after bariatric surgery of obese patients (310). IL-6 is associated with type 2 diabetes (284;285) and has been shown to inhibit insulin signalling pathways in the liver (287;288) and adipocytes (286).

For skeletal muscle cells, i*n vitro* studies showed that a rather brief challenge of minutes to few hours with recombinant IL-6 (about 200 ng/ml) enhanced insulin-stimulated Akt phosphorylation in rat L6 myotubes (311) and primary human myotubes (20 ng/ml) (312;313). IL-6 treatment of rat L6 myotubes increased basal and insulin-stimulated glucose uptake and translocation of GLUT4 to the plasma membrane after 5-120 min (1-100ng/ml) (290). Furthermore, IL-6 rapidly and markedly increased AMP-activated protein kinase (AMPK) and increased fatty acid oxidation (290;314). On the other hand, incubation of the rat L6 myotubes with 200 ng/ml recombinant IL-6 induced insulin resistance on the level of diminished Akt phosphorylation after 96 h (311) and in primary human myotubes after 48 h (128).

These findings support the hypothesis that the myokine IL-6 is important for muscle metabolism during contraction, whereas the chronic elevation of IL-6 released from

adipocytes may induce insulin resistance. Additionally, Weigert et al. propose a different influence of IL-6 depending on the target tissue: In energy-supplying tissues like the liver and fat the insulin signal is attenuated, whereas in energy-utilizing tissues like the skeletal muscle insulin action is improved (289).

PEDF and DDP4 as adipo-myokines

Within this study we validated PEDF and DDP4 as novel myokines (222), (Table 4). Both proteins were identified within an adipocyte secretome study (115) and analyzed as adipokines by our group before (131;133).

PEDF serum levels are increased in subjects with obesity (315), insulin resistance (316), and type 2 diabetes (317) and significantly decline after weight loss (316). *In vitro* studies revealed that PEDF impairs insulin signalling (131). We found that PEDF is also a myokine and its secretion of primary human myotubes is increased after contraction induced by EPS (222), while an acute bout of exercise decreased PEDF serum levels (222), which was just recently shown by Oberbach et al. as well (318). Post-exercise to strength training transcription of muscle PEDF was increased in human (277), indicating that the enhanced PEDF expression in response to exercise is rather a long-term effect. PEDF is one of the most abundant proteins secreted by primary human adipocytes (115;131), and myotubes secrete PEDF at significantly lower levels compared to adipocytes (Table 2). Thus, we assume that serum PEDF levels mainly originate from adipose tissue and PEDF secreted from skeletal myotubes upon contraction acts rather in an auto/paracrine manner within the muscle.

DPP4 was mainly considered to be secreted from small intestine. Lamers et al. showed that DPP4 is secreted from primary human adipocytes and we could now show that it is also secreted from skeletal muscle myotubes, although it was not regulated by contraction (222). Increased DPP4 serum levels strongly correlate with adipocyte volume and parameters of the metabolic syndrome, while DPP4 levels decrease after weight reduction (133). Substantial DPP4 activity is found in plasma because of a soluble form of DPP4 lacking the cytoplasmic tail and the transmembrane region. The function of the soluble form remains poorly understood and it is unknown if the process of DPP4 release from cell membranes is regulated

or not. In this study, neither inflammatory cytokines like TNFα, MCP-1, IFNγ and IL-6 nor contraction could alter the secretion of DPP4 (222).

Table 4: Newly described adipo-myokines. DPP, dipeptidylpeptidase; MCP, monocyte chemotactic protein; PEDF, pigment epithelium derived factor; n.d., not described; ✓, association has been shown in indicated publications; x, association has not been shown.

Adipo-Myokine	*Associated with obesity*	*Associated with insulin resistance/T2D*	*Contraction-Regulated*	*Associated with improved glucose metabolism*
DPP4	✓ Serum DPP4 correlates with adipocyte size (133)	✓ DPP4 impairs insulin signalling in fat and muscle cells (133)	x DDP4 was not contraction-regulated in vitro (220)	n.d.
MCP-1	✓ Serum MCP-1 is increased in obesity (126)	✓ Promotes insulin resistance (126;132)	✓ Contraction-regulated in vitro (222), increased after resistance training (227)	n.d.
PEDF	✓ PEDF serum levels increased in obesity (316;317)	✓ PEDF serum levels associated with insulin resistance (315-317;319), PEDF promotes insulin resistance (131)	✓ Contraction-regulated in vitro (222), decreased serum levels after acute bout of exercise (222;318)	n.d.

Leptin

Originally, leptin was described as adipokine which controls food intake (320). It is synthesized and released in response to increased energy storage in adipose tissue (320-322). Leptin was detected in several tissues including skeletal muscle (323). Recently, Wolsk et al. published that human skeletal muscle releases leptin *in vivo* (324). Leptin secretion from muscle was measured by insertion of catheters to the femoral artery and vein draining the skeletal muscle as well as an epigastric vein graining the abdominal subcutaneous adipose tissue. The authors measured a leptin release of 0.8 ± 0.3 ng min^{-1} 100 g $tissue^{-1}$ from adipose

tissue and 0.5 ± 0.1 ng min^{-1} 100 g $tissue^{-1}$ form skeletal muscle. From these results the authors conclude that the contribution of whole body leptin production could be substantial greater from skeletal muscle compared to fat due to the greater muscle mass in lean subjects. Earlier work also observed leptin release from human skeletal muscle tissue and subcutaneous adipose explants, with more than ten times less secretion from muscle explants compared to fat explants (325). Here, we measured the leptin release of differentiated primary human adipocytes and myotubes. The myotubes secrete leptin in much smaller concentrations than the adipocytes. Even in concentrated supernatants of myotubes leptin secretion was barely detectable and contraction induced by EPS had no effect on leptin secretion (Raschke, unpublished observation). Nevertheless, increasing evidence revealed inter- and intramuscular accumulation of non-myogenic cell types, which might contribute to the observed leptin secretion of skeletal muscle tissue. Preadipocytes of unknown origin are observed in skeletal muscle (326;327) and macrophage accumulation is slightly enhanced in skeletal muscle in obese type 2 diabetic subjects or in elderly individuals (328).

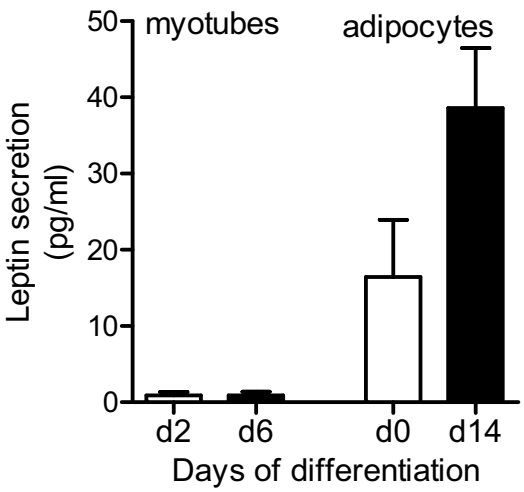


Figure 8: Leptin secretion from primary human myotubes and adipocytes. Primary human skeletal muscle cells and preadipocytes were differentiatiated in vitro for 6 and 14 days, respectively. Supernatants were collected on day 2/6 and day 0/14 of differentiation, respectively. Leptin secretion was measured by ELISA , n ≥ 8.

Taken together, the results of this study lead to the conclusion that one protein can be a myokine as well as an adipokine, indeed two sides of the same coin. As Paracelsus (1493-1541) already coined the famous phrase; “dosis sola facit venenum”, (“Only the dose makes the poison”). This might also be true for adipo-myokines. Findings support the hypothesis that the myokines are essential for muscle metabolism during contraction (273), whereas the chronic elevation of adipokines released from adipocytes may induce adverse effects, even leading to insulin resistance (157).

Additionally, in healthy, normal weight subjects skeletal muscle is the largest tissue in the human body, accounting for 40-50 % of total human body mass, while body fat accounts for 20-35 %. In obese subjects the percentage of total body fat increases to 40-60 % resulting in an increased secretion of pro-inflammatory adipokines, while the percentage of proteins secreted from skeletal muscle is decreased. Thus in the obese state the beneficial effect of myokines decreases, while the adverse effects of long lasting increased in pro-inflammatory cytokines are enhanced.

3.5 Identification of novel contraction-regulated myokines

In addition to 52 novel myokines, the cytokine antibody arrays of primary human myotubes identified 48 contraction-regulated myokines (222). Four of these myokines were already described, namely IL-6, IL-7, IL-8 and VEGF. Among others, MCP-1 and PAI-1 were identified as contraction-regulated myokines. MCP-1 and PAI-1 resemble IL-6, which was recognized as inflammatory cytokine for a long time and then found to be an exercise factor with a positive impact on muscle physiology.

MCP-1

MCP-1 is a chemokine and member of the small inducible cytokine family. It plays a crucial role in the recruitment of monocytes and T lymphocytes into tissues (329;330). It was described to be produced in isolated adipocytes, associated with adiposity and reduced after weight loss in morbid obese subjects (331). MCP-1-induced macrophage infiltration in

adipose tissue leads to a chronic state of low-grade inflammation (332), which is linked to insulin resistance. Additionally, in vitro data demonstrate that this factor has the ability to induce insulin resistance in adipocytes and skeletal muscle cells (132), (Table 4).

Here, we describe MCP-1 as contraction-regulated myokine. Secretion of MCP-1 was significantly upregulated compared to control (1.55 ± 0.33) (222). Increased MCP-1 mRNA expression in skeletal muscle was reported in elderly individuals following one bout of resistance exercise (333) and in young men after a repeated eccentric exercise bout (334). Immunohistochemistry analysis of muscle biopsies colocalized MCP-1 with resident macrophage and satellite cell populations, suggesting that alterations in cytokine signalling between these cell populations may play a role in muscle adaptation to exercise (334).

PAI-1

PAI-1 is the primary physiological inhibitor of tissue-type plasminogen activator and urokinase to promote fibrinolysis (the breakdown of blood clots) (335). Increased PAI-1 has been linked not only to thrombosis and fibrosis but also to obesity and insulin resistance (336;337), which could be prevented in mice lacking PAI-1 (338). Studies revealed that plasma levels of PAI-1 were closely correlated with visceral adiposity but not with subcutaneous adiposity in human subjects, suggesting that an enhanced expression of the PAI-1 gene in visceral fat may increase plasma levels and may have a role in the development of diseases in obesity (339).

Norheim et al. showed that strength training increases transcription of PAI-1 mRNA expression in skeletal muscle of healthy young men and proved the secretion of PAI-1 from primary skeletal myotubes *in vitro* in the basal state (277). Here we describe that PAI-1 is secreted from primary myotubes in a contraction-regulated manner (222). Up to now, the acute effect of PAI-1 secretion during exercise has not been studied. PAI-1 deficiency also enhanced basal and insulin-stimulated glucose uptake in adipose cells *in vitro* (338). It might be speculated that increased PAI-1 serum levels inhibit glucose uptake in adipose tissue, and thus higher glucose uptake by skeletal muscle.

Follistatin-like 1

Our 2D-PAGE/MALDI-MS data clearly demonstrate that follistatin-like 1 (Fstl1) is secreted by human skeletal muscle cells, which is in line with three proteomics studies performed in murine and rat cell lines (213;215;216). Fstl1 is the smallest member of the SPARC protein family and a secreted glycoprotein of 45-55 kDa that, despite limited homology, has been grouped in the follistatin family of proteins. It was not contraction-regulated by EPS of primary human myotubes. However, an acute bout of exercise of healthy young men increased Fstl1 serum levels significantly (340). Additionally, Norheim et al. observed a significant increase in *M. vastus lateralis* and *M. trapezius* after 11 weeks of strength training (277). We speculated that Fstl1 might have an insulin-sensitizing effect, since Ouchi et al. observed increased basal Akt phosphorylation in endothelial cells after adenoviral transduction with Fstl1 (341). However, treatment of human myotubes with recombinant Fstl1 had no effect on basal or insulin-stimulated Akt phophorylation (340). Treatment of myocytes with recombinant Fst1 enhanced AMPK phosphorylation in a dose- and time-dependent manner (342). It might be speculated that increased Fstl1 serum levels after exercise lead to an additional activation of AMPK in target tissues (342) and enhance downstream signalling. Additionally, Fstl1 promotes endothelial cell migration and differentiation into vascular-like structures (341) and could support vascularization of skeletal muscle after exercise. Thus, we identified Fslt1 as myokine which is increased in serum levels after exercise and that may contribute to health benefits associated with physical activity.

3.6 Myokines with a positive endocrine effect

Nowadays, the reports of novel contraction-regulated myokines increase, but research elaborating the influence of myokines on skeletal muscle or whole-body metabolism is just at the beginning. Up to now, for many cytokines the beneficial effect on glucose metabolism or other metabolic parameter has not been shown (Table 3, Table 4).

IL-6 - the prototype myokine increases glucose disposal and fat oxidation

For the long studied myokine-prototype IL-6 some evidence is published that it might have systemic effects. One study reported, acute IL-6 administration in physiological concentrations does not impair whole-body glucose disposal, net leg-glucose uptake, or endogenous glucose production in resting healthy young men (343). Another study observed that IL-6 infusion in healthy humans increases glucose disposal without affecting the complete suppression of endogenous glucose production during a hyperinsulinemic-euglycemic clamp (187). This insulin-sensitizing effect of IL-6, without influencing glucose output from the liver, indicates that the main effect of IL-6 on insulin-stimulated glucose metabolism is likely to occur in peripheral tissues and might just affect skeletal muscle itself or adipose tissue as well. Infusion of rhIL-6 in physiological concentrations into healthy humans increases whole body fat oxidation (291). IL-6 was described as a potent modulator of fat metabolism in humans, increasing fat oxidation and fatty acid reesterification without causing hypertriacylglyceridemia (291) and IL-6 knock-out mice develop late onset obesity and impaired glucose tolerance (344). In summary, IL-6 is released by contracting human skeletal muscle and seems to have a beneficial effect on insulin-stimulated glucose disposal and fatty acid oxidation after acute stimulation.

Irisin – a novel contraction-regulated myokine driving white fat to brown fat in mice

Just recently, a novel contraction-regulated myokine was published, which has drawn our attention. PGC-1a stimulates oxidative metabolism in skeletal muscle and is considered to mediate many of the downstream molecular events induced by exercise. Using a combination of gene expression arrays and computer-based prediction of protein secretion approaches, Boström et al. identified that overexpression of PGC1α in mice muscle as well as exercise induces the expression of the FNDC5 gene (193), which encodes a transmembrane protein. Teufel et al. described FNDC5 as a protein containing a signal peptide, fibronectin type III repeats, and a hydrophobic region encoding the transmembrane domain (345). The membrane protein is cleaved and the extracellular protein part is secreted as novel messenger molecule called irisin (193). In this study, viral delivery of FNDC5 caused a browning of the

subcutaneous white adipose tissue depot, stimulated oxygen consumption, and diminished diet-induced weight gain and metabolic dysfunction in mice (193). Irisin is proposed to bind to an undetermined receptor on the surface of preadipocytes of white adipose tissue inducing thermogenic mechanisms, which improves whole body energy balance in mice (193).

Nowadays, brown adipose tissue has drawn the attention as novel therapeutic target to treat obesity and metabolic disease like type 2 diabetes. Brown adipose tissue is specialized in energy expenditure, while white adipose tissue is the primary site of triglyceride storage. In order to maintain body temperature in a cold environment, brown adipose tissue is primarily a thermogenic tissue that burns fat to generate heat (346), through the mitochondrial protein UCP1 (uncoupling protein 1). This protein uncouples the proton electrochemical gradient generated by respiration leading to the dissipation of heat. UCP1 knock-out mice are cold sensitive and tend to obesity, even in mice fed a control diet (347), whereas experimental conditions capable of increasing the extent of brown adipose tissue reduce obesity (348). It has been shown that certain traditional white adipose tissues have the capacity to shift to brown adipocytes by chronic rosiglitazone treatment (349), bone morphogenetic factor 7 (BMP7) (348) and inhibition of PI3K signalling by overexpression of its natural repressor, PTEN (350). Increased UCP1 expression in white adipocytes after external stimuli is described as a brown in white (`brite`) phenotype. Although the mechanism is not completely understood, irisin induced the expression of UCP1 and other brown-adipocyte associated genes partly via increased PPARα expression leading to increased energy expenditure in mice (193).

Although, the initial description of irisin was focused on mice, the highly conserved amino acid sequence was the reason to hope that exogenously administered irisin could have a therapeutic potential in the treatment of obesity and diabetes. In our model system, we observed that irisin protein level in the supernatants of human cells is ten times less compared to murine cells (351). The analysis of genomic, mRNA, EST and SNP data show that FNDC5, the gene coding for the precursor of irisin, is mutated in the start codon in humans which might lead to lower protein expression (351).

Insulin stimulates glucose uptake in brown adipocytes through mechanisms similar to insulin-sensitive tissues described in chapter 1.1. Additionally, glucose uptake in myocytes and brown adipocytes is also stimulated by norephinephrine. Assumed that this pathway is not affected in subjects with metabolic syndrome, an increase in energy expenditure and glucose uptake by brown adipocytes could delay the enhanced glucose levels and thus development of insulin resistance.

Work by our group revealed evidence against the beneficial effect of irisin in humans (351). We used primary human preadipocytes isolated from subcutaneous adipose tissue and differentiated these cells in the presence of recombinant BMP7, FNDC5 or irisin. In this study we could observe a `britening` effect of adipocytes after the treatment with BMP7, with the strongest effect in CD137 high expressing cells. CD137 is a novel marker for preadipocytes which are capable of britening after stimulation (352). However, neither recombinant FNDC5 nor the cleaved protein irisin had an effect on the britening of adipocytes in CD137 high expressing human adipocytes. Our findings raise the question if the beneficial effect of irisin observed in mice can be transferred to humans (351).

Kelley raised in his comment on the irisin-study an evident question; why would physical activity induce a program that burns fat stores (353)? Boström et al. gave the explanation that increased irisin secretion with exercise may be evolved as a consequence of muscle contraction during shivering. Muscle secretion of a hormone that rapidly increases thermogenic capacity during cold might provide a broader, more robust defense against hyperthermia. However, also De Matteis et al. observed browning of the visceral fat in rats after treadmill running by a supposed white-to-brown transdifferentiation (354). Nevertheless, it remains paradoxial why exercising muscle would increase the secretion of a myokine which depletes fuel stores.

3.7 Outlook

Within this thesis a novel model for skeletal muscle contraction was established to study cellular mechanisms involved in the beneficial effect of exercise. One major finding was that contractile activity of human skeletal myotubes inhibits pro-inflammatory signalling induced by adipokines. Current literature discusses several pathways, which might be involved in the anti-inflammatory effect of contraction. CaM inhibitors prevent NFκB activation due to the prevention of inducible IκBα phosphorylation and downstream targets of AMPK block NFκB activation.

Future studies should address the more detailed explanation how calcium signalling or AMPK activity influence anti-inflammatory signalling.

To elucidate, single pathways can be inhibited during contraction. CaM can be inhibited by Ca^{2+}-chelators like BAPTA-AM or by verapamil, a blocker for Ca^{2+}-channels. The phosphorylation and subsequent activation of AMPK can be inhibited by cell-permeable pyrrazolopyrimidine called compound c. These experiments would provide a novel approach for investigating the molecular mechanisms that mediate the beneficial effects of muscle contraction more detailed.

The used protocol is a unique tool for investigating skeletal muscle contraction. However, using a frequency of 1 Hz, pulse duration of 2 ms and an intensity of 11.5 V for 4-24 hours is just one possibility to mimic exercise *in vitro*. Exercise physiology is a complex topic and not just the acute effect has a positive influence on muscle physiology. As described for insulin sensitivity, the adaptive processes take place in the post-exercise phase. Additionally, contractile activity did not influence fatty acid metabolism and lipotoxic effect of palmitic acid. To activate lipid metabolism glucose concentration in the medium could be diminished.

Therefore, additional EPS protocols are necessary to address different effects of exercise.

Cytokines can be released by myocytes and adipocytes. These adipo-myokines are described with contradictory results regarding whole-body metabolism.

Future studies should discriminate the long-term and the short-term of cytokines on skeletal muscle and adipocytes.

Myokines itself might not only have autocrine and paracrine effects in the tissue of origin, several of them might also be distributed by the circulation and affect other tissues in an endocrine manner. These adipo-myokines might participate in a bi-directional crosstalk between skeletal muscle and adipose tissue. Therefore, human studies are necessary and should be carefully planned with regard to the intensity of exercise.

In conclusion, the established contraction model provides a tool for further investigation of underlying signalling pathways that mediate the beneficial effects of muscle contraction. However, further analyses are necessary to elucidate the mechanisms in detail more. The model will help to clarify the potential of exercise to combat insulin resistance.

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Eidesstattliche Erklärung

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

(Silja Raschke)

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