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Identification and Characterization of Novel Myokines

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„Erfahrungen sind Maßarbeit. Sie passen nur dem, der sie macht."

-Carlo Levi-

Summary

Nowadays, the prevalence of type 2 diabetes is increasing worldwide and reaches epidemic proportions already. Physical inactivity and obesity are closely associated with muscle insulin resistance, which is a major risk factor for the pathogenesis of type 2 diabetes. In the last few years research has shown that obesity leads to a chronic low-grade inflammation within adipose tissue and affects the insulin sensitivity of other organs and tissues. In contrast, regular physical activity does not only prevent obesity, but also considerably improves insulin sensitivity and skeletal muscle metabolism. Skeletal muscle has recently been identified as an endocrine organ that produces and releases cytokines and other peptides, which have been named "myokines". Considering that skeletal muscle is the largest organ in the human body and the discovery that contracting skeletal muscle secretes proteins sets a novel paradigm: skeletal muscle is an endocrine organ producing and releasing myokines in response to contraction, which can influence metabolism in other tissues and organs.

Therefore, the starting point of the present work was to identify and characterize novel contraction-induced myokines *in vitro* and *in vivo,* and to analyze their biological functions in human skeletal muscle cells. We demonstrated for the first time that the myokine follistatin-like protein 1 (Fstl1) is differentiation-dependently expressed and released by human skeletal muscle cells. Furthermore, we show that an acute bout of exercise performed by healthy young men increased Fstl1 serum levels significantly. However, electrical pulse stimulation of primary human myotubes revealed no regulation by *in vitro* contraction. However, treatment of human myotubes with different concentrations of recombinant Fstl1 had no effect on basal or insulin-stimulated Akt or GSK-3 phosphorylation.

With comprehensive proteomic profiling of the human myocyte secretome our working group identified chitinase-3-like protein 1 (CHI3L1) as a novel myokine. Moreover, by using four independent exercise studies we clearly demonstrated that circulating CHI3L1 levels as well as muscle CHI3L1 mRNA expression was up-regulated after acute exercise. Therefore, we classified CHI3L1 as a novel contraction-regulated myokine. Furthermore, incubation of cultured human myoblasts with CHI3L1 protein leads to a strong activation of p44/42, p38 MAPK and Akt as well as enhanced myoblast proliferation. Our findings suggest that CHI3L1 is a contraction-induced myokine, potentially acting in an autocrine or paracrine fashion to induce myocyte proliferation for restructuring of skeletal muscle after exercise. In addition, CHI3L1 treatment diminished the TNFα-induced inflammation in differentiated myotubes. Also, impaired insulin action at the level of Akt and glycogen synthase kinase

(GSK) 3α/ß phosphorylation and insulin-stimulated glucose uptake was normalized by CHI3L1. In conclusion, the novel myokine CHI3L1 which is induced by pro-inflammatory cytokines and exercise can counteract TNFα-mediated inflammation and insulin resistance in human skeletal muscle cells, potentially involving an auto/paracrine mechanism.

Exercise is accompanied by a decrease in intramuscular oxygen levels resulting in an increased HIF-1α protein abundance. HIF-1α is a master regulator of gene expression and could play an important role in skeletal muscle function and metabolism. Thus we examined the role of oxygen in insulin- and contraction-stimulated glucose metabolism in primary human skeletal muscle cells. We show that exposure of these cells to a lower oxygen tension (7% O2) in combination with electrical pulse stimulation (EPS) for 24 h improves insulin action at the level of insulin signaling and glucose uptake. Furthermore, the combination of 7% O2 and EPS resulted in an enhanced IL-6 production and secretion as well as HIF-1α and GLUT4 protein expression. In contrast, knockdown of HIF-1α totally inhibits the insulin- and contraction-induced glucose uptake. Additionally, human skeletal muscle HIF-1α mRNA expression was enhanced after acute and chronic exercise and positively correlates with whole body insulin sensitivity of the participants. The ability to induce HIF-1α by exercise was reduced in pre-diabetic subjects. In conclusion, we demonstrated that HIF-1α is involved in the regulation of glucose metabolism in human skeletal muscle induced by both stimuli, insulin and contraction. It may be anticipated that the insulin-sensitizing effect of exercise is at least partly a result of HIF-1α-regulated gene expression of the insulin signaling cascade and related downstream metabolic targets.

Taken together, our findings may provide a better understanding for the endocrine role of the skeletal muscle producing and releasing myokines, which potentially influence skeletal muscle metabolism itself and/or contribute to regulate the functions of other tissues in an inter-organ crosstalk. In addition, we demonstrated that skeletal muscle HIF-1α is a critical determinant for insulin- and exercise-regulated glucose metabolism in human skeletal muscle and could be used as a therapeutically relevant target to improve insulin sensitivity in human.

Zusammenfassung

Die Anzahl der Typ 2 Diabetiker steigt weltweit rapide an und erreicht mittlerweile epidemische Ausmaße. Gefördert wird die Pathogenese des Typ 2 Diabetes durch die Kombination von Adipositas und Bewegungsmangel. Beides führt zu einer Insulinresistenz des Skeletmuskels, welche zu einer der wichtigsten Ursachen für die Entwicklung des Typ 2 Diabetes zählt. Die Forschung der letzten Jahre hat gezeigt, dass Adipositas zu einer subklinischen, chronischen Inflammation des Fettgewebes führt, welche die Insulinwirkung im Fettgewebe selbst als auch in anderen Organen und Geweben negativ beeinflusst. Regelmäßige körperliche Bewegung hingegen verbessert nicht nur die Energiebilanz sondern auch die Insulinsensitivität des Skelettmuskels und dient daher zur Vorbeugung des Typ 2 Diabetes. Aktuelle Forschung deutet darauf hin, dass der kontrahierende Skelettmuskel Hormone freisetzt, welche Myokine (griechisch „Mys“: Muskel und „kinos“: Bewegung) genannt werden einen positiven Einfluss auf die Funktion der Skelettmuskulatur ausüben. Jedoch können diese Myokine auch endokrin wirken und entzündungshemmende, Knochen stärkende und neuroprotektive Effekte vermitteln. Dies stellt einen Paradigmenwechsel der Bedeutung der Skelettmuskulatur für die Gesundheit dar, in der der Skelettmuskel nicht ausschließlich für die Umwandlung von chemischer Energie in kinetische Energie verantwortlich ist, sondern auch als endokrines Organ berücksichtigt wird.

Aus diesem Grunde war das Ziel dieser Arbeit, neue kontraktions-regulierte Myokine zu identifizieren und ihre potentiellen auto-/parakrinen Effekte auf die Skelettmuskulatur zu untersuchen. In dieser Arbeit konnte erstmalig gezeigt werden, dass das Myokin Follistatin-like protein 1 (Fstl1) in humanen Skelettmuskelzellen differenzierungsabhängig exprimiert und sekretiert wird. Jedoch konnten wir keine Verbesserung der Insulinwirkungen durch Fstl1 Behandlung in humanen Skelettmuskelzellen beobachten. Darüber hinaus führte die Kontraktion von Skelettmuskelzellen *in vitro* nicht zu einer erhöhten Expression oder Freisetzung von Fstl1. Dennoch konnte gezeigt werden, dass durch 60 minütiges Fahrradfahren der Serumlevel von Fstl1 signifikant erhöht wird. Somit bleibt die Frage offen, welches Gewebe oder Organ für die erhöhte Freisetzung nach sportlicher Betätigung verantwortlich ist. Darüber hinaus konnte in dieser Arbeit erstmalig Chitinase-3-like protein 1 (CHI3L1) als ein neues kontraktions-reguliertes Myokin beschrieben werden. Durch verschiedene Sportstudien konnten wir zeigen, dass die Expression im Muskel sowie die zirkulierende Konzentration von CHI3L1 nach körperlicher Aktivität signifikant zunahmen. Desweitern konnten wir belegen, das CHI3L1 die Proliferation von Skelettmuskel-

Vorläuferzellen fördert und somit die Muskeladaption nach körperlicher Belastung verbessert. Darüber hinaus schützt CHI3L1 die Skelettmuskulatur vor Inflammation und der daraus resultierender Insulinresistenz. CHI3L1 ist somit ein neues kontraktions-reguliertes Myokin, welches entscheidende Prozesse im Muskel fördert, die zu einer Verbesserung der Funktion und des Metabolismus innerhalb der Skelettmuskulatur führen.

Seit längerem ist bekannt, dass die Sauerstoffkonzentration einen erheblichen Einfluss auf die Funktion der Skelettmuskulatur hat und dass körperliche Aktivität unter reduzieren Sauerstoffbedingungen die Insulinsensitivität von Typ 2 Diabetikern viel effektiver verbessert als körperliche Aktivität unter normalen (21%) Sauerstoffbedingungen. Der zugrundeliegende Mechanismus dieser Verbesserung und die Rolle der Skelettmuskulatur in diesem Szenario sind jedoch weitestgehend unerforscht. Wir untersuchten in dieser Arbeit, ob die Kombination von reduzierten Sauerstoffbedingungen und Muskelkontraktion die Insulinsensitivität im Skelettmuskel verbessert. Hierfür etablierten wir ein *in vitro* System, in welchem die Muskelkontraktion von primären human Skelettmuskelzellen unter reduzierten Sauerstoffbedingungen durchgeführt werden konnte. Hierbei konnte gezeigt werden, dass Muskelkontraktion unter reduzierten (7% vs. 21%) Sauerstoffkonzentrationen zu einer Verbesserung der Insulinsensitivität in Bezug auf den Insulin Signalweg und die Insulin-induzierte Glukoseaufnahme führt. Darüber hinaus führte die Kombination aus 7% Sauerstoff und Muskelkontraktion zu einer erhöhten Expression von GLUT4 und dem Transkriptionsfaktor HIF-1α. Durch die Hemmung der Genexpression von HIF-1α konnten wir zeigen, dass HIF-1α essential für den Insulin- und Kontraktions-regulierten Glukosemetabolismus im Skelettmuskel ist. Des Weitern konnten wir zeigen, dass die Expression von HIF-1α im Skelettmuskel durch körperliche Aktivität reguliert wird und die Expression positiv mit der Insulinsensitivität der Probanden korreliert. Infolgedessen kann man vermuten, dass die Insulin Sensibilisierung durch regelmäßiger körperlicher Aktivität zum Teil von HIF-1α reguliert wird.

Zusammenfassend kann gesagt werden, dass der Skelettmuskel Myokine produziert und freisetzt, die einen positiven Einfluss auf den Metabolismus und die Funktion des Skelettmuskel haben, indem sie die Muskelregeneration und das Wachstum nach körperlicher Belastung fördern und den Muskel vor dem negativen Einfluss von pro-inflammatorischen Cytokinen schützen. Des Weitern konnte gezeigt werden, dass der Transkriptionsfaktor HIF-1α ein essentieller Faktor für den Insulin- und Kontraktions-regulierten Glukosemetabolismus im Skelettmuskel ist und somit ein potentielles Target für die Behandlung des Typ 2 Diabetikers darstellen könnte.

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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
BMI	Body mass index
CaM	Calmodulin
CaMKKβ	$Ca^{2+/}$Calmodulin-dependent kinase kinase β
CHI3L1	Chitinase-3-like protein 1
CBP	CREB (cyclic AMP response element–binding protein)-binding protein
DAG	Diacylglycerol
EPS	Electrical pulse stimulation
ERK	Extracellular signal-regulated kinase
FFA	Free fatty acids
Fstl1	Follistatin-like protein 1
GLUT	Glucose transporter
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase 3
HbA1c	glycated hemoglobin
HIF-1α	Hypoxia-inducible factor 1-alpha
HIF-1β	Aryl hydrocarbon receptor nuclear translocator
HRE	hormone response element
IGF	insulin-like growth factor
IκB	Inhibitors of κB

IKK	IκB kinase
IL	Interleukin
IR	Insulin receptor
IRS	Insulin receptor substrate
LIF	Leukemia inhibitory factor
LKB	Tumor suppressor kinase
MAPK	p38 mitogen activated protein kinase
MCP-1	Monocyte chemotactic protein-1
mTOR	Mammalian target of rapamycin
NFκB	Nuclear factor kappa B
NRF	Nuclear respiratory factor
OXPHOS	Oxidative phosphorylation
PAGE	polyacrylamid gel electrophoresis
PAI-1	Plasminogen activator inhibitor 1
PAR2	Protease activated receptor 2
PDK	Phosphoinositide-dependent protein kinase
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
TBC1D1	(tre-2/USP6, BUB2, cdc16) domain family member 1
TNFα	Tumor necrosis factor-alpha
TNFR	Tumor necrosis factor receptor

1. General Introduction

Throughout the world diabetes afflicts over 347 million people (4.75% of the world population), and epidemiological estimates from the World Health Organization extrapolate that diabetes will be the 7th leading cause of death in 2030. In 2014, 9% of adults 18 years and older had diabetes. Type 2 diabetes (T2D) comprises 90% of people with diabetes around the world and is largely the result of excess body weight and physical inactivity. Thus, diabetes is rapidly being recognized as a public health threat rising to epidemic proportions. The total estimated cost worldwide of diagnosed diabetes in 2012 was €220 billion, including €157 billion in direct medical costs and €63 billion in reduced productivity. While the rate of diabetes increases it has long been recognized that regular physical activity has important health benefits for people with type 2 diabetes.

Acute exercise positively moderates glucose homeostasis by enhancing glucose transport and insulin action in working skeletal muscle, the major tissue for total glucose disposal (1). Regular physical activity increases the protein level of glucose transporter 4 (GLUT4), oxidative capacity, mitochondrial enzyme content, and alters fiber type in skeletal muscle. Moreover, the profile of secreted molecules is influenced which generates an anti-inflammatory environment, thus providing an additional mechanism for exercise-mediated improvements in insulin sensitivity (1-5). Collectively, these effects help to explain the strong epidemiological evidences that regular physical activity prevents or delays the onset of T2D (4;6). Despite the physiological importance of exercise in regulating skeletal muscle metabolism, the molecular mechanisms underlying these fundamental phenomena are only partly understood. Therefore, determining the comprehensive mechanism will unravel essential knowledge of this complex physiological process and may provide novel targets for treatment of T2D. In the following sections, I will briefly introduce the current literature on skeletal muscle function in relation to exercise and diabetes research.

1.1 Glucose uptake in skeletal muscle

Carbohydrates, especially glucose, are an important source of energy for most living organisms. Tissues such as the brain need glucose constantly, and low blood concentrations of glucose can cause seizures, loss of consciousness, and death (1). However, chronically elevated blood glucose levels can result in blindness, renal failure, cardiac and peripheral vascular disease, and neuropathy (7-11). Therefore, regulation of the blood glucose level is of high importance and needs to be maintained within a narrow range. Despite periods of feeding and fasting, plasma glucose remains between 4 and 7 mM in metabolically normal individuals (12). This is achieved by hormonal regulation of peripheral glucose uptake and hepatic glucose production. During fasting, most of the glucose in the blood is supplied by liver and is utilized in the brain independently of insulin. After a meal, the elevation in blood glucose level rapidly stimulates insulin secretion by the ß-cells of the pancreas, which results in increased glucose transport within minutes, metabolism, and storage by skeletal muscle and adipocytes. In addition, insulin inhibits hepatic gluconeogenesis as well as glucagon secretion by pancreatic alpha cells and lipolysis in adipose tissue, thereby lowering serum free fatty acid concentrations, which also contributes to a decline in hepatic glucose production.

Skeletal muscle is the major site of insulin-stimulated glucose disposal in humans; less glucose is transported into adipose tissue (1). In addition, it is known since 120 years that skeletal muscle contraction is an additional stimulus for skeletal muscle glucose uptake (13-16). The intensity and duration of exercise are the primary determinants of skeletal muscle glucose uptake during exercise and blood glucose levels account for up to 40% of oxidative metabolism during exercise, when muscle glycogen is depleted (5;14). The highest glucose uptake is observed when insulin and muscle contraction act together indicating an additive effect of both stimuli (17). Finally, the identification and characterization of the insulin- and contraction-regulated glucose transporter isoform 4 (GLUT4) in 1989 opened the way for a deeper understanding of the molecular pathways involved in skeletal muscle glucose uptake (18-20).

Glucose uptake by skeletal muscle is regulated at several levels and occurs by facilitated diffusion dependent on blood glucose concentration, presence of glucose transporters (GLUT) in the cell surface membrane and the inward diffusion gradient for glucose. Fourteen GLUT proteins are expressed in the human body and they are categorized into three classes based on sequence similarity (21). All GLUT proteins appear to possess 12 transmembrane segments, a

single site of N-linked glycosylation, a relatively large central cytoplasmic linker domain, and exhibit topologies with both their N- and C- termini positioned in the cytoplasm (22). Several GLUTs are expressed in skeletal muscle but two isoforms of the GLUT family play a decisive role. GLUT4, as mentioned above, is important for insulin- and contraction-regulated glucose uptake while the ubiquitously expressed GLUT1 isoform is responsible for constitutive glucose delivery during resting and fasting (5;23).

Once glucose has been transported across the sarcolemma, it is phosphorylated to glucose 6-phosphate (G-6-P) in a reaction catalyzed by hexokinase (1). This is the first step in the metabolism of glucose via either the glycolytic and oxidative pathway responsible for energy generation or conversion to glycogen (1). Phosphorylation of glucose is another site of regulation for glucose uptake because phosphorylated hexoses are charged and thus more difficult to transport out of a cell, which will maintain the diffusion gradient for glucose (1). In addition, accumulation of G-6-P inhibits the function of hexokinase and hence regulates the glucose uptake indirectly (24).

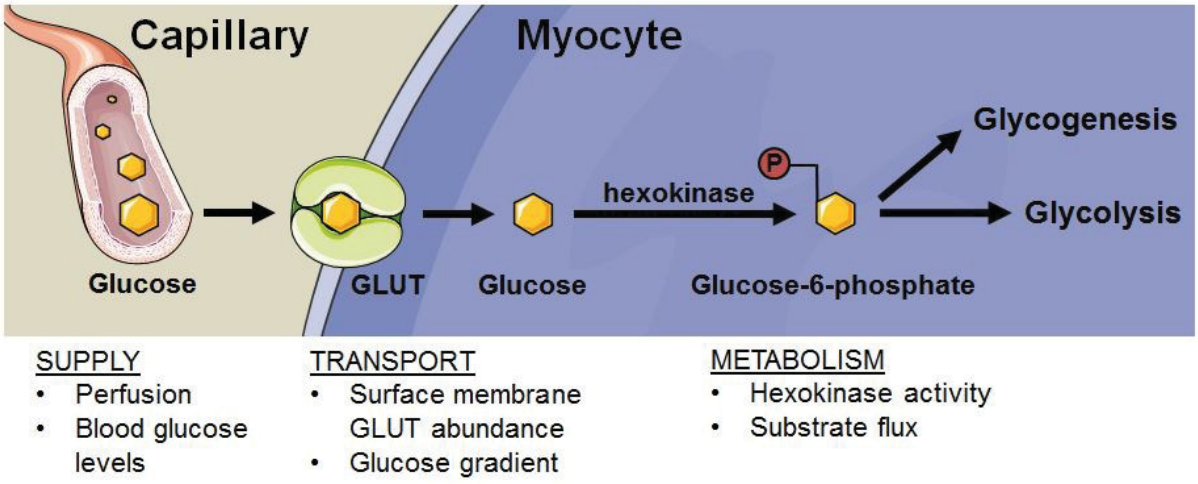

Figure 1: Potential sites of regulation of skeletal muscle glucose uptake. Glucose diffuses from the capillary to the muscle surface membranes, is transported across these membranes by facilitated diffusion, and is irreversibly phosphorylated in the myocyte, provided that there is a glucose concentration gradient. Each of these steps is tightly coupled. Shown below each step are the main mechanisms of regulation. Adapted from (25) with modifications and new illustration.

To summarize, glucose uptake in skeletal muscle is coordinated by the blood glucose level, the transport by GLUTs through the cell membrane, and metabolism of glucose. The

following sections will explain in detail the underlying molecular mechanism for skeletal muscle glucose uptake induced by insulin, muscle contraction and finally by hypoxia.

1.1.1 Insulin-stimulated glucose uptake in skeletal muscle

Insulin-stimulated glucose uptake into skeletal muscle is a fundamental response of the body essential for nutrient utilization. A core mechanism is the rapid mobilization and translocation of glucose transporter GLUT4 to the plasma membrane (7).

Insulin signaling is mediated by a complex, highly integrated network that controls several processes. Insulin activates two main signaling pathways: the phosphatidylinositol 3-kinase (PI3K)–Akt/protein kinase B (PKB) pathway, which is responsible for most of the metabolic actions of insulin, and the Ras–mitogen-activated protein kinase (MAPK) pathway, which regulates expression of genes and cooperates with the PI3K pathway to control cell growth and differentiation (26). The insulin-mediated MAPK signaling pathway is reviewed by Avruch et al. in detail (27).

Insulin signaling is initiated by binding of insulin to its cell surface receptor. The insulin receptor is a tetrameric protein consisting of two α- and two β- glycoprotein subunits linked by disulfide bonds and is located on the cell membrane (28). Insulin binds to the extracellular α-subunit leading to de-repression of the kinase activity in the β-subunit, followed by trans-phosphorylation of the β-subunits and a conformational change that further increases kinase activity (29). Auto-phosphorylation of the insulin receptor results in tyrosine phosphorylation of insulin receptor substrates (IRSs) including IRS1, IRS2, IRS3, IRS4, Gab1 and Shc (29;30). Binding of IRSs to the regulatory subunit of phosphoinositide 3-kinase (PI3K) via Src homology 2 (SH2) domains results in activation of PI3K, which phosphorylates membrane phospholipids and phosphatidylinositol 4,5-bisphosphate (PIP2) in the 3’ position (31). This complex activates the 3-phosphoinisitide-dependent protein kinase (PDK-1) (32;33). PDK-1 phosphorylates two proteins that have been shown to be essential key factors for insulin-stimulated glucose transport; first protein kinase B (PKB or Akt) and second the atypical protein kinase C (aPKC) (34-37). PDK-1 leads to conformational changes and subsequent phosphorylation of Akt on Thr308, resulting in partial activation of Akt (38). 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) (39). The Akt substrate of 160 kD (AS160), which is a Rab-GAP protein, links the insulin signal to GLUT4 translocation from intracellular stores to the plasma membrane (40-42). Insulin-induced AS160 phosphorylation leads to an inactivation of the Rab-GAP activity (43;44). Rab small G proteins on the GLUT4 vesicles turn into an active, GTP-bound state. Activation of Rab proteins induce processes that lead to the mobilization and fusion of GLUT4 into the plasma membrane (7;45;46). This complex network is summarized in figure 2.

1.1.2 Contraction-stimulated glucose uptake in skeletal muscle

The molecular signaling mechanisms that lead to GLUT4 translocation initiated by muscle contraction are not as well understood as the insulin-mediated GLUT4 translocation. It is generally believed that muscle contraction stimulates GLUT4 translocation via a molecular mechanism distinct from that of insulin (7;47-52). However, these two pathways at least partially converge in their distal parts, and there are now some signaling molecules known that are involved in GLUT4 translocation and are activated by both stimuli, like AS160 (7;53-57). Perhaps this convergence explains the observation that the most pronounced glucose uptake is achieved when insulin and muscle contraction act together (7;17).

Muscle contraction is a multifactorial process including changes in energy status (increased AMP/ATP ratio) and increases in intracellular Ca^{2+} levels. Therefore, many investigators speculate that these processes initiate one or more intracellular signaling pathways that coordinately act to increase GLUT4 translocation and glucose uptake in response to physical activity. In the following sections, I will discuss the signaling proteins that have been implicated in this complex process including AMPK, serine/threonine kinase 11 (also known as liver kinase B1, LKB1), Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) and AS160.

An important step in understanding how muscle contraction affects glucose transport was the identification of adenosine monophosphate (AMP) as an activator of AMP activated protein kinase (AMPK) (58). During the last years, research has been conducted to elucidate the role of AMPK in contraction-stimulated glucose transport (59-63). AMPK is a heterotrimeric protein that is activated by a complex mechanism involving an increase in the AMP/ATP ratio, allosteric modification and phosphorylation by up-stream kinases (64;65). Until now, two kinases were identified acting up-stream of AMPK, namely LKB1 and CaMKKβ (66-68).

Studies using the AMP-analogue 5-aminoidazole-4-carboxamide ribonuclease (AICAR) have demonstrated that activation of AMPK induced muscle glucose uptake (69;70). In contrast, data obtained from mouse models of attenuated AMPK activity have demonstrated that inhibition of AMPK has no or just a small effect on contraction-induced glucose uptake (71-73). Furthermore, muscle-specific ablation of LKB1 only partially inhibits contraction-stimulated glucose uptake (74;75). Increases in cellular Ca^{2+} are a fundamental part of muscle contraction, and recent studies have shown that $Ca^{2+/}$calmodulin signaling and CaMKs are important for the contraction-induced glucose uptake by the muscle (76;77). CaMMK activates AMPK in response to a rise in cytosol Ca^{2+} (78). 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 (79).

Once activated by AMP and up-stream kinases, AMPK switches on catabolic pathways that generate ATP while it inactivates anabolic pathways and other ATP-consuming processes (80). Furthermore, activated AMPK has been shown to phosphorylate AS160 in response to AICAR and contraction in skeletal muscle. In addition, mutation of different phosphorylation sites in the per-arnt-sim domain (PAS) of AMPK inhibits both insulin- and contraction-induced glucose uptake (81-83). AS160 is known to regulate insulin-stimulated GLUT4 translocation in adipocytes and skeletal muscle cells (44;84;85). Collectively, these data suggest that AS160 may serve as a point of convergence for both insulin- and contraction-dependent signaling in the regulation of glucose uptake by skeletal muscle.

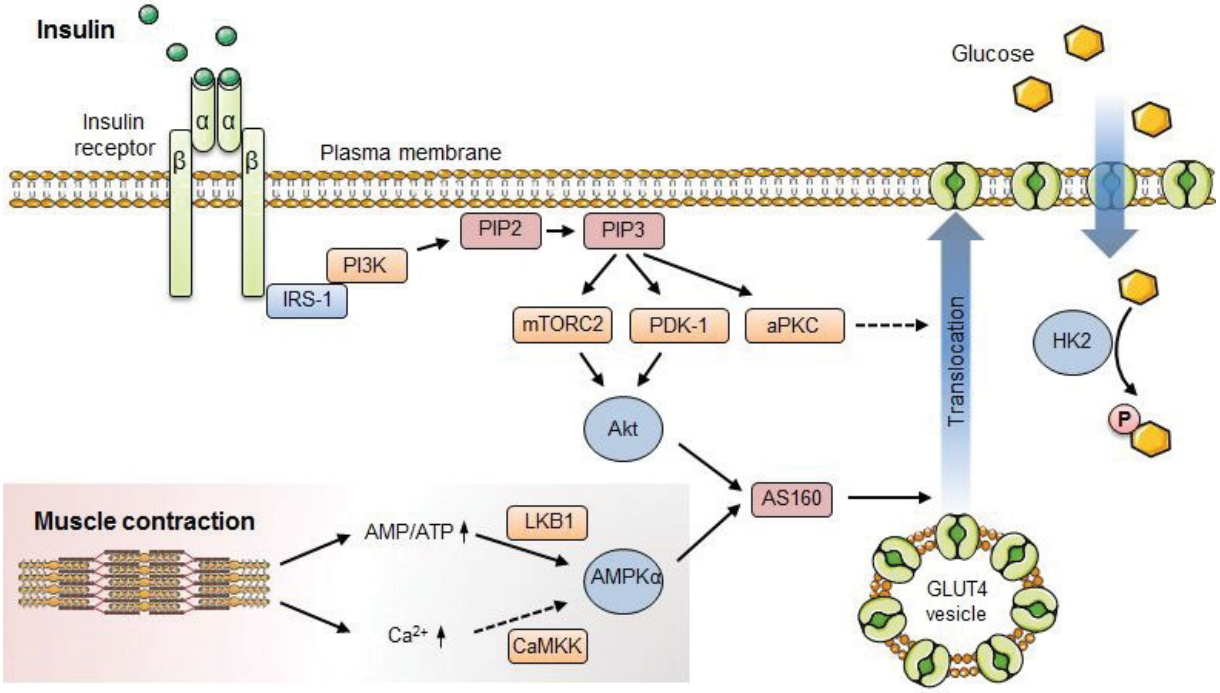

Figure 2: Proposed model for signaling pathways mediating insulin- and contraction-regulated GLUT4 translocation in skeletal muscle: Insulin binding leads to phosphorylation of the insulin receptor with subsequent activation of insulin receptor substrate 1 (IRS-1) and phosphatidylinostitol 3-kinase (PI3K). Downstream of PI3K the protein kinases, 3-phosphoinisitide-dependent protein kinase (PDK-1) and mammalian target of rapamycin 2 (mTORC2) are activated and phosphorylate Akt. Akt then regulates activation of Akt Substrate 160 kDa (AS160) to mediated insulin-stimulated GLUT4 translocation. Also, the atypical protein kinase C (aPKC) have been identified to stimulated GLUT4 translocation. Contraction-stimulated glucose uptake is mediated by Ca2+/calmodulin-dependent protein kinase kinases (CaMKK), LKB1 and AMP-activated protein kinase (AMPK), hexokinase 2 (HK2).

1.1.3 Hypoxia-stimulated glucose uptake in skeletal muscle

Skeletal muscle, like all oxidative tissues of the body, is critically dependent on the supply of oxygen to maintain energetic and redox homeostasis (86). ATP can be synthesized in skeletal muscle in an oxygen-dependent manner in the mitochondria via oxidative phosphorylation, which utilizes substrates such as glycolytically-derived pyruvate, fatty acids, amino acids and ketone bodies (86). Moreover, ATP can be generated in an oxygen-independent manner in the cytosol via glycolysis which is followed by conversion of the resulting pyruvate to lactate (86).

Whatever the origin of the hypoxic stimulus may be (environmental or pathological), the ultimate consequence is an inadequate O_2 delivery/availability at the tissue level implying that

tissue demand exceeds its O_2 supply (86). All nucleated cells in the human body are able to sense O_2 and to respond to O_2 deficiency in order to maintain homeostasis. The main mediator of cellular hypoxia is the hypoxia inducible factor (HIF) pathway, discovered by the group of Semenza (87;88). Like other tissues resting skeletal muscle homeostasis is challenged during hypoxic exposure, either acutely or chronically. However, in contrast to other tissues, skeletal muscle function may be further compromised if exercise is superimposed during hypoxic exposure.

HIF-1 is a heterodimeric protein belonging to the basic helix-loop-helix-PAS family of transcription factors (89). This protein is composed of two subunits: HIF-1α, which has a short half-life and is highly sensitive to oxygen, and HIF-1ß (or ARNT: aryl hydrocarbon nuclear receptor), which is constitutively expressed and remarkably insensitive to oxygen levels (89). Although the HIF family comprises two other members, HIF-2 and HIF-3, only HIF-1 is known to play a very general role in signaling hypoxia, whereas induction of HIF-2 with hypoxia is restricted to certain cells and HIF-3 function is still incompletely understood (87;88). During normoxia, HIF-1α is degraded through hydroxylation (87;88). This process involves prolyl hydroxylases acting on HIF-1α oxygen-dependent degradation domain (ODDD) (88). Once it has been hydroxylated, HIF-1α binds to von Hippel Lindau (VHL) protein resulting in proteasomal degradation of HIF-1α (88).

Under hypoxic conditions, HIF-1α degradation is blocked because hydroxylation is inhibited. HIF-1α protein therefore accumulates allowing for its binding to ARNT (HIF-1ß) and hence the formation of a HIF-1 complex, which translocates into the nucleus and can recognize hypoxia responsive elements (HRE) located in the promoter of target genes (88). The interaction between HIF-1 and HRE ultimately triggers the transcription of the target genes. To date, more than 1000 HIF-1 downstream genes have been identified (87;90). Those genes, which mainly enable the cell to cope with oxygen stress, are involved in erythropoiesis/iron metabolism, angiogenesis, and cell proliferation/ survival and glucose metabolism, respectively (88). For example, glucose transporters 1-3 and hexokinase 2 are target genes of the HIF-1 complex. In addition, GLUT4, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), citrate synthase (CS), and few other genes were discussed to be regulated by HIF-1 (91).

One particular aspect of skeletal muscle is a high expression of HIF-1α protein in this tissue even at normoxic conditions suggesting that HIF-1 could have potential function in muscle homeostasis in normoxia (92). Predominantly, it has been concluded that muscle contraction

and hypoxia stimulate glucose transport via the same signaling pathway, independent of the action of insulin (93). The ability of hypoxia to stimulate glucose disposal independent of contractile activity has been documented in animal models and *in vitro* using isolated human skeletal muscle (94-97). The research in humans has also demonstrated an increased dependence on blood glucose during exposure to high altitude (4300 m; ∼12% O2) (98). Very recently, Sakagami et al. have demonstrated that knockdown of HIF-1α strongly reduced insulin-stimulated glucose uptake in murine skeletal muscle cells (99). Furthermore, AS160 phosphorylation and GLUT4 trafficking was increased in C2C12 cells overexpressing constitutively active HIF-1α (99).

Taken together, hypoxia, more precisely HIF-1α, is a determinant of glucose uptake in skeletal muscle cells and hence a possible target to alleviate impaired glucose metabolism in e.g. T2D.

1.2 Diabetes and insulin resistance

Diabetes is recognized as a group of heterogeneous disorders with the common elements of hyperglycemia and glucose intolerance resulting from defects in either insulin secretion, insulin action, or the combination (100). Depending on its etiology and clinical presentation diabetes can be mainly subdivided into type 1 and type 2 diabetes. Type 1 diabetes is also known as insulin-dependent, juvenile-onset, or immune-mediated diabetes since it develops as a consequence of an autoimmune destruction of the insulin-producing β-cells in the pancreas (101). While it accounts for 5-10 % of all diabetes cases today, the incidence of type 1 diabetes is increasing. The reasons for this are unclear but are likely to be mainly due to changes in environmental risk factors such as increased weight development, increased maternal age at delivery, and possibly also aspects of diet and exposure to certain viral infections that may initiate autoimmunity or accelerate an already ongoing β- cell destruction (102).

T2D is accounting for more than 90 % of diabetes cases, and is characterized by hyperglycemia resulting from impaired insulin secretion of pancreatic ß-cells and impaired insulin action in peripheral tissues as liver, adipose tissue and skeletal muscle, or both (103). Usually it takes years before the two defects, impaired peripheral insulin action and impaired insulin secretion, result in the manifestation of type 2 diabetes. However, long before the manifestation, impaired fasting glucose as well as impaired glucose tolerance has been found to identify individuals at high risk for progression towards diabetes (104). In these

individuals, hyperglycemia at levels below the current diagnostic thresholds for diabetes is now recognized to be sufficient to cause functional and pathological changes in various tissues, especially the heart and circulatory system (104-106). These pathological changes remain unrecognized since they occur without clinical symptoms.

The American Diabetes Association (ADA) and the World Health Organisation (WHO) propose several diagnostic criteria for diabetes. These include a fasting plasma glucose of ≥ 126 mg/dl, or hyperglycaemia of ≥ 200 mg/dl, or plasma glucose levels of ≥ 200 mg/dl two hours after an oral glucose load (107). Nowadays it is recognized that T2D is accompanied by a range of associated metabolic disorders like obesity, hypertension, and dyslipidemia. These metabolic conditions constitute risk factors for the development of each other and are collectively described as the metabolic syndrome. T2D patients commonly have a combination of obesity, dyslipidemia, hypertension, and insulin resistance, especially an increased body mass index (BMI) has been identified to increase the risk of developing T2D in a dose-dependent manner (103;107;108). It has been shown that the prevalence of T2D in overweight or obese adults (BMI ≥ 25) is up to 7 times higher compared to normal-weight controls (BMI 18.5-24.9) and that morbidly obese patients (BMI ≥35) have a 20 times increased risk to develop T2D (108;109). In addition, it is well-established that also physical inactivity increases the risk of T2D (110) and interestingly, inactivity unequivocally has also been shown to increase the morbidity and mortality rates of associated chronic disorders (111;112). Moreover, chronic systemic inflammation, which is involved in the pathophysiology of insulin resistance and T2D, is associated with physical inactivity independent of BMI (113). Therefore, a sedentary lifestyle is a major risk factor for many chronic pathologies like obesity, insulin resistance and T2D (114). Increasing the amount of physical activity effectively prevents the development of chronic diseases (111;112) and exercise is an excellent therapeutic intervention in conditions such as obesity, insulin resistance and T2D. In terms of efficacy, exercise can be as beneficial as the drugs that are prescribed for many of these conditions (4). The mechanisms that mediate the therapeutic effect of exercise and the pathological changes elicited by a sedentary lifestyle remain enigmatic (115). Therefore, understanding the multiple mechanisms involved in the disturbance of proper insulin action and hence the development of skeletal muscle insulin resistance will provide the basis to develop new strategies to improve insulin sensitivity or prevent insulin resistance.

1.2.1 Skeletal muscle insulin resistance

Insulin resistance is a widely used but rather inexact term. It describes a condition in which insulin becomes inefficient to lower the blood glucose level due to a failure of insulin-sensitive tissues to adequately respond to the circulating insulin. Insulin resistance is not a disease but rather a metabolic dysfunction characterized by a large range of decreased values in the insulin sensitivity index (e.g. fasting blood glucose levels) throughout a population of individuals. It refers to the fact that peripheral target tissues including skeletal muscle, liver, and adipose tissue do not respond sufficiently to physiological insulin concentrations. Hence, a normal insulin concentration is not able to maintain glucose homeostasis. In such a scenario, skeletal muscle and adipose tissue take up less glucose from the blood after a meal, and in hepatocytes insulin inappropriately suppresses the production of glucose through gluconeogenesis and glycogenolysis which results in hyperglycemia (107). Insulin resistance of skeletal muscle is a major problem because it is this tissue which is mainly responsible for the postprandial glucose disposal from the circulation (up to 80%) (1). These pathological changes in insulin-mediated glucose uptake and utilization are central defects in the majority of T2D patients, which are often present even years before the disease is diagnosed (116).

In order to improve whole-body glucose homeostasis, much attention has focused on understanding the molecular mechanism for the development of insulin resistance in skeletal muscle (117). Several studies have demonstrated impaired insulin action on skeletal muscle glucose uptake in T2D patients (118-120). Therefore, intensive research has focused on the question whether reduced insulin-mediated glucose transport in skeletal muscle of T2D subjects results from alterations in the insulin signaling pathway or from alterations in translocation of GLUT4 to the cell surface. Early steps in the insulin signaling cascade including the insulin receptor, IRS-1, PI3K and Akt phosphorylation are critical events leading to insulin-regulated glucose uptake. It was shown that insulin receptor, IRS-1 and PI3K phosphorylation in skeletal muscle is reduced in patients with T2D (31;121;122). In addition, knockdown or depletion of Akt (isoform Akt2/PKBβ) in mice causes insulin resistance (123). This result was extended to humans by the finding that a mutation in the gene encoding Akt2/PKBβ results in severe insulin resistance (124). Furthermore, several studies demonstrated reduced insulin-stimulated Akt phosphorylation in skeletal muscle from insulin-resistant or T2D subjects (125-127).

In addition, phosphorylation of Ser/Thr sites of the insulin receptor and IRS1/2 likely represents one of the most important mechanisms involved in insulin resistance (128-131). There are more than 70 Ser/Thr residues in IRS proteins subjected to phosphorylation, and numerous Ser/Thr kinases have been implicated as potential trigger of insulin resistance including c-Jun N-terminal kinase-1 (JNK1), inhibitory- κ B kinase β (IKK β), p70 – S6 kinase 1 (S6K1), and extracellular signal regulated kinase- 1 (ERK1) (129;132). In line with these observations, experiments using knockout of JNK1, IKK β, and ERK1 have been shown to prevent insulin resistance in animals exposed to high-fat diet (133-135).

The ultimate response of skeletal muscle to insulin is a proper translocation of GLUT4 to the plasma membrane. Interestingly, GLUT4 protein expression is reduced in skeletal muscle of morbidly obese subjects, while in lean T2D subjects total GLUT4 protein expression is not altered (136-138). In these cases reduced glucose uptake may be related to impaired translocation, resulting in a reduced glucose uptake. Accordingly, abnormal cellular localization of GLUT4 in skeletal muscle from insulin-resistant subjects as well as reduced insulin-stimulated GLUT4 translocation in skeletal muscle from T2D patients have been demonstrated (120;139). In summary, a deeper understanding of intracellular signaling pathways which lead to GLUT4 translocation, could result in new approaches for the treatment of insulin resistance. Therapies that improve the recruitment of glucose transporters to the cell surface are likely to reduce the morbidity associated with T2D and may prevent the development of diabetes in individuals with high risk (117). The molecular mechanisms leading to skeletal muscle insulin resistance remain incompletely understood and controversial due to numerous players and the multifactorial nature of events involved in its development.

Obesity is often accompanied by elevated levels of circulating free fatty acids and induces activation of JNK, IKK, and IRS-1 Ser-307 phosphorylation (140). The fatty acid palmitate plays a particular role in promoting insulin resistance as it induces endoplasmic reticulum (ER) stress, cytokine production, and activates JNK (141). In addition, palmitate activates NF-κB signaling while inhibition of this pathway reverses lipid-induced insulin resistance (142). The lipid metabolite diacylglycerol (DAG) has also been shown to induce insulin resistance. Increased muscle DAG leads to muscle insulin resistance by activating PKC-θ and inducing IRS-1 Ser-307 phosphorylation (143).

In addition, obesity and a sedentary lifestyle are often characterized by a chronic low-grade inflammatory state, which is considered as a key component in promoting insulin resistance

(2). Adipose tissue expansion occurs in response to caloric overload and is associated with an increase in immune cell infiltration and a subsequent pro-inflammatory response (144). Adipocytes and macrophages are capable of secreting pro-inflammatory cytokines that induce insulin resistance. Elevated secretion of the chemokine MCP-1 by adipocytes have been shown to induce insulin resistance in skeletal muscle cells (145;146). Increased secretion of cytokines, such as TNF-α, IL1β, or IL-6, by both immune cells and adipocytes induces insulin resistance via multiple mechanisms, including activation of Ser/Thr kinases and reduction of IRS-1 and GLUT4 expression (129;133). Another driving factor in obesity-associated inflammation is caused by activation of Toll-like receptor (TLR), especially activation of TLR-4. TLRs belong to the innate immune system and are generally activated by pathogen-associated molecular patterns such as LPS, and induce inflammation via activation of the NF-κB pathway (147;148). TLRs are ubiquitously expressed and TLR-4 is elevated in skeletal muscle of patients with obesity and insulin resistance (148). Interestingly, saturated fatty acids (FA) can also activate this pathway, indicating a potential role for these receptors in obesity-driven inflammation (149).

Furthermore, mitochondrial dysfunction and reactive oxygen species (ROS) formation may also induce skeletal muscle insulin resistance. Low levels of ROS can enhance insulin action, but high concentration of ROS causes oxidative stress (150). ROS formation occurs as a by-product of the electron transport chain and is a major consequence of mitochondrial dysfunction (150). Increased ROS levels have been observed in obese and diabetic states and can be caused by an increased metabolite flux into mitochondria, alterations in mitochondrial proteins, and reduced expression of antioxidant enzymes (151;152). Increased oxidative stress leads to the activation of stress kinases that induce insulin resistance by serine phosphorylation of IRS proteins (151;152). Besides the aspect of ROS-mediated insulin resistance, altered mitochondrial dynamics in the form of increased mitochondrial fission leads to insulin resistance and can be rescued by inhibiting fission, which decreases the activity of p38 MAP kinase and increases IRS-1 and Akt activation (153).

In conclusion, the complexity of this signaling system is essential to mediate the variety of biological responses to insulin. Many steps are negatively regulated by action of phosphatases or inhibitory proteins. One of the big challenges remaining is to decode the complexity of the pathogenesis of insulin resistance. In rare cases, the cause is genetic, but in most others insulin resistance is triggered by cellular perturbations, such as lipotoxicity, inflammation, mitochondrial dysfunction, and ER stress which leads to deregulation of genes and inhibitory

protein modifications, those resulting in impaired insulin action. A better understanding of the causes and mechanisms leading to insulin resistance will be essential for a more effective treatment of type-2 diabetes and associated diseases.

1.2.2 Effects of physical activity on skeletal muscle insulin sensitivity

Regular physical activity leads to a number of adaptations in skeletal muscle that allow the muscle to more efficiently utilize substrate for ATP production and thus become more resistant to fatigue (154). Improved insulin action after a single bout of exercise was first demonstrated in 1982 using perfused isolated rat hindquarters (155). In that study, Richter et al. have shown that after treadmill running the ability of insulin to stimulate glucose uptake as well as glycogen synthesis was markedly increased (156). Furthermore, the insulin sensitizing effect of prior exercise is mainly observed in glycogen-depleted muscle suggesting that this phenomenon is locally related to contracting muscle (157). In addition, it was demonstrated that following exercise, a residual effect on glucose uptake is still measured 1-2 hours after completing the exercise even in the absence of insulin whereas 2.5 hours after exercises increased glucose uptake can only be measured in the presence of insulin (158;159). In addition to the acute effect on glucose transport, the sensitivity of skeletal muscle to insulin is markedly increased for more than 48 hours in human subjects (160;161). Despite 30 years of research aiming to characterize the connection between exercise and improved insulin action, the underlying mechanisms are still poorly understood.

Consistent with the observation that improved insulin action is mainly observed in glycogen-depleted muscle, it is believed that this finding is due to a local contraction-related mechanism. This hypothesis is supported by the finding that improved insulin sensitivity in rodents is still observed after contraction of one leg by electrical stimulation of the sciatic nerve followed by measurement of insulin sensitivity in both legs by hindquarter perfusion (157). This early findings in rodents have subsequently been supported by human studies using one-legged exercise models (161;162). Previously, it was assumed 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 (162;163). Also human studies reported increased insulin sensitivity after exercises without simultaneous increase in proximal insulin signaling (164;165). In contrast, a few studies

observed increased insulin-induced Akt phosphorylation after exercise (166;167). Taken together, it seems more relevant to focus on steps that are commonly used by exercise and insulin stimulation, and in addition may control glucose uptake. There is considerable evidence that regular physical activity increases the expression of GLUT4 protein level in skeletal muscle (162;168-170). Furthermore, translocation of intracellular GLUT4 vesicles to the cell surface is increased after chronic exercise (163;171). Therefore, current research tries to elucidate the role of distal signaling components in the insulin signaling pathway such as atypical protein kinase C, AS160 and TBC1D1, which leads to glucose uptake. These candidates are all influenced by insulin and exercise and might regulate the insulin-sensitizing effect of regular physical activity.

1.2.3 The effect of altitude hypoxia on glucose homeostasis

Altitude is characterized by hypoxia, low temperature, and low atmospheric pressure. Many researchers focused on hypoxic aspects of physiological responses since this condition exerts a potent effect on human metabolism.

Acclimation to an altitude of 4000 m has been found to decline blood glucose levels in parallel with a higher glucose turnover, both at rest and during exercise (172). The increased skeletal muscle glucose uptake is accompanied by enhanced insulin sensitivity (94;173). Likewise, it was reported in obese people that after being at high altitude (2600 m), the HbA1c level decreased significantly 4 weeks after the stay (174).
A number of studies have investigated glucose and insulin sensitivity in response to exercise under hypoxic conditions in subjects with T2D. Mackenzie et al. have shown that 1 h of exercise while breathing 14.8% O2 (corresponding to an altitude of appr. 2,800 m) increased glucose disposal and decreased blood glucose levels and HOMA-IR (Homeostasis Model Assessment of Insulin Resistance) to a greater extent than normoxic exercise in subjects with uncomplicated T2D [169]. Schobersberger et al. found reductions in glucose concentrations and HOMA-IR after oral glucose loading following 3 weeks of exercise at an altitude of 1,700 m in subjects with metabolic syndrome, four of whom had T2D [172].

In addition, a recently published study has shown that acute hypoxic training improved glucose tolerance while exercise at hypoxic conditions further ameliorated insulin sensitivity in T2D patients (175). Another study demonstrated that glucose tolerance enhancement 4 h

after exposure to hypoxic exercise can be attributed to improvements in peripheral insulin sensitivity in sedentary males with T2D (176). Moreover, it has been described that hypoxic exercise increased GLUT4 levels in patients with T2D (177).

Taken together, hypoxia and exercise show an additive effect on insulin sensitivity, suggesting that insulin signaling and insulin-dependent glucose uptake might be up-regulated following hypoxic exercise. Acute hypoxic exercise could improve glycemic control in patients with insulin resistance or T2D to a greater extent than exercise under normoxic conditions. Hence, a hypoxic stimulus might be a promising and practical tool for clinical use in obese, insulin-resistant and T2D patients to reduce blood glucose levels and improve insulin sensitivity.

1.2.4 Physical activity and low-grade systemic inflammation

Enlargement of adipose tissue as a consequence of a sedentary lifestyle and excessive energy intake results in a local inflammatory response in the visceral adipose tissue, infiltration of immune cells, and local and systemic increases of pro-inflammatory cytokines. Thus, obesity, insulin resistance and T2D are associated with a chronic low-grade systemic inflammation, and regular physical activity protects against a number of chronic diseases which are characterized by inflammation (2;178;179).

It is well established that regular physical activity reduces chronic low-grade inflammation and improves insulin action in subjects with insulin resistance and T2D. Cross-sectional studies have shown a strong inverse association between the level of physical activity and systemic low-grade inflammation (180-183). These observations might be explained by an anti-inflammatory effect of regular exercise which could be mediated via different mechanisms. Regular physical activity results in enhanced energy utilization thus leading to reduction of body weight and visceral fat mass which is an important source of pro-inflammatory cytokines (144;184). This may explain in part the reduction of inflammatory markers such as CRP following long-term exercise (183;184). Furthermore, it is well accepted that exercise decreases expression of TLR2 and TLR4 in immune cells and skeletal muscle (185). Interestingly, activation of TLR in skeletal muscle by factors such as lipopolysaccharide (LPS), heat shock protein 60 or free FAs has been suggested to participate in the development of insulin resistance (185;186). Moreover, due to exercise-induced

increases of adrenaline, cortisol, and other factors with immunomodulatory effects, physical activity may also have beneficial effects on the function of the immune system (187).

Starkie et al. investigated the hypothesis that acute exercise induces a direct anti-inflammatory response (188). Healthy subjects received LPS-endotoxin to induce low-grade inflammation during resting or after 2.5 hours bicycling. In resting subjects LPS-endotoxin administration resulted in a strong increase in systemic inflammation. Interestingly, in the exercising subjects the pro-inflammatory response, at the level of TNFα induction, was totally blunted. These data are further supported by *in vitro* experiments using electrical pulse stimulation (EPS) of primary human myotubes which induces contraction of the cells (189). Lambernd et al. demonstrated that EPS prevents inflammatory responses induced by various treatments due to blocking the pro-inflammatory signaling pathway (189). In conclusion, these data supporting the idea that physical activity and muscle contraction mediates an anti-inflammatory environment.

1.3 Skeletal muscle as an endocrine organ

The skeletal muscle is the largest organ in the human body in normal weight individuals and plays a key role in locomotion for maintaining the physical activities in human life. Skeletal muscles have additional properties as an elaborately energy production and consumption system that influences whole body energy metabolism. More recently, skeletal muscle has gained considerable interest as an endocrine organ (190;191). Therefore, researchers have tried to identify a contraction-induced humoral factor, an "exercises factor", that could be at least partly responsible for the health-promoting effects of physical activity, which protects against major chronic, low-grade inflammatory diseases like T2D, insulin resistance, the metabolic syndrome and many others. Cytokines and other peptides that are produced, expressed, and released by skeletal muscle cells and exert either paracrine or endocrine effects should be classified as "myokines" (192). IL-6 was the first myokine which fulfilled all criteria of being a myokine (192). During the last years several novel contraction-regulated myokines have been identified and characterized, e.g. interleukin 8 (IL-8), IL-7, IL-15, brain-derived neurotrphic factor (BDNF) and leukemia inhibitory factor (LIF) (2;191;193;194). Myokines are crucial for proper skeletal muscle function and homeostasis and are part of a complex network of inter-organ communication with e.g. liver and adipose tissue (Figure 3). In the following sections I will briefly introduce the current literature on this important area.

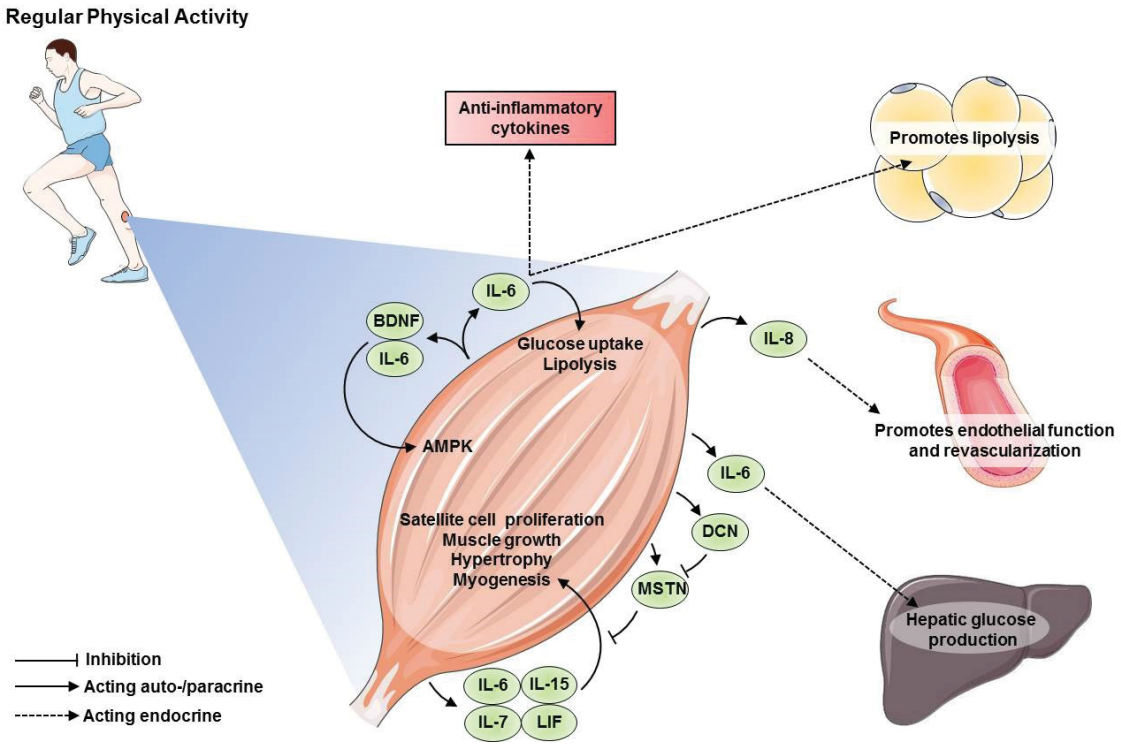

Figure 3: Selected myokines and their auto-/paracrine or endocrine functions. LIF, IL-6, IL-7 and IL-15 promote muscle growth and regeneration in response to exercise. Myostatin (MSTN) inhibits muscle hypertrophy and exercise provokes the release of myostatin inhibitor, decorin (DCN). BDNF and IL-6 are involved in AMPK-activation, and IL-6 enhances lipolysis and insulin-stimulated glucose uptake. IL-6 appears to have systemic effects on the liver and adipose tissue to enhance hepatic glucose production and lipolysis. Furthermore, IL-6 promotes the production of anti-inflammatory cytokines. IL-8 improves endothelial function and revascularization of ischaemic vessels.

1.3.1 Skeletal muscle and myokines

The best characterized myokine is interleukin 6 (IL-6), which is profoundly up-regulated by muscle contraction (145;149). The finding that contracting skeletal muscle releases significant amounts of IL-6 into the circulation during prolonged exercise was a milestone in this research field and proved skeletal muscle as an endocrine organ (195). Up to now, IL-6 is the most prominent muscle-derived myokine, which was demonstrated to be upregulated in the circulation after exercise without muscle damage (196;197). The level of circulating IL-6 increases in an exponential way in response to exercise and declines in the post-exercise period (3;191;198). The magnitude by which plasma levels increase is related to exercise duration, intensity, glycogen status, and the muscle mass involved in the mechanical work (199;200). IL-6 acts in an auto-/paracrine fashion within skeletal muscle and endocrine in a hormone-like fashion to mediate metabolic and anti-inflammatory effects (191;201). In

skeletal muscle, IL-6 signals via gp130Rb/IL-6 receptor agonist (IL-6Ra), which results in activation of AMPK and/or phosphoinositide 3-kinase (202-204). These pathways mediate increased glucose uptake, GLUT4 translocation and glycogen synthesis on acute exposure to IL-6. In addition, *in vitro* studies have shown that IL-6 increases skeletal muscle fatty acid oxidation and lipolysis via AMPK activation (205;206). Moreover, it was demonstrated that IL-6 is necessary for adult hypertrophic muscle growth *in vivo,* and muscle-produced IL-6 is a major regulator of satellite cell proliferation and myonuclear accretion (207).

IL-6 works also in an endocrine fashion to increase hepatic glucose production during exercise and lipolysis in adipose tissue (208-210). Moreover, IL-6 promotes glucagon-like peptide-1 (GLP-1) secretion and production in intestinal L-cells and pancreatic alpha cells thereby leading to improved beta cell insulin secretion and glucose tolerance (211). These data could explain the increase of GLP-1 plasma level during exercise, which may be mediated by skeletal muscle-derived IL-6 (211-213). Since the effect of GLP-1 on insulin secretion is glucose-dependent, IL-6–induced GLP-1 release will not acutely affect insulin secretion in healthy subjects during exercise but rather promote insulin secretion during a post-exercise meal (211). Furthermore, it is known that IL-6 released by contracting skeletal muscle triggers an anti-inflammatory cascade by inducing the production of the anti-inflammatory cytokines IL-10, IL-1 receptor antagonist (IL-1ra) and soluble TNF receptor (sTNFR) (2;191;201). Moreover, data suggest that IL-6 inhibits the production of pro-inflammatory TNFα. Starkie et al. has reported that in healthy humans elevation of plasma IL-6 level by acute exercise or infusion of IL-6 blunts an LPS-endotoxin-mediated increase of TNFα (188).

Leukemia inhibitory factor (LIF), another member of the IL-6 superfamily has been classified as a myokine (193). LIF has multiple biological functions within skeletal muscle such as induction of satellite cell proliferation, which is essential for muscle hypertrophy and regeneration after muscle damage (193). LIF is also a contraction-induced myokine and LIF mRNA expression is strongly up-regulated after acute exercise (193;214).

More recently, interleukin 7 (IL-7) was described as a novel exercise-regulated myokine (194). IL-7 belongs to the interleukin superfamily 2 and is required for T-cell and B-cell development but its relevance in non-immune cells has not been sufficiently explored. A study by Haugen et al. has shown that IL-7 is expressed and secreted by primary human

skeletal muscle cells (194). *In vitro* experiments suggested that IL-7 increased migration of satellite cells without affecting their proliferation (194).

Another member of the interleukin superfamily 2 is interleukin 15 (IL-15) which has been identified as an anabolic factor, and is highly expressed in skeletal muscle (215). Furthermore, IL-15 has been suggested to play a role in muscle-adipose tissue interaction (216). In differentiated human skeletal muscle cells, IL-15 induces an increase in accumulation of the protein myosin heavy chain (MHC), suggesting IL-15 as an anabolic factor in muscle growth, and IL-15 stimulates myogenic differentiation (217;218). However, the regulatory role of muscle contraction with regard to IL-15 is unclear.

Interleukin 8 (IL-8) is a known chemokine that primarily attracts neutrophils. In addition to its chemokine properties, IL-8 acts as an angiogenic factor (219). The plasma concentration of IL-8 increases in response to exhaustive exercise such as running, which involves eccentric muscle contractions, whereas no increase in plasma IL-8 in relation to concentric exercise was found (3;190). However, when measuring the arterial-venous concentration difference across a concentrically exercising limb, a small and transient net release of IL-8 was detectible, which did not result in an increase in the systemic IL-8 plasma concentration (220). The fact that high local IL-8 expression occurs in contracting muscle with only a small and transient net release may indicate that muscle-derived IL-8 acts locally and exerts its effect in an autocrine or paracrine fashion (220). IL-8 induces its chemotactic effects via the chemokine receptor CXCR1, whereas CXCR2, which is expressed by human microvascular endothelial cells, is the receptor responsible for IL-8-induced angiogenesis (221-223). The expression of the IL-8 receptor CXCR2 is enhanced in human skeletal muscle biopsies after concentric exercise and the increase in CXCR2 protein is seen not only in the muscle fibers but also to a larger extent in the vascular endothelium, suggesting that it may play a role in angiogenesis (224).

Brain-derived neurotrophic factor (BDNF) also has been described as an exercise-induced myokine (225), although the protein and its receptor are most abundantly expressed in the brain (226). In humans it should be noted that 70–80% of plasma BDNF originates from the brain during both rest and after exercise, suggesting the brain as the major source of this factor (227). It might be that muscle-derived BDNF acts primarily within skeletal muscle tissue, e.g. inducing lipid oxidation via AMPK activation (225), whereas brain-derived BDNF may act more systemically and is potentially involved in beneficial effects of exercise with regard to Alzheimer´s disease, depression or impaired cognitive function (228).

In addition, the myokine myostatin is a member of the transforming growth factor beta (TGF-β) superfamily that negatively regulates skeletal muscle size (229). Importantly, myostatin is antagonized by different factors such as follistatin (230) and decorin (231). Interestingly, decorin mRNA expression is enhanced in skeletal muscle after chronic exercise combining strength and endurance training (232). A study by Heinemeier et al. reported increase decorin mRNA expression in skeletal muscle following an acute enducance exercise (233), and decorin plasma levels are increased after acute resistance exercise (232).

Taken together, myokines are key elements of the multi-organ crosstalk communication network, and they play a prominent role in mediating the health-promoting effects of regular physical activity. Their effects involve substrate oxidation, lipid partitioning, reduction of inflammation, and improved pancreatic beta cell function. Furthermore, myokines play an essential role for skeletal muscle function by controlling skeletal muscle differentiation and growth, and regulating glucose and fat metabolism. The list of new myokines is continuously increasing, whereas the physiological impact of this multitude of molecules remains largely unexplored (234;235).

1.4 Objectives

As emphasized in the previous sections, the mechanisms involved in the beneficial effects of exercise attract increasing attention because of continuously increasing prevalence of T2D. Understanding these mechanisms regulated by muscle-contraction can potentially be used to develop effective and personalized exercise interventions and may be also useful in identifying relevant targets for drug development. 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.

Exercise, and especially the contracting muscle, is indeed a source of numerous "drug-like" molecules. Myokines are key elements for skeletal muscle function and play an important role in the multi-organ crosstalk communication network by mediating at least partially the health-promoting effects of regular physical activity. Some myokines act locally within skeletal muscle via a paracrine mechanism to promote muscle growth and regeneration in response to exercise. Progress in proteomics and other techniques has allowed identifying a myriad of novel myokines, whereas the physiological impact of this multitude of molecules remains poorly unknown. Therefore, the aim of the present work is to identify and characterize novel contraction-induced myokines *in vitro* and *in vivo* and analyze their biological functions in human skeletal muscle cells.

- Follistatin-like protein 1 (Fstl1) was identified as a myokine in rodents, but its existence and physiological role in humans was completely unknown. Therefore, the first objective of this thesis was to investigate the expression and regulation of Fstl1 in human skeletal muscle cells and to assess the impact of exercise on circulating Fstl1 levels. Furthermore, we investigated potential paracrine effects of Fstl1 *in vitro*.
- Using a comprehensive proteomic profiling of the human skeletal muscle secretome, chitinase-3-like protein 1 (CHI3L1) was identified by our group as a novel myokine. Therefore, the next objective of this thesis was to analyze the expression and regulation of CHI3L1 in human skeletal muscle cells. Moreover, we investigated potential auto-/paracrine effects of CHI3L1 in the context of skeletal muscle inflammation, insulin signaling, skeletal muscle growth and repair. Finally, we analyzed the role of exercise for CHI3L1 production.

As mentioned above, hypoxia and exercise have shown an additive effect on insulin sensitivity suggesting that insulin signaling and insulin-dependent glucose uptake might be up-regulated following hypoxic exercise. Acute hypoxic exercise could improve glycemic control in patients with T2D or insulin resistance to a greater extent than exercise under normoxic conditions. However, there is no consensus in these areas, most likely as a consequence of the variations in degree and duration of hypoxic exposure, as well as the broad range of experimental parameters used as markers of metabolic processes. Finally, the impact of the combination of contraction and hypoxia regarding insulin action in the skeletal muscle at the molecular level has never been investigated. We hypothesized that the combination of hypoxia and contraction would improve skeletal muscle insulin sensitivity and metabolism. Therefore, the aim was to establish an *in vitro* model of hypoxic exercise.

- In an attempt to resolve some of the controversies, we established a novel *in vitro* model of human skeletal muscle cell contraction at reduced oxygen levels by combining our well-established EPS model with the BioSpherix hypoxia working chamber. Furthermore, we analyzed the effect of different oxygen levels with or without EPS on insulin- and contraction-regulated glucose metabolism and tried to identify novel targets of insulin sensitivity in human skeletal muscle cells. Finally, we tried to translate these novel targets to the *in vivo* setting.

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

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***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.**

***Index Terms*— Follistatin-like protein 1, Skeletal muscle cells, Myokine, Exercise, Electrical pulse stimulation**

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I. 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 (1;2). 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 (3). 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 (4). 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 (5).

Follistatin-like 1 (Fstl1), also known as TSC-36, is a secreted glycoprotein and belongs to the follistatin family of proteins (6). Follistatin family members bind to TGF-ß super-family proteins and inhibit their functions (7). Oshima *et al.* have shown Fstl1 to be a cardiokine which is upregulated in ischemic injured and hypertrophic hearts of mice (8). 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 (9). 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.The aim of this study was to characterize CHI3L1 as a novel myokine and analyze potential auto- and/or paracrine effects in primary human skeletal muscle cells.

II. 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 (10). 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 sixwell dishes using a C-Dish in combination with a C-Pace pulse generator (C-Pace 100, IonOptix, Milton MA), as recently described by us . 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 2 ms and intensity of 11.5 V for 2 to 24 h. 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 (11). 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 the manufacturer's 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's protocol.

RNA isolation and RT-PCR: Total RNA was isolated using TriPure Isolation Reagent (Roche). RNA concentration and purity was measured using a NanoDrop 2000 (Thermo Scientific). One microgram of RNA was reverse transcribed using 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. 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 triplicates using a 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, 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 % VO_2max 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.

III. RESULTUS

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 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 concentration is affected by acute exercise: We analyzed the impact of acute exercise on Fstl1 serum concentration in eight human male subjects. We observed a significant 1.2fold increase of Fstl1 serum level 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 concentration was significantly reduced after a rest period of 120 min compared to post-exercise 0 min and 30 min and normalized at the basal level (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).

IV. 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

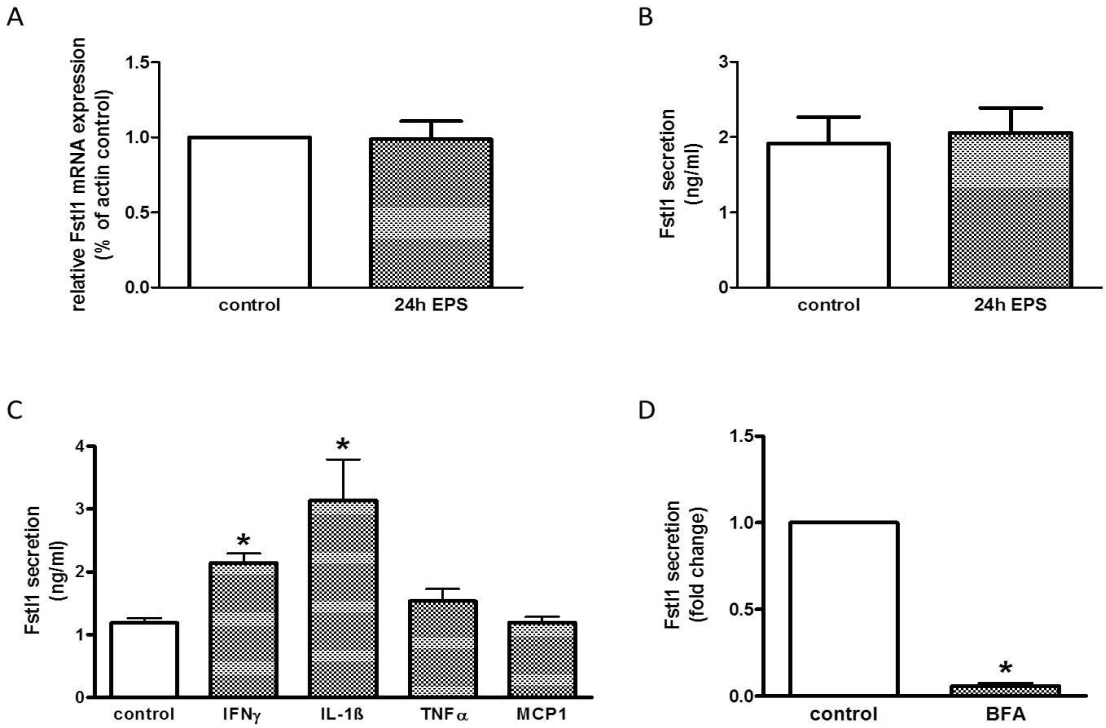

Figure 1: Comparison of Fstl1 secretion with and without EPS and regulation of Fstl1 secretion by different cytokines. (A) Comparison of Fstl1 mRNA expression in non-contracted skeletal muscle cells (control) and after 24 h EPS (1 Hz, 2 ms, 11.5 V). Fstl1 mRNA expression was analyzed by real time PCR 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. (D) Supernatants of control and brefeldin A treated cells were measured by ELISA and shown as fold change to control. Data are mean values ± SEM, n=8-10, * $p<0.05$ vs. control.

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 (12). In this study, we show that Fstl1 is expressed and secreted by primary human skeletal 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 (13). 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 (14). 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 (15). In addition, induction of Fstl1 by IL-1ß and LPS via an NFκB-dependent pathway has been reported (16). 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 (17). 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 (17). 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.

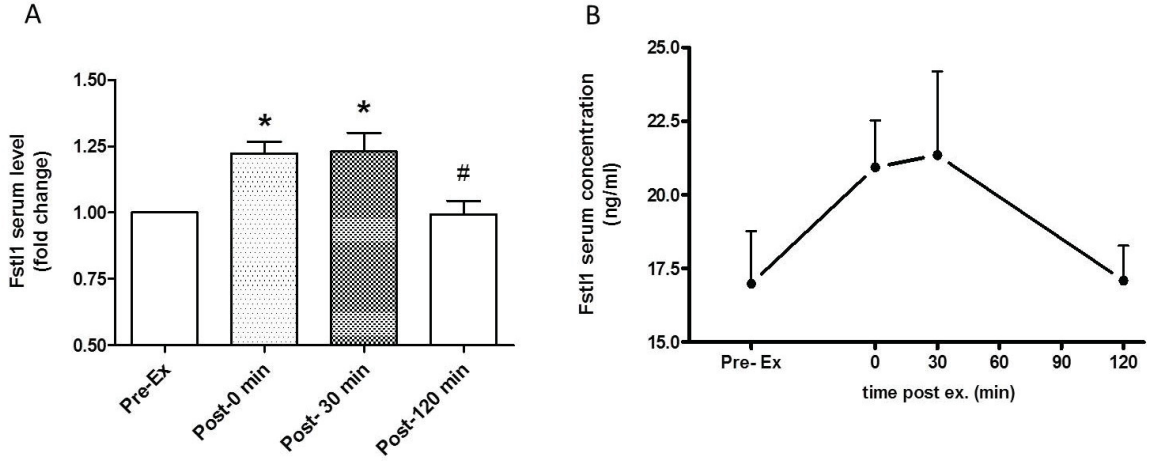

Figure 2: Fstl1 serum concentration is up-regulated after exercise. A baseline blood sample was taken before start of the exercise (Pre-Ex). Eight healthy lean male subjects cycled at 70 % VO_2max for 60 min and then rested for 2 h. Blood was sampled immediately after the exercise session (Post-0 min) 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 (18-21). An acute increase of IL-6 after exercise, for example, increases insulin-stimulated glucose uptake *in vitro* and may enhance fatty acid oxidation (22). 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

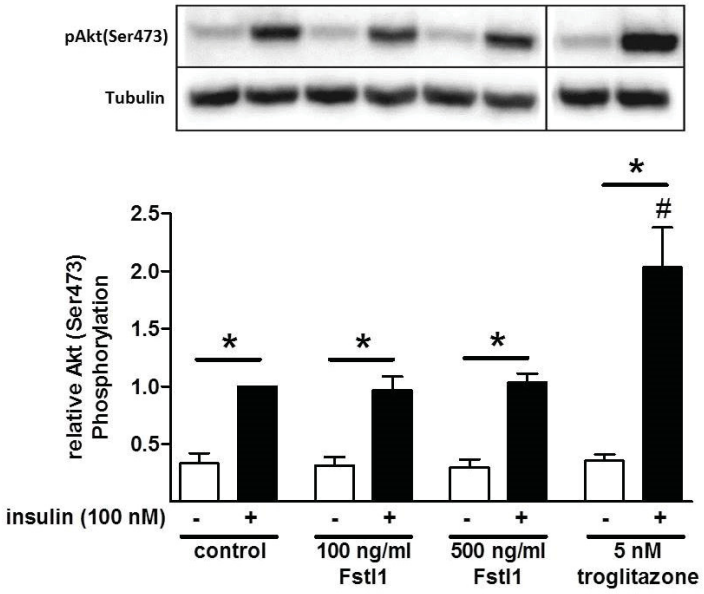

Figure 3: Effects of Fstl1 on Akt signaling in human skeletal muscle cells. Myotubes from different donors were cultured with two concentrations of Fstl1 (100 ng/ml and 500 ng/ml) or with troglitazone (5 nM) as positive control for 24 h. After acute stimulation with 100 nM insulin, total cell lysates 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.

myocardium has been reported (8). 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 (23). 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 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.

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	secretion
(days)	(relative Fstl1 mRNA expression)	(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.

V. REFERENCES

1. Broholm C, Laye MJ, Brandt C, Vadalasetty R, Pilegaard H, Pedersen BK, Scheele C. 2011. LIF is a contraction-induced myokine stimulating human myocyte proliferation. J Appl Physiol 111: 251-259.
2. Carey AL, Steinberg GR, Macaulay SL, Thomas WG, Holmes AG, Ramm G, Prelovsek O, Hohnen-Behrens C, Watt MJ, James DE, Kemp BE, Pedersen BK, Febbraio MA. 2006. Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase. Diabetes 55: 2688-2697.
3. Clutter SD, Wilson DC, Marinov AD, Hirsch R. 2009. Follistatin-like protein 1 promotes arthritis by up-regulating IFN-gamma. J Immunol 182: 234-239.
4. Dietze D, Koenen M, Rohrig K, Horikoshi H, Hauner H, Eckel J. 2002. Impairment of insulin signaling in human skeletal muscle cells by co-culture with human adipocytes. Diabetes 51: 2369-2376.
5. Hansen J, Brandt C, Nielsen AR, Hojman P, Whitham M, Febbraio MA, Pedersen BK, Plomgaard P. 2011. Exercise induces a marked increase in plasma follistatin: evidence that follistatin is a contraction-induced hepatokine. Endocrinology 152: 164-171.
6. Lambernd S, Taube A, Schober A, Platzbecker B, Gorgens SW, Schlich R, Jeruschke K, Weiss J, Eckardt K, Eckel J. 2012. Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways. Diabetologia 55: 1128-1139.
7. Matthews VB, Astrom MB, Chan MH, Bruce CR, Krabbe KS, Prelovsek O, Akerstrom T, Yfanti C, Broholm C, Mortensen OH, Penkowa M, Hojman P, Zankari A, Watt MJ, Bruunsgaard H, Pedersen BK, Febbraio MA. 2009. Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. Diabetologia 52: 1409-1418.
8. Nielsen AR, Mounier R, Plomgaard P, Mortensen OH, Penkowa M, Speerschneider T, Pilegaard H, Pedersen BK. 2007. Expression of interleukin-15 in human skeletal muscle effect of exercise and muscle fibre type composition. J Physiol 584: 305-312.
9. Norheim F, Raastad T, Thiede B, Rustan AC, Drevon CA, Haugen F. 2011. Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training. Am J Physiol Endocrinol Metab 301: E1013-E1021.
10. Ogura Y, Ouchi N, Ohashi K, Shibata R, Kataoka Y, Kambara T, Kito T, Maruyama S, Yuasa D, Matsuo K, Enomoto T, Uemura Y, Miyabe M, Ishii M, Yamamoto T, Shimizu Y, Walsh K, Murohara T. 2012. Therapeutic Impact of Follistatin-like 1 on Myocardial Ischemic Injury in Preclinical Animal Models. Circulation 126: 1728-38.
11. Oshima Y, Ouchi N, Sato K, Izumiya Y, Pimentel DR, Walsh K. 2008. Follistatin-like 1 is an Akt-regulated cardioprotective factor that is secreted by the heart. Circulation 117: 3099-3108.
12. Ouchi N, Oshima Y, Ohashi K, Higuchi A, Ikegami C, Izumiya Y, Walsh K. 2008. Follistatin-like 1, a secreted muscle protein, promotes endothelial cell function and revascularization in ischemic tissue through a nitric-oxide synthase-dependent mechanism. J Biol Chem 283: 32802-32811.
13. Pedersen BK. 2011. Exercise-induced myokines and their role in chronic diseases. Brain Behav Immun 25: 811-816.
14. Pedersen BK. 2012. Muscular interleukin-6 and its role as an energy sensor. Med Sci Sports Exerc 44: 392-396.
15. Pedersen BK, Febbraio MA. 2012. Muscles, exercise and obesity: skeletal muscle as a secretory organ. Nat Rev Endocrinol 8: 457-465.
16. Rosenberg MI, Georges SA, Asawachaicharn A, Analau E, Tapscott SJ. 2006. MyoD inhibits Fstl1 and Utrn expression by inducing transcription of miR-206. J Cell Biol 175: 77-85.
17. Shi Y, Massague J. 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113: 685-700.
18. Shibanuma M, Mashimo J, Mita A, Kuroki T, Nose K. 1993. Cloning from a mouse osteoblastic cell line of a set of transforming-growth-factor-beta 1-regulated genes, one of which seems to encode a follistatin-related polypeptide. Eur J Biochem 217: 13-19.
19. Sumitomo K, Kurisaki A, Yamakawa N, Tsuchida K, Shimizu E, Sone S, Sugino H. 2000. Expression of a TGF-beta1 inducible gene, TSC-36, causes growth inhibition in human lung cancer cell lines. Cancer Lett 155: 37-46.
20. Wilson DC, Marinov AD, Blair HC, Bushnell DS, Thompson SD, Chaly Y, Hirsch R. 2010. Follistatin-like protein 1 is a mesenchyme-derived inflammatory protein and may represent a biomarker for systemic-onset juvenile rheumatoid arthritis. Arthritis Rheum 62: 2510-2516.
21. Wolsk E, Mygind H, Grondahl TS, Pedersen BK, van Hall G. 2010. IL-6 selectively stimulates fat metabolism in human skeletal muscle. Am J Physiol Endocrinol Metab 299: E832-E840.
22. Yoon JH, Song P, Jang JH, Kim DK, Choi S, Kim J, Ghim J, Kim D, Park S, Lee H, Kwak D, Yea K, Hwang D, Suh PG, Ryu SH. 2011. Proteomic analysis of tumor necrosis factor-alpha (TNF-alpha)-induced L6 myotube secretome reveals novel TNF-alpha-dependent myokines in diabetic skeletal muscle. J Proteome Res 10: 5315-5325.

ABBREVIATIONS AND ACRONYMS

(EPS) electrical pulse stimulation; (Fstl1) follistatin-like 1; (IFNγ) interferon gamma; (IL) interleukin; (LPS) lipopolysaccharide; (MCP1) monocyte chemotactic protein-1; (SDS-PAGE) sodium dodecyl sulfate polyacrylamide gel electrophoresis; (TGF-ß) transforming growth factor beta; (TNFα) tumor necrosis factor alpha

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CONFLICTS OF INTEREST

The authors report no declarations of interest.

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	Analysed data: 70%
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	Wrote the manuscript: 70%
	Reviewed / edited manuscript: 70%
Author:	1st author (shared)

2.2 Chitinase-3-like protein 1 protects skeletal muscle from TNFα-induced inflammation and insulin resistance

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Abstract— **CHI3L1 is a glycoprotein consisting of 383 amino acids with a molecular mass of 40 kDa, and its serum level is elevated in inflammatory diseases. Although CHI3L1 is described as a biomarker of inflammation, the function of this protein is not completely understood. Here, we examined the regulation of CHI3L1 in primary human skeletal muscle cells. Moreover, we analyzed potential autocrine effects of CHI3L1. We show that myotubes express CHI3L1 in a differentiation-dependent manner. Furthermore, pro-inflammatory cytokines up-regulate CHI3L1 expression (6-fold) and release (3-fold). Importantly, CHI3L1 treatment blocked tumor necrosis factor α (TNFα) -induced inflammation by inhibiting NF-κB activation in skeletal muscle cells. We show that this effect is mediated via protease activated receptor 2 (PAR2). In addition, CHI3L1 treatment diminished the TNFα-induced expression and secretion of IL-8, MCP1 and IL-6. Also, impaired insulin action at the level of Akt and glycogen synthase kinase (GSK) 3α/ß phosphorylation and insulin-stimulated glucose uptake was normalized by CHI3L1. In conclusion, the novel myokine CHI3L1 which is induced by pro-inflammatory cytokines can counteract TNFα-mediated inflammation and insulin resistance in human skeletal muscle cells, potentially involving an auto/paracrine mechanism.**

Index Terms— **Chitinase-3-like protein 1, Skeletal muscle cells, Myokines, Inflammation**

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I. INTRODUCTION

Inflammation plays an important role in the development of peripheral insulin resistance in type 2 diabetes [1]. Elevated levels of the pro-inflammatory cytokine tumor necrosis factor α (TNFα) are found in plasma [2], adipose tissue [3] and skeletal muscle [4] in patients with whole body insulin resistance. TNFα infusion in healthy subjects rapidly induces skeletal muscle insulin resistance by disrupting insulin signaling to GLUT4 translocation [5]. Furthermore, it could be demonstrated that TNFα directly induces insulin resistance in primary human skeletal muscle cells [5]. Thus, the identification and characterization of novel molecules which are able to reduce TNFα-mediated inflammation will certainly be an important approach to prevent the development of peripheral insulin resistance.

Chitinase-3-like protein 1 (CHI3L1; also known as YKL-40) is a heparin- and chitin-binding glycoprotein and was originally discovered in mouse breast cancer cells [6]. CHI3L1 is a member of the protein family of chi-lectins (chitinase-like lectins) [7]. The members of this protein family are structurally related to the family 18 glycohydrolases. Based on a substitution of the catalytic essential glutamic acid residue in the active site of this protein, CHI3L1 has no enzymatic activity [8]. CHI3L1 is expressed in a variety of cells including macrophages, chondrocytes, fibroblasts, vascular smooth muscle cells and endothelial cells [9]. The expression of CHI3L1 has been shown to be regulated by various pro-inflammatory cytokines including interleukin (IL)-6, interferon γ (IFNγ), IL-1ß and TNFα [9]. Elevated serum levels of CHI3L1 are found in a number of human diseases characterized by acute or chronic inflammation and tissue remodeling [9]. For example, circulating levels of CHI3L1 are increased in patients with type 2 diabetes and positively correlate with insulin resistance while no correlation with body mass index was found [10]. Importantly, elevated CHI3L1 levels are a predictor of overall and cardiovascular mortality [11]. However, understanding the biological roles of CHI3L1 is still in its beginning. CHI3L1 has been shown to act as a growth factor for skin and fetal fibroblasts and to promote bronchial smooth muscle cell proliferation and migration by a protease activated receptor 2 (PAR2)-dependent pathway [12;13]. Immune response studies have linked CHI3L1 to a down-regulation of the inflammatory mediators matrix metalloprotease (MMP) 1, MMP3 and IL-8, suggesting a protective influence under innate immune response condition [14]. Knockout studies of the mouse orthologue of CHI3L1 (breast regression protein 39) revealed a significant reduction in the antigen-induced Th2 inflammatory response. In addition, it has been shown that epithelial CHI3L1 rescues the Th2 responses in these animals [15]. Taken together, these observations suggest that CHI3L1 may play a protective

role in inflammatory environments, limiting degradation of the extracellular matrix and thus controlling tissue damage. However, the biological function of CHI3L1 is not completely understood.

The aim of this study was to characterize CHI3L1 as a novel myokine and analyze potential auto- and/or paracrine effects in primary human skeletal muscle cells.

II. MATERIAL AND METHODS

Materials: 2-Deoxy-D-[^{14}C] glucose (2-DOG) and L-[^{14}C] glucose were purchased from GE Healthcare (Uppsala, Sweden). Liquid scintillation Aquasafe 300 plus from Zinsser Analytic (Frankfurt, Germany) was used for glucose uptake assays. TNFα was provided by Sigma-Aldrich (Munich, Germany). IL-1ß, IL-6 and IFNγ were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Recombinant human CHI3L1 was provided by R&D Systems (Wiesbaden, Germany). Primary human skeletal muscle cells and supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). Horse serum for differentiation medium was provided by Gibco (Berlin, Germany). Anti-phospho Akt(Ser473), anti-phospho Akt(Thr308), anti-phospho GSK3α/β(Ser21/Ser9), anti-phospho NF-κB (p65), total Akt and total GSK3α/β were supplied by Cell Signalling Technology (Frankfurt, Germany). Anti-CHI3L1, anti-tubulin and anti-ß-actin were obtained from Abcam (Cambridge, U.K.). Anti-PAR2 was purchased by Santa Cruz Biotechnology (Heidelberg, Germany). PAR2 agonist (H-Ser-Leu-Ile-Gly-Lys-Val-NH2) was from by Bachem (Bubendorf, Switzerland). The specific IKK inhibitor (I229) was kindly provided by Sanofi-Aventis (Frankfurt, Germany). All other chemicals were of the highest analytical grade commercially available and purchased from Sigma.

Culture of human skeletal muscle cells: Primary human skeletal muscle cells isolated from seven healthy Caucasian donors (4 males 16, 21, 41 and 47 of age; 3 females 25, 33 and 37 of age) were supplied as proliferating myoblasts and cultured as described before [16]. For an individual experiment, myoblasts were seeded in six-well culture dishes and 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. For the differentiation experiments serum-free medium was used during the complete differentiation period.

Generation of adipocyte-conditioned medium (CM): Adipose tissue samples were obtained from subcutaneous fat of normal or moderately overweight women (BMI 27.9±0.9 kg/m^2, age 26–44 years). The procedure for obtaining adipose tissue was approved by the Ethics Committee of the Heinrich-Heine University, Düsseldorf, 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 [16;17]. After 15 days, 70–90 % of the pre-adipocytes differentiated to mature adipocytes as defined by accumulation of lipid droplets. These mature adipocytes were then used to generate CM by incubation with αMEM for 48 h [17].

Immunoblotting: Skeletal muscle cells were treated as indicated and lysed in buffer containing 50 mmol/L HEPES (pH 7.4), 1 % Triton X-100, PhosStop, and Complete Protease Inhibitor cocktail (Roche, Penzberg, Germany). After incubation for 2 h at 4°C, the suspension was centrifuged at 15,000 rpm for 15 min. 7 µg of total cell lysates were separated by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked in TBS containing 0.1 % Tween-20 and 5 % nonfat dry milk and incubated over night with the appropriate antibodies. After washing, membranes were incubated with secondary horseradish peroxidase-coupled antibody and processed for enhanced chemiluminescence detection using Immobilion Western detection reagents (Millipore, Schwalbach, Germany). Signals were visualized and evaluated on a VersaDoc 4000 MP BioRad (Munich, Germany) work station and analyzed by Quantity One analysis software (Version 4.6.7).

Analyses of supernatants: During differentiation supernatants from human skeletal muscle cells were collected after indicated time points, and CHI3L1 protein concentration was determined using the CHI3L1 Quantikine ELISA from R&D Systems (Wiesbaden, Germany). IL-8 and IL-6 concentrations in the supernatants were analyzed by ELISA kits from Gen-Probe (Bedford, MA), and MCP1 was detecting using an ELISA kit from RayBiotech (Norcross, GA, USA).

Treatment of skeletal muscle cells: Differentiated skeletal muscle cells were incubated with IFNγ (10 ng/ml), TNFα (50 pg/ml or 20 ng/ml), and IL-1ß (10 ng/ml) as well as with CM for 24 h, and CHI3L1 secretion and total protein levels were assessed by Western Blot. To analyze the activation of NF-κB subunit p65, myotubes were pre-incubated with CHI3L1 (100 ng/ml) and trypsin (50 nmol/L), respectively, for 30 min followed by stimulation with TNFα (50 pg/ml) or IL-ß (5 ng/ml) for 10 min. For neutralization studies we used an azide-free PAR2 antibody from Santa Cruz (SAM11-L). Cells were pre-incubated with the antibody (25 µg/ml) or PAR2-activating peptide (PAR-AP) (100 ng/ml) for 30 min followed by CHI3L1 and TNFα treatment. I229, a specific IκB kinase complex inhibitor targeting both IKKα and IKKß [18], was dissolved in DMSO as a 10 mmol/L stock solution, and was further diluted in sterile αMEM medium to a final concentration of 10 µmol/L. All controls of experiments involving the IKK-inhibitor were treated with 0.1% DMSO alone. To induce insulin resistance, cells were treated with TNFα (20 ng/ml) for 3 h. Subsequently, cells were stimulated with insulin (100 nmol/L) for 10 min.

Immunofluorescence staining: For immunecyto-chemistry, human skeletal muscle cells were cultured on 12x12 mm coverslips. After 6 days of differentiation, myotubes were washed with PBS and fixed with 3 % formaldehyde for 15 min at room temperature.

Afterwards, cells were washed three times with PBS for 5 min and incubated with blocking solution (PBS containing 1 % BSA and 0.1 % Triton X-100) for 1 h at room temperature. Mouse anti-human sarcomeric alpha-actinin and rabbit anti-human CHI3L1 (Abcam, Cambridge, UK) were diluted 1:100 in blocking solution and incubated at 4°C over night. After 3 washes for 5 min with PBS cells were incubated with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 555 (Life Technologies, Carlsbad, CA) diluted 1:500 in blocking solution for 1 h at room temperature. Nuclei were stained with 1 µg/ml DAPI (Life Technologies) in PBS for 10 min at room temperature. Finally, coverslips were mounted with ProLong Gold Antifade reagent (Life Technologies). Cells were analyzed using a Zeiss fluorescence microscope (Oberkochen, Germany) equipped with a Axio Cam MRc5. Images were acquired with AxioVision rel. 4.3 with equal settings for contrast/brightness and merged by use of Image J software.

Glucose uptake: Differentiated human skeletal muscle cells were pre-incubated with 100 ng/ml CHI3L1, TNFα (20 ng/ml) or the combination of both factors. Afterwards, the cells were stimulated with 100 nmol/L insulin for 30 min. Then, 2-DOG (9.25 kBq/ml per well) was added and uptake was measured for 2 h. The experiment was terminated by repeated washing with ice-cold 0.25 µmol/L cytochalasin B and then, cells were lysed with 1 mol/L NaOH. The radioactivity of the lysates was counted in a liquid scintillation counter (Beckman, Munich, Germany). Values were corrected for non-specific uptake as measured after incubation with L-[^{14}C] glucose.

RNA isolation and RT-PCR: Cells were lysed with TriPure Isolation Reagent (Roche) and total RNA was isolated using a RNeasy kit from Qiagen (Hilden, Germany). Concentration and purity was measured using a NanoDrop 2000 (Thermo Scientific, Bremen, Germany). 1 µg of RNA was reverse transcribed using Omniscript RT Kit (Qiagen). CHI3L1 (Hs_CHI3L1_1_SG) and TNFα (Hs_TNF_1_SG) mRNA expression were measured using pre-designed primers (Quantitect Primer Assay, Qiagen) and GoTaq qPCR Master Mix (Promega, Mannheim, Germany) in a SYBR Green-based Real-Time PCR. Samples were run in triplicates using a 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 ß-actin.

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.

III. RESULTUS

The novel myokine CHI3L1 is expressed in a differentiation-dependent manner. We first characterized CHI3L1 as a novel myokine and studied its expression and release by human skeletal muscle cells. CHI3L1 protein levels are significantly reduced during skeletal muscle cell differentiation. Compared to myoblasts corresponding to day 0 of differentiation, differentiated myotubes of day 6 show a ~ 80% decrease of CHI3L1 mRNA expression and a significant reduced protein abundance (~ 56% decrease) (Fig. 1A and B). Also the release of CHI3L1 was significantly decreased during differentiation. While in the supernatant of skeletal muscle cells differentiated for two days a CHI3L1 concentration of 1.27±0.26 ng/ml was measured, the supernatant of myotubes differentiated for six days contained a concentration of 0.56±0.17 ng/ml (Fig. 1C). To validate the differentiation of the cells we analyzed different markers of skeletal muscle differentiation. During differentiation, we observed a significant increase of protein levels of insulin receptor ß subunit (IR-ß subunit), total myosin heavy chain (MHC) and a characteristic expression of myogenin (Fig. 1D-F).

CHI3L1 is induced by pro-inflammatory signaling. After 24 h incubation of undifferentiated (myoblast) and differentiated (myotubes) human skeletal muscle cells with CM, which was shown before to induce pro-inflammatory signaling [19], we observed a strong induction of CHI3L1 protein in human myotubes. CHI3L1 protein increased up to 6-fold compared to control and was even 3.1-fold higher compared to CM-treated myoblast (Fig. 2A). In contrast, after CM treatment of myoblasts only a minor induction of CHI3L1 was observed (Fig. 2A). To confirm that CHI3L1 is produced by human skeletal muscle cells, immunofluorescence staining of CHI3L1 and sarcomeric α-actinin was performed. The results show that CHI3L1 is located within myotubes (Fig. 2B a-h) thereby supporting the notion that CHI3L1 is a myokine. In addition, the fluorescence signal of CHI3L1 was enhanced after 24 h CM treatment compared to the control situation (Fig. 2B b and f).

Moreover, we assessed the effect of selected pro-inflammatory cytokines on CHI3L1 expression and release. Low dose TNFα (50 pg/ml) treatment significantly increased CHI3L1 mRNA expression (1.5-fold after 6 h, and 2.1-fold after 24 h), protein level (2.4-fold after 24 h) and secretion (0.84±0.13 ng/ml up to 1.62±0.25 ng/ml) after 24 h treatment (Fig. 2B-D). In addition, treatment with high doses of TNFα (20 ng/ml) produced a substantially more prominent increased of CHI3L1 mRNA expression (4.2-fold) and secretion (0.80±0.11 ng/ml up to 3.75±0.50 ng/ml), but only after 24 h treatment (Suppl. Fig. S1A and B). Moreover, the increased CHI3L1 mRNA expression is significantly reduced after 48 h TNFα treatment compared to 24 h, whereas CHI3L1 secretion remains unaltered and raised up to 6.51±1.13 ng/ml (Suppl. Fig. S1A and B). Furthermore, the pro-inflammatory cytokines IFNγ, IL-6 and IL-1ß increased CHI3L1 protein level 2-fold and

induced a significantly augmented release of CHI3L1 after 24 h incubation (Suppl. Fig. S2A and B). In contrast, the anti-inflammatory cytokine IL-4 showed no significant influence on CHI3L1 mRNA expression or secretion (Suppl. Fig. S3A and B).

Next, to investigate whether CHI3L1 expression and release is under control of the NF-κB pathway we treated differentiated myotubes with TNFα, the IKK inhibitor I229, and the combination of both (Fig. 2C-E). I229 significantly reduced basal CHI3L1 mRNA expression after 6 h which was not further altered up to 24 h (Fig. 2C). TNFα-induced CHI3L1 mRNA expression after 6 h and 24 h was completely blocked by I229 (Fig. 2C). I229 tends to reduce CHI3L1 basal protein levels (Fig. 2D), but completely inhibited TNFα-induced CHI3L1 production after 24 h treatment (Fig. 2D). Also, the secretion of CHI3L1 is enhanced by TNFα while I229 treatment reduced the secretion to the level of untreated control cells (Fig. 2E).

CHI3L1 abolishes TNFα-induced NF-κB activation via a PAR2-dependent mechanism. To investigate the physiological role of CHI3L1 in an inflammatory setting we treated human skeletal muscle cells with TNFα and IL-1ß, respectively, for 10 min and observed a strong induction of NF-κB activation as measured by phosphorylation of the p65 subunit (Fig. 3A and B). However, pre-incubation with CHI3L1 for 30 min prevented this TNFα-induced p65 phosphorylation (Fig. 3A). The same effect was obtained by pre-incubation with trypsin, suggesting involvement of the PAR2 receptor in this process (Fig. 3A). In contrast, IL-1ß-induced p65 phosphorylation was unaffected by CHI3L1 or trypsin pre-incubation as shown in Fig. 3B.

We next investigated the expression of PAR2, the putative receptor for CHI3L1 in human skeletal muscle cells. Using quantitative Real-Time PCR and Western Blot analysis we show here that PAR2 is expressed both at mRNA and protein level. PAR2 is differentiation-dependent regulated with a low level in myoblast and a strong up-regulation during differentiation resulting in a 5-fold higher mRNA level and a 3fold higher protein level in myotubes compared to myoblasts (Fig. 4A and B). Furthermore, we observed that CM and TNFα treatment, respectively, for 24 h are able to increase PAR2 protein abundance by 1.5-fold (Fig. 4C).

To confirm that CHI3L1 acts via a PAR2-dependent mechanism we used a blocking antibody (anti-PAR2) against PAR2. Pre-incubation with anti-PAR2 antibody completely abolished the protective effect of CHI3L1 on TNFα-induced p65 phosphorylation (Fig. 4D). The

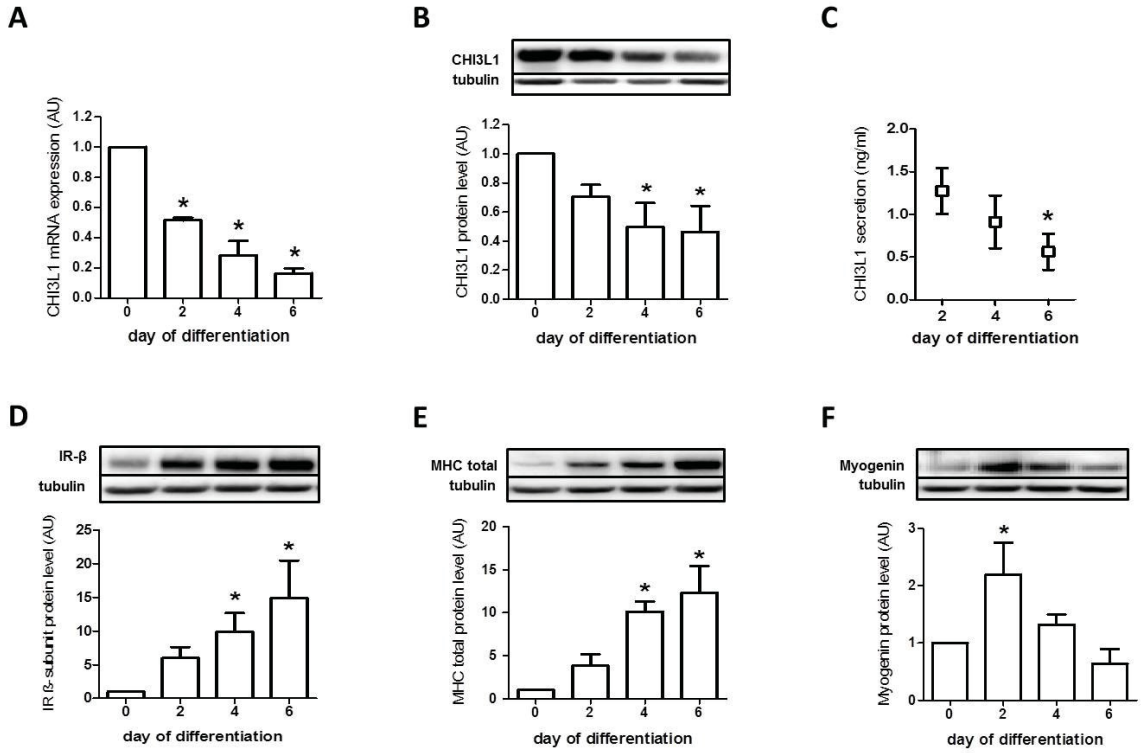

Figure 1: Differentiation-dependent CHI3L1 expression and secretion
(**A**) Primary human skeletal muscle cells were differentiated for the indicated time points and CHI3L1 mRNA expression was analyzed by Real-Time PCR and normalized to ß-actin expression. (**B**) CHI3L1 protein levels during differentiation were analyzed by Western Blot. (**C**) CHI3L1 protein secretion was analyzed by ELISA at the indicated day of differentiation. (**D-F**) Three differentiation markers of human skeletal muscle differentiation were analyzed during differentiation by Western Blot, namely IR ß subunit (**D**), total MHC (**E**) and myogenin (**F**). All data are normalized to tubulin and expressed relative to day 0. Data are mean values ± SEM, n= 5-7, * $p<0.05$ vs. day 0 and day 2 for CHI3L1 secretion, respectively. AU = arbitrary units.

antibody alone or in combination with TNFα had no effect on NF-κB activation. In addition, we repeated the signaling, human skeletal muscle cells were incubated with TNFα, CHI3L1 or with a combination of both

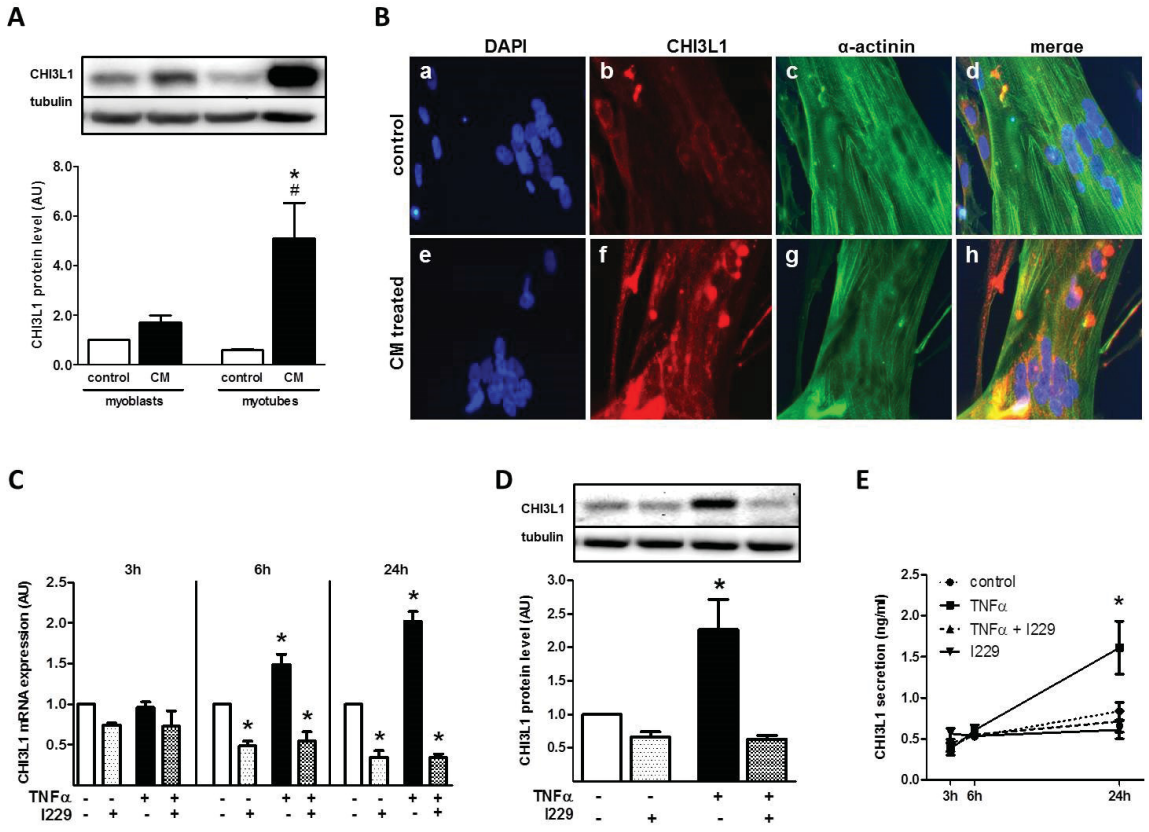

Figure 2: Up-regulation of CHI3L1 protein level and secretion by inflammatory cytokines
(A) Undifferentiated (myoblast) and differentiated (myotubes) human skeletal muscle cells were treated with CM for 24 h, and CHI3L1 protein levels were analyzed by Western Blot. Data are normalized to tubulin and expressed as fold over control. **(B)** Localization of CHI3L1 in control human myotubes (a-d, upper panel) and effects of CM treatment on CHI3L1 induction (e-f, lower panel) were analyzed by immunofluorescence staining. Cells were fixed and stained for CHI3L1 (b and f; red) and sarcomeric α-actinin (c and g; green). Nuclei were stained with DAPI (a and e). In d and h merged images of control and CM-treated myotubes are shown. Magnification ×80. (**C**) Differentiated skeletal muscle cells were treated with TNFα (50 pg/ml) and the IKK-inhibitor I229 (10 µmol/L) for the indicated time points. CHI3L1 mRNA expression was analyzed by Real-Time PCR and normalized to ß-actin expression. (**D**) CHI3L1 protein levels were analyzed by Western Blot after treatment with TNFα (50 pg/ml), I229 (IKK-inhibitor) (10 µmol/l) or in combination of both for 24 h. Data are normalized to tubulin and expressed as fold over control. (**E**) CHI3L1 protein secretion was analyzed by ELISA after treatment with TNFα (50 pg/ml), I229 (IKK-inhibitor) (10 µmol/l) or in combination of both. All data are mean values ± SEM, n=5-8, * $p<0.05$ vs. control and # $p<0.05$ vs. myoblasts treated with CM.

experiments in undifferentiated myoblasts since these cells possess a very low basal PAR2 expression as shown above (Fig. 4A, B and D). 10 min of TNFα treatment induced a strong p65 phosphorylation in human myoblast. However, pre-incubation with CHI3L1 was not able to prevent this TNFα-induced p65 phosphorylation (Fig. 4E). Moreover, we used the PAR2 agonist SLIGKV-amide (PAR2-AP), which corresponds to the tethered ligand sequence of human PAR2, to treat human myotubes. Pre-incubation with PAR2-AP also significantly reduced TNFα-induced p65 phosphorylation (Suppl. Fig. S4).

CHI3L1 prevents TNFα-induced secretion of MCP1, IL-8 and IL-6. To determine whether CHI3L1 is also able to block physiological effects of TNFα beyond factors for 24 h, and secretion of the well-known NF-κB targets MCP1, IL-8 and IL-6 were analyzed. As shown in Fig. 5, secretion of these three cytokines was strongly up-regulated by TNFα treatment while CHI3L1 itself had no effect. MCP1 secretion was increased by 7.4-fold after TNFα treatment. While in supernatants from untreated myotubes 120±54 pg/ml of MCP1 was measured, 889±270 pg/ml was found after TNFα treatment (Fig. 5A). The combination of TNFα and CHI3L1 significantly reduced the secretion of MCP1 by 58 % compared to TNFα alone (Fig. 5A). For IL-8 secretion, we observed an 8-fold increased secretion after TNFα treatment (control: 231±95 pg/ml vs. TNFα: 1532±145 pg/ml), while the combination with CHI3L1 reduced this effect by 45 % (Fig. 5B). IL-6 secretion was

4.5-fold up-regulated by TNFα (control: 99±26 pg/ml vs. TNFα 450±134 pg/ml), while simultaneous treatment with CHI3L1 completely inhibited TNFα-induced IL-6 secretion (Fig. 5C).

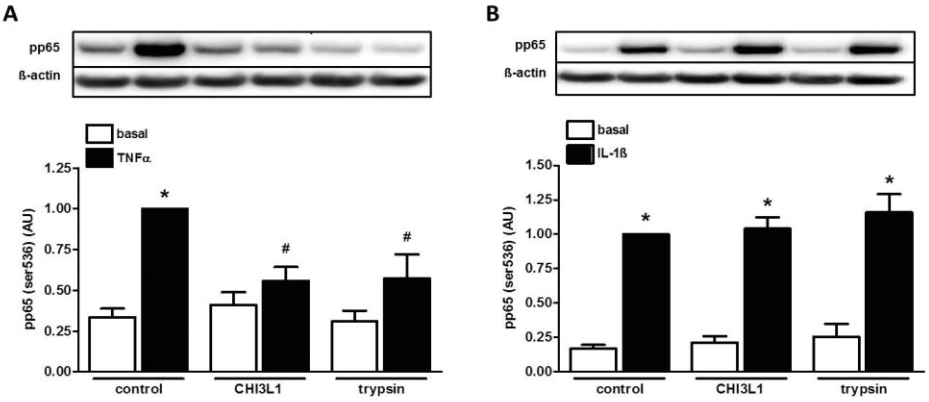

Figure 3: CHI3L1 blocks TNFα- but not IL-1β -induced NF-κB activation
(A) Differentiated human skeletal muscle cells were treated for 10 min with TNFα (50 pg/ml) or (**B**) IL-1β (5 ng/ml), and in combination with CHI3L1 (100 ng/ml) and trypsin (50 nmol/L), respectively. CHI3L1 and trypsin were added 30 min before TNFα stimulation. Phosphorylation of NF-κB subunit p65 was assessed by Western Blot. Data are normalized to β-actin and expressed relative to TNFα treatment. Data are mean values ± SEM, n=3-5, * $p<0.05$ vs. TNFα, # $p<0.05$ vs. corresponding condition without TNFα and IL-1β treatment, respectively.

CHI3L1 normalizes TNFα-induced insulin resistance. Acute stimulation of primary human skeletal muscle cells with insulin strongly increased Akt(Ser473) and Akt(Thr308) phosphorylation as shown in Fig. 6A-C. Insulin action was unaltered in cells treated with CHI3L1 alone, while TNFα treatment impaired insulin action on Akt(Ser473) and Akt(Thr308) phosphorylation by ~45 %. In the combination of TNFα and CHI3L1 insulin-mediated Akt(Ser473) and Akt(Thr308) phosphorylation were completely restored (Fig. 6A-C). Next, we assessed the effect of TNFα +/- CHI3L1 on phosphorylation of the Akt substrate GSK3. Insulin increased phosphorylation of GSK3α at Ser21 and GSK3ß at Ser9 in myotubes (Fig. 6D-F) while the response to insulin was unaffected by CHI3L1. TNFα treatment completely inhibited insulin-mediated GSK3α phosphorylation and significantly reduced GSK3ß phosphorylation while CHI3L1 pre-incubation restored the normal insulin effect (Fig. 6D-F).
Insulin-stimulated glucose uptake was monitored to analyze potential effects of CHI3L1 treatment on a physiological end-point of insulin. Stimulation with insulin increased the glucose uptake by ~50 % (Fig. 6G). Insulin action was unaltered in cells treated with CHI3L1 alone, while TNFα treatment impaired the insulin-stimulated glucose uptake (Fig. 6G). In the combination of TNFα and CHI3L1, insulin-stimulated glucose uptake was completely restored (Fig. 6G).

IV. DISCUSSION

Several studies have investigated the expression of CHI3L1 in a variety of cell types and in pathological conditions [20], however the biological function of CHI3L1 is only poorly understood. In this study we characterized CHI3L1 as a novel myokine which is up-regulated by pro-inflammatory cytokines and show its protective function regarding TNFα-induced inflammation and insulin resistance in primary human skeletal muscle cells.
CHI3L1 was originally described in mouse breast cancer cells [6]. Further studies have shown that it is also expressed and secreted by a variety of cells, e.g. by human synovial cells [7;21], chondrocytes [7], smooth muscle cells [22], neutrophils [23] and macrophages [24]. Here, we show that CHI3L1 is also expressed and released by primary human skeletal muscle cells thereby adding CHI3L1 to the growing list of myokines [25]. Western Blot, ELISA and Real-Time PCR analyses revealed that expression and release of CHI3L1 is higher in undifferentiated myoblasts compared to human myotubes. CHI3L1 has been described as a late macrophage differentiation marker and the expression of CHI3L1 is under the control of the transcriptional factor Sp1 [26]. Interestingly, it has been shown that differentiation of skeletal muscle cells and MyoD down-regulate Sp1 [27]. Therefore, the down-regulation of CHI3L1 is potentially related to down-regulation of Sp1 during myogenesis leading to decreased CHI3L1 promoter activity and hence decreased CHI3L1 levels in myotubes.
Furthermore, we observed a strong induction of CHI3L1 by different inflammatory stimuli. We show in this study that CM, which contains the secretory output of adipocytes, as well as the inflammatory cytokines TNFα, IFNγ, IL-6 and IL-1β are able to increase CHI3L1 protein level and secretion dramatically. In addition, we were able to show that basal CHI3L1 mRNA expression is reduced by the specific IKK-inhibitor I229 [18]. Furthermore, TNFα-induced CHI3L1 mRNA expression is completely blocked by using I229 thus indicating that induction and enhanced secretion of CHI3L1 are controlled by NF-κB activity in primary human skeletal muscle cells. This finding is in line with results from Recklies et al. showing that TNFα- and IL-1β-induced CHI3L1 expression as well as the constitutive expression and secretion of CHI3L1 is controlled by NF-κB activity in chondrocytes [28]. Furthermore, they have described an active binding site for NF-κB in the human CHI3L1 promotor region [28]. In addition, Baht et al. have shown that CHI3L1 expression is regulated by TNFα-stimulated

NF-κB activation in different cancer cell lines [29]. TNFα causes recruitment of the p65 and p50 subunits of NF-κB to the CHI3L1 promotor in all cell types [29]. However, based on our time course experiment we cannot exclude an indirect action of TNFα, in that other targets of TNFα promote NF-κB activation, which finally leads to augmented CHI3L1 expression.

Interestingly, myotubes which have lower basal CHI3L1 protein level compared to myoblasts are much more sensitive to an inflammatory stimulus such as CM. Upon stimulation, myotubes produced much more CHI3L1 than myoblasts despite the down-regulation of CHI3L1 during differentiation. Therefore, it may be speculated that CHI3L1 plays an important role in conditions of inflammation in skeletal muscle thus representing a kind of "rescue myokine" that is produced on demand.

The main finding of this study is the inhibition of TNFα-induced inflammation and insulin resistance in primary human skeletal muscle cells by CHI3L1. Recklies et al. have shown that CHI3L1 reduces TNFα- and IL-1β-mediated activation of p38 and SPAK/JNK MAPKs as well as cytokine-induced MMP1, MMP3 and IL-8 production in fibroblasts [14]. However, they did not observe effects on TNFα-induced NF-κB activation [14]. In our study using primary human skeletal muscle cells, we report that CHI3L1 inhibits TNFα-induced NF-κB activation as shown by p65 phosphorylation. This effect is specific for TNFα since IL-1β-induced NF-κB activation was not affected by CHI3L1 treatment. To study underlying molecular pathways we assessed the expression of the CHI3L1 receptor PAR2 [13;30]. Bara et al. have shown that CHI3L1 increases bronchial smooth muscle cell proliferation and migration through a PAR2-dependent pathway [13]. Furthermore, Chinni et al. have reported PAR2 expression in mouse skeletal myoblasts and demonstrated that activation of this receptor leads to

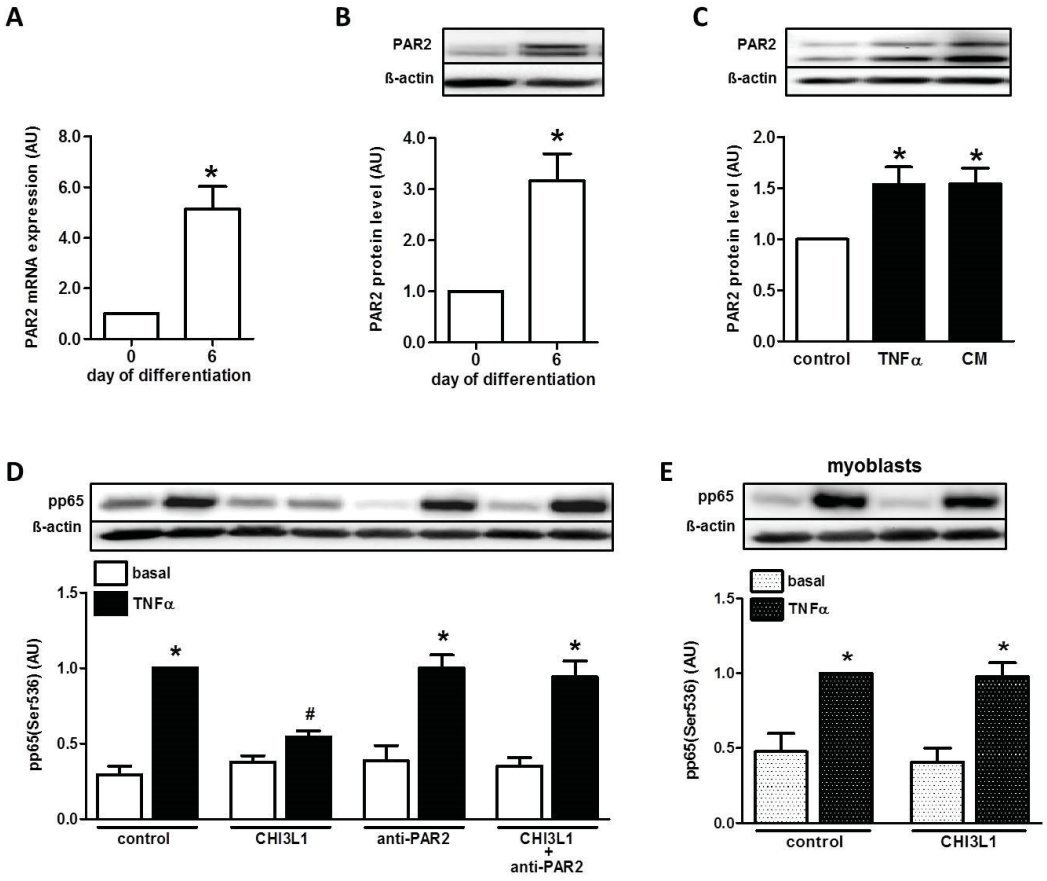

Figure 4: CHI3L1 acts via a PAR2-dependent mechanism

(**A**) PAR2 mRNA expression and (**B**) protein levels from myoblasts (day 0) and myotubes (day 6) were analyzed by Real-Time PCR and Western Blot and normalized to ß-actin expression. (**C**) Differentiated skeletal muscle cells were treated with TNFα (50 pg/ml) or CM for 24 h, and PAR2 protein levels were assessed by Western Blot. Data are mean values ± SEM, n=5-7, * $p<0.05$ vs. day 0 or control. (**D**) Differentiated skeletal muscle cells were treated for 10 min with TNFα (50 pg/ml), TNFα in combination with CHI3L1 (100 ng/ml) and anti-PAR2 (25 µg/ml), respectively, and with a combination of all factors. CHI3L1 and anti-PAR2 were added 30 min before TNFα stimulation. Phosphorylation of p65 was assessed by Western Blot. Data are normalized to β-actin and expressed relative to TNFα treatment. Data are mean values ± SEM, n=6, * $p<0.05$ vs. corresponding condition without TNFα treatment. # $p<0.05$ vs. control with TNFα treatment. (**E**) Undifferentiated myoblasts were treated with TNFα (50 pg/ml), CHI3L1 (100 ng/ml) and with a combination of both, respectively, as described before. Phosphorylation of p65 was assessed by Western Blot. Data are normalized to β-actin and expressed relative to control. Data are mean values ± SEM, n=6, * $p<0.05$ vs. control.

stimulation of myoblast proliferation [31]. By using Western-Blot and Real-Time PCR analyses, we show here for the first time that PAR2 is expressed in human skeletal muscle cells. Moreover, we clearly demonstrate that the effect of CHI3L1 on TNFα-induced NF-κB activation is PAR2-dependent using four different approaches: I) use of a blocking antibody directed against PAR2 that prevented the effects of CHI3L1, II) use of trypsin, a well-known activator of PAR2, that mimicked the effect of CHI3L1, III) use of an PAR2 agonist (SLIGKV-NH_2) as an additional positive control, and IV) repetition of the experiments in human myoblasts with a very low expression of PAR2 and therefore no effect of CHI3L1 on TNFα-induced NF-κB activation. PARs belong to the G-protein coupled receptor family that is activated by protease-mediated cleavage of the N-terminus of the receptor [32]. Various studies have shown that trypsin cleaves PAR2 at residue $R^{34} > S^{35}$ to expose a hexametric-tethered peptide (SLIGKV) that binds to conserved regions in the extracellular second loop of the receptor to initiate signaling [33]. However, synthetic peptides with almost identical amino acid sequence like the tethered peptide are also able to activate PAR2 signaling [34]. Recently, Bara et al. (13) have shown that CHI3L1 activates PAR2. They also used anti-PAR2 antibody to block the receptor and lenti-viral-mediated shRNA to knockdown PAR2. Both approaches decreased CHI3L1-induced cell proliferation and migration as well as effects induced by the synthetic hexameric-peptide SLIGKV-NH_2 which specifically activates PAR2 signaling [13]. However, the exact mechanism by which CHI3L1 activates PAR2 is still unknown. In addition to PAR2, CHI3L was recently shown to also bind to the interleukin-13 receptor α2 (IL-13Rα2) [35]. He et al. reported that CHI3L1 activates mitogen-activated protein kinase (ERK), Akt, and Wnt/ß-catenin signaling as well as TGF-ß1 production via IL-13Rα2 [35]. However, their data indicate that other receptors may exist because in some of their experimental systems the elimination of IL-13Rα2 only partially abrogated the specific CHI3L1 effector response. Therefore, it may be speculated that CHI3L1 signals via different pathways in a cell and/or tissue specific way. Future studies are required to investigate whether CHI3L1 also signals via IL-13Rα2 in human skeletal muscle cells.

It is widely recognized that PAR2 signaling is implicated in various pathological conditions including chronic arthritis, inflammatory pain and colitis as well as skin disorders such as type IV and contact dermatitis [36]. However, there are also evidences supporting an anti-inflammatory role of PAR2 signaling. A study by McIntosh et al. has shown that activation of PAR2 mediates inhibition of TNFα-stimulated JNK activation by disrupting the binding of tumor necrosis factor receptor 1 (TNFR1) to receptor-interacting serine/threonine-protein kinase 1 (RIP) and tumor necrosis factor receptor type 1-associated DEATH domain (TRADD) in the human skin epithelial cell line NCTC2544 [37]. Nichols et al. have suggested that PAR2-enhanced inflammatory processes are ß-arrestin-dependent whereas the protective effect of PAR2 may be ß-arrestin independent [38]. In our study we show that activation of PAR2 by CHI3L1 has anti-inflammatory properties with respect to TNFα-mediated inflammation in human skeletal muscle. Further studies have to show the exact mechanism underlying the duality of PAR2-mediated signaling.

The physiological response of most cell types to TNFα involves enhanced production of cytokines such as IL-6, IL-8 and MCP1 which play a key role in inflammatory processes associated with conditions of insulin resistance [39]. We demonstrate in this study that treatment of human skeletal muscle cells with TNFα in the presence of CHI3L1 for 24 h prevents the inflammatory response induced by TNFα. In addition, we show that CHI3L1 protects skeletal muscle cells from TNFα-induced insulin resistance. It is has been demonstrated before that stimulation of primary human skeletal muscle cells with TNFα can directly induce insulin resistance [5]. This effect can be inhibited by blocking the NF-κB signaling pathway [40]. Based on our data we suggest that the effects of CHI3L1 on TNFα-induced responses are explained by activation of PAR2 resulting in impaired

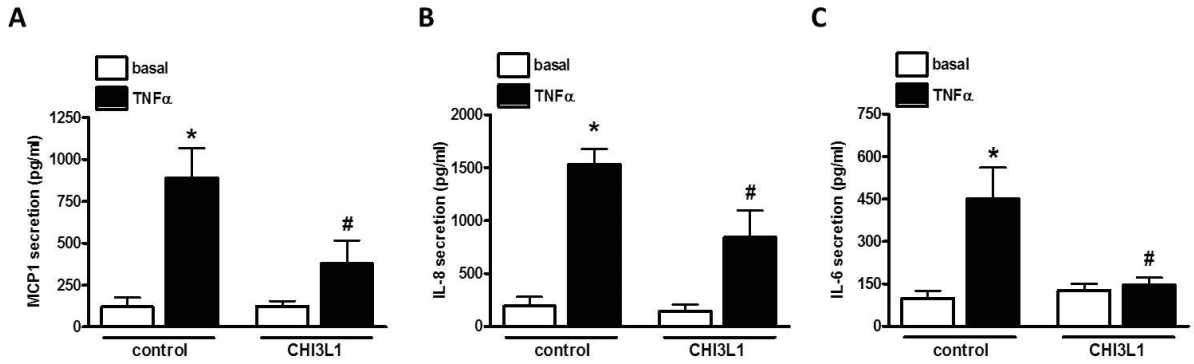

Figure 5: CHI3L1 diminishes TNFα-induced MCP1, IL-8 and IL-6 secretion
Differentiated human skeletal muscle cells were treated with TNFα (50 pg/ml), CHI3L1 (100 ng/ml) or with a combination of both for 24 h. Supernatants were collected and MCP1 (**A**), IL-8 (**B**) and IL-6 concentration (**C**) was assessed by ELISA. Data are mean values ± SEM, n=6, *p<0.05 vs. control and # p<0.05 vs. TNFα treatment.

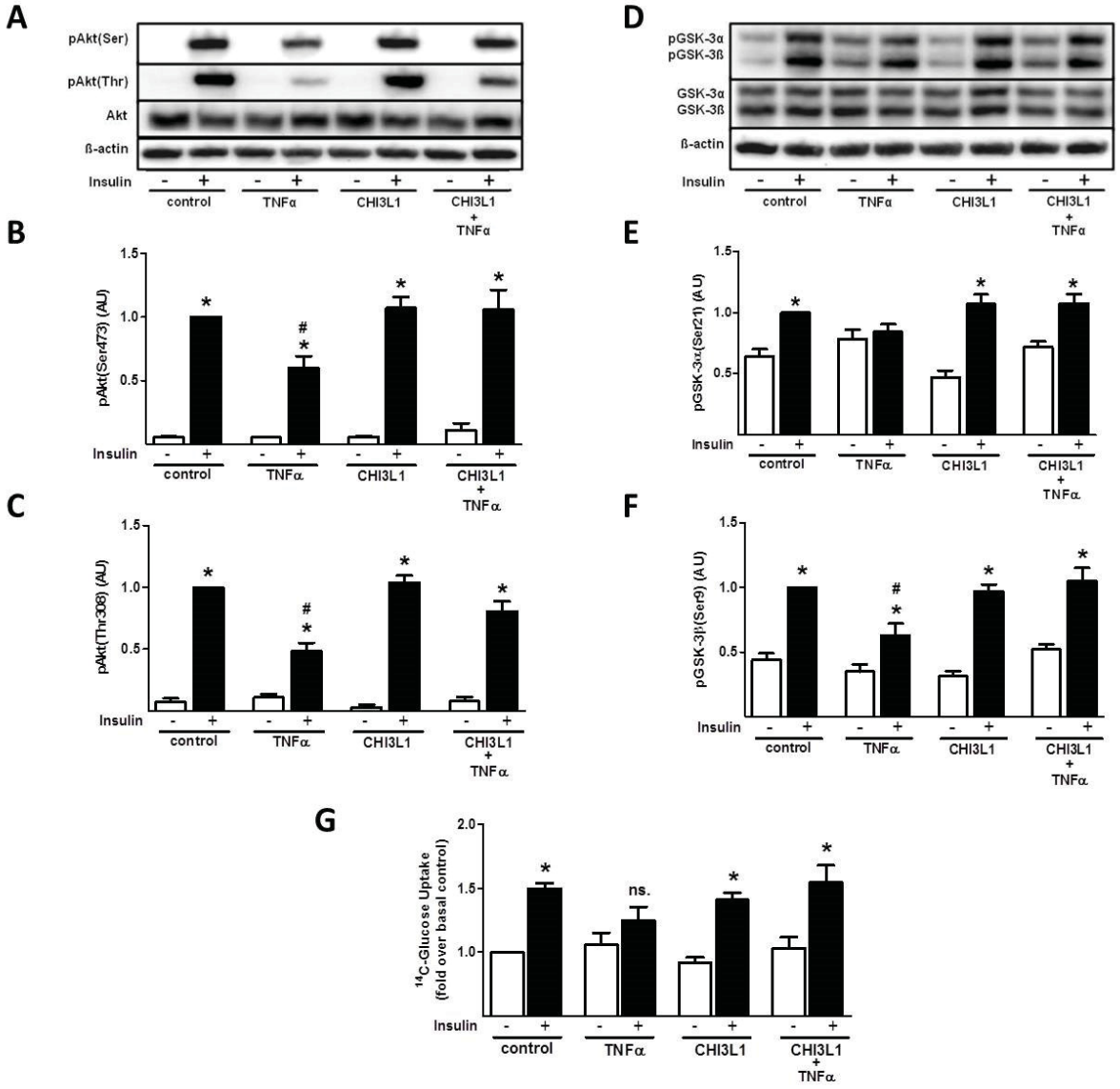

Figure 6: CHI3L1 normalizes TNFα-induced insulin resistance
Differentiated human skeletal muscle cells were treated with TNFα (20 ng/ml), CHI3L1 (100 ng/ml) or simultaneous with both for 3 h followed by insulin stimulation (100 nmol/L) for 10 min. (**A**) Representative Western Blot images show phosphorylation of Akt(Ser473), Akt(Thr308) as well as total Akt protein level after the indicated treatment. (**B**) Quantification of Akt(Ser473) and (**C**) Akt(Thr308) phosphorylation. (**D**) Representative Western Blot images show phosphorylation of GSK-3α(Ser21) and GSK-3ß(Ser9) as well as total GSK-3α/ß protein level after indicated treatment. (**E**) Quantification of GSK-3α(Ser21) and (**F**) GSK-3ß(Ser9) phosphorylation. Data are normalized to β-actin and expressed relative to control. Data are mean values ± SEM, n=4-7, *p<0.05 vs. corresponding basal and #p<0.05 vs. control insulin treatment. (**G**) Cells were treated with TNFα (20 ng/ml), CHI3L1 (100 ng/ml) or simultaneously with both agents for 3 h followed by insulin stimulation (100 nmol/L) for 30 min. Glucose uptake was assessed for 2 h. Data are mean values ± SEM, n=3, *p<0.05 vs. corresponding basal.

TNFα downstream signaling and thus reduced NF-κB signaling.

The induction of CHI3L1 in response to inflammatory cytokines provides a negative feedback loop to control the action of TNFα, and thereby to locally reduce the inflammatory response in skeletal muscle (summarized in Fig. 7). High TNFα concentrations induce insulin resistance in skeletal muscle and impair whole-body glucose sensitivity in humans. Thus, the identification and characterization of novel molecules which are able to reduce TNFα-mediated inflammation will certainly be an important approach to prevent the development of peripheral insulin resistance.

In conclusion, the novel myokine CHI3L1 is induced by pro-inflammatory cytokines and counteracts TNFα-mediated inflammation and insulin resistance in human skeletal muscle. CHI3L1 acts via a PAR2-dependent mechanism potentially in an auto- and/or paracrine way. Hence, we suggest CHI3L1 to be an autoprotective factor that is induced to protect the skeletal muscle from negative impact of TNFα.

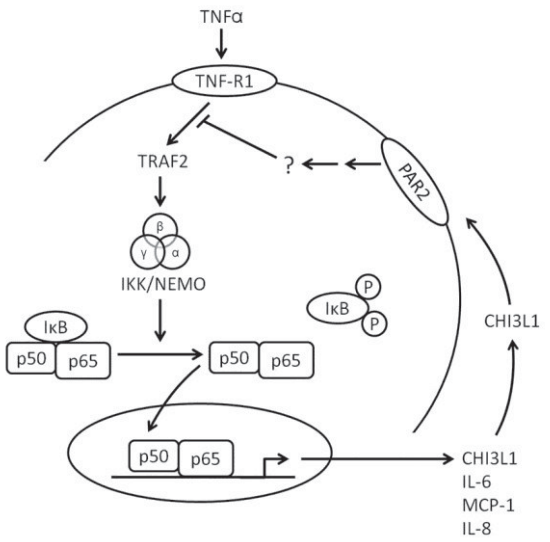

Figure 7: Proposed model for the negative feedback control of TNFα by CHI3L1 in human skeletal muscle cells

TNFα binds to the TNFR1 thus leading to activation of NF-κB that results in the enhanced expression of CHI3L1, IL-6, IL-8 and MCP1. Increased levels of CHI3L1 then activate PAR2 signaling leading to the inhibition of TNFα-induced NF-κB activation by a yet unidentified mechanism. This finally leads to a suppression of CHI3L1, IL-6, IL-8 and MCP-1 expression.

V. REFERENCES

1. Glass, C. K. and Olefsky, J. M. (2012) Inflammation and lipid signaling in the etiology of insulin resistance Cell Metab. **15**, 635-645
2. Mishima, Y., Kuyama, A., Tada, A., Takahashi, K., Ishioka, T., and Kibata, M. (2001) Relationship between serum tumor necrosis factor-alpha and insulin resistance in obese men with Type 2 diabetes mellitus Diabetes Res.Clin.Pract. **52**, 119-123
3. Sell, H., Dietze-Schroeder, D., and Eckel, J. (2006) The adipocyte-myocyte axis in insulin resistance Trends Endocrinol.Metab. **17**, 416-422
4. Saghizadeh, M., Ong, J. M., Garvey, W. T., Henry, R. R., and Kern, P. A. (1996) The expression of TNF alpha by human muscle. Relationship to insulin resistance J.Clin.Invest. **97**, 1111-1116
5. Plomgaard, P., Bouzakri, K., Krogh-Madsen, R., Mittendorfer, B., Zierath, J. R., and Pedersen, B. K. (2005) Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation Diabetes. **54**, 2939-2945
6. Morrison, B. W. and Leder, P. (1994) neu and ras initiate murine mammary tumors that share genetic markers generally absent in c-myc and int-2-initiated tumors Oncogene. **9**, 3417-3426
7. Hakala, B. E., White, C., and Recklies, A. D. (1993) Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family J.Biol.Chem. **268**, 25803-25810
8. Fusetti, F., Pijning, T., Kalk, K. H., Bos, E., and Dijkstra, B. W. (2003) Crystal structure and carbohydrate-binding properties of the human cartilage glycoprotein-39 J.Biol.Chem. **278**, 37753-37760
9. Lee, C. G., Da Silva, C. A., Dela Cruz, C. S., Ahangari, F., Ma, B., Kang, M. J., He, C. H., Takyar, S., and Elias, J. A. (2011) Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury Annu.Rev.Physiol. **73**, 479-501
10. Nielsen, A. R., Erikstrup, C., Johansen, J. S., Fischer, C. P., Plomgaard, P., Krogh-Madsen, R., Taudorf, S., Lindegaard, B., and Pedersen, B. K. (2008) Plasma YKL-40: a BMI-independent marker of type 2 diabetes Diabetes. **57**, 3078-3082
11. Rathcke, C. N., Raymond, I., Kistorp, C., Hildebrandt, P., Faber, J., and Vestergaard, H. (2010) Low grade inflammation as measured by levels of YKL-40: association with an increased overall and cardiovascular mortality rate in an elderly population Int.J.Cardiol. **143**, 35-42
12. Recklies, A. D., White, C., and Ling, H. (2002) The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways Biochem.J. **365**, 119-126
13. Bara, I., Ozier, A., Girodet, P. O., Carvalho, G., Cattiaux, J., Begueret, H., Thumerel, M., Ousova, O., Kolbeck, R., Coyle, A. J., Woods, J., Tunon de Lara, J. M., Marthan, R., and Berger, P. (2012) Role of YKL-40 in bronchial smooth muscle remodeling in asthma Am.J.Respir.Crit Care Med. **185**, 715-722
14. Ling, H. and Recklies, A. D. (2004) The chitinase 3-like protein human cartilage glycoprotein 39 inhibits cellular responses to the inflammatory cytokines interleukin-1 and tumour necrosis factor-alpha Biochem.J. **380**, 651-659
15. Lee, C. G., Hartl, D., Lee, G. R., Koller, B., Matsuura, H., Da Silva, C. A., Sohn, M. H., Cohn, L., Homer, R. J., Kozhich, A. A., Humbles, A., Kearley, J., Coyle, A., Chupp, G., Reed, J., Flavell, R. A., and Elias, J. A. (2009) Role of breast regression protein 39 (BRP-39)/chitinase 3-like-1 in Th2 and IL-13-induced tissue responses and apoptosis J.Exp.Med. **206**, 1149-1166
16. Sell, H., Laurencikiene, J., Taube, A., Eckardt, K., Cramer, A., Horrighs, A., Arner, P., and Eckel, J. (2009) Chemerin is a novel adipocyte-derived factor inducing insulin resistance in primary human skeletal muscle cells Diabetes. **58**, 2731-2740
17. Sell, H., Eckardt, K., Taube, A., Tews, D., Gurgui, M., Echten-Deckert, G., and Eckel, J. (2008) Skeletal muscle insulin resistance induced by adipocyte-conditioned medium: underlying mechanisms and

reversibility Am.J.Physiol Endocrinol.Metab. **294**, E1070-E1077
18. Ivashchenko, Y., Kramer, F., Schafer, S., Bucher, A., Veit, K., Hombach, V., Busch, A., Ritzeler, O., Dedio, J., and Torzewski, J. (2005) Protein kinase C pathway is involved in transcriptional regulation of C-reactive protein synthesis in human hepatocytes Arterioscler.Thromb.Vasc.Biol. **25**, 186-192
19. Dietze, D., Ramrath, S., Ritzeler, O., Tennagels, N., Hauner, H., and Eckel, J. (2004) Inhibitor kappaB kinase is involved in the paracrine crosstalk between human fat and muscle cells Int.J.Obes.Relat Metab Disord. **28**, 985-992
20. Mygind, N. D., Iversen, K., Kober, L., Goetze, J. P., Nielsen, H., Boesgaard, S., Bay, M., Johansen, J. S., Nielsen, O. W., Kirk, V., and Kastrup, J. (2013) The inflammatory biomarker YKL-40 at admission is a strong predictor of overall mortality J.Intern.Med. **273**, 205-216
21. Nyirkos, P. and Golds, E. E. (1990) Human synovial cells secrete a 39 kDa protein similar to a bovine mammary protein expressed during the non-lactating period Biochem.J. **269**, 265-268
22. Shackelton, L. M., Mann, D. M., and Millis, A. J. (1995) Identification of a 38-kDa heparin-binding glycoprotein (gp38k) in differentiating vascular smooth muscle cells as a member of a group of proteins associated with tissue remodeling J.Biol.Chem. **270**, 13076-13083
23. Volck, B., Price, P. A., Johansen, J. S., Sorensen, O., Benfield, T. L., Nielsen, H. J., Calafat, J., and Borregaard, N. (1998) YKL-40, a mammalian member of the chitinase family, is a matrix protein of specific granules in human neutrophils Proc.Assoc.Am.Physicians. **110**, 351-360
24. Renkema, G. H., Boot, R. G., Au, F. L., Donker-Koopman, W. E., Strijland, A., Muijsers, A. O., Hrebicek, M., and Aerts, J. M. (1998) Chitotriosidase, a chitinase, and the 39-kDa human cartilage glycoprotein, a chitin-binding lectin, are homologues of family 18 glycosyl hydrolases secreted by human macrophages Eur.J.Biochem. **251**, 504-509
25. Raschke, S., Eckardt, K., Bjorklund, H. K., Jensen, J., and Eckel, J. (2013) Identification and validation of novel contraction-regulated myokines released from primary human skeletal muscle cells PLoS.One. **8**, e62008
26. Rehli, M., Niller, H. H., Ammon, C., Langmann, S., Schwarzfischer, L., Andreesen, R., and Krause, S. W. (2003) Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation J.Biol.Chem. **278**, 44058-44067
27. Vinals, F., Fandos, C., Santalucia, T., Ferre, J., Testar, X., Palacin, M., and Zorzano, A. (1997) Myogenesis and MyoD down-regulate Sp1. A mechanism for the repression of GLUT1 during muscle cell differentiation J.Biol.Chem. **272**, 12913-12921
28. Recklies, A. D., Ling, H., White, C., and Bernier, S. M. (2005) Inflammatory cytokines induce production of CHI3L1 by articular chondrocytes J.Biol.Chem. **280**, 41213-41221
29. Bhat, K. P., Pelloski, C. E., Zhang, Y., Kim, S. H., deLaCruz, C., Rehli, M., and Aldape, K. D. (2008) Selective repression of YKL-40 by NF-kappaB in glioma cell lines involves recruitment of histone deacetylase-1 and -2 FEBS Lett. **582**, 3193-3200
30. Liu, C., Li, Q., Zhou, X., Kolosov, V. P., and Perelman, J. M. (2013) The chitinase-like protein YKL-40 increases mucin5AC production in human bronchial epithelial cells Exp.Cell Res. **319**, 2866-2873
31. Chinni, C., de Niese, M. R., Jenkins, A. L., Pike, R. N., Bottomley, S. P., and Mackie, E. J. (2000) Protease-activated receptor-2 mediates proliferative responses in skeletal myoblasts J.Cell Sci. **113 Pt 24:4427-33.**, 4427-4433
32. Xiang, Y., Masuko-Hongo, K., Sekine, T., Nakamura, H., Yudoh, K., Nishioka, K., and Kato, T. (2006) Expression of proteinase-activated receptors (PAR)-2 in articular chondrocytes is modulated by IL-1beta, TNF-alpha and TGF-beta Osteoarthritis.Cartilage. **14**, 1163-1173
33. Soh, U. J., Dores, M. R., Chen, B., and Trejo, J. (2010) Signal transduction by protease-activated receptors Br.J.Pharmacol. **160**, 191-203
34. Barry, G. D., Suen, J. Y., Le, G. T., Cotterell, A., Reid, R. C., and Fairlie, D. P. (2010) Novel agonists and antagonists for human protease activated receptor 2 J.Med.Chem. **53**, 7428-7440
35. He, C. H., Lee, C. G., Dela Cruz, C. S., Lee, C. M., Zhou, Y., Ahangari, F., Ma, B., Herzog, E. L., Rosenberg, S. A., Li, Y., Nour, A. M., Parikh, C. R., Schmidt, I., Modis, Y., Cantley, L., and Elias, J. A. (2013) Chitinase 3-like 1 regulates cellular and tissue responses via IL-13 receptor alpha2 Cell Rep. **4**, 830-841
36. Rothmeier, A. S. and Ruf, W. (2012) Protease-activated receptor 2 signaling in inflammation Semin.Immunopathol. **34**, 133-149
37. McIntosh, K., Cunningham, M. R., Cadalbert, L., Lockhart, J., Boyd, G., Ferrell, W. R., and Plevin, R. (2010) Proteinase-activated receptor-2 mediated inhibition of TNFalpha-stimulated JNK activation - A novel paradigm for G(q/11) linked GPCRs Cell Signal. **22**, 265-273
38. Nichols, H. L., Saffeddine, M., Theriot, B. S., Hegde, A., Polley, D., El Mays, T., Vliagoftis, H., Hollenberg, M. D., Wilson, E. H., Walker, J. K., and DeFea, K. A. (2012) beta-Arrestin-2 mediates the proinflammatory effects of proteinase-activated receptor-2 in the airway Proc.Natl.Acad.Sci.U.S.A. **109**, 16660-16665
39. Marino, M., Scuderi, F., Provenzano, C., and Bartoccioni, E. (2011) Skeletal muscle cells: from local inflammatory response to active immunity Gene Ther. **18**, 109-116

40. Austin, R. L., Rune, A., Bouzakri, K., Zierath, J. R., and Krook, A. (2008) siRNA-mediated reduction of inhibitor of nuclear factor-kappaB kinase prevents tumor necrosis factor-alpha-induced insulin resistance in human skeletal muscle Diabetes. **57**, 2066-2073

ABBREVIATIONS AND ACRONYMS

2-DOG, 2-Deoxy-D-[14C] glucose; CM, adipocyte-conditioned medium; αMEM, α-modified Eagle's; CHI3L1, chitinase-3-like protein 1; EPS, electrical pulse stimulation; GSK3, glycogen synthase kinase 3; IL-13Rα2, interleukin-13 receptor α2; MCP1, monocyte chemotactic protein-1; PAR2, protease activated receptor 2; PAR-AP, PAR2-activating peptide; TNFα, tumor necrosis factor α; IL, interleukin; IFNγ, interferon γ

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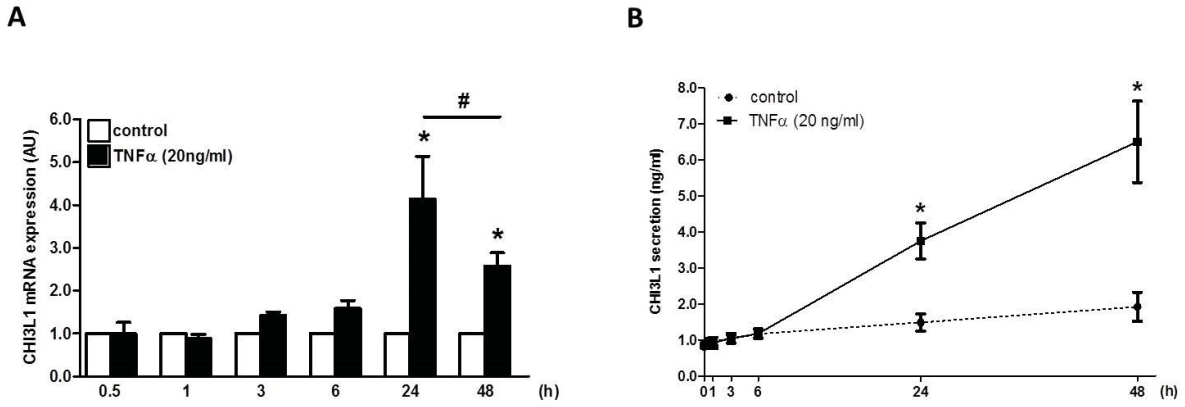
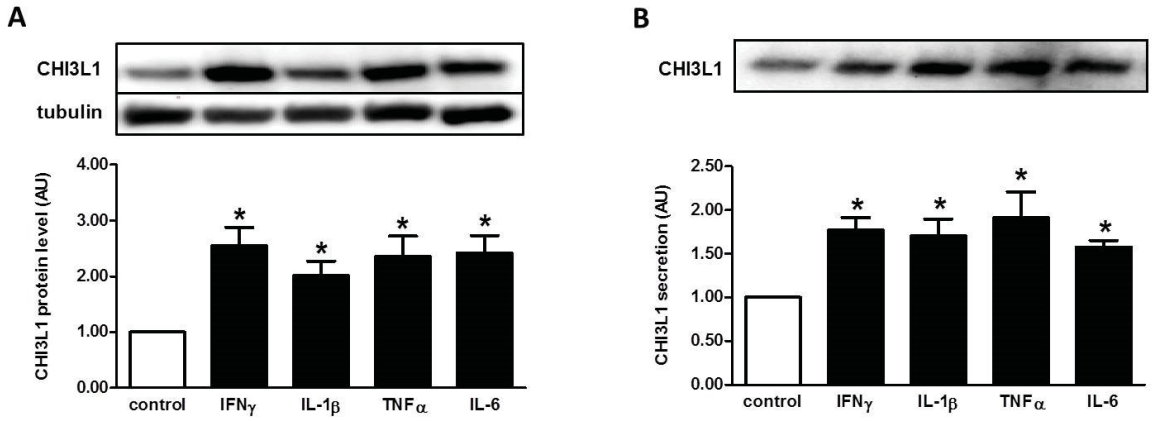
CONTRIBUTION STATEMENT

SWG and KE contributed to the concept, acquired, analyzed and interpreted data, wrote the manuscript and had the main responsibility together with JE. ME performed research and contributed to analysis and interpretation of data. NT 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.

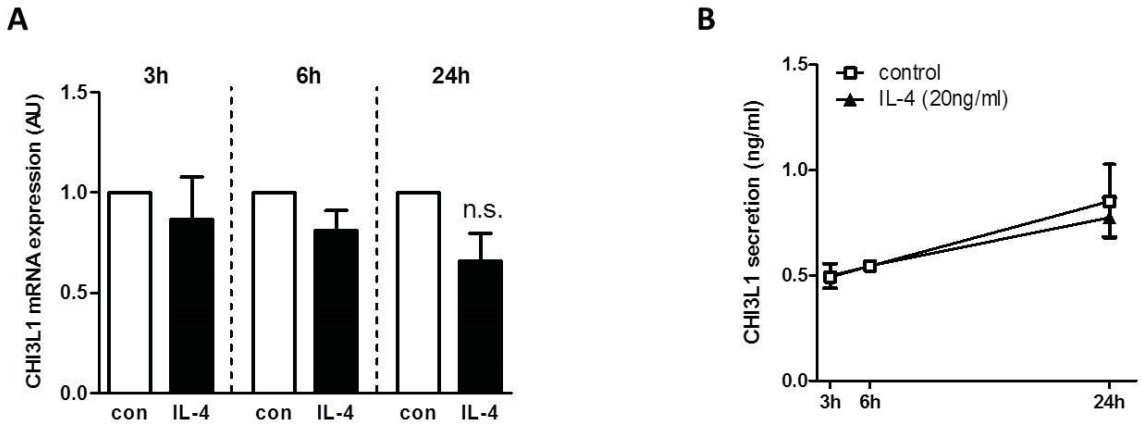
Supplement



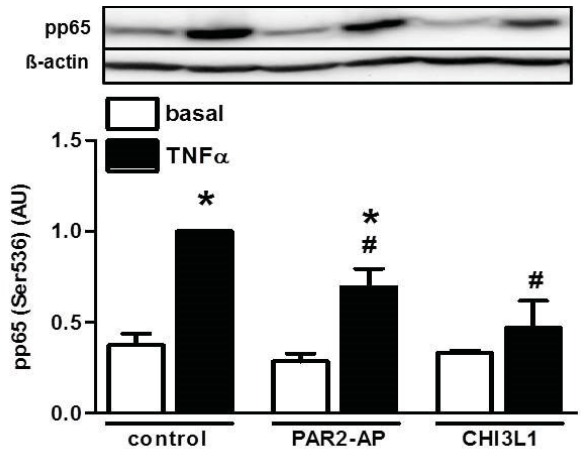
Suppl. Fig. 1: Induction of CHI3L1 mRNA expression and secretion by TNFα
(A) Differentiated skeletal muscle cells were treated with TNFα (20 ng/ml) for the indicated time points. CHI3L1 mRNA expression was analyzed by Real-Time PCR and normalized to ß-actin expression. (B) CHI3L1 protein secretion was analyzed by ELISA after treatment with TNFα (20 ng/ml). All data are mean values ± SEM, n=3, * $p<0.05$ vs. corresponding control and # $p<0.05$ TNFα-treatment 24 h vs. 48 h.



Suppl. Fig. 2: Induction of CHI3L1 protein level and secretion by inflammatory cytokines
(A) Differentiated human skeletal muscle cells were treated with IFNγ (10 ng/ml), TNFα (50 pg/ml), IL-1β (10 ng/ml) or IL-6 (5 ng/ml), respectively, for 24 h, and CHI3L1 protein levels were assessed by Western Blot. Data are normalized to tubulin and expressed as fold over control. (B) CHI3L1 secretion from primary human skeletal muscle cells after the indicated treatments was assessed by Western Blot. CHI3L1protein levels in the supernatants are normalized to the protein level of the corresponding lysates. All data are mean values ± SEM, n=5-8, * $p<0.05$ vs. control.



Suppl. Fig. 3: CHI3L1 expression and secretion is unaffected by IL-4 treatment
(A) Differentiated human skeletal muscle cells were treated with IL-4 (20 ng/ml) for the indicated time points, and CHI3L1 mRNA expression was analyzed by Real-Time PCR and normalized to ß-actin expression. (B) Cells were treated with IL-4 (20 ng/ml) for the indicated time points, and CHI3L1 protein secretion was analyzed by ELISA. All data are mean values ± SEM, n=4.



Suppl. Fig. 4: PAR2-agonist and CHI3L1 reduce TNFα-mediated NF-κB activation
Differentiated skeletal muscle cells were treated for 10 min with TNFα (50 pg/ml), in combination with CHI3L1 (100 ng/ml) and PAR2-agonist (PAR2-AP) (0.1 mmol/L), respectively. CHI3L1 and PAR2-agonist were added 30 min before TNFα stimulation. Phosphorylation of p65 was assessed by Western Blot. Data are normalized to β-actin and expressed relative to TNFα treatment. Data are mean values ± SEM, n=4, * $p<0.05$ vs. corresponding condition without TNFα treatment. # $p<0.05$ vs. control with TNFα treatment.

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	Performed experiments: 100%
	Analysed data: 100%
	Contributed to discussion: 100%
	Wrote the manuscript: 90%
	Reviewed / edited manuscript: 100%
Author:	1st author (shared)

2.3 Myokines in insulin resistance and type 2 diabetes

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Abstract— **Skeletal muscle represents the largest organ in non-obese subjects and is now considered as an active endocrine organ releasing a host of so-called myokines. These myokines are part of a complex multi-organ crosstalk network that mediates communication between muscle, the liver, adipose tissue, the brain and other organs. Recent data suggest that myokines regulated by muscle contraction may play a key role in mediating the health-promoting effects of regular physical activity. Despite hundreds of myokines recently described in proteomic studies, rather limited knowledge currently exists regarding the specific role of these myokines for preventing insulin resistance, inflammation, and associated metabolic dysfunction. In addition, several myokines exert both local and endocrine functions, but in many cases the contribution of physical activity to the systemic level of these molecules has remained unexplored. Very recently, novel myokines like irisin which is supposed to induce a white-to-brown shift in adipocytes, have gained considerable interest as therapeutical targets. In this review, we summarize the most recent findings on the role of myokines in regulating substrate metabolism and insulin sensitivity. We further explore the role of myokines in the regulation of inflammation and provide a critical assessment of irisin and other myokines regarding drug target development.**

Index Terms— **Exercise, Inflammation, Insulin resistance, Metabolism, Myokines, Skeletal muscle, Therapeutical targets, Type 2 diabetes**

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I. INTRODUCTION

Insulin resistance and/or type 2 diabetes which are characterized by a range of metabolic disturbances such as hyperinsulinemia, enhanced hepatic gluconeogenesis, impaired glucose uptake, metabolic inflexibility and mitochondrial dysfunction, are often associated with a deficit of regular physical exercise and a sedentary lifestyle. Data from bed-rest studies performed in healthy subjects support the hypothesis that physical inactivity itself is one of the main causes in the development of metabolic disturbances.

Skeletal muscle (SkM) accounts for ~40% of body weight and constitutes the largest organ in non-obese subjects. Nowadays, SkM is recognized as an endocrine organ, and proteins expressed by and released from SkM have been termed myokines [1]. In three studies using primary human myotubes and proteomic approaches, hundreds of myokines have been identified including more than 50 novel myokines [2-4].

It is supposed that contractile activity of SkM impacts on its secretory function, which may link physical activity to the health-promoting effects of exercise. Myokines are part of a complex communication network within the body and play a pivotal role in the crosstalk between SkM and other organs such as adipose tissue, liver, and pancreas. Although several hundreds of myokines have been identified now, information about their regulation by contraction or other stimuli is lacking in most cases. However, for a number of myokines such as angiopoietin-like 4, interleukin (IL)-6, IL-15, and myostatin it has been shown that their expression in SkM is enhanced and their concentrations in plasma are increased after exercise [5].

An increased energy expenditure and hence an improved energy balance may explain in part the positive effects of regular exercise. However, myokines released by contracting SkM may play a key role in mediating the beneficial effects of physical exercise [6]. In support of this idea, several studies investigating the effect of physical inactivity have shown a rapid decline in insulin sensitivity, decreased insulin-stimulated glucose uptake and GLUT4 protein content in human SkM [7]. Moreover, physical inactivity was shown to impact on partitioning of saturated fatty acids (FAs) towards storage resulting in enhanced incorporation of palmitate into intramuscular lipids and decreased palmitate oxidation (reviewed in [8]). Interestingly, transcriptional analysis of human SkM after nine days of bed-rest revealed expression changes of >4500 genes, and a total of 54% of genes in the oxidative phosphorylation pathway including peroxisome proliferator-activated receptor (PPAR) gamma coactivator 1α (PGC1α) were downregulated [9]. These data support the notion that physical inactivity *per se* is a main factor contributing to the onset of numerous chronic diseases. So far, these studies mostly have focused on expression changes of key players of insulin signaling and glucose/FA metabolism. However, it would be also very interesting to study how physical inactivity impacts on expression and release of myokines, since information on this topic is currently unavailable.

This review aims to summarize our current knowledge about myokines and their role in mediating metabolic regulation and adaptation to physical activity as well as

their impact on inflammation. Finally, we will discuss the potential of certain myokines as therapeutical targets to treat metabolic disorders associated with a sedentary lifestyle.

II. MYOKINES AND METABOLIC REGULATION

Myokines are very likely involved in the crosstalk between SkM and other tissues such as liver, adipose tissue, pancreas and gut. Therefore, their autocrine, paracrine, and/or endocrine effects with regard to metabolic regulation are an important focus of research since they may help to overcome the above described impairments associated with insulin resistance.

A. Myokines and glucose metabolism

For only a few myokines effects on glucose metabolism have been described so far. Most data are available for IL-6 which has been intensively studied during the past decades. Upon muscle contraction, IL-6 is released into the circulation depending on intensity and duration of exercise [10] and the energy status of a muscle, as determined by pre-exercise glycogen content [11]. This leads to an acute increase of IL-6 plasma levels during exercise, and myofibers have been identified to be the main source of IL-6 under this condition [12].

It has been suggested that contraction-induced expression of IL-6 is independent of the IKK/NFκB signaling pathway [13]. Studies have shown that after exercise the NFκB signaling pathway is not activated and binding of p65 subunit of NFκB to the IL-6 promoter decreases with skeletal muscle contraction [14], thus supporting this idea. Results obtained in C2C12 myotubes and mice suggest that contraction-induced IL-6 expression is mediated by activation of c-Jun NH2-terminal kinase (JNK) and activator protein-1 [14].

A study using C2C12 myotubes has shown that the effect of IL-6 on muscle insulin sensitivity depends on the duration of exposure [15]. Acute IL-6 treatment increased glucose uptake while chronic exposure resulted in insulin resistance due to activation of JNK and impairment of insulin signaling on the level of IRS-1 [15]. Interestingly, *in vivo* animal studies revealed that chronically elevated IL-6 serum levels induced by either treatment with recombinant protein [16] or using a transgenic approach [17] surprisingly improved whole body glucose tolerance and insulin sensitivity which might by linked to augmentation of central leptin action.

In vitro studies have shown that in myotubes IL-6 signals via gp130Rb/IL-6Ra which results in activation of AMP-kinase (AMPK) and/or PI3-kinase. These pathways mediate increased glucose uptake, GLUT4 translocation, and glycogen synthesis upon acute exposure to IL-6 (Tab. 1) [18-20]. Interestingly, ingestion of glucose during exercise blunts IL-6 secretion from contracting muscle [21] and a low pre-exercise muscular glycogen level augments the induction of IL-6 expression and release by exercise [11,22]. Exercise-induced adaptation in SkM also includes an increase of pre-exercise muscular glycogen content. This observation may explain why the increase of contraction-induced IL-6 expression and secretion is diminished after a period of regular endurance training. Moreover, regular training decreases basal plasma level of IL-6 and increases basal IL-6 receptor mRNA expression in SkM resulting in enhanced

Table 1 Selected myokines and their effects related to metabolism

Myokine	Auto/paracrine action	Endocrine action
BDNF	↑ fatty acid oxidation [46]	no data availabe regarding endocrine metabolic effects of muscle-derived BDNF
IL-6	↑ GLUT4 translocation [19,20] ↑ glucose uptake [15,19] ↑ glycogen synthesis [18,20] ↑ fatty acid oxidation [18-20] ↑ lipolysis [32-34]	Liver: ↑ glucose production [23] Intestine: ↑ GLP-1 secretion of L-cells [52] Pancreas: ↑ beta cell proliferation [52] ↑ GLP-1 secretion of alpha cells [52] ↑ proliferation and ↓ apoptosis of alpha cells [51]
IL-13	↑ glucose uptake [28] ↑ glucose oxidation [28] ↑ glycogen synthesis [28]	Liver: ↓ glucose production [29]
IL-15	↑ fatty acid oxidation [43]	Adipose tissue: ↓ lipid accumulation [44,45] ↑ adiponectin secretion [44]
Irisin	no data availabe	Adipose tissue: ↑ white-to-brown shift [98,111] No effect on browning [100]
FGF-21	↑ glucose uptake [99,136]	Adipose tissue: ↑ white-to-brown shift [99] ↑ glucose uptake [99] ↑ adiponectin secretion [127,128] Liver: ↑ fatty acid oxidation [137] ↑ gluconeogenesis [137]

IL-6 sensitivity [13]. Interestingly, Jiang et al. have recently demonstrated that myotubes from type 2 diabetic patients are resistant to the acute effect of IL-6 on glucose metabolism [20].

Infusion studies in humans using recombinant IL-6 have suggested a stimulation of endogenous glucose production by IL-6 during exercise [23] (Tab.1). However, this effect seems to be related to another yet unidentified factor released during contraction because IL-6 infusion alone is not able to increase hepatic glucose production in resting subjects [24]. Interestingly, IL-6-deficient mice displayed similar level of blood glucose compared to wild-type controls after a non-exhaustive exercise, suggesting that at least in mice IL-6 is not necessary for hepatic glucose production during exercise [25]. Furthermore, animal studies have shown that IL-6 impairs the ability of insulin to suppress hepatic glucose production [26,27] while this is not the case in humans [19].

IL-13 was recently identified as novel myokine that is released by human myotubes [28]. Interestingly, type 2 diabetic subjects have significantly reduced serum levels of IL-13 compared to controls, and myotubes from these patients secrete 75% less IL-13 than myotubes from controls. Furthermore, Jiang et al. suggested a role of IL-13 for SkM glucose metabolism by showing that treatment of myotubes with this protein increased basal glucose uptake and oxidation, and glycogen synthesis (Tab. 1) [28]. Moreover, a study using IL-13-deficient mice has suggested an implication for IL-13 in suppression of hepatic glucose production [29]. So far, data regarding the regulation of IL-13 release and expression by exercise are not available.

B. Myokines and FA metabolism

Stimulation of FA oxidation is another important metabolic adaptation to physical activity that may be mediated via myokines. Human studies have shown that an acute increase of circulating IL-6 by infusion results in increased systemic FA oxidation followed by an increase in systemic lipolysis [30,31]. Interestingly, no effect on adipose tissue lipolysis was found [31] suggesting that IL-6 primary acts locally within muscle tissue on FA metabolism. As summarized in Tab. 1, i*n vitro* studies have shown that IL-6 increases myotube FA oxidation as well as lipolysis via AMPK activation [32,33]. Recently, a study using isolated mouse muscle reported that IL-6 induces intramuscular lipolysis in glycolytic, but not in oxidative muscle pointing towards a fiber-type specific action of IL-6 [34]. The molecular background of this observation still needs to be determined.

Furthermore, IL-15 which is expressed in SkM and released by myotubes [2,35] potentially has a role in lipid metabolism. Conflicting results have been reported concerning the regulation of IL-15 expression in SkM and circulating IL-15 upon exercise. While one study reported no change of IL-15 mRNA expression after acute resistance training [36], Nielsen et al. observed a twofold increase in SkM which, however, was not accompanied by increased muscular IL-15 protein level or plasma IL-15 [37]. Other studies have reported elevated circulating IL-15 level after a single bout of resistance or endurance exercise and after eight weeks of resistance training [38-40]. Lower levels of IL-15 have been reported in type 2 diabetic patients compared to controls [41]. Moreover, a negative association has been found in humans between plasma IL-15 levels and total adipose tissue mass [6] and IL-15 has been found to be

Table 2 Comparison of three myokines which are discussed as therapeutical targets.
AT= adipose tissue

	FNDC5/Irisin	FGF21	FSTL1
Expression in multiple tissue panels	muscle > heart > AT [105] heart > muscle > AT [100]	liver>>thymus [138] liver (48h fasted) = muscle [114]	heart>>AT [139]
Induction of mRNA by exercise	→ No effect (11 human studies) ↑ 1.3fold [102] ↑ 1.4fold [103] ↑ 1.4fold [104] ↑ 2fold [98]	→ No effect [117]	↑ 1.7 fold [4] ↑ 2.6 fold [4]
Basal circulating levels	0.04 – 2158 ng/ml [108,110]	143-569 pg/ml [116,118]	16.9 ng/ml [131]
Effect of exercise on circulating levels	→ No effect (11 human studies) ↑ 1.2 fold [104-106]	↓ 0.4fold [116] → No effect [117] ↑ 1.7fold [118] ↑ 2fold [119]	↑ 1.2 fold [131]

decreased in obese subjects compared with lean controls [42].

IL-15 was reported to increase FA oxidation in isolated rat *extensor digitorum longus* muscle via induction of PPARδ expression [43] and to inhibit lipid accumulation in preadipocytes [44] (Tab. 1). Overexpression of IL-15 in SkM resulted in reduced body fat suggesting a potential role in the regulation of fat mass [6,45].

Brain-derived neurotrophic factor (BDNF) is another myokine that may impact on FA metabolism. BDNF mRNA expression and protein has been detected in human SkM and C2C12 cells [46], and our group recently reported BDNF in the supernatant of human myotubes [2]. While in patients with type 2 diabetes circulating levels of BDNF are decreased independently of obesity [47], exercise has been demonstrated to increase serum BDNF level as well as mRNA and protein expression in human SkM [46]. However, it has been shown in humans that 70-80% of circulating BDNF originates from the brain during both rest and after exercise suggesting the brain as major source of this factor [48]. *In vitro* studies using C2C12 cells have shown that BDNF increases FA oxidation (Tab. 1) via activation of AMPK and phosphorylation of acetyl-CoA carboxylase [46]. While BDNF has no direct effect on glucose uptake or gluconeogenesis in primary cultured hepatocytes, L6 myotubes or 3T3-L1 adipocytes [49], a potential impact on lipolysis has not been studied so far.

C. Myokines and insulin secretion

Recent studies have suggested a potential crosstalk between SkM and pancreas. Using supernatants from insulin-sensitive vs. insulin-resistant (due to TNFα treatment) human myotubes for incubation of human and rat primary beta cells, Boukazkri et al. found a differential impact on these cells [50]. Supernatant of normal myotubes increased proliferation of beta cells and glucose-stimulated insulin secretion (GSIS). However, supernatant of insulin-resistant myotubes increased apoptosis and decreased proliferation independently from TNFα while blunting of GSIS was shown to be a TNFα-mediated effect. Further analyses revealed that the panel of myokines expressed and released is different between insulin-sensitive and insulin-resistant myotubes. Several myokines were induced by TNFα-treatment such as VEGF, CXCL1, IL-8, and IL-6. However, the authors were not able to show which myokines mediate the observed effects on beta cells.

Recent data from Ellingsgaard et al. have shown that IL-6 increases proliferation of both alpha and beta cells and prevents apoptosis of alpha cells due to metabolic stress (Tab. 1) [51]. Moreover, IL-6 promotes glucagon-like peptide-1 (GLP-1) secretion and production in intestinal L-cells and pancreatic alpha cells thereby leading to improved beta cell insulin secretion and glucose tolerance [52]. These data could explain the increase of GLP-1 plasma level during exercise [52-54], which may be mediated by SkM-derived IL-6. Since the effect of GLP-1 on insulin secretion is glucose-dependent, IL-6–induced GLP-1 release will not acutely affect insulin secretion in healthy subjects during exercise but rather promote insulin secretion during a post-exercise meal [52]. Furthermore, an interaction between GLP-1 and IL-6 in the brain has been recently described showing that activation of central GLP-1 receptors increase IL-6 expression in the hypthalamus and hindbrain. These data point towards a role of central IL-6 in mediating the anorexic and body weight loss effects of GLP-1 receptor activation [55].

Fractalkine (CX3CL1) described by Henningsen et al. as myokine [56] might also play a role in the crosstalk to the pancreas. This chemokine has been reported to be associated with obesity, insulin resistance, and type 2 diabetes [57]. However, another study has found no differences in circulating fractalkine levels between type 2 diabetic patients and controls. Plomgaard et al. recently reported increased fractalkine mRNA expression and protein in SkM of healthy subjects after an acute bout of exercise as well as elevated circulating levels [58]. Data from Lee et al. have shown that treatment of islets with fractalkine increased intracellular Ca(2+) and potentiated insulin secretion in both mouse and human islets. Moreover, expression of fractalkine in islets of mice was decreased by aging and high-fat diet/obesity [59]. Further studies are needed to understand the impact of fractalkine on pancreatic function.

The potential role of myokines in the regulation of inflammation

Enlargement of adipose tissue as a consequence of a sedentary lifestyle and excessive energy intake results in a local inflammatory response in the visceral adipose tissue, infiltration of immune cells, and local and systemic increases of pro-inflammatory cytokines and adipokines. Thus, obesity, insulin resistance and type 2 diabetes are associated with a chronic low-grade systemic inflammation [60], and regular physical activity protects against a number of chronic diseases which are characterized by inflammation [61,62]. While regular physical exercise has been shown to reduce basal levels of inflammatory markers [63,64], physical inactivity results in elevated levels of inflammatory markers [65,66]. Whether certain myokines may be directly implicated in the anti-inflammatory effects of exercise is currently under investigation.

III. THE ANTI-INFLAMMATORY EFFECTS OF EXERCISE

It is known that regular physical activity reduces inflammation and improves insulin resistance. Cross-sectional studies have shown a strong inverse association between the level of physical activity and systemic low-grade inflammation [67-70]. These observations might be explained by an anti-inflammatory effect of regular exercise which could be mediated via different mechanisms. The scheme presented in fig. 1 summarizes the impact of physical activity levels on the systemic inflammation status and which mechanisms are potentially involved. Regular physical activity and an active lifestyle results in enhanced energy utilization thus leading to reduction of body weight and visceral fat mass

which is an important source of pro-inflammatory cytokines [60,71]. This may explain in part the reduction of inflammatory markers such as CRP following long-term exercise [70,72]. In addition, it has been shown that exercise decreases expression of toll-like receptor (TLR) 2 and TLR4 in immune cells and SkM [73-75] while even short-term bed rest increases expression of TLR4 [66]. Importantly, activation of TLR in SkM by factors such as lipopolysaccharide (LPS), heat shock protein 60 or free FAs has been suggested to participate in the development of insulin resistance [73,76]. Moreover, due to exercise-induced increases of adrenaline, cortisol, and other factors with immunomodulatory effects, physical activity may also have beneficial effects on the function of the immune system [75].

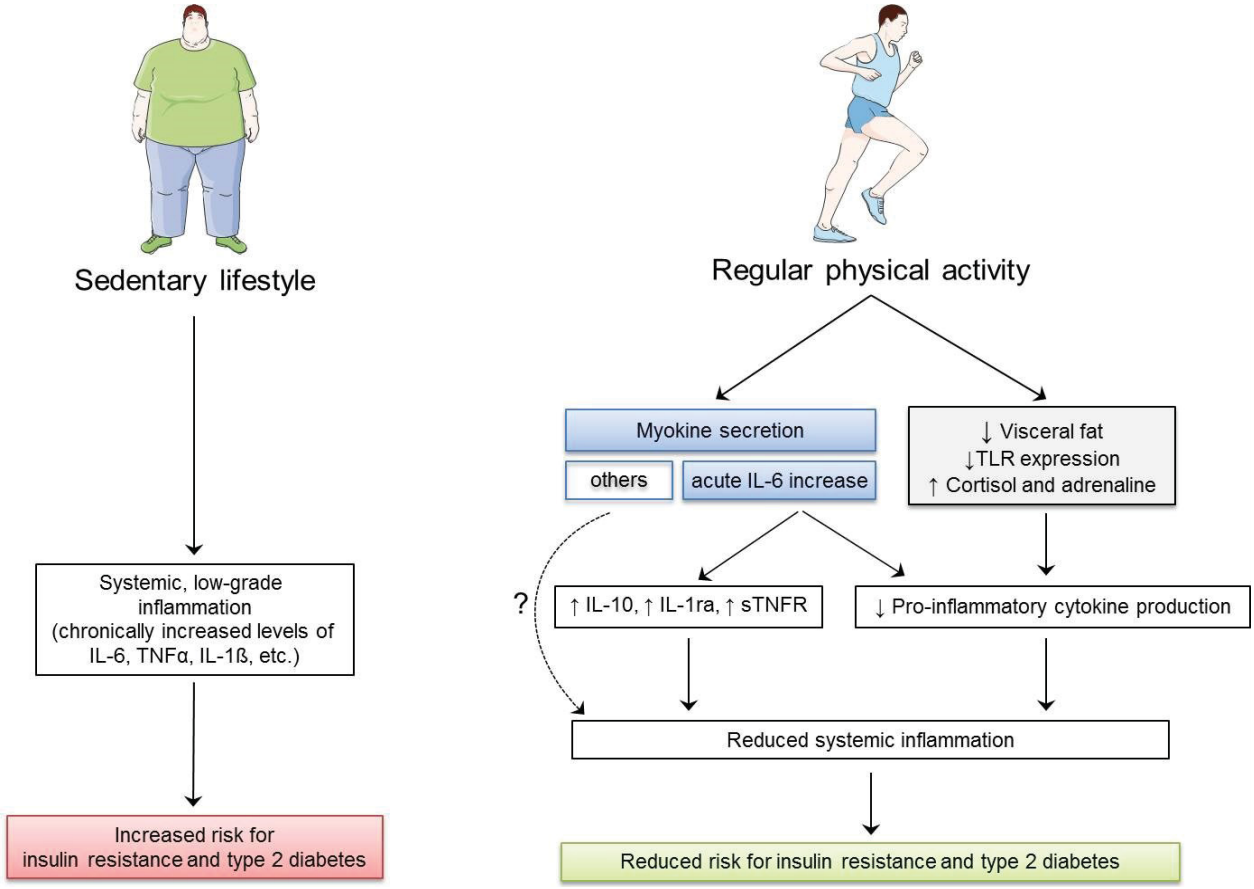

Fig. 1 Impact of physical activity level on inflammatory status and risk of insulin resistance and/or type 2 diabetes. On the one hand, a sedentary lifestyle combined with obesity is often associated with systemic low-grad inflammation and an increased risk of insulin resistance and type 2 diabetes. On the other hand, an active lifestyle with regular physical activity decreases the risk to develop insulin resistance and type 2 diabetes. This is in part mediated due to beneficial effects of exercise on systemic inflammation status. Exercise-induced acute increase of IL-6 results in enhanced release of anti-inflammatory cytokines such as IL-10, IL-1ra and sTNFR that reduces systemic inflammation. Currently, it is not known whether other myokines may also impact directly or indirectly on systemic inflammation. In addition, physical activity reduces visceral adipose tissue, TLR expression in immune cells and skeletal muscle, and increases release of adrenaline and cortisol which is known to have potent anti-inflammatory effects. In summary, these events finally result in reduced production of pro-inflammatory cytokines thereby contributing to reduced systemic inflammation and decreased risk to develop insulin resistance and type 2 diabetes.

Starkie et al. investigated the hypothesis that acute exercise induces a direct anti-inflammatory response [77]. Healthy subjects received LPS-endotoxin to induce low-grade inflammation during resting or after 2.5h bicycling. In resting subjects LPS-endotoxin administration resulted in a strong increase of plasma TNFα. Interestingly, in the exercising subjects the TNFα response to LPS-endotoxin was totally blunted, thus supporting the idea that physical activity mediates an anti-inflammatory activity. These data are further supported by *in vitro* experiments using electrical pulse stimulation (EPS) of primary human myotubes which induces contraction of the cells [78]. Our group has demonstrated that EPS prevents inflammatory responses induced by variuos treatments due to blocking the pro-inflammatory NFκB signaling pathway [78].

A. Myokines and anti-inflammatory effects

While IL-6 is acutely increased in the circulation in response to exercise [79] patients with insulin resistance, obesity and type 2 diabetes display chronically elevated IL-6 serum levels. [80-82]. An important source of circulating IL-6 in conditions of obesity is the expanding visceral adipose tissue. Expression of IL-6 by macrophages within the adipose tissue is dependent on activation of the NFκB signaling pathway, whereas intramuscular IL-6 expression is regulated by different signaling cascades involving Ca(2+), nuclear factor of

activated T cells [83], calcineurin [84], p38 MAPK signaling [85] and JNK/AP1 [14]. These observations led to the question about the functional role of IL-6 after exercise and in inflammatory conditions (reviewed in detail by Pedersen et al. [13]).

It is known that IL-6 released by contracting SkM triggers an anti-inflammatory cascade by inducing the production of the anti-inflammatory cytokines IL-10, IL-1 receptor antagonist (IL-1ra) and soluble TNF receptor (sTNFR) [13]. Moreover, data suggest that IL-6 inhibits the production of pro-inflammatory TNFα. Starkie et al. has reported that in healthy humans elevation of plasma IL-6 level by acute exercise or infusion of IL-6 blunts an LPS-endotoxin-mediated increase of TNFα [77].

The presence of IL-10, IL-1ra, and sTNFR in the circulation after exercise contributes to the anti-inflammatory effect of exercise (Fig. 1). IL-10 inhibits the production of pro-inflammatory factors such as IL-1α, IL-1ß, TNFα, IL-8 and MIPα [86]. IL-1ra is a member of the IL-1 family, binds to the IL-1 receptor, and inhibits the intracellular signal transduction thereby reducing the pro-inflammatory cascade induced by IL-1ß [87]. sTNFR is the naturally occurring inhibitor of TNFα [86].

Very recently, our group identified chitinase-3-like protein 1 (CHI3L1) as a novel myokine [88]. Interestingly, plasma CHI3L1 levels are up-regulated in patients with insulin resistance and type 2 diabetes independent of obesity [89]. However, the physiological role and source of circulating CHI3L1 remains largely unknown. Our *in vitro* experiments have shown that expression and release of CHI3L1 is stimulated by pro-inflammatory cytokines such as TNFα. We provide evidence that CHI3L1 protects myotubes from TNFα-induced insulin resistance and inflammation by inhibiting NFκB activation, and therefore suggest CHI3L1 to be an auto-protective factor that is induced upon demand to protect SkM from negative impact of TNFα [88].

Interestingly, similar effects were observed for fractalkine. Data recently presented by Plomgaard et al. have shown that fractalkine also prevents TNFα-induced insulin resistance by blocking the NFκB signaling pathway [58]. Furthermore, circulating levels were increased by acute exercise and TNFα-infusion [58]. *In vitro* data using human myotubes have shown that TNFα-treatment induces fractalkine mRNA expression. However, analysis of myotube-conditioned media found no elevated release of fractalkine after TNFα-treatment [50] which could mean that SkM may not be the main source of circulating fractalkine after TNFα-infusion. Fractalkine is also produced by adipose tissue and its serum level is positively associated with obesity, insulin resistance, and type 2 diabetes [57]. Taken together, these data indicate that myokines such as IL-6, CHI3L1 and fractalkine may be involved in mediating the anti-inflammatory effects of exercise and thus in mediating the beneficial effect of exercise on health.

In contrast to the observation that regular moderate exercise reduces chronic inflammation, high intensive training causes a temporary depression of various aspects of immune function and an increase in systemic inflammation for a certain post-exercise period (~3-24h) [61]. After a very intensive exercise such as marathon running, TNFα and IL-1β level increase in response to muscle damage [79,90,91]. It is well established that muscle repair and regeneration after acute muscle injury involves tissue-remodeling and growth-promoting local inflammation. The initial inflammatory response is required for a positive outcome of muscle repair [92].

In conclusion, it is obvious that regular physical activity is useful for prevention and therapy of various pathological conditions. For type 2 diabetic patients, a combination of both endurance and strength training was suggested as the most beneficial exercise training to improve glycemic control [93]. To understand the underlying mechanism of the anti-inflammatory effect of exercise in more detail, future efforts may also need to focus on the endocrine effects of myokines on immune cells.

IV. MYOKINES AS THERAPEUTICAL TARGETS

Due to improved understanding of myokine biology, the interest in contraction-regulated myokines as therapeutical targets has grown significantly. One additional strategy currently discussed is to increase brown adipose tissue (BAT) mass, its activity or to promote browning of white adipose tissue (WAT). This idea is based on the finding that the activity of BAT correlates with decreased BMI and adipose tissue mass and augmented energy expenditure [94-97]. Two myokines have been suggested to activate a white-to-brown shift in adipocytes, namely irisin [98] and fibroblast growth factor 21 (FGF21) [99]. Moreover, FGF21 enhances insulin sensitivity, and follistatin-like 1 (FSTL1) has been described as a cardio-protective myokine. Therefore, these three myokines are discussed here regarding their potential as therapeutical targets (see text box 1).

A. Irisin - does it keep its promises?

Boström et al. observed an enhanced FNDC5 mRNA expression in SkM of a muscle-specific PGC1α-overexpressing mouse model [98]. Data of the group indicated a cleaving of the extracellular part of this protein which acts as a novel signaling peptide, called irisin. Based on Western blot analysis circulating irisin was detected in both mice and humans and found increased after exercise. However, the antibody used in these analyses is not able to detect irisin since it binds to the non-secreted portion of FNDC5 [100,101]. Therefore, these data need to be reconsidered and re-assessed using independent experimental approaches.

Up to now, a total number of nine publications have described 15 different exercise studies in humans with different duration and types of exercise. Out of these, only in four cohorts an increase of FNDC5 mRNA expression in SkM by 1.3-2fold was observed [98,102-104] and three studies reported increased circulating irisin levels after exercise [104-106] (Tab. 2).

Our group has recently published an analysis of genomic DNA, mRNA and expressed sequence tags indicating that *FNDC5*, the gene encoding the precursor of irisin, is

present in rodents and most primates [100]. However, in humans we observed a mutation in the conserved start codon ATG to ATA. HEK293 cells transfected with a human FNDC5 construct with ATA as start codon only expressed 1% full-length protein compared to human FNDC5 with ATG. Thus, the mutation results in very low translation efficiency of full-length FNDC5. Based on our findings, we propose that irisin should be barely detectable in human samples [100].

Nevertheless, at least 18 studies from various groups quantified the level of irisin protein in plasma [103,105,107-109]. All these studies have relied on commercially available EIA kits which have not been fully validated. Basal circulating irisin levels in control subjects differ markedly ranging from 0.04 ng/ml [110] to 2158 ng/ml [108] (Tab. 2). Most importantly, none of the antibodies has been tested for cross-reacting proteins in plasma [101]. Future studies are urgently required to identify FNDC5 protein species potentially present in human serum.

Nevertheless, the hypothesis described by Boström et al. seems promising: irisin as a circulating factor that induces a white-to-brown shift in adipocytes thereby enhancing whole body energy expenditure. This principle was described comprehensively for mouse-derived preadipocytes and mice treated with recombinant FNDC5 [98,111]. Even though irisin might not be expressed in human cells: could it be a potential drug for humans since downstream signaling pathways might still exist? Recently, we have shown that primary human preadipocytes isolated from subcutaneous adipose tissue exhibit differentiation and a white-to-brown shift when treated with bone morphogenetic protein-7. However, we and others have detected no effects of recombinant irisin or FNDC5 obtained from different sources on the induction of a white-to-brown shift [100,112]. Therefore, the original discovery proposing FNDC5/irisin as a contraction-regulated browning factor and its use as a therapeutical needs to be reconsidered.

B. FGF21 – effects on insulin sensitivity and browning

FGF21 is a member of the fibroblast growth factor super family, a large family of proteins involved in cell proliferation, growth, and differentiation. In type 2 diabetic patients serum levels of FGF21 are significantly higher compared to healthy controls [113]. Izumiya et al. have described FGF21 as an Akt-regulated myokine due to an insulin-mediated increase of FGF21 protein level and secretion, and a PI3-kinase inhibitor-mediated prevention of these effects using C2C12 myotubes [114]. Additionally, a hyperinsulinemic-euglycemic clamp study in young healthy men has revealed an induction of FGF21 mRNA in SkM and increased plasma levels [115]. These data may link insulin-activated Akt to enhanced FGF21 secretion from SkM.

Circulating FGF21 serum levels have been studied in three human exercise studies with diverging results (Tab. 2). While three months combined aerobic and resistance exercise diminished FGF21 serum levels [116], two months endurance training had no effect on FGF21 serum levels and mRNA expression in SkM [117]. Most interestingly, two weeks of daily endurance training and an acute bout of endurance exercise enhanced circulating FGF21 levels accompanied by increased serum levels of free FAs [118,119]. However, in mice Kim et al. have observed no increase of FGF21 mRNA expression in *soleus* and *gastrocnemius* muscle and epididymal WAT after an acute bout of exercise while hepatic FGF21 mRNA expression was significantly enhanced. Therefore, the authors have suggested that hepatic FGF21 contributes to increased serum levels after exercise most likely due to exercise-induced lipolysis [119]. Thus, future studies have to clarify whether FGF21 is a contraction-regulated myokine by investigating changes of FGF21 mRNA expression and protein level in SkM which could result in increased FGF21 serum levels after exercise.

Given that hepatic FGF21 mRNA expression is dramatically induced by fasting [120,121], Izumiya et al. used 48-h fasted liver tissues in comparison to gastrocnemius muscle and reported similar levels of FGF21 protein in both tissues [114]. Although it is a challenge to determine the extact tissue or even the cell type within a certain tissue as a major source of circulating proteins, the current data presume that SkM might may be an additional important source of circulating FGF21. Most likely, FGF21 serum levels depend on whole body metabolic status.

It has been demonstrated that FGF21 acts on several tissues by affecting carbohydrate and lipid metabolism, enhancing insulin sensitivity, decreasing triglyceride concentrations and causing weight loss (reviewed by Cuevas-Ramos et al. [122]). FGF21 administration improves metabolic phenotypes such as increased fasting glucose, insulin and triacylglycerol levels in different obese mice model and diabetic rhesus monkeys [123-126]. Additionally, experiments with FGF21-treated mice indicated that it regulates whole-body insulin responsiveness by enhanced glucose uptake in SkM, WAT and BAT and improved suppression of WAT lipolysis is [99]. Furthermore, FGF21 promotes a white-to-brown shift of WAT in mice treated with recombinant FGF21 [99] as demonstrated by increased oxygen consumption in WAT and increased mRNA expression of typical markers of brite adipogenesis [99].

The results of two recent studies indicate that the beneficial effects of FGF21 are not merely mediated by the protein itself [127,128] given that in mice FGF21 stimulates the production and secretion of the adipocyte-specific hormone adiponectin in WAT. Adiponectin seems to play a central role for the effects of FGF21 on whole body metabolism and insulin sensitivity since these effects were almost completely abolished in adiponectin knockout mice [127,128]. Of note, IL-15 is another contraction-regulated myokine that stimulates secretion of adiponectin, and IL-15 treatment improves glucose homeostasis and insulin sensitivity in obese mice [44].

Based on experimental data showing beneficial effects of FGF21 in the context of obesity, a study was initiated to investigate the effects of the FGF21 analog LY2405319 in obese type 2 diabetic patients [129]. LY2405319 has

been shown to have favorable effects on both body weight and fasting insulin levels. Moreover, it results in a shift to a potentially less atherogenic apolipoprotein concentration profile [129]. Thus, FGF21 or FGF21 analogs seem to be interesting therapeutical targets.

However, currently no information are available concerning circulating levels of FSTL1 in insulin resistant and/or type 2 diabetic patients, and more data obtained in human models are necessary to evaluate the potential of FSTL1 as a therapeutical target.

Key facts: myokines as therapeutical targets
▪ A new exercise-regulated myokine called irisin has been identified that induces browning of subcutaneous WAT in mice. However, translation to the human situation remains unclear. ▪ FGF21 enhances insulin sensitivity by increasing circulating adiponectin levels. Moreover, it promotes a white-to-brown shift in mice. The FGF21 analog LY2405319 is a promising candidate as novel therapeutical approach. ▪ The cardio-protective protein FSTL1 might play an important role in the pathophysiological responses to cardiovascular stress which is closely associated with type 2 diabetes.

Text box 1

C. Follistatin-like 1 – a cardio-protective cytokine

FSTL1, also known as TSC36, is a secreted glycoprotein that has limited homology with other follistatin family proteins that classically act as binding partners of the TGFβ protein family. FSTL1 is secreted by SkM and based on initial data it has been discussed as a contraction-regulated myokine (Tab. 2). Most interestingly, it is upregulated via Akt signaling. Ouchi et al. have proposed that FSTL1 can be designated as a myokine that acts on vascular endothelial cells [130]. In murine SkM, overexpression of FSTL1 was shown to promote endothelial cell function and revascularization in ischemic tissue. Moreover, FSTL1 secretion of primary human myotubes is enhanced by pro-inflammatory cytokines such as IL-1β and interferon γ [131]. It might be speculated that enhanced FSTL1 secretion of myotubes counteracts the harmful effects of proinflammatory adipokines on the muscular vasculature. However, SkM is not the only tissue expressing and releasing FSTL1. Proteomic approaches have identified FSTL1 also in the supernatant of primary human adipocytes [132], in supernatants of human endothelial cells [130] and in lysates of murine cardiac myocytes [133].

FSTL1 is described as a cardio-protective cytokine. Its expression is induced in the heart by ischemic insults and systemic administration of FSTL1 protects the heart from ischemia/reperfusion injury [134]. Shimano et al. have described that cardiac FSTL1 functions as an auto-/paracrine regulatory factor that antagonizes myocyte hypertrophic growth and the loss of ventricular performance in response to pressure overload [133]. In preclinical animal models of ischemia/reperfusion FSTL1 was demonstrated to prevent myocardial ischemia/injury by inhibiting apoptosis and inflammatory responses due to modulation of AMPK [135]. Therefore, FSTL1 appears to be a clinically relevant factor that participates in pathophysiological responses to cardiovascular stress which is closely associated with type 2 diabetes.

V. CONCLUSION

Myokines are key elements of the multi-organ crosstalk communication network, and they play a prominent role in mediating the health-promoting effects of regular physical activity. This involves substrate oxidation, lipid partitioning, reduced inflammation, and improved pancreatic beta cell function. The list of new myokines is continuously increasing, whereas the physiological impact of this multitude of molecules remains largely unexplored. This is complicated by differences in the local and systemic concentrations of many myokines, and additionally by a substantial overlap of myokines and adipokines. The comprehensive analysis of the myokine network will help to identify drugable targets in the future.

VI. REFERENCES

1. Febbraio MA, Pedersen BK (2005) Contraction-induced myokine production and release: is skeletal muscle an endocrine organ? Exerc.Sport Sci.Rev. 33: 114-119
2. Raschke S, Eckardt K, Holven KB, Jensen J, Eckel J (2013) Identification and Validation of Novel Contraction-Regulated Myokines Released from Primary Human Skeletal Muscle Cells. PLoS ONE 8: e62008
3. Hartwig S, Raschke S, Knebel B, et al (2013) Secretome profiling of primary human skeletal muscle cells. Biochim.Biophys.Acta
4. Norheim F, Raastad T, Thiede B, Rustan AC, Drevon CA, Haugen F (2011) Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training. Am.J.Physiol Endocrinol.Metab 301: E1013-E1021
5. Raschke S, Eckel J (2013) Adipo-myokines: two sides of the same coin-mediators of inflammation and mediators of exercise. Mediators.Inflamm. 2013: 320724

6. Pedersen BK, Febbraio MA (2012) Muscles, exercise and obesity: skeletal muscle as a secretory organ. Nat Rev.Endocrinol. 8: 457-465
7. Thyfault JP, Booth FW (2011) Lack of regular physical exercise or too much inactivity. Curr.Opin.Clin.Nutr.Metab Care 14: 374-8
8. Bergouignan A, Rudwill F, Simon C, Blanc S (2011) Physical inactivity as the culprit of metabolic inflexibility: Evidences from bed-rest studies. J Appl.Physiol 11: 1201-10
9. Alibegovic AC, Sonne MP, Hojbjerre L, et al (2010) Insulin resistance induced by physical inactivity is associated with multiple transcriptional changes in skeletal muscle in young men. Am.J Physiol Endocrinol.Metab 299: E752-63
10. Steensberg A, van Hall G, Osada T, Sacchetti M, Saltin B, Klarlund PB (2000) Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. J Physiol 529 Pt 1: 237-242
11. Steensberg A, Febbraio MA, Osada T, et al (2001) Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content. J.Physiol 537: 633-639
12. Hiscock N, Chan MH, Bisucci T, Darby IA, Febbraio MA (2004) Skeletal myocytes are a source of interleukin-6 mRNA expression and protein release during contraction: evidence of fiber type specificity. FASEB J. 18: 992-994
13. Pedersen BK, Febbraio MA (2008) Muscle as an endocrine organ: focus on muscle-derived interleukin-6. Physiol Rev. 88: 1379-1406
14. Whitham M, Chan MH, Pal M, et al (2012) Contraction-induced interleukin-6 gene transcription in skeletal muscle is regulated by c-Jun terminal kinase/activator protein-1. J.Biol.Chem. 287: 10771-10779
15. Nieto-Vazquez I, Fernandez-Veledo S, De Alvaro C, Lorenzo M (2008) Dual role of interleukin-6 in regulating insulin sensitivity in murine skeletal muscle. Diabetes 57: 3211-3221
16. Holmes AG, Mesa JL, Neill BA, et al (2008) Prolonged interleukin-6 administration enhances glucose tolerance and increases skeletal muscle PPARalpha and UCP2 expression in rats. J.Endocrinol. 198: 367-374
17. Sadagurski M, Norquay L, Farhang J, D'Aquino K, Copps K, White MF (2010) Human IL6 enhances leptin action in mice. Diabetologia 53: 525-535
18. Al-Khalili L, Bouzakri K, Glund S, Lonnqvist F, Koistinen HA, Krook A (2006) Signaling specificity of interleukin-6 action on glucose and lipid metabolism in skeletal muscle. Mol.Endocrinol. 20: 3364-3375
19. Carey AL, Steinberg GR, Macaulay SL, et al (2006) Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase. Diabetes 55: 2688-2697
20. Jiang LQ, Duque-Guimaraes DE, Machado UF, Zierath JR, Krook A (2012) Altered Response of Skeletal Muscle to IL-6 in Type 2 Diabetic Patients. Diabetes 62: 355-61
21. Febbraio MA, Steensberg A, Keller C, et al (2003) Glucose ingestion attenuates interleukin-6 release from contracting skeletal muscle in humans. J.Physiol 549: 607-612
22. Keller C, Steensberg A, Pilegaard H, et al (2001) Transcriptional activation of the IL-6 gene in human contracting skeletal muscle: influence of muscle glycogen content. FASEB J. 15: 2748-2750
23. Febbraio MA, Hiscock N, Sacchetti M, Fischer CP, Pedersen BK (2004) Interleukin-6 is a novel factor mediating glucose homeostasis during skeletal muscle contraction. Diabetes 53: 1643-1648
24. Steensberg A, Fischer CP, Sacchetti M, et al (2003) Acute interleukin-6 administration does not impair muscle glucose uptake or whole-body glucose disposal in healthy humans. J.Physiol 548: 631-638
25. Fritsche L, Hoene M, Lehmann R, et al (2010) IL-6 deficiency in mice neither impairs induction of metabolic genes in the liver nor affects blood glucose levels during fasting and moderately intense exercise. Diabetologia 53: 1732-1742
26. Kim HJ, Higashimori T, Park SY, et al (2004) Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action in vivo. Diabetes 53: 1060-1067
27. Klover PJ, Zimmers TA, Koniaris LG, Mooney RA (2003) Chronic exposure to interleukin-6 causes hepatic insulin resistance in mice. Diabetes 52: 2784-2789
28. Jiang LQ, Franck N, Egan B, et al (2013) Autocrine role of interleukin-13 on skeletal muscle glucose metabolism in Type 2 diabetic patients involves microRNA let-7. Am J Physiol Endocrinol Metab
29. Stanya KJ, Jacobi D, Liu S, et al (2013) Direct control of hepatic glucose production by interleukin-13 in mice. J.Clin.Invest 123: 261-271
30. van HG, Steensberg A, Sacchetti M, et al (2003) Interleukin-6 stimulates lipolysis and fat oxidation in humans. J.Clin.Endocrinol.Metab 88: 3005-3010
31. Wolsk E, Mygind H, Grondahl TS, Pedersen BK, van HG (2010) IL-6 selectively stimulates fat metabolism in human skeletal muscle. Am J Physiol Endocrinol Metab 299: E832-E840

32. Petersen EW, Carey AL, Sacchetti M, et al (2005) Acute IL-6 treatment increases fatty acid turnover in elderly humans in vivo and in tissue culture in vitro. Am J Physiol Endocrinol Metab 288: E155-E162
33. Kelly M, Gauthier MS, Saha AK, Ruderman NB (2009) Activation of AMP-activated protein kinase by interleukin-6 in rat skeletal muscle: association with changes in cAMP, energy state, and endogenous fuel mobilization. Diabetes 58: 1953-1960
34. Macdonald TL, Wan Z, Frendo-Cumbo S, Dyck DJ, Wright DC (2013) IL-6 and epinephrine have divergent fiber type effects on intramuscular lipolysis. J Appl.Physiol (1985.)
35. Peterson JM, Pizza FX (2009) Cytokines derived from cultured skeletal muscle cells after mechanical strain promote neutrophil chemotaxis in vitro. J.Appl.Physiol 106: 130-137
36. Nieman DC, Davis JM, Brown VA, et al (2004) Influence of carbohydrate ingestion on immune changes after 2 h of intensive resistance training. J Appl.Physiol 96: 1292-1298
37. Nielsen AR, Mounier R, Plomgaard P, et al (2007) Expression of interleukin-15 in human skeletal muscle effect of exercise and muscle fibre type composition. J Physiol 584: 305-312
38. Tamura Y, Watanabe K, Kantani T, Hayashi J, Ishida N, Kaneki M (2011) Upregulation of circulating IL-15 by treadmill running in healthy individuals: is IL-15 an endocrine mediator of the beneficial effects of endurance exercise? Endocr.J 58: 211-215
39. Yeo NH, Woo J, Shin KO, Park JY, Kang S (2012) The effects of different exercise intensity on myokine and angiogenesis factors. J.Sports Med Phys.Fitness 52: 448-454
40. Riechman SE, Balasekaran G, Roth SM, Ferrell RE (2004) Association of interleukin-15 protein and interleukin-15 receptor genetic variation with resistance exercise training responses. J Appl.Physiol (1985.) 97: 2214-2219
41. Al-Shukaili A, Al-Ghafri S, Al-Marhoobi S, Al-Abri S, Al-Lawati J, Al-Maskari M (2013) Analysis of inflammatory mediators in type 2 diabetes patients. Int.J Endocrinol 2013: 976810
42. Barra NG, Reid S, MacKenzie R, et al (2010) Interleukin-15 contributes to the regulation of murine adipose tissue and human adipocytes. Obesity.(Silver.Spring) 18: 1601-1607
43. Almendro V, Busquets S, Ametller E, et al (2006) Effects of interleukin-15 on lipid oxidation: disposal of an oral [(14)C]-triolein load. Biochim.Biophys.Acta 1761: 37-42
44. Quinn LS, Strait-Bodey L, Anderson BG, Argiles JM, Havel PJ (2005) Interleukin-15 stimulates adiponectin secretion by 3T3-L1 adipocytes: evidence for a skeletal muscle-to-fat signaling pathway. Cell Biol.Int. 29: 449-457
45. Quinn LS, Anderson BG, Strait-Bodey L, Stroud AM, Argiles JM (2009) Oversecretion of interleukin-15 from skeletal muscle reduces adiposity. Am.J.Physiol Endocrinol.Metab 296: E191-E202
46. Matthews VB, Astrom MB, Chan MH, et al (2009) Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. Diabetologia 52: 1409-1418
47. Krabbe KS, Nielsen AR, Krogh-Madsen R, et al (2007) Brain-derived neurotrophic factor (BDNF) and type 2 diabetes. Diabetologia 50: 431-438
48. Rasmussen P, Brassard P, Adser H, et al (2009) Evidence for a release of brain-derived neurotrophic factor from the brain during exercise. Exp.Physiol 94: 1062-1069
49. Tsuchida A, Nakagawa T, Itakura Y, et al (2001) The effects of brain-derived neurotrophic factor on insulin signal transduction in the liver of diabetic mice. Diabetologia 44: 555-566
50. Bouzakri K, Plomgaard P, Berney T, Donath MY, Pedersen BK, Halban PA (2011) Bimodal effect on pancreatic beta-cells of secretory products from normal or insulin-resistant human skeletal muscle. Diabetes 60: 1111-1121
51. Ellingsgaard H, Ehses JA, Hammar EB, et al (2008) Interleukin-6 regulates pancreatic alpha-cell mass expansion. Proc.Natl.Acad.Sci.U.S.A 105: 13163-13168
52. Ellingsgaard H, Hauselmann I, Schuler B, et al (2011) Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells. Nat.Med. 17: 1481-1489
53. Ueda SY, Yoshikawa T, Katsura Y, Usui T, Nakao H, Fujimoto S (2009) Changes in gut hormone levels and negative energy balance during aerobic exercise in obese young males. J Endocrinol 201: 151-159
54. Martins C, Robertson MD, Morgan LM (2008) Effects of exercise and restrained eating behaviour on appetite control. Proc.Nutr.Soc. 67: 28-41
55. Shirazi R, Palsdottir V, Collander J, et al (2013) Glucagon-like peptide 1 receptor induced suppression of food intake, and body weight is mediated by central IL-1 and IL-6. Proc.Natl.Acad.Sci.U.S.A 110: 16199-16204
56. Henningsen J, Rigbolt KT, Blagoev B, Pedersen BK, Kratchmarova I (2010) Dynamics of the skeletal muscle secretome during myoblast differentiation. Mol.Cell Proteomics. 9: 2482-2496
57. Shah R, Hinkle CC, Ferguson JF, et al (2011) Fractalkine is a novel human adipochemokine associated with type 2 diabetes. Diabetes 60: 1512-1518

58. Plomgaard P, Hansen J, Bianda V, Pedersen BK, Bouzakri K (2013) Fractalkine is a novel myokine which protects myotubes from TNF-alpha induced insulin resistance. Diabetologia 56: 79
59. Lee YS, Morinaga H, Kim JJ, et al (2013) The fractalkine/CX3CR1 system regulates beta cell function and insulin secretion. Cell 153: 413-425
60. Sell H, Habich C, Eckel J (2012) Adaptive immunity in obesity and insulin resistance. Nat.Rev.Endocrinol. 8: 709-716
61. Gleeson M (2007) Immune function in sport and exercise. J.Appl.Physiol 103: 693-699
62. Pedersen BK, Saltin B (2006) Evidence for prescribing exercise as therapy in chronic disease. Scand.J.Med.Sci.Sports 16 Suppl 1: 3-63
63. Donges CE, Duffield R, Drinkwater EJ (2010) Effects of resistance or aerobic exercise training on interleukin-6, C-reactive protein, and body composition. Med.Sci.Sports Exerc. 42: 304-313
64. Christiansen T, Paulsen SK, Bruun JM, Pedersen SB, Richelsen B (2010) Exercise training versus diet-induced weight-loss on metabolic risk factors and inflammatory markers in obese subjects: a 12-week randomized intervention study. Am J Physiol Endocrinol Metab 298: E824-E831
65. Fischer CP, Berntsen A, Perstrup LB, Eskildsen P, Pedersen BK (2007) Plasma levels of interleukin-6 and C-reactive protein are associated with physical inactivity independent of obesity. Scand.J.Med.Sci.Sports 17: 580-587
66. Drummond MJ, Timmerman KL, Markofski MM, et al (2013) Short-term bed rest increases TLR4 and IL-6 expression in skeletal muscle of older adults. Am.J.Physiol Regul.Integr.Comp Physiol 305: R216-R223
67. King DE, Carek P, Mainous AG, III, Pearson WS (2003) Inflammatory markers and exercise: differences related to exercise type. Med.Sci.Sports Exerc. 35: 575-581
68. Abramson JL, Vaccarino V (2002) Relationship between physical activity and inflammation among apparently healthy middle-aged and older US adults. Arch.Intern.Med. 162: 1286-1292
69. Geffken DF, Cushman M, Burke GL, Polak JF, Sakkinen PA, Tracy RP (2001) Association between physical activity and markers of inflammation in a healthy elderly population. Am.J.Epidemiol. 153: 242-250
70. Mattusch F, Dufaux B, Heine O, Mertens I, Rost R (2000) Reduction of the plasma concentration of C-reactive protein following nine months of endurance training. Int.J.Sports Med. 21: 21-24
71. de Lemos ET, Oliveira J, Pinheiro JP, Reis F (2012) Regular physical exercise as a strategy to improve antioxidant and anti-inflammatory status: benefits in type 2 diabetes mellitus. Oxid.Med.Cell Longev. 2012: 741545
72. Stewart LK, Flynn MG, Campbell WW, et al (2007) The influence of exercise training on inflammatory cytokines and C-reactive protein. Med.Sci.Sports Exerc. 39: 1714-1719
73. Francaux M (2009) Toll-like receptor signalling induced by endurance exercise. Appl.Physiol Nutr.Metab 34: 454-458
74. Lambert CP, Wright NR, Finck BN, Villareal DT (2008) Exercise but not diet-induced weight loss decreases skeletal muscle inflammatory gene expression in frail obese elderly persons. J Appl.Physiol (1985.) 105: 473-478
75. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA (2011) The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. Nat.Rev.Immunol. 11: 607-615
76. Liang H, Hussey SE, Sanchez-Avila A, Tantiwong P, Musi N (2013) Effect of lipopolysaccharide on inflammation and insulin action in human muscle. PLoS ONE 8: e63983
77. Starkie R, Ostrowski SR, Jauffred S, Febbraio M, Pedersen BK (2003) Exercise and IL-6 infusion inhibit endotoxin-induced TNF-alpha production in humans. FASEB J. 17: 884-886
78. Lambernd S, Taube A, Schober A, et al (2012) Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways. Diabetologia 55: 1128-1139
79. Pedersen BK, Akerstrom TC, Nielsen AR, Fischer CP (2007) Role of myokines in exercise and metabolism. J.Appl.Physiol 103: 1093-1098
80. Dandona P, Aljada A, Bandyopadhyay A (2004) Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol. 25: 4-7
81. Duncan BB, Schmidt MI, Pankow JS, et al (2003) Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study. Diabetes 52: 1799-1805
82. Kern PA, Ranganathan S, Li C, Wood L, Ranganathan G (2001) Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. Am.J.Physiol Endocrinol.Metab 280: E745-E751
83. Im SH, Rao A (2004) Activation and deactivation of gene expression by Ca2+/calcineurin-NFAT-mediated signaling. Mol.Cells 18: 1-9
84. Banzet S, Koulmann N, Sanchez H, et al (2007) Contraction-induced interleukin-6 transcription in rat slow-type muscle is partly dependent on calcineurin activation. J.Cell Physiol 210: 596-601

85. Chan MH, McGee SL, Watt MJ, Hargreaves M, Febbraio MA (2004) Altering dietary nutrient intake that reduces glycogen content leads to phosphorylation of nuclear p38 MAP kinase in human skeletal muscle: association with IL-6 gene transcription during contraction. FASEB J. 18: 1785-1787
86. Petersen AM, Pedersen BK (2005) The anti-inflammatory effect of exercise. J.Appl.Physiol 98: 1154-1162
87. Dinarello CA (2000) The role of the interleukin-1-receptor antagonist in blocking inflammation mediated by interleukin-1. N.Engl.J.Med. 343: 732-734
88. Görgens SW, Eckardt K, Elsen M, Tennagels N, Eckel J (2014) Chitinase-3-like protein 1 protects skeletal muscle from TNFalpha-induced inflammation and insulin resistance. Biochem.J. [Epub ahead of print]
89. Nielsen AR, Erikstrup C, Johansen JS, et al (2008) Plasma YKL-40: a BMI-independent marker of type 2 diabetes. Diabetes 57: 3078-3082
90. Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK (1999) Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. J Physiol 515 (Pt 1): 287-291
91. Bernecker C, Scherr J, Schinner S, Braun S, Scherbaum WA, Halle M (2013) Evidence for an exercise induced increase of TNF-alpha and IL-6 in marathon runners. Scand.J Med.Sci.Sports 23: 207-214
92. Paulsen G, Mikkelsen UR, Raastad T, Peake JM (2012) Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise? Exerc.Immunol.Rev. 18: 42-97
93. Sigal RJ, Kenny GP, Boule NG, et al (2007) Effects of aerobic training, resistance training, or both on glycemic control in type 2 diabetes: a randomized trial. Ann.Intern.Med. 147: 357-369
94. Cypess AM, Lehman S, Williams G, et al (2009) Identification and importance of brown adipose tissue in adult humans. N.Engl.J.Med. 360: 1509-1517
95. Ouellet V, Routhier-Labadie A, Bellemare W, et al (2011) Outdoor temperature, age, sex, body mass index, and diabetic status determine the prevalence, mass, and glucose-uptake activity of 18F-FDG-detected BAT in humans. J.Clin.Endocrinol.Metab 96: 192-199
96. Zingaretti MC, Crosta F, Vitali A, et al (2009) The presence of UCP1 demonstrates that metabolically active adipose tissue in the neck of adult humans truly represents brown adipose tissue. FASEB J. 23: 3113-3120
97. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, et al (2009) Cold-activated brown adipose tissue in healthy men. N.Engl.J.Med. 360: 1500-1508
98. Bostrom P, Wu J, Jedrychowski MP, et al (2012) A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature 481: 463-468
99. Camporez JP, Jornayvaz FR, Petersen MC, et al (2013) Cellular Mechanisms by Which FGF21 Improves Insulin Sensitivity in Male Mice. Endocrinology 154: 3099-3109
100. Raschke S, Elsen M, Gassenhuber H, et al (2013) Evidence against a Beneficial Effect of Irisin in Humans. PLoS.ONE. 8: e73680
101. Erickson HP (2013) Irisin and FNDC5 in retrospect: An exercise hormone or a transmembrane receptor? Adipocyte. 2: 289-293
102. Timmons JA, Baar K, Davidsen PK, Atherton PJ (2012) Is irisin a human exercise gene? Nature 488: E9-10
103. Pekkala S, Wiklund P, Hulmi JJ, et al (2013) Are Skeletal Muscle FNDC5 Gene Expression and Irisin Release Regulated by Exercise and Related to Health? J.Physiol
104. Norheim F, Langleite TM, Hjorth M, et al (2013) The effects of acute and chronic exercise on PGC-1alpha, irisin and browning of subcutaneous adipose tissue in human. FEBS J
105. Huh JY, Panagiotou G, Mougios V, et al (2012) FNDC5 and irisin in humans: I. Predictors of circulating concentrations in serum and plasma and II. mRNA expression and circulating concentrations in response to weight loss and exercise. Metabolism 61: 1725-1738
106. Kraemer RR, Shockett P, Webb ND, Shah U, Castracane VD (2013) A Transient Elevated Irisin Blood Concentration in Response to Prolonged, Moderate Aerobic Exercise in Young Men and Women. Horm.Metab Res.
107. Gouni-Berthold I, Berthold HK, Huh JY, et al (2013) Effects of lipid-lowering drugs on irisin in human subjects in vivo and in human skeletal muscle cells ex vivo. PLoS.ONE. 8: e72858
108. Moreno-Navarrete JM, Ortega F, Serrano M, et al (2013) Irisin is expressed and produced by human muscle and adipose tissue in association with obesity and insulin resistance. J.Clin.Endocrinol.Metab 98: E769-E778
109. Stengel A, Hofmann T, Goebel-Stengel M, Elbelt U, Kobelt P, Klapp BF (2013) Circulating levels of irisin in patients with anorexia nervosa and different stages of obesity--correlation with body mass index. Peptides 39: 125-130
110. Choi YK, Kim MK, Bae KH, et al (2013) Serum irisin levels in new-onset type 2 diabetes. Diabetes Res.Clin.Pract. 100: 96-101
111. Zhang Y, Li R, Meng Y, et al (2013) Irisin Stimulates Browning of White Adipocytes through Mitogen-Activated Protein Kinase p38 MAP Kinase and ERK MAP Kinase Signaling. Diabetes

112. Lee D, Zhou Y, Tu M, et al (2013) Irisin Does Not Induce Browning of Mouse or Human Adipocytes. Diabetes 62: A25
113. Cheng X, Zhu B, Jiang F, Fan H (2011) Serum FGF-21 levels in type 2 diabetic patients. Endocr.Res. 36: 142-148
114. Izumiya Y, Bina HA, Ouchi N, Akasaki Y, Kharitonenkov A, Walsh K (2008) FGF21 is an Akt-regulated myokine. FEBS Lett. 582: 3805-3810
115. Hojman P, Pedersen M, Nielsen AR, et al (2009) Fibroblast growth factor-21 is induced in human skeletal muscles by hyperinsulinemia. Diabetes 58: 2797-80
116. Yang SJ, Hong HC, Choi HY, et al (2011) Effects of a three-month combined exercise programme on fibroblast growth factor 21 and fetuin-A levels and arterial stiffness in obese women. Clin.Endocrinol.(Oxf) 75: 464-469
117. Besse-Patin A, Montastier E, Vinel C, et al (2013) Effect of endurance training on skeletal muscle myokine expression in obese men: identification of apelin as a novel myokine. Int.J.Obes.(Lond)
118. Cuevas-Ramos D, Almeda-Valdes P, Meza-Arana CE, et al (2012) Exercise increases serum fibroblast growth factor 21 (FGF21) levels. PLoS.ONE. 7: e38022
119. Kim KH, Kim SH, Min YK, Yang HM, Lee JB, Lee MS (2013) Acute exercise induces FGF21 expression in mice and in healthy humans. PLoS.ONE. 8: e63517
120. Badman MK, Pissios P, Kennedy AR, Koukos G, Flier JS, Maratos-Flier E (2007) Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. Cell Metab 5: 426-437
121. Inagaki T, Dutchak P, Zhao G, et al (2007) Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. Cell Metab 5: 415-425
122. Cuevas-Ramos D, Aguilar-Salinas CA, Gomez-Perez FJ (2012) Metabolic actions of fibroblast growth factor 21. Curr.Opin.Pediatr. 24: 523-529
123. Coskun T, Bina HA, Schneider MA, et al (2008) Fibroblast growth factor 21 corrects obesity in mice. Endocrinology 149: 6018-6027
124. Kharitonenkov A, Shiyanova TL, Koester A, et al (2005) FGF-21 as a novel metabolic regulator. J.Clin.Invest 115: 1627-1635
125. Kharitonenkov A, Wroblewski VJ, Koester A, et al (2007) The metabolic state of diabetic monkeys is regulated by fibroblast growth factor-21. Endocrinology 148: 774-781
126. Xu J, Lloyd DJ, Hale C, et al (2009) Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice. Diabetes 58: 250-259
127. Holland WL, Adams AC, Brozinick JT, et al (2013) An FGF21-adiponectin-ceramide axis controls energy expenditure and insulin action in mice. Cell Metab 17: 790-797
128. Lin Z, Tian H, Lam KS, et al (2013) Adiponectin mediates the metabolic effects of FGF21 on glucose homeostasis and insulin sensitivity in mice. Cell Metab 17: 779-789
129. Gaich G, Chien JY, Fu H, et al (2013) The Effects of LY2405319, an FGF21 Analog, in Obese Human Subjects with Type 2 Diabetes. Cell Metab 18: 333-340
130. Ouchi N, Oshima Y, Ohashi K, et al (2008) Follistatin-like 1, a secreted muscle protein, promotes endothelial cell function and revascularization in ischemic tissue through a nitric-oxide synthase-dependent mechanism. J.Biol.Chem. 283: 32802-32811
131. Gorgens SW, Raschke S, Holven KB, Jensen J, Eckardt K, Eckel J (2013) Regulation of follistatin-like protein 1 expression and secretion in primary human skeletal muscle cells. Arch.Physiol Biochem. 119: 75-80
132. Lehr S, Hartwig S, Lamers D, et al (2012) Identification and validation of novel adipokines released from primary human adipocytes. Mol.Cell Proteomics. 11: M111
133. Shimano M, Ouchi N, Nakamura K, et al (2011) Cardiac myocyte follistatin-like 1 functions to attenuate hypertrophy following pressure overload. Proc.Natl.Acad.Sci.U.S.A 108: E899-E906
134. Oshima Y, Ouchi N, Sato K, Izumiya Y, Pimentel DR, Walsh K (2008) Follistatin-like 1 is an Akt-regulated cardioprotective factor that is secreted by the heart. Circulation 117: 3099-3108
135. Ogura Y, Ouchi N, Ohashi K, et al (2012) Therapeutic impact of follistatin-like 1 on myocardial ischemic injury in preclinical models. Circulation 126: 1728-1738
136. Mashili FL, Austin RL, Deshmukh AS, et al (2011) Direct effects of FGF21 on glucose uptake in human skeletal muscle: implications for type 2 diabetes and obesity. Diabetes Metab Res.Rev. 27: 286-297
137. Potthoff MJ, Inagaki T, Satapati S, et al (2009) FGF21 induces PGC-1alpha and regulates carbohydrate and fatty acid metabolism during the adaptive starvation response. Proc.Natl.Acad.Sci.U.S.A 106: 10853-10858
138. Nishimura T, Nakatake Y, Konishi M, Itoh N (2000) Identification of a novel FGF, FGF-21, preferentially expressed in the liver. Biochim.Biophys.Acta 1492: 203-206

139. Adams DC, Karolak MJ, Larman BW, Liaw L, Nolin JD, Oxburgh L (2010) Follistatin-like 1 regulates renal IL-1beta expression in cisplatin nephrotoxicity. Am.J.Physiol Renal Physiol 299: F1320-F1327

ABBREVIATIONS AND ACRONYMS

AMP-activated protein kinase, AMPK; brain-derived neurotrophic factor, BDNF; brown adipose tissue, BAT; chitinase-3-like protein 1, CHI3L1; c-Jun NH2-terminal kinase, JNK; electrical pulse stimulation, EPS; fatty acid, FA; fibroblast growth factor 21, FGF21; follistatin-like 1, FSTL1; glucagon-like peptide-1, GLP-1; glucose-stimulated insulin secretion, GSIS; IL-1 receptor antagonist, IL-1ra; interleukin, IL; lipopolysaccharide, LPS; peroxisome proliferator-activated receptor, PPAR; peroxisome proliferator-activated receptor gamma coactivator 1α, PGC1α; skeletal muscle, SkM; soluble TNF receptor, sTNFR; toll-like receptor, TLR; white adipose tissue, WAT

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CONTRIBUTION STATEMENT

All authors were responsible for the conception, design and drafting of the manuscript, and approved the final version for publication.

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2.4 The exercise-regulated myokine CHI3L1 stimulates human myocyte proliferation

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Abstract—**Chitinase-3-like protein 1 (CHI3L1) is involved in tissue remodeling and inflammatory processes. Plasma levels are elevated in patients with insulin resistance and T2DM. We recently showed that CHI3L1 and its receptor protease activated receptor 2 (PAR-2) are expressed in skeletal muscle. Activation of PAR-2 by CHI3L1 protects against TNFα-induced inflammation and insulin resistance. However, the effect of exercise on CHI3L1 and PAR-2 signaling remains unknown. The aim of this work was to study the impact of exercise on CHI3L1 production and the effect of CHI3L1/PAR-2 signaling on skeletal muscle growth and repair. Three human exercise studies were used to measure CHI3L1 plasma levels (n = 32). In addition, muscle and adipose tissue CHI3L1 mRNA expression was measured in response to acute and long-term exercise (n = 24). Primary human skeletal muscle cells were differentiated in vitro and electrical pulse stimulation was applied. In addition, myoblasts were incubated with CHI3L1 protein and activation of MAP kinase signaling as well as proliferation was measured. Circulating CHI3L1 levels and muscle CHI3L1 mRNA were increased after acute exercise. In addition, CHI3L1 mRNA expression as well as CHI3L1 secretion was enhanced in electrically stimulated cultured myotubes. Incubation of cultured human myoblasts with CHI3L1 protein leads to a strong activation of p44/42, p38 MAPK and Akt as well as enhanced myoblast proliferation. Conclusion: Our findings suggest that CHI3L1 is a contraction-induced myokine, potentially acting in an autocrine or paracrine fashion to induce myocyte proliferation for restructuring of skeletal muscle after exercise.**

Index Terms— **Skeletal muscle, Exercise, Chitinase-3-like protein 1, PAR-2**

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I. INTRODUCTION

The health-promoting effects of physical activity are well known, and regular exercise has been shown to protect against development of various chronic diseases such as obesity, insulin resistance and type 2 diabetes (T2DM) (1;2). However, our knowledge on underlying mechanisms mediating the protective effects of physical activity remains incomplete.

Skeletal muscle is an important endocrine organ releasing hundreds of myokines upon contraction. These molecules may be partly responsible for the beneficial effects of exercise on health (1). Myokines can affect skeletal muscle metabolism in an auto/paracrine fashion as shown for IL-6 or IL-15 (3). Other myokines such as myostatin, follistatin, decorin, LIF and IL-7 are involved in the regulation and adaptation of skeletal muscle mass in response to work load and regular exercise (3;4). Moreover, regular physical activity has been shown to reduce basal levels of inflammatory markers and myokines, which may be at least partly responsible for the anti-inflammatory effects of exercise. Thus, IL-6, which is released by contracting skeletal muscle, triggers an anti-inflammatory cascade by inducing production of the anti-inflammatory cytokines IL-10, IL-1 receptor antagonist (IL-1ra) and soluble TNF receptor (sTNFR) (1).

Recently, our group identified chitinase-3-like protein 1 (CHI3L1) as a novel myokine (5). CHI3L1, also known as YKL-40, was originally discovered in mouse breast cancer cells (6) and is expressed in many cell types including macrophages, chondrocytes, fibroblasts, vascular smooth muscle cells and endothelial cells (7). We have shown that expression and release of skeletal muscle CHI3L1 is stimulated by pro-inflammatory cytokines such as TNFα and IL-6 (5). Moreover, our data provide evidence that CHI3L1 protects myotubes from TNFα-induced insulin resistance and inflammation by inhibiting NFκB activation. Hence, CHI3L1 could be an auto-protective factor that is induced upon demand to protect skeletal muscle cells from negative impact of TNFα (5). The receptor for CHI3L1, namely PAR-2, is expressed in human and rodent skeletal muscle cells (5;11). Interestingly, it was shown that activation of PAR-2 mediates a proliferative response of murine skeletal myoblasts, which is essential for muscle hypertrophy and regeneration (11). Plasma CHI3L1 levels are elevated in patients with insulin resistance and T2DM (8). However, the physiological role and main sources of circulating CHI3L1 under various conditions remain largely unknown.

We hypothesized that contraction-regulated CHI3L1 production and CHI3L1/PAR-2 signaling may impact skeletal muscle growth and repair. Furthermore, we assessed potential differences of CHI3L1 and PAR-2 expression in skeletal muscle and adipose tissue from control and dysglemic subjects and the effect of exercise intervention. We identify the CHI3L1/PAR-2 axis as a novel player in muscle growth and repair.

II. MATERIAL AND METHODS

Materials: Purified human CHI3L1 was provided by Quidel Corporation (San Diego, CA). Primary human skeletal muscle cells (hSkMC) and supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). Anti-phospho p38 MAPK (Thr180/Tyr182), anti-phospho Akt (Ser473) and anti-phospho p44/42 MAPK (Thr202/Tyr204) were supplied by Cell Signalling Technology (Frankfurt, Germany). Anti-CHI3L1, anti-tubulin and anti-ß-actin were obtained from Abcam (Cambridge, U.K.). Anti-PAR-2 was purchased by Santa Cruz Biotechnology (Heidelberg, Germany). PAR-2 agonist (H-Ser-Leu-Ile-Gly-Lys-Val-NH2) and the specific PAR-2 antagonist GB83 were provided by Bachem (Bubendorf, Switzerland). All other chemicals were of the highest analytical grade commercially available and purchased from Sigma.

Culture of human skeletal muscle cells: Primary hSkMC isolated from 7 healthy Caucasian donors (3 males 16, 41 and 47 of age; 4 females 16, 25, 33 and 37 of age) were supplied as proliferating myoblasts and cultured as described before (5). Myoblasts were seeded in 6-well culture dishes and cultured in growth medium (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. Serum-free medium was used during the complete differentiation period.

Electrical Pulse Stimulation (EPS): Differentiated myotubes were subjected to EPS using a C-Dish in combination with a C-Pace pulse generator (C-Pace 100, IonOptix, Milton, MA, USA), as recently described (9). Cells were stimulated with a frequency of 1 Hz, a pulse duration of 2 ms, and an intensity of 11.5 V for 24 h.

Culture of skeletal muscle cells: Human myoblasts were incubated with recombinant CHI3L1 (50, 100, 250 and 500 ng mL-1) for 10, 30 and 120 min and lysed to generate protein samples for SDS-PAGE. In addition, samples for isolation of RNA were prepared. For proliferation studies, cells were incubated with CHI3L1 (250 ng mL-1) for 24 h with or without GB-83 (100 nmol L-1). The specific PAR-2 inhibitor GB-83 was dissolved in DMSO to produce a stock solution of 100 mmol L-1, which was further diluted in sterile αMEM medium for a final concentration of 100 nmol L-1. All controls for experiments involving the PAR-inhibitor were incubated with 0.1% (v/v) DMSO alone.

Immunoblotting: hSkMC were incubated as indicated and lysed in a buffer containing 50 mmol L-1 HEPES (pH 7.4), 1% TritonX, PhosStop, and Complete Protease Inhibitor cocktail (Roche, Penzberg, Germany). After incubation for 2 h at 4°C, the suspension was centrifuged at 15,000 rpm for 15 min. 7 µg of total cell lysates were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. Then, membranes were blocked with Tris-buffered saline (TBS) containing 0.1% (v/v) Tween-20 and 5% (wt/v) non-fat dry milk and incubated over night with the appropriate antibodies to analyze phosphorylation of p42/44, p38 and Akt. After washing, membranes were incubated with secondary horseradish peroxidase-coupled antibody and processed for enhanced chemi-luminescence detection using Immobilion Western detection reagents (Millipore, Schwalbach, Germany). Signals were visualized and evaluated on a VersaDoc 4000 MP BioRad (Munich, Germany) work station and analyzed by Quantity One analysis software (Version 4.6.7).

RNA isolation and RT-PCR: Cells were lysed with RLT lysisbuffer (Qiagen) and total RNA was isolated using the RNeasy kit from Qiagen (Hilden, Germany). Concentration and purity were measured using a NanoDrop 2000 (Thermo Scientific, Bremen, Germany). 1 µg of RNA was reversely transcribed using Omniscript RT Kit (Qiagen). CHI3L1 (Hs_CHI3L1_1_SG) and PAR-2 (Hs_ F2RL1_1_SG) mRNA expression were measured using pre-designed primers (Quantitect Primer Assay, Qiagen) and GoTaq qPCR Master Mix (Promega, Mannheim, Germany) in a SYBR Green-based quantitative real-time PCR. All analyses were performed in triplicates using a StepOne plus sequence detection system for real-time PCR (Applied Biosystems). Melting curve analyses of the PCR products were used to verify their specificity and identity. Target mRNA levels were normalized relative to ß-actin.

***In vitro* human myoblast proliferation:** To monitor DNA synthesis, 2.500 hSkMC per 15 mm² well were seeded in 96-well culture dishes and allowed to attach for 24 h. Cells were incubated with CHI3L1 alone or in combination with the PAR-2 antagonist GB-83 in the presence of BrdU (10 µM) for 24 h. All controls for experiments involving PAR-2 antagonist GB-83 were incubated with DMSO (0.1% v/v) alone. A chemiluminescent BrdU ELISA (Roche, Mannheim, Germany) was used to determine proliferation according to the manufacturer's protocol. Signals were visualized and evaluated on a LUMI Imager work station (Boehringer, Mannheim, Germany).

***In vitro* human skeletal muscle scratch assay:** Cells were plated in 6-well plates and maintained in growth medium. At 80 to 90% confluence, a scratch was created in the center of the cell monolayer using a sterile plastic pipette tip. Immediately thereafter, the cells were washed with PBS to remove floating cell debris and re-incubated for an additional 24 h with either serum-free αMEM medium (control), growth medium (positive control), or serum-free αMEM medium containing recombinant CHI3L1 (250 ng mL-1). The ability of cells to proliferate and migrate into the wounded area was assessed by comparing phase-contrast micrographs captured at 0, 6 and 24 h of 8 marked points along the wounded area in

each well. The wounded area was calculated by tracing along the border of the wound using ImageJ 1.48v image analysis software, and the percentage wound closure was calculated using the following equation:

$$\frac{[Wounded\ Area\ (0h) - Wounded\ Area\ (6\ or\ 24h)]}{Wound\ Area\ (0h)} \times 100 = \%\ Wound\ Closure$$

Human exercise studies: All participants were informed about the project, procedures and rights before signing an agreement form. The studies were approved by the Regional Committee for Medical and Health Research Ethics, Region Sør-Øst-Norge, Norway (2011/927b).

Human study I - acute endurance exercise: Eight well-trained healthy lean male volunteers (BMI 23.1±0.6 kg/m2) participated in the study. A baseline blood sample was taken before start of the exercise. Subjects cycled at 70% VO2max for 60 min and blood was sampled immediately after the exercise session.

Human study II – acute strength exercise: Eleven well-trained healthy male volunteers (BMI 24.4±0.6 kg/m2) were recruited for the study. A baseline blood sample was taken before start of the exercise. A warm-up before the strength training session was conducted by 5-7 min cycling on stationary bike. The strength training session was composed of 7 types exercises performed in three sets at a load corresponding to 8 RM (the weight that could be lifted maximal 8 times). The exercises were leg press, leg curls, bench press, pull-down, sitting shoulder press, cable-flies, and laying rowing. New set of exercises started every 3 min. Blood was sampled immediately after the exercise session.

Human study III - 12 weeks exercise intervention study: Healthy, sedentary men (40–65 years) were recruited in two groups I) participants with BMI of 23.5 ± 2.0 kg/m2 and normal glucose metabolism (control group, n = 13) and II) overweight participants (BMI 29.0 ± 2.4 kg/m2) with abnormal glucose metabolism (dysglycemic group, n = 11). Abnormal glucose metabolism was defined as fasting glucose ≥ 5.6 mmol L-1 and/or impaired glucose tolerance (2 h serum glucose ≥ 7.8 mmol L-1). Two subjects with a BMI>28 but with normal fasting and 2 h serum glucose levels were excluded. The participants were subjected to combined strength- and endurance-training for 12 weeks as described previously (4;10). A 45 min bicycle session at 70% VO2max was performed before and after the 12 weeks training intervention as an acute exercise challenge (10). Blood and skeletal muscle samples were taken before, directly after, and 2 h after the acute exercise, before as well as after 12 weeks of training. Blood samples were taken by standard antecubital venous puncture, kept on ice until centrifugation and stored at -80°C until further analysis. Biopsies from m. vastus lateralis were taken and immediately transferred to RNA-later (Qiagen, Limburg, Netherlands), kept overnight at 4°C, and transferred to -80°C. For one subject no muscle biopsies were taken at 2 h post exercise before as well as after the intervention. In addition, a single subcutaneous adipose tissue biopsy in the periumbilical region was taken ~30 min after the acute exercise session, before as well as after the intervention period. Subcutaneous abdominal adipose tissue biopsies were obtained from 13 controls and 11 dysglycemic subjects before and after 12 weeks of training, frozen immediately in liquid nitrogen and stored at -80°C until further processing.

Sample analyses: CHI3L1 concentration in plasma was assessed using a human CHI3L1 ELISA (R&D Systems) according to the manufacturer´s protocol. Frozen human muscle biopsies and subcutaneous adipose tissue biopsies were crushed to powder in a liquid nitrogen-cooled mortar using a pestle. QIAzol Lysis Reagent (Qiagen) was added and the samples were homogenized using a TissueRuptor (Qiagen). Total RNA was then isolated by miRNeasy Mini Kit (Qiagen). All mRNA samples were deep-sequenced using the Illumina HiSeq 2000 system with multiplexed design. Illumina HiSeq RTA (real-time analysis) v1.17.21.3 was used for real-time analysis during the sequencing run. Reads passing Illumina's recommended parameters were demultiplexed using CASAVA v1.8.2. For pre-alignment quality checks we used the software FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The mean library size was 44.1 millions unstranded single-ended reads with no difference between groups or time points. No batch effects are present. Reads alignment was done using Tophat v2.0.8 (11), Samtools v0.1.18 (12) and Bowtie v2.1.0 (13) with default settings against the UCSC hg19 annotated transcriptome and genome dated 14th of May 2013. Post-alignment quality checks were done using the Integrative Genome Viewer 2.3 (14;15) and BEDtools v2.19.1 (16). Reads counted by gene feature were performed using the intersection strict mode in HTSeq 0.6.1 (17).

Statistics: Statistical evaluation was done by Student's t-tests for paired or unpaired observations and one-way ANOVA (post hoc Bonferroni multiple comparison test). For differential expression analyses of CHI3L1 and PAR-2 using mRNA sequencing, edgeR v3.4.2 (18) was used to calculate statistical significance. A p-value of <0.05 was considered statistically significant. Normalized gene expression levels are presented in fragments per kB mapped reads (FPKM). Filtering strategies, quality checks and generalized linear model construction were performed in R v3.0.3 following the edgeR developers' recommendations (www.bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf). Correlation analyses were performed in Prism5 (GraphPad, LA Jolla, CA). Pearson's correlations were used on normally distributed parameters. Data are presented as means ± SEM.

III. RESULTUS

Acute exercise increases plasma CHI3L1 levels and skeletal muscle CHI3L1 mRNA expression: To investigate the effect of acute and chronic exercise on circulating CHI3L1 levels, we analyzed samples of three independent human exercise studies. In the first acute

endurance exercise study, the mean baseline level of plasma CHI3L1 was 16.6 ± 1.2 ng mL-1 before the exercise bout (Fig. 1A). Cycling at 70% VO2max for 60 min significantly increased plasma CHI3L1 levels up to 21.1 ± 1.9 ng mL-1 in well-trained healthy male volunteers (Fig. 1A). Similarly, in study II plasma CHI3L1 levels significantly increased immediately after an acute strength exercise session from 19.2 ± 1.1 to 22.3 ± 1.7 ng mL-1 (Fig. 1B). Furthermore, cycling at 70% VO2max for 45 min significantly enhanced circulating CHI3L1 levels in control subjects (20.7 ± 2.3 vs. 23.8 ± 1.2 ng mL-1, study III) (Fig 1C). Overall, acute exercise significantly increased plasma CHI3L1 levels from 19.5 ± 1.1 to 22.8 ± 1.2 ng mL-1 in 32 subjects. The increase of plasma CHI3L1 after acute exercise positively correlates with the increase of plasma IL-6 (Fig. 1I). In line with these results, muscle CHI3L1 mRNA expression was 3.6-fold enhanced immediately after acute endurance exercise followed by a decline after 2 h of rest (Fig. 1E). In contrast, a combined strength and endurance training intervention for 12 weeks had no effect on plasma CHI3L1 levels as well as skeletal muscle CHI3L1 mRNA expression (Fig. 1D and F).

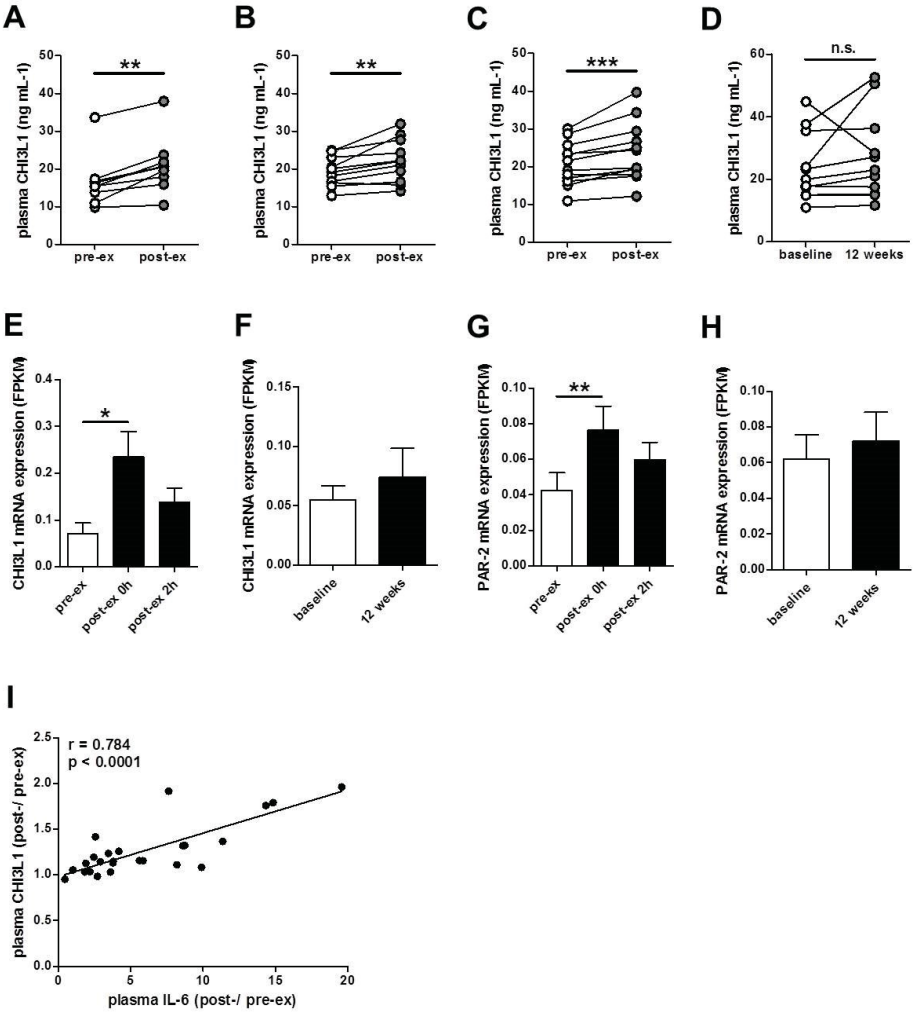

Figure 1: Acute exercise induced CHI3L1 in skeletal muscle and plasma *in vivo.* (**A**) **Human study I - acute endurance exercise:** Nine well-trained healthy lean male volunteers cycled for 1 h at 70% VO_2max and plasma levels of CHI3L1 were measured before (pre-ex; white circles) and immediately after the exercise bout (post-ex; grey circles). CHI3L1 plasma levels are shown individually for each subject. (**B**) **Human study II – acute strength exercise:** Eleven well-trained healthy lean male volunteers completed a strength training session composed of 7 exercises performed in three sets at a load corresponding to 8 RM and plasma levels of CHI3L1 were measured. (**C-H**) **Human study III - 12 weeks exercise intervention study:** Changes in skeletal muscle *CHI3L1* and *PAR-2* expression as well as CHI3L1 plasma concentrations in healthy sedentary men (40–65 years) in response to acute and long-term exercise (n = 13). Skeletal muscle biopsies were obtained before (pre-ex), immediately after (post-ex 0 h) and 2 h after exercise (post-ex 2 h) of 45- min ergometer cycling (70% VO_2max). Biopsies were taken at baseline and after the long-term training period of 12 weeks. Samples were processed for mRNA sequencing and normalized gene expression levels are expressed in fragments per kB mapped reads (FPKM). Bars depict means ± SEM *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ between pre-exercise values and immediately after exercise. (**I**) Linear regression analysis of CHI3L1 increased immediately after exercise and IL-6 increase. The r correlation coefficient and P values were obtained using Pearson's correlation. Statistical evaluation is indicated in the graph (n = 32).

Likewise, skeletal muscle PAR-2 mRNA expression was significantly up-regulated after acute exercise (1.9-fold), whereas 12 weeks training intervention had no effect (Fig. 1G-H).

Electrical pulse stimulation induces CHI3L1 expression and secretion: In addition to our in vivo studies, we monitored CHI3L1 expression and secretion in contracting primary hSkMC. EPS for 24 h significantly increased CHI3L1 mRNA expression levels as well as CHI3L1 protein levels (Fig. 2A and B). CHI3L1 levels in the supernatant increased from 1.1 ± 0.1 to 1.9 ± 0.2 ng mL-1 after EPS (Fig. 2C). As expected, 24 h of EPS also strongly increased IL-6 release and IL-6 mRNA expression (Suppl. 1A and B). Interestingly, the increase of CHI3L1 secretion was positively correlated with the increase of IL-6 secretion after EPS (Fig. 2F). Moreover, PAR-2 mRNA expression as well as PAR-2 protein levels were significantly up-regulated after 24 h EPS (Fig. 2D, E).

CHI3L1 induces MAPK and Akt signaling in a time- and concentration-dependent manner: To investigate potential auto- or paracrine effects of CHI3L1 on signaling in primary hSkMC, we incubated human myoblasts from several donors with purified human CHI3L1 protein and measured the activation of p44/42, p38 MAPK and Akt. CHI3L1 incubation strongly enhanced phosphorylation of all three kinases in a concentration-dependent manner (Fig. 3A-D). 50 ng mL-1 CHI3L1 was sufficient to increase significantly the phosphorylation of p42/44 after 10 min. By further increasing the concentration of CHI3L1 we reached up to 6.5-fold activation of p44/42 compared to untreated cells (Fig. 3A, B). p38 MAPK and Akt phosphorylation was significantly increased by either 100 or 250 ng mL-1 CHI3L1 (2.5-fold and 1.8-fold) respectively (Fig. 3A, C, D). Furthermore, CHI3L1-induced phosphorylation of p44/42, p38 MAPK and Akt was transient and declined after 30 and 120 min.

CHI3L1 induces myoblast proliferation and signaling via PAR-2: Because myoblast proliferation is important in the adaptation of skeletal muscle to altered work load and repair, we assessed the functional consequences of CHI3L1-induced signaling at the level of myoblast proliferation. Incubation with 250 ng mL-1 CHI3L1 for

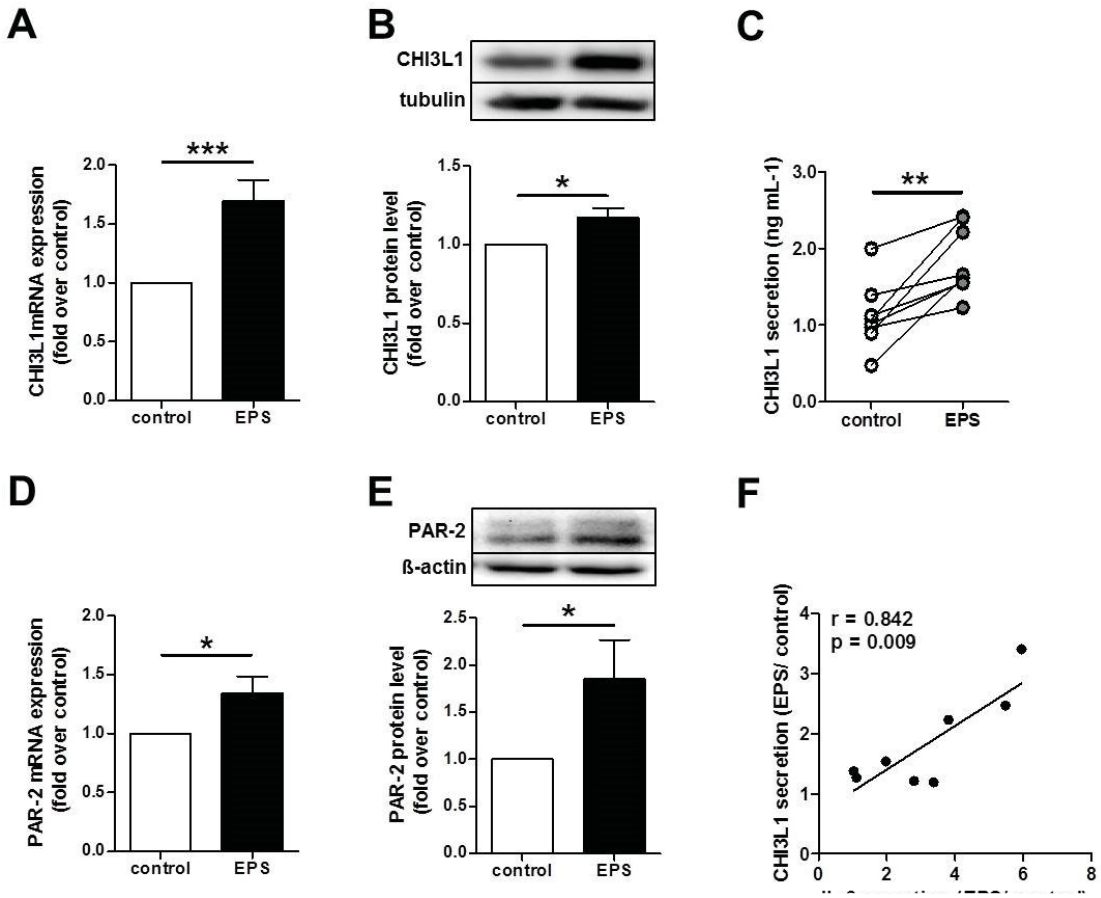

Figure 2: Contraction-regulated expression and secretion of CHI3L1 *in vitro*. (**A**) Comparison of *CHI3L1* mRNA expression in non-contracted hSkMC (control) and after 24 h EPS (EPS). *CHI3L1* mRNA expression was analyzed by real-time PCR and data were normalized to β-actin. (**B**) CHI3L1 protein levels of control and contracted myotubes were analyzed by Western blotting. Representative images are shown. (**C**) Supernatants of non-contracted and contracted myotubes were analyzed by ELISA. CHI3L1 secretion is shown separately for each experiment. (**D**) *PAR-2* mRNA expression was analyzed by real-time PCR and data were normalized to β-actin. (**E**) PAR-2 protein levels of control and contracting myotubes. All data are mean values ± SEM, n=6-8, $*P < 0.05$ and $***P < 0.001$. (**F**) Linear regression analysis of CHI3L1 increase after EPS and IL-6 increase. The r correlation coefficient and P values were obtained using Pearson's correlation. Statistical evaluation is indicated in the graph ($n = 8$).

24 h significantly increased the proliferation of human myoblasts as assessed by BrdU incorporation, cell number counting and total protein content (Fig. 4A, B, D). Incubation with 500 ng mL-1 CHI3L1 as well as 5 % fetal calf serum (FCS, positive control) induced a significant ~1.8-fold increase in cell proliferation within 24 h. Furthermore, pre-incubation with the specific PAR-2 antagonist GB-83 totally inhibited CHI3L1-induced proliferation and phosphorylation of p44/42 and Akt (Fig. 4C, E and F). To further evaluate the importance of PAR-2 for CHI3L1-mediated p44/42 and Akt signaling in human myoblast, we incubated the cells with a specific PAR-2 agonist (SLIGKV) and the endogenous PAR-2 ligand trypsin for 10 min. As expected, the PAR-2 agonist as well as trypsin significantly induced p44/42 and Akt phosphorylation (Suppl. 2A, B).

Scratch wound closure of human skeletal muscle cells is enhanced by CHI3L1 *in vitro*: To further assess the physiological relevance of CHI3L1/PAR-2-induced human myoblast proliferation, which is essential for muscle development, regeneration and repair, we examined the effect of CHI3L1 in a scratch assay in

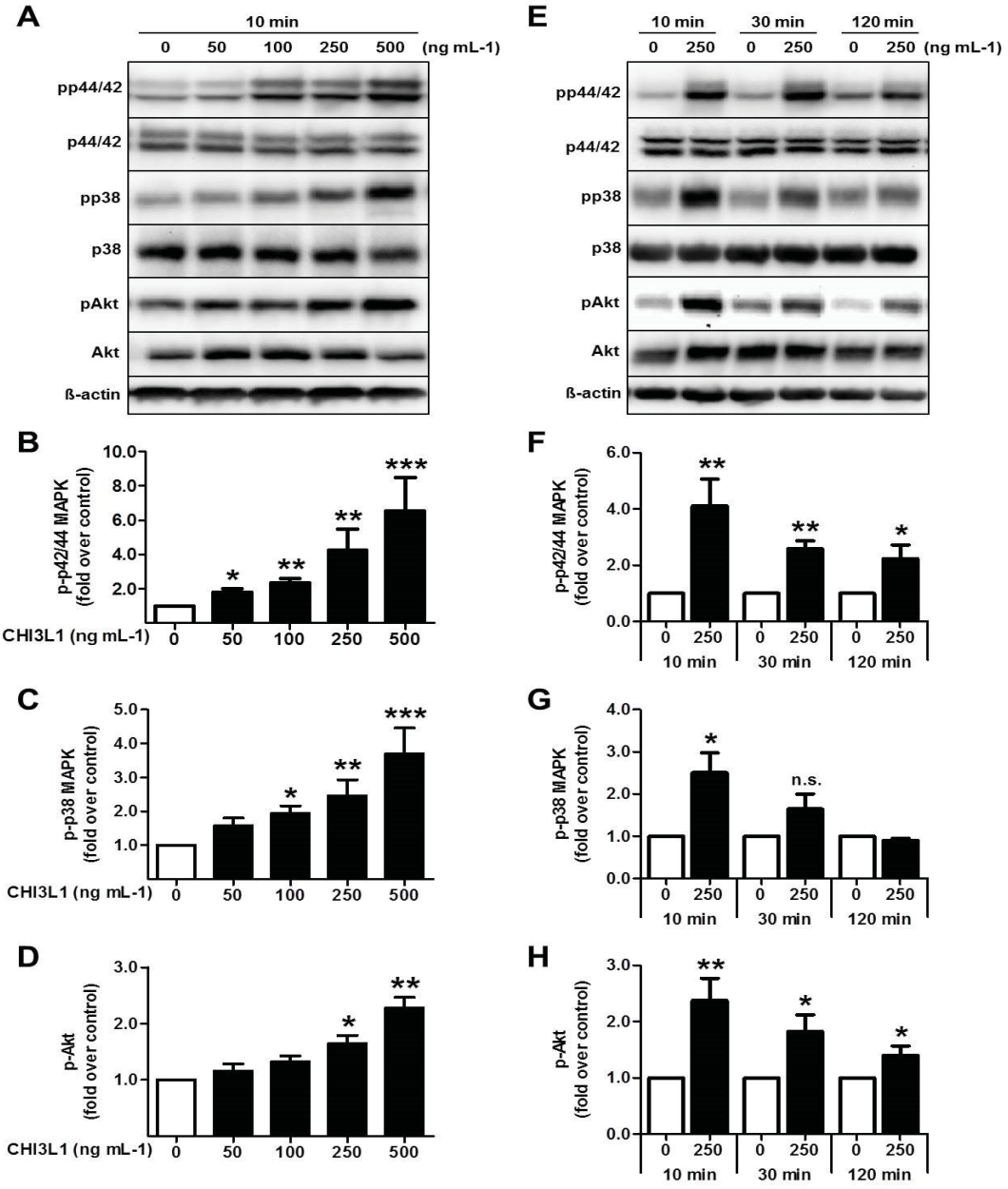

Figure 3: CHI3L1 induced MAPK and Akt signaling. (**A-D**) Undifferentiated hSkMC were incubated for 10 min with different CHI3L1 concentrations (50, 100, 250 and 500 ng mL-1) and phosphorylation of p44/42, p38 and Akt was assessed by Western blotting. (**E-H**) Undifferentiated hSkMC were incubated with 250 ng mL-1 CHI3L1 for different time points (10, 30 and 120 min) and phosphorylation of p44/42, p38 and Akt was assessed by Western blotting. Representative images are shown. Data represent mean values ± SEM, n=5-8, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 between control and CHI3L1-incubated cells.

undifferentiated and differentiated hSkMC. Primary human myoblast were grown to confluence in monolayers, and their ability to proliferate or migrate into and across an injured area of the monolayer was followed up to 6 and 24 h. The percentage wound closure was 63% in myoblasts grown in serum-free αMEM medium after 24 h. Both CHI3L1-incubated cells and cells cultured in growth medium (positive control) significantly reduced the wound size compared to control cells (79% and 96%, respectively) (Fig. 5A and C). Accordingly, the number of cells that migrated into the wounded area was significantly higher in CHI3L1-incubated cells and the positive controls (1.9-fold and 5.1-fold, respectively) as compared to control cells (Fig. 5E).

To assess the role of p44/42 activation in CHI3L1-induced wound healing, we measured the phosphorylation of p44/42 after 6 and 24 h (Suppl. 3A and B). Incubation with both CHI3L1 and growth medium were able to induce p44/42 phosphorylation for up to 6 h, while after 24 h only cells grown in growth medium showed significantly enhanced p44/42 activation (Suppl. 3A and B).

In addition to analyzing undifferentiated myoblasts, migration of cells into a wounded area generated within a monolayer of differentiated myotubes was also investigated, which mimics in vivo skeletal muscle injury to an even greater extent. Primary hSkMC were differentiated for 5 days and their ability to migrate into the injured area was followed for 6 and 24 h. Control cells hardly migrated into the wounded area within 24 h (37% wound closure), whereas both CHI3L1-treated cells and cells grown in growth medium significantly reduced the wound area size by 62% and 86%, respectively (Fig. 5B and D). In line with these results, cells incubated with

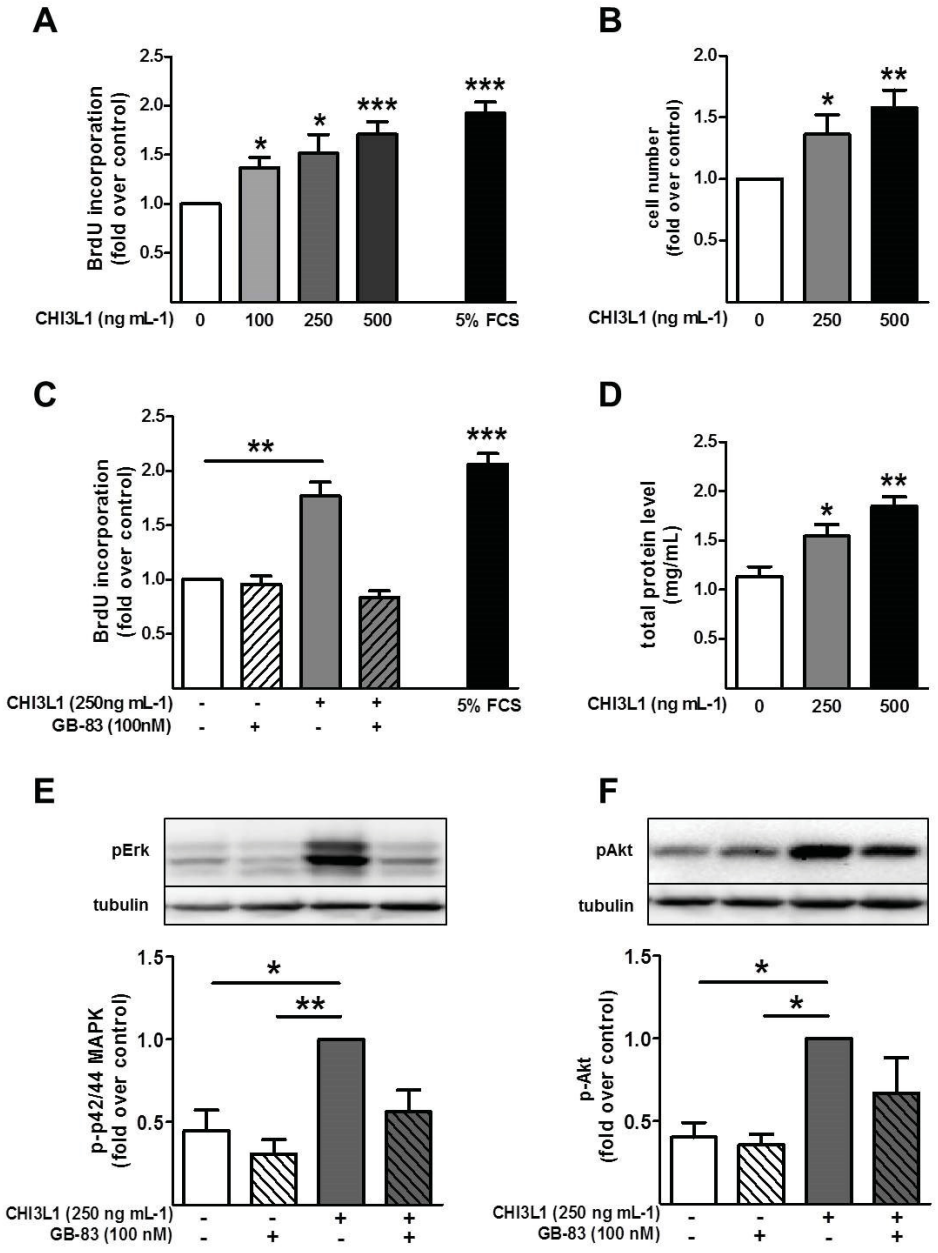

Figure 4: CHI3L1/PAR-2 induced proliferation. (**A**) Undifferentiated hSkMC were incubated with CHI3L1 in different concentrations (as indicated) for 24 h. Proliferation of human myoblasts was determined by measuring the incorporation of BrdU into DNA. (**B**) Human myoblasts were incubated with CHI3L1 (as indicated) for 24 h and cell number was determined. (**C**) Human myoblasts were incubated with CHI3L1 alone or in combination with PAR-2 inhibitor GB-83 for 24 h and proliferation was determined by measuring the incorporation of BrdU into DNA. (**D**) Human myoblasts were incubated with CHI3L1 (as indicated) for 24 h and total protein content was measured by Bradford protein assay. (**E** and **F**) Human myoblasts were incubated with CHI3L1 alone or in combination with PAR-2 inhibitor GB-83 for 10 min and the phosphorylation of p44/42 and Akt was analyzed by Western blotting. Representative images are shown. Data represent mean values ± SEM, n=5-8, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 between control and CHI3L1-treated cells.

CHI3L1 and growth medium, the number of cells that migrated into the wounded area within 24 h was significantly increased compared to control cells (Fig. 5F). Interestingly, CHI3L1 protein levels were ~2-fold higher in cells subjected to the scratch assay compared to control cells (Fig. 5G).

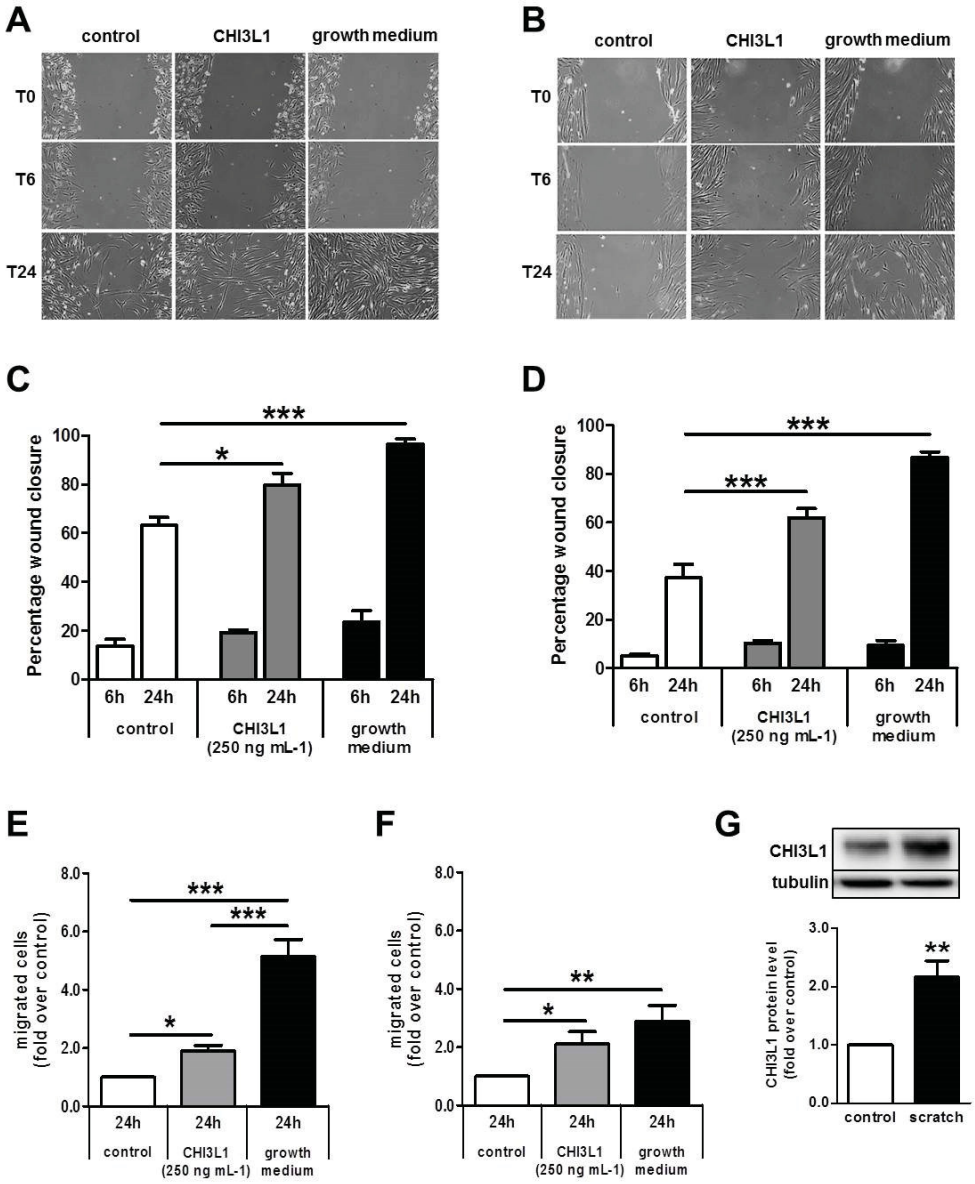

Figure 5: CHI3L1 treatment enhanced cell migration. (**A**) Scratch assay of undifferentiated hSkMC. Wounding (~1 mm wide) was performed after cells became confluent, and cells were incubated with CHI3L1 or growth medium as a positive control. Migration was followed over time (0 h, 6 h and 24 h). (**C**) Quantification represents the percentage wound closure as means ± SEM compared to the original wound (set as 0 %). (**E**) Quantification of cell number migrating into the wounded area after 24 h shown as fold change over control. (**B**) Scratch assay of differentiated hSkMC. Wounding was performed after 5 days of differentiation and cells were incubated with CHI3L1 or growth medium. Migration was followed over time (0 h, 6 h and 24 h). (**D**) Graphic represents the wound width as the means ± SEM of the % of the closure of original wound. (**F**) Quantification represents the number of cells migrating into the wounded area after 24 h. (**G**) Differentiated hSkMC were scratched and CHI3L1 protein level in cell lysates were analyzed by Western blotting. Data represent mean values ± SEM, n=4-6, $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ as indicated.

CHI3L1 has no effect on human skeletal muscle differentiation: Based on the observation that CHI3L1 and PAR-2 were strongly regulated by skeletal muscle differentiation (5), we investigated whether induction of CHI3L1/PAR-2 signaling might have an effect on myogenesis. We incubated primary hSkMC during differentiation with 250 ng mL-1 CHI3L1 and monitored the expression of different markers of skeletal muscle cell differentiation. CHI3L1-incubation had no effect on the expression of selected differentiation marker (Fig. 6A-C). During differentiation, we observed a significant increase of protein levels of insulin receptor ß subunit (IR-ß subunit), total myosin heavy chain (MHC) and a characteristic expression of myogenin in both CHI3L1-

incubated cells as well as in control cells (Fig. 6A-C). Furthermore, myogenin and MHCIIa mRNA expression were unaffected by CHI3L1-incubation (Fig. 6E and F). CHI3L1 protein level declined strongly during differentiation and CHI3L1-incubation had no effect on its own expression (Fig. 6D).

Skeletal muscle CHI3L1 mRNA expre ssion is altered in dysglycemic subjects: Circulating CHI3L1 levels are elevated in patients with chronic inflammation and therefore might be useful as a potential biomarker for insulin resistance and T2DM (19). However, the effect of an abnormal glucose metabolism before onset of T2DM on CHI3L1 expression has not been investigated so far. Thus, we measured skeletal muscle and adipose tissue CHI3L1 mRNA expression as well as plasma levels in dysglycemic subjects and controls. Skeletal muscle CHI3L1 mRNA expression was lower in the dysglycemic group compared to the control group at baseline (P = 0.056). In contrast, adipose tissue CHI3L1 mRNA expression was slightly, but not significantly, higher in dysglycemic subjects at baseline, and after 12 weeks of training (Fig. 7B). Acute induction of skeletal muscle CHI3L1 mRNA expression immediately after exercise tended to be delayed in the dysglycemic group before the 12 weeks exercise intervention (Suppl. 4A). After the intervention period we observed no significant difference between the groups with respect to acute exercise-induced CHI3L1 mRNA expression in skeletal muscle (Suppl. 4C).

However, no difference in basal circulating CHI3L1 levels was observed between the two groups (Fig. 7C). In addition, plasma CHI3L1 levels significantly increased in the dysglycemic group in response to acute exercise to the same extent as in the control group (Suppl. 4B). Skeletal muscle PAR-2 mRNA expression was slightly but not significantly lower in the dysglycemic group as compared to control group (Fig. 7D). Interestingly, PAR-2 mRNA expression was not detectable in biopsies from subcutaneous adipose tissue obtained from the periumbilical region in any of the subjects.

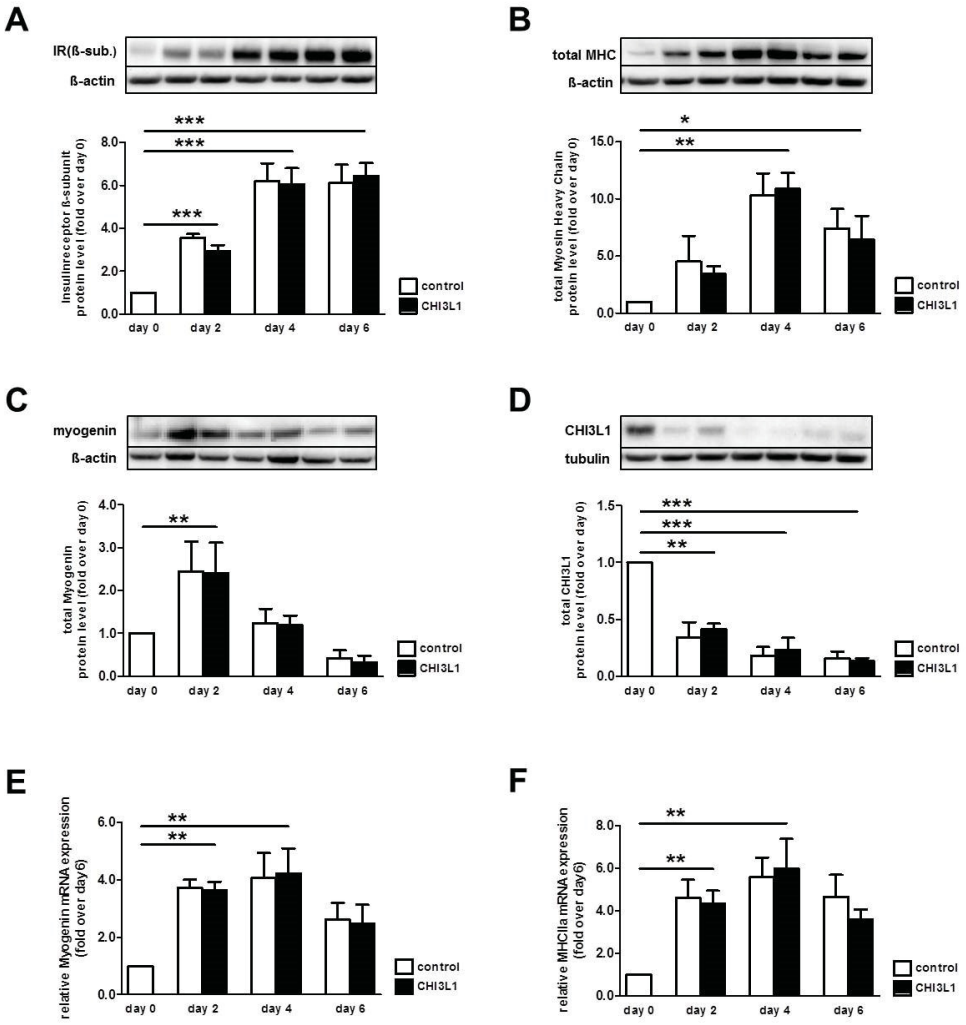

Figure 6: CHI3L1 incubation had no effect on myogenesis. Primary hSkMC were differentiated for the indicated times with or without CHI3L1 (250 ng mL-1) and protein abundance of insulin receptor ß-subunit (**A**), total myosin heavy chain (**B**), myogenin (**C**), CHI3L1 (**D**), and mRNA expression of *myogenin* (**E**) and *MHCIIa* (**F**) was measured. Myogenin and MHCIIa mRNA expression was determined by real time-PCR and normalized to β-actin expression. Data represent mean values ± SEM, n=3, **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 as indicated.

IV. DISCUSSION

In the present study we show that acute endurance and strength exercise increase circulating CHI3L1 levels. These observations are in accordance with a recent study showing that marathon running increased the levels of circulating CHI3L1 (20). However, Johansen et al. observed no effect on circulating CHI3L1 levels after physical exercise at moderate intensity for 20 min (21). It is possible that a higher intensity and/or a longer duration

are necessary for induction of circulating CHI3L1 levels in response to acute exercise. Interestingly, we observed that the increase of plasma CHI3L1 levels positively correlates with the increase of plasma IL-6. Recently, we reported that IL-6 was able to induce CHI3L1 production in human skeletal muscle cells (5). Moreover, Nielsen et al. observed that IL-6 but not TNFα infusion increased plasma levels of CHI3L1 in healthy young men (22). Thus, it is possible that the increase of IL-6 after exercise indirectly enhances skeletal muscle-derived CHI3L1 release. Further experiments are necessary to elaborate the exact molecular mechanism and pathways promoting enhanced secretion of skeletal muscle-derived CHI3L1 during exercise. Overall, we measured an average plasma CHI3L1 concentration of 21 ng mL-1 with a maximum value of 52 ng mL-1. There are no established normal ranges for circulating CHI3L1 in healthy persons. The two most extensive studies of healthy subjects both agree on a maximum serum value of about 125 ng mL-1 (23;24). Several studies have reported significantly higher CHI3L1 values in serum compared to plasma (25). The difference is probably caused by a small release of CHI3L1 from activated neutrophils during coagulation (26). Moreover, also different immunoassays can give different CHI3L1 levels. We also investigated a potential regulation of CHI3L1 mRNA expression in skeletal muscle of subjects participating in acute and chronic exercise programs. Our results indicate that CHI3L1 mRNA expression is enhanced after acute exercise, whereas 12 weeks of training do not alter CHI3L1 mRNA expression in control subjects. To our knowledge there is no other study monitoring short- or long-term effects of exercise on skeletal muscle CHI3L1 mRNA expression. To validate further CHI3L1 as a contraction-induced myokine, we monitored its regulation in vitro by our well-established EPS model for human skeletal muscle cell contraction (9). The finding that CHI3L1 is released from differentiated contracting myotubes in culture supports the notion that CHI3L1 is indeed a contraction-regulated myokine.

Skeletal muscle hypertrophy and regeneration depend on the addition of new myonuclei by activation, proliferation, and fusion of satellite cells to adult muscle fibers (27). Molecules released from skeletal muscle itself have been shown to increase satellite cell proliferation (28). Here we show that physiological concentrations of CHI3L1 induced p44/42, p38 MAPK and Akt signaling, promoting enhanced myoblast proliferation. Recently, we showed that CHI3L1 signals via PAR-2 in human skeletal muscle cells (5). Indeed, using a specific PAR-2 antagonist we were able to abolish completely CHI3L1-induced p44/42, p38 MAPK and Akt activation. In line with our results, Bara et al. demonstrated that CHI3L1 increased bronchial smooth muscle cell proliferation and migration through PAR-2-, Akt-, p44/42-, and p38-dependent mechanisms (29). As activation of p44/42 has

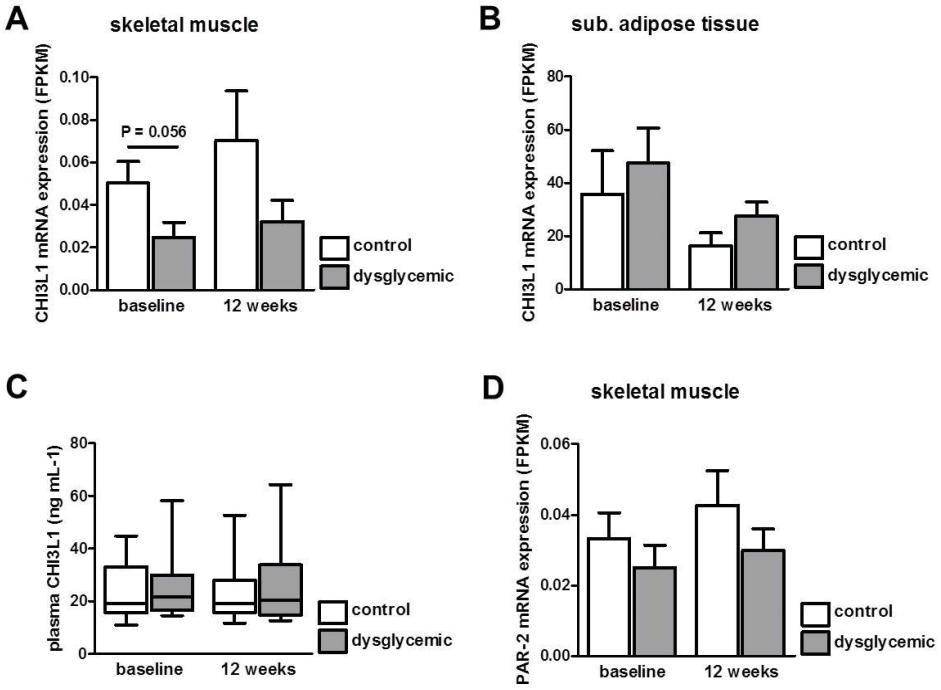

Figure 7: Long-term exercise induced CHI3L1 differently in skeletal muscle and adipose tissue of healthy controls and dysglycemic subjects. (**A** and **B**) Changes of *CHI3L1* expression in *m. vastus lateralis* and subcutaneous fat in healthy (control; white bars) and dysglycemic men (dysglycemic; grey bars) in response to chronic exercise. Samples were obtained before (baseline) and after the training period of 12 weeks. (**C**) CHI3L1 plasma levels were analyzed by ELISA before and after 12 weeks of training in controls and dysglycemic subjects. (**D**) Changes of *PAR-2* mRNA expression in skeletal muscle of controls and dysglycemic subjects were analyzed. Samples were processed for mRNA expression analysis by mRNA sequencing and normalized gene expression levels are presented in fragments per kB mapped reads (FPKM). n=24, **P* < 0.05 as indicated.

been closely associated with cellular proliferation, protein synthesis, and wound healing (30), it is tempting to speculate that the p44/42 pathway is critically involved in mediating enhanced myoblast proliferation and improved skeletal muscle wound healing induced by CHI3L1. These observations are in accordance with previous studies showing that activation of PAR-2 mediates proliferative responses in murine skeletal muscle cells (30;31). In addition, we showed that CHI3L1 is induced by inflammation and counteracts TNFα-mediated inflammation and insulin resistance in hSkMC (5). It is well established that muscle repair and regeneration following acute muscle injury involves a tissue-remodeling, growth-promoting, local inflammation (1). The initial inflammatory response is required for a positive outcome of muscle repair (32). Hence, it is possible that CHI3L1 operates locally in skeletal muscle tissue to induce myoblast proliferation and reduce inflammation, hereby providing a feedback loop for skeletal muscle to regulate its own growth and regeneration for adaptation to exercise.

Muscle regeneration has not been studied in detail in obese and type 2 diabetic humans, and mechanisms leading to decreased muscle regeneration in obesity remain elusive. Here we show that CHI3L1 expression is slightly reduced in skeletal muscle of dysglycemic subjects, hence providing a possible explanation how disturbed glucose metabolism negatively impacts skeletal muscle maintenance.

Notably, CHI3L1 serum levels are increased in patients with insulin resistance and T2DM independent of BMI (8;19;33). However, the main source causing the systemic elevated CHI3L1 levels in this pathological condition has not been identified so far. Interestingly, we observed a higher CHI3L1 mRNA expression in the adipose tissue compared to the skeletal muscle. This observation might be related to an increasing accumulation of macrophages within adipose tissue occurring in the early development of obesity (34). Iwata et al. has shown that CHI3L1 is exclusively secreted from macrophages among the cells of the stromal vascular fraction (SVF) obtained from human adipose tissue (35). Therefore, the adipose tissue could be responsible for the chronic systemic elevated CHI3L1 levels in pathological conditions, whereas acute exercise-mediated induction of CHI3L1 might be acting within the skeletal muscle tissue to induce myoblast proliferation and reduce inflammation.

In conclusion, CHI3L1 is produced during exercise and might contribute to muscle adaption following exercise by stimulating myoblast proliferation, a process important for muscle regeneration. In consequence, we hypothesize that the function of CHI3L1, as a contraction-induced myokine, is that of a mitogenic growth factor affecting myoblasts in an auto- or paracrine fashion.

V. REFERENCES

1. Eckardt,K, Gorgens,SW, Raschke,S, Eckel,J: Myokines in insulin resistance and type 2 diabetes. Diabetologia 57:1087-1099, 2014
2. Knowler,WC, Barrett-Connor,E, Fowler,SE, Hamman,RF, Lachin,JM, Walker,EA, Nathan,DM: Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med 346:393-403, 2002
3. Pedersen,BK, Febbraio,MA: Muscles, exercise and obesity: skeletal muscle as a secretory organ. Nat Rev Endocrinol 8:457-465, 2012
4. Kanzleiter,T, Rath,M, Gorgens,SW, Jensen,J, Tangen,DS, Kolnes,AJ, Kolnes,KJ, Lee,S, Eckel,J, Schurmann,A, Eckardt,K: The myokine decorin is regulated by contraction and involved in muscle hypertrophy. Biochem Biophys Res Commun 450:1089-1094, 2014
5. Gorgens,SW, Eckardt,K, Elsen,M, Tennagels,N, Eckel,J: Chitinase-3-like protein 1 protects skeletal muscle from TNFalpha-induced inflammation and insulin resistance. Biochem J 459:479-488, 2014
6. Morrison,BW, Leder,P: neu and ras initiate murine mammary tumors that share genetic markers generally absent in c-myc and int-2-initiated tumors. Oncogene 9:3417-3426, 1994
7. Lee,CG, Da Silva,CA, Dela Cruz,CS, Ahangari,F, Ma,B, Kang,MJ, He,CH, Takyar,S, Elias,JA: Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. Annu Rev Physiol 73:479-501, 2011
8. Nielsen,AR, Erikstrup,C, Johansen,JS, Fischer,CP, Plomgaard,P, Krogh-Madsen,R, Taudorf,S, Lindegaard,B, Pedersen,BK: Plasma YKL-40: a BMI-independent marker of type 2 diabetes. Diabetes 57:3078-3082, 2008
9. Lambernd,S, Taube,A, Schober,A, Platzbecker,B, Gorgens,SW, Schlich,R, Jeruschke,K, Weiss,J, Eckardt,K, Eckel,J: Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways. Diabetologia 55:1128-1139, 2012
10. Norheim,F, Hjorth,M, Langleite,TM, Lee,S, Holen,T, Bindesboll,C, Stadheim,HK, Gulseth,HL, Birkeland,KI, Kielland,A, Jensen,J, Dalen,KT, Drevon,CA: Regulation of angiopoietin-like protein 4 production during and after exercise. Physiol Rep 2: 2014
11. Kim,D, Pertea,G, Trapnell,C, Pimentel,H, Kelley,R, Salzberg,SL: TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol 14:R36, 2013
12. Li,H, Handsaker,B, Wysoker,A, Fennell,T, Ruan,J, Homer,N, Marth,G, Abecasis,G, Durbin,R: The Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078-2079, 2009
13. Langmead,B, Trapnell,C, Pop,M, Salzberg,SL: Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10:R25, 2009
14. Thorvaldsdottir,H, Robinson,JT, Mesirov,JP: Integrative Genomics Viewer (IGV): high-

performance genomics data visualization and exploration. Brief Bioinform 14:178-192, 2013
15. Robinson,JT, Thorvaldsdottir,H, Winckler,W, Guttman,M, Lander,ES, Getz,G, Mesirov,JP: Integrative genomics viewer. Nat Biotechnol 29:24-26, 2011
16. Quinlan,AR, Hall,IM: BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 26:841-842, 2010
17. Anders,S, Pyl,PT, Huber,W: HTSeq-a Python framework to work with high-throughput sequencing data. Bioinformatics 2014
18. Robinson,MD, McCarthy,DJ, Smyth,GK: edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139-140, 2010
19. Syed Ikmal,SI, Zaman,HH, Vethakkan,SR, Wan Ahmad,WA: Potential biomarkers of insulin resistance and atherosclerosis in type 2 diabetes mellitus patients with coronary artery disease. Int J Endocrinol 2013:698567, 2013
20. Vuolteenaho,K, Leppanen,T, Kekkonen,R, Korpela,R, Moilanen,E: Running a marathon induces changes in adipokine levels and in markers of cartilage degradation - novel role for resistin. PLoS One 9:e110481, 2014
21. Johansen,JS, Lottenburger,T, Nielsen,HJ, Jensen,JE, Svendsen,MN, Kollerup,G, Christensen,IJ: Diurnal, weekly, and long-time variation in serum concentrations of YKL-40 in healthy subjects. Cancer Epidemiol Biomarkers Prev 17:2603-2608, 2008
22. Nielsen,AR, Plomgaard,P, Krabbe,KS, Johansen,JS, Pedersen,BK: IL-6, but not TNF-alpha, increases plasma YKL-40 in human subjects. Cytokine 55:152-155, 2011
23. Mygind,ND, Iversen,K, Kober,L, Goetze,JP, Nielsen,H, Boesgaard,S, Bay,M, Johansen,JS, Nielsen,OW, Kirk,V, Kastrup,J: The inflammatory biomarker YKL-40 at admission is a strong predictor of overall mortality. J Intern Med 273:205-216, 2013
24. Garnero,P, Piperno,M, Gineyts,E, Christgau,S, Delmas,PD, Vignon,E: Cross sectional evaluation of biochemical markers of bone, cartilage, and synovial tissue metabolism in patients with knee osteoarthritis: relations with disease activity and joint damage. Ann Rheum Dis 60:619-626, 2001
25. Hogdall,EV, Johansen,JS, Kjaer,SK, Price,PA, Blaakjaer,J, Hogdall,CK: Stability of YKL-40 concentration in blood samples. Scand J Clin Lab Invest 60:247-251, 2000
26. Johansen,JS: Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue remodelling, fibroses and cancer. Dan Med Bull 53:172-209, 2006
27. Hawley,JA, Hargreaves,M, Joyner,MJ, Zierath,JR: Integrative Biology of Exercise. Cell 159:738-749, 2014
28. Broholm,C, Laye,MJ, Brandt,C, Vadalasetty,R, Pilegaard,H, Pedersen,BK, Scheele,C: LIF is a contraction-induced myokine stimulating human myocyte proliferation. J Appl Physiol (1985) 111:251-259, 2011
29. Bara,I, Ozier,A, Girodet,PO, Carvalho,G, Cattiaux,J, Begueret,H, Thumerel,M, Ousova,O, Kolbeck,R, Coyle,AJ, Woods,J, Tunon de Lara,JM, Marthan,R, Berger,P: Role of YKL-40 in bronchial smooth muscle remodeling in asthma. Am J Respir Crit Care Med 185:715-722, 2012
30. Borensztajn,K, Stiekema,J, Nijmeijer,S, Reitsma,PH, Peppelenbosch,MP, Spek,CA: Factor Xa stimulates proinflammatory and profibrotic responses in fibroblasts via protease-activated receptor-2 activation. Am J Pathol 172:309-320, 2008
31. Chinni,C, de Niese,MR, Jenkins,AL, Pike,RN, Bottomley,SP, Mackie,EJ: Protease-activated receptor-2 mediates proliferative responses in skeletal myoblasts. J Cell Sci 113 Pt 24:4427-4433, 2000
32. Paulsen,G, Mikkelsen,UR, Raastad,T, Peake,JM: Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise? Exerc Immunol Rev 18:42-97, 2012
33. Rathcke,CN, Johansen,JS, Vestergaard,H: YKL-40, a biomarker of inflammation, is elevated in patients with type 2 diabetes and is related to insulin resistance. Inflamm Res 55:53-59, 2006
34. Sell,H, Habich,C, Eckel,J: Adaptive immunity in obesity and insulin resistance. Nat Rev Endocrinol 8:709-716, 2012
35. Iwata,T, Kuwajima,M, Sukeno,A, Ishimaru,N, Hayashi,Y, Wabitsch,M, Mizusawa,N, Itakura,M, Yoshimoto,K: YKL-40 secreted from adipose tissue inhibits degradation of type I collagen. Biochem Biophys Res Commun 388:511-516, 2009

ABBREVIATIONS AND ACRONYMS

CHI3L1, Chitinase-3-like protein 1; EPS, electrical pulse stimulation; MAPK, Mitogen-activated protein kinase; MHC, myosin heavy chain; PAR-2, protease activated receptor 2; hSkMC, human skeletal muscle cells; TNFα, tumor necrosis factor alpha; IL, interleukin

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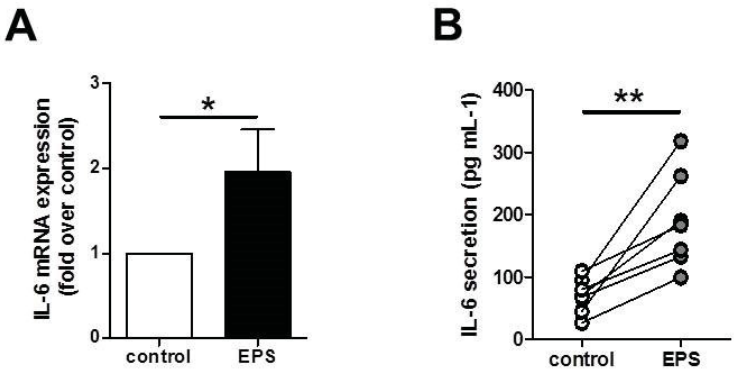
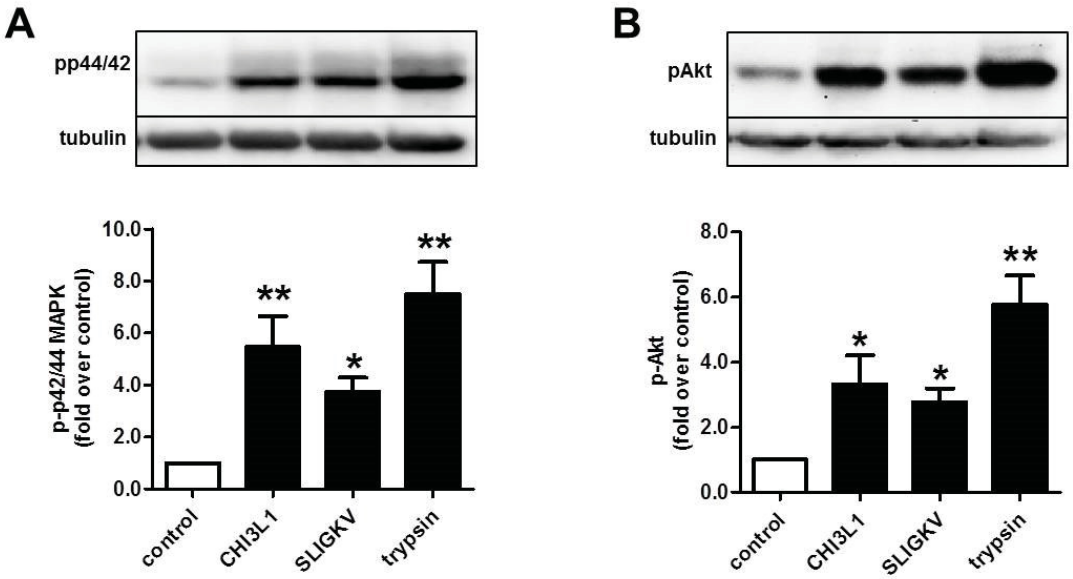
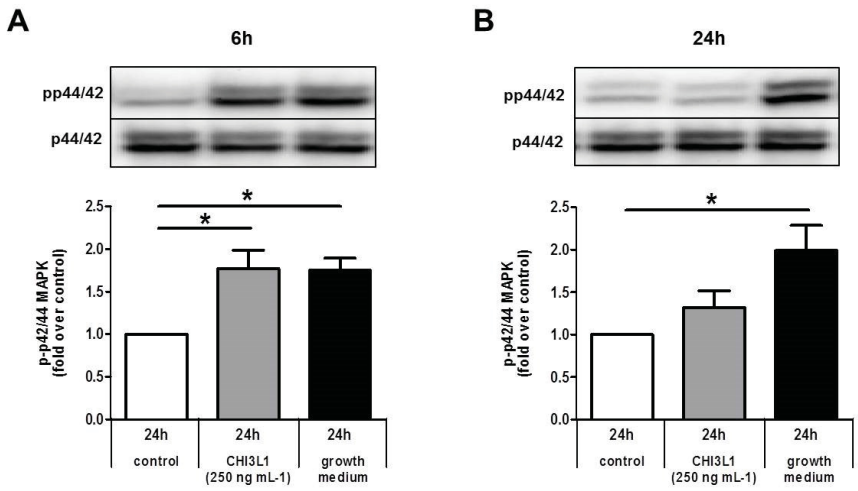
Supplement



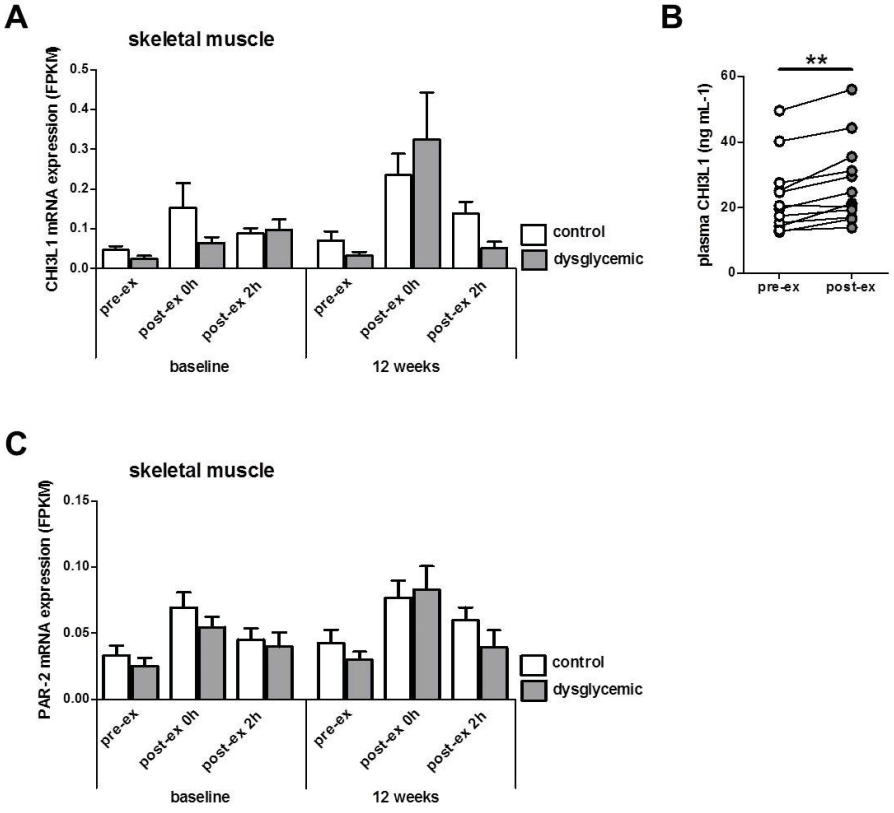
Supplement Figure 1: Contraction-regulated expression and secretion of IL-6 *in vitro*. (**A**) Comparison of IL-6 mRNA expression in non-contracted hSkMC (control) and after 24 h EPS (EPS). IL-6 mRNA expression was analyzed by real-time PCR and data were normalized to β-actin. (**B**) Supernatants of non-contracted (white circles) and contracted myotubes (grey circles) were analyzed by ELISA. IL-6 secretion is shown separately for each experiment. Data represent mean values ± SEM, n=4-7, **P* < 0.05 and ***P* < 0.01 as indicated.



Supplement Figure 2: CHI3L1, SLIGKV and trypsin-induced MAPK and Akt signaling. (**A, B**) Undifferentiated hSkMC were incubated for 10 min with CHI3L1 (250 ng ml-1), SLIGKV (200 µmol/L) and trypsin (50 nmol/L) and phosphorylation of p44/42 and Akt were assessed by Western blotting. Representative images are shown. Data represent mean values ± SEM, n=4-6, **P* < 0.05 and ***P* < 0.01 vs control.



Supplement Figure 3: CHI3L1 and growth medium enhanced MAPK signaling. (**A, B**) Undifferentiated hSkMC were cultured in growth medium or with CHI3L1 (250 ng mL-1) for 6 or 24 h and phosphorylation of p44/42 was analyzed by Western Blot. Representative images are shown. Data represent mean values ± SEM, n=3, **P* < 0.05 as indicated.



Supplement Figure 4: Human study III - 12 weeks exercise intervention study: Changes in skeletal muscle *CHI3L1* and *PAR-2* expression in sedentary healthy controls and dysglycemic subjects in response to acute and chronic exercise. (**A, C**) Skeletal muscle biopsies were obtained before (pre-ex), immediately after (post-ex 0 h) and 2 h after exercise (post-ex 2 h) of 45- min ergometer cycling (70% VO_2max) both at baseline and after 12 weeks of training (12 weeks). Samples were processed for mRNA sequencing and normalized gene expression levels are in fragments per kB mapped reads (FPKM). Bars depict means ± SEM. (**B**) CHI3L1 plasma levels of the dysglycemic group were measured by ELISA before (pre-ex) and after exercise (post-ex) and are shown separately for each subject (n=11, ***P* < 0.01 as indicated).

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	Performed experiments: 80%
	Analysed data: 75%
	Contributed to discussion: 90%
	Wrote the manuscript: 90%
Author:	1st author

2.5 Exercise and regulation of adipokine and myokine production

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Abstract— **Skeletal muscle and white adipose tissue are the largest organs in the human body and both tissues act as endocrine organs capable of secreting many bioactive molecules. There has been some confusion about nomenclature and we suggest that the name myokine be restricted to a protein or molecule secreted from myocytes, whereas the term adipokine should be used to describe proteins and molecules secreted from adipocytes. In fact, many myokines are also produced by adipocytes and we propose to name them adipo-myokines. Many adipo-myokines produced by skeletal muscle or adipose tissue are influenced by exercise. Therefore, it is likely that adipo-myokines may contribute in the mediation of the health benefits of exercise and physical inactivity probably leads to an altered adipo-myokine profile, which could provide a potential mechanism for the association between sedentary behavior and many chronic diseases. Within this review, we evaluate the effects of acute and chronic exercise on myokine, adipokine and adipo-myokine production. By using the adipo-myokine concept and including both skeletal muscle and adipose tissue, an attempt is made to gain a global view on the beneficial effects of different exercise programs and the underlying pathways.**

Index Terms— **Adipokines, Myokines, Adipo-myokines, Exercise, Skeletal muscle, Adipose tissue**

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I. INTRODUCTION

Skeletal muscle plays a key role in postural control, locomotion and other physiological tasks requiring mechanical activity based on muscle fiber contraction. As the largest organ in the body, the energy production and consumption by skeletal muscle is fundamental for metabolic control and homeostasis. More recently, skeletal muscle has gained considerable interest as an endocrine organ and the release of myokines from contracting muscle is assumed to be at least partly responsible for the health-promoting effects of physical activity[1], that protects against major chronic, low-grade inflammatory diseases like type 2 diabetes, insulin resistance, the metabolic syndrome and many others. Myokines are part of a complex network of inter-organ communication and exert both auto-/paracrine and endocrine effects[1]. The most extensively studied myokine is IL-6, which is profoundly up-regulated during acute exercise and assumed to play a key role in the anti-inflammatory effect of acute exercise[2]. However, it is now evident that skeletal muscle secretes hundreds of myokines, with many of them being regulated by muscle contraction[3]. At present, for the majority of these molecules the biological function has remained elusive. An additional complexity results from the broad spectrum of physical activity used in lifestyle intervention studies and profound differences in the exercise training programs. The **textbox** summarizes some of the major acute and chronic exercise training programs that were used in the studies considered for this review.

In addition to being the paradigm myokine, IL-6 is a pro-inflammatory adipo-cytokine and produced by adipose tissue in substantial amounts. In fact, many myokines are also produced by adipose tissue and we suggested to call them adipo-myokines[4] (see **Figure 1**). These molecules are mediators of exercise and mediators of inflammation, most likely exerting as of yet incompletely understood time- and concentration-dependent functions. To gain a more comprehensive view on the molecular pathways involved in physical activity, studies of myokines, adipokines and adipo-myokines, including those produced by adipose tissue are required. In fact, exercise training has been reported to reduce central adiposity independent of overall weight changes[5], and this may represent an additional mechanism of the anti-inflammatory action of acute exercise. Unfortunately, only very limited information is available regarding direct effects of exercise on adipokine and adipo-myokine production.

In this review, by focusing on the adipo-myokine concept and including both skeletal muscle and adipose tissue, an attempt is made to gain a more global view of the beneficial effects of different exercise programs and the underlying pathways. The general approach for selecting published studies is outlined in **Figure 2**. Thus, we only considered human studies and molecules that are detectable in the circulation. This does not exclude auto-/paracrine effects of adipo-myokines, but emphasizes the crosstalk between fat and muscle and the potential mediators of this process.

II. SKELETAL MUSCLE AS THE SOURCE OF MYOKINES AND ADIPO-MYOKINES AFTER ACUTE AND CHRONIC EXERCISE

Interleukin 6 (IL-6) is the prototype of an adipo-myokine meaning that it is expressed and released by both skeletal muscle and adipose tissue[4]. It acts in an auto-/paracrine fashion within skeletal muscle and endocrine in a hormone-like fashion to mediate metabolic and anti-inflammatory effects[6]. IL-6 has been shown to trigger an anti-inflammatory cascade by promoting the induction of anti-inflammatory factors, such as IL-10 and IL-1ra (IL-1 receptor agonist), and to inhibit the production of the pro-inflammatory cytokines IL-1ß and TNFα[1]. Moreover, IL-6 plays a role in hypertrophic muscle growth[7]. Skeletal muscle releases IL-6 into the circulation in response to acute muscle contractions. The increase of circulating IL-6 in response to acute exercise occurs in an exponential manner and the maximum is reached at the end of the exercise session[6]. The magnitude by which IL-6 levels increase is related to the type of exercise, its duration, intensity, and the amount of muscle mass engaged in the exercise but is not affected by muscle damage[6;8;9]. Moreover, pre-exercise muscle glycogen stores impact on the amount of IL-6 released during exercise[10;11]. The most pronounced increase of plasma IL-6 has been observed with running, which involves several large muscle groups[8]. Based on data from several epidemiological studies, a negative association between the extent of regular physical activity and the basal plasma IL-6 levels have been described; basal plasma IL-6 concentration is more closely associated with physical inactivity than other cytokines associated with the metabolic syndrome[8]. However, data on the effect of regular exercise on circulating IL-6 concentrations are more controversial. Aerobic training for 10 months with overweight elderly subjects (age ≥ 64) reduced basal plasma levels of IL-6, whereas strength training had no effect[12]. A study with severely obese humans that used a combination of hypocaloric diet and regular physical activity for 15 weeks, reported decreased basal plasma levels of IL-6 and IL-6 mRNA expression in skeletal muscle along with a 13% reduction of body weight [13]. However, other studies observed no changes in circulating IL-6 concentrations in response to chronic exercise[14;15]. In summary, IL-6 is released from contracting human skeletal muscle, and exercise-induced IL-6 has metabolic effects in human, and those include effects on insulin-stimulated glucose disposal and fatty acid oxidation.

Another member of the IL-6 superfamily has been classified as a myokine, namely leukemia inhibitory factor (LIF). LIF has multiple biological functions such as induction of satellite cell proliferation, which is essential for muscle hypertrophy and regeneration after muscle damage[16]. Broholm et al. reported in young healthy volunteers that LIF mRNA expression in skeletal muscle increased following acute aerobic exercise (4.5-fold)[17] and heavy resistance exercise (9–fold)[16]. However, LIF protein level in skeletal muscle tissue was not changed[17], and LIF could not be detected in plasma in the same study[16]. Due to the paucity of data, future studies should address regulation of plasma LIF levels during acute or chronic exercise to determine whether the increased LIF mRNA expression in the muscle also is reflected in the circulation.

More recently, interleukin 7 (IL-7) was described as a novel exercise-regulated myokine. It belongs to the interleukin superfamily 2 and is required for T-cell and B-cell development but its relevance in non-immune cells has not been sufficiently explored. A study by Haugen et al. has shown that IL-7 is expressed and secreted by primary human skeletal muscle cells[18]. *In vitro* experiments suggested that IL-7 increased migration of satellite cells without affecting their proliferation[18]. There are only a few studies which have examined the effect of acute or chronic exercise on circulating IL-7 concentrations. Andersson et al. observed a robust increase in plasma IL-7 levels following a 90-min soccer games in elite female soccer players[19]. In young healthy men, acute resistance exercise increased plasma IL-7 levels 30 min post-exercise, while 12 weeks of chronic resistance training had no effect on circulating IL-7 concentrations[20]. Skeletal muscle mRNA expression of IL-7 was reported to be increased after 11 weeks of strength training in healthy males[18]. It has been concluded that IL-7 is an exercise-regulated myokine and that it may play a role in the regulation of muscle cell development.

Another member of the interleukin superfamily 2 is interleukin 15 (IL-15). The potential effect of exercise on IL-15 expression and secretion by skeletal muscle is still unclear. In marathon runners, Nieman et al. found no alteration of IL-15 mRNA expression in skeletal muscle immediately after a 3-hour treadmill run[21]. Similarly, another study performed with healthy, physically active men reported no difference in skeletal muscle IL-15 mRNA expression and circulating IL-15 after 3 hours of cycling[22]. Furthermore, chronic endurance training for 12 weeks had no effect on plasma IL-15 levels and skeletal muscle mRNA expression. Interestingly, increased IL-15 protein content was found in skeletal muscle in this study[22]. In contrast, Tamura et al. have shown that 30-min treadmill running at 70% of maximum heart rate promoted a significant increase in circulating IL-15 levels in untrained healthy young men[23]. Another study reported increased plasma IL-15 levels directly after acute resistance exercise in young, healthy but previously inactive volunteers, whereas no change of plasma IL-15 concentrations was observed after chronic resistance training for 10 weeks[24]. In support of these results, a study by Nielsen et al. described increased skeletal muscle mRNA expression of IL-15 after acute heavy resistance exercise in healthy, normally active men. However, no changes in skeletal muscle IL-15 protein content or plasma IL-15 were found [25]. Studies investigating the effect of exercise on IL-15 in overweight and obese humans are still missing. So far, it remains an open question which type of exercise is able to impact on IL-15 and how important are duration and intensity of the training program, as well as pre-exercise activity level and health status of the participants.

Myostatin is a member of the transforming growth factor beta (TGF-β) superfamily that negatively regulates skeletal muscle size, and was the first described myokine[26].

chronic exercise and are playing a role in exercise-related restructuring processes of skeletal muscle.

Brain-derived neurotrophic factor (BDNF) also has been described as an exercise-induced myokine[41], although the

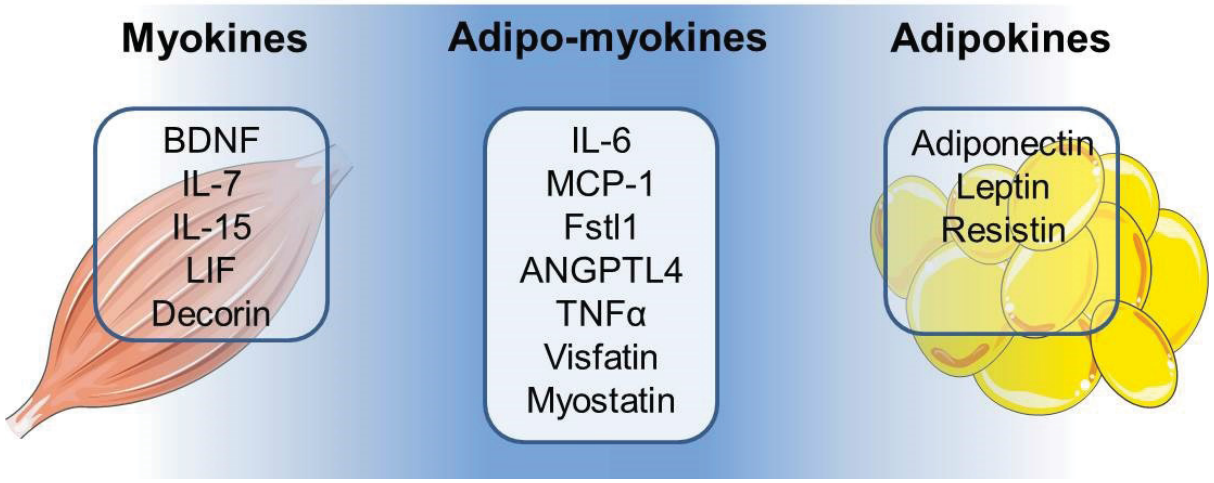

Figure 1: The adipo-myokine concept

A search of original articles in PubMed was performed for the major exercise-regulated myokines and adipokines to identify molecules that were produced and secreted in both tissues. The term adipo-myokines was used for proteins fulfilling both of these criteria. The search terms we used were "skeletal muscle" or "adipose tissue","myokine" or"adipokine" and "exercise".

When subjected to moderate aerobic exercise training for 6 months, overweight or obese men displayed a reduction of skeletal muscle expression and circulating levels of myostatin[27]. Moreover, acute endurance[28] or resistance exercise[29-31] was shown to attenuate myostatin mRNA expression in skeletal muscle. Importantly, myostatin is antagonized by different factors such as follistatin[32] and decorin[33]. Following acute resistance exercise, Dalbo et al. have found no effect on skeletal muscle follistatin mRNA expression in lean young and old men[31] while another study performed with postmenopausal women reported enhanced follistatin mRNA expression[30]. In addition, it was recently shown that decorin mRNA expression is enhanced in skeletal muscle after chronic exercise combining strength and endurance training[34]. A study by Heinemeier et al. reported increased decorin mRNA expression in skeletal muscle following an acute endurance exercise[35], and decorin plasma levels were increased after acute resistance exercise[34].

Another antagonist of proteins of the TGF-β super family is follistatin-like (FSTL1) which is a secreted glycoprotein belonging to the follistatin family of proteins[36]. Studies indicate that FSTL1 may have beneficial effects on ischemia–reperfusion injury in muscle and heart tissue associated with anti-apoptosis[37;38]. We have recently shown that FSTL1 is a myokine expressed and secreted by primary human skeletal muscle cells. Moreover, FSTL1 plasma levels were increased after acute endurance exercise performed by young healthy men[39]. In line with these results, Norheim et al. reported elevated FSTL1 mRNA expression in skeletal muscle from healthy male volunteers after chronic strength training[40]. We can conclude that members of TGF-β superfamily and their specific antagonist are strongly regulated by acute and

protein and its receptor are most abundantly expressed in the brain[42]. Acute endurance exercise increases plasma BDNF levels[41;43;44], specifically with high-intensity exercise[45]. In addition, Matthews et al. found significantly increased BDNF mRNA and protein abundance in skeletal muscle after acute endurance exercise[41]. Moreover, circulating BDNF concentrations increased in response to chronic endurance training[46]. On the other hand, a study using resistance exercise could not find an effect on BDNF plasma levels after acute or chronic exercise[47]. In humans, it should be noted that 70–80% of plasma BDNF originates from the brain during both rest and after exercise, suggesting the brain as the major source of this factor[48]. It might be that muscle-derived BDNF acts primarily within skeletal muscle tissue, e.g. inducing lipid oxidation via AMPK activation[41], whereas brain-derived BDNF may act more systemically and is potentially involved in the apparent beneficial effects of exercise with regard to Alzheimer´s disease, depression or impaired cognitive function[49].

Angiopoietin-like protein 4 (ANGPTL4) is detected in skeletal muscle as well as in adipose tissue and is therefore classified as adipo-myokine which is also regulated by exercise[50]. Importantly, the induction of plasma ANGPTL4 after exercise may depend on the increase of free fatty acids. In healthy, untrained male volunteers circulating ANGPTL4 was increased after acute endurance exercise[50]. Catoire et al. observed significantly increased plasma ANGPTL4 levels in healthy men after one-legged cycling exercise[51]. Interestingly, ANGPTL4 mRNA is more highly induced in the non-exercising leg than in the exercising leg[51]. However, plasma ANGPTL4 levels were not changed after 2 weeks of intense endurance exercise training, or by 12 weeks of endurance training[51]. In line with these

results, Norheim et al. reported that acute endurance exercise significantly increased plasma ANGPTL4 levels and skeletal muscle ANGPTL4 mRNA. However, a chronic exercise intervention combining strength and endurance training for 12 weeks had no effect on basal plasma ANGPTL4 concentrations and skeletal muscle ANGPTL4 mRNA content[52]. In conclusion, ANGPTL4 is highly induced in muscle in response to exercise. However, adipose tissue and the liver may contribute more than muscle to the exercise-induced increase in circulating ANGPTL4[52].

The chemokine MCP-1 is another adipo-myokine which is regulated by exercise in muscle. Among other functions, it plays an important role in the recruitment of monocytes and T lymphocytes into tissues[53]. Acute resistance exercise strongly increased MCP-1 mRNA expression in skeletal muscle of young male volunteers two hours post-exercise[54]. These results are supported by another study that observed a significant increase in MCP-1 protein level in skeletal muscle after acute resistance training in young and elderly healthy subjects[55]. Furthermore, cycling at 70% VO_{2max} for 40 min enhanced MCP-1 mRNA expression in skeletal muscle of lean, obese and type 2 diabetic subjects[56]. Kraemer et al. observed in young lean males increased plasma MCP-1 levels after an acute bout of resistance exercise that was normalized 30 min post-exercise[20]. Another study found increased MCP-1 plasma levels after acute treadmill running at different intensities with significantly higher plasma levels of MCP-1 following high-intensity compared to moderate-intensity trials[57]. These results suggest that the exercise-induced production of MCP-1 is more influenced by the intensity of exercise than by exercise-induced muscle damage, but it is not clear what role MCP-1 plays under exercising conditions. With regard to chronic resistance training, no effect on MCP-1 protein expression in the muscle of young and elderly healthy subjects was observed after 12 weeks[55]. Moreover, plasma MCP-1 levels were not changed after 12 weeks of low-intensity resistance training in a study with sedentary, lean, elderly women[58]. However, other studies observed a decrease in MCP-1 plasma levels after chronic strength training which could be related to the observed reduction of body fat mass[13;59].

The pro-inflammatory molecule TNFα is an early mediator of local inflammatory responses as well as initiator of the systemic acute phase response. It is produced by adipose tissue, and circulating TNFα levels are positively correlated with body fat mass[60]. However, TNFα mRNA is also detectable in skeletal muscle but no difference in its expression was found between overweight and lean subjects[61] or type 2 diabetics and BMI-matched controls[62] while elevated TNFα mRNA content in skeletal muscle was reported in elderly compared to young humans[63]. Interestingly, Febbraio et al. demonstrated that TNFα is not released by skeletal muscle after acute exercise in either healthy subjects or patients with type 2 diabetes[64]. In contrast, chronic resistance or endurance training significantly reduced TNFα mRNA and protein in skeletal muscle but had no effect on circulating TNFα concentrations[63;65]. More importantly, a large body of evidence shows an inverse relationship between plasma TNFα levels and the amount of physical activity even in healthy lean subjects as reviewed by Golbidi et al.[66]. In contrast to the observation that regular moderate exercise reduces pro-inflammatory cytokines such as TNFα, high intensive training cause a temporary depression of various aspects of immune function and an increase in systemic inflammation during the post-exercise period (~3-24 hours). TNFα levels increase only during a very intensive exercise (such as marathon running) in response to muscle damage[67]. It is well established that muscle repair and regeneration following acute muscle injury involves a tissue-remodeling, growth-promoting local inflammation. The initial inflammatory response seems to be required for the positive muscle repair process[68].

Nicotinamide phosphoribosyl transferase (NAMPT) is an ubiquitously expressed NAD biosynthetic enzyme that occurs either in an intracellular (iNAMPT) or extracellular (eNAMPT/visfatin) form[69]. In mammals, NAMPT is responsible for the first and rate-limiting step in the conversion of nicotinamide to nicotinamide dinucleotide (NAD^+) in the NAD^+ salvage pathway. Costford et al. reported a twofold higher NAMPT expression in skeletal muscle of athletes as compared to sedentary obese, non-obese, and type 2 diabetic subjects[70]. Moreover, 3 weeks of endurance training enhanced NAMPT mRNA expression and protein content more than twofold in skeletal muscle of non-obese sedentary individuals. A study by Brandauer et al. using one-legged endurance exercise training for 3 weeks confirmed these results and reported a specific up-regulation of NAMPT in the exercising leg[71]. Interestingly, acute exercise (3 hours of cycling at 60% VO_{2max}) had no effect on skeletal muscle NAMPT expression and circulating eNAMPT/visfatin was found unchanged[72]. However, an acute bout of high-intensity exercise was shown to increase plasma eNAMPT/visfatin immediately after the challenge[73] suggesting that acute release of eNAMPT/visfatin could be intensity-dependent. Interestingly, Costford et al. found that skeletal muscle NAMPT protein was negatively correlated with body fat, while in obesity and type 2 diabetes circulating eNAMPT/visfatin were reported to be elevated compared to controls[70]. In addition, several chronic exercise intervention studies conducted with obese volunteers reported reductions in plasma eNAMPT/visfatin levels[74-76]. Taken together, these observations suggest a potential impact of the adipose tissue on circulating levels of eNAMPT/visfatin and an independent regulation of intracellular NAMPT and circulating eNAMPT/visfatin, which awaits further investigations.

Although adiponectin is a classical adipokine, and therefore will be discussed in more detail in the next section, it is also expressed in skeletal muscle[77;78]. In obesity and insulin resistance, plasma adiponectin levels are lower, whereas skeletal muscle expression of adiponectin receptors AdipoR1 and AdipoR2 are

increased[79]. A study by Bluher et al. found that chronic endurance training increased both plasma adiponectin levels as well as expression of AdipoR1 and AdipoR2 in skeletal muscle of overweight/obese subjects with impaired glucose metabolism and in lean healthy controls. However, in severely obese subjects a combination of endurance exercise and diet increased plasma adiponectin levels but had no effect on AdipoR1 and AdipoR2 mRNA expression in skeletal muscle[13]. Interestingly, patients with chronic heart failure displayed elevated adiponectin and lower AdipoR1 mRNA expression in skeletal muscle which was normalized by a combined endurance and resistance exercise training for four months[80]. So far, no other studies have investigated the regulation of adiponectin expression in skeletal muscle with exposure to various types of exercise in different groups of patients.

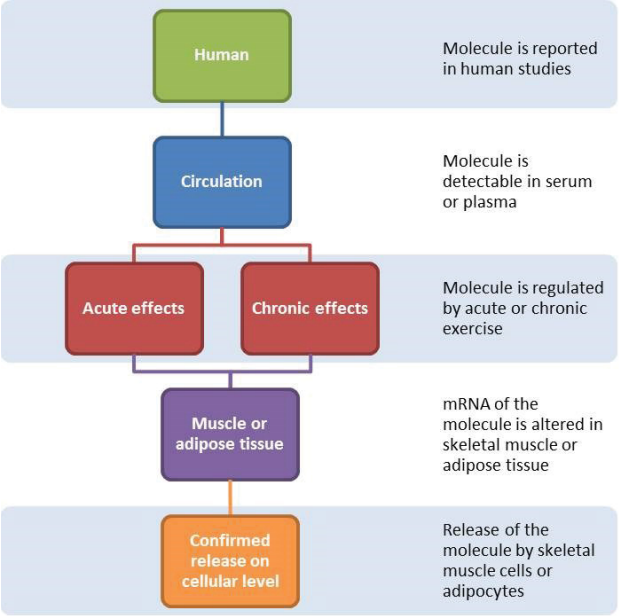

Figure 2: Scheme for the approach to selecting published studies
A search of original articles in PubMed was performed to identify myokines, adipokines or adipo-myokines, which satisfied all of the following criteria: The molecule must be human and detectable in serum or plasma. Furthermore, the molecule must be regulated by either acute or chronic exercise and expressed in skeletal muscle or adipose tissue. The protein has to be secreted by adipocytes or myocytes. The search terms we used were "skeletal muscle" or "adipose tissue","myokine" or"adipokine" and "exercise".

Leptin is a classical adipokine that has also been found to be expressed in skeletal muscle[81]. More importantly, release of leptin by human skeletal muscle has been described by two studies. Wolsk et al. have shown that leptin is released by human skeletal muscle *in vivo*[82] and reported a release of ~0.8 ng per min per 100 g tissue from adipose tissue and ~0.5 ng per min per 100 g tissue from skeletal muscle. These data suggest that the contribution of skeletal muscle to whole-body leptin production could be substantial in lean humans because of the greater muscle mass compared to fat mass. In contrast, Lappas et al. observed a more than 10-fold higher release of adiponectin from adipose tissue explants as compared to skeletal muscle explants[83].
Another set of genes that are highly expressed in cultured myotubes encodes proteins related to extracellular matrix (ECM) in the resting state. In particular p roteoglycans and proteins related to their metabolism like secreted protein, acidic and rich in cysteine (SPARC), collagen alpha-1, lumican, gelsolin, cathepsin B, D, H, and L1, fibronectin, tissue inhibitor metalloproteinase 1 and extracellular matrix protein 1, are expressed also in muscle biopsies and are enhanced during strength training[40]. The enhanced expression of these ECM-related proteins is likely due to restructuring of the muscle tissue in response to increased mechanical demands. Moreover, some glycoproteins like serglycin may regulate storage of numerous proteases, growth factors and chemokines and could be important in addition to their structural functions in ECM[84].
In summary, skeletal muscle tissues produce a wide range of different molecules *in vivo* that can be classified as myokines or adipo-myokines.

III. EXERCISE AND THE PRODUCTION OF ADIPOKINES BY ADIPOSE TISSUE

While numerous studies have investigated the effect of different types of exercise training on circulating levels of adipose tissue-derived factors, only a few have addressed the adipose tissue-specific expression of these factors.
Adiponectin is one of the best characterized classical adipokines and has been shown to increase fatty acid oxidation and glucose uptake in skeletal muscle and inhibit gluconeogenesis in liver[85]. In macrophages, adiponectin inhibits expression and secretion of TNFα while increasing the production of anti-inflammatory cytokines such as IL-10[86]. Moreover, it exerts cardio-protective effects and is inversely related to BMI[87]. Studies on the regulation of adiponectin by exercise have shown divergent results, although most studies indicate no effect of exercise. Two reports have investigated adiponectin mRNA expression in adipose tissue following acute exercise and found either increased[88] or decreased[61] expression after cycling in overweight, obese and lean subjects with no difference between the groups. Also, plasma adiponectin levels remained unchanged after acute exercise atmoderate intensity in healthy[89;90] or overweight/obese participants[90-93]. On the other hand, Saunders et al. reported increased adiponectin plasma levels following an acute bout of exercise in sedentary obese men[94]. With regard to chronic exercise intervention, most studies using endurance type training with moderate intensity reported no change in plasma levels of adiponectin[89;95-98], while mRNA expression in adipose tissue was found either increased[95;97] or unmodified[98]. In contrast, two studies that used chronic endurance exercise protocols reported increased plasma adiponectin levels in obese young women[99] and in overweight patients with impaired glucose metabolism[79] after the intervention period. In studies using resistance

exercise for chronic intervention, the expression of adiponectin mRNA in adipose tissue as well as circulating levels were found unchanged in obese men[100] and women[101]. Interestingly, Fatouros et al. reported in overweight elderly men that resistance training at high intensity for 6 months increased plasma adiponectin levels while a moderate intensity training had no effect[102]. Similar results were reported for acute resistance exercise in overweight elderly men[103]. These data suggest that the intensity of exercise may be an important factor in the regulation of adiponectin concentration in plasma.

Leptin is another classical adipokine predominantly secreted from adipocytes into the circulation to regulate energy homeostasis[104]. Following acute endurance exercise, leptin mRNA in adipose tissue was found unchanged[105] or decreased in lean and overweight subjects[61]. Moreover, plasma leptin levels analyzed immediately after an exercise challenge were found to be no different compared to pre-exercise levels[90;106;107], while several studies have shown a delayed decrease of circulating leptin levels in healthy active men 24 and 48 hours post-exercise[101;108-110]. On the other hand, acute resistance exercise at different intensities had no effect on circulating leptin levels in overweight elderly men[103]. Also, pre-exercise training status of the participants has no effect on leptin levels after a single weight training session[107]. Following chronic intervention of ≥ 12 weeks leptin mRNA expression in adipose tissue remained unchanged after endurance training in obese subjects[95;98]. Leptin plasma concentrations are mostly found to be decreased[98;99;106;111] but also body weight and fat mass of the participants were reduced after the intervention periods. However, Polak et al. reported declined leptin plasma level which remained significant after adjusting for BMI and fat mass[98], suggesting independent effects of the training besides reduction of body weight. Moreover, studies using resistance training reported decreased plasma leptin levels without changes in body weight and fat mass and no effect on adipose tissue mRNA expression of leptin[100;101]. So far, the available data suggest that plasma leptin levels are decreased in response to exercise training while adipose tissue mRNA expression seems to be not affected.

TNFα is a major inflammatory cytokine that is highly expressed in adipose tissue in obese conditions and plays a role in the pathogenesis of insulin resistance. Following acute exercise, TNFα mRNA expression in adipose tissue was found to be unchanged in overweight, obese and lean subjects[61;88]. Interestingly, Christiansen et al. reported higher TNFα mRNA expression in the post exercise period, 2.5 hours after completing the exercise bout. With regard to circulating TNFα levels either no effect of acute exercise was found[61;93;112] or increased plasma TNFα concentrations were reported[88;90]. Several studies on chronic exercise using either endurance[95;98] or resistance training[100] found no effect on adipose tissue mRNA expression of TNFα, whereas one study reported lower TNFα mRNA expression in obese humans[113]. Circulating TNFα levels were mostly reported to be reduced[99;101;114] by chronic exercise. However, not all studies conducted on obese humans have found an effect of long-term exercise on plasma TNFα concentrations[98;111;115]. In summary, chronic exercise seems to be able to reduce circulating TNFα levels without an effect on adipose tissue expression.

As discussed above, IL-6 is the proto-type of an adipo-myokine. It is well established that the increase of circulating IL-6 upon exercise is mainly due to its release from skeletal muscle[6]. However, subjects with insulin resistance, obesity and type 2 diabetes display chronically elevated serum levels of IL-6[116-118]. An important source of circulating IL-6 in obesity is the expanding visceral adipose tissue mass. Expression of IL-6 by macrophages within the adipose tissue is dependent on activation of the NFκB signaling pathway whereas intramuscular IL-6 expression is regulated by different signaling cascades[1]. To clarify a potential contribution of adipose tissue to increased IL-6 levels after acute endurance exercise, Hojbjerre et al. have analyzed microdialysates from abdominal adipose tissue in overweight and lean males[61]. Interestingly, they observed increased IL-6 release from adipose tissue in the post-exercise phase but not during exercise in both groups. Moreover, IL-6 mRNA expression in adipose tissue was increased during and post-exercise. Similar results were observed by Christiansen[88]. Following chronic endurance training with moderate weight loss, no reduction of IL-6 mRNA expression in adipose tissue of obese subjects was found and basal IL-6 concentrations remained unaffected[95;98]. In the case of chronic resistance exercise, no reduction of adipose tissue IL-6 mRNA expression or basal circulating IL-6 in obese subjects was observed[100;101]. Another study that combined chronic endurance training and a hypocaloric diet reported decreased IL-6 mRNA expression in adipose tissue as well as reduced plasma IL-6 levels[13]. However, it has to be mentioned that this lifestyle intervention also decreased body weight by 13% and that the reduction of IL-6 mRNA expression and circulating levels could be related to the reduced fat mass.

In addition to the above discussed adipokines/adipo-myokines, only a few studies have investigated other adipose tissue-derived factors in response the exercise. Resistin is an inflammatory biomarker and potential mediator of obesity-associated diseases. It is positively correlated to percent fat mass and waist circumference, and evidence suggests that this adipokine causes endothelial dysfunction by promoting oxidative stress and down-regulating the production of nitric oxide[107;119]. Acute endurance training had no effect on circulating resistin levels up to 48 h post-exercise in overweight males[91]. Similarly, in another study with lean and overweight volunteers, resistin mRNA expression in adipose tissue was not affected[61]. A lifestyle intervention combining chronic endurance training and hypocaloric diet with obese patients for three months had also no effect on plasma resistin concentrations[120]. Interestingly, a study using acute resistance exercise observed that the exercise effect on plasma resistin levels was dependenton the training status of the subjects. While a reduction was observed in participants that performed regular weight

training (≥ 1 h, three times / week, for 6 months before the study), no effect was found in sedentary males or in active runners (running ≥ 15 miles / week for 6 months before the study)[107].

The expression of NAMPT/Visfatin in human adipose tissue has been shown by several groups[72;121]. So far, only one study has investigated adipose tissue NAMPT expression after exercise and reported an increase after an

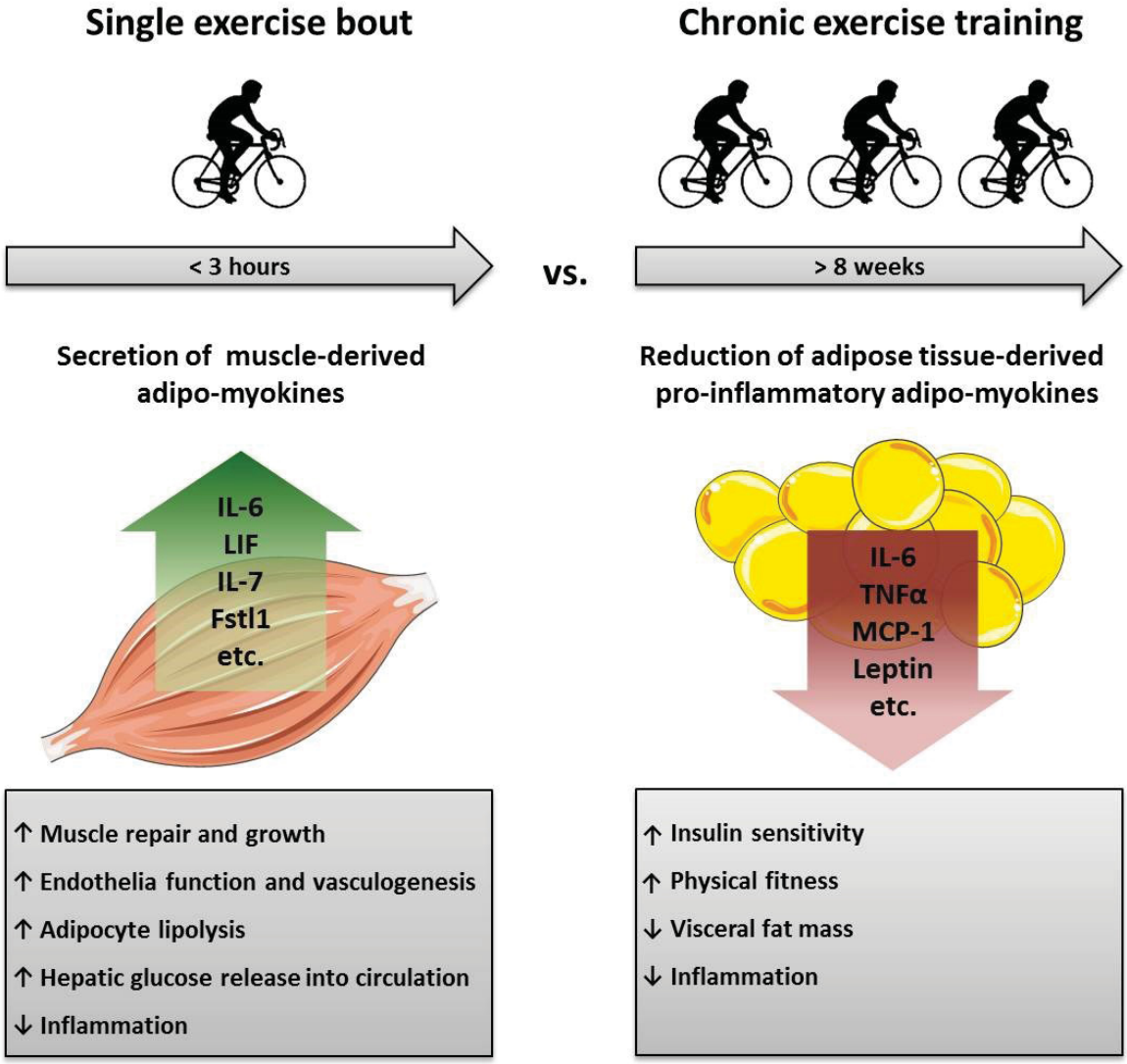

Figure 3: Differential effects of acute and chronic exercise
After acute exercise, a high number of myokines are secreted by skeletal muscle exerting a variety of endocrine effects. Acute induction of myokines like myostatin, IL-7, decorin and LIF are involved in the regulation of muscle hypertrophy and may play a role in exercise-related restructuring of skeletal muscle. The high level of circulating IL-6 after exercise induces an anti-inflammatory environment by inducing the production of IL-1ra and IL-10, but also inhibits TNFα production. Furthermore, IL-6 has metabolic effects, by affecting insulin-stimulated glucose disposal and fatty acid oxidation. The myokine FSTL1 has protective effects on ischemia–reperfusion injury in muscle and heart tissue. One the other hand, regular exercise training induces a reduction of adipose tissue-derived pro-inflammatory cytokines like IL-6, TNFα and MCP-1, which are associated with low-grade systemic inflammation and a reduction of whole-body insulin sensitivity. Exercise training has been reported to reduce central adiposity independent of overall weight changes, and this may represent an additional mechanism of the anti-inflammatory action of chronic exercise training.

With regard to MCP-1, which has been discussed above in more detail, studies have found no effect of chronic endurance training on adipose tissue mRNA expression despite reductions of circulating MCP-1 levels[95]. Also, chronic resistance training in obese women left MCP-1 mRNA expression in adipose tissue unaltered but circulating levels were not measured in this study[101].

acute bout of endurance training which was not accompanied by elevated plasma eNAMPT/visfatin concentrations. Reports on the effect of chronic exercise on circulating eNAMPT/visfatin levels are not consistent. Obese non-diabetic subjects were reported to have higher eNAMPT/visfatin levels compared to controls which were reduced by 12 weeks of aerobic[75;76] or aerobic plus resistance exercise[74] accompanied by decreased body

weight. However, Jorge et al reported increased plasma eNAMPT/visfatin levels after 12 weeks of exercise (endurance training, resistance training or a combination of both) in obese type 2 diabetic patients. Importantly, body weight was not changed in the course of this study. Based on these data it is most likely that exercise-induced reduction of plasma eNAMPT/visfatin is the result of weight loss and body composition changes.

Few reports are available on ANGPTL4 in human adipose tissue with exercise. In a study by Norheim et al., it was reported that 30 min after an acute bout of endurance exercise, ANGPTL4 mRNA expression was significantly higher in overweight dysglycemic subjects compared to lean controls at the beginning of the intervention study[52]. Interestingly, they found that ANGPTL4 mRNA expression was much higher in adipose tissue than muscle. Following 12 weeks of combined endurance and resistance training, adipose tissue mRNA expression of ANGPTL4 as well as basal plasma levels were unchanged in both groups. In contrast, another chronic endurance training study in obese but otherwise healthy participants found significantly reduced mRNA expression in adipose tissue but increased circulating ANGPTL4 levels[122]. Clearly, more studies are required to understand the regulation of ANGPTL4 in response to various types of exercise in different types of subjects as well as the contribution of the different tissues to circulating ANGPTL4.

IV. CONCLUSION

There are hundreds of secretory proteins released from skeletal muscle as well as adipose tissue with an array of biological effects on most organs in the body. It is a striking observation that many of the myokines and adipokines are expressed and secreted from many different tissues. It seems as if there are very few of these adipo-myokines that are exclusively expressed in one organ. However, considering that both adipose tissue and skeletal muscle tissues are often closely associated with the same phenotypes or outcomes, it is likely that the pattern of signal molecules released from these two tissues have marked physiological effects of essential importance for health as well as well-being. The original expectation that we would be able to describe discrete signatures of secretory proteins from muscle and adipose has to be replaced by a much more complicated model.

A single bout of exercise is predominantly characterized by the secretion of myokines and adipo-myokines by the working skeletal muscles, which exert a variety of autocrine and endocrine effects (Figure 3). Acute induction of myokines like myostatin, IL-7, decorin and LIF are involved in the regulation of muscle growth and may play a role in exercise-related restructuring of skeletal muscle. Muscle-derived IL-6 has metabolic effects on insulin-stimulated glucose disposal and fatty acid oxidation. Furthermore, acute high levels of circulating IL-6 provide an anti-inflammatory environment after exercise by induction of IL-1ra and IL-10, and inhibition of TNFα production. One the other hand, regular exercise training is associated with reduced levels of adipose tissue-derived pro-inflammatory cytokines, which are linked tolow-grade systemic inflammation and low whole-body insulin sensitivity (Figure 3).Chronic exercise reduces visceral fat mass and has been reported to reduce central adiposity independent of overall weight changes. The anti-inflammatory effects of regular exercise training may be mediated by both, the reduction of body fat mass and the induction of an anti-inflammatory environment associated with each single bout of exercise. In summary, regular exercise reduces the risk of chronic metabolic diseases and various mechanism may contribute to this beneficial effect, including decreased production of adipose tissue-derived pro-inflammatory cytokines and increased production of anti-inflammatory myokines from contracting muscle.

V. REFERENCES

1. Eckardt,K, Gorgens,SW, Raschke,S, Eckel,J: Myokines in insulin resistance and type 2 diabetes. *Diabetologia* 57:1087-1099, 2014
2. Pedersen,BK, Febbraio,MA: Muscles, exercise and obesity: skeletal muscle as a secretory organ. *Nat Rev Endocrinol* 8:457-465, 2012
3. Raschke,S, Eckardt,K, Bjorklund,HK, Jensen,J, Eckel,J: Identification and validation of novel contraction-regulated myokines released from primary human skeletal muscle cells. *PLoS One* 8:e62008, 2013
4. Raschke,S, Eckel,J: Adipo-myokines: two sides of the same coin--mediators of inflammation and mediators of exercise. *Mediators Inflamm* 2013:320724, 2013
5. Lee,S, Kuk,JL, Davidson,LE, Hudson,R, Kilpatrick,K, Graham,TE, Ross,R: Exercise without weight loss is an effective strategy for obesity reduction in obese individuals with and without Type 2 diabetes. *J Appl Physiol (1985)* 99:1220-1225, 2005
6. Pedersen,BK: Muscle as a secretory organ. *Compr Physiol* 3:1337-1362, 2013
7. Serrano,AL, Baeza-Raja,B, Perdiguero,E, Jardi,M, Munoz-Canoves,P: Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. *Cell Metab* 7:33-44, 2008
8. Fischer,CP: Interleukin-6 in acute exercise and training: what is the biological relevance? *Exerc Immunol Rev* 12:6-33, 2006
9. Willoughby,DS, McFarlin,B, Bois,C: Interleukin-6 expression after repeated bouts of eccentric exercise. *Int J Sports Med* 24:15-21, 2003
10. Keller,C, Steensberg,A, Pilegaard,H, Osada,T, Saltin,B, Pedersen,BK, Neufer,PD: Transcriptional activation of the IL-6 gene in human contracting skeletal muscle: influence of muscle glycogen content. *FASEB J* 15:2748-2750, 2001
11. Steensberg,A, Febbraio,MA, Osada,T, Schjerling,P, van,HG, Saltin,B, Pedersen,BK: Interleukin-6 production in contracting human

skeletal muscle is influenced by pre-exercise muscle glycogen content. *J Physiol* 537:633-639, 2001

12. Kohut,ML, McCann,DA, Russell,DW, Konopka,DN, Cunnick,JE, Franke,WD, Castillo,MC, Reighard,AE, Vanderah,E: Aerobic exercise, but not flexibility/resistance exercise, reduces serum IL-18, CRP, and IL-6 independent of beta-blockers, BMI, and psychosocial factors in older adults. *Brain Behav Immun* 20:201-209, 2006
13. Bruun,JM, Helge,JW, Richelsen,B, Stallknecht,B: Diet and exercise reduce low-grade inflammation and macrophage infiltration in adipose tissue but not in skeletal muscle in severely obese subjects. *Am J Physiol Endocrinol Metab* 290:E961-E967, 2006
14. Rokling-Andersen,MH, Reseland,JE, Veierod,MB, Anderssen,SA, Jacobs,DR, Jr., Urdal,P, Jansson,JO, Drevon,CA: Effects of long-term exercise and diet intervention on plasma adipokine concentrations. *Am J Clin Nutr* 86:1293-1301, 2007
15. Bruunsgaard,H, Bjerregaard,E, Schroll,M, Pedersen,BK: Muscle strength after resistance training is inversely correlated with baseline levels of soluble tumor necrosis factor receptors in the oldest old. *J Am Geriatr Soc* 52:237-241, 2004
16. Broholm,C, Laye,MJ, Brandt,C, Vadalasetty,R, Pilegaard,H, Pedersen,BK, Scheele,C: LIF is a contraction-induced myokine stimulating human myocyte proliferation. *J Appl Physiol (1985)* 111:251-259, 2011
17. Broholm,C, Mortensen,OH, Nielsen,S, Akerstrom,T, Zankari,A, Dahl,B, Pedersen,BK: Exercise induces expression of leukaemia inhibitory factor in human skeletal muscle. *J Physiol* 586:2195-2201, 2008
18. Haugen,F, Norheim,F, Lian,H, Wensaas,AJ, Dueland,S, Berg,O, Funderud,A, Skalhegg,BS, Raastad,T, Drevon,CA: IL-7 is expressed and secreted by human skeletal muscle cells. *Am J Physiol Cell Physiol* 298:C807-C816, 2010
19. Andersson,H, Bohn,SK, Raastad,T, Paulsen,G, Blomhoff,R, Kadi,F: Differences in the inflammatory plasma cytokine response following two elite female soccer games separated by a 72-h recovery. *Scand J Med Sci Sports* 20:740-747, 2010
20. Kraemer,WJ, Hatfield,DL, Comstock,BA, Fragala,MS, Davitt,PM, Cortis,C, Wilson,JM, Lee,EC, Newton,RU, Dunn-Lewis,C, Hakkinen,K, Szivak,TK, Hooper,DR, Flanagan,SD, Looney,DP, White,MT, Volek,JS, Maresh,CM: Influence of HMB Supplementation and Resistance Training on Cytokine Responses to Resistance Exercise. *J Am Coll Nutr* 33:247-255, 2014
21. Nieman,DC, Davis,JM, Henson,DA, Walberg-Rankin,J, Shute,M, Dumke,CL, Utter,AC, Vinci,DM, Carson,JA, Brown,A, Lee,WJ, McAnulty,SR, McAnulty,LS: Carbohydrate ingestion influences skeletal muscle cytokine mRNA and plasma cytokine levels after a 3-h run. *J Appl Physiol (1985)* 94:1917-1925, 2003
22. Rinnov,A, Yfanti,C, Nielsen,S, Akerstrom,TC, Peijs,L, Zankari,A, Fischer,CP, Pedersen,BK: Endurance training enhances skeletal muscle interleukin-15 in human male subjects. *Endocrine* 45:271-278, 2014
23. Tamura,Y, Watanabe,K, Kantani,T, Hayashi,J, Ishida,N, Kaneki,M: Upregulation of circulating IL-15 by treadmill running in healthy individuals: is IL-15 an endocrine mediator of the beneficial effects of endurance exercise? *Endocr J* 58:211-215, 2011
24. Riechman,SE, Balasekaran,G, Roth,SM, Ferrell,RE: Association of interleukin-15 protein and interleukin-15 receptor genetic variation with resistance exercise training responses. *J Appl Physiol (1985)* 97:2214-2219, 2004
25. Nielsen,AR, Mounier,R, Plomgaard,P, Mortensen,OH, Penkowa,M, Speerschneider,T, Pilegaard,H, Pedersen,BK: Expression of interleukin-15 in human skeletal muscle effect of exercise and muscle fibre type composition. *J Physiol* 584:305-312, 2007
26. McPherron,AC, Lawler,AM, Lee,SJ: Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* 387:83-90, 1997
27. Hittel,DS, Axelson,M, Sarna,N, Shearer,J, Huffman,KM, Kraus,WE: Myostatin Decreases with Aerobic Exercise and Associates with Insulin Resistance. *Med Sci Sports Exerc* 42:2023-9, 2010
28. Harber,MP, Crane,JD, Dickinson,JM, Jemiolo,B, Raue,U, Trappe,TA, Trappe,SW: Protein synthesis and the expression of growth-related genes are altered by running in human vastus lateralis and soleus muscles. *Am J Physiol Regul Integr Comp Physiol* 296:R708-R714, 2009
29. Kim,JS, Cross,JM, Bamman,MM: Impact of resistance loading on myostatin expression and cell cycle regulation in young and older men and women. *Am J Physiol Endocrinol Metab* 288:E1110-E1119, 2005
30. Dieli-Conwright,CM, Spektor,TM, Rice,JC, Sattler,FR, Schroeder,ET: Hormone therapy and maximal eccentric exercise alters myostatin-related gene expression in postmenopausal women. *J Strength Cond Res* 26:1374-1382, 2012
31. Dalbo,VJ, Roberts,MD, Sunderland,KL, Poole,CN, Stout,JR, Beck,TW, Bemben,M, Kerksick,CM: Acute loading and aging effects on myostatin pathway biomarkers in human skeletal muscle after three sequential bouts of resistance exercise. *J Gerontol A Biol Sci Med Sci* 66:855-865, 2011
32. Amthor,H, Nicholas,G, McKinnell,I, Kemp,CF, Sharma,M, Kambadur,R, Patel,K: Follistatin complexes Myostatin and antagonises Myostatin-

mediated inhibition of myogenesis. *Dev Biol* 270:19-30, 2004
33. Miura,T, Kishioka,Y, Wakamatsu,J, Hattori,A, Hennebry,A, Berry,CJ, Sharma,M, Kambadur,R, Nishimura,T: Decorin binds myostatin and modulates its activity to muscle cells. *Biochem Biophys Res Commun* 340:675-680, 2006
34. Kanzleiter,T, Rath,M, Gorgens,SW, Jensen,J, Tangen,DS, Kolnes,AJ, Kolnes,KJ, Lee,S, Eckel,J, Schurmann,A, Eckardt,K: The myokine decorin is regulated by contraction and involved in muscle hypertrophy. *Biochem Biophys Res Commun* 450:1089-1094, 2014
35. Heinemeier,KM, Bjerrum,SS, Schjerling,P, Kjaer,M: Expression of extracellular matrix components and related growth factors in human tendon and muscle after acute exercise. *Scand J Med Sci Sports* 23:e150-e161, 2013
36. Shi,Y, Massague,J: Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* 113:685-700, 2003
37. Ouchi,N, Oshima,Y, Ohashi,K, Higuchi,A, Ikegami,C, Izumiya,Y, Walsh,K: Follistatin-like 1, a secreted muscle protein, promotes endothelial cell function and revascularization in ischemic tissue through a nitric-oxide synthase-dependent mechanism. *J Biol Chem* 283:32802-32811, 2008
38. Ogura,Y, Ouchi,N, Ohashi,K, Shibata,R, Kataoka,Y, Kambara,T, Kito,T, Maruyama,S, Yuasa,D, Matsuo,K, Enomoto,T, Uemura,Y, Miyabe,M, Ishii,M, Yamamoto,T, Shimizu,Y, Walsh,K, Murohara,T: Therapeutic impact of follistatin-like 1 on myocardial ischemic injury in preclinical models. *Circulation* 126:1728-1738, 2012
39. Gorgens,SW, Raschke,S, Holven,KB, Jensen,J, Eckardt,K, Eckel,J: Regulation of follistatin-like protein 1 expression and secretion in primary human skeletal muscle cells. *Arch Physiol Biochem* 119:75-80, 2013
40. Norheim,F, Raastad,T, Thiede,B, Rustan,AC, Drevon,CA, Haugen,F: Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training. *Am J Physiol Endocrinol Metab* 301:E1013-E1021, 2011
41. Matthews,VB, Astrom,MB, Chan,MH, Bruce,CR, Krabbe,KS, Prelovsek,O, Akerstrom,T, Yfanti,C, Broholm,C, Mortensen,OH, Penkowa,M, Hojman,P, Zankari,A, Watt,MJ, Bruunsgaard,H, Pedersen,BK, Febbraio,MA: Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. *Diabetologia* 52:1409-1418, 2009
42. Huang,EJ, Reichardt,LF: Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci* 24:677-736, 2001
43. Ferris,LT, Williams,JS, Shen,CL: The effect of acute exercise on serum brain-derived neurotrophic factor levels and cognitive function. *Med Sci Sports Exerc* 39:728-734, 2007
44. Gold,SM, Schulz,KH, Hartmann,S, Mladek,M, Lang,UE, Hellweg,R, Reer,R, Braumann,KM, Heesen,C: Basal serum levels and reactivity of nerve growth factor and brain-derived neurotrophic factor to standardized acute exercise in multiple sclerosis and controls. *J Neuroimmunol* 138:99-105, 2003
45. Schmidt-Kassow,M, Schadle,S, Otterbein,S, Thiel,C, Doehring,A, Lotsch,J, Kaiser,J: Kinetics of serum brain-derived neurotrophic factor following low-intensity versus high-intensity exercise in men and women. *Neuroreport* 23:889-893, 2012
46. Griffin,EW, Mullally,S, Foley,C, Warmington,SA, O'Mara,SM, Kelly,AM: Aerobic exercise improves hippocampal function and increases BDNF in the serum of young adult males. *Physiol Behav* 104:934-941, 2011
47. Goekint,M, De Pauw,K, Roelands,B, Njemini,R, Bautmans,I, Mets,T, Meeusen,R: Strength training does not influence serum brain-derived neurotrophic factor. *Eur J Appl Physiol* 110:285-293, 2010
48. Rasmussen,P, Brassard,P, Adser,H, Pedersen,MV, Leick,L, Hart,E, Secher,NH, Pedersen,BK, Pilegaard,H: Evidence for a release of brain-derived neurotrophic factor from the brain during exercise. *Exp Physiol* 94:1062-1069, 2009
49. Mattson,MP: Energy intake and exercise as determinants of brain health and vulnerability to injury and disease. *Cell Metab* 16:706-722, 2012
50. Kersten,S, Lichtenstein,L, Steenbergen,E, Mudde,K, Hendriks,HF, Hesselink,MK, Schrauwen,P, Muller,M: Caloric restriction and exercise increase plasma ANGPTL4 levels in humans via elevated free fatty acids. *Arterioscler Thromb Vasc Biol* 29:969-974, 2009
51. Catoire,M, Alex,S, Paraskevopulos,N, Mattijssen,F, Evers-van,G, I, Schaart,G, Jeppesen,J, Kneppers,A, Mensink,M, Voshol,PJ, Olivecrona,G, Tan,NS, Hesselink,MK, Berbee,JF, Rensen,PC, Kalkhoven,E, Schrauwen,P, Kersten,S: Fatty acid-inducible ANGPTL4 governs lipid metabolic response to exercise. *Proc Natl Acad Sci U S A* 111:E1043-E1052, 2014
52. Norheim,F, Hjorth,M, Langleite,TM, Lee,S, Holen,T, Bindesboll,C, Stadheim,HK, Gulseth,HL, Birkeland,KI, Kielland,A, Jensen,J, Dalen,KT, Drevon,CA: Regulation of angiopoietin-like protein 4 production during and after exercise. *Physiol Rep* 2: 2014
53. Qin,S, LaRosa,G, Campbell,JJ, Smith-Heath,H, Kassam,N, Shi,X, Zeng,L, Buthcher,EC, Mackay,CR: Expression of monocyte chemoattractant protein-1 and interleukin-8 receptors on subsets of T cells: correlation with transendothelial chemotactic potential. *Eur J Immunol* 26:640-647, 1996

54. Vella,L, Caldow,MK, Larsen,AE, Tassoni,D, Della Gatta,PA, Gran,P, Russell,AP, Cameron-Smith,D: Resistance exercise increases NF-kappaB activity in human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 302:R667-R673, 2012
55. Della Gatta,PA, Garnham,AP, Peake,JM, Cameron-Smith,D: Effect of exercise training on skeletal muscle cytokine expression in the elderly. *Brain Behav Immun* 39:80-86, 2014
56. Tantiwong,P, Shanmugasundaram,K, Monroy,A, Ghosh,S, Li,M, DeFronzo,RA, Cersosimo,E, Sriwijitkamol,A, Mohan,S, Musi,N: NF-kappaB activity in muscle from obese and type 2 diabetic subjects under basal and exercise-stimulated conditions. *Am J Physiol Endocrinol Metab* 299:E794-E801, 2010
57. Peake,JM, Suzuki,K, Hordern,M, Wilson,G, Nosaka,K, Coombes,JS: Plasma cytokine changes in relation to exercise intensity and muscle damage. *Eur J Appl Physiol* 95:514-521, 2005
58. Ogawa,K, Sanada,K, Machida,S, Okutsu,M, Suzuki,K: Resistance exercise training-induced muscle hypertrophy was associated with reduction of inflammatory markers in elderly women. *Mediators Inflamm* 2010:171023, 2010
59. Glintborg,D, Christensen,LL, Kvorning,T, Larsen,R, Brixen,K, Hougaard,DM, Richelsen,B, Bruun,JM, Andersen,M: Strength training and testosterone treatment have opposing effects on migration inhibitor factor levels in ageing men. *Mediators Inflamm* 2013:539156, 2013
60. Eckardt,K, Gorgens,SW, Raschke,S, Eckel,J: Myokines in insulin resistance and type 2 diabetes. *Diabetologia* 57:1087-1099, 2014
61. Hojbjerre,L, Rosenzweig,M, Dela,F, Bruun,JM, Stallknecht,B: Acute exercise increases adipose tissue interstitial adiponectin concentration in healthy overweight and lean subjects. *Eur J Endocrinol* 157:613-623, 2007
62. Plomgaard,P, Nielsen,AR, Fischer,CP, Mortensen,OH, Broholm,C, Penkowa,M, Krogh-Madsen,R, Erikstrup,C, Lindegaard,B, Petersen,AM, Taudorf,S, Pedersen,BK: Associations between insulin resistance and TNF-alpha in plasma, skeletal muscle and adipose tissue in humans with and without type 2 diabetes. *Diabetologia* 50:2562-2571, 2007
63. Greiwe,JS, Cheng,B, Rubin,DC, Yarasheski,KE, Semenkovich,CF: Resistance exercise decreases skeletal muscle tumor necrosis factor alpha in frail elderly humans. *FASEB J* 15:475-482, 2001
64. Febbraio,MA, Steensberg,A, Starkie,RL, McConell,GK, Kingwell,BA: Skeletal muscle interleukin-6 and tumor necrosis factor-alpha release in healthy subjects and patients with type 2 diabetes at rest and during exercise. *Metabolism* 52:939-944, 2003
65. Gielen,S, Adams,V, Mobius-Winkler,S, Linke,A, Erbs,S, Yu,J, Kempf,W, Schubert,A, Schuler,G, Hambrecht,R: Anti-inflammatory effects of exercise training in the skeletal muscle of patients with chronic heart failure. *J Am Coll Cardiol* 42:861-868, 2003
66. Golbidi,S, Laher,I: Exercise induced adipokine changes and the metabolic syndrome. *J Diabetes Res* 2014:726861, 2014
67. Pedersen,BK, Akerstrom,TC, Nielsen,AR, Fischer,CP: Role of myokines in exercise and metabolism. *J Appl Physiol* 103:1093-1098, 2007
68. Paulsen,G, Mikkelsen,UR, Raastad,T, Peake,JM: Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise? *Exerc Immunol Rev* 18:42-97, 2012
69. Revollo,JR, Korner,A, Mills,KF, Satoh,A, Wang,T, Garten,A, Dasgupta,B, Sasaki,Y, Wolberger,C, Townsend,RR, Milbrandt,J, Kiess,W, Imai,S: Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab* 6:363-375, 2007
70. Costford,SR, Bajpeyi,S, Pasarica,M, Albarado,DC, Thomas,SC, Xie,H, Church,TS, Jubrias,SA, Conley,KE, Smith,SR: Skeletal muscle NAMPT is induced by exercise in humans. *Am J Physiol Endocrinol Metab* 298:E117-E126, 2010
71. Brandauer,J, Vienberg,SG, Andersen,MA, Ringholm,S, Risis,S, Larsen,PS, Kristensen,JM, Frosig,C, Leick,L, Fentz,J, Jorgensen,S, Kiens,B, Wojtaszewski,JF, Richter,EA, Zierath,JR, Goodyear,LJ, Pilegaard,H, Treebak,JT: AMP-activated protein kinase regulates nicotinamide phosphoribosyl transferase expression in skeletal muscle. *J Physiol* 591:5207-5220, 2013
72. Frydelund-Larsen,L, Akerstrom,T, Nielsen,S, Keller,P, Keller,C, Pedersen,BK: Visfatin mRNA expression in human subcutaneous adipose tissue is regulated by exercise. *Am J Physiol Endocrinol Metab* 292:E24-E31, 2007
73. Ghanbari-Niaki,A, Saghebjoo,M, Soltani,R, Kirwan,JP: Plasma visfatin is increased after high-intensity exercise. *Ann Nutr Metab* 57:3-8, 2010
74. Choi,KM, Kim,JH, Cho,GJ, Baik,SH, Park,HS, Kim,SM: Effect of exercise training on plasma visfatin and eotaxin levels. *Eur J Endocrinol* 157:437-442, 2007
75. Haus,JM, Solomon,TP, Marchetti,CM, O'Leary,VB, Brooks,LM, Gonzalez,F, Kirwan,JP: Decreased visfatin after exercise training correlates with improved glucose tolerance. *Med Sci Sports Exerc* 41:1255-1260, 2009
76. Lee,KJ, Shin,YA, Lee,KY, Jun,TW, Song,W: Aerobic exercise training-induced decrease in plasma visfatin and insulin resistance in obese female adolescents. *Int J Sport Nutr Exerc Metab* 20:275-281, 2010
77. Delaigle,AM, Jonas,JC, Bauche,IB, Cornu,O, Brichard,SM: Induction of adiponectin in skeletal muscle by inflammatory cytokines: in vivo and in vitro studies. *Endocrinology* 145:5589-5597, 2004

78. Van Berendoncks,AM, Garnier,A, Beckers,P, Hoymans,VY, Possemiers,N, Fortin,D, Martinet,W, Van,H, V, Vrints,CJ, Ventura-Clapier,R, Conraads,VM: Functional adiponectin resistance at the level of the skeletal muscle in mild to moderate chronic heart failure. *Circ Heart Fail* 3:185-194, 2010
79. Bluher,M, Bullen,JW, Jr., Lee,JH, Kralisch,S, Fasshauer,M, Kloting,N, Niebauer,J, Schon,MR, Williams,CJ, Mantzoros,CS: Circulating adiponectin and expression of adiponectin receptors in human skeletal muscle: associations with metabolic parameters and insulin resistance and regulation by physical training. *J Clin Endocrinol Metab* 91:2310-2316, 2006
80. Van Berendoncks,AM, Garnier,A, Beckers,P, Hoymans,VY, Possemiers,N, Fortin,D, Van,H, V, Dewilde,S, Vrints,CJ, Ventura-Clapier,R, Conraads,VM: Exercise training reverses adiponectin resistance in skeletal muscle of patients with chronic heart failure. *Heart* 97:1403-1409, 2011
81. Wang,J, Liu,R, Hawkins,M, Barzilai,N, Rossetti,L: A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. *Nature* 393:684-688, 1998
82. Wolsk,E, Mygind,H, Grondahl,TS, Pedersen,BK, van,HG: Human skeletal muscle releases leptin in vivo. *Cytokine* 60:667-673, 2012
83. Lappas,M, Yee,K, Permezel,M, Rice,GE: Release and regulation of leptin, resistin and adiponectin from human placenta, fetal membranes, and maternal adipose tissue and skeletal muscle from normal and gestational diabetes mellitus-complicated pregnancies. *J Endocrinol* 186:457-465, 2005
84. Kolset,SO, Pejler,G: Serglycin: a structural and functional chameleon with wide impact on immune cells. *J Immunol* 187:4927-4933, 2011
85. Yamauchi,T, Iwabu,M, Okada-Iwabu,M, Kadowaki,T: Adiponectin receptors: a review of their structure, function and how they work. *Best Pract Res Clin Endocrinol Metab* 28:15-23, 2014
86. Moschen,AR, Wieser,V, Tilg,H: Adiponectin: key player in the adipose tissue-liver crosstalk. *Curr Med Chem* 19:5467-5473, 2012
87. Han,SH, Quon,MJ, Kim,JA, Koh,KK: Adiponectin and cardiovascular disease: response to therapeutic interventions. *J Am Coll Cardiol* 49:531-538, 2007
88. Christiansen,T, Bruun,JM, Paulsen,SK, Olholm,J, Overgaard,K, Pedersen,SB, Richelsen,B: Acute exercise increases circulating inflammatory markers in overweight and obese compared with lean subjects. *Eur J Appl Physiol* 2013
89. Bobbert,T, Wegewitz,U, Brechtel,L, Freudenberg,M, Mai,K, Mohlig,M, Diederich,S, Ristow,M, Rochlitz,H, Pfeiffer,AF, Spranger,J: Adiponectin oligomers in human serum during acute and chronic exercise: relation to lipid metabolism and insulin sensitivity. *Int J Sports Med* 28:1-8, 2007
90. Ferguson,MA, White,LJ, McCoy,S, Kim,HW, Petty,T, Wilsey,J: Plasma adiponectin response to acute exercise in healthy subjects. *Eur J Appl Physiol* 91:324-329, 2004
91. Jamurtas,AZ, Theocharis,V, Koukoulis,G, Stakias,N, Fatouros,IG, Kouretas,D, Koutedakis,Y: The effects of acute exercise on serum adiponectin and resistin levels and their relation to insulin sensitivity in overweight males. *Eur J Appl Physiol* 97:122-126, 2006
92. Bouassida,A, Lakhdar,N, Benaissa,N, Mejri,S, Zaouali,M, Zbidi,A, Tabka,Z: Adiponectin responses to acute moderate and heavy exercises in overweight middle aged subjects. *J Sports Med Phys Fitness* 50:330-335, 2010
93. Numao,S, Katayama,Y, Hayashi,Y, Matsuo,T, Tanaka,K: Influence of acute aerobic exercise on adiponectin oligomer concentrations in middle-aged abdominally obese men. *Metabolism* 60:186-194, 2011
94. Saunders,TJ, Palombella,A, McGuire,KA, Janiszewski,PM, Despres,JP, Ross,R: Acute exercise increases adiponectin levels in abdominally obese men. *J Nutr Metab* 2012:148729, 2012
95. Christiansen,T, Paulsen,SK, Bruun,JM, Pedersen,SB, Richelsen,B: Exercise training versus diet-induced weight-loss on metabolic risk factors and inflammatory markers in obese subjects: a 12-week randomized intervention study. *Am J Physiol Endocrinol Metab* 298:E824-E831, 2010
96. Hulver,MW, Zheng,D, Tanner,CJ, Houmard,JA, Kraus,WE, Slentz,CA, Sinha,MK, Pories,WJ, MacDonald,KG, Dohm,GL: Adiponectin is not altered with exercise training despite enhanced insulin action. *Am J Physiol Endocrinol Metab* 283:E861-E865, 2002
97. Moghadasi,M, Mohebbi,H, Rahmani-Nia,F, Hassan-Nia,S, Noroozi,H: Effects of short-term lifestyle activity modification on adiponectin mRNA expression and plasma concentrations. *Eur J Sport Sci* 13:378-385, 2013
98. Polak,J, Klimcakova,E, Moro,C, Viguerie,N, Berlan,M, Hejnova,J, Richterova,B, Kraus,I, Langin,D, Stich,V: Effect of aerobic training on plasma levels and subcutaneous abdominal adipose tissue gene expression of adiponectin, leptin, interleukin 6, and tumor necrosis factor alpha in obese women. *Metabolism* 55:1375-1381, 2006
99. Kondo,T, Kobayashi,I, Murakami,M: Effect of exercise on circulating adipokine levels in obese young women. *Endocr J* 53:189-195, 2006
100. Klimcakova,E, Polak,J, Moro,C, Hejnova,J, Majercik,M, Viguerie,N, Berlan,M, Langin,D, Stich,V: Dynamic strength training improves insulin sensitivity without altering plasma levels

and gene expression of adipokines in subcutaneous adipose tissue in obese men. *J Clin Endocrinol Metab* 91:5107-5112, 2006

101. Phillips,MD, Patrizi,RM, Cheek,DJ, Wooten,JS, Barbee,JJ, Mitchell,JB: Resistance training reduces subclinical inflammation in obese, postmenopausal women. *Med Sci Sports Exerc* 44:2099-2110, 2012
102. Fatouros,IG, Tournis,S, Leontsini,D, Jamurtas,AZ, Sxina,M, Thomakos,P, Manousaki,M, Douroudos,I, Taxildaris,K, Mitrakou,A: Leptin and adiponectin responses in overweight inactive elderly following resistance training and detraining are intensity related. *J Clin Endocrinol Metab* 90:5970-5977, 2005
103. Fatouros,IG, Chatzinikolaou,A, Tournis,S, Nikolaidis,MG, Jamurtas,AZ, Douroudos,II, Papassotiriou,I, Thomakos,PM, Taxildaris,K, Mastorakos,G, Mitrakou,A: Intensity of resistance exercise determines adipokine and resting energy expenditure responses in overweight elderly individuals. *Diabetes Care* 32:2161-2167, 2009
104. Munzberg,H, Morrison,CD: Structure, production and signaling of leptin. *Metabolism* 2014
105. Keller,P, Keller,C, Steensberg,A, Robinson,LE, Pedersen,BK: Leptin gene expression and systemic levels in healthy men: effect of exercise, carbohydrate, interleukin-6, and epinephrine. *J Appl Physiol (1985)* 98:1805-1812, 2005
106. Perusse,L, Collier,G, Gagnon,J, Leon,AS, Rao,DC, Skinner,JS, Wilmore,JH, Nadeau,A, Zimmet,PZ, Bouchard,C: Acute and chronic effects of exercise on leptin levels in humans. *J Appl Physiol (1985)* 83:5-10, 1997
107. Varady,KA, Bhutani,S, Church,EC, Phillips,SA: Adipokine responses to acute resistance exercise in trained and untrained men. *Med Sci Sports Exerc* 42:456-462, 2010
108. Essig,DA, Alderson,NL, Ferguson,MA, Bartoli,WP, Durstine,JL: Delayed effects of exercise on the plasma leptin concentration. *Metabolism* 49:395-399, 2000
109. Olive,JL, Miller,GD: Differential effects of maximal- and moderate-intensity runs on plasma leptin in healthy trained subjects. *Nutrition* 17:365-369, 2001
110. Yang,CB, Chuang,CC, Kuo,CS, Hsu,CH, Tsao,TH: Effects of an acute bout of exercise on serum soluble leptin receptor (sOB-R) levels. *J Sports Sci* 32:446-451, 2014
111. O'Leary,VB, Marchetti,CM, Krishnan,RK, Stetzer,BP, Gonzalez,F, Kirwan,JP: Exercise-induced reversal of insulin resistance in obese elderly is associated with reduced visceral fat. *J Appl Physiol (1985)* 100:1584-1589, 2006
112. Harris,RA, Padilla,J, Hanlon,KP, Rink,LD, Wallace,JP: The flow-mediated dilation response to acute exercise in overweight active and inactive men. *Obesity (Silver Spring)* 16:578-584, 2008
113. Baturcam,E, Abubaker,J, Tiss,A, Abu-Farha,M, Khadir,A, Al-Ghimlas,F, Al-Khairi,I, Cherian,P, Elkum,N, Hammad,M, John,J, Kavalakatt,S, Lehe,C, Warsame,S, Behbehani,K, Dermime,S, Dehbi,M: Physical exercise reduces the expression of RANTES and its CCR5 receptor in the adipose tissue of obese humans. *Mediators Inflamm* 2014:627150, 2014
114. Abubaker,J, Tiss,A, Abu-Farha,M, Al-Ghimlas,F, Al-Khairi,I, Baturcam,E, Cherian,P, Elkum,N, Hammad,M, John,J, Kavalakatt,S, Khadir,A, Warsame,S, Dermime,S, Behbehani,K, Dehbi,M: DNAJB3/HSP-40 cochaperone is downregulated in obese humans and is restored by physical exercise. *PLoS One* 8:e69217, 2013
115. Nicklas,BJ, Ambrosius,W, Messier,SP, Miller,GD, Penninx,BW, Loeser,RF, Palla,S, Bleecker,E, Pahor,M: Diet-induced weight loss, exercise, and chronic inflammation in older, obese adults: a randomized controlled clinical trial. *Am J Clin Nutr* 79:544-551, 2004
116. Dandona,P, Aljada,A, Bandyopadhyay,A: Inflammation: the link between insulin resistance, obesity and diabetes. *Trends Immunol* 25:4-7, 2004
117. Duncan,BB, Schmidt,MI, Pankow,JS, Ballantyne,CM, Couper,D, Vigo,A, Hoogeveen,R, Folsom,AR, Heiss,G: Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study. *Diabetes* 52:1799-1805, 2003
118. Kern,PA, Ranganathan,S, Li,C, Wood,L, Ranganathan,G: Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab* 280:E745-E751, 2001
119. Codoner-Franch,P, Alonso-Iglesias,E: Resistin: Insulin resistance to malignancy. *Clin Chim Acta* 438C:46-54, 2014
120. de Luis,DA, Aller,R, Izaola,O, Sagrado,MG, Conde,R: Influence of ALA54THR polymorphism of fatty acid binding protein 2 on lifestyle modification response in obese subjects. *Ann Nutr Metab* 50:354-360, 2006
121. Berndt,J, Kloting,N, Kralisch,S, Kovacs,P, Fasshauer,M, Schon,MR, Stumvoll,M, Bluher,M: Plasma visfatin concentrations and fat depot-specific mRNA expression in humans. *Diabetes* 54:2911-2916, 2005
122. Cullberg,KB, Christiansen,T, Paulsen,SK, Bruun,JM, Pedersen,SB, Richelsen,B: Effect of weight loss and exercise on angiogenic factors in the circulation and in adipose tissue in obese subjects. *Obesity (Silver Spring)* 21:454-460, 2013

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2.6 HIF-1α is a critical determinant for insulin- and contraction-regulated glucose uptake in human skeletal muscle

submitted to Diabetes, Mai 2015

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Abstract— **Skeletal muscle insulin resistance is the hallmark of type 2 diabetes, and develops long before the onset of the disease. It is well accepted that physical activity improves glycemic control but the knowledge on underlying mechanisms mediating the beneficial effects remains incomplete. Exercise is accompanied by a decrease in intramuscular oxygen levels resulting in induction of HIF-1α. HIF-1α is a master regulator of gene expression and could play an important role in skeletal muscle metabolism. We examined the role of O2 in insulin- and contraction-stimulated glucose metabolism in primary human skeletal muscle cells. We show that exposure to 7% O2 in combination with electrical pulse stimulation (EPS) improves insulin action. Furthermore, the combination of 7% O2 and EPS resulted in an enhanced IL-6 secretion as well as HIF-1α and GLUT4 protein expression. Knockdown of HIF-1α totally inhibits the insulin- and contraction-induced glucose uptake, whereas up-regulation of HIF-1α by CoCl2, strongly enhanced insulin signaling. Moreover, human skeletal muscle HIF-1α mRNA expression was enhanced after acute and chronic exercise and negatively correlated with fasting blood glucose levels. In conclusion, we demonstrate that HIF-1α is involved in the regulation of glucose metabolism in human skeletal muscle induced by both insulin and contraction.**

Index Terms— **Skeletal muscle, Exercise, Hypoxia, HIF-1α**

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I. INTRODUCTION

The health-promoting effects of physical activity are well known, and regular exercise has been shown to protect against the development of various chronic diseases such as obesity, insulin resistance and type 2 diabetes (T2DM) (1;2). However, our knowledge on underlying mechanisms mediating the beneficial effects of physical activity remains incomplete.

Skeletal muscle is the largest organ involved in postprandial peripheral glucose disposal and hence a target for approaches that aim to improve insulin sensitivity in conditions of insulin resistance such as obesity and T2DM (1). Binding of insulin to the insulin receptor on the surface of skeletal muscle cells induces insulin receptor substrate-1 (IRS-1) phosphorylation and IRS-1-asscociated phosphatidyl-inositol 3-phosphate kinase activation. This cascade affects activation of downstream targets such as protein kinase B (Akt), which further activates the translocation of glucose transporter 4 (GLUT4) (3). Moreover, in skeletal muscle GLUT4 translocation occurs in response to contractile activity, even in the absence of insulin (4). The most prominent GLUT4 translocation and resulting glucose uptake is observed when insulin and muscle contraction act together, indicating an additive effect of both stimuli (5). Therefore, an adequate GLUT4 expression and translocation is fundamental for skeletal muscle glucose uptake.

The GLUT4 gene regulatory region is well described and contains several responsive elements to transcriptional factors. An E-box domain, which can bind members of the basic helix loop helix (bHLH) family including the hypoxia inducible factor 1α (HIF 1α), is also present (6). HIF-1α is a heterodimeric transcription factor composed of HIF-1α and HIF-1ß subunits (7). In response to cellular hypoxia HIF-1α is activated to regulate the transcription of more than 1000 target genes, e.g. vascular endothelial growth factors (VEGF), glycolytic enzymes and glucose transporters (8). The detailed mechanism for activation of HIF-1α and its regulation by oxygen has been reviewed in detail by Schofield and Ratcliffe (9).

One particular aspect of skeletal muscle is that HIF-1α protein is highly expressed in this tissue even in normoxic conditions, suggesting that HIF-1α could have functional impact on in muscle homeostasis in normoxia (10). We investigated the effect of different oxygen levels in combination with electrical pulse stimulation (EPS) on insulin-stimulated signaling and glucose uptake in primary human skeletal muscle cells, to identify conditions which are associated with enhanced insulin action. Furthermore, we examined the role of HIF-1α in insulin- and contraction-stimulated glucose uptake, and analyzed the effect of an exercise intervention on the

expression of HIF-1α in skeletal muscle in control and dysglycemic subjects. Our data suggest that HIF-1α is a critical determinant for glucose metabolism in human skeletal muscle cells, and that the insulin-sensitizing effect of exercise is at least partly a result of HIF-1α regulated gene expression of the insulin signaling cascade and related downstream metabolic targets.

II. MATERIAL AND METHODS

Materials: 2-deoxy-D-[14C] glucose (2-DOG) and L-[14C] glucose were purchased from GE Healthcare (Uppsala, Sweden). Liquid scintillation Aquasafe 300 plus from Zinsser Analytic (Frankfurt, Germany) was used for glucose uptake assays. Primary human skeletal muscle cells (hSkMC) and supplement pack for growth medium were obtained from PromoCell (Heidelberg, Germany). All other chemicals were of the highest analytical grade commercially available and purchased from Sigma or Applichem.

Culture of human skeletal muscle cells: Primary hSkMC isolated from 8 healthy Caucasian donors (4 males 16, 21, 41 and 47 of age; 4 females 16, 25, 33 and 37 of age) were supplied as proliferating myoblasts and cultured as described before (11). For an individual experiment, myoblasts were cultured in growth medium (α-modified Eagle's (αMEM)/Ham's F-12 medium containing growth medium supplement pack) up to near-confluence. The cells were then differentiated in αMEM until day 6 of differentiation.

Electrical Pulse Stimulation (EPS): Differentiated myotubes were subjected to EPS treatment using a C-Dish in combination with a C-Pace pulse generator (C-Pace 100, IonOptix, Milton, MA, USA), as recently described (12). Cells were stimulated with a frequency of 1 Hz, a pulse duration of 2 ms and an intensity of 11.5 V for 24 h.

Combination of EPS and reduced oxygen tension: Differentiated myotubes were cultured for 24 h with or without EPS in an atmosphere containing 21%, 7% or 2% O2 supplemented with 5% CO2 and respective concentrations of nitrogen in an Xvivo hypoxia chamber system (Biospherix, US).

Glucose uptake: Differentiated hSkMC were stimulated with 100 nmol/L insulin for 30 min. Then, 2-DOG (9.25 kBq/ml per well) was added and uptake was measured for 2 h. The radioactivity of the lysates was counted in a liquid scintillation counter (Beckman, Munich, Germany). Values were corrected for non-specific uptake as measured after incubation with L-[14C] glucose.

RT-PCR and Western Blotting: These studies were performed as previously described (11). Total RNA was isolated using the RNeasy kit from Qiagen (Hilden, Germany). GLUT4 (Hs_SLC2A4_1_SG), GLUT1 (Hs_SLC2A1_1_SG) and HIF-1A (Hs_HIF1A_1_SG) mRNA expression were measured using pre-designed primers (Quantitect Primer Assay, Qiagen) and GoTaq qPCR Master Mix (Promega, Mannheim, Germany) in a SYBR Green-based quantitative real-time PCR. HIF-1α, anti-phospho Akt (Ser473), anti-phospho AMPKα (Thr172), anti-phospho Akt (Thr308), anti-phospho AS160 (Thr642), total Akt and total AS160 were supplied by Cell Signalling Technology (Frankfurt, Germany).

Silencing of HIF-1α: Silencing experiments were performed by using FlexiTube siRNA HIF1A (SI05073250) and HiPerfect (Qiagen) according to the manufacturers' instructions. At day 4 of differentiation hSkMC were incubated with 40 nM siRNA and HiPerfect. To control for unspecific effects, control cells were treated with AllStars Negative Control siRNA (QIAGEN, 1027280).

Human study - 12 weeks exercise intervention study: All participants were informed about the project, procedures and rights before signing an agreement form. The studies were approved by the Regional Committee for Medical and Health Research Ethics, Region North, Norway (2011/927b).

Physically inactive men (40–65 years) were recruited in two groups I) healthy participants with a BMI of 23.5 ± 2.0 kg/m2 and normal glucose metabolism (control group, n = 12) and II) participants with a BMI of 29.0 ± 2.4 kg/m2) with abnormal glucose metabolism (dysglycemic group, n = 11). Abnormal glucose metabolism was defined as fasting glucose ≥ 5.6 mmol/L and/or impaired glucose tolerance (2 h serum glucose ≥ 7.8 mmol/L). Two subjects with a BMI>28 kg/m2 but with normal fasting and 2 h serum glucose levels were excluded. One control subject was excluded from the statistical analyses, because HIF-1α mRNA expression deviated strongly (Grubbs' test; $p<0.0001$). The participants were subjected to a combined strength and endurance training program for 12 weeks as described previously (13;14). A 45 min bicycle session at 70% VO2max was performed before and after the 12-weeks training period as an acute exercise challenge (13). Blood and skeletal muscle samples were taken before, directly after, and 2 h after the acute exercise before as well as after 12 weeks of training. Biopsies from m. vastus lateralis were taken and immediately transferred to RNA-later (Qiagen, Limburg, Netherlands), kept overnight at 4°C, and transferred to -80°C. For one subject no muscle biopsies were taken at 2 h post exercise before as well as after the intervention.

High throughput mRNA sequencing and differential expression analysis: Skeletal muscle biopsies sampled before the start of acute exercise at baseline and after 12 weeks of training were deep-sequenced in 6 batches using the Illumina HiSeq 2000 system (Illumina, San Diego, USA) with multiplexing at the Norwegian Sequencing Centre, University of Oslo. Samples in different groups and time points were multiplexed between batches to avoid batch effects. Illumina HiSeq RTA (real-time analysis) v1.17.21.3 (Illumina) was used for real-time analysis during the sequencing. Reads passing Illumina's recommended parameters were demultiplexed using CASAVA v1.8.2 (Illumina). For prealignment quality checks we used the software FastQC v0.10.1. (Babraham Bioinformatics, Cambridge, UK). To avoid low quality data negatively influencing downstream analysis, reads were trimmed on the 3′-end and only the first 51 bp from the 5′-end of each read were kept for further analysis. The

mean library size was ~44 million unstranded 51 bp single-ended reads with no difference between groups or time points. Base composition in bases 1–12 showed patterns typical for RNA-seq and bases 13-51 were evenly distributed. All base positions were of high quality (Phred score >30). Alignment of sequenced cDNA reads to the RefSeqGene database as transcriptome reference and quantitative analysis of the transcripts was performed as described by Li et al. (2014) (16).

endpoint assay at different oxygen levels, alone or in combination with EPS, to assess the effect of different O_2 levels on human skeletal muscle insulin sensitivity. Therefore, we cultured differentiated myotubes (Suppl. Fig. 1) at four different oxygen levels (21%, 14%, 7% and 2%) with or without EPS for 24 h and analyzed basal and insulin-stimulated glucose uptake (Fig. 1 a, c and e). EPS at 21% O_2 significantly increased basal and insulin-stimulated glucose uptake (Fig.1 a). However, the

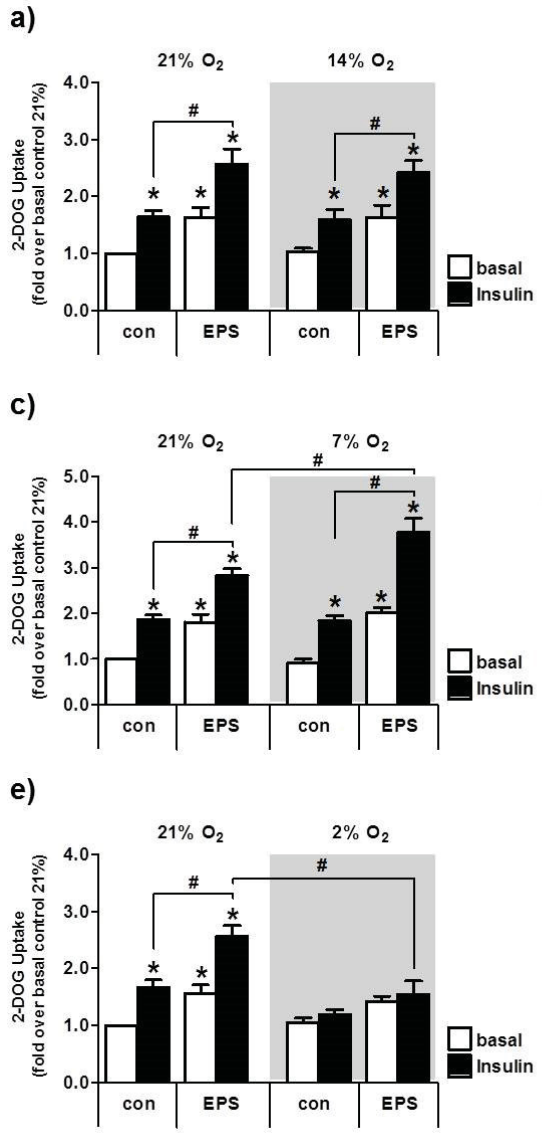

Figure 1: EPS in combination with lower oxygen levels enhanced insulin-stimulated glucose uptake: (**a**, **c** and **e**) Differentiated myotubes were cultured at 21%, 14%, 7% or 2% O_2 with or without EPS for 24h followed by insulin stimulation (100 nmol/L) for 30 min. Glucose uptake was assessed for 2 h. (**b**) Lactate concentrations were measured in the supernatants after EPS at 21% and 7% O_2. (**d**) Representative Western Blot image and quantification of the phosphorylation of AMPKα(Thr172) after indicated treatment. Data are mean values ± SEM, n=4-7, *p<0.05 vs. basal control 21% O_2 and $^{\#}$p<0.05 as indicated. AU = arbitrary units.

Presentation of data and statistics: Statistical evaluation was done by Student's t-tests for paired or unpaired observations and two-way ANOVA (post hoc Bonferroni multiple comparison test). Correlation analyses were performed in Prism5 (GraphPad, LA Jolla, CA). Pearson's correlation coefficient was used on normal distributed parameters. Data are presented as means ± SEM.

III. RESULTUS

Changes in oxygen tension alters insulin action in human skeletal muscle cells: We first performed insulin-stimulated glucose uptake studies as a biological

combination of EPS and a reduced oxygen level of 14% had no additive effects on basal and insulin-stimulated glucose uptake compared to EPS at 21% O_2 (Fig. 1 a). Also, 14% O_2 without EPS showed no significant differences compared to 21% O_2 (Fig. 1 a). EPS in combination with 7% O_2 strongly increased insulin-stimulated glucose uptake compared to EPS at 21% O_2, whereas the basal glucose uptake showed no significant difference (Fig. 1 c). Cells cultured at 2% O_2 showed no response to insulin with respect to glucose uptake (Fig. 1 e). Moreover, using the combination of 2% O_2 and EPS we observed a total inhibition of insulin- and EPS-induced glucose uptake (Fig. 1 e).

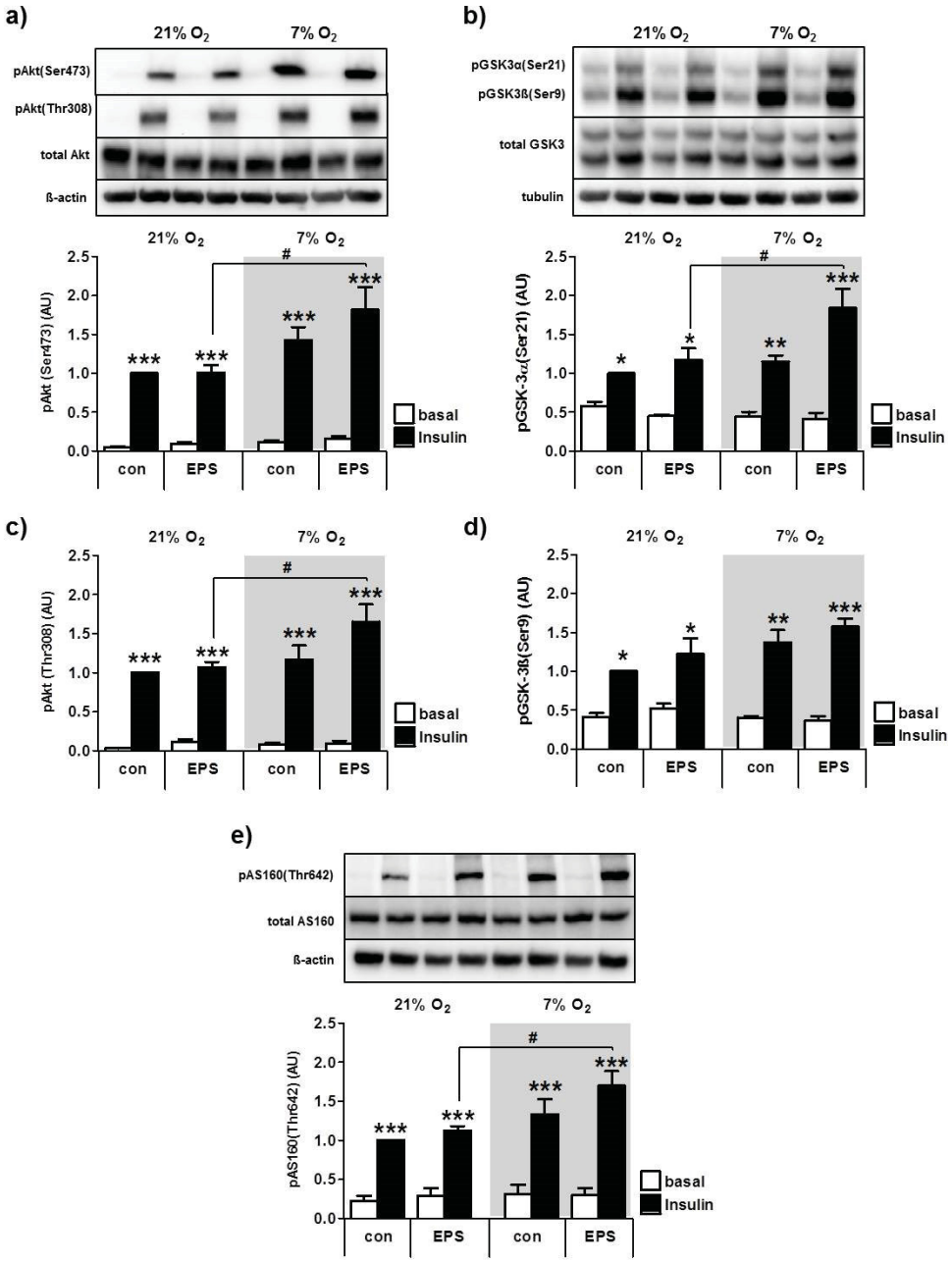

Figure 2: EPS and 7% O_2 enhanced insulin signaling: (**a** and **c**) Representative Western Blot images show phosphorylation of Akt(Ser473), Akt(Thr308) as well as total Akt protein level after indicated treatment. Quantification of Akt(Ser473) and Akt(Thr308) phosphorylation. (**b** and **d**) Representative Western Blot images show phosphorylation of GSK-3α(Ser21) and GSK-3ß(Ser9) as well as total GSK-3α/ß protein level after indicated treatment. Quantification of GSK-3α(Ser21) and GSK-3ß(Ser9) phosphorylation. (**e**) Representative Western Blot images and quantification of AS160(Thr642) phosphorylation after indicated treatment. Data are normalized to β-actin and expressed relative to control. Data are mean values ± SEM, n=4-5, *p<0.05, **p<0.005 and ***p<0.0001 vs. corresponding basal, #p<0.05 as indicated. AU = arbitrary units.

Because the combination of 7% O_2 and EPS significantly increased insulin-stimulated glucose uptake, all further experiments were performed using these conditions. To exclude that 7% O_2 had any effect on the contractile activity, we measured phosphorylation of AMPKα and the production of lactate (Fig. 1 b and d). AMPKα activation was significantly increased at both O_2 conditions after EPS (Fig. 1 d). However, EPS in combination with 7% O_2 enhanced the EPS-induced lactate release (Fig. 1 b).

To elucidate the underlying mechanism of regulation of insulin-stimulated glucose uptake by combination of 7% O_2 and EPS, we analyzed the key components of insulin signal transduction in skeletal muscle cells. In accordance with an enhanced insulin-stimulated glucose uptake, we also observed increased insulin signaling (Fig. 2 a-e). 7% O_2 in combination with EPS enhanced insulin-stimulated Akt(Ser473), Akt(Thr308), GSK-3α(Ser21) and AS160(Thr642) phosphorylation compared to EPS at 21% O_2 (Fig 2 a-e). However, insulin-stimulated GSK-3ß(Ser9) phosphorylation was not altered at 7% O_2 (Fig. 2 d).

EPS and 7% O_2 induce HIF-1α and GLUT4 expression: Reduced oxygen levels and muscle contraction are well known stimuli for HIF-1α induction. Therefore, we analyzed HIF-1α protein level as well as HIF-1α mRNA expression (Fig. 3 a-c). After 8h incubation we observed a significant increase of HIF-1α protein levels at 7% O_2 and in the combination of 7% O_2 with EPS (Fig. 3 a). The elevated HIF-1 α protein levels remained high up to 24h in the combined setting (Fig. 3 b) while HIF-1α mRNA expression was decreased (Fig. 3 c). The well-known HIF-1α target gene GLUT1 was not regulated at these conditions (Fig. 3 d). Interestingly, GLUT4 protein level as well as GLUT4 mRNA expression were significant up-regulated in the combined setting of 7% O2 and EPS (Fig. 3 e and f).

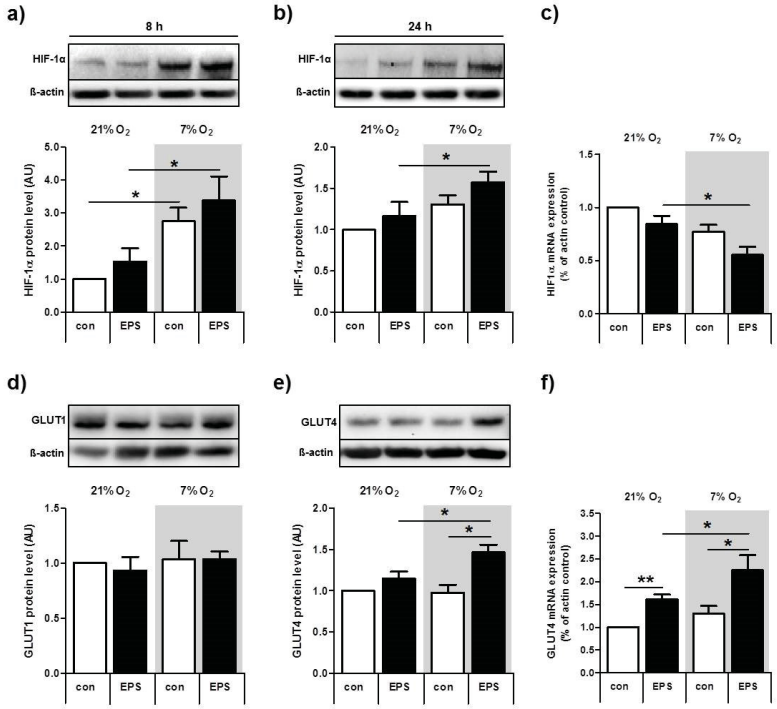

Figure 3: EPS and 7% O_2 induced HIF-1α and GLUT4: (**a** and **b**) Quantification and representative Western Blot images show HIF-1α protein level after 8 and 24h . (**d**) Representative Western Blot images and quantification of GLUT1 protein level after indicated treatment. (**e**) Quantification and representative Western Blot images show GLUT4 protein level after indicated treatment. Data are normalized to β-actin and expressed relative to control. (**c** and **f**) Comparison of HIF-1α and GLUT4 mRNA expression. mRNA expression was analyzed by real-time PCR and data were normalized to β-actin. Data are mean values ± SEM, n=3-5, *p<0.05 and **p<0.001 as indicated. AU = arbitrary units.

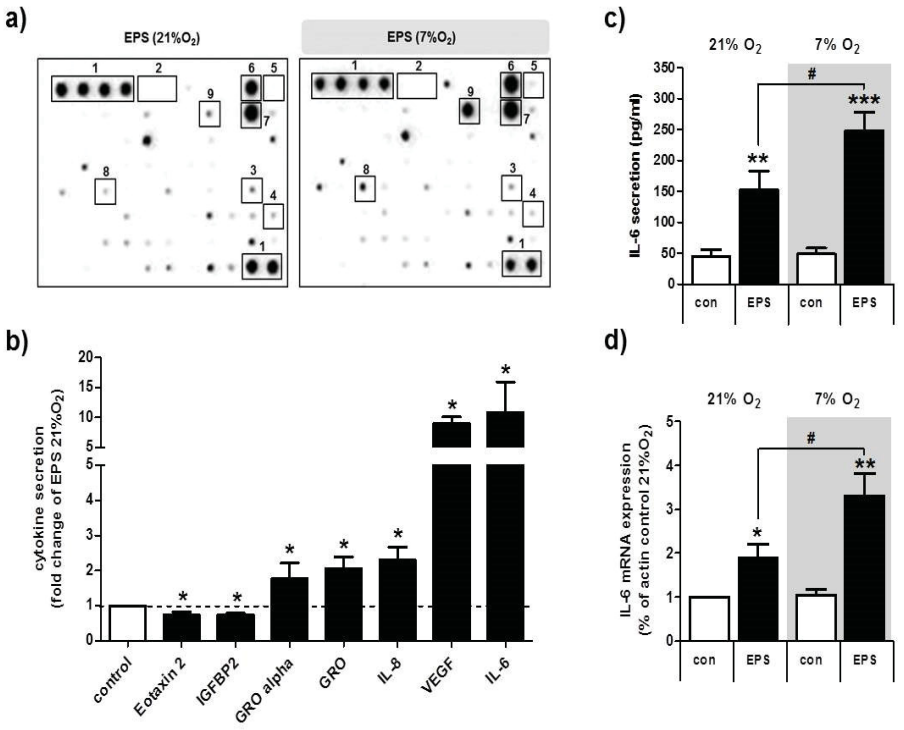

Figure 4: EPS and 7%O_2 altered myokine secretion: Supernatants of EPS-treated cells at 21% or 7% O_2 were collected after 24h and analyzed as described in Material and Methods. (**a**) Cytokine antibody array membranes #5 after incubation with supernatants of EPS-treated cells are shown. The encircled areas reflect spots corresponding to the myokines shown in Fig.4 **b**. 1 = positive control; 2 = negative control; 3 = Eotaxin-2; 4 = IGFBP2; 5 = Gro alpha; 6 = Gro; 7 = IL-8; 8 = VEGF and 9 = IL-6. n = 4, *p<0.05 vs. EPS at 21% O_2. (**c**) IL-6 protein concentration was additionally analyzed by ELISA. (**d**) IL-6 mRNA expression was analyzed by real-time PCR and data were normalized to β-actin. Data are mean values ± SEM, n=7-8, *p<0.05, **p<0.005 and ***p<0.0001 vs. corresponding condition without EPS, #p<0.05 as indicated.

EPS and 7% O_2 alter myokine secretion: To further characterize the impact of reduced oxygen levels in combination with EPS, we analyzed the secretion of various myokines via a human cytokine antibody array (Fig. 4 a and b). 40 of the 80 cytokines were detectable in all experiments (Fig. 4 a and b; Suppl. Fig. 2). The strongest effects were observed for IL-6 and the well-known HIF-1α target gene VEGF-A (Fig. 4 a and b). EPS at 7% O_2 enhanced the secretion of IL-6 (~14-fold) and VEGF-A (~9-fold) compared to EPS at 21% O_2 (Fig. 4 b). Validation by ELISA confirmed the enhanced IL-6 secretion (Fig. 4 c). In addition, IL-6 mRNA expression was significantly higher in the combined setting of 7% O_2 and EPS vs. 21% O_2 and EPS (Fig. 4 d).

Loss of HIF-1α impairs insulin action in human skeletal muscle cells: To characterize HIF-1α as an important regulator of human skeletal muscle metabolism, we silenced HIF-1α and analyzed insulin signaling and glucose uptake in hSkMC. Silencing of HIF-1α by siRNA strongly down-regulated HIF-1α protein level (~60%) and mRNA expression (~50%) (Fig. 5 a and d). Also, the HIF-1α target genes GLUT1 and GLUT4 were down-regulated after silencing of HIF-1α (Fig. 5 b, c, e and f) whereas the untargeted siRNA

control (scramble) had no effect on HIF-1α, GLUT1 or GLUT4 protein expression (Fig. a-f). Furthermore, insulin receptor ß-subunit and 14-3-3 protein expression was not effected by HIF-1α silencing (Suppl. Fig. 4 a and b).

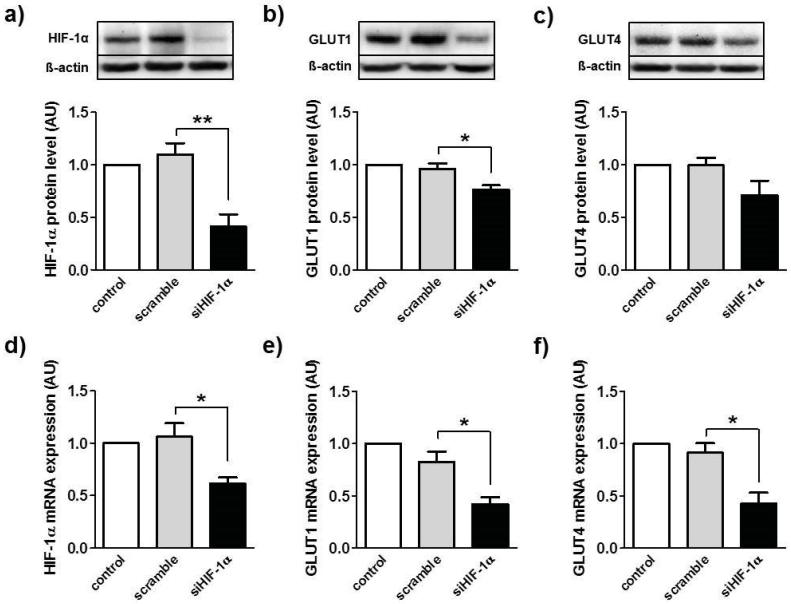

Figure 5: Knock-down of HIF-1α reduced GLUT1 and GLUT4 expression: (**a, b** and **c**) Quantification and representative Western Blot images show HIF-1α, GLUT1 and GLUT4 protein levels of untreated cells (control) or after treatment with 40 nM AllStars Negative Control siRNA (scramble) or HIF-1α siRNA (siHIF-1α). Data are normalized to β-actin and expressed relative to control. Data are mean values ± SEM, n=5, *p<0.05 and **p<0.005 as indicated. (**d, e** and **f**) HIF-1α, GLUT1 and GLUT4 mRNA expression was analyzed by real-time PCR and data were normalized to β-actin. Data are mean values ± SEM, n=5, *p<0.05 as indicated.

To examine the effect of HIF-1α silencing on insulin action, we treated the cells with insulin for 10 min and analyzed the insulin signaling pathway and glucose uptake. Interestingly, insulin-induced Akt(Ser), Akt(Thr) and AS160 phosphorylation were significantly reduced in siHIF-1α treated cells (Fig 6 a-c). In accordance with the decreased signaling, insulin-stimulated glucose uptake was totally inhibited by HIF-1α silencing (Fig. 6 d). To clarify if HIF-1α silencing has any effect on contraction-induced glucose uptake, we analyzed basal and insulin-stimulated glucose uptake after 24 h EPS. Interestingly, in addition to the insulin-induced glucose uptake also EPS-stimulated glucose uptake was totally blunted after HIF-1α silencing (Fig. 6 e). Moreover, insulin-treatment after EPS was not able to further increase the glucose uptake (Fig. 6 e). To exclude that HIF-1α has any effect on contractile activity, we measured phosphorylation of AMPKα and IL-6 secretion (Suppl. Fig. 5 a and b). EPS-induced IL-6 secretion as well as activation of AMPKα after EPS treatment was not affected by the loss of HIF-1α (Suppl. Fig. 5 a and b).

To verify that HIF-1α mediates the enhanced insulin action in the combined treatment of reduced O_2 levels and EPS, we silenced HIF-1α and cultured the cells for 24 h at 7%O_2 with or without EPS. Again, the combined setting of 7% O2 and EPS strongly induced insulin-stimulated glucose uptake whereas siHIF-1α-treated cells showed no response to insulin or muscle contraction (Fig. 6 f).

Finally, we tested whether an elevated HIF-α protein level improves insulin signaling. By using cobalt chloride ($COCl_2$) we were able to substantially augment the HIF-1α protein level (Suppl. Fig. 3 a). Under these conditions, we observed a strong increase in insulin-mediated Akt phosphorylation (Suppl. Fig. 3 b).

Exercise intervention increases HIF-1alpha expression: We analyzed HIF-1α mRNA expression in skeletal muscle biopsies after acute and chronic exercise from healthy subjects (control) and subjects with abnormal glucose metabolism (pT2D). Baseline HIF-1α mRNA expression was not different between both groups (Fig. 7 a). Interestingly, 12 weeks training composed of two endurance bicycle sessions (60 min) and two whole-body strength-training sessions (60 min) per week, significantly increased HIF-1α mRNA expression in both groups (Fig. 7 a). However, the expression of HIF-1α mRNA was slightly but not significantly reduced in the pT2D group (Fig. 7 a). To analyze acute induction of muscle HIF-1α, a 45-min bicycle session at 70% of VO_2max was performed before and after the 12-week training period as an acute work challenge. At baseline, HIF-1α mRNA expression was significantly up-regulated after acute exercise (Fig. 7 b). Moreover, the acute induction of HIF-1α was significantly higher after the 12-week training period (Fig. 7 b). To confirm our hypothesis that HIF-1α is an important factor for glucose metabolism *in vivo*, blood glucose and insulin levels of all subjects were related to HIF-1α mRNA expression. Skeletal muscle HIF-1α mRNA expression was negatively correlated with fasting blood glucose levels and showed a tendency for fasting insulin levels and HOMAR-IR (Fig. 7 c, d and e).

IV. DISCUSSION

By using a combination of 7% O_2 and EPS, we established and characterized a novel *in vitro* model for human skeletal muscle contraction at reduced oxygen levels which results in improved insulin sensitivity of the cells. The normal tissue O_2 level in adult skeletal muscle, measured by direct microelectrode analysis, varies between 1.8 to 10.5% depending on exact electrode placement (17). Csete et al. have shown that both skeletal

muscle satellite cell proliferation as well as survival of mature muscle fibers was increased at more physiologic (6%) O_2 levels compared to normal (21%) O_2 concentration used in most cell cultures (18). In addition, Storch et al. have shown that myogenesis of primary fiber cultures after 5-azacytidine treatment was enhanced at reduced O_2 levels compared to traditional cell culture O_2 conditions (19). Taken together, these observations indicate that the reduction of O_2 levels *in vitro* improves skeletal muscle function and metabolism which may be partly explained by the more physiological O_2 concentrations (2-11%) compared to the hyperoxic O_2 levels (20-21%) which are used in traditional cell cultures.

Interestingly, we show here that HIF-1α protein levels were elevated in the combined setting of reduced O_2 level and EPS, which resulted in enhanced insulin action, indicating that HIF-1α plays a potential role in the glucose metabolism in human skeletal muscle cells. In addition, knockdown of HIF-1α resulted in a severe reduction of insulin- and contraction-induced glucose uptake as well as insulin signaling, whereas up-regulation of HIF-1α protein by $COCl_2$ enhanced insulin signaling. Furthermore, HIF-1α siRNA-treated cells showed a reduced expression of GLUT1 and GLUT4. In line with our results, Sakagami et al. have demonstrated that knockdown of HIF-1α strongly reduced insulin-stimulated glucose uptake in murine skeletal muscle cells (20). Furthermore, AS160 phosphorylation and GLUT4 trafficking was increased in C_2C_{12} cells overexpressing constitutively active HIF-1α (20). In excellent agreement with *in vitro* analysis, HIF-1α+/- mice show reduced

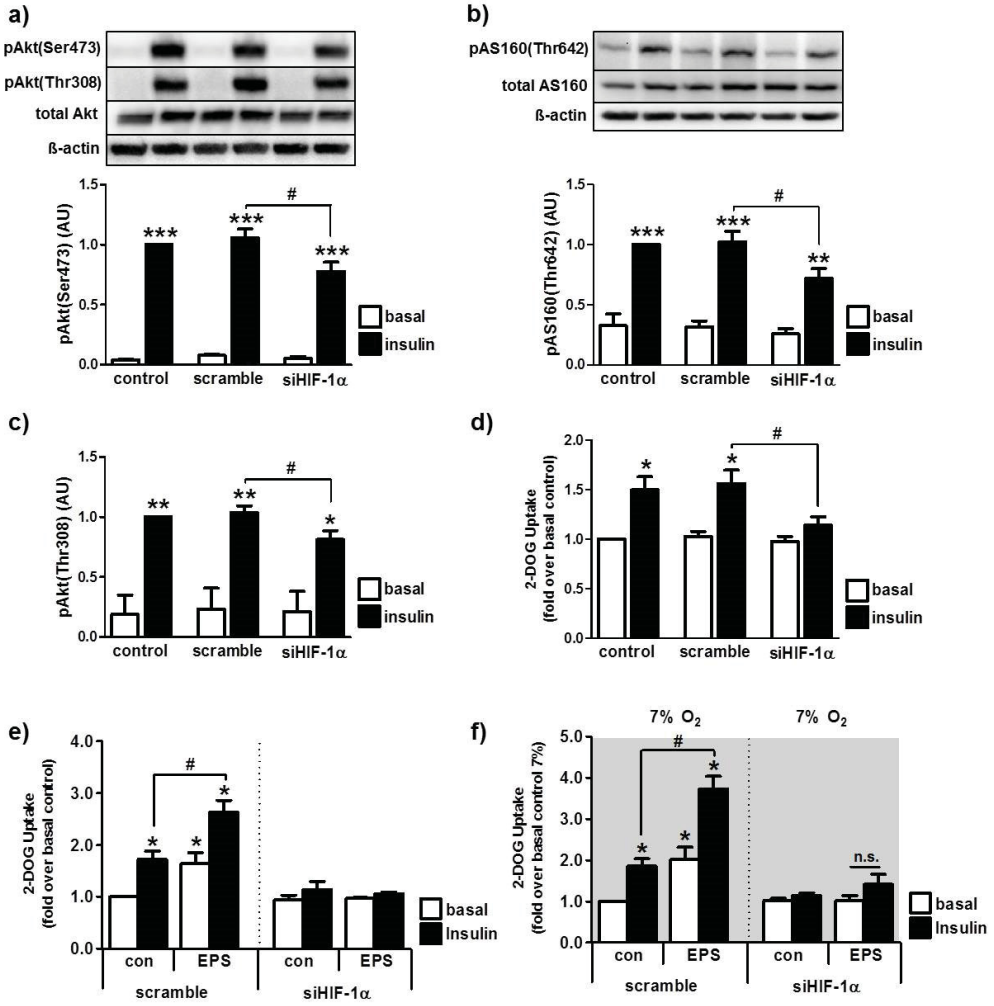

Figure 6: Knock-down of HIF-1α inhibited insulin- and contraction-induced glucose uptake: (**a** and **c**) Representative Western Blot images show phosphorylation of Akt(Ser473), Akt(Thr308) as well as total Akt protein level after indicated treatment. Quantification of Akt(Ser473) and Akt(Thr308) phosphorylation after indicated treatment. (**b**) Representative Western Blot images show phosphorylation of AS160(Thr642) and total AS160 protein levels after indicated treatment. Quantification of AS160(Thr642) phosphorylation. Data are normalized to β-actin and expressed relative to control. Data are mean values ± SEM, n=4. (**d**) Glucose uptake was assessed for 2 h. Data are mean values ± SEM n=7. (**e**) Glucose uptake after silencing experiments in combination with EPS-treatment. (**f**) siRNA-transfected cells were cultured at 7% O_2 with or without EPS for 24h followed by insulin stimulation (100 nmol/L) for 30 min. Glucose uptake was assessed for 2 h. Data are mean values ± SEM n=4, *p<0.05, **p<0.005 and ***p<0.0001 vs. corresponding basal and $^{\#}$p<0.05 as indicated.

glucose uptake under insulin-stimulated conditions (24). In addition, phosphorylation of AS160 in skeletal muscle isolated from HIF-1α+/- mice was reduced compared with specimens from wild-type animals (24). In summary, these data indicate HIF-1α as an important regulator of insulin-mediated glucose metabolism in skeletal muscle.

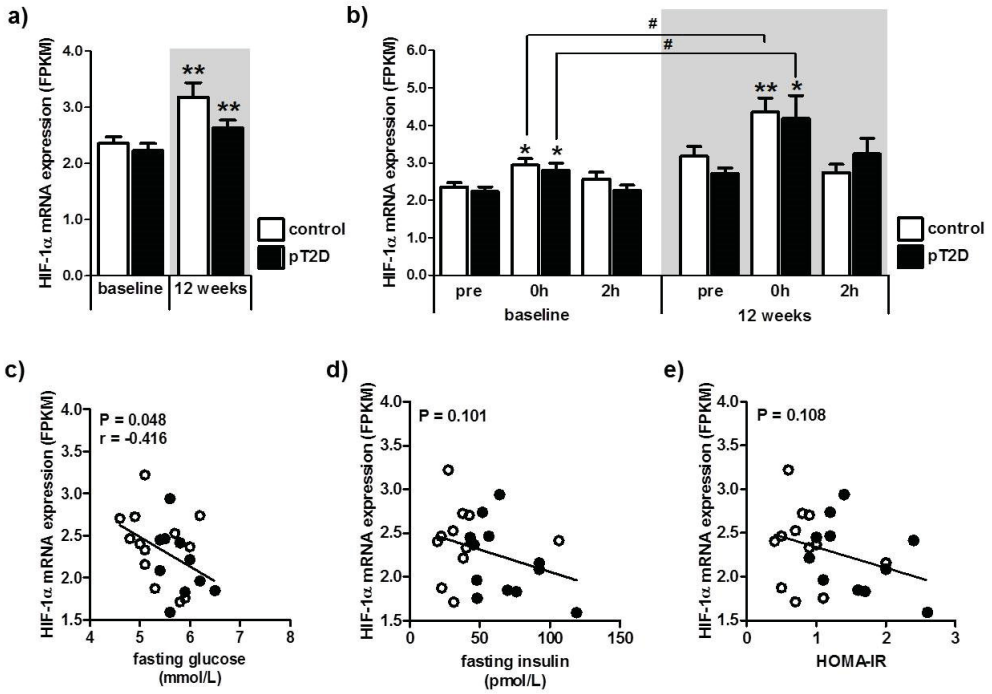

Figure 7: HIF-1α mRNA in *m. vastus lateralis* is regulated by exercise: Changes in *m. vastus lateralis* of HIF-1α mRNA expression in healthy (control; with bars, n=12) and pre-diabetic men (pT2D; black bars, n=11) in response to acute and chronic exercise. Samples were obtained before (pre), immediately after (0h), and after 2 h after exercise (2h) of 45-min ergometer cycling (70% VO_2max) at baseline and after 12 weeks training. Muscle biopsies were processed for mRNA expression analysis by mRNA sequencing. (**a**) Muscle HIF-1α mRNA expression at baseline and after 12 weeks training in control and pT2D group. (**b**) Changes in muscle HIF-1α mRNA expression after acute exercises at baseline and after 12 weeks of training. *p<0.05 and **p<0.005 between pre-exercise values and immediately (0h) or 2 h (2h) post exercise. #p<0.05 as indicated. Correlation of muscle HIF-1α mRNA and (**c**) fasting blood glucose levels, (**d**) fasting insulin levels as well as (**e**) HOMAR-IR. Statistical evaluation is indicated in the graphs. The r correlation coefficient and P values were obtained using Pearson's correlation.

The mechanism underlying HIF-1α-mediated regulation of Akt or AS160 phosphorylation is still unknown. The well-known recognized function of HIF-1α is its transcriptional regulation, and therefore it is most unlikely that HIF-1α directly affects the phosphorylation process of Akt or downstream targets like AS160. More than one thousand target genes were reported to be regulated by HIF-1α (7). Interestingly, 14-3-3 proteins, which interact with AS160 to regulate its function, might be regulated by hypoxia and therefore maybe also a target gene of HIF-1α (21;22). Therefore, we analyzed the expression of all seven 14-3-3 (ß/α, γ, ε, η, ζ/δ, θ/τ and σ) isoforms in HIF-1α siRNA-treated and EPS-treated (7% O_2) cells. However, we observed no significant differences (Suppl. Fig. 2 b). Further experiments are necessary to evaluate the underlying mechanism of how the transcription factor HIF-1α impacts the insulin signaling cascade.

Furthermore, we demonstrated for the first time that the loss of HIF-1α totally abolished the contraction-induced glucose uptake in human skeletal muscle cells. However, the EPS-mediated AMPK activation was not affected by loss of HIF-1α. In accordance, it could be demonstrated that knockout of HIF-1α abolished glucose uptake by the AMPK activator AICAR in mouse skeletal muscle cells (24). AMPK-mediated AS160 phosphorylation and GLUT4 translocation are involved in contraction/exercise-induced glucose uptake by skeletal muscle (25). Therefore, HIF-1α might play a role in glucose uptake by exercising muscles as well.

One of the aims of this study was to investigate the effect of acute and chronic exercise on skeletal muscle HIF-1α mRNA expression in healthy normoglycemic and dysglycemic subjects. Others have shown that skeletal muscle HIF-1α mRNA was up-regulated after a single bout of exercise (23;24). In line with these results, we observed a significant increase in muscle HIF-1α mRNA expression after a single bout of exercise as well as after long term exercise in control subjects. In contrast, we show that chronic induction of muscle HIF-1α mRNA expression by exercise was slightly reduced in subjects with abnormal glucose metabolism. Alterations of hypoxia-sensitive genes have been reported previously in diabetic rats (25). In addition, Fadini et al. have shown that streptozotocin-treated rats were not able to induce HIF-1α in skeletal muscle after ischaemia, whereas control animals showed a strong up-regulation (26).

Furthermore, we observed a correlation between skeletal muscle HIF-1α mRNA expression and fasting blood glucose levels, which might suggest that skeletal muscle HIF-1α expression is coupled to whole body glucose metabolism in humans. Interestingly, Gunton et al. showed that patients with T2D have decreased HIF-1β mRNA levels in pancreatic tissue, which results in changes of HIF-1 activity (27). In addition, a human genetic polymorphism that results in the substitution of serine for proline at residue 582 of HIF-1α is associated with T2D (28). Hence, it might be speculated that the inability to induce HIF-1α or changes in HIF-1 activity may contribute to the pathogenesis of diabetes.

Exercising is accompanied with severe oxygen deprivation in the muscle, and thus it is easy to imagine a central role for HIF-1α during muscular response to endurance training (29). Furthermore, growth factors like the HIF-1α target gene VEGF, have been proposed to be of importance in the angiogenesis process in skeletal muscle (30). It is well accepted that endurance training increases the capillarization of skeletal muscle (31;32). Therefore, skeletal muscle HIF-1α is a potential target to promote improvements in perfusion of ischemic limb in patients with peripheral artery disease and intermittent claudication (33). The implication of our results could extend the therapeutic possibilities of overexpressing constitutively active HIF-1α in the skeletal muscle with respect to the improvements in insulin sensitivity.

One the other hand, HIF-1α is implicated in many cancers where it frequently promotes the expression of pro-tumorigenic pathways (34;35). Furthermore, HIF-1α is directly linked to metabolic dysfunction in adipose tissues under hypoxic conditions and HIF-1α inhibition in the adipocytes leads to significant metabolic improvement (36). It is important to keep in mind that HIF-1α has different biological functions depending on the target tissue or organ and the physiological or pathophysiological condition in which it is enrolled.

In summary, we demonstrate that a reduction of the oxygen level to 7% and the combination with muscle contraction improves insulin action in human skeletal muscle cells. Furthermore, we have shown that HIF-1α plays a critical role in this setting and our data indicate that HIF-1α is an important determinant in insulin- and contraction-induced glucose uptake in human skeletal muscle cells. Additionally, we observed that muscle HIF-1α mRNA is regulated by acute and chronic exercise and muscle HIF-1α expression negatively correlated with blood glucose levels. Therefore, we hypothesize that skeletal muscle HIF-1α could be used as a therapeutically relevant target to improve insulin sensitivity in human.

V. REFERENCES

1. Eckardt,K, Gorgens,SW, Raschke,S, Eckel,J: Myokines in insulin resistance and type 2 diabetes. *Diabetologia* 57:1087-1099, 2014
2. Knowler,WC, Barrett-Connor,E, Fowler,SE, Hamman,RF, Lachin,JM, Walker,EA, Nathan,DM: Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med* 346:393-403, 2002
3. Leto,D, Saltiel,AR: Regulation of glucose transport by insulin: traffic control of GLUT4. *Nat Rev Mol Cell Biol* 13:383-396, 2012
4. Nesher,R, Karl,IE, Kipnis,DM: Dissociation of effects of insulin and contraction on glucose transport in rat epitrochlearis muscle. *Am J Physiol* 249:C226-C232, 1985
5. Richter,EA, Hargreaves,M: Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiol Rev* 93:993-1017, 2013
6. Santalucia,T, Moreno,H, Palacin,M, Yacoub,MH, Brand,NJ, Zorzano,A: A novel functional co-operation between MyoD, MEF2 and TRalpha1 is sufficient for the induction of GLUT4 gene transcription. *J Mol Biol* 314:195-204, 2001
7. Semenza,GL: Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. *Trends Pharmacol Sci* 33:207-214, 2012
8. Semenza,GL: Oxygen sensing, homeostasis, and disease. *N Engl J Med* 365:537-547, 2011
9. Schofield,CJ, Ratcliffe,PJ: Oxygen sensing by HIF hydroxylases. *Nat Rev Mol Cell Biol* 5:343-354, 2004
10. Lundby,C, Calbet,JA, Robach,P: The response of human skeletal muscle tissue to hypoxia. *Cell Mol Life Sci* 66:3615-3623, 2009
11. Gorgens,SW, Eckardt,K, Elsen,M, Tennagels,N, Eckel,J: Chitinase-3-like protein 1 protects skeletal muscle from TNFalpha-induced inflammation and insulin resistance. *Biochem J* 459:479-488, 2014
12. Lambernd,S, Taube,A, Schober,A, Platzbecker,B, Gorgens,SW, Schlich,R, Jeruschke,K, Weiss,J, Eckardt,K, Eckel,J: Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways. *Diabetologia* 55:1128-1139, 2012
13. Norheim,F, Hjorth,M, Langleite,TM, Lee,S, Holen,T, Bindesboll,C, Stadheim,HK, Gulseth,HL, Birkeland,KI, Kielland,A, Jensen,J, Dalen,KT, Drevon,CA: Regulation of angiopoietin-like protein 4 production during and after exercise. *Physiol Rep* 2: 2014
14. Kanzleiter,T, Rath,M, Gorgens,SW, Jensen,J, Tangen,DS, Kolnes,AJ, Kolnes,KJ, Lee,S, Eckel,J, Schurmann,A, Eckardt,K: The myokine decorin is regulated by contraction and involved in muscle hypertrophy. *Biochem Biophys Res Commun* 450:1089-1094, 2014
15. DeFronzo,RA, Tobin,JD, Andres,R: Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 237:E214-E223, 1979
16. Li,H, Handsaker,B, Wysoker,A, Fennell,T, Ruan,J, Homer,N, Marth,G, Abecasis,G, Durbin,R: The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 25:2078-2079, 2009

17. Greenbaum,AR, Etherington,PJ, Manek,S, O'Hare,D, Parker,KH, Green,CJ, Pepper,JR, Winlove,CP: Measurements of oxygenation and perfusion in skeletal muscle using multiple microelectrodes. *J Muscle Res Cell Motil* 18:149-159, 1997
18. Csete,M, Walikonis,J, Slawny,N, Wei,Y, Korsnes,S, Doyle,JC, Wold,B: Oxygen-mediated regulation of skeletal muscle satellite cell proliferation and adipogenesis in culture. *J Cell Physiol* 189:189-196, 2001
19. Storch,TG: Oxygen concentration regulates 5-azacytidine-induced myogenesis in C3H/10T1/2 cultures. *Biochim Biophys Acta* 1055:126-129, 1990
20. Sakagami,H, Makino,Y, Mizumoto,K, Isoe,T, Takeda,Y, Watanabe,J, Fujita,Y, Takiyama,Y, Abiko,A, Haneda,M: Loss of HIF-1alpha impairs GLUT4 translocation and glucose uptake by the skeletal muscle cells. *Am J Physiol Endocrinol Metab* 306:E1065-E1076, 2014
21. Chen,S, Synowsky,S, Tinti,M, MacKintosh,C: The capture of phosphoproteins by 14-3-3 proteins mediates actions of insulin. *Trends Endocrinol Metab* 22:429-436, 2011
22. Cao,WD, Kawai,N, Miyake,K, Zhang,X, Fei,Z, Tamiya,T: Relationship of 14-3-3zeta (zeta), HIF-1alpha, and VEGF expression in human brain gliomas. *Brain Tumor Pathol* 31:1-10, 2014
23. Ameln,H, Gustafsson,T, Sundberg,CJ, Okamoto,K, Jansson,E, Poellinger,L, Makino,Y: Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. *FASEB J* 19:1009-1011, 2005
24. Lima,GA, Anhe,GF, Giannocco,G, Nunes,MT, Correa-Giannella,ML, Machado,UF: Contractile activity per se induces transcriptional activation of SLC2A4 gene in soleus muscle: involvement of MEF2D, HIF-1a, and TRalpha transcriptional factors. *Am J Physiol Endocrinol Metab* 296:E132-E138, 2009
25. Marfella,R, D'Amico,M, Di,FC, Piegari,E, Nappo,F, Esposito,K, Berrino,L, Rossi,F, Giugliano,D: Myocardial infarction in diabetic rats: role of hyperglycaemia on infarct size and early expression of hypoxia-inducible factor 1. *Diabetologia* 45:1172-1181, 2002
26. Fadini,GP, Sartore,S, Schiavon,M, Albiero,M, Baesso,I, Cabrelle,A, Agostini,C, Avogaro,A: Diabetes impairs progenitor cell mobilisation after hindlimb ischaemia-reperfusion injury in rats. *Diabetologia* 49:3075-3084, 2006
27. Gunton,JE, Kulkarni,RN, Yim,S, Okada,T, Hawthorne,WJ, Tseng,YH, Roberson,RS, Ricordi,C, O'Connell,PJ, Gonzalez,FJ, Kahn,CR: Loss of ARNT/HIF1beta mediates altered gene expression and pancreatic-islet dysfunction in human type 2 diabetes. *Cell* 122:337-349, 2005
28. Yamada,N, Horikawa,Y, Oda,N, Iizuka,K, Shihara,N, Kishi,S, Takeda,J: Genetic variation in the hypoxia-inducible factor-1alpha gene is associated with type 2 diabetes in Japanese. *J Clin Endocrinol Metab* 90:5841-5847, 2005
29. Richardson,RS, Noyszewski,EA, Kendrick,KF, Leigh,JS, Wagner,PD: Myoglobin O2 desaturation during exercise. Evidence of limited O2 transport. *J Clin Invest* 96:1916-1926, 1995
30. Hoier,B, Hellsten,Y: Exercise-induced capillary growth in human skeletal muscle and the dynamics of VEGF. *Microcirculation* 21:301-314, 2014
31. Andersen,P, Henriksson,J: Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise. *J Physiol* 270:677-690, 1977
32. Ingjer,F: Effects of endurance training on muscle fibre ATP-ase activity, capillary supply and mitochondrial content in man. *J Physiol* 294:419-432, 1979
33. Creager,MA, Olin,JW, Belch,JJ, Moneta,GL, Henry,TD, Rajagopalan,S, Annex,BH, Hiatt,WR: Effect of hypoxia-inducible factor-1alpha gene therapy on walking performance in patients with intermittent claudication. *Circulation* 124:1765-1773, 2011
34. Koh,MY, Spivak-Kroizman,T, Venturini,S, Welsh,S, Williams,RR, Kirkpatrick,DL, Powis,G: Molecular mechanisms for the activity of PX-478, an antitumor inhibitor of the hypoxia-inducible factor-1alpha. *Mol Cancer Ther* 7:90-100, 2008
35. Palayoor,ST, Mitchell,JB, Cerna,D, Degraff,W, John-Aryankalayil,M, Coleman,CN: PX-478, an inhibitor of hypoxia-inducible factor-1alpha, enhances radiosensitivity of prostate carcinoma cells. *Int J Cancer* 123:2430-2437, 2008
36. Sun,K, Halberg,N, Khan,M, Magalang,UJ, Scherer,PE: Selective inhibition of hypoxia-inducible factor 1alpha ameliorates adipose tissue dysfunction. *Mol Cell Biol* 33:904-917, 2013

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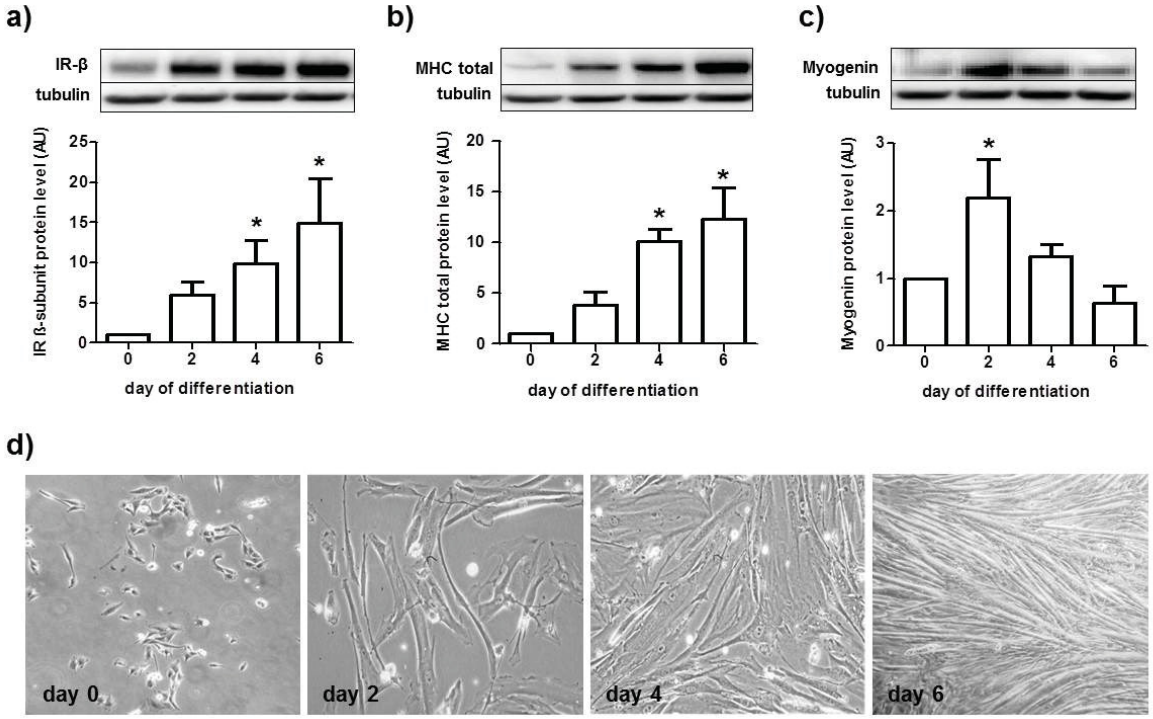
CONTRIBUTION STATEMENT

SWG contributed to the concept, acquired, analyzed and interpreted data, wrote the manuscript and had the main responsibility together with JE. KE, MH, JF, FN, TH, SL, TML, KIB, and CAD performed research and contributed to analysis and interpretation of data. JE and KE 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.

Supplement

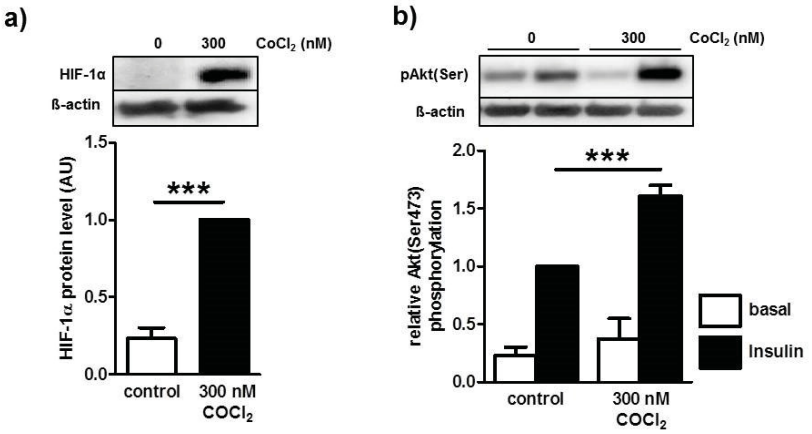


Supplement Figure 1: Differentiation of primary human skeletal muscle cells. (a - c) Primary human skeletal muscle cells were differentiated for the indicated time points and insulin receptor subunit ß (IR-ß), total myosin heavy chain (MHC) and myogenin protein levels were analyzed by Western Blot. Data are mean values ± SEM, n= 5, * $p<0.05$ vs. day 0. AU = arbitrary units. Representative photos were taken at day 0, day 2, day 4 and day 6 following start of the differentiation process.

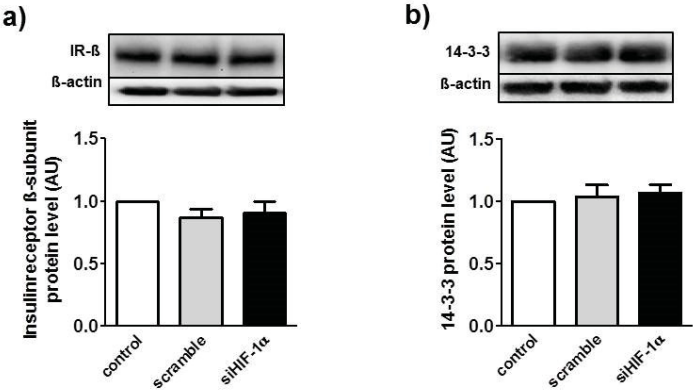
Nr.	Gene symbol	Protein name	Short name	Fold vs. EPS 21% O_2
1	**IL6**	**Interleukin 6**	**IL-6**	**14.89 (±8.66)***
2	**VEGFA**	**Vascular endothelial growth factor A**	**VEGF-A**	**8.99 (±2.11)***
3	CXCL12	Stromal cell-derived factor 1	SDF-1	5.06 (±4.92)
4	CXCL9	Monokine induced by interferon-gamma	MIG	3.64 (±2.53)
5	**IL8/CXCL8**	**Interleukin 8**	**IL-8**	**2.31 (±0.37)***
6	**CXCL1/CXCL2/CXCL3**	**Growth-regulated alpha protein alpha/ beta/ delta**	**GRO**	**2.06 (±0.33)***
7	LTA	Tumor necrosis factor alpha	TNFß	2.03 (±1.33)
8	OSM	Oncostatin M	OSM	1.96 (±0.58)
9	**CXCL1**	**Growth-regulated alpha protein alpha**	**GROα**	**1.94 (±0.37)***
10	IL1A	Interleukin 1 alpha	IL-1α	1.64 (±0.37)
11	IFNG	Interferon gamma	IFNγ	1.55 (±0.64)
12	CCL17	Thymus and activation-regulated chemokine	TARC	1.54 (±0.67)
13	CCL2	Monocyte Chemoattractant Protein 1	MCP-1	1.47 (±0.20)
14	CSF1	Macrophage colony-stimulating factor	M-CSF	1.45 (±0.39)
15	KITLG	Stem cell factor	SCF	1.42 (±0.53)
16	IL2	Interleukin 2	IL-2	1.35 (±0.39)
17	TNFSF14	Tumor necrosis factor ligand superfamily member 14	LIGHT	1.22 (±0.58)
18	CCL5	Regulated on activation, normal T cell expressed and secreted	RANTES	1.21 (±0.16)
19	IL1B	Interleukin 1 beta	IL-1ß	1.16 (±0.23)
20	IL10	Interleukin 10	IL-10	1.10 (±0.11)
21	HGF	Hepatocyte growth factor	HGF	1.08 (±0.20)
22	FGF9	Fibroblast growth factor 9	FGF-9	1.03 (±0.30)
23	CCL23	CK-beta-8	CKB-8	1.02 (±0.31)
24	TIMP1	Tissue inhibitor of metalloproteinases 1	TIMP-1	1.02 (±0.32)
25	TGFB2	Transforming growth factor-beta 2	TGFß-2	1.00 (±0.21)
26	IL3	Interleukin 3	IL-3	0.97 (±0.14)
27	BDNF	Brain-derived neurotrophic factor	BDNF	0.95 (±0.15)
28	GDNF	Glial cell line-derived neurotrophic factor	GDNF	0.92 (±0.24)
29	TNF	Tumor necrosis factor alpha	TNFα	0.90 (±0.36)
30	LIF	Leukemia inhibitory factor	LIF	0.90 (±0.26)
31	Ppbp	Neutrophil activating peptide 2	NAP-2	0.89 (±0.13)
32	IGFBP1	Insulin-like growth factor binding protein 1	IGFBP1	0.89 (±0.08)
33	PGF	Placental growth factor	PLGF	0.85 (±0.22)
34	**CCL4**	**Macrophage inflammatory protein 1 beta**	**MIP-1ß**	**0.83 (±0.05)***
35	TIMP2	Tissue inhibitor of metalloproteinases 2	TIMP-2	0.83 (±0.23)
36	CXCL10	Interferon gamma-induced protein 10	IP-10	0.79 (±0.23)
37	**IGFBP2**	**Insulin-like growth factor binding protein 2**	**IGFBP2**	**0.78 (±0.05)*****
38	**IL16**	**Interleukin 16**	**IL-16**	**0.78 (±0.08)***
39	NTF3	Neurotrophin 3	NT-3	0.77 (±0.11)
40	**CCL24**	**Eosinophil chemotactic protein 2**	**Eotaxin-2**	**0.75 (±0.06)****

mean (±sem)

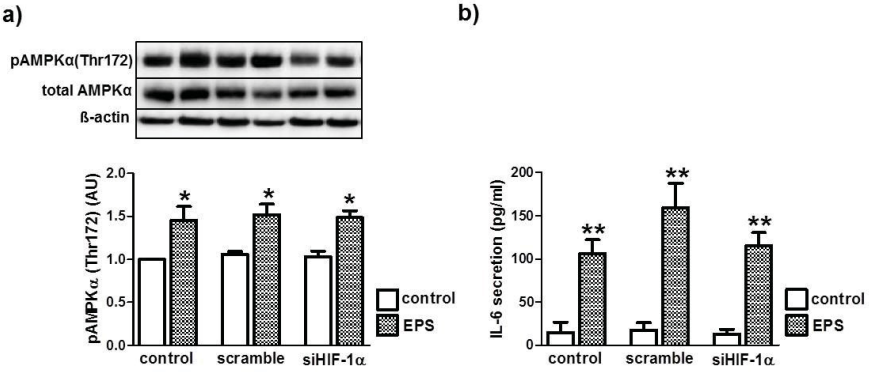
Supplement Figure 2: List of all detectible cytokines. Supernatants of EPS-treated myotubes cultured at 21% or 7% O_2 were collected after 24 h and analyzed as described in Material and Methods.



Supplement Figure 3: Up-regulation of HIF-1α by $COCl_2$ enhanced insulin signaling: (**a** and **b**) Quantification and representative Western Blot images show HIF-1α protein level and Akt(Ser473) phosphorylation of untreated cells (control) or after treatment with 300 nM cobalt chloride ($COCl_2$) for 24 h followed by insulin stimulation (100 nM) for 10 min. Data are normalized to β-actin and expressed relative to control. Data are mean values ± SEM, n=4, ***p<0.0001 as indicated.



Supplement Figure 4: Knock-down of HIF-1α does not influence insulin receptor ß-subunit or 14-3-3 protein abundance: (**a** and **b**) Quantification and representative Western Blot images show insulin receptor ß-subunit and 14-3-3 protein levels of untreated cells (control) or after treatment with 40 nM AllStars Negative Control siRNA (scramble) or HIF-1α siRNA (siHIF-1α). Data are normalized to β-actin and expressed relative to control. Data are mean values ± SEM, n=5.



Supplement Figure 5: Knock-down of HIF-1α does not influence EPS-induced AMPKα phosphorylation or IL-6 secretion: (**a**) Quantification and representative Western Blot images show phosphorylation of AMPKα of untreated cells (control) or after treatment with 40 nM AllStars Negative Control siRNA (scramble) or HIF-1α siRNA (siHIF-1α), with or without EPS treatment for 24 h. Data are normalized to β-actin and expressed relative to control. Data are mean values ± SEM, n=5, *p<0.05, vs. corresponding control. (**b**) IL-6 levels in the supernatants were measured by human IL-6 ELISA. Data are normalized to total protein level. Data are mean values ± SEM, n=5, **p<0.005, vs. corresponding control.

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	Analysed data: 75%
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Author:	1st author

3 General Discussion

3.1 Identification and characterization of novel contraction-induced 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. Recently, a novel research concept has been suggested that proposes the skeletal muscle as an endocrine organ releasing so-called myokines, which might in part be responsible for the beneficial effect of exercise on health (2;190;201). Several myokines are known to have both local and endocrine functions, however, in many cases the contribution of physical activity to systemic levels of these molecules remains incompletely unexplored. For providing a relevant contribution to answer this open question we aimed to identify myokines released by contracting myotubes followed by *in vitro* characterization. Finally, we analyzed the impact of exercise on systemic levels. In the present thesis, we identified and characterized two novel exercise-regulated myokines, namely follistatin-like protein 1 (Fstl1) and chitinase-3-like protein 1 (CHI3L1), which are produced, expressed and released by skeletal muscle. Our findings may provide a better understanding for the endocrine role of the skeletal muscle producing and releasing myokines, which potentially influence skeletal muscle metabolism itself and/or contribute to regulate the functions of other tissues in an inter-organ crosstalk.

The following chapter will provide a summary of some of the major findings and associate them to the context of recent findings. I will briefly introduce Fstl1 as an exercise-regulated myokine followed by a more detailed statement about the novel myokine CHI3L1 and its functions on skeletal muscle homeostasis.

3.1.1 Follistatin-like protein 1

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 into the follistatin family of proteins (236). Recently, Kenneth Walsh has identified Fstl1 as a novel myokine (237). Myogenic Akt signaling or ischemic hind limb surgery led to the induction of Fstl1 in murine skeletal muscle and increased circulating levels of Fstl1 (237). However, the expression and secretion of Fstl1 by human skeletal muscle cells as well as the impact of exercise on circulating Fstl1 levels have not yet been investigated.

Applying RT-PCR, Western Blot and ELISA analyses of supernatant our data clearly demonstrate that Fstl1 is expressed and secreted by human skeletal muscle cells (238). In addition, Fstl1 expression and secretion is up-regulated during differentiation and pro-inflammatory cytokines like interferon gamma (IFNγ) and interleukin 1 beta (IL-1ß) significantly induce Fstl1 secretion.

Similar to exercising skeletal muscle *in vivo*, electrical pulse stimulation (EPS) induced contractile activity in human skeletal muscle cells *in vitro* as well as activation of AMPK and induction of IL-6 secretion (189). Furthermore, EPS of human skeletal muscle cells results in clearly visible contraction and formation of striated patterns typical of sarcomeric structures, as visualized by immunofluorescence staining of α-actinin and electron microscopy (figure 4). However, electrical pulse stimulation (EPS) of primary human myotubes revealed no regulation of Fstl1 expression by *in vitro* contraction.

In contrast, we demonstrated that an acute bout of exercise performed by healthy young men increased Fstl1 serum levels significantly (238). Plasma Fstl1 concentration increased from 16.9±3.5 ng/ml pre-exercise to 20.1±3.1 ng/ml immediately after exercise. Additionally, Norheim et al. observed a significant increase of Fstl1 mRNA expression in *m. vastus lateralis* and *m. trapezius* after 11 weeks of strength training (239). Taken together, these data indicate that Fstl1 is regulated by exercise *in vivo*, but future studies are necessary to demonstrate whether the contracting skeletal muscle itself is the source of elevated circulating levels during exercise.

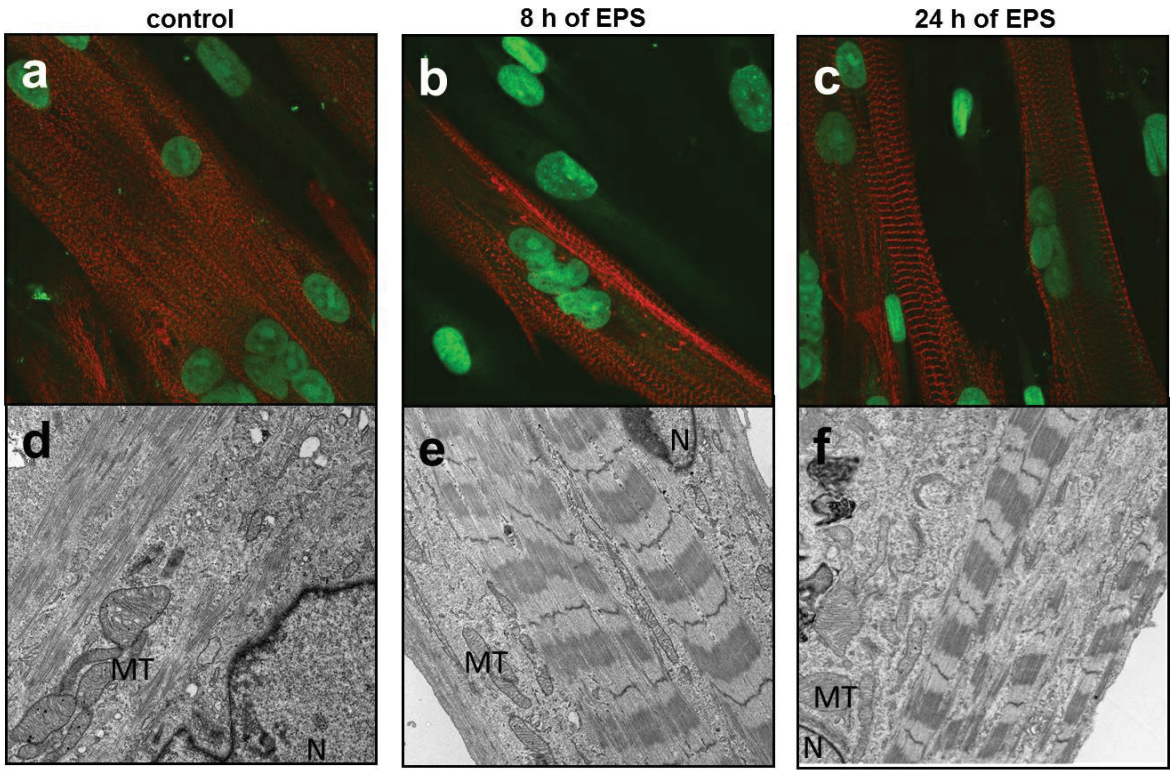

Figure 4: Effect of EPS on sarcomere structure assembly in human skeletal myotubes. a) The cells were fixed and analysed for localization of sarcomeric α-actinin without (control), and after 8 (b) and 24 h (c) of EPS (1 Hz, 2 ms, 11.5 V) by immunofluorescence staining. Red, sarcomeric α-actinin; green, 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. Adapted from (183).

Based on data by Ouchi et al. who observed increased Akt phosphorylation in endothelial cells after adenoviral transduction with Fstl1 (237) we hypothesized that Fstl1 might have an insulin-sensitizing effect. Akt is required for the insulin-induced translocation of GLUT4 to the plasma membrane. Furthermore, glycogen synthase kinase 3 (GSK-3) is inhibited upon phosphorylation by Akt, which results in increased glycogen synthesis. Therefore, Akt is an important determinant of skeletal muscle glucose metabolism regulated by insulin. However, treatment of human myotubes with different concentrations of recombinant Fstl1 had no effect on basal or insulin-stimulated Akt or GSK-3 phosphorylation (238). Despite using recombinant Fstl1 proteins obtained from three independent companies we observed no effect. In contrast, Shimano et al. demonstrated that treatment of cardiac myocytes with

recombinant Fst1 enhanced AMPK and ACC phosphorylation in a dose- and time dependent manner (240).

It might be speculated that increased Fstl1 serum levels after exercise may be involved in further activation of AMPK in target tissues and enhance downstream signaling. It has been shown that Fstl1 also promotes endothelial cell migration and differentiation into vascular-like structures and hence could support enhanced vascularization of skeletal muscle after exercise (237). It is well accepted that increased skeletal muscle vascularization is associated with improved glucose tolerance and insulin sensitivity (241). We identified Fstl1l to be an exercise-regulated myokine which is increased in serum levels after acute exercise and suggest that it may contribute to the health benefits associated with physical activity.

3.1.2 Chitinase-3-like protein 1

In a search for new bone proteins, the glycoprotein CHI3L1 was identified in 1989 to be secreted *in vitro* in large amounts by the human osteosarcoma cell line MG63 (242;243). The protein is also named YKL-40 based on its three N-terminal amino acids tyrosine (Y), lysine (K) and leucine (L) and its molecular weight of 40 kDa (242). In the last few years there have been a growing number of publications concerning CHI3L1/YKL-40, which is complicated by the fact that the protein has several names including CHI3L1, YKL-40, human cartilage glycoprotein-39 (HC gp39), breast regressing protein 39 Kd (brp-39), 38-kDa heparin-binding glycoprotein (gp38k), Chondrex, and 40 kDa mammary gland protein (MGP-40) (244). In this thesis the protein is named CHI3L1.

Molecular mechanism leading to CHI3L1 production

In 1997 the human gene encoding CHI3L1 was isolated (245). It is assigned to chromosome 1q31-q32, consists of 10 exons and spans about 8 kilo bases of genomic DNA (245). There are probably two independent transcription start sites and the promotor sequence contains binding sites for several known factors such as PU.1, Sp1, Sp3, USF, AML-1 and C/EBP proteins (245). It was further described that the Sp1-family transcription factors seem to have a predominant role in controlling CHI3L1 promotor activity (246). The transcriptional up-regulation of CHI3L1 during human macrophage differentiation has been described (246). In addition, we demonstrated that CHI3L1 transcription is also down-regulated during human skeletal muscle differentiation (247). Interestingly, it has been shown that differentiation of skeletal muscle cells and MyoD down-regulate Sp1 (248). Therefore, the down-regulation of CHI3L1 is potentially related to down-regulation of Sp1 during myogenesis leading to decreased CHI3L1 promoter activity and hence decreased CHI3L1 levels in myotubes. In addition, the expression of CHI3L1 is regulated by various cytokines and hormones, including IL-6, IL-13, IFNγ, vasopressin, and parathyroid hormone-related protein (249). The inflammatory cytokines TNF-α and IL-1ß also stimulate the expression of CHI3L1 in articular chondrocytes (250). Furthermore, the constitutive expression and secretion of CHI3L1 is controlled by NF-κB activity in chondrocytes (250). Additionally, the authors have described an active binding site for NF-κB in the human CHI3L1 promotor region (250). In line with these findings, Baht et al. have shown that CHI3L1 expression is regulated by TNFα-stimulated NF-κB activation in different cancer cell lines (251). TNFα causes recruitment of

the p65 and p50 subunits of NF-κB to the CHI3L1 promotor in all cell types (251). In accordance, we demonstrated that CHI3L1 expression and enhanced secretion are controlled by NF-κB activity in primary human skeletal muscle cells (247). Taken together, CHI3L1 transcription is regulated by a variety of pro-inflammatory cytokines and these mechanisms may contribute to the dysregulation of CHI3L1 seen in a number of human diseases characterized by acute or chronic inflammation and tissue remodeling.

Systemic levels

There is no established normal range for circulating CHI3L1 in healthy humans (252). Several studies have used serum CHI3L1 with maximum value set at the 95% percentile. The two largest studies of healthy subjects both agree on a maximum serum value around 125 ng/mL (252). In another study the value was higher but the controls in this study were older when compared to the two other studies (253). Since CHI3L1 increases with age, it is important to compare CHI3L1 with an age-corrected CHI3L1 scale (243;254). The currently published levels of circulating CHI3L1 can differ between comparable patient populations which could be related to the use of either plasma or serum samples. Several studies have reported significantly higher CHI3L1 values in serum compared to plasma (255). The difference is probably caused by a small release of CHI3L1 from activated neutrophils during coagulation (249). Moreover, the use of different immunoassays can give different CHI3L1 levels.

Increased circulating levels of CHI3L1 in plasma or serum samples have been found in different cancer types, diabetes mellitus, pulmonary, and chronic inflammatory, pulmonary and acute infectious diseases (256). Circulating CHI3L1 levels are a predictor of all-cause mortality in the elderly and have a significant association with rates of overall and cardiovascular mortality (257). Several studies have documented elevated CHI3L1 levels in both patients with type 1 and type 2 diabetes (T2D) independent of body mass index (BMI), and CHI3L1 is associated with increasing albuminuric levels as a measure of impaired kidney function (258-260). It is interesting that a recent study demonstrated identically increased CHI3L1 levels in patients with T2D with or without ischemic heart disease (IHD), and in patients with IHD alone (261). This indicates that probably the underling vascular disease and not the diabetes could cause elevated CHI3L1 levels (261). In addition, circulating CHI3L1 has been demonstrated to be associated with degree of obesity and CHI3L1 decreased after weight loss (258). In line with these findings, we observed a positive correlation between adipose tissue CHI3L1 mRNA expression and circulating levels (unpublished data).

Moreover, the expression of adipose tissue CHI3L1 mRNA positively correlates with BMI and weight, whereas skeletal muscle CHI3L1 mRNA expression shows no correlation with any maker of obesity (unpublished data). Finally, circulating CHI3L1 levels positively correlate with BMI and weight (unpublished data). These data indicate that adipose tissue may contribute to systemically elevated circulating levels of CHI3L1 in conditions of obesity. Moreover, expansion of the visceral adipose tissue may contribute to increased inflammation within the tissue (144). This could be due to an accumulation of macrophages within adipose tissue, in particular the hypertrophic type of obesity, which is associated with reduced production of the anti-inflammatory factor adiponectin (144). The number of macrophages within adipose tissue has been shown to correlate with insulin resistance, indicating a link between inflammation, insulin resistance and T2D (144;262).

Sources of CHI3L1

Several different cell types of ectodermal, mesodermal, and endodermal origin express CHI3L1 mRNA and protein *in vitro* and *in vivo* under specific conditions (263). Macrophages, neutrophils, chondrocytes, fibroblast, vascular smooth muscle cells, endothelial cells, hepatic cells as well as colonic, ductal, and airway epithelia cells have been shown to express CHI3L1 (249). CHI3L1 is not expressed in monocytes and marginally expressed in monocyte-derived dendritic cells, but is strongly induced at late stages of macrophage differentiation (246). *In vivo* CHI3L1 mRNA and protein expression are found in a subpopulation of macrophages in different tissues with inflammation and extracellular matrix remodeling, e.g. macrophages in inflamed synovial membranes from patients with rheumatoid arthritis or osteoarthritis express high levels of CHI3L1 mRNA and protein (264;265). In addition, CHI3L1 expression in cells of the stroma vascular fraction originating from visceral adipose tissue is higher compared to those from subcutaneous adipose tissue. Immunofluorescence staining revealed that CHI3L1 is exclusively expressed in macrophages among stroma vascular fraction cells and is not expressed by adipocytes (266). Recently, we added human skeletal muscle cells to the list of cell types which express and release CHI3L1 (247). Interestingly, CHI3L1 levels decline during skeletal muscle differentiation but inflammatory cytokines or mechanical stress strongly induce CH3L1 expression (247;267). In conclusion, CHI3L1 is derived from different cells types and tissues and is regulated by a variety of physiological and pathophysiological conditions indicating that the induction and function of CHI3L1 seems to be restricted to a local auto-/paracrine function.

Sources of contraction-induced CHI3L1

Our data clearly demonstrated that CHI3L1 is a novel myokine, produced, expressed, and released by human skeletal muscle cells (267). Furthermore, we show that circulating CHI3L1 was increased after acute exercise in three independent exercise studies (267). Also skeletal muscle CHI3L1 mRNA expression was strongly up-regulated after a single bout of exercise (267). To further validate CHI3L1 as a contraction-induced myokine we analyzed its regulation *in vitro* by using our well-established EPS model for human skeletal muscle cell contraction. The finding that CHI3L1 is released from differentiated contracting myotubes in culture supports the notion that CHI3L1 is indeed a contraction-regulated myokine. These observations are in accordance with a very recent study showing that marathon running increased the levels of circulating CHI3L1 (268). In contrast, Johansen et al. observed no effect on serum CHI3L1 levels after physical exercise at moderate intensity for 20 min (269). It is possible that a higher intensity and/or a longer duration are necessary for the induction of elevated plasma CHI3L1 levels in response to acute exercise. Interestingly, unpublished data show that heavy exhausting exercise strongly induces circulating CHI3L1 levels. These data support the notion that the intensity and/or the duration of exercise are important determinants for the increase of circulating CHI3L1.

Table 1. Effect of acute exercise on plasma CHI3L1 in humans

Literature	Type of exercise	Duration	Intensity	Pre-exercise (mean ± sem)	Post-exercise (mean ± sem)	Increase (%)
Johansen SJ et al. 2008 (242)	bicycling	25 min	N/D	35-64 ng/ml	35-64 ng/ml	no change
Görgens SW et al. 2015 (Study 4)	bicycling	45 min	70% VO_2max	19.1 ± 1.8 ng/ml	22.3 ± 2.4 ng/ml	16 %
Görgens SW et al. 2015 (Study 4)	bicycling	60 min	75% VO_2max	16.6 ± 2.4 ng/ml	21.0 ± 4.7 ng/ml	28 %
Vuolteenaho K et al. 2014 (262)	marathon run	3.20 – 3.42 h	N/D	63.2 ± 9.3 ng/ml	99.2 ± 11.2 ng/ml	56 %
unpublished	bicycling	1 – 2.5 h	until exhausting	16.9 ± 2.1 ng/ml	38.9 ± 7.4 ng/ml	235 %

Shown is the relation between exercise mode (bicycling and marathon running), exercise duration, and plasma CHI3L1 increase (fold change from pre-exercise level in percentage). N/D = no data.

Table 1 summarizes all available human exercise studies that measured CHI3L1 in plasma before and after exercise. These data indicate that the response of CHI3L1 to exercise is

sensitive to the exercise duration. The strongest effect was observed after bicycling until exhausting, which lasts in some cases up to 2.5 hours. In addition, marathon running which lasts several hours was also a strong stimulus for the increase in circulating CHI3L1.

The CHI3L1-response is also sensitive to the exercise intensity, which indirectly represents the muscle mass involved in the contractile activity. Since our *in vitro* data demonstrated that the contracting myotube *per se* is a source of CHI3L1, it is not surprising that bicycling, which involves several large muscle groups of the lower extremities, seems to be the type of exercise in which the most prominent CHI3L1 increase has been found. In addition, we observed that the increase of plasma CHI3L1 levels positively correlates with the increase of plasma IL-6. Recently, we published that IL-6 is able to induce CHI3L1 production in human skeletal muscle cells (247). In addition, Nielsen et al. observed that IL-6 but not TNFα infusion increased plasma levels of CHI3L1 in healthy young men (270). It is known that exercise duration is the single most important factor determining the post-exercise IL-6 plasma amplitude (271). Therefore, it could be possible that the increase of IL-6 after exercise indirectly enhances skeletal muscle-derived CHI3L1 release.

Biological role of CHI3L1

CHI3L1 is a secreted glycoprotein suggesting that its sites of actions are most likely to be extracellular (272). The protease activated receptor 2 (PAR-2) has been proposed as a specific cell-surface receptor for CHI3L1 (272). The biological function of CHI3L1 is not yet clear but several possible functions, especially growth promoting properties, have been proposed (249).

In vitro studies have shown that CHI3L1 in physiological concentrations increases proliferation of guinea pig chondrocytes, rabbit chondrocytes and synovial cells, and that CHI3L1 increases proteoglycan synthesis of guinea pig and rabbit chondrocytes (273). Recklies et al. found that CHI3L1 increased growth rates of three fibroblastic cell lines derived from human osteoarthritic synovium, fetal lung and adult skin (274). The magnitude of the response of CHI3L1 stimulation on synovial cells and skin fibroblast on incorporation of [H^3]thymidine into cellular DNA was similar to that elicited by insulin-like growth factor 1 (IGF-1), and CHI3L1 and IGF-1 work synergistically in stimulating the growth of fibroblasts (274). CHI3L1 initiates MAP kinase and PI-3K signaling cascades in fibroblasts leading to phosphorylation of both ERK1/2 MAP kinase and Akt-mediated signaling cascade, which are

associated with control of mitogenesis (274). This suggests a role of CHI3L1 as an anti-apoptotic protein (274). The PI-3K pathway, and in particular the phosphorylation of Akt, is strongly associated with cell survival (275). In the beginning of 2012, Bara et al. proposed that PAR-2 could be a potential receptor for CHI3L1. They demonstrated that CHI3L1 increased bronchial smooth muscle cell proliferation and migration through PAR-2, Akt-, ERK-, and p38-dependent mechanisms (272). In addition, Liu et al. found that CHI3L1 increased mRNA and protein expression of mucin5AC in a dose- and time-dependent manner, in association with phosphorylation of ERK and NF-κB reflecting their activation (276). These responses were significantly suppressed by knockdown of PAR-2 with specific small interfering RNA (276).

Protease-activated receptors (PARs) comprise a family of receptors (PAR-1, PAR-2, PAR-3 and PAR-4) that are uniquely activated by the proteolytic cleavage of their extracellular portion (277). This cleavage unmasks a new N-terminus, which serves as a tethered ligand that binds to the second extracellular domain of the protein resulting in a variety of cellular responses (278). PAR-2 can be activated by tryptase and trypsin, but not thrombin. Experimentally, PAR-2 can also be activated by synthetic peptides that mimic the neo-amino terminus of the cleaved receptor (279). Such agonist peptides are useful for the study of PARs without using proteases that also have biological functions unrelated to PAR activation. Once activated, PAR-2 can elicit a variety of cellular responses through multiple signaling cascade pathways (280).

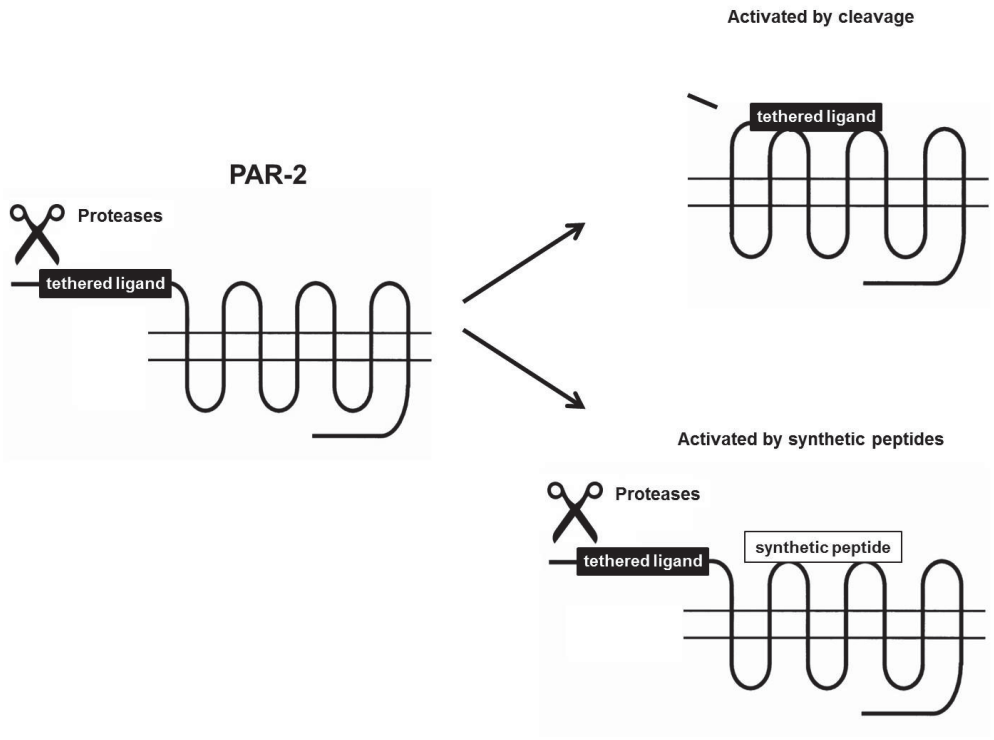

Figure 5: Different mechanisms of PAR-2 activation: Proteases cleave the peptide bond, liberating the new N-terminus, which then serves as a tethered ligand that binds to the receptor thereby triggering transmembrane signaling. In addition, a synthetic peptide corresponding to the tethered ligand for PAR-2 specifically activates PAR-2 in the absence of proteases. PAR-2, protease-activated receptor; (modified from (274))

PAR-2 has been proposed to be important in inflammation and proliferation processes (281-286). Based mainly on the properties of exogenous peptide agonists together with some knockout mouse studies, activation of PAR-2 has been predicted to be pro-inflammatory in arthritis (287-290), pancreatitis (291;292), gastric and kidney cells (293-296). One the other hand, PAR-2 activation also triggered anti-inflammatory effects in airways inflammation (297-299). Increased expression of PAR-2 associated with proliferation has been found in many types of cancer cells (300;301). In 2000, Chinni et al. demonstrated for the first time that PAR-2 is expressed by murine skeletal muscle cells both *in vivo* and *in vitro* (302). Furthermore, they show that activation of PAR-2 leads to stimulation of myoblast proliferation (302). In line with these findings, our data clearly demonstrate that PAR-2 is also expressed in human skeletal muscle cells. Therefore, we hypothesize that muscle-derived CHI3L1 increased after exercise may be related to induction of myoblast proliferation via PAR-2 signaling.

Muscle-derived CHI3L1 promotes myoblast proliferation

Our data have shown that CHI3L1 is primarily expressed by myoblasts and not by mature myotubes (247). Thus, it seems that CHI3L1 has the potential to affect satellite cells or myoblasts rather than mature muscle fibers.

The ability for skeletal muscle regeneration in response to exercise, injury or diseases is of crucial importance (303). Skeletal muscle regeneration depends on the addition of new myonuclei by activation, proliferation, and fusion of satellite cells to adult skeletal muscle fibers (304-306). Satellite cell myogenesis is a complex process that begins with the commitment of an embryonic precursor to the myogenic lineage followed by the proliferation of these committed myoblasts, the differentiation of myoblasts, and finally fusion of several myoblasts to form a multinucleated myotube (307 ;308). The myotube matures and becomes specialized for its particular function with the contractile apparatus and forms the myofibre. The common hypothesis is that satellite cells in mammals are derived from somites, transitory mesoderm-derived structures formed in pairs on either side of the neural tube (307;308). Somites then differentiate into dermomyotome and sclerotome, and mesodermal cells become specified as skeletal muscle (309;310). The process of satellite cell myogenesis is controlled by several myogenic transcription factors (muscle regulatory factors, MRFs) (308). Paired-box protein 7 (Pax7) maintains a population of quiescent satellite cells and together with myogenic factor 5 (Myf5) plays a determining role in the expansion and activation of myoblasts (308;311;312). When satellite cells are activated they rapidly raise the expression of myoblast determination protein (MyoD) (313). Afterwards, myogenin and myocte enhancer factor 2 (Mef2) drive the differentiation and fusion to form a multinucleated myotube together with a variety of regulatory and structural muscle genes typical for skeletal muscle myocytes (313;314). Finally, muscle-specific regulator factor 4 (Mrf4) acts as a myogenic determination factor and is necessary for the hypertrophy of the growing myofiber (311). This highly ordered sequence of different transcription factors is illustrated in figure 6.

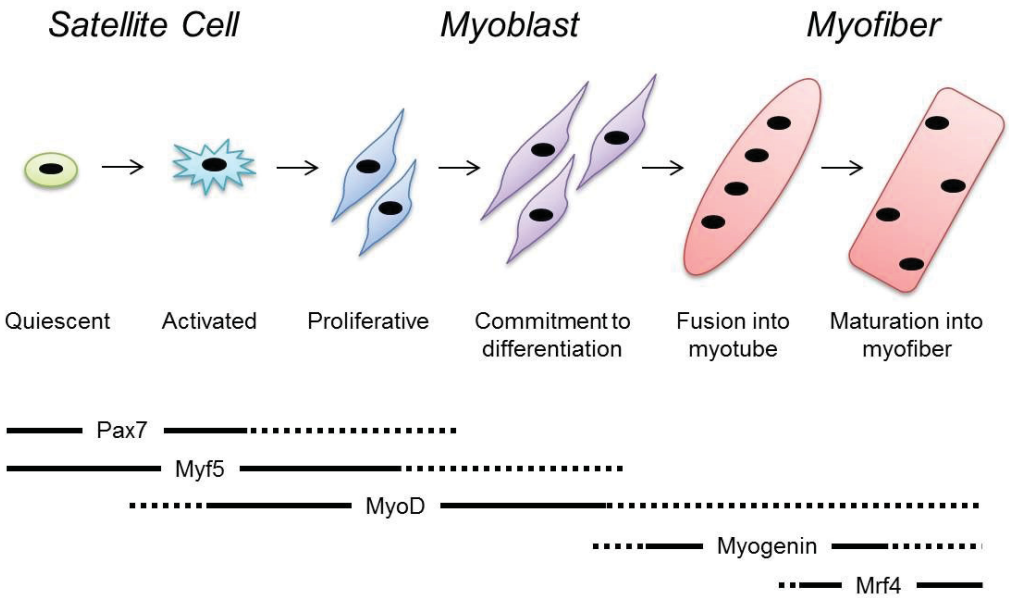

Figure 6: Scheme of satellite cell myogenesis and transcription factors typical for each stage. Satellite cells are quiescent in adult muscle and can be activated by several stimuli. Once activated, satellite cells divide to produce satellite cell-derived myoblasts that further proliferate before committing to differentiation and fusing to myotubes, which then mature into myofiber. Pax7 and Myf5 transcription factors are expressed in quiescent and activated satellite cells. MyoD is highly expressed in proliferative myoblast, whereas myogenin later marks the commitment of differentiation. Finally, the expression of Mrf4 is typical for the late stage of satellite cell myogenesis and promotes the maturation and hypertrophy of the growing muscle myofiber. Adapted from (315) and (316) with own modifications.

Maintenance of the balance between satellite cell differentiation and self-renewal is required for muscle homeostasis (303). A defect in self-renewal ability leads to a decrease in satellite cell number, resulting in depletion of the satellite cell pool as well as in reduced muscle regeneration capacity (303). Molecules released from skeletal muscle itself have been shown to increase satellite cell and myoblast proliferation (193;214). Here, we show that muscle-derived CHI3L1 induced p44/42, p38 MAPK and Akt signaling resulting in enhanced myoblast proliferation. Recently, we have shown that CHI3L1 signals via PAR-2 in human skeletal muscle cells (247). Indeed, using a specific PAR-2 antagonist we were able to completely abolish CHI3L1-induced p44/42, p38 MAPK and Akt activation. In line with our results, Bara et al. demonstrated that CHI3L1 increased bronchial smooth muscle cell proliferation and migration through PAR-2-, Akt-, p44/42-, and p38-dependent mechanisms (272). As activation of p44/42 has been closely associated with cellular proliferation and protein synthesis, it is tempting to speculate that the p44/42 pathway is critically involved in mediating the enhanced myoblast proliferation induced by CHI3L1. These observations are in

accordance with previous studies showing that activation of PAR-2 mediates proliferative responses in murine skeletal muscle cells (302). Thus, the possibility exists that proliferative effects of CHI3L1 on myoblasts are closely linked to a role of CHI3L1 in muscle repair and regeneration.

In conclusion, depending on the type and duration of exercise, muscle adaptations may involve processes such as muscle growth and regeneration. CHI3L1 is produced during exercise and might contribute to muscle adaption following exercise by stimulating myoblast proliferation, a process important for muscle hypertrophy and regeneration. In consequence, we hypothesize that the function of CHI3L1, as a contraction-induced myokine, is that of a mitogenic growth factor affecting nearby myoblast in a paracrine function (Figure 7).

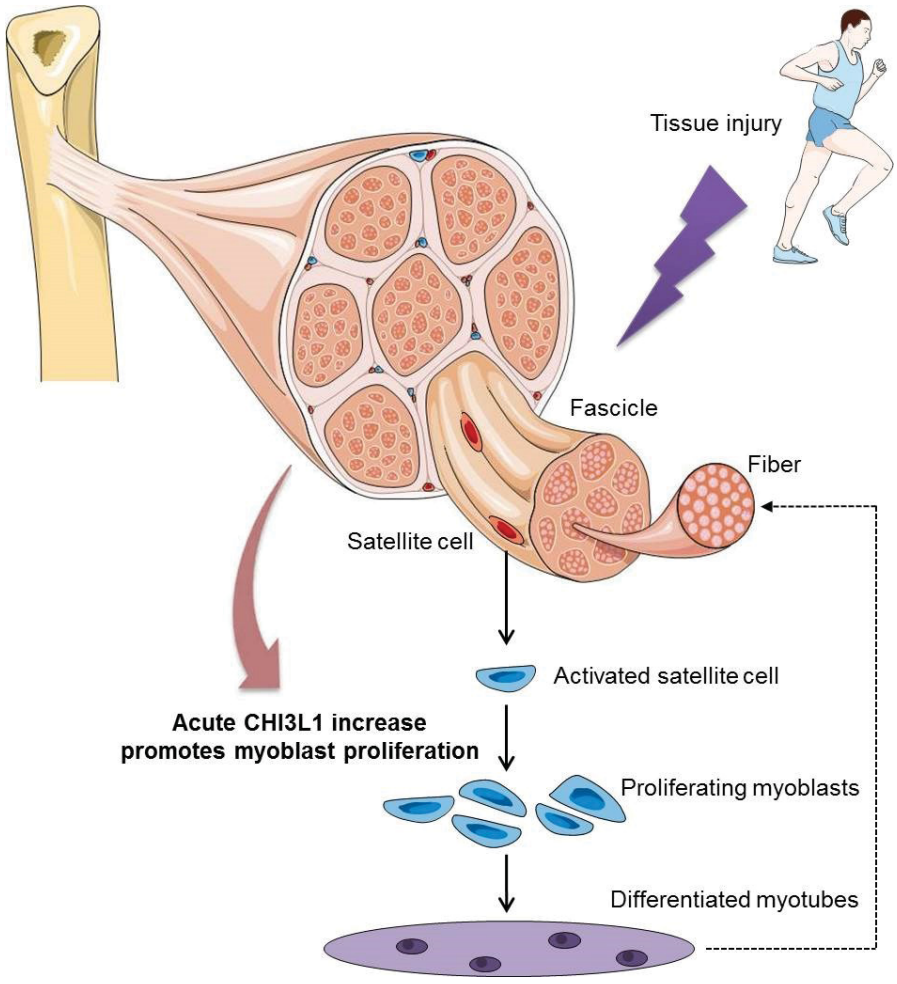

Figure 7: Skeletal muscle injury and repair. Satellite cells are muscle-resident stem cells which are located underneath the basal lamina of myofibers and are normally quiescent. Upon muscle injury, e.g. induced by heavy exercise, satellite cells become activated, start to proliferate as myoblasts, and subsequently fuse and differentiate into myotubes that later grow thereby replacing damaged muscle. Acute induction of CHI3L1 by exercise or muscle damage promotes the proliferation of myoblasts.

The anti-inflammatory effect of CHI3L1/PAR-2

It is widely recognized that PAR-2 signaling is implicated in various pathological conditions including chronic arthritis, inflammatory pain and colitis as well as skin disorders such as type IV and contact dermatitis, as mentioned above (291;295;296;301). However, there are also evidences supporting an anti-inflammatory role of PAR-2 signaling. A study by McIntosh et al. has shown that activation of PAR-2 mediates inhibition of TNFα-stimulated JNK activation by disrupting the binding of tumor necrosis factor receptor 1 (TNFR1) to receptor-interacting serine/threonine-protein kinase 1 (RIP) and tumor necrosis factor receptor type 1-associated DEATH domain (TRADD) in the human skin epithelial cell line NCTC2544 (317). Nichols et al. have suggested that PAR-2-enhanced inflammatory processes are ß-arrestin-dependent, whereas the protective effect of PAR-2 may be ß-arrestin independent (318). We characterized CHI3L1 as a novel myokine which is up-regulated by exercise and pro-inflammatory cytokines, and show its protective function regarding TNFα-induced inflammation and insulin resistance in primary human skeletal muscle cells (247;267). Recklies et al. have shown that CHI3L1 reduces TNFα- and IL-1β-mediated activation of p38 and SPAK/JNK MAPKs as well as cytokine-induced MMP1, MMP3 and IL-8 production in fibroblasts (274). However, they did not observe effects on TNFα-induced NF-κB activation. In our studies using primary human skeletal muscle cells, we report that CHI3L1 inhibits TNFα-induced NF-κB activation as shown by p65 phosphorylation. In addition, we clearly demonstrate that the effect of CHI3L1 on TNFα-induced NF-κB activation is PAR-2-dependent. However, the exact mechanism by which CHI3L1 activates PAR-2 is still unknown.

The physiological response of most cell types to TNFα involves enhanced production of cytokines such as IL-6, IL-8 and MCP1 which play a key role in inflammatory processes associated with conditions of insulin resistance (319). We demonstrate that treatment of human skeletal muscle cells with TNFα in the presence of CHI3L1 for 24 h prevents the inflammatory response induced by TNFα. In addition, we show that CHI3L1 protects skeletal muscle cells from TNFα-induced insulin resistance. It is has been demonstrated before that stimulation of primary human skeletal muscle cells with TNFα can directly induce insulin resistance (319;320). This effect can be inhibited by blocking the NF-κB signaling pathway (321). Based on our data we suggest that the effects of CHI3L1 on TNFα-induced responses are explained by activation of PAR-2 resulting in impaired TNFα downstream signaling and thus reduced NF-κB signaling. The induction of CHI3L1 in response to inflammatory

cytokines provides a negative feedback loop to control the action of TNFα, and thereby to locally reduce the inflammatory response in skeletal muscle. High TNFα concentrations induce insulin resistance in skeletal muscle and impair whole-body glucose sensitivity in humans. Thus, the identification and characterization of novel molecules which are able to reduce TNFα-mediated inflammation will certainly be an important approach to prevent the development of peripheral insulin resistance. In conclusion, the novel myokine CHI3L1 is induced by pro-inflammatory cytokines as well as exercise and counteracts TNFα-mediated inflammation and insulin resistance in human skeletal muscle. CHI3L1 acts via a PAR-2-dependent mechanism potentially in an auto- and/or paracrine way. Hence, we suggest CHI3L1 to be an auto-protective factor that is induced to protect skeletal muscle from a negative impact of TNFα.

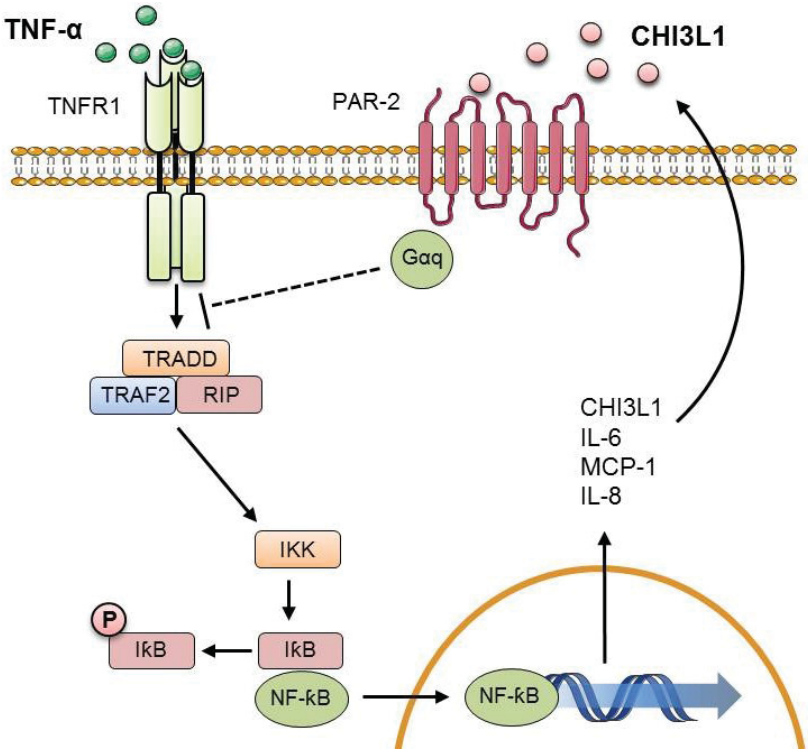

Figure 8: Proposed model for the negative-feedback control of TNFα by CHI3L1 in human skeletal muscle cells. TNFα binds to the TNFR1 thus leading to the activation of NF-κB that results in the enhanced expression of CHI3L1, IL-6, MCP-1 and IL-8. Increased levels of CHI3L1 then activate PAR-2 signaling leading to the inhibition of TNFα-induced NF-κB activation by an yet unidentified mechanism. This finally leads to a suppression of CHI3L1, IL-6, MCP-1 and IL-8 expression. Tumor necrosis factor receptor 1 (TNFR1); receptor-interacting serine/threonine-protein kinase 1 (RIP); tumor necrosis factor receptor type 1-associated DEATH domain (TRADD); inhibitor of NF-κB (IκB); IκB kinase (IKK).

In contrast to the observation that regular moderate exercise reduces chronic inflammation, high intensive training causes a temporary depression of various aspects of immune function and an increase in systemic inflammation for a certain post-exercise period (~3-24h) (322). After a very intensive exercise such as marathon running, TNFα and IL-1β levels increase in response to muscle damage (323;324). It is well established that muscle repair and regeneration after acute muscle injury involves tissue-remodeling and growth-promoting local inflammation (324). The initial inflammatory response is required for a positive outcome of muscle repair (325). Hence, it is possible that CHI3L1 operates locally in skeletal muscle tissue to induce myoblast proliferation and reduce inflammation, hereby providing a feedback loop for skeletal muscle to regulate its own growth and regeneration for adaptation to exercise.

3.2 HIF-1α and skeletal muscle function

In this thesis experiments are presented that were performed to gain a better understanding of the role of HIF-1α in skeletal muscle homeostasis, metabolism and function. This chapter will give a brief summary of major findings and set them in context with recent literature. It will finish with an outlook on current and future studies aimed to better understand the activity of HIF-1α in skeletal muscle with regard to insulin action and glucose metabolism.

HIF-1α in skeletal muscle

Like in virtually all cells, skeletal muscle tissue is able to respond to hypoxia through the HIF-1 pathway (87;88). Animal studies have demonstrated that 1 h of systemic hypoxia is sufficient to increase HIF-1α protein expression in skeletal muscle (326). In accordance with this, when leg oxygen delivery is impaired following blood flow restriction in humans, HIF-1α protein levels are known to be increased (327). In addition to this classical mode of activation of HIF-1α by hypoxia, evidence suggests that various cellular stimuli such as cytokines, metabolites in the tricarboxylic acid cycle such as fumarate, reactive oxygen species, and various kinase cascades activate HIF-1α even under normoxic conditions (328-332). However, it is worth noting that the contribution of these regulators is only partially understood and that their influence on HIF-1 stabilization is far less than the dramatic induction caused by hypoxia (330; 331). One particular important aspect of skeletal muscle is a high HIF-1α protein level even in normoxic conditions (92). Therefore, it is very plausible that HIF-1 plays a critical role in the regulation of cellular functions not only at hypoxic conditions, but also in conditions such as metabolic disturbance and inflammation. Insulin has been shown to induce HIF-1α and its target gene expression in skeletal muscle cells under normoxic conditions, indicating an involvement of HIF-1α in mediating a part of insulin action (99).

The normal tissue O_2 level in adult skeletal muscle, in general measured by direct microelectrode analysis, varies between 1.8 to 10.5% depending on exact electrode placement (333). Csete et al. have shown that both skeletal muscle satellite cell proliferation as well as survival of mature muscle fibers was increased at more physiologic (6%) O_2 levels compared to normal (21%) O_2 concentration used in most cell cultures (334). In addition, Storch et al. have shown that myogenesis of primary fiber cultures after 5-azacytidine treatment was enhanced at reduced O_2 levels compared to traditional cell culture O_2 conditions (335). By using a combination of 7% O_2 and EPS we established and characterized a novel *in vitro*

model for human skeletal muscle contraction at reduced oxygen levels resulting in improved insulin sensitivity of the cells, which is accompanied by induction of HIF-1α. Taken together, these observations indicate that the reduction of O_2 levels *in vitro* improves skeletal muscle function and metabolism which may be partly explained by the more physiological O_2 concentrations (2-11%) compared to the hyperoxic O_2 levels (20-21%) which are used in traditional cell cultures.

Skeletal muscle HIF-1α expression in response to exercise

Physical exercise *per se* may also challenge muscle oxygen homeostasis (336). Accordingly, an alteration in HIF-1 signaling is expected. In support of this increased HIF-1α protein expression in human skeletal muscle following an acute bout of normoxic exercise as reported (327). In line with these results, we observed an increase in skeletal muscle HIF-1α mRNA expression after a single bout of normoxic endurance exercise. If a single bout of exercise is associated with muscle hypoxia, it may be suggested that the repetition of exercise potentially challenges muscle oxygen homeostasis even more. The effect of chronic exercise on skeletal muscle function has been extensively investigated. Angiogenesis and alterations of metabolic control are well-known adaptations to endurance training (337). HIF-1 may play a central role in this setting, since HIF-1 modulates vascular endothelial growth factor as well as several glycolytic enzymes (337). We demonstrated that 12 weeks training composed of two endurance bicycle sessions and two whole-body strength-training sessions per week significantly increased skeletal muscle HIF-1α mRNA expression. In contrast, Lindholm et al. demonstrated that HIF inhibitors (prolyl hydroxylase PHD1-3) are up-regulated in skeletal muscle of elite endurance athletes (338). They propose that an up-regulation of these negative regulators may lead to a subsequent attenuation of the HIF response, which may represent a functional switch toward a higher capacity to activate the oxidative system in response to endurance training. Moreover, a recent study shows that endurance training actually reduces muscle HIF-1α signaling (339). In this study skeletal muscle biopsies were obtained from a trained (5 days/week during 4 weeks) and untrained leg from the same human subject before and immediately after a 3 hour two-legged knee extensor exercise bout, where legs exercised at the same absolute workload. Interestingly, in the untrained leg the exercise bout induced an increase of HIF-1α mRNA expression, whereas HIF-1α mRNA levels were not altered at any time point in the trained leg. These data could explain our observation that HIF-1α mRNA expression was increased after long-term exercise because the subjects in our study were sedentary before the exercise intervention.

Furthermore, in HIF-1α null and wild-type mice, skeletal muscles were shown to respond similarly to endurance training, suggesting that the HIF-1 pathway is not essential for endurance training (92;339;340). To draw clear-cut conclusions regarding the effect of long-term endurance exercise on the induction of HIF-1, future studies are needed to clearly clarify if HIF-1α is essential for endurance training.

However, how does skeletal muscle respond to exercise in hypoxia if exercise is superimposed and hence combines the effects of exercise and hypoxia on HIF-1 signaling? Combining acute hypoxia and acute exercise results in higher HIF-1α protein expression than with exercise alone (327). The effect of hypoxic exercise training on HIF-1α mRNA content in human skeletal muscle has been investigated in two studies (341;342). Vogt et al. demonstrated that HIF-1α mRNA was increased by hypoxic but not by normoxic training measured in muscle biopsies obtained 24 h after the last exercise stimulus. In accordance, we show that HIF-1α protein levels were elevated in the combined setting of reduced O_2 level and EPS, whereas EPS under normoxic conditions was not able to enhance HIF-1α expression or stabilization.

Taken together, these data indicate that HIF-1α can be activated in skeletal muscle during muscle contraction in hypoxia, highlighting its possible functional role on target genes involved in angiogenesis and/or energy metabolism. The acute response of hypoxia and exercise on gene expression in human skeletal muscle is at present poorly described. Therefore, our *in vitro* model of human skeletal muscle contracting under reduced oxygen levels gained a better understanding of the role of HIF-1α in skeletal muscle. This *in vitro* model represents a unique platform to analyze the HIF-1 pathway at the molecular level.

HIF-1α and skeletal muscle glucose metabolism

Additionally, we investigated the role of HIF-1α for insulin- and contraction-stimulated glucose uptake in skeletal muscle cells and demonstrated an implication of HIF-1α in regulation of insulin signaling as well as glucose uptake, which may indicate a previously unknown aspect of HIF-1α in determination of glucose metabolism in skeletal muscle. By using a combination of 7% O_2 and EPS we established and characterized a novel *in vitro* model for human skeletal muscle contraction at reduced oxygen levels which results in improved insulin sensitivity of the cells. Insulin signaling at the level of Akt, GSK-3 and

AS160 phosphorylation was increased in contracting cells cultured at more physiological (7%) O_2 concentration compared to non-physiologic (21%) O_2 concentration as used in traditional cell cultures. Furthermore, combination of 7% O_2 and EPS enhanced insulin-stimulated glucose uptake, whereas basal glucose uptake was unaffected. Interestingly, GLUT4 and not the well-known HIF-1α target gene GLUT1, was significantly up-regulated in the combined treatment. In addition, knockdown of HIF-1α resulted in severe reduction of insulin-induced glucose uptake as well as impaired insulin signaling. In line with our results, Sakagami et al. have demonstrated that knockdown of HIF-1α strongly reduced insulin-stimulated glucose uptake and AS160 phosphorylation in murine skeletal muscle cells (343). Furthermore, AS160 phosphorylation and GLUT4 trafficking was increased in C_2C_{12} cells overexpressing constitutively active HIF-1α (343). In excellent agreement with *in vitro* analysis, HIF-$1\alpha^{+/-}$ mice show reduced glucose uptake under insulin-stimulated conditions (99). In addition, phosphorylation of AS160 in skeletal muscle isolated from HIF-$1\alpha^{+/-}$ mice was reduced compared with specimens from wild-type animals (99).

Furthermore, we have demonstrated that loss of HIF-1α in human skeletal muscle cells resulted in reduction of contraction-induced glucose uptake. EPS treatment doubled the glucose uptake in control cells, whereas HIF-1α siRNA-treated cells showed no increased glucose uptake in response to EPS. However, the EPS-mediated AMPK activation was not affected by loss of HIF-1α. In accordance, it could be demonstrated that knockout of HIF-1α abolished glucose uptake by the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) in mouse skeletal muscle cells (99). AMPK-mediated AS160 phosphorylation and GLUT4 translocation are involved in contraction/exercise-induced glucose uptake by skeletal muscle (56). Therefore, HIF-1α might play a role in glucose uptake by exercising muscles as well.

Taken together, our data indicate HIF-1α as an important regulator of glucose metabolism in skeletal muscle. The mechanism underlying HIF-1α-mediated up-regulation of Akt and AS160 phosphorylation is still elusive. The most widely recognized functional aspect of HIF-1α is a transcriptional regulation, and so far evidence demonstrating kinase activities in HIF-1α has not been found. Therefore, it is unlikely that HIF-1α directly affects the phosphorylation process of Akt and AS160. Given the fact that introduction of a constitutively active form of HIF-1α mimics enhanced phosphorylation of AS160, we may speculate about a rather indirect model in which a HIF-1 target gene product may regulate phosphorylation of AS160, e.g. via an allosteric mechanism. More than one thousand target

genes were reported to be regulated by HIF-1α (344). Interestingly, 14-3-3 proteins, which interact with AS160 to regulate its function, might be regulated by hypoxia and therefore may also be a target gene of HIF-1α (345;346). Therefore, we analyzed the expression of all seven 14-3-3 (ß/α, γ, ε, η, ζ/δ, θ/τ and σ) isoforms in HIF-1α siRNA-treated and EPS-treated (7% O_2) cells. However, we observed no significant differences. Further experiments are necessary to evaluate the underlying mechanism of how the transcription factor HIF-1α impacts the insulin signaling cascade.

One of the aims of this study was to investigate the effect of acute and chronic exercise on skeletal muscle HIF-1α mRNA expression in healthy normoglycemic and dysglycemic subjects. Others have shown that skeletal muscle HIF-1α mRNA was up-regulated after a single bout of exercise in lean healthy controls (347;348). In line with these results, we observed a significant increase in muscle HIF-1α mRNA expression after a single bout of exercise as well as after long term exercise in control subjects. In contrast, to the best of our knowledge, we show for the first time that the induction of muscle HIF-1α mRNA expression by long term exercise is inhibited in subjects with abnormal glucose metabolism. Alterations of hypoxia-sensitive genes have been reported previously in diabetic rats; e.g. it was shown that myocardial infarct size in rats was increased in hyperglycemic conditions and was associated with a reduced expression of the HIF-1α gene (349). In addition, Fadini et al. have shown that streptozotocin-treated rats were not able to induce HIF-1α in skeletal muscle after ischaemia, whereas control animals showed a strong up-regulation (350). Furthermore, we observed a strong correlation between skeletal muscle HIF-1α mRNA expression and whole body insulin sensitivity. High skeletal muscle HIF-1α mRNA expression was associated with lower blood glucose and higher glucose infusion rate (GIR). Interestingly, Gunton et al. showed that patients with T2D have decreased HIF-1β mRNA levels in pancreatic tissue, which results in changes of HIF-1 activity (351). In addition, a human genetic polymorphism that results in the substitution of serine for proline at residue 582 of HIF-1α is associated with T2D (352). Hence, it might be speculated that the inability to induce HIF-1α or changes in HIF-1 activity may contribute to the pathogenesis of diabetes.

In summary, we demonstrated that a reduction of the oxygen level to 7% and the combination with muscle contraction improved insulin action in primary human skeletal muscle cells. Furthermore, we have shown that HIF-1α plays a critical role in this setting and our data indicate that HIF-1α is an important determinant in insulin- and contraction-induced glucose uptake in human skeletal muscle cells. Moreover, skeletal muscle HIF-1α mRNA expression

was up-regulated by acute and chronic exercise in healthy subjects. In contrast, the exercise-induced up-regulation of HIF-1α was blunted in pre-diabetic subjects. Additionally, we observed that muscle HIF-1α mRNA expression is positively correlated with whole body insulin sensitivity. Therefore, we hypothesize that skeletal muscle HIF-1α is a critical determinant for insulin- and exercise-regulated glucose metabolism in human skeletal muscle and could be used as a therapeutically relevant target to improve insulin sensitivity in human.

3.3 Perspectives

The myokine follistatin-like protein 1

In this thesis we characterized Fstl1 as a novel exercise-regulated myokine, which is expressed, produced and secreted by human skeletal muscle cells. However, muscle-contraction *in vitro* induced by electrical pulse stimulation was not able to enhance Fstl1 expression or release. Furthermore, stimulation of human skeletal muscle cells with recombinant protein did not lead to the activation of Akt, AMPKα or ACC. Therefore future studies are necessary in order to clarify the following points:

- There is a need to clarify which organ, tissue or cell type is responsible for the enhanced circulating Fstl1 levels after exercise. In this respect it may be helpful to analyze biopsies from different tissues (e.g. skeletal muscle or adipose tissue) before and after exercise to investigate the expression of Fstl1. Furthermore, blood samples obtained from the arterial-femoral venous of the exercising leg could be examined with regard to clarify whether the working muscle is responsible for the exercise-induced increase in Fstl1 plasma concentration.
- The biological function of Fstl1 in skeletal muscle is still unknown. Therefore, the identification of the specific receptor could be helpful to understand the biological function of Fstl1 in more detail and could provide information of relevant target tissues.

The myokine chitinase-3-like protein 1

We show for the first time, that CHI3L1 is expressed, produced and released from contracting muscle cells and circulating CHI3L1 levels are up-regulated by acute exercise. Therefore, we classified CHI3L1 as a novel contraction-regulated myokine. Furthermore, we show that CHI3L1 induces myoblast proliferation and protects the skeletal muscle cells from TNFα-induced inflammation and insulin resistance. However, elevated CHI3L1 levels are found in many diseases characterized by acute and chronic inflammation. In addition, CHI3L1 is produced by several cell types and the expression is up-regulated in many pathological conditions indicating that CHI3L1 has multiple functions depending on the target tissue and the physiological or pathological state. Therefore, future studies are needed to assess the following issues:

- It is necessary to clarify which tissue or organ is responsible for chronically elevated CHI3L1 circulating levels in various pathological conditions. To figure this out, it

would be helpful to use tissue-specific CHI3L1 knock-out mice (e.g. adipose tissue or macrophages) to gain a better understanding of depot-specific CHI3L1 on systemic levels.

- The biological role of CHI3L1 in many tissues and organs is still unknown, therefore future studies should investigate the impact of CHI3L1 in other cell types like adipocytes, hepatocytes or macrophages under physiological and pathological conditions.

HIF-1α and skeletal muscle glucose metabolism

In this thesis we established a novel model to study skeletal muscle contraction under reduced oxygen tensions. We demonstrated that 7% O_2 and the combination with muscle contraction improved insulin action in primary human skeletal muscle cells. Furthermore, we provide clear evidences that HIF-1α is an important regulator for insulin- and contraction-mediated glucose metabolism in human skeletal muscle cells. However, the mechanism underlying HIF-1α-mediated regulation of the insulin signaling pathway is still unknown. Furthermore, the impact of HIF-1α on skeletal muscle glucose metabolism *in vivo* in addition to enhanced vascularization has never been investigated. Therefore, we actually plan to focus on the following:

- A genome-wide gene expression profiling approach will be used based on total RNA-sequencing of HIF-1α overexpressing cells to identify novel targets, connecting HIF-1α to the insulin signaling pathway.
- Overexpression of HIF-1α in murine skeletal muscle will be performed to confirm the role of HIF-1α as a key regulator of skeletal muscle function *in vivo*.

Reference List

1. Shepherd,PR, Kahn,BB: Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus. *N Engl J Med* 341:248-257, 1999
2. Eckardt,K, Gorgens,SW, Raschke,S, Eckel,J: Myokines in insulin resistance and type 2 diabetes. *Diabetologia* 57:1087-1099, 2014
3. Febbraio,MA, Pedersen,BK: Contraction-induced myokine production and release: is skeletal muscle an endocrine organ? *Exerc Sport Sci Rev* 33:114-119, 2005
4. Knowler,WC, Barrett-Connor,E, Fowler,SE, Hamman,RF, Lachin,JM, Walker,EA, Nathan,DM: Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. *N Engl J Med* 346:393-403, 2002
5. Richter,EA, Hargreaves,M: Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiol Rev* 93:993-1017, 2013
6. Tuomilehto,J, Lindstrom,J, Eriksson,JG, Valle,TT, Hamalainen,H, Ilanne-Parikka,P, Keinanen-Kiukaanniemi,S, Laakso,M, Louheranta,A, Rastas,M, Salminen,V, Uusitupa,M: Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance. *N Engl J Med* 344:1343-1350, 2001
7. Sano,H, Kane,S, Sano,E, Miinea,CP, Asara,JM, Lane,WS, Garner,CW, Lienhard,GE: Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem* 278:14599-14602, 2003
8. Shin,ES, Sorenson,CM, Sheibani,N: Diabetes and retinal vascular dysfunction. *J Ophthalmic Vis Res* 9:362-373, 2014
9. Lovre,D, Fonseca,V: Benefits of timely basal insulin control in patients with type 2 diabetes. *J Diabetes Complications* 29:295-301, 2015
10. Zhao,J, Randive,R, Stewart,JA: Molecular mechanisms of AGE/RAGE-mediated fibrosis in the diabetic heart. *World J Diabetes* 5:860-867, 2014
11. Ryu,TY, Park,J, Scherer,PE: Hyperglycemia as a risk factor for cancer progression. *Diabetes Metab J* 38:330-336, 2014
12. Saltiel,AR, Kahn,CR: Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414:799-806, 2001
13. Ahlborg,G, Felig,P, Hagenfeldt,L, Hendler,R, Wahren,J: Substrate turnover during prolonged exercise in man. Splanchnic and leg metabolism of glucose, free fatty acids, and amino acids. *J Clin Invest* 53:1080-1090, 1974
14. Ahlborg,G, Wahren,J, Felig,P: Splanchnic and peripheral glucose and lactate metabolism during and after prolonged arm exercise. *J Clin Invest* 77:690-699, 1986
15. An,D, Toyoda,T, Taylor,EB, Yu,H, Fujii,N, Hirshman,MF, Goodyear,LJ: TBC1D1 regulates insulin- and contraction-induced glucose transport in mouse skeletal muscle. *Diabetes* 59:1358-1365, 2010
16. Chauveau M, Kauffmann M.: Experiences pour la determination du coefficient de l'activite nutritive et resporatoire des muscle en repos et en travail. *C R Acad Sci* 104:1125-1131: 1887
17. Lauritzen,HP, Schertzer,JD: Measuring GLUT4 translocation in mature muscle fibers. *Am J Physiol Endocrinol Metab* 299:E169-E179, 2010
18. Birnbaum,MJ: Identification of a novel gene encoding an insulin-responsive glucose transporter protein. *Cell* 57:305-315, 1989
19. Charron,MJ, Brosius,FC, III, Alper,SL, Lodish,HF: A glucose transport protein expressed predominately in insulin-responsive tissues. *Proc Natl Acad Sci U S A* 86:2535-2539, 1989
20. James,DE, Strube,M, Mueckler,M: Molecular cloning and characterization of an insulin-regulatable glucose transporter. *Nature* 338:83-87, 1989
21. Mueckler,M, Thorens,B: The SLC2 (GLUT) family of membrane transporters. *Mol Aspects Med* 34:121-138, 2013
22. Mueckler,M, Caruso,C, Baldwin,SA, Panico,M, Blench,I, Morris,HR, Allard,WJ, Lienhard,GE, Lodish,HF: Sequence and structure of a human glucose transporter. *Science* 229:941-945, 1985

23. Kasahara,M, Hinkle,PC: Reconstitution and purification of the D-glucose transporter from human erythrocytes. *J Biol Chem* 252:7384-7390, 1977
24. Vestergaard,H: Studies of gene expression and activity of hexokinase, phosphofructokinase and glycogen synthase in human skeletal muscle in states of altered insulin-stimulated glucose metabolism. *Dan Med Bull* 46:13-34, 1999
25. Rose,AJ, Richter,EA: Skeletal muscle glucose uptake during exercise: how is it regulated? *Physiology (Bethesda)* 20:260-270, 2005
26. Piwien-Pilipuk,G, Huo,JS, Schwartz,J: Growth hormone signal transduction. *J Pediatr Endocrinol Metab* 15:771-786, 2002
27. Avruch,J: Insulin signal transduction through protein kinase cascades. *Mol Cell Biochem* 182:31-48, 1998
28. Kido,Y, Nakae,J, Accili,D: Clinical review 125: The insulin receptor and its cellular targets. *J Clin Endocrinol Metab* 86:972-979, 2001
29. Taniguchi,CM, Emanuelli,B, Kahn,CR: Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 7:85-96, 2006
30. White,MF: The IRS-signaling system: a network of docking proteins that mediate insulin and cytokine action. *Recent Prog Horm Res* 53:119-138, 1998
31. Boucher,J, Kleinridders,A, Kahn,CR: Insulin receptor signaling in normal and insulin-resistant states. *Cold Spring Harb Perspect Biol* 6: 2014
32. Farese,RV, Sajan,MP, Standaert,ML: Insulin-sensitive protein kinases (atypical protein kinase C and protein kinase B/Akt): actions and defects in obesity and type II diabetes. *Exp Biol Med (Maywood)* 230:593-605, 2005
33. Sale,EM, Sale,GJ: Protein kinase B: signalling roles and therapeutic targeting. *Cell Mol Life Sci* 65:113-127, 2008
34. Kanzaki,M, Mora,S, Hwang,JB, Saltiel,AR, Pessin,JE: Atypical protein kinase C (PKCzeta/lambda) is a convergent downstream target of the insulin-stimulated phosphatidylinositol 3-kinase and TC10 signaling pathways. *J Cell Biol* 164:279-290, 2004
35. Bandyopadhyay,G, Standaert,ML, Galloway,L, Moscat,J, Farese,RV: Evidence for involvement of protein kinase C (PKC)-zeta and noninvolvement of diacylglycerol-sensitive PKCs in insulin-stimulated glucose transport in L6 myotubes. *Endocrinology* 138:4721-4731, 1997
36. Wang,Q, Somwar,R, Bilan,PJ, Liu,Z, Jin,J, Woodgett,JR, Klip,A: Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol Cell Biol* 19:4008-4018, 1999
37. Cartee,GD, Wojtaszewski,JF: Role of Akt substrate of 160 kDa in insulin-stimulated and contraction-stimulated glucose transport. *Appl Physiol Nutr Metab* 32:557-566, 2007
38. Alessi,DR, James,SR, Downes,CP, Holmes,AB, Gaffney,PR, Reese,CB, Cohen,P: Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol* 7:261-269, 1997
39. Sarbassov,DD, Guertin,DA, Ali,SM, Sabatini,DM: Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307:1098-1101, 2005
40. Kane,S, Sano,H, Liu,SC, Asara,JM, Lane,WS, Garner,CC, Lienhard,GE: A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain. *J Biol Chem* 277:22115-22118, 2002
41. Eguez,L, Lee,A, Chavez,JA, Miinea,CP, Kane,S, Lienhard,GE, McGraw,TE: Full intracellular retention of GLUT4 requires AS160 Rab GTPase activating protein. *Cell Metab* 2:263-272, 2005
42. Hajduch,E, Litherland,GJ, Hundal,HS: Protein kinase B (PKB/Akt)--a key regulator of glucose transport? *FEBS Lett* 492:199-203, 2001
43. Miinea,CP, Sano,H, Kane,S, Sano,E, Fukuda,M, Peranen,J, Lane,WS, Lienhard,GE: AS160, the Akt substrate regulating GLUT4 translocation, has a functional Rab GTPase-activating protein domain. *Biochem J* 391:87-93, 2005
44. Sano,H, Kane,S, Sano,E, Miinea,CP, Asara,JM, Lane,WS, Garner,CW, Lienhard,GE: Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation. *J Biol Chem* 278:14599-14602, 2003

45. Kaddai,V, Le Marchand-Brustel,Y, Cormont,M: Rab proteins in endocytosis and Glut4 trafficking. *Acta Physiol (Oxf)* 192:75-88, 2008
46. Dugani,CB, Klip,A: Glucose transporter 4: cycling, compartments and controversies. *EMBO Rep* 6:1137-1142, 2005
47. Goodyear,LJ, Giorgino,F, Balon,TW, Condorelli,G, Smith,RJ: Effects of contractile activity on tyrosine phosphoproteins and PI 3-kinase activity in rat skeletal muscle. *Am J Physiol* 268:E987-E995, 1995
48. Goodyear,LJ, King,PA, Hirshman,MF, Thompson,CM, Horton,ED, Horton,ES: Contractile activity increases plasma membrane glucose transporters in absence of insulin. *Am J Physiol* 258:E667-E672, 1990
49. Lee,AD, Hansen,PA, Holloszy,JO: Wortmannin inhibits insulin-stimulated but not contraction-stimulated glucose transport activity in skeletal muscle. *FEBS Lett* 361:51-54, 1995
50. Lund,S, Holman,GD, Schmitz,O, Pedersen,O: Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin. *Proc Natl Acad Sci U S A* 92:5817-5821, 1995
51. Ploug,T, Galbo,H, Richter,EA: Increased muscle glucose uptake during contractions: no need for insulin. *Am J Physiol* 247:E726-E731, 1984
52. Wallberg-Henriksson,H, Holloszy,JO: Contractile activity increases glucose uptake by muscle in severely diabetic rats. *J Appl Physiol Respir Environ Exerc Physiol* 57:1045-1049, 1984
53. Bruss,MD, Arias,EB, Lienhard,GE, Cartee,GD: Increased phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle in response to insulin or contractile activity. *Diabetes* 54:41-50, 2005
54. Frosig,C, Pehmoller,C, Birk,JB, Richter,EA, Wojtaszewski,JF: Exercise-induced TBC1D1 Ser237 phosphorylation and 14-3-3 protein binding capacity in human skeletal muscle. *J Physiol* 588:4539-4548, 2010
55. Funai,K, Cartee,GD: Inhibition of contraction-stimulated AMP-activated protein kinase inhibits contraction-stimulated increases in PAS-TBC1D1 and glucose transport without altering PAS-AS160 in rat skeletal muscle. *Diabetes* 58:1096-1104, 2009
56. Kramer,HF, Witczak,CA, Taylor,EB, Fujii,N, Hirshman,MF, Goodyear,LJ: AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle. *J Biol Chem* 281:31478-31485, 2006
57. Kramer,HF, Witczak,CA, Fujii,N, Jessen,N, Taylor,EB, Arnolds,DE, Sakamoto,K, Hirshman,MF, Goodyear,LJ: Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle. *Diabetes* 55:2067-2076, 2006
58. Barnes,BR, Ryder,JW, Steiler,TL, Fryer,LG, Carling,D, Zierath,JR: Isoform-specific regulation of 5' AMP-activated protein kinase in skeletal muscle from obese Zucker (fa/fa) rats in response to contraction. *Diabetes* 51:2703-2708, 2002
59. Hayashi,T, Hirshman,MF, Kurth,EJ, Winder,WW, Goodyear,LJ: Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* 47:1369-1373, 1998
60. Koistinen,HA, Zierath,JR: Regulation of glucose transport in human skeletal muscle. *Ann Med* 34:410-418, 2002
61. Hutber,CA, Hardie,DG, Winder,WW: Electrical stimulation inactivates muscle acetyl-CoA carboxylase and increases AMP-activated protein kinase. *Am J Physiol* 272:E262-E266, 1997
62. Musi,N, Fujii,N, Hirshman,MF, Ekberg,I, Froberg,S, Ljungqvist,O, Thorell,A, Goodyear,LJ: AMP-activated protein kinase (AMPK) is activated in muscle of subjects with type 2 diabetes during exercise. *Diabetes* 50:921-927, 2001
63. Wojtaszewski,JF, Birk,JB, Frosig,C, Holten,M, Pilegaard,H, Dela,F: 5'AMP activated protein kinase expression in human skeletal muscle: effects of strength training and type 2 diabetes. *J Physiol* 564:563-573, 2005
64. Hardie,DG, Carling,D, Carlson,M: The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu Rev Biochem* 67:821-855, 1998
65. Kemp,BE, Mitchelhill,KI, Stapleton,D, Michell,BJ, Chen,ZP, Witters,LA: Dealing with energy demand: the AMP-activated protein kinase. *Trends Biochem Sci* 24:22-25, 1999

66. Woods,A, Johnstone,SR, Dickerson,K, Leiper,FC, Fryer,LG, Neumann,D, Schlattner,U, Wallimann,T, Carlson,M, Carling,D: LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. *Curr Biol* 13:2004-2008, 2003
67. Hawley,SA, Pan,DA, Mustard,KJ, Ross,L, Bain,J, Edelman,AM, Frenguelli,BG, Hardie,DG: Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab* 2:9-19, 2005
68. Woods,A, Dickerson,K, Heath,R, Hong,SP, Momcilovic,M, Johnstone,SR, Carlson,M, Carling,D: Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metab* 2:21-33, 2005
69. Merrill,GF, Kurth,EJ, Hardie,DG, Winder,WW: AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. *Am J Physiol* 273:E1107-E1112, 1997
70. Sylow,L, Jensen,TE, Kleinert,M, Mouatt,JR, Maarbjerg,SJ, Jeppesen,J, Prats,C, Chiu,TT, Boguslavsky,S, Klip,A, Schjerling,P, Richter,EA: Rac1 is a novel regulator of contraction-stimulated glucose uptake in skeletal muscle. *Diabetes* 62:1139-1151, 2013
71. Fujii,N, Hirshman,MF, Kane,EM, Ho,RC, Peter,LE, Seifert,MM, Goodyear,LJ: AMP-activated protein kinase alpha2 activity is not essential for contraction- and hyperosmolarity-induced glucose transport in skeletal muscle. *J Biol Chem* 280:39033-39041, 2005
72. Jorgensen,SB, Viollet,B, Andreelli,F, Frosig,C, Birk,JB, Schjerling,P, Vaulont,S, Richter,EA, Wojtaszewski,JF: Knockout of the alpha2 but not alpha1 5'-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. *J Biol Chem* 279:1070-1079, 2004
73. Mu,J, Brozinick,JT, Jr., Valladares,O, Bucan,M, Birnbaum,MJ: A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell* 7:1085-1094, 2001
74. Koh,HJ, Arnolds,DE, Fujii,N, Tran,TT, Rogers,MJ, Jessen,N, Li,Y, Liew,CW, Ho,RC, Hirshman,MF, Kulkarni,RN, Kahn,CR, Goodyear,LJ: Skeletal muscle-selective knockout of LKB1 increases insulin sensitivity, improves glucose homeostasis, and decreases TRB3. *Mol Cell Biol* 26:8217-8227, 2006
75. Sakamoto,K, McCarthy,A, Smith,D, Green,KA, Grahame,HD, Ashworth,A, Alessi,DR: Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. *EMBO J* 24:1810-1820, 2005
76. Hinkley,JM, Ferey,JL, Brault,JJ, Smith,CA, Gilliam,LA, Witczak,CA: Constitutively active CaMKKalpha stimulates skeletal muscle glucose uptake in insulin-resistant mice in vivo. *Diabetes* 63:142-151, 2014
77. Mohankumar,SK, Taylor,CG, Zahradka,P: Domain-dependent modulation of insulin-induced AS160 phosphorylation and glucose uptake by Ca2+/calmodulin-dependent protein kinase II in L6 myotubes. *Cell Signal* 24:302-308, 2012
78. Pfisterer,SG, Mauthe,M, Codogno,P, Proikas-Cezanne,T: Ca2+/calmodulin-dependent kinase (CaMK) signaling via CaMKI and AMP-activated protein kinase contributes to the regulation of WIPI-1 at the onset of autophagy. *Mol Pharmacol* 80:1066-1075, 2011
79. Jensen,TE, Rose,AJ, Jorgensen,SB, Brandt,N, Schjerling,P, Wojtaszewski,JF, Richter,EA: Possible CaMKK-dependent regulation of AMPK phosphorylation and glucose uptake at the onset of mild tetanic skeletal muscle contraction. *Am J Physiol Endocrinol Metab* 292:E1308-E1317, 2007
80. Shirwany,NA, Zou,MH: AMPK: a cellular metabolic and redox sensor. A minireview. *Front Biosci (Landmark Ed)* 19:447-474, 2014
81. Kramer,HF, Witczak,CA, Fujii,N, Jessen,N, Taylor,EB, Arnolds,DE, Sakamoto,K, Hirshman,MF, Goodyear,LJ: Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle. *Diabetes* 55:2067-2076, 2006
82. Treebak,JT, Glund,S, Deshmukh,A, Klein,DK, Long,YC, Jensen,TE, Jorgensen,SB, Viollet,B, Andersson,L, Neumann,D, Wallimann,T, Richter,EA, Chibalin,AV, Zierath,JR, Wojtaszewski,JF: AMPK-mediated AS160 phosphorylation in skeletal muscle is dependent on AMPK catalytic and regulatory subunits. *Diabetes* 55:2051-2058, 2006

83. Kramer,HF, Witczak,CA, Taylor,EB, Fujii,N, Hirshman,MF, Goodyear,LJ: AS160 regulates insulin- and contraction-stimulated glucose uptake in mouse skeletal muscle. *J Biol Chem* 281:31478-31485, 2006
84. Thong,FS, Dugani,CB, Klip,A: Turning signals on and off: GLUT4 traffic in the insulin-signaling highway. *Physiology (Bethesda)* 20:271-284, 2005
85. Bruss,MD, Arias,EB, Lienhard,GE, Cartee,GD: Increased phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle in response to insulin or contractile activity. *Diabetes* 54:41-50, 2005
86. Horscroft,JA, Murray,AJ: Skeletal muscle energy metabolism in environmental hypoxia: climbing towards consensus. *Extrem Physiol Med* 3:19, 2014
87. Semenza,GL: Hypoxia-inducible factors in physiology and medicine. *Cell* 148:399-408, 2012
88. Semenza,GL: Hypoxia-inducible factor 1 (HIF-1) pathway. *Sci STKE* 2007:cm8, 2007
89. Ratcliffe,PJ, O'Rourke,JF, Maxwell,PH, Pugh,CW: Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. *J Exp Biol* 201:1153-1162, 1998
90. Rhim,T, Lee,DY, Lee,M: Hypoxia as a target for tissue specific gene therapy. *J Control Release* 172:484-494, 2013
91. Ponsot,E, Dufour,SP, Zoll,J, Doutrelau,S, N'Guessan,B, Geny,B, Hoppeler,H, Lampert,E, Mettauer,B, Ventura-Clapier,R, Richard,R: Exercise training in normobaric hypoxia in endurance runners. II. Improvement of mitochondrial properties in skeletal muscle. *J Appl Physiol (1985)* 100:1249-1257, 2006
92. Lundby,C, Calbet,JA, Robach,P: The response of human skeletal muscle tissue to hypoxia. *Cell Mol Life Sci* 66:3615-3623, 2009
93. Fisher,JS, Gao,J, Han,DH, Holloszy,JO, Nolte,LA: Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. *Am J Physiol Endocrinol Metab* 282:E18-E23, 2002
94. Azevedo,JL, Jr., Carey,JO, Pories,WJ, Morris,PG, Dohm,GL: Hypoxia stimulates glucose transport in insulin-resistant human skeletal muscle. *Diabetes* 44:695-698, 1995
95. Cartee,GD, Douen,AG, Ramlal,T, Klip,A, Holloszy,JO: Stimulation of glucose transport in skeletal muscle by hypoxia. *J Appl Physiol (1985)* 70:1593-1600, 1991
96. Chiu,LL, Chou,SW, Cho,YM, Ho,HY, Ivy,JL, Hunt,D, Wang,PS, Kuo,CH: Effect of prolonged intermittent hypoxia and exercise training on glucose tolerance and muscle GLUT4 protein expression in rats. *J Biomed Sci* 11:838-846, 2004
97. Chou,SW, Chiu,LL, Cho,YM, Ho,HY, Ivy,JL, Ho,CF, Kuo,CH: Effect of systemic hypoxia on GLUT4 protein expression in exercised rat heart. *Jpn J Physiol* 54:357-363, 2004
98. Brooks,GA, Butterfield,GE, Wolfe,RR, Groves,BM, Mazzeo,RS, Sutton,JR, Wolfel,EE, Reeves,JT: Increased dependence on blood glucose after acclimatization to 4,300 m. *J Appl Physiol (1985)* 70:919-927, 1991
99. Sakagami,H, Makino,Y, Mizumoto,K, Isoe,T, Takeda,Y, Watanabe,J, Fujita,Y, Takiyama,Y, Abiko,A, Haneda,M: Loss of HIF-1alpha impairs GLUT4 translocation and glucose uptake by the skeletal muscle cells. *Am J Physiol Endocrinol Metab* 306:E1065-E1076, 2014
100. Alberti,KG, Zimmet,PZ: Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 15:539-553, 1998
101. Bluestone,JA, Herold,K, Eisenbarth,G: Genetics, pathogenesis and clinical interventions in type 1 diabetes. *Nature* 464:1293-1300, 2010
102. Akerblom,HK, Vaarala,O, Hyoty,H, Ilonen,J, Knip,M: Environmental factors in the etiology of type 1 diabetes. *Am J Med Genet* 115:18-29, 2002
103. Inzucchi,SE: Clinical practice. Diagnosis of diabetes. *N Engl J Med* 367:542-550, 2012
104. Meigs,JB, Muller,DC, Nathan,DM, Blake,DR, Andres,R: The natural history of progression from normal glucose tolerance to type 2 diabetes in the Baltimore Longitudinal Study of Aging. *Diabetes* 52:1475-1484, 2003
105. Meigs,JB, Nathan,DM, D'Agostino,RB, Sr., Wilson,PW: Fasting and postchallenge glycemia and cardiovascular disease risk: the Framingham Offspring Study. *Diabetes Care* 25:1845-1850, 2002

106. Smith,NL, Barzilay,JI, Shaffer,D, Savage,PJ, Heckbert,SR, Kuller,LH, Kronmal,RA, Resnick,HE, Psaty,BM: Fasting and 2-hour postchallenge serum glucose measures and risk of incident cardiovascular events in the elderly: the Cardiovascular Health Study. *Arch Intern Med* 162:209-216, 2002
107. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 31 Suppl 1:S55-S60, 2008
108. Klein,S, Sheard,NF, Pi-Sunyer,X, Daly,A, Wylie-Rosett,J, Kulkarni,K, Clark,NG: Weight management through lifestyle modification for the prevention and management of type 2 diabetes: rationale and strategies. A statement of the American Diabetes Association, the North American Association for the Study of Obesity, and the American Society for Clinical Nutrition. *Am J Clin Nutr* 80:257-263, 2004
109. Mokdad,AH, Ford,ES, Bowman,BA, Dietz,WH, Vinicor,F, Bales,VS, Marks,JS: Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001. *JAMA* 289:76-79, 2003
110. Pedersen,BK: The diseasome of physical inactivity--and the role of myokines in muscle--fat cross talk. *J Physiol* 587:5559-5568, 2009
111. Erikssen,G, Liestol,K, Bjornholt,J, Thaulow,E, Sandvik,L, Erikssen,J: Changes in physical fitness and changes in mortality. *Lancet* 352:759-762, 1998
112. Hu,FB, Willett,WC, Li,T, Stampfer,MJ, Colditz,GA, Manson,JE: Adiposity as compared with physical activity in predicting mortality among women. *N Engl J Med* 351:2694-2703, 2004
113. Handschin,C, Spiegelman,BM: The role of exercise and PGC1alpha in inflammation and chronic disease. *Nature* 454:463-469, 2008
114. Stanford,KI, Goodyear,LJ: Exercise and type 2 diabetes: molecular mechanisms regulating glucose uptake in skeletal muscle. *Adv Physiol Educ* 38:308-314, 2014
115. Handschin,C, Spiegelman,BM: The role of exercise and PGC1alpha in inflammation and chronic disease. *Nature* 454:463-469, 2008
116. Eriksson,J, Koranyi,L, Bourey,R, Schalin-Jantti,C, Widen,E, Mueckler,M, Permutt,AM, Groop,LC: Insulin resistance in type 2 (non-insulin-dependent) diabetic patients and their relatives is not associated with a defect in the expression of the insulin-responsive glucose transporter (GLUT-4) gene in human skeletal muscle. *Diabetologia* 35:143-147, 1992
117. Roberts,CK, Hevener,AL, Barnard,RJ: Metabolic syndrome and insulin resistance: underlying causes and modification by exercise training. *Compr Physiol* 3:1-58, 2013
118. Zierath,JR: In vitro studies of human skeletal muscle: hormonal and metabolic regulation of glucose transport. *Acta Physiol Scand Suppl* 626:1-96, 1995
119. Zierath,JR, Krook,A, Wallberg-Henriksson,H: Insulin action and insulin resistance in human skeletal muscle. *Diabetologia* 43:821-835, 2000
120. Zierath,JR, He,L, Guma,A, Odegoard,WE, Klip,A, Wallberg-Henriksson,H: Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. *Diabetologia* 39:1180-1189, 1996
121. Meerza,D, Naseem,I, Ahmed,J: Pharmacology of signaling pathways: in type 2 diabetes. *Diabetes Metab Syndr* 7:180-185, 2013
122. Mukherjee,B, Hossain,CM, Mondal,L, Paul,P, Ghosh,MK: Obesity and insulin resistance: an abridged molecular correlation. *Lipid Insights* 6:1-11, 2013
123. Cho,H, Mu,J, Kim,JK, Thorvaldsen,JL, Chu,Q, Crenshaw,EB, III, Kaestner,KH, Bartolomei,MS, Shulman,GI, Birnbaum,MJ: Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* 292:1728-1731, 2001
124. George,S, Rochford,JJ, Wolfrum,C, Gray,SL, Schinner,S, Wilson,JC, Soos,MA, Murgatroyd,PR, Williams,RM, Acerini,CL, Dunger,DB, Barford,D, Umpleby,AM, Wareham,NJ, Davies,HA, Schafer,AJ, Stoffel,M, O'Rahilly,S, Barroso,I: A family with severe insulin resistance and diabetes due to a mutation in AKT2. *Science* 304:1325-1328, 2004
125. Krook,A, Roth,RA, Jiang,XJ, Zierath,JR, Wallberg-Henriksson,H: Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. *Diabetes* 47:1281-1286, 1998
126. Kim,YB, Kotani,K, Ciaraldi,TP, Henry,RR, Kahn,BB: Insulin-stimulated protein kinase C lambda/zeta activity is reduced in skeletal muscle of humans with obesity and type 2 diabetes: reversal with weight reduction. *Diabetes* 52:1935-1942, 2003

127. Mackenzie,RW, Elliott,BT: Akt/PKB activation and insulin signaling: a novel insulin signaling pathway in the treatment of type 2 diabetes. *Diabetes Metab Syndr Obes* 7:55-64, 2014
128. Aguirre,V, Uchida,T, Yenush,L, Davis,R, White,MF: The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem* 275:9047-9054, 2000
129. Hotamisligil,GS, Peraldi,P, Budavari,A, Ellis,R, White,MF, Spiegelman,BM: IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 271:665-668, 1996
130. Paz,K, Hemi,R, LeRoith,D, Karasik,A, Elhanany,E, Kanety,H, Zick,Y: A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. *J Biol Chem* 272:29911-29918, 1997
131. Um,SH, Frigerio,F, Watanabe,M, Picard,F, Joaquin,M, Sticker,M, Fumagalli,S, Allegrini,PR, Kozma,SC, Auwerx,J, Thomas,G: Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* 431:200-205, 2004
132. Boura-Halfon,S, Zick,Y: Phosphorylation of IRS proteins, insulin action, and insulin resistance. *Am J Physiol Endocrinol Metab* 296:E581-E591, 2009
133. Hirosumi,J, Tuncman,G, Chang,L, Gorgun,CZ, Uysal,KT, Maeda,K, Karin,M, Hotamisligil,GS: A central role for JNK in obesity and insulin resistance. *Nature* 420:333-336, 2002
134. Yuan,M, Konstantopoulos,N, Lee,J, Hansen,L, Li,ZW, Karin,M, Shoelson,SE: Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. *Science* 293:1673-1677, 2001
135. Bost,F, Aouadi,M, Caron,L, Even,P, Belmonte,N, Prot,M, Dani,C, Hofman,P, Pages,G, Pouyssegur,J, Le Marchand-Brustel,Y, Binetruy,B: The extracellular signal-regulated kinase isoform ERK1 is specifically required for in vitro and in vivo adipogenesis. *Diabetes* 54:402-411, 2005
136. Handberg,A, Vaag,A, Damsbo,P, Beck-Nielsen,H, Vinten,J: Expression of insulin regulatable glucose transporters in skeletal muscle from type 2 (non-insulin-dependent) diabetic patients. *Diabetologia* 33:625-627, 1990
137. Dohm,GL, Elton,CW, Friedman,JE, Pilch,PF, Pories,WJ, Atkinson,SM, Jr., Caro,JF: Decreased expression of glucose transporter in muscle from insulin-resistant patients. *Am J Physiol* 260:E459-E463, 1991
138. Aksnes,AK, Hjeltnes,N, Wahlstrom,EO, Katz,A, Zierath,JR, Wallberg-Henriksson,H: Intact glucose transport in morphologically altered denervated skeletal muscle from quadriplegic patients. *Am J Physiol* 271:E593-E600, 1996
139. Garvey,WT, Maianu,L, Zhu,JH, Brechtel-Hook,G, Wallace,P, Baron,AD: Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance. *J Clin Invest* 101:2377-2386, 1998
140. Yoshizaki,T, Milne,JC, Imamura,T, Schenk,S, Sonoda,N, Babendure,JL, Lu,JC, Smith,JJ, Jirousek,MR, Olefsky,JM: SIRT1 exerts anti-inflammatory effects and improves insulin sensitivity in adipocytes. *Mol Cell Biol* 29:1363-1374, 2009
141. Winnay,JN, Kahn,CR: PI 3-kinase regulatory subunits as regulators of the unfolded protein response. *Methods Enzymol* 490:147-158, 2011
142. Sinha,S, Perdomo,G, Brown,NF, O'Doherty,RM: Fatty acid-induced insulin resistance in L6 myotubes is prevented by inhibition of activation and nuclear localization of nuclear factor kappa B. *J Biol Chem* 279:41294-41301, 2004
143. Shulman,GI: Ectopic fat in insulin resistance, dyslipidemia, and cardiometabolic disease. *N Engl J Med* 371:1131-1141, 2014
144. Sell,H, Habich,C, Eckel,J: Adaptive immunity in obesity and insulin resistance. *Nat Rev Endocrinol* 8:709-716, 2012
145. Sell,H, Eckel,J: Monocyte chemotactic protein-1 and its role in insulin resistance. *Curr Opin Lipidol* 18:258-262, 2007

146. Sell,H, Dietze-Schroeder,D, Kaiser,U, Eckel,J: Monocyte chemotactic protein-1 is a potential player in the negative cross-talk between adipose tissue and skeletal muscle. *Endocrinology* 147:2458-2467, 2006
147. Francaux,M: Toll-like receptor signalling induced by endurance exercise. *Appl Physiol Nutr Metab* 34:454-458, 2009
148. Kim,JK: Fat uses a TOLL-road to connect inflammation and diabetes. *Cell Metab* 4:417-419, 2006
149. Lee,BC, Lee,J: Cellular and molecular players in adipose tissue inflammation in the development of obesity-induced insulin resistance. *Biochim Biophys Acta* 1842:446-462, 2014
150. Besse-Patin,A, Estall,JL: An Intimate Relationship between ROS and Insulin Signalling: Implications for Antioxidant Treatment of Fatty Liver Disease. *Int J Cell Biol* 2014:519153, 2014
151. Zephy,D, Ahmad,J: Type 2 diabetes mellitus: Role of melatonin and oxidative stress. *Diabetes Metab Syndr* 2014
152. Alfadda,AA, Sallam,RM: Reactive oxygen species in health and disease. *J Biomed Biotechnol* 2012:936486, 2012
153. Jheng,HF, Tsai,PJ, Guo,SM, Kuo,LH, Chang,CS, Su,IJ, Chang,CR, Tsai,YS: Mitochondrial fission contributes to mitochondrial dysfunction and insulin resistance in skeletal muscle. *Mol Cell Biol* 32:309-319, 2012
154. Rockl,KS, Hirshman,MF, Brandauer,J, Fujii,N, Witters,LA, Goodyear,LJ: Skeletal muscle adaptation to exercise training: AMP-activated protein kinase mediates muscle fiber type shift. *Diabetes* 56:2062-2069, 2007
155. Frosig,C, Richter,EA: Improved insulin sensitivity after exercise: focus on insulin signaling. *Obesity (Silver Spring)* 17 Suppl 3:S15-S20, 2009
156. Richter,EA, Ruderman,NB, Gavras,H, Belur,ER, Galbo,H: Muscle glycogenolysis during exercise: dual control by epinephrine and contractions. *Am J Physiol* 242:E25-E32, 1982
157. Richter,EA, Garetto,LP, Goodman,MN, Ruderman,NB: Enhanced muscle glucose metabolism after exercise: modulation by local factors. *Am J Physiol* 246:E476-E482, 1984
158. Ivy,JL, Holloszy,JO: Persistent increase in glucose uptake by rat skeletal muscle following exercise. *Am J Physiol* 241:C200-C203, 1981
159. Garetto,LP, Richter,EA, Goodman,MN, Ruderman,NB: Enhanced muscle glucose metabolism after exercise in the rat: the two phases. *Am J Physiol* 246:E471-E475, 1984
160. Mikines,KJ, Sonne,B, Farrell,PA, Tronier,B, Galbo,H: Effect of physical exercise on sensitivity and responsiveness to insulin in humans. *Am J Physiol* 254:E248-E259, 1988
161. Dela,F, Mikines,KJ, von,LM, Secher,NH, Galbo,H: Effect of training on insulin-mediated glucose uptake in human muscle. *Am J Physiol* 263:E1134-E1143, 1992
162. Wojtaszewski,JF, Hansen,BF, Kiens,B, Richter,EA: Insulin signaling in human skeletal muscle: time course and effect of exercise. *Diabetes* 46:1775-1781, 1997
163. Hansen,PA, Nolte,LA, Chen,MM, Holloszy,JO: Increased GLUT-4 translocation mediates enhanced insulin sensitivity of muscle glucose transport after exercise. *J Appl Physiol (1985)* 85:1218-1222, 1998
164. Wojtaszewski,JF, Hansen,BF, Gade, Kiens,B, Markuns,JF, Goodyear,LJ, Richter,EA: Insulin signaling and insulin sensitivity after exercise in human skeletal muscle. *Diabetes* 49:325-331, 2000
165. Frosig,C, Sajan,MP, Maarbjerg,SJ, Brandt,N, Roepstorff,C, Wojtaszewski,JF, Kiens,B, Farese,RV, Richter,EA: Exercise improves phosphatidylinositol-3,4,5-trisphosphate responsiveness of atypical protein kinase C and interacts with insulin signalling to peptide elongation in human skeletal muscle. *J Physiol* 582:1289-1301, 2007
166. Arias,EB, Kim,J, Funai,K, Cartee,GD: Prior exercise increases phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 292:E1191-E1200, 2007
167. Funai,K, Schweitzer,GG, Sharma,N, Kanzaki,M, Cartee,GD: Increased AS160 phosphorylation, but not TBC1D1 phosphorylation, with increased postexercise insulin sensitivity in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 297:E242-E251, 2009

168. Goodyear,LJ, Hirshman,MF, Valyou,PM, Horton,ES: Glucose transporter number, function, and subcellular distribution in rat skeletal muscle after exercise training. *Diabetes* 41:1091-1099, 1992
169. Houmard,JA, Shinebarger,MH, Dolan,PL, Leggett-Frazier,N, Bruner,RK, McCammon,MR, Israel,RG, Dohm,GL: Exercise training increases GLUT-4 protein concentration in previously sedentary middle-aged men. *Am J Physiol* 264:E896-E901, 1993
170. Hughes,VA, Fiatarone,MA, Fielding,RA, Kahn,BB, Ferrara,CM, Shepherd,P, Fisher,EC, Wolfe,RR, Elahi,D, Evans,WJ: Exercise increases muscle GLUT-4 levels and insulin action in subjects with impaired glucose tolerance. *Am J Physiol* 264:E855-E862, 1993
171. Maarbjerg,SJ, Sylow,L, Richter,EA: Current understanding of increased insulin sensitivity after exercise - emerging candidates. *Acta Physiol (Oxf)* 202:323-335, 2011
172. Brooks,GA, Butterfield,GE, Wolfe,RR, Groves,BM, Mazzeo,RS, Sutton,JR, Wolfel,EE, Reeves,JT: Increased dependence on blood glucose after acclimatization to 4,300 m. *J Appl Physiol (1985)* 70:919-927, 1991
173. Frisancho,AR, Frisancho,HG, Milotich,M, Brutsaert,T, Albalak,R, Spielvogel,H, Villena,M, Vargas,E, Soria,R: Developmental, genetic, and environmental components of aerobic capacity at high altitude. *Am J Phys Anthropol* 96:431-442, 1995
174. Lippl,FJ, Neubauer,S, Schipfer,S, Lichter,N, Tufman,A, Otto,B, Fischer,R: Hypobaric hypoxia causes body weight reduction in obese subjects. *Obesity (Silver Spring)* 18:675-681, 2010
175. Mackenzie,R, Maxwell,N, Castle,P, Brickley,G, Watt,P: Acute hypoxia and exercise improve insulin sensitivity (S(I) (2*)) in individuals with type 2 diabetes. *Diabetes Metab Res Rev* 27:94-101, 2011
176. Louis,M, Punjabi,NM: Effects of acute intermittent hypoxia on glucose metabolism in awake healthy volunteers. *J Appl Physiol (1985)* 106:1538-1544, 2009
177. Cauza,E, Strehblow,C, Metz-Schimmerl,S, Strasser,B, Hanusch-Enserer,U, Kostner,K, Dunstan,D, Fasching,P, Haber,P: Effects of progressive strength training on muscle mass in type 2 diabetes mellitus patients determined by computed tomography. *Wien Med Wochenschr* 159:141-147, 2009
178. Gleeson,M: Immune function in sport and exercise. *J Appl Physiol (1985)* 103:693-699, 2007
179. Pedersen,BK, Saltin,B: Evidence for prescribing exercise as therapy in chronic disease. *Scand J Med Sci Sports* 16 Suppl 1:3-63, 2006
180. King,DE, Carek,P, Mainous,AG, III, Pearson,WS: Inflammatory markers and exercise: differences related to exercise type. *Med Sci Sports Exerc* 35:575-581, 2003
181. Abramson,JL, Vaccarino,V: Relationship between physical activity and inflammation among apparently healthy middle-aged and older US adults. *Arch Intern Med* 162:1286-1292, 2002
182. Geffken,DF, Cushman,M, Burke,GL, Polak,JF, Sakkinen,PA, Tracy,RP: Association between physical activity and markers of inflammation in a healthy elderly population. *Am J Epidemiol* 153:242-250, 2001
183. Mattusch,F, Dufaux,B, Heine,O, Mertens,I, Rost,R: Reduction of the plasma concentration of C-reactive protein following nine months of endurance training. *Int J Sports Med* 21:21-24, 2000
184. de Lemos,ET, Oliveira,J, Pinheiro,JP, Reis,F: Regular physical exercise as a strategy to improve antioxidant and anti-inflammatory status: benefits in type 2 diabetes mellitus. *Oxid Med Cell Longev* 2012:741545, 2012
185. Francaux,M: Toll-like receptor signalling induced by endurance exercise. *Appl Physiol Nutr Metab* 34:454-458, 2009
186. Liang,H, Hussey,SE, Sanchez-Avila,A, Tantiwong,P, Musi,N: Effect of lipopolysaccharide on inflammation and insulin action in human muscle. *PLoS One* 8:e63983, 2013
187. Gleeson,M, Bishop,NC, Stensel,DJ, Lindley,MR, Mastana,SS, Nimmo,MA: The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. *Nat Rev Immunol* 11:607-615, 2011
188. Starkie,R, Ostrowski,SR, Jauffred,S, Febbraio,M, Pedersen,BK: Exercise and IL-6 infusion inhibit endotoxin-induced TNF-alpha production in humans. *FASEB J* 17:884-886, 2003

189. Lambernd,S, Taube,A, Schober,A, Platzbecker,B, Gorgens,SW, Schlich,R, Jeruschke,K, Weiss,J, Eckardt,K, Eckel,J: Contractile activity of human skeletal muscle cells prevents insulin resistance by inhibiting pro-inflammatory signalling pathways. *Diabetologia* 55:1128-1139, 2012
190. Pedersen,BK, Febbraio,MA: Muscles, exercise and obesity: skeletal muscle as a secretory organ. *Nat Rev Endocrinol* 8:457-465, 2012
191. Pedersen,BK, Febbraio,MA: Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiol Rev* 88:1379-1406, 2008
192. Pedersen,BK, Akerstrom,TC, Nielsen,AR, Fischer,CP: Role of myokines in exercise and metabolism. *J Appl Physiol (1985)* 103:1093-1098, 2007
193. Broholm,C, Laye,MJ, Brandt,C, Vadalasetty,R, Pilegaard,H, Pedersen,BK, Scheele,C: LIF is a contraction-induced myokine stimulating human myocyte proliferation. *J Appl Physiol (1985)* 111:251-259, 2011
194. Haugen,F, Norheim,F, Lian,H, Wensaas,AJ, Dueland,S, Berg,O, Funderud,A, Skalhegg,BS, Raastad,T, Drevon,CA: IL-7 is expressed and secreted by human skeletal muscle cells. *Am J Physiol Cell Physiol* 298:C807-C816, 2010
195. Steensberg,A, van,HG, Osada,T, Sacchetti,M, Saltin,B, Klarlund,PB: Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *J Physiol* 529 Pt 1:237-242, 2000
196. Croisier,JL, Camus,G, Venneman,I, Deby-Dupont,G, Juchmes-Ferir,A, Lamy,M, Crielaard,JM, Deby,C, Duchateau,J: Effects of training on exercise-induced muscle damage and interleukin 6 production. *Muscle Nerve* 22:208-212, 1999
197. Ostrowski,K, Rohde,T, Asp,S, Schjerling,P, Pedersen,BK: Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. *J Physiol* 515 (Pt 1):287-291, 1999
198. Starkie,RL, Arkinstall,MJ, Koukoulas,I, Hawley,JA, Febbraio,MA: Carbohydrate ingestion attenuates the increase in plasma interleukin-6, but not skeletal muscle interleukin-6 mRNA, during exercise in humans. *J Physiol* 533:585-591, 2001
199. Steensberg,A, Febbraio,MA, Osada,T, Schjerling,P, van,HG, Saltin,B, Pedersen,BK: Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content. *J Physiol* 537:633-639, 2001
200. Febbraio,MA, Pedersen,BK: Muscle-derived interleukin-6: mechanisms for activation and possible biological roles. *FASEB J* 16:1335-1347, 2002
201. Pedersen,BK, Fischer,CP: Beneficial health effects of exercise--the role of IL-6 as a myokine. *Trends Pharmacol Sci* 28:152-156, 2007
202. Al-Khalili,L, Bouzakri,K, Glund,S, Lonnqvist,F, Koistinen,HA, Krook,A: Signaling specificity of interleukin-6 action on glucose and lipid metabolism in skeletal muscle. *Mol Endocrinol* 20:3364-3375, 2006
203. Carey,AL, Steinberg,GR, Macaulay,SL, Thomas,WG, Holmes,AG, Ramm,G, Prelovsek,O, Hohnen-Behrens,C, Watt,MJ, James,DE, Kemp,BE, Pedersen,BK, Febbraio,MA: Interleukin-6 increases insulin-stimulated glucose disposal in humans and glucose uptake and fatty acid oxidation in vitro via AMP-activated protein kinase. *Diabetes* 55:2688-2697, 2006
204. Jiang,LQ, Duque-Guimaraes,DE, Machado,UF, Zierath,JR, Krook,A: Altered response of skeletal muscle to IL-6 in type 2 diabetic patients. *Diabetes* 62:355-361, 2013
205. Petersen,EW, Carey,AL, Sacchetti,M, Steinberg,GR, Macaulay,SL, Febbraio,MA, Pedersen,BK: Acute IL-6 treatment increases fatty acid turnover in elderly humans in vivo and in tissue culture in vitro. *Am J Physiol Endocrinol Metab* 288:E155-E162, 2005
206. Kelly,M, Gauthier,MS, Saha,AK, Ruderman,NB: Activation of AMP-activated protein kinase by interleukin-6 in rat skeletal muscle: association with changes in cAMP, energy state, and endogenous fuel mobilization. *Diabetes* 58:1953-1960, 2009
207. Serrano,AL, Baeza-Raja,B, Perdiguero,E, Jardi,M, Munoz-Canoves,P: Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. *Cell Metab* 7:33-44, 2008
208. Fritsche,L, Hoene,M, Lehmann,R, Ellingsgaard,H, Hennige,AM, Pohl,AK, Haring,HU, Schleicher,ED, Weigert,C: IL-6 deficiency in mice neither impairs induction of metabolic

genes in the liver nor affects blood glucose levels during fasting and moderately intense exercise. *Diabetologia* 53:1732-1742, 2010
209. Kim,HJ, Higashimori,T, Park,SY, Choi,H, Dong,J, Kim,YJ, Noh,HL, Cho,YR, Cline,G, Kim,YB, Kim,JK: Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action in vivo. *Diabetes* 53:1060-1067, 2004
210. Klover,PJ, Zimmers,TA, Koniaris,LG, Mooney,RA: Chronic exposure to interleukin-6 causes hepatic insulin resistance in mice. *Diabetes* 52:2784-2789, 2003
211. Ellingsgaard,H, Hauselmann,I, Schuler,B, Habib,AM, Baggio,LL, Meier,DT, Eppler,E, Bouzakri,K, Wueest,S, Muller,YD, Hansen,AM, Reinecke,M, Konrad,D, Gassmann,M, Reimann,F, Halban,PA, Gromada,J, Drucker,DJ, Gribble,FM, Ehses,JA, Donath,MY: Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells. *Nat Med* 17:1481-1489, 2011
212. Ueda,SY, Yoshikawa,T, Katsura,Y, Usui,T, Nakao,H, Fujimoto,S: Changes in gut hormone levels and negative energy balance during aerobic exercise in obese young males. *J Endocrinol* 201:151-159, 2009
213. Martins,C, Robertson,MD, Morgan,LM: Effects of exercise and restrained eating behaviour on appetite control. *Proc Nutr Soc* 67:28-41, 2008
214. Broholm,C, Mortensen,OH, Nielsen,S, Akerstrom,T, Zankari,A, Dahl,B, Pedersen,BK: Exercise induces expression of leukaemia inhibitory factor in human skeletal muscle. *J Physiol* 586:2195-2201, 2008
215. Grabstein,KH, Eisenman,J, Shanebeck,K, Rauch,C, Srinivasan,S, Fung,V, Beers,C, Richardson,J, Schoenborn,MA, Ahdieh,M, .: Cloning of a T cell growth factor that interacts with the beta chain of the interleukin-2 receptor. *Science* 264:965-968, 1994
216. Argiles,JM, Lopez-Soriano,J, Almendro,V, Busquets,S, Lopez-Soriano,FJ: Cross-talk between skeletal muscle and adipose tissue: a link with obesity? *Med Res Rev* 25:49-65, 2005
217. Furmanczyk,PS, Quinn,LS: Interleukin-15 increases myosin accretion in human skeletal myogenic cultures. *Cell Biol Int* 27:845-851, 2003
218. Quinn,LS, Haugk,KL, Damon,SE: Interleukin-15 stimulates C2 skeletal myoblast differentiation. *Biochem Biophys Res Commun* 239:6-10, 1997
219. Waugh,DJ, Wilson,C, Seaton,A, Maxwell,PJ: Multi-faceted roles for CXC-chemokines in prostate cancer progression. *Front Biosci* 13:4595-4604, 2008
220. Akerstrom,T, Steensberg,A, Keller,P, Keller,C, Penkowa,M, Pedersen,BK: Exercise induces interleukin-8 expression in human skeletal muscle. *J Physiol* 563:507-516, 2005
221. Bek,EL, McMillen,MA, Scott,P, Angus,LD, Shaftan,GW: The effect of diabetes on endothelin, interleukin-8 and vascular endothelial growth factor-mediated angiogenesis in rats. *Clin Sci (Lond)* 103 Suppl 48:424S-429S, 2002
222. Koch,AE, Polverini,PJ, Kunkel,SL, Harlow,LA, DiPietro,LA, Elner,VM, Elner,SG, Strieter,RM: Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* 258:1798-1801, 1992
223. Norrby,K: Interleukin-8 and de novo mammalian angiogenesis. *Cell Prolif* 29:315-323, 1996
224. Frydelund-Larsen,L, Penkowa,M, Akerstrom,T, Zankari,A, Nielsen,S, Pedersen,BK: Exercise induces interleukin-8 receptor (CXCR2) expression in human skeletal muscle. *Exp Physiol* 92:233-240, 2007
225. Matthews,VB, Astrom,MB, Chan,MH, Bruce,CR, Krabbe,KS, Prelovsek,O, Akerstrom,T, Yfanti,C, Broholm,C, Mortensen,OH, Penkowa,M, Hojman,P, Zankari,A, Watt,MJ, Bruunsgaard,H, Pedersen,BK, Febbraio,MA: Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. *Diabetologia* 52:1409-1418, 2009
226. Huang,EJ, Reichardt,LF: Neurotrophins: roles in neuronal development and function. *Annu Rev Neurosci* 24:677-736, 2001
227. Rasmussen,P, Brassard,P, Adser,H, Pedersen,MV, Leick,L, Hart,E, Secher,NH, Pedersen,BK, Pilegaard,H: Evidence for a release of brain-derived neurotrophic factor from the brain during exercise. *Exp Physiol* 94:1062-1069, 2009
228. Mattson,MP: Energy intake and exercise as determinants of brain health and vulnerability to injury and disease. *Cell Metab* 16:706-722, 2012

229. McPherron,AC, Lawler,AM, Lee,SJ: Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature* 387:83-90, 1997
230. Amthor,H, Nicholas,G, McKinnell,I, Kemp,CF, Sharma,M, Kambadur,R, Patel,K: Follistatin complexes Myostatin and antagonises Myostatin-mediated inhibition of myogenesis. *Dev Biol* 270:19-30, 2004
231. Miura,T, Kishioka,Y, Wakamatsu,J, Hattori,A, Hennebry,A, Berry,CJ, Sharma,M, Kambadur,R, Nishimura,T: Decorin binds myostatin and modulates its activity to muscle cells. *Biochem Biophys Res Commun* 340:675-680, 2006
232. Kanzleiter,T, Rath,M, Gorgens,SW, Jensen,J, Tangen,DS, Kolnes,AJ, Kolnes,KJ, Lee,S, Eckel,J, Schurmann,A, Eckardt,K: The myokine decorin is regulated by contraction and involved in muscle hypertrophy. *Biochem Biophys Res Commun* 450:1089-1094, 2014
233. Heinemeier,KM, Bjerrum,SS, Schjerling,P, Kjaer,M: Expression of extracellular matrix components and related growth factors in human tendon and muscle after acute exercise. *Scand J Med Sci Sports* 23:e150-e161, 2013
234. Catoire,M, Kersten,S: The search for exercise factors in humans. *FASEB J* 2015
235. Weigert,C, Lehmann,R, Hartwig,S, Lehr,S: The secretome of the working human skeletal muscle--a promising opportunity to combat the metabolic disaster? *Proteomics Clin Appl* 8:5-18, 2014
236. Sylva,M, Moorman,AF, van den Hoff,MJ: Follistatin-like 1 in vertebrate development. *Birth Defects Res C Embryo Today* 99:61-69, 2013
237. Ouchi,N, Oshima,Y, Ohashi,K, Higuchi,A, Ikegami,C, Izumiya,Y, Walsh,K: Follistatin-like 1, a secreted muscle protein, promotes endothelial cell function and revascularization in ischemic tissue through a nitric-oxide synthase-dependent mechanism. *J Biol Chem* 283:32802-32811, 2008
238. Gorgens,SW, Raschke,S, Holven,KB, Jensen,J, Eckardt,K, Eckel,J: Regulation of follistatin-like protein 1 expression and secretion in primary human skeletal muscle cells. *Arch Physiol Biochem* 119:75-80, 2013
239. Norheim,F, Raastad,T, Thiede,B, Rustan,AC, Drevon,CA, Haugen,F: Proteomic identification of secreted proteins from human skeletal muscle cells and expression in response to strength training. *Am J Physiol Endocrinol Metab* 301:E1013-E1021, 2011
240. Shimano,M, Ouchi,N, Nakamura,K, van,WB, Ohashi,K, Asaumi,Y, Higuchi,A, Pimentel,DR, Sam,F, Murohara,T, van den Hoff,MJ, Walsh,K: Cardiac myocyte follistatin-like 1 functions to attenuate hypertrophy following pressure overload. *Proc Natl Acad Sci U S A* 108:E899-E906, 2011
241. Akerstrom,T, Laub,L, Vedel,K, Brand,CL, Pedersen,BK, Lindqvist,AK, Wojtaszewski,JF, Hellsten,Y: Increased skeletal muscle capillarization enhances insulin sensitivity. *Am J Physiol Endocrinol Metab* 307:E1105-E1116, 2014
242. Johansen,JS, Williamson,MK, Rice,JS, Price,PA: Identification of proteins secreted by human osteoblastic cells in culture. *J Bone Miner Res* 7:501-512, 1992
243. Johansen,JS, Lottenburger,T, Nielsen,HJ, Jensen,JE, Svendsen,MN, Kollerup,G, Christensen,IJ: Diurnal, weekly, and long-time variation in serum concentrations of YKL-40 in healthy subjects. *Cancer Epidemiol Biomarkers Prev* 17:2603-2608, 2008
244. Lee,CG, Da Silva,CA, Dela Cruz,CS, Ahangari,F, Ma,B, Kang,MJ, He,CH, Takyar,S, Elias,JA: Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annu Rev Physiol* 73:479-501, 2011
245. Rehli,M, Krause,SW, Andreesen,R: Molecular characterization of the gene for human cartilage gp-39 (CHI3L1), a member of the chitinase protein family and marker for late stages of macrophage differentiation. *Genomics* 43:221-225, 1997
246. Rehli,M, Niller,HH, Ammon,C, Langmann,S, Schwarzfischer,L, Andreesen,R, Krause,SW: Transcriptional regulation of CHI3L1, a marker gene for late stages of macrophage differentiation. *J Biol Chem* 278:44058-44067, 2003
247. Gorgens,SW, Eckardt,K, Elsen,M, Tennagels,N, Eckel,J: Chitinase-3-like protein 1 protects skeletal muscle from TNFalpha-induced inflammation and insulin resistance. *Biochem J* 459:479-488, 2014

248. Vinals,F, Fandos,C, Santalucia,T, Ferre,J, Testar,X, Palacin,M, Zorzano,A: Myogenesis and MyoD down-regulate Sp1. A mechanism for the repression of GLUT1 during muscle cell differentiation. *J Biol Chem* 272:12913-12921, 1997
249. Johansen,JS: Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue remodelling, fibroses and cancer. *Dan Med Bull* 53:172-209, 2006
250. Recklies,AD, Ling,H, White,C, Bernier,SM: Inflammatory cytokines induce production of CHI3L1 by articular chondrocytes. *J Biol Chem* 280:41213-41221, 2005
251. Bhat,KP, Pelloski,CE, Zhang,Y, Kim,SH, deLaCruz,C, Rehli,M, Aldape,KD: Selective repression of YKL-40 by NF-kappaB in glioma cell lines involves recruitment of histone deacetylase-1 and -2. *FEBS Lett* 582:3193-3200, 2008
252. Kastrup,J: Can YKL-40 be a new inflammatory biomarker in cardiovascular disease? *Immunobiology* 217:483-491, 2012
253. Garnero,P, Piperno,M, Gineyts,E, Christgau,S, Delmas,PD, Vignon,E: Cross sectional evaluation of biochemical markers of bone, cartilage, and synovial tissue metabolism in patients with knee osteoarthritis: relations with disease activity and joint damage. *Ann Rheum Dis* 60:619-626, 2001
254. Johansen,JS, Bojesen,SE, Mylin,AK, Frikke-Schmidt,R, Price,PA, Nordestgaard,BG: Elevated plasma YKL-40 predicts increased risk of gastrointestinal cancer and decreased survival after any cancer diagnosis in the general population. *J Clin Oncol* 27:572-578, 2009
255. Hogdall,EV, Johansen,JS, Kjaer,SK, Price,PA, Blaakjaer,J, Hogdall,CK: Stability of YKL-40 concentration in blood samples. *Scand J Clin Lab Invest* 60:247-251, 2000
256. Ostergaard,C, Johansen,JS, Benfield,T, Price,PA, Lundgren,JD: YKL-40 is elevated in cerebrospinal fluid from patients with purulent meningitis. *Clin Diagn Lab Immunol* 9:598-604, 2002
257. Johansen,JS, Pedersen,AN, Schroll,M, Jorgensen,T, Pedersen,BK, Bruunsgaard,H: High serum YKL-40 level in a cohort of octogenarians is associated with increased risk of all-cause mortality. *Clin Exp Immunol* 151:260-266, 2008
258. Hempen,M, Kopp,HP, Elhenicky,M, Hobaus,C, Brix,JM, Koppensteiner,R, Schernthaner,G, Schernthaner,GH: YKL-40 is elevated in morbidly obese patients and declines after weight loss. *Obes Surg* 19:1557-1563, 2009
259. Nielsen,AR, Erikstrup,C, Johansen,JS, Fischer,CP, Plomgaard,P, Krogh-Madsen,R, Taudorf,S, Lindegaard,B, Pedersen,BK: Plasma YKL-40: a BMI-independent marker of type 2 diabetes. *Diabetes* 57:3078-3082, 2008
260. Rathcke,CN, Vestergaard,H: YKL-40--an emerging biomarker in cardiovascular disease and diabetes. *Cardiovasc Diabetol* 8:61, 2009
261. Thomsen,SB, Rathcke,CN, Zerahn,B, Vestergaard,H: Increased levels of the calcification marker matrix Gla Protein and the inflammatory markers YKL-40 and CRP in patients with type 2 diabetes and ischemic heart disease. *Cardiovasc Diabetol* 9:86, 2010
262. Mathieu,P, Lemieux,I, Despres,JP: Obesity, inflammation, and cardiovascular risk. *Clin Pharmacol Ther* 87:407-416, 2010
263. Johansen,JS, Hoyer,PE, Larsen,LA, Price,PA, Mollgard,K: YKL-40 protein expression in the early developing human musculoskeletal system. *J Histochem Cytochem* 55:1213-1228, 2007
264. Kirkpatrick,RB, Emery,JG, Connor,JR, Dodds,R, Lysko,PG, Rosenberg,M: Induction and expression of human cartilage glycoprotein 39 in rheumatoid inflammatory and peripheral blood monocyte-derived macrophages. *Exp Cell Res* 237:46-54, 1997
265. Baeten,D, Boots,AM, Steenbakkers,PG, Elewaut,D, Bos,E, Verheijden,GF, Berheijden,G, Miltenburg,AM, Rijnders,AW, Veys,EM, De,KF: Human cartilage gp-39+,CD16+ monocytes in peripheral blood and synovium: correlation with joint destruction in rheumatoid arthritis. *Arthritis Rheum* 43:1233-1243, 2000
266. Iwata,T, Kuwajima,M, Sukeno,A, Ishimaru,N, Hayashi,Y, Wabitsch,M, Mizusawa,N, Itakura,M, Yoshimoto,K: YKL-40 secreted from adipose tissue inhibits degradation of type I collagen. *Biochem Biophys Res Commun* 388:511-516, 2009
267. Gorgens,SW, Hjorth,M, Eckardt,K, Wichert,S, Norheim,F, Holen,T, Lee,S, Langleiter,T, Birkeland,KI, Stadheim,HK, Kolnes,KJ, Tangen,DS, Jensen,J, Drevon,CA, Eckel,J:

CHI3L1/PAR-2 signaling promotes muscle growth and repair in response to exercise. *Acta Physiol (Oxf)* submitted: 2015

268. Vuolteenaho,K, Leppanen,T, Kekkonen,R, Korpela,R, Moilanen,E: Running a marathon induces changes in adipokine levels and in markers of cartilage degradation--novel role for resistin. *PLoS One* 9:e110481, 2014
269. Johansen,JS, Lottenburger,T, Nielsen,HJ, Jensen,JE, Svendsen,MN, Kollerup,G, Christensen,IJ: Diurnal, weekly, and long-time variation in serum concentrations of YKL-40 in healthy subjects. *Cancer Epidemiol Biomarkers Prev* 17:2603-2608, 2008
270. Nielsen,AR, Plomgaard,P, Krabbe,KS, Johansen,JS, Pedersen,BK: IL-6, but not TNF-alpha, increases plasma YKL-40 in human subjects. *Cytokine* 55:152-155, 2011
271. Fischer,CP: Interleukin-6 in acute exercise and training: what is the biological relevance? *Exerc Immunol Rev* 12:6-33, 2006
272. Bara,I, Ozier,A, Girodet,PO, Carvalho,G, Cattiaux,J, Begueret,H, Thumerel,M, Ousova,O, Kolbeck,R, Coyle,AJ, Woods,J, Tunon de Lara,JM, Marthan,R, Berger,P: Role of YKL-40 in bronchial smooth muscle remodeling in asthma. *Am J Respir Crit Care Med* 185:715-722, 2012
273. De,CF, Gaufillier,S, Bonnaud,A, Sabatini,M, Lesur,C, Pastoureau,P: YKL-40 (cartilage gp-39) induces proliferative events in cultured chondrocytes and synoviocytes and increases glycosaminoglycan synthesis in chondrocytes. *Biochem Biophys Res Commun* 285:926-931, 2001
274. Recklies,AD, White,C, Ling,H: The chitinase 3-like protein human cartilage glycoprotein 39 (HC-gp39) stimulates proliferation of human connective-tissue cells and activates both extracellular signal-regulated kinase- and protein kinase B-mediated signalling pathways. *Biochem J* 365:119-126, 2002
275. Kumar,A, Rajendran,V, Sethumadhavan,R, Purohit,R: AKT kinase pathway: a leading target in cancer research. *ScientificWorldJournal* 2013:756134, 2013
276. Liu,C, Li,Q, Zhou,X, Kolosov,VP, Perelman,JM: The chitinase-like protein YKL-40 increases mucin5AC production in human bronchial epithelial cells. *Exp Cell Res* 319:2866-2873, 2013
277. Rothmeier,AS, Ruf,W: Protease-activated receptor 2 signaling in inflammation. *Semin Immunopathol* 34:133-149, 2012
278. Kyriazis,I, Ellul,J, Katsakiori,P, Panayiotakopoulos,G, Flordellis,C: The multiple layers of signaling selectivity at protease-activated receptors. *Curr Pharm Des* 18:161-174, 2012
279. Shpacovitch,V, Feld,M, Hollenberg,MD, Luger,TA, Steinhoff,M: Role of protease-activated receptors in inflammatory responses, innate and adaptive immunity. *J Leukoc Biol* 83:1309-1322, 2008
280. Soh,UJ, Dores,MR, Chen,B, Trejo,J: Signal transduction by protease-activated receptors. *Br J Pharmacol* 160:191-203, 2010
281. Niu,QX, Chen,HQ, Chen,ZY, Fu,YL, Lin,JL, He,SH: Induction of inflammatory cytokine release from human umbilical vein endothelial cells by agonists of proteinase-activated receptor-2. *Clin Exp Pharmacol Physiol* 35:89-96, 2008
282. Park,GH, Jeon,SJ, Ko,HM, Ryu,JR, Lee,JM, Kim,HY, Han,SH, Kang,YS, Park,SH, Shin,CY, Ko,KH: Activation of microglial cells via protease-activated receptor 2 mediates neuronal cell death in cultured rat primary neuron. *Nitric Oxide* 22:18-29, 2010
283. Nishibori,M, Mori,S, Takahashi,HK: Physiology and pathophysiology of proteinase-activated receptors (PARs): PAR-2-mediated proliferation of colon cancer cell. *J Pharmacol Sci* 97:25-30, 2005
284. Nishibori,M: Physiology and pathophysiology of proteinase-activated receptors (PARs): Preface. *J Pharmacol Sci* 97:7, 2005
285. Arisawa,T, Tahara,T, Shibata,T, Nagasaka,M, Nakamura,M, Kamiya,Y, Fujita,H, Takagi,T, Hasegawa,S, Wang,FY, Hirata,I, Nakano,H: Promoter hypomethylation of protease-activated receptor 2 associated with carcinogenesis in the stomach. *J Gastroenterol Hepatol* 22:943-948, 2007
286. Iwaki,K, Shibata,K, Ohta,M, Endo,Y, Uchida,H, Tominaga,M, Okunaga,R, Kai,S, Kitano,S: A small interfering RNA targeting proteinase-activated receptor-2 is effective in suppression of tumor growth in a Panc1 xenograft model. *Int J Cancer* 122:658-663, 2008

287. Ferrell,WR, Lockhart,JC, Kelso,EB, Dunning,L, Plevin,R, Meek,SE, Smith,AJ, Hunter,GD, McLean,JS, McGarry,F, Ramage,R, Jiang,L, Kanke,T, Kawagoe,J: Essential role for proteinase-activated receptor-2 in arthritis. *J Clin Invest* 111:35-41, 2003
288. Kelso,EB, Ferrell,WR, Lockhart,JC, Elias-Jones,I, Hembrough,T, Dunning,L, Gracie,JA, McInnes,IB: Expression and proinflammatory role of proteinase-activated receptor 2 in rheumatoid synovium: ex vivo studies using a novel proteinase-activated receptor 2 antagonist. *Arthritis Rheum* 56:765-771, 2007
289. Lam,FF: Role of protease-activated receptor 2 in joint inflammation. *Arthritis Rheum* 56:3514-3517, 2007
290. McIntosh,KA, Plevin,R, Ferrell,WR, Lockhart,JC: The therapeutic potential of proteinase-activated receptors in arthritis. *Curr Opin Pharmacol* 7:334-338, 2007
291. Maeda,K, Hirota,M, Kimura,Y, Ichihara,A, Ohmuraya,M, Sugita,H, Ogawa,M: Proinflammatory role of trypsin and protease-activated receptor-2 in a rat model of acute pancreatitis. *Pancreas* 31:54-62, 2005
292. Hirota,M, Ohmuraya,M, Baba,H: The role of trypsin, trypsin inhibitor, and trypsin receptor in the onset and aggravation of pancreatitis. *J Gastroenterol* 41:832-836, 2006
293. Hansen,KK, Sherman,PM, Cellars,L, Andrade-Gordon,P, Pan,Z, Baruch,A, Wallace,JL, Hollenberg,MD, Vergnolle,N: A major role for proteolytic activity and proteinase-activated receptor-2 in the pathogenesis of infectious colitis. *Proc Natl Acad Sci U S A* 102:8363-8368, 2005
294. Cottrell,GS, Amadesi,S, Pikios,S, Camerer,E, Willardsen,JA, Murphy,BR, Caughey,GH, Wolters,PJ, Coughlin,SR, Peterson,A, Knecht,W, Pothoulakis,C, Bunnett,NW, Grady,EF: Protease-activated receptor 2, dipeptidyl peptidase I, and proteases mediate Clostridium difficile toxin A enteritis. *Gastroenterology* 132:2422-2437, 2007
295. Vesey,DA, Kruger,WA, Poronnik,P, Gobe,GC, Johnson,DW: Proinflammatory and proliferative responses of human proximal tubule cells to PAR-2 activation. *Am J Physiol Renal Physiol* 293:F1441-F1449, 2007
296. Vesey,DA, Hooper,JD, Gobe,GC, Johnson,DW: Potential physiological and pathophysiological roles for protease-activated receptor-2 in the kidney. *Nephrology (Carlton)* 12:36-43, 2007
297. Cocks,TM, Sozzi,V, Moffatt,JD, Selemidis,S: Protease-activated receptors mediate apamin-sensitive relaxation of mouse and guinea pig gastrointestinal smooth muscle. *Gastroenterology* 116:586-592, 1999
298. Cocks,TM, Fong,B, Chow,JM, Anderson,GP, Frauman,AG, Goldie,RG, Henry,PJ, Carr,MJ, Hamilton,JR, Moffatt,JD: A protective role for protease-activated receptors in the airways. *Nature* 398:156-160, 1999
299. D'Agostino,G, La,RG, Russo,R, Sasso,O, Iacono,A, Esposito,E, Raso,GM, Cuzzocrea,S, Lo,VJ, Piomelli,D, Meli,R, Calignano,A: Acute intracerebroventricular administration of palmitoylethanolamide, an endogenous peroxisome proliferator-activated receptor-alpha agonist, modulates carrageenan-induced paw edema in mice. *J Pharmacol Exp Ther* 322:1137-1143, 2007
300. Matej,R, Mandakova,P, Netikova,I, Pouckova,P, Olejar,T: Proteinase-activated receptor-2 expression in breast cancer and the role of trypsin on growth and metabolism of breast cancer cell line MDA MB-231. *Physiol Res* 56:475-484, 2007
301. Arisawa,T, Tahara,T, Shibata,T, Nagasaka,M, Nakamura,M, Kamiya,Y, Fujita,H, Takagi,T, Hasegawa,S, Wang,FY, Hirata,I, Nakano,H: Promoter hypomethylation of protease-activated receptor 2 associated with carcinogenesis in the stomach. *J Gastroenterol Hepatol* 22:943-948, 2007
302. Chinni,C, de Niese,MR, Jenkins,AL, Pike,RN, Bottomley,SP, Mackie,EJ: Protease-activated receptor-2 mediates proliferative responses in skeletal myoblasts. *J Cell Sci* 113 Pt 24:4427-4433, 2000
303. Cassano,M, Quattrocelli,M, Crippa,S, Perini,I, Ronzoni,F, Sampaolesi,M: Cellular mechanisms and local progenitor activation to regulate skeletal muscle mass. *J Muscle Res Cell Motil* 30:243-253, 2009

304. Ceafalan,LC, Popescu,BO, Hinescu,ME: Cellular players in skeletal muscle regeneration. *Biomed Res Int* 2014:957014, 2014
305. Chang,NC, Rudnicki,MA: Satellite cells: the architects of skeletal muscle. *Curr Top Dev Biol* 107:161-181, 2014
306. Juhas,M, Bursac,N: Engineering skeletal muscle repair. *Curr Opin Biotechnol* 24:880-886, 2013
307. Armand,O, Boutineau,AM, Mauger,A, Pautou,MP, Kieny,M: Origin of satellite cells in avian skeletal muscles. *Arch Anat Microsc Morphol Exp* 72:163-181, 1983
308. Buckingham,M: Skeletal muscle formation in vertebrates. *Curr Opin Genet Dev* 11:440-448, 2001
309. Braun,T, Rudnicki,MA, Arnold,HH, Jaenisch,R: Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell* 71:369-382, 1992
310. Rudnicki,MA, Schnegelsberg,PN, Stead,RH, Braun,T, Arnold,HH, Jaenisch,R: MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* 75:1351-1359, 1993
311. Kassar-Duchossoy,L, Gayraud-Morel,B, Gomes,D, Rocancourt,D, Buckingham,M, Shinin,V, Tajbakhsh,S: Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. *Nature* 431:466-471, 2004
312. Kassar-Duchossoy,L, Giacone,E, Gayraud-Morel,B, Jory,A, Gomes,D, Tajbakhsh,S: Pax3/Pax7 mark a novel population of primitive myogenic cells during development. *Genes Dev* 19:1426-1431, 2005
313. Fuchtbauer,EM, Westphal,H: MyoD and myogenin are coexpressed in regenerating skeletal muscle of the mouse. *Dev Dyn* 193:34-39, 1992
314. Grounds,MD, Garrett,KL, Beilharz,MW: The transcription of MyoD1 and myogenin genes in thymic cells in vivo. *Exp Cell Res* 198:357-361, 1992
315. Miller,JB, Schaefer,L, Dominov,JA: Seeking muscle stem cells. *Curr Top Dev Biol* 43:191-219, 1999
316. Zammit,PS, Partridge,TA, Yablonka-Reuveni,Z: The skeletal muscle satellite cell: the stem cell that came in from the cold. *J Histochem Cytochem* 54:1177-1191, 2006
317. McIntosh,K, Cunningham,MR, Cadalbert,L, Lockhart,J, Boyd,G, Ferrell,WR, Plevin,R: Proteinase-activated receptor-2 mediated inhibition of TNFalpha-stimulated JNK activation - A novel paradigm for G(q/11) linked GPCRs. *Cell Signal* 22:265-273, 2010
318. Nichols,HL, Saffeddine,M, Theriot,BS, Hegde,A, Polley,D, El-Mays,T, Vliagoftis,H, Hollenberg,MD, Wilson,EH, Walker,JK, DeFea,KA: beta-Arrestin-2 mediates the proinflammatory effects of proteinase-activated receptor-2 in the airway. *Proc Natl Acad Sci U S A* 109:16660-16665, 2012
319. Agrawal,NK, Kant,S: Targeting inflammation in diabetes: Newer therapeutic options. *World J Diabetes* 5:697-710, 2014
320. Plomgaard,P, Bouzakri,K, Krogh-Madsen,R, Mittendorfer,B, Zierath,JR, Pedersen,BK: Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. *Diabetes* 54:2939-2945, 2005
321. Austin,RL, Rune,A, Bouzakri,K, Zierath,JR, Krook,A: siRNA-mediated reduction of inhibitor of nuclear factor-kappaB kinase prevents tumor necrosis factor-alpha-induced insulin resistance in human skeletal muscle. *Diabetes* 57:2066-2073, 2008
322. Gleeson,M: Immune function in sport and exercise. *J Appl Physiol (1985)* 103:693-699, 2007
323. Ostrowski,K, Rohde,T, Asp,S, Schjerling,P, Pedersen,BK: Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. *J Physiol* 515 (Pt 1):287-291, 1999
324. Bernecker,C, Scherr,J, Schinner,S, Braun,S, Scherbaum,WA, Halle,M: Evidence for an exercise induced increase of TNF-alpha and IL-6 in marathon runners. *Scand J Med Sci Sports* 23:207-214, 2013
325. Paulsen,G, Mikkelsen,UR, Raastad,T, Peake,JM: Leucocytes, cytokines and satellite cells: what role do they play in muscle damage and regeneration following eccentric exercise? *Exerc Immunol Rev* 18:42-97, 2012

326. Stroka,DM, Burkhardt,T, Desbaillets,I, Wenger,RH, Neil,DA, Bauer,C, Gassmann,M, Candinas,D: HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. *FASEB J* 15:2445-2453, 2001
327. Ameln,H, Gustafsson,T, Sundberg,CJ, Okamoto,K, Jansson,E, Poellinger,L, Makino,Y: Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. *FASEB J* 19:1009-1011, 2005
328. Isaacs,JS, Jung,YJ, Mole,DR, Lee,S, Torres-Cabala,C, Chung,YL, Merino,M, Trepel,J, Zbar,B, Toro,J, Ratcliffe,PJ, Linehan,WM, Neckers,L: HIF overexpression correlates with biallelic loss of fumarate hydratase in renal cancer: novel role of fumarate in regulation of HIF stability. *Cancer Cell* 8:143-153, 2005
329. Haddad,JJ, Land,SC: A non-hypoxic, ROS-sensitive pathway mediates TNF-alpha-dependent regulation of HIF-1alpha. *FEBS Lett* 505:269-274, 2001
330. McMahon,S, Charbonneau,M, Grandmont,S, Richard,DE, Dubois,CM: Transforming growth factor beta1 induces hypoxia-inducible factor-1 stabilization through selective inhibition of PHD2 expression. *J Biol Chem* 281:24171-24181, 2006
331. Page,EL, Robitaille,GA, Pouyssegur,J, Richard,DE: Induction of hypoxia-inducible factor-1alpha by transcriptional and translational mechanisms. *J Biol Chem* 277:48403-48409, 2002
332. Palazon,A, Goldrath,AW, Nizet,V, Johnson,RS: HIF transcription factors, inflammation, and immunity. *Immunity* 41:518-528, 2014
333. Greenbaum,AR, Etherington,PJ, Manek,S, O'Hare,D, Parker,KH, Green,CJ, Pepper,JR, Winlove,CP: Measurements of oxygenation and perfusion in skeletal muscle using multiple microelectrodes. *J Muscle Res Cell Motil* 18:149-159, 1997
334. Csete,M, Walikonis,J, Slawny,N, Wei,Y, Korsnes,S, Doyle,JC, Wold,B: Oxygen-mediated regulation of skeletal muscle satellite cell proliferation and adipogenesis in culture. *J Cell Physiol* 189:189-196, 2001
335. Storch,TG: Oxygen concentration regulates 5-azacytidine-induced myogenesis in C3H/10T1/2 cultures. *Biochim Biophys Acta* 1055:126-129, 1990
336. Richardson,RS, Noyszewski,EA, Kendrick,KF, Leigh,JS, Wagner,PD: Myoglobin O2 desaturation during exercise. Evidence of limited O2 transport. *J Clin Invest* 96:1916-1926, 1995
337. Hoppeler,H, Howald,H, Conley,K, Lindstedt,SL, Claassen,H, Vock,P, Weibel,ER: Endurance training in humans: aerobic capacity and structure of skeletal muscle. *J Appl Physiol (1985)* 59:320-327, 1985
338. Lindholm,ME, Fischer,H, Poellinger,L, Johnson,RS, Gustafsson,T, Sundberg,CJ, Rundqvist,H: Negative regulation of HIF in skeletal muscle of elite endurance athletes: a tentative mechanism promoting oxidative metabolism. *Am J Physiol Regul Integr Comp Physiol* 307:R248-R255, 2014
339. Lundby,C, Gassmann,M, Pilegaard,H: Regular endurance training reduces the exercise induced HIF-1alpha and HIF-2alpha mRNA expression in human skeletal muscle in normoxic conditions. *Eur J Appl Physiol* 96:363-369, 2006
340. Mason,S, Johnson,RS: The role of HIF-1 in hypoxic response in the skeletal muscle. *Adv Exp Med Biol* 618:229-244, 2007
341. Vogt,M, Puntschart,A, Geiser,J, Zuleger,C, Billeter,R, Hoppeler,H: Molecular adaptations in human skeletal muscle to endurance training under simulated hypoxic conditions. *J Appl Physiol (1985)* 91:173-182, 2001
342. Ookawara,T, Suzuk,K, Haga,S, Ha,S, Chung,KS, Toshinai,K, Hamaoka,T, Katsumura,T, Takemasa,T, Mizuno,M, Hitomi,Y, Kizaki,T, Suzuki,K, Ohno,H: Transcription regulation of gene expression in human skeletal muscle in response to endurance training. *Res Commun Mol Pathol Pharmacol* 111:41-54, 2002
343. Sakagami,H, Makino,Y, Mizumoto,K, Isoe,T, Takeda,Y, Watanabe,J, Fujita,Y, Takiyama,Y, Abiko,A, Haneda,M: Loss of HIF-1alpha impairs GLUT4 translocation and glucose uptake by the skeletal muscle cells. *Am J Physiol Endocrinol Metab* 306:E1065-E1076, 2014
344. Semenza,GL: Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. *Trends Pharmacol Sci* 33:207-214, 2012

345. Chen,S, Synowsky,S, Tinti,M, MacKintosh,C: The capture of phosphoproteins by 14-3-3 proteins mediates actions of insulin. *Trends Endocrinol Metab* 22:429-436, 2011
346. Cao,WD, Kawai,N, Miyake,K, Zhang,X, Fei,Z, Tamiya,T: Relationship of 14-3-3zeta (zeta), HIF-1alpha, and VEGF expression in human brain gliomas. *Brain Tumor Pathol* 31:1-10, 2014
347. Ameln,H, Gustafsson,T, Sundberg,CJ, Okamoto,K, Jansson,E, Poellinger,L, Makino,Y: Physiological activation of hypoxia inducible factor-1 in human skeletal muscle. *FASEB J* 19:1009-1011, 2005
348. Lima,GA, Anhe,GF, Giannocco,G, Nunes,MT, Correa-Giannella,ML, Machado,UF: Contractile activity per se induces transcriptional activation of SLC2A4 gene in soleus muscle: involvement of MEF2D, HIF-1a, and TRalpha transcriptional factors. *Am J Physiol Endocrinol Metab* 296:E132-E138, 2009
349. Marfella,R, D'Amico,M, Di,FC, Piegari,E, Nappo,F, Esposito,K, Berrino,L, Rossi,F, Giugliano,D: Myocardial infarction in diabetic rats: role of hyperglycaemia on infarct size and early expression of hypoxia-inducible factor 1. *Diabetologia* 45:1172-1181, 2002
350. Fadini,GP, Sartore,S, Schiavon,M, Albiero,M, Baesso,I, Cabrelle,A, Agostini,C, Avogaro,A: Diabetes impairs progenitor cell mobilisation after hindlimb ischaemia-reperfusion injury in rats. *Diabetologia* 49:3075-3084, 2006
351. Gunton,JE, Kulkarni,RN, Yim,S, Okada,T, Hawthorne,WJ, Tseng,YH, Roberson,RS, Ricordi,C, O'Connell,PJ, Gonzalez,FJ, Kahn,CR: Loss of ARNT/HIF1beta mediates altered gene expression and pancreatic-islet dysfunction in human type 2 diabetes. *Cell* 122:337-349, 2005
352. Yamada,N, Horikawa,Y, Oda,N, Iizuka,K, Shihara,N, Kishi,S, Takeda,J: Genetic variation in the hypoxia-inducible factor-1alpha gene is associated with type 2 diabetes in Japanese. *J Clin Endocrinol Metab* 90:5841-5847, 2005

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Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis“ an der Heinrich-Heine-Universität Düsseldorf erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

(Sven Wolfgang Görgens)

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