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The role of NF-κB activation in fatty acid-induced insulin resistance in skeletal muscle

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CONTENTS

CHAPTER 1

General introduction

GENERAL INTRODUCTION

Currently, around 250 million people are having diabetes type 2 worldwide, and it is estimated that this number will rise to over 400 million in 2030 due to an endemic increase in the prevalence of obesity [1].

Insulin resistance, which can be defined as a reduced ability of cells to respond to physiological levels of insulin, plays an important role in the development of type 2 diabetes mellitus and the metabolic syndrome. The importance of skeletal muscle in the disease is clear, since it is responsible for more than 80% of total insulin-stimulated glucose uptake and skeletal muscle insulin resistance precedes the clinical diagnosis of the disease [2]. In the last two decades, it has become clear that increased free fatty acids (FFA) levels in plasma are associated with skeletal muscle insulin resistance. Most insulin resistant subjects have increased plasma FFA concentrations and acute elevations of FFAs by lipid infusion resulted in skeletal muscle insulin resistance within hours in rodents [3, 4] and humans [5-7], whereas the induction of insulin resistance in skeletal muscle after feeding high-fat diets to rats was induced within weeks [8, 9].

The molecular basis of skeletal muscle insulin resistance is still not elucidated, but substantial evidence supports a role for inflammatory signaling in insulin resistance in response to increased FA levels. Therefore, in the next paragraphs the molecular basis of insulin-mediated glucose uptake in skeletal muscle will be discussed as well as the potential involvement of inflammation in skeletal muscle insulin resistance.

INSULIN-INDUCED GLUCOSE UPTAKE IN SKELETAL MUSCLE

Insulin, discovered in 1922, is an anabolic hormone produced by the pancreas. A main function is to help regulating blood glucose levels after food intake by stimulating glucose uptake and storage in the form of glycogen in peripheral tissues such as skeletal muscle. However, apart from stimulating glucose uptake, insulin has more functions in skeletal muscle, such as glycogen formation, stimulating amino acid uptake and protein synthesis, and decreasing proteolysis. These processes are however beyond the scope of this thesis.

In peripheral tissues, glucose uptake occurs via glucose transporters (GLUT). Currently, thirteen different glucose transporters have been described, of which seven are expressed in human muscle [10]. However, only GLUT4 has the unique characteristic to reside in intracellular vesicles when no insulin is triggering the insulin receptor, which translocate acutely to the plasma membrane in response to insulin.

The insulin receptor is a transmembrane receptor belonging to the receptor tyrosine kinase superfamily and consists of two α- and two β-subunits. After binding of insulin to the α-subunits, the resulting change in the receptor configuration leads to autophosphorylation of specific tyrosine residues in the β-subunits, which constitute the intracellular part of the receptor [11]. Following activation, insulin receptor substrate proteins (IRS-1 to 4) are recruited to the insulin receptor and bind to the trans-phosphorylated receptor at tyrosine docking sites. Subsequently, the IRS proteins become phosphorylated at multiple tyrosine residues via the activated receptor kinase. The IRS proteins have no intrinsic catalytic activity themselves, but the tyrosine phosphorylation provides docking sites for signaling molecules with a Src homology 2 (SH2) domain [12]. IRS-1 appears to be the most important IRS isoform for insulin action [13, 14].

One of the SH2 domain containing signaling molecules that interact with tyrosine phosphorylated IRS is phosphatidylinositol 3-kinase (PI 3-kinase). PI 3-kinase is a heterodimeric protein, consisting of two subunits: a p85 adaptor subunit and a p110 catalytic subunit. Both SH2 domains of the adaptor subunit have to be occupied with tyrosine phosphorylated YxxM motifs for PI 3-kinase activation. This interaction leads to translocation of PI 3-kinase to the plasma membrane and positions PI3K in proximity to its substrate, phosphotidylinositol-(4,5)bisphosphate (PIP_2). This catalyzes the conversion of plasma membrane localized PIP_2 to the second messenger phosphotidylinositol-(3,4,5)triphosphate (PIP_3) [15]. PIP_3 recruits various serine kinases to the plasma membrane, including phosphoinositide-dependant kinase (PDK-1), protein kinase B (PKB or Akt) and atypical protein kinase C (PKC) ζ and λ. These molecules all contain a pleckstrin homology (PH) domain, which is needed for interaction with phospholipids within the plasma membrane [16, 17]. It has also been shown that PDK-1 is able to phosphorylate and activate other serine kinases, like Akt. PDK-1 mediated Akt phosphorylation occurs at one of the two regulatory sites of akt; i.e. Thr308, which is localized in the kinase domain [18]. Recently, the mammalian target of rapamycin complex-2 (mTORC2) protein kinase has been found to be responsible for phosphorylation of the second regulatory site of Akt, i.e. Ser473 in the C-terminal regulatory domain [19]. The importance of mTORC2 for glucose homeostasis in muscle has been shown in mice with a muscle-specific deletion of Rictor (an essential component of mTORC2). Both insulin-stimulated Akt phosphorylation and glucose uptake were reduced in muscle from these mice [20].

There is a substantial amount of evidence for a key role for Akt in insulin-induced GLUT4 translocation and glucose uptake, based on studies involving over-expression of constitutively active forms of Akt [21-24] and on studies depleting Akt using small interference RNA [25-27] in adipose and muscle cells. Also isolated skeletal muscle from Akt deficient mice showed a reduction in insulin-induced glucose uptake [28]. Furthermore, expression of a kinase inactive and

phosphorylation-deficient Akt-mutant in L6 myoblasts resulted in inhibition of insulin induced GLUT4 translocation [29]. Although there are three isoforms of Akt, which share a high degree of sequence homology, Akt2 seems to be specifically required for insulin-induced glucose uptake [28, 30].

Akt is able to phosphorylate various downstream targeting proteins. One of these proteins that was recently discovered in 3T3L1 adipocytes is the 160 kDa Akt substrate (AS160). It has been demonstrated that this Rab-GTPase-activating protein (Rab-GAP) contained two Akt phosphorylation sites that are regulated in reponse to insulin [31, 32]. Also in skeletal muscle AS160 appeared to be important in insulin signaling [33].

Studies in muscle cells and in adipocytes concluded that AS160 phosphorylation is required for GLUT4 translocation and linked to the inactivation of its Rab-GAP activity [32, 34-36]. Rab proteins, which lie downstream of AS160 in skeletal muscle cells [35], are GTPases involved in the regulation of many membrane trafficking processes [37]. The GTP bound form of Rab proteins is considered the “active state” and the GDP bound form the “inactive state.” So, insulin-induced phosphorylation of AS160, leading to inactivation of Rab-GAP leads to stabilization of target Rab proteins in the GTP-bound form, which is thought to promote traffic of GLUT4 storage vesicles (GSV).

The process of insulin-stimulated translocation of GLUT4 from GLUT4 storage vesicles to the cell surface is a multistep process involving sorting, vesicular transport along cytoskeletal elements, docking, and fusion with the cell surface membrane. Currently, extensive research is done on the exact mechanisms. For a description of the current knowledge in this field we refer to recent reviews [38-40].

Although many studies support an important role for PI3-kinase signaling in insulin-dependent glucose uptake [41-44], a PI3-kinase independent pathway also has been proposed as a parallel insulin-induced pathway regulating GLUT4 translocation [45, 46]. This pathway involves the tyrosine phosphorylation of the proto-oncogene Cbl. This is initiated by the recruitment of Cbl to the activated insulin receptor via the adaptor proteins APS and CAP (Cbl associated adaptor protein). Following tyrosine phosphorylation, the Cbl-CAP complex translocates to lipid raft microdomains by interacting with flotillin, a protein present in caveolae. Expression of a CAP mutant which lacks the ability to bind cbl inhibits Cbl translocation and insulin-induced glucose uptake. In these lipid rafts, Cbl recruits the adaptor protein CrKII and the guanosine exchange factor C3G. When present in the caveolae, C3G activates the GTP binding protein TC10. Activation of TC10 was postulated to be the essential event in this pathway that contributes to complete insulin-induced GLUT4 translocation and glucose uptake, in parallel with the PI3-kinase pathway [47, 48].

However, the importance of this parallel pathway to total insulin dependent glucose uptake remains to be determined, as dominant-negative TC10 mutants do

not interfere with insulin-induced GLUT4 translocation in skeletal muscle cells [49] and different RNAi studies in 3T3 adipocytes also do not support a role of the TC10 pathway in insulin-stimulated glucose uptake [26]. Furthermore, the generation of Cbl knockout mice did not result in a difference in insulin sensitivity [50]. The insulin signaling pathway in skeletal muscle cells is summarized in Figure 1. Defects in the insulin-signaling pathway result in insulin resistance. Moreover, a reduced insulin-stimulated tyrosine phosphorylation of IRS-1, PI3-kinase activity, Akt phosphorylation, AS160 phosphorylation, GLUT4 translocation may all contribute to a reduction in insulin-mediated glucose uptake, or insulin resistance in skeletal muscle.

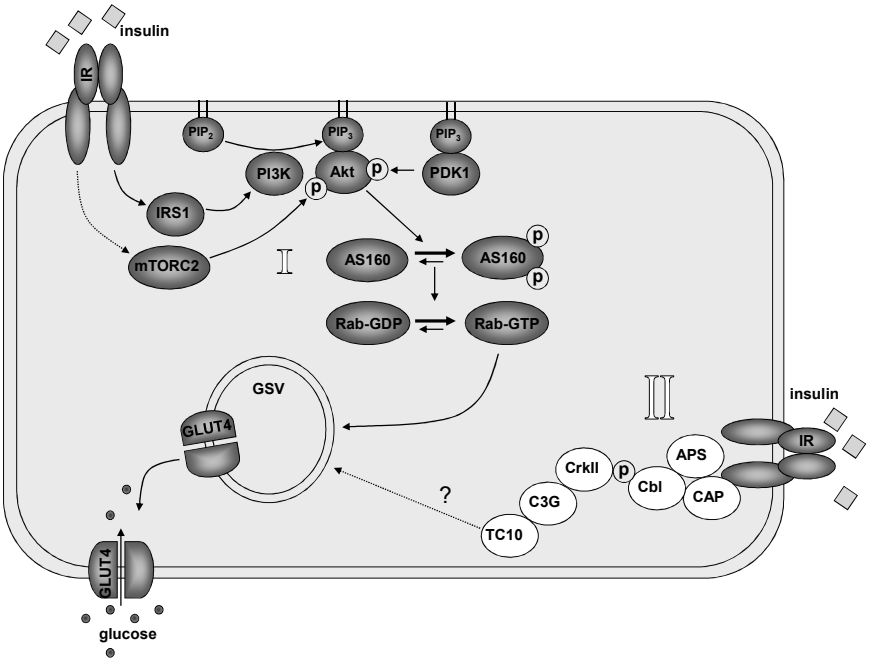

Figure 1: Insulin signaling pathway leading to GLUT4 translocation in muscle. (I) Insulin stimulates the insulin receptor (IR), leading to tyrosine phosphorylation of insulin receptor substrate (IRS)-1 and subsequent recruitment of phosphatidylinositol 3-kinase (PI3K). This results in the generation of the second messenger PIP3, leading to the recruitment of Akt and subsequent phosphorylation by the upstream mediators PDK1 and mTORC2. Activated Akt phosphorylates Akt substrate 160 (AS160), thereby inactivating its Rab-GAP domain and preventing the hydrolysis of GTP to GDP by Rab proteins. This results in processes promoting the translocation of GLUT4 to the cell surface. (II) This PI3K-independent APS-Cbl-TC10 pathway likely plays a minor role in insulin-stimulated GLUT4 translocation. For further details, see text. CAP, cbl associated adapter protein; GAP, GTPase activating protein; GSV, GLUT4 storage vesicle; mTORC2, mammalian target of rapamycin complex 2; PDK1, phosphoinositide-dependant kinase; PIP2, phosphotidylinositol-(4,5)bisphosphate; PIP3, phosphotidylinositol-(3,4,5)triphosphate.

FATTY ACID-INDUCED INSULIN RESISTANCE IN SKELETAL MUSCLE

It is generally accepted that in most obese subjects increased circulating levels of FFAs are present and many studies have shown that an acute or chronic elevation of FFA causes insulin resistance in both diabetic and non-diabetic patients [7, 51, 52].

Adipose tissue is the main lipid storage depot and is important in buffering the daily influx of dietary fat. During prolonged dietary excess and obesity, the postprandial buffering capacity for lipid storage in adipose tissue is exceeded and the sensitivity to insulin-mediated suppression of lipolysis is lost. Thus, FAs are released to the circulation, which leads to an oversupply of FAs to non-adipose tissue, primarily skeletal muscle [9, 53].

It has been proposed that these FFAs can induce insulin resistance in skeletal muscle by intracellular inflammatory signaling directly after binding to toll-like receptors, or after lipid metabolite accumulation inside the myocytes (Figure 2).

TOLL-LIKE RECEPTORS

Toll-like receptors (TLRs) are a family of evolutionary conserved receptors that recognize pathogen-associated molecular patterns (PAMPs) (reviewed in [54, 55]). They play an important role in the innate immune system, since they activate pro-inflammatory signaling pathways in response to this pattern-recognition. Following ligand binding, the subunits of the TLR receptor associate which leads to the intra-cellular formation of a complex containing adaptor proteins of the myeloid differentiation factor 88 (MyD88) family. This interaction leads to a downstream signaling cascade resulting in the activation of the Nuclear Factor-κB (NF-κB) pathway and consequent transcription of a variety of pro-inflammatory genes. Besides the NF-κB pathway, TLRs are able to activate the JNK and SOCS pathways, which have both been implicated in insulin resistance. TLRs are expressed in multiple tissues. The main site of TLR expression is on cells of the immune system like macrophages, but TLRs, and in particular TLR2 and TLR4, are also found on other tissues, for example in fat and muscle tissue where they can initiate signal transduction [56-58].

TLR4, the best-characterized TLR, binds the ligand lipopolysaccharide (LPS), which is a component of gram-negative bacteria cell walls. It was shown that addition of saturated fatty acids (SFAs), the main constituent of the lipid part of LPS, Lipid A, was sufficient to trigger TLR4 activation in a macrophage cell line [59]. A recent study presents TLR4 as the link between innate immunity, fatty acids and insulin resistance [60]. Saturated fatty acids (myristate, palmitate and stearate) can activate the NF-κB pathway and IL-6 and TNF-α expression in adipocytes and macrophages *in vitro* in a TLR4 dependent manner. In adipocytes of TLR4 knockout

mice, SFA-induced NF-κB activation and cytokine production was blunted. The TLR4 mediated activating effects were most pronounced with SFAs and absent with n-3 long chain polyunsaturated fatty acids. Furthermore, in contrast to wild type mice, lipid infusion in TLR4 knockout mice did not result in insulin resistance in skeletal muscle, which pointed to an important role of TLR4 in obesity-induced insulin resistance *in vivo* [60, 61]. Furthermore, mice with a loss-of-function mutation in TLR4 showed a reduction in the development of diet-induced insulin resistance, coinciding with decreased NF-κB and JNK activation by high-fat diet in muscle, fat and liver. Isolated muscle from these mice were protected from SFA-induced insulin resistance and isolated muscle from control mice could be protected against insulin resistance and activation of JNK and NF-κB via an antagonist monoclonal TLR4 antibody [62]. In L6 myotubes, blocking TLR4 reduced palmitate-induced inhibition of glucose uptake [61]. Recently, activation of TLR2 in skeletal muscle was postulated as a potential mechanism for palmitate-induced insulin resistance in cultured skeletal muscle cells. Specifically, a TLR2 antagonist antibody and RNAi-induced inhibition of TLR2 expression both inhibited palmitate-induced insulin resistance, measured by a reduced Akt phosphorylation in C2C12 cells [63].

These studies demonstrate that SFAs may cause insulin resistance via inflammatory signaling resulting from extra-cellular binding and activation of TLRs by SFA.

INTRAMYOCELLULAR LIPID METABOLITE ACCUMULATION

Within skeletal muscle cells, the FAs become activated to acyl-coA derivates and a major part is esterified to triglycerides. Evidence about the role of intramyocellular triglycerides (IMTG) in muscle insulin resistance was obtained by NMR spectroscopy studies showing a strong relationship between the accumulation of IMTG content and insulin resistance in human [64, 65]. Also high-fat diet intervention studies in rodents showed associations between the development of insulin resistance and the accumulation of IMTG [66]. However, several observations [67-70] resulted in the conclusion that it is unlikely that increased IMTG themselves are responsible for the defects in insulin signaling. When acyl-CoA derivates are not esterified/stored as IMTG, they can undergo β-oxidation or can be esterified into DAG or metabolized into ceramides [71]. Currently it is believed that IMTG may represent a storage form for FAs, while the accumulation of lipid metabolites like diacylglycerol (DAG) and ceramides [5, 53, 65, 72-75] are more directly responsible for the observed FA-induced decreased insulin action in skeletal muscle.

Ceramides

Ceramides can be generated by *de novo* synthesis from long-chain FAs or by hydrolysis of sphingomyelins [76]. *De novo* synthesis by serine palmitoyltransferase (SPT) is dependent on the availability of long-chain SFAs and serine. Although SPT is specific for SFAs, the incorporation of the second FA in ceramide can originate from SFA or unsaturated FA. Sphingomyelinase-induced hydrolysis of sphingomyelin, a common phospholipid in the plasma membrane, is another way in which ceramides can accumulate in muscle [77].

Many studies have proposed ceramides in the development of insulin resistance: increased ceramide levels have been demonstrated in tissues of insulin-resistant animals and in the skeletal muscle of lipid-infused humans and obese insulin resistant humans [78-80]. It has been indicated that the ceramides act downstream of IRS-1 signaling, at the level of Akt activation [75]. Inhibition of SPT reversed palmitate-mediated reduction in insulin-induced Akt phosphorylation and glucose uptake in L6 myotubes [81]. Remarkably, sustained inhibition of ceramides increased DAG accumulation and induced impaired insulin signaling via IRS-1.

Altogether, although there is evidence from *in vitro* and animal studies to suggest that ceramides may play a role in muscular insulin resistance, it remains unclear whether ceramides directly influence insulin sensitivity in human skeletal muscle *in vivo* [82].

Diacylglycerol and protein kinase C

DAG has been found to accumulate in muscles of rodent models of HFD-induced insulin resistance, obese Zucker (fa/fa) rats, and in obese individuals [83-85]. Excess of long chain acyl-coAs in muscle from high-fat fed animals can esterify to glycerol-3-phosphate to generate DAG [86]. DAG is able to activate both conventional and novel protein kinase C (PKC).

In rats, lipid infusion resulted in an increase in intracellular DAG concentrations in muscle, which was associated with protein kinase C (PKC)-θ activation and an increased inhibitory IRS-1 mediated serine phosphorylation. It was hypothesized that intracellular fatty acid metabolites, like DAG, could be involved in FA-induced insulin resistance by means of activation of a serine kinase cascade involving PKC, resulting in the phosphorylation of serine on IRS-1 [73, 87, 88]. In several studies it was postulated that PKCs are involved in skeletal muscle insulin resistance [85, 89] and that decreased insulin-signaling may be caused by IRS-1 serine/threonine phosphorylation by PKC [73, 90]. Indeed, PKC-θ knockout mice are protected against fat-induced insulin resistance [91]. Activated PKC directly phosphorylates skeletal muscle IRS1 at Ser101 [90], but PKCs also act upstream of stress kinases IκBα kinase β (IKK) and c-Jun NH2-terminal kinase (JNK), and may mediate negative serine phosphorylation of IRS1 through these kinases [92, 93].

NUCLEAR FACTOR-κB

Skeletal muscle insulin resistance following FA-induced lipid metabolite accumulation as well as FA-induced TLR-activation has been suggested to rely on activation of the NF-κB signaling pathway. Under basal conditions, NF-κB is present in the cytoplasm in an inactive state, bound to its inhibitory protein inhibitor κBα (IκBα). After stimulation, an intracellular signaling cascade is initiated, resulting in the activation of the serine kinase inhibitor κB kinase (IKK). This leads to phosphorylation and subsequent degradation of IκBα. NF-κB is then released from its inhibitory protein and translocates to the nucleus, where it regulates the transcription of pro-inflammatory genes by binding to its cognate DNA sequence in promoters and enhancers of their corresponding genes (reviewed in [94]).

Itani *et al.* demonstrated that an acute elevation in plasma FFA in humans after a six hour lipid infusion not only induced insulin resistance, accumulation of DAG, and an increase in PKC activity, but also a reduction in IκBα in skeletal muscle [5]. The latter is a sign of increased NF-κB activation, and suggested that accumulation of DAG could lead to insulin resistance via activation of the NF-κB pathway. Moreover, several studies in rodents and humans indeed show that fat-induced insulin resistance is associated with NF-κB activation in skeletal muscle [95, 96]. An obesity-inducing diet for 6 weeks or acute hyperlipidemia (lipid infusion) resulted in a reduction of IκBα levels in skeletal muscle in rats [95]. Another study in rats showed that consumption of an obesity-inducing HFD for 3 months resulted in a reduction of IκBα levels in skeletal muscle, which was associated with reduced insulin signaling [96]. As decreased IκB levels may reflect increased NF-κB activity, this suggested that the observed muscle insulin resistance resulted from increased transcriptional activity of NF-κB.

Another potential mechanism via which the NF-κB pathway may cause decreased insulin signaling in muscle involves IRS-1 serine phosphorylation by IKK-2. Studies in obese rodents showed that pharmacological inhibition of IKK-2 by high doses salicylate could improve the lipid-induced reduction in PI3-kinase activity and glucose uptake in skeletal muscle. Furthermore, they also showed that partial inactivation of IKK by using heterozygous (IKK-2 +/-) knockout mice, prevented fat-induced insulin resistance in skeletal muscle [97, 98]. These studies pointed to IKK-2 as a likely candidate for FA-induced insulin resistance [97]. A role for IKK-2 in insulin resistance was confirmed by *in vitro* studies, which revealed that IKK is able to phosphorylate IRS-1 at serine residues [3]. A recent study in rats did show increased IKK-2 activity in skeletal muscle after three weeks of high-fat feeding, which coincided with a reduced insulin-stimulated glucose uptake in the gastrocnemius muscle [99].

However, conflicting results have been found regarding the requirement of muscular NF-κB activation in FA-induced whole body and skeletal muscle insulin

resistance. Activation of NF-κB in skeletal muscle by muscle-specific transgenic expression of activated IKK in mice (MIKK mice) did not result in insulin resistance (measured by glucose tolerance testing and determining *ex vivo* glucose uptake in isolated extensor digitorum longus muscles) [100]. However, it might be possible that lifelong activation of NF-κB triggered compensatory mechanisms to restore insulin sensitivity. Muscle-specific inhibition of NF-κB or muscle-specific deletion of IKK-2 did not improve whole body insulin resistance in obese mice [100, 101]. Muscle-specific IKK-2 deficient mice showed no protection against insulin resistance after gold thioglucose-induced or high-fat diet-induced obesity [101]. Furthermore, muscle-specific inhibition of NF-κB by transgenic expression of the IκBα superrepressor (MISR mice) did not protect against the development of obesity-induced insulin resistance, following a high fat diet for 3 months [100].

In contrast, *in vitro* studies in L6 skeletal muscle cells, circumventing possible inter-organ effects, provided evidence that NF-κB activation was causally related to FA-induced insulin resistance. In that study, it was demonstrated that palmitate-induced insulin resistance could be prevented by pharmacological blocking of IKK and NF-κB nuclear translocation [102]. Altogether, so far studies on the role of NF-kappaB in FA-induced skeletal muscle insulin resistance yielded controversial results.

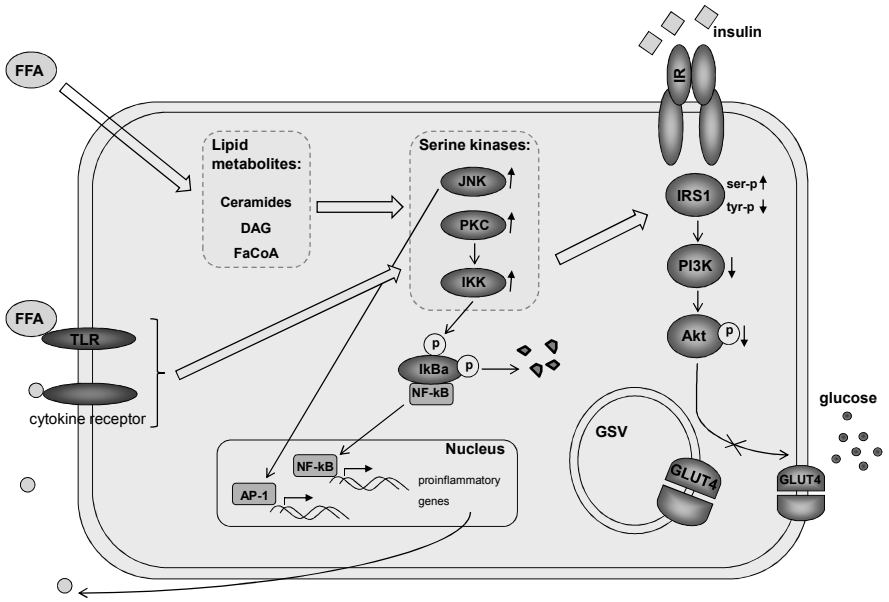

Figure 2: Summary of inflammatory mechanisms involved in skeletal muscle insulin resistance. An increase in serum free fatty acids (FFA) can result from an excess in dietary fat. FFAs can stimulate toll-like receptors. Furtehrmore, an increased FA uptake in skeletal muscle can lead to an intramyocellular accumulation of triglycerides. Subsequently, lipid metabolites like diacylglycerol, long-chain acyl-coenzyme A and ceramides may activate several serine/threonine kinases like PKC, IKK and JNK. Subsequent serine phosphorylation of IRS proteins results in a reduced tyrosine phosphorylation and an impairment of insulin signaling, ultimately leading to a reduced insulin-stimulated glucose uptake. Furthermore, IKK activation results in activation of the transcription factor NF-κB and subsequent production of pro-inflammatory cytokines. In this way, skeletal muscle insulin resistance may also be induced in an autocrine way.

AIMS AND OUTLINE OF THE THESIS

The main aim of this thesis was to evaluate the effects of different fatty acids on insulin sensitivity and NF-κB activation in skeletal muscle cells, and to investigate the potential causality between both parameters.

Palmitic acid (C16:0) is able to induce insulin resistance and NF-κB activation in muscle cells [103, 104]. To evaluate the effects of other saturated fatty acids (SFAs) differing in chain length on their ability to affect insulin sensitivity and NF-κB activation in skeletal muscle cells *in vitro*, two well-established models of cultured skeletal muscle cells were used (**chapter 2**). Myoblasts from the rat L6 and the mouse C2C12 cell lines were differentiated into myotubes and incubated with the medium-chain length SFAs caprylic acid (C8:0) and lauric acid (C12:0), and with the long-chain length SFAs palmitic acid (C16:0) and stearic acid (C18:0). After this, insulin sensitivity and activation of the NF-κB pathway was determined. In **chapter 3** the supposed association between insulin resistance and NF-κB activation is further addressed. We evaluated the effects of long-chain FAs, differing in their degree of saturation and configuration. FAs from the same backbone (C16 and C18) were compared with their mono-unsaturated [palmitoleic acid (C16:1), oleic acid (C18:1), elaidic acid (*trans*-9 C18:1), and vaccenic acid (*trans*-11 C18:1)] and poly-unsaturated [linoleic acid (C18:2), linolenic acid (C18:3), *cis*-9, *trans*-11 conjugated linoleic acid (CLA) and *trans*-10, *cis*-12 CLA] counterparts.

Changes in muscle mass induce changes in total glucose disposal. Furthermore, the contribution of skeletal muscle to glucose homeostasis is not only determined by insulin-mediated GLUT4 translocation, but also by differences in GLUT4 expression. Since it is known that GLUT4 expression is increased with muscle differentiation and that cytokines reduce muscle differentiation via incereased NF-κB signaling, we investigated the effects of the most potent NF-κB activating FAs from the previous chapters on muscle differentiation and GLUT4 expression (**chapter 4**).

Definitive experiments to address the causality of NF-κB in palmitic acid-induced insulin resistance are described in **chapter 5**. Both pharmacological and genetic approaches were used to investigate a possible cuasality. Furthermore, the role of lipid metabolite accumulation in inducing NF-κB activation and differences in insulin sensitivity in skeletal muscle were investigated by interfering with DAG metabolism and FA oxidation.

Chapter 6 provides an overview of the current literature on the role of inflammatory signaling in skeletal muscle insulin resistance. Both intracellular inflammatory signaling in muscle cells as well as inflammatory processes originating from other organs and their effects on skeletal muscle insulin resistance are discussed. Furthermore, potential dietary interventions to improve or prevent

insulin resistance in muscle via interfering with inflammatory signaling are described.

The insights resulting from the studies conducted in this thesis are integrated in **chapter 7**, and recommendations for future research to further unravel the mechanisms of insulin resistance based on this thesis are discussed.

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CHAPTER 2

Saturated fatty acid-induced NF-κB activation and insulin resistance in skeletal muscle are chain length dependent

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ABSTRACT

The saturated fatty acid (SFA) palmitate (C16:0) induces insulin resistance in cultured skeletal muscle cells, which may be related to NF-κB activation. The aim of this study was to evaluate if other SFAs also exert these effects on skeletal muscle and whether these relate to chain length. Therefore, we incubated L6 and C2C12 skeletal muscle cells with four different fatty acids, caprylate (C8:0), laurate (C12:0), palmitate (C16:0), and stearate (C18:0), to study effects on GLUT4 translocation, deoxyglucose uptake, and NF-κB activation. Incubation of L6 cells with the long-chain FAs C16:0 and C18:0 reduced insulin-stimulated GLUT4 translocation and deoxyglucose uptake, whereas L6 cells incubated with the medium-chain FAs C8:0 and C12:0 remained insulin sensitive. Besides increasing NF-κB DNA binding activity in both L6 and C2C12 cells, C16:0 also induced NF-κB transcriptional activity. C18:0 showed comparable effects, whereas the SFAs with shorter chain lengths were not able to elevate NF-κB transcriptional activity. Collectively, these results demonstrate that SFA-induced NF-κB activation coincides with insulin resistance and depends on FA chain length.

INTRODUCTION

The prevalence of both type 2 diabetes and the metabolic syndrome is rapidly increasing [1]. Insulin resistance, defined as a decreased sensitivity of target tissues to increase glucose uptake in response to insulin, is recognized as a major contributor to the pathogenesis of these disorders. Skeletal muscle is the predominant site of insulin-induced glucose uptake and therefore an important tissue to analyze mechanisms underlying insulin resistance and to evaluate effects of (dietary) interventions. Many studies have shown that an elevation in plasma free fatty acids (FAs) is associated with insulin resistance, indicating FA-induced insulin resistance [2-6]. Local or systemic inflammation has been implicated in this process, since circulating levels of several proinflammatory cytokines are elevated in type 2 diabetic patients [7] and increased mRNA and protein expression levels of the cytokine tumor necrosis factor-α (TNFα) have been demonstrated in skeletal muscle of insulin-resistant and diabetic subjects [8]. Moreover, it has been proposed that a relationship exists between activation of the transcription factor nuclear factor kappa B (NF-κB), regulating the expression of many proinflammatory mediators, and FA-induced insulin resistance [2, 9-10]. Different studies in cultured skeletal muscle cells provided evidence that the saturated FA palmitate (C16:0) induces insulin resistance [11-14] and activates the NF-κB pathway, which resulted in the production of pro-inflammatory cytokines interleukin-6 (IL-6) and TNF-α [15-17]. NF-κB activation and insulin resistance may be causally related since the palmitate-induced insulin resistance in cultured skeletal muscle cells was prevented by blocking NF-κB activation [18].

Under basal conditions, NF-κB is present in the cytoplasm in an inactive state, bound to its inhibitory protein inhibitor κBα. After stimulation, an intracellular signaling cascade is initiated, resulting in the activation of the serine kinase inhibitor κB kinase. This leads to phosphorylation and subsequent degradation of IκBα. NF-κB is then released from its inhibitory protein and translocates to the nucleus, where it regulates the transcription of proinflammatory genes by binding to its cognate DNA sequence in promoters and enhancers of their corresponding genes (reviewed in Ref. [19]).

Despite all the available data, the exact mechanisms underlying the observed relation between inflammation and palmitate-induced insulin resistance in skeletal muscle are not completely clear. Because the effects of FAs on insulin sensitivity in skeletal muscle have so far been investigated only by measuring glucose uptake or activation of signaling proteins upstream of the glucose transporter 4 (GLUT4), but never on the level of GLUT4 translocation itself, we have studied whether FA-induced insulin resistance is apparent at the level of GLUT4 translocation.

Although the actions of palmitate on insulin resistance and inflammation have been investigated, a systematic evaluation of the effects of other FAs,

classified according to chain length, on insulin resistance and inflammation is lacking. This is quite remarkable, because our Western diet also contains substantial amounts of medium-chain length FAs (MCFAs) such as laurate (C12:0) in dairy products. Improving our knowledge on inflammatory and insulin (de)sensitizing properties of FAs will aid in composing a healthy diet.

Therefore, the goal of this study was to investigate the role of FA chain length on insulin sensitivity, measured as GLUT4 translocation and deoxyglucose uptake, in relation to NF-κB activity in cultured skeletal muscle cells.

MATERIALS AND METHODS

Cell culture

The C2C12 murine skeletal muscle cell line (ATCC CRL1772; ATCC, Manassas, VA), stably transfected with the 6κB-TK-luciferase, was used for the assessment of NFκB transcriptional activity, as previously described [20]. In brief, C2C12 cells were plated (1 x 10^4 cells/cm^2) on Matrigel (Becton-Dickinson Labware, Bedford, MA) coated (1:50 in DMEM) dishes, as described previously [21]. C2C12 myoblasts were cultured in growth medium, composed of low-glucose Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; both from Gibco-Invitrogen, Rockville, MD) and 9% (vol/vol) fetal bovine serum (FBS; PAA Laboratories). To induce differentiation, growth medium was replaced by differentiation medium, containing DMEM with 0.5% (vol/vol) heat-inactivated FBS and antibiotics. As a positive control for NF-κB transcriptional activity, murine TNFα (10 ng/ml) was added for 4 h.

The L6 rat skeletal muscle cell line, stably transfected with a construct encoding GLUT4 with an exofacial *myc* epitope (L6-GLUT4*myc*), was kindly provided by Dr. Amira Klip from the Hospital for Sick Children, Toronto, ON, Canada. L6 myoblasts were cultured in growth medium, composed of α-MEM (Gibco-Invitrogen, Rockville, MD) containing 9% (vol/vol) FBS and antibiotics. The plating density used for the experiments was 2 x 10^4/cm^2. After 24 h of culturing in growth medium, differentiation was induced by replacing growth medium with differentiation medium containing α-MEM with 2% (vol/vol) heat-inactivated FBS and antibiotics.

All experiments for both cell lines were performed in 5 day-differentiated myotubes.

Fatty acid incubations

Stock solutions of 40 mmol/l were made in ethanol for all FAs [caprylate (C8:0), laurate (C12:0), palmitate (C16:0), and stearate (C18:0); all from Sigma, St. Louis, MO]. Before being applied to the cells, FAs were conjugated to bovine serum albumin (BSA) by diluting the FA solution 1:100 (for C2C12) or 1:200 (for L6) with differentiation medium containing 1% (wt/vol) FA-free BSA (Sigma). Solutions were filter-sterilized for use in experiments. Vehicle controls contained 1 (C2C12) or 0.5% (L6) (vol/vol) ethanol and 1% (wt/vol) BSA.

Surface GLUT4myc detection

L6 myotubes cultured in 24-well plates with or without FA preincubation were serum deprived for 3 h prior to incubation with 25 nmol/l insulin (Sigma) for 20 min at 37°C. Cells were washed three times with ice-cold PBS, followed by a blocking step with 5% (vol/vol) goat serum (Gibco-Invitrogen, Rockville, MD) in PBS for 10 min at 4°C. Cells were incubated with anti-myc monoclonal antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) in 3% (vol/vol) goat serum containing PBS for 1 h at 4°C. Subsequently, a fixation step of 3% (vol/vol) paraformaldehyde (Sigma) in PBS for 10 min at 4°C was followed by incubation with 50 mmol/l NH_4Cl to remove all remaining paraformaldehyde. After washing with ice-cold PBS, cells were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:1,000; Vector Laboratories, Burlingame, CA) for 45 min at 4°C. Cells were washed five times, and to quantify the amount of GLUT4*myc* present at the cell surface, 1 ml of *o*-phenylenediamine dihydrochloride reagent (Sigma) was added at room temperature for ≤30 min. The reaction was stopped by adding 250 μl of 3 mol/l HCl. Subsequently, a 200-μl aliquot of the mixture was transferred to a 96-well plate, and absorbance was measured at 492 nm [22].

[^{3}H]deoxyglucose uptake

After experimental treatments, deoxyglucose uptake was determined as described previously [23]. In brief, myotubes with or without FA preincubation were serum deprived for 3 h prior to incubation with 25 nmol/l insulin (Sigma) for 15 min at 37°C in glucose-free medium. Label was added {final concentration of 20 μmol/l [^{3}H]deoxyglucose (0.2 μCi/ml [^{3}H]deoxyglucose)} and incubated for 20 min at 37°C. Glucose transport was stopped by washing three times with an ice-cold stop-solution containing 0.2 mmol/l phloretin, and cells were harvested in 0.5 ml 0.05 mol/l NaOH. Cell-associated radioactivity was determined by scintillation counting.

Electrophoretic mobility shift analysis

To determine DNA binding activity of NF-κB, complexes binding to an oligonucleotide containing an NF-κB consensus sequence were analyzed. Nuclear extracts were isolated to analyze NF-κB DNA binding. To this end, cells were harvested following experimental treatments and lysed on ice in 400 μl of buffer containing 20 mmol/l HEPES, pH 7.8, 20 mmol/l KCl, 4 mmol/l $MgCl_2$, 0.2 mmol/l EDTA, 1 mmol/l DTT, 0.2 mmol/l sodiumorthovanadate, 0.4 mmol/l phenylmethyl sulfonylfluoride, 0.3 μg/ml leupeptin and 0.2 mmol/l NaFl for 15 min. Subsequently, 25 μl of 10% Nonidet P-40 was added and samples were vortexed for 15 s, followed by centrifugation (14,000 rpm for 30 s). Supernatants were removed and pelleted nuclei were washed with the previously mentioned buffers and resuspended with a buffer containing 100 mmol/l HEPES, pH 7.8, 100 mmol/l KCl, 600 mmol/l NaCl, 0.2 mmol/l EDTA, 20% glycerol, 1 mmol/l DTT, 0.2 mmol/l sodiumorthovanadate, 0.667 mmol/l phenylmethyl sulfonylfluoride, and 0.2 mmol/l NaFl. Nuclei were mixed vigorously for 20 min at 4 °C, using a rotating platform and centrifuged (14,000 rpm for 5 min), and samples were stored at -20°C (for protein concentration determination) and -80°C (for DNA binding activity measurements). Seven micrograms of nuclear cell extracts were used per binding reaction, and protein-DNA complexes were resolved on a 5% polyacrylamide gel in 0.25X Tris-borate-EDTA buffer at 160 V for 2 h. Gels were dried and exposed to film (X-Omat Blue XB-1; Kodak, Rochester, NY). Shifted complexes were quantified by phosphoimager analysis (Bio-Rad). To determine the presence of RelA (p65) by supershift analysis, nuclear extracts were pre-incubated with an antibody specific to the RelA subunit of NF-κB (Santa Cruz Biotechnology).

NF-κB transcriptional activity

To determine NF-κB transcriptional activity, luciferase activity was measured in C2C12 cells as described previously [21]. Cells were lysed in luciferase lysis buffer and stored at -80°C. Luciferase activity (Promega, Madison, WI) was measured according to the manufacturers' instructions and expressed relative to the concentration of the soluble protein fraction.

Statistical analysis

SPSS (version 11.5) was used for statistical analysis. Values for NF-κB transcriptional activity, NF-κB DNA-binding, and cell surface GLUT4*myc* measurements were analyzed by one-way ANOVA, and the various treatment groups were compared post hoc with the Bonferroni test.

RESULTS

Palmitate induces NF-κB activity in skeletal muscle cells

To determine the effects of palmitate (C16:0) on NF-κB DNA binding in L6 myotubes, we first performed time- and dose-response studies. After already 8 h of incubation with 200 μmol/l palmitate, a significant ~3.5-fold increase of NF-κB DNA binding over basal levels was found, which was sustained ≤24 h (Figure 1, A and B). To determine the lowest activating dose of C16:0 in our cell model, a dose-response experiment was performed at the 16-h time point. C16:0 concentrations <200 μmol/l did not activate NF-κB DNA binding (Figure 1C). Therefore, subsequent incubations of L6 myotubes with FAs were performed for 16 h with a concentration of 200 μmol/l. To investigate whether NF-κB DNA binding was accompanied by NF-κB-mediated transcription, following C16:0 incubation, NF-κB transcriptional activity was measured in C2C12 myotubes stably transfected with an NF-κB-sensitive reporter construct. Figure 1D shows that C16:0 induced NF-κB transactivation in skeletal muscle. By performing an NF-κB DNA-binding ELISA, we confirmed that C16:0 induces NF-κB activation in both cell lines (data not shown). Collectively, these data demonstrate that C16:0 induces NF-κB DNA binding and transcriptional activity in cultured skeletal muscle cells.

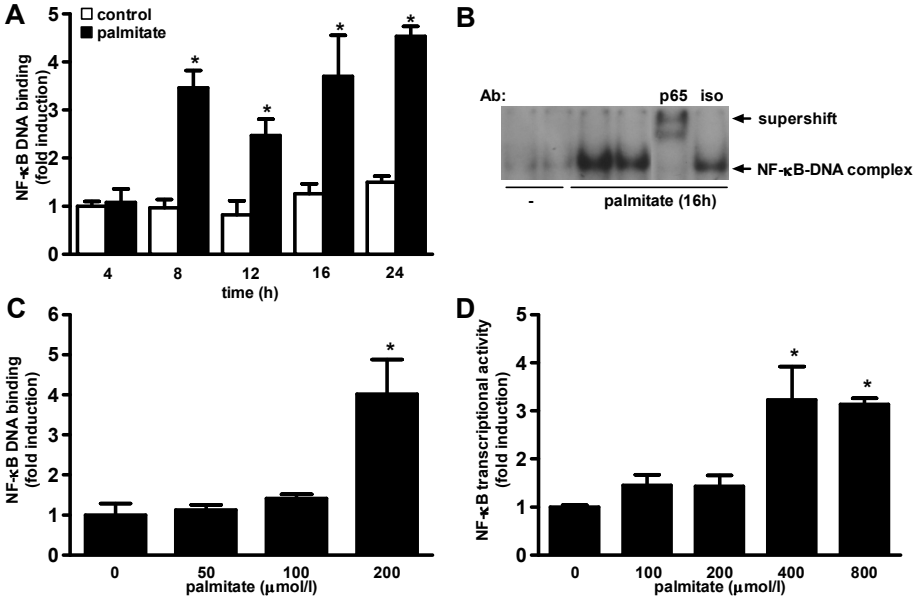

Figure 1: Palmitate induces NF-κB DNA-binding and transcriptional activity in myotubes. L6 myoblasts were differentiated for 5 days and incubated with (black bars) and without (open bars) 200 μmol/l palmitate for the indicated time (**A**) or 16 h (**B**) or incubated with indicated amounts of palmitate at the 16-h time point (**C**). Nuclear extracts were prepared at the indicated times and assessed for DNA-binding activity to a consensus NF-κB oligonucleotide by electrophoretic mobility shift assay. Relative DNA-binding activity was determined by phosphor-imager analysis and expressed as fold induction. To determine the presence of RelA (p65) by supershift analysis, nuclear extracts were preincubated with an antibody specific to the p65 subunit of NF-κB or an isotype control antibody (iso). **D**: alternatively, C2C12 myoblasts stably transfected with the 6κB-TK-luciferase construct were differentiated for 5 days and incubated with the indicated amounts of palmitate for 24 h. Lysates were prepared for assessment of luciferase activity. Values were corrected for protein content and expressed as fold induction over control. Statistically significant differences between the indicated treatment and corresponding BSA-treated control (*P < 0.05) were determined by one-way ANOVA. Data shown are representative examples of 2 or 3 independent experiments.

Long chain FAs reduce insulin-induced GLUT4 translocation and deoxyglucose uptake in skeletal muscle cells

The well-established L6 cell line, stably transfected with GLUT4*myc* cDNA [22], was used to assess whether induction of NF-κB activity by C16:0 was associated with decreased insulin sensitivity. Insulin stimulation resulted in a reproducible 2 - 2.6-fold increase in GLUT4 levels at the cell surface in the BSA-treated myotubes in all experiments (Figure 2). However, preincubation with C16:0 (200 μmol/l, 16 h) decreased the insulin-stimulated GLUT4 translocation by ~68% compared with BSA-

treated myotubes, whereas basal GLUT4 surface levels were unaffected by C16:0 (Figure 2, *top left*). These data demonstrate that C16:0, besides elevating NF-κB activity, also induces insulin resistance in L6 myotubes.

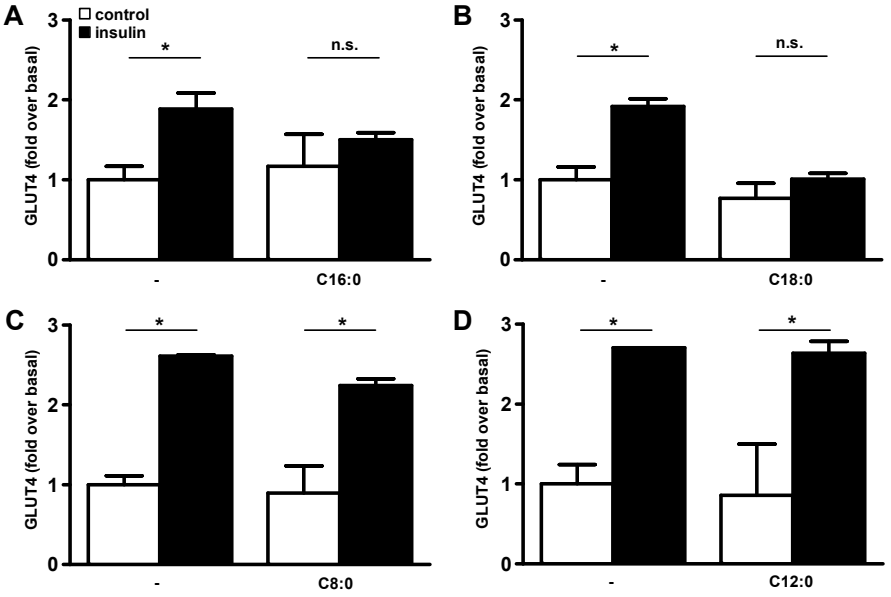

Figure 2: Palmitate (C16:0) and stearate (C18:0), but not caprylate (C8:0) and laurate (C12:0), reduce insulin-induced glucose transporter 4 (GLUT4) translocation in myotubes. Five-day differentiated L6-GLUT4*myc* myotubes were incubated with and without the indicated fatty acids (200 μmol/, 16 h) prior to insulin stimulation (25 nmol/l, 15 min; open bars, no insulin stimulation; black bars, insulin stimulation). GLUT4 presence in the cell membrane was measured as described in 'materials and methods' and was expressed as fold induction over basal (BSA-treated group without insulin). Statistically significant differences between the insulin-stimulated group and corresponding control group (* $P < 0.05$) were determined by one-way ANOVA. Data shown are representative examples of 3 or more independent experiments. ns, not significant.

We next evaluated whether the insulin-desensitizing effects of palmitate were replicated following preincubation with other saturated FAs. Stearate (C18:0), another long-chain fatty acid, and two medium-chain fatty acids, caprylate (C8:0) and laurate (C12:0), were chosen. Comparable with the C16:0-treated cells, C18:0 reduced insulin-dependent GLUT4 translocation ~69% compared with the BSA-treated myotubes (Figure 2, *top right*). In contrast, L6 myotubes incubated with C8:0 and C12:0 still caused a ~2.5-fold and ~2.6-fold increase in cell surface GLUT4 after insulin stimulation (Figure 2, *bottom, left* and *right*, respectively). Since this response is similar to that of vehicle controls, the myotubes remained insulin sensitive after C8:0 and C12:0 incubation.

To investigate whether the effects on GLUT4 translocation were reflected by similar changes in glucose transport, deoxyglucose uptake was assessed for the same series of FAs (Figure 3). In vehicle-treated myotubes, insulin stimulation increased deoxyglucose uptake 2.5-fold. Pretreatment with C18:0 reduced insulin-induced deoxyglucose uptake from 2.5- to 1.6-fold, whereas C16:0 almost completely abolished insulin-induced deoxyglucose uptake. C16:0 and C18:0 decreased the net insulin-stimulated deoxyglucose uptake by ~80 and ~60%, respectively, compared with control cells. Unlike C16:0 and C18:0, C8:0 and C12:0 had no effects on insulin-stimulated deoxyglucose uptake compared with control cells.

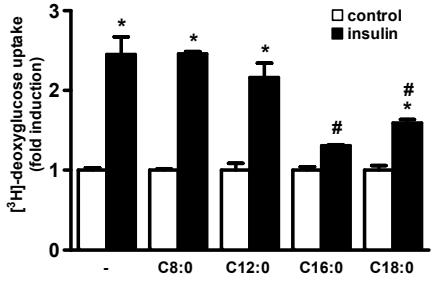

Figure 3: C16:0 and C18:0, but not C8:0 and C12:0, reduce insulin-induced deoxyglucose uptake in myotubes. Five-day differentiated L6-GLUT4*myc* myotubes were incubated with and without the indicated fatty acids (200 μmol/l, 16 h) prior to stimulation with 25 nmol/l insulin for 15 min (open bars, no insulin stimulation; black bars, insulin stimulation). 2-Deoxyglucose uptake was measured as described in 'materials and methods', and insulin-stimulated glucose uptake was expressed as fold induction over own basal (non-insulin stimulated) glucose uptake. Basal and insulin-stimulated glucose uptake are represented by the open and black bars, respectively. Statistically significant differences between basal and insulin-stimulated conditions for all fatty acids were determined by one-way ANOVA (* $P < 0.05$) or for insulin-stimulated glucose uptake between the indicated fatty acid treatment and vehicle control (# $P < 0.05$). Data shown are representative examples of 3 independent experiments.

Together, these data show that only the long-chain saturated FAs C16:0 and C18:0, but not the medium-chain length FAs C8:0 and C12:0, induced insulin resistance in L6 myotubes.

Long chain FAs increase NF-κB transcriptional activity in skeletal muscle cells

To investigate if saturated FA-induced insulin resistance showed the same pattern in terms of FA specificity towards NF-κB activation, we determined the effect of incubation of C8:0, C12:0, and C18:0 on NF-κB DNA binding and transcriptional activity. Incubation of L6 and C2C12 myotubes with the medium-chain length FAs C8:0 and C12:0 did not result in an elevation of NF-κB DNA binding and transcriptional activity. In contrast, the long-chain length FAs C16:0 and C18:0

showed an ~3.7- and ~2.8-fold induction of NF-κB DNA binding in L6 myotubes, and an ~3- and ~2.5-fold induction of NF-κB transcriptional activity in C2C12 myotubes over control levels, respectively (Figure 4). Thus, saturated FA-induced insulin resistance is evident only in response to long-chain FA, but not to medium-chain FA, and occurs in association with NF-κB activation.

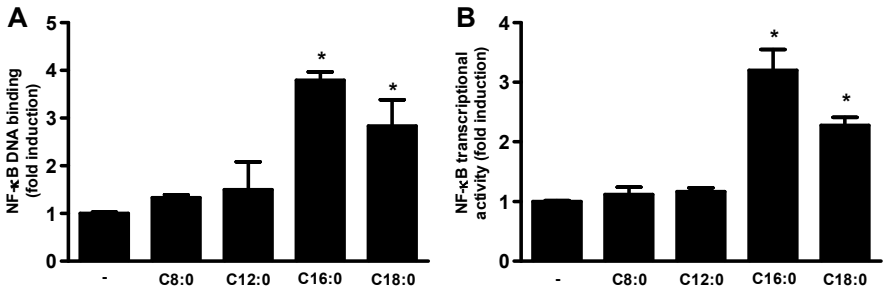

Figure 4: C16:0 and C18:0, but not C8:0 and C12:0, increase NF-κB DNA binding and transcriptional activity in myotubes. Five-day differentiated L6 and C2C12 myotubes were incubated with the indicated fatty acids (200 μmol/l, 16 h and 400 μmol/l, 24 h). Experiments were performed and analyzed, and results are expressed as described in Figure 1. Statistically significant differences between indicated treatment and BSA-treated control (*$P < 0.01$) were determined by one-way ANOVA. Data shown are representative examples of 2 or 3 independent experiments.

DISCUSSION

It is generally accepted that the saturated FA (SFA) palmitate (C16:0) induces insulin resistance in cultured skeletal muscle cells [11-14], which was postulated to be associated with NF-κB activation [18]. This was also confirmed by our results. However, it has never been systematically evaluated if other SFAs exert similar effects on skeletal muscle and whether these effects relate to FA chain length. In the present study, we showed that incubation of skeletal muscle cells with the long-chain SFAs C16:0 and stearate (C18:0) both resulted in a reduction of insulin-induced GLUT4 translocation and glucose uptake. Furthermore, this effect was associated with NF-κB activation. In contrast, the medium-chain SFA caprylate (C8:0) and laurate (C12:0) did not induce a change in GLUT4 translocation and glucose uptake, nor did it activate NF-κB in skeletal muscle cells. These data illustrate that SFA-induced insulin resistance and NF-κB activation depend on chain length of the FA.

Stimulation of skeletal muscle cells with insulin results in the translocation of the glucose transporter GLUT4 from intracellular vesicles to the cell membrane, thereby enabling elevated glucose uptake in the cells [24]. Although C2C12 cells have been used extensively in unraveling the insulin signaling cascade, analysis of

insulin-induced GLUT4 translocation and glucose uptake has been problematic. This may be attributed to low GLUT4 protein expression or defects in the GLUT4 translocation machinery in C2C12 cells [25-26]. Therefore, we analyzed the effects on GLUT4 translocation in another well-established skeletal muscle cell line, i.e., the rat L6 cells [22, 27]. Using this cell line, we are the first to demonstrate reduced insulin-dependent glucose uptake and GLUT4 translocation as part of FA-induced insulin resistance in the same model. Interpretation of data relying on detection of endogenous GLUT4 translocation to assess insulin sensitivity is limited by possible effects of FAs on GLUT4 expression, as have been reported for C16:0 [15]. In contrast, changes in GLUT4 cell surface presence observed in our study can be directly attributed to alterations in insulin-induced GLUT4 translocation, since this GLUT4 is constitutively expressed [27], and only ectopically expressed GLUT4 is detected. Therefore, our results conclusively demonstrate that the long-chain fatty acids (LCFAs) C16:0 and C18:0 inhibit GLUT4 translocation. These findings are in accordance with our deoxyglucose uptake measurements and with earlier studies showing in C2C12 cells that palmitate and longer LCFAs (C18:0, C20:0, and C24:0), but not the MCFAs (C12:0 and C14:0), blocked insulin signaling upstream of GLUT4 translocation, i.e., insulin-induced phosphorylation and activation of Akt [11]. This is also in line with our findings regarding the absence of any effect of the MCFAs on GLUT4 translocation and glucose uptake.

Several studies in primary skeletal muscle cells [17] and C2C12 [15-16] and L6 myotubes [18] have shown C16:0-induced NF-κB DNA binding activity. In the latter study [18], it was even concluded, on the basis of pharmacological blockers of NF-κB translocation, that NF-κB activation is required for C16:0-induced insulin resistance. Using the C2C12 NF-κB reporter cell line [20], we have now shown that C16:0 and C18:0 induce NF-κB transcriptional activity in myotubes, which is in agreement with findings that C16:0 induces NF-κB dependent TNFα and IL-6 mRNA and protein expression [15-17] in skeletal muscle. Furthermore, the stimulatory effect of C18:0 incubation on IL-6 mRNA expression was also in line with our data showing increased NF-κB activation by this long-chain FA [17]. To our knowledge, differential effects of FA varying in chain length on NF-κB activation in skeletal muscle cells have not been shown before. However, in the insulin-sensitive 3T3-L1 adipocyte cells, C16:0, but not C12:0, was reported to increase both NF-κB transcriptional activity and IL-6 release [28].

One of the proposed mechanisms linking FA-induced insulin resistance to NF-κB activation in skeletal muscle is the accumulation of lipid metabolites, including diacylglycerol (DAG) and ceramides, [2, 4, 11-12, 29-31]. DAG and ceramides are able to activate protein kinase C (PKC), a protein that has been linked to insulin resistance in cell lines [14], rodents [6, 30, 32] and humans [2, 33]. Furthermore, it is also known that PKC can activate NF-κB. Thus, an oversupply of FAs in skeletal muscle may lead to accumulation of lipid metabolites, thereby activating the NF-κB

pathway. Therefore, a possible explanation for the differential effects we have found for medium-chain and long-chain SFAs on insulin resistance might relate to the metabolism of these FAs within the skeletal muscle cell. MCFAs do not require the carnitine palmitoyltransferase system, which is necessary for LCFA transport over the mitochondrial membrane. This means that MCFAs are preferentially oxidized by the mitochondria [34]. This could be a reason why long-chain SFAs are more available for incorporation into ceramides and DAG, thereby potentially activating PKC and the NF-κB pathway. This hypothesis is supported by our data, since we found differential effects of MCFAs and LCFAs not only on insulin action but also on NF-κB activation in our skeletal muscle models. Furthermore, incubation with FAs C16:0 and C18:0 indeed resulted in ceramide and DAG accumulation, whereas C12:0 incubation showed no effects on ceramide and DAG concentrations [11].

Recently, activation of Toll-like receptors (TLRs) was postulated as potential mechanism for C16:0-induced insulin resistance in skeletal muscle. Mice lacking TLR4 have been shown to be partially protected against FA-induced insulin resistance in skeletal muscle [35-36]. Furthermore, in L6 myotubes it has been shown that pharmacological blocking of TLR4 prevented C16:0-induced insulin resistance [36]. In C2C12 myotubes, TLR2-mediated C16:0-induced insulin resistance was shown in combination with activation of NF-κB, measured as NF-κB DNA binding [37] already after 4-6 h. Although we have not measured NF-κB DNA binding in the C2C12 myotubes at earlier time points, both DNA binding and transcriptional activation of NF-κB were apparent at 24 h. In addition, in L6 myotubes, we detected NF-κB DNA binding after 8 h of incubation with C16:0. Differences in the kinetics of C16:0-induced NF-κB activation and insulin resistance have been reported in L6, C2C12, and primary cell lines [11, 13, 15, 17-18, 37-38]. These discrepancies between studies may be explained by differences in cell model, incubation time and concentration of the FAs used. Therefore, at this moment we cannot exclude TLR activation as possible mechanism for FA-induced insulin resistance in skeletal muscle. Elaborating on this, it is tempting to speculate that, in light of the data we present here, medium-chain length SFAs did not induce insulin resistance because they lack TLR-activating potential and therefore do not affect NF-κB. Further studies are required to address this possibility.

Apart from potential insight in mechanisms of LCFA-induced insulin resistance, our study suggests that substitution of C16:0 and C18:0, FAs that are highly abundant in our Western diet, for medium-chain FAs may be an approach to prevent nutrition-associated insulin resistance. A similar application has been suggested for oleate (C18:1), which even appears to antagonize the effects of C16:0 [39]. Whether the MCFAs presented in the present paper are also able to antagonize the negative effects of C16:0 on insulin sensitivity needs further study.

In conclusion, we have shown that saturated FA-induced insulin resistance, reflected by reduced insulin-stimulated GLUT4 translocation and glucose uptake, occurs only in response to LCFAs but not MCFAs and is associated with NF-κB activation. The exact mechanisms underlying these differential effects of SFAs on insulin resistance and NF-κB activation remain to be investigated. This knowledge can ultimately be used in developing dietary intervention strategies to combat insulin resistance.

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CHAPTER 3

Trans fatty acid-induced NF-κB activation does not induce insulin resistance in cultured murine skeletal muscle cells

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ABSTRACT

Long-chain saturated fatty acids such as palmitic acid induce insulin resistance and NF-κB activation in skeletal muscle cells. Here we investigated the effects of long-chain fatty acid (FA) saturation and configuration on NF-κB activity and insulin sensitivity in cultured skeletal muscle cells. Of all tested unsaturated FAs, only elaidic acid (threefold), cis9, trans11-CLA (threefold) and trans10, cis12-CLA (13-fold) increased NF-κB transactivation in myotubes. This was not accompanied by decreased insulin sensitivity (measured as insulin-induced glucose uptake and GLUT4 translocation). We therefore conclude that FA-induced NF-κB activation is not sufficient for the induction of insulin resistance in skeletal muscle cells.

INTRODUCTION

Although the molecular mechanisms underlying the pathogenesis of insulin resistance are still not fully understood, different lines of evidence support the notion that elevated concentrations of plasma free fatty acids (FA) play an important role [1-3]. A relation between the activation of the transcription factor-κB (NF-κB) and FA-induced insulin resistance in skeletal muscle in humans has been proposed [1]. In addition, studies in cultured skeletal muscle cells have shown that the saturated FA palmitate (C16:0) not only induces insulin resistance [4-7] but also activates the NF-κB pathway, which results in the production of pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α [8-10]. Still, a causal relationship between NF-κB activation and FA-induced skeletal muscle insulin resistance is disputable [11-15]. Recently, we reported that the differential effects of SFAs on NF-κB activation and insulin resistance in skeletal muscle cells depend on FA chain length [16]. Incubation with palmitic acid (C16:0) and stearic acid (C18:0) resulted in insulin resistant skeletal muscle cells showing increased NF-κB activation, while incubation with the shorter saturated FAs caprylic acid (C8:0) and lauric acid (C12:0) did not trigger these effects. Effects of individual FAs with the same long-chain length, but with differences in the degree of saturation or in *cis-trans* configuration have not yet been systematically evaluated. To investigate if the association between long-chain SFA-induced NF-κB activation and insulin resistance in skeletal muscle cells can be generalized to all long-chain FAs (≥16 carbon atoms), we investigated the effect of saturation and configuration on long chain FA-induced NF-κB activity in relation to insulin sensitivity in cultured skeletal muscle cells.

MATERIALS AND METHODS

Cell culture

The C2C12 murine skeletal muscle cell line (ATCC CRL1772; Manassas, VA), stably transfected with the 6κB-TK-luciferase, was used for the assessment of NF-κB transcriptional activity as described previously [16-18].

The L6 rat skeletal muscle cell line, stably transfected with a construct encoding GLUT4 with an exofacial *myc* epitope (L6-GLUT4*myc*) was kindly provided by Dr. Amira Klip from the Hospital for Sick Children, Toronto, ON, Canada. Cells were cultured and [^{3}H]-deoxyglucose uptake, GLUT4 translocation and NF-κB DNA binding by electrophoretic mobility shift analysis (EMSA) was determined as described previously [16]. All experiments for both cell lines were performed in 5-day differentiated myotubes.

Fatty acid incubations

Stock solutions of 40 mmol/l were made in ethanol for all FAs (palmitic, palmitoleic, stearic, oleic, elaidic, vaccenic, linoleic, α-linolenic, arachidonic acid, EPA, DHA (all from Sigma, St. Louis, MO) and *cis*-9, *trans*-11 CLA and *trans*-10, *cis*-12 CLA (Bio-connect, The Netherlands). FAs were conjugated to bovine serum albumin (BSA) [16] prior to addition to the cells. For every type of measurement, the experimental set-up was chosen to include all comparisons between saturated and unsaturated FA as reported.

Statistical analysis

SPSS (version 16.0) was used for statistical analysis. Values for NF-κB transcriptional activity and [^{3}H]-deoxyglucose uptake measurements were analyzed by one-way ANOVA, and the various treatment groups (N = 3) were compared post hoc with Bonferroni correction, in which a p value of ≤ 0.05 was considered significant.

RESULTS AND DISCUSSION

To test whether the previously reported effects of long-chain SFAs on NF-κB activation in skeletal muscle [9, 11, 16] are also present after incubation with monounsaturated (MUFAs) and polyunsaturated FAs (PUFAs), we incubated C2C12 myotubes, stably transfected with an NF-κB sensitive reporter construct, with a variety of FAs. The concentrations FA used in this study are within the range of those found in the plasma of healthy and diabetic subjects [19-21].

The incubation time and FA concentration were optimized for palmitic acid [16] and used for all other FAs tested. In contrast to palmitic acid (C16:0) and stearic acid (C18:0), their mono-unsaturated counterparts [palmitoleic (C16:1) and oleic (C18:1) acid] did not induce NF-κB transactivation in C2C12 skeletal muscle cells (Figure 1A). Also poly-unsaturated FAs with the same chain length as stearic acid [linoleic (C18:2) and α-linolenic (C18:3) acid] did not show these effects. The absence of activation of NF-κB for these FAs was confirmed in another skeletal muscle cell line, by performing an EMSA for the assessment of RelA DNA binding in L6 myotubes (Figure 1B).

Deoxyglucose uptake increased ~4-fold following insulin stimulation of vehicle treated myotubes (Figure 1C). Pretreatment with the different mono- and polyunsaturated FAs resulted in a similar increase in insulin-induced deoxyglucose uptake (between 3.5- and 4-fold increase compared to their respective vehicle controls), while C16:0 and C18:0 decreased insulin-stimulated deoxyglucose uptake by ~60 and ~80% respectively, compared with vehicle control. These results were confirmed by GLUT4 translocation measurements (Figure 1D) and are in line with

the notion that NF-κB activation and the occurrence of insulin resistance occur simultaneously, suggesting that NF-κB activation may be causally related to long-chain SFA induced insulin resistance.

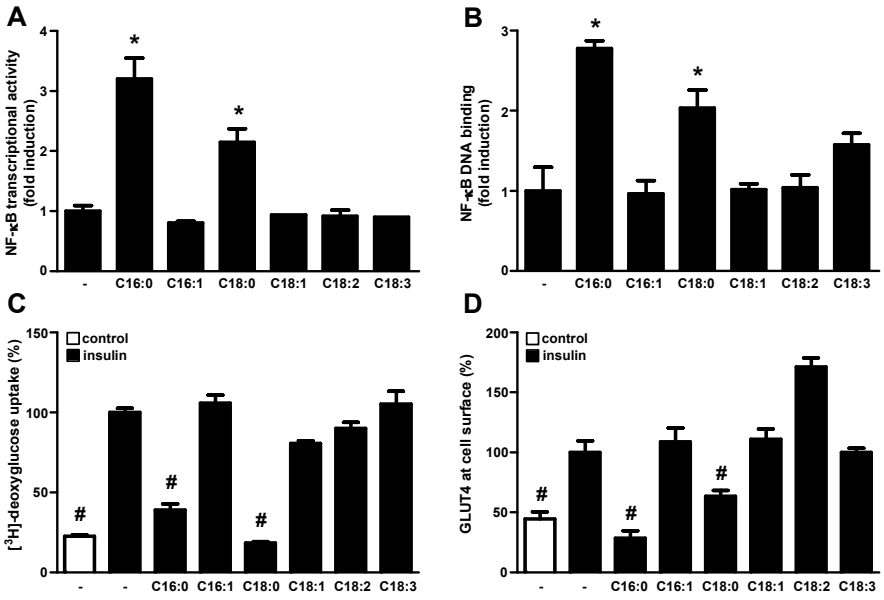

Figure 1: NF-κB transcriptional activity and insulin sensitivity in skeletal muscle cells after incubation with C16 or C18 FAs differing in degree of saturation. C2C12 myoblasts stably transfected with the 6κB-TK-luciferase construct were differentiated for 5 days and incubated with 400 μmol/l of the indicated FAs for 24 h (A). Lysates were prepared for assessment of luciferase activity. Values were corrected for protein content and expressed as fold-induction over control. Alternatively, 5 days differentiated L6-GLUT4*myc* myotubes were incubated with and without the indicated fatty acids (200 μmol/l, 16 h). Nuclear extracts were prepared and assessed for RelA DNA binding activity to a consensus NF-κB oligonucleotide by EMSA (B). Relative DNA binding activity was determined by phosphor-imager analysis and expressed as fold induction. Furthermore, 2-deoxyglucose uptake (C) and GLUT4 translocation (D) were measured after stimulation with 25 nmol/l insulin for 15 min in 5 days differentiated L6-GLUT4myc myotubes. Data are expressed as percentage of insulin-stimulated glucose uptake or GLUT4 translocation in absence of FA. Basal and insulin-stimulated conditions are represented by the open and black bars, respectively. * $p < 0.05$ versus control. # $p < 0.05$ versus insulin-stimulated control. Data shown are representative examples of 3 independent experiments.

These results are supported by several *in vitro* studies in L6 cells, C2C12 cells or primary myotubes, where no insulin desensitizing and NF-κB activating effects were found for C16:1, C18:1 and C18:2 [4-6, 10, 22]. However, Sinha *et al.* [11] reported an increased NF-κB DNA binding and insulin resistance in L6 myotubes after incubating with C16:0 or C18:2, whereas no effects were found for C18:3 incubation. A possible explanation for this discrepancy may lie in the fact that the experiments by Sinha and co-workers were performed for 6h, while the other

studies mentioned, including ours, evaluated effects after longer exposure time points.

Besides the *cis*-unsaturated FA described so far, unsaturated FA in our diet can have a *trans* configuration. The main sources of dietary *trans*-MUFA include industrially hydrogenated oils, and dairy products and meat containing ruminant fat. The most abundant MUFA *trans*-FAs found in these groups are elaidic acid (*trans*-9 C18:1) and vaccenic acid (*trans*-11 C18:1), respectively [23]. Of these two tested *trans* isomers of oleic acid, only the incubation of myotubes with elaidic acid resulted in an increased (~3-fold) NF-κB transactivation compared with vehicle-treated myotubes (Figure 2A). This might suggest that the position of the *trans* double bound determines the potential of a specific FA to activate NF-κB.

Well-known isomers of linoleic acid are the geometric and positional isomers *cis*-9, *trans*-11 conjugated linoleic acid (CLA) and *trans*-10, *cis*-12 CLA. We found an increased NF-κB transactivation (~three-fold) after culturing the cells with *cis*-9, *trans*-11 CLA, which was comparable with the magnitude of the effects of C16:0 and C18:0. Since a single double bound at the *cis*-9 (oleic acid) or *trans*-11 (vaccenic acid) position of the C18-backbone did not result in an increased NF-κB transactivation, it is remarkable that the combination of these two double bounds in *cis*-9, *trans*-11 CLA caused an increased NF-κB transactivation. Strikingly, *trans*-10, *cis*-12 CLA provoked a ~13-fold increase in NF-κB transcriptional activity, which is even higher than could maximally be achieved with the pro-inflammatory cytokine TNF-α (10 ng/ml), the prototypical inducer of NF-κB (routinely five to tenfold induction after 4 h incubation, data not shown). In addition, activation of NF-κB after incubation with *trans*-10, *cis*-12 CLA was confirmed by performing an EMSA for the assessment of RelA DNA binding in L6 myotubes (Figure 2B).

The fact that elaidic acid and *cis*-9, *trans*-11 CLA did not reveal increased DNA binding might be due to transient DNA binding, as bi-phasic NF-κB activation in skeletal muscle has been reported, and the use of a reporter construct is less sensitive to transient changes in NF-κB transactivation [24]. Another explanation could be that the demonstrated increase in NF-κB transactivation is based on a mechanism independent from increased DNA binding, for example protein kinase A induced phosphorylation of NF-κB [25].

In spite of the considerable differences in NF-κB activity between the tested C18 *cis* and *trans*-FAs, and in particular between *cis*-C18:2 and C18:2 *trans*-10, *cis*-12 CLA, differential effects were not observed on insulin-stimulated glucose uptake (Figure 2C) or GLUT4 translocation (Figure 2D), which were not affected by any of these isomers.

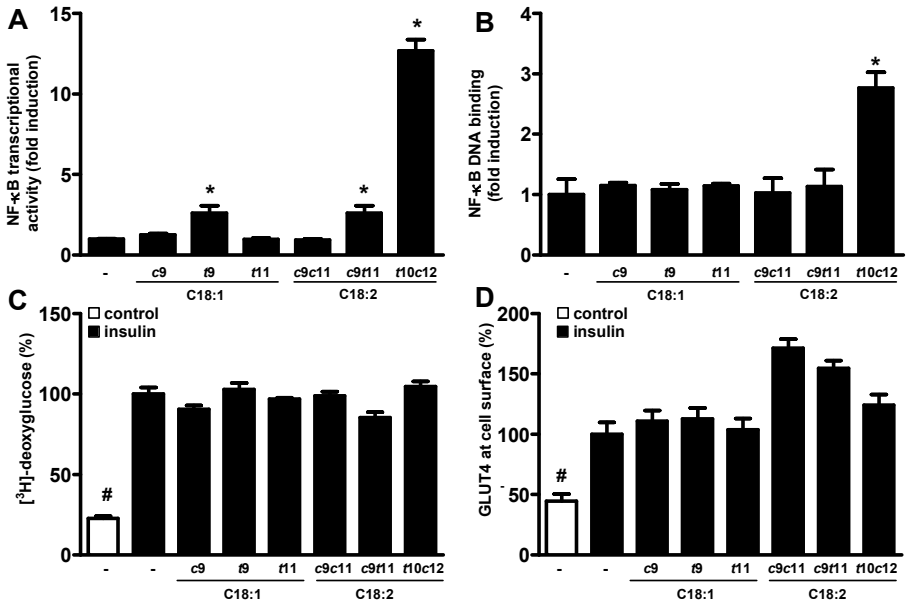

Figure 2: NF-κB transcriptional activity and insulin sensitivity in skeletal muscle cells after incubation with geometric and positional C18:1 or C18:2 isomers. C2C12 myoblasts stably transfected with the 6κB-TK-luciferase construct were differentiated for 5 days and incubated with 400 μmol/l of the indicated FAs for 24 h (A). Lysates were prepared for assessment of luciferase activity. Values were corrected for protein content and expressed as fold-induction over control. Alternatively, 5 days differentiated L6-GLUT4*myc* myotubes were incubated with and without the indicated fatty acids (200 μmol/l, 16 h). Nuclear extracts were prepared and assessed for RelA DNA binding activity to a consensus NF-κB oligonucleotide by EMSA (B). Relative DNA binding activity was determined by phosphor-imager analysis and expressed as fold induction. Furthermore, 2-deoxyglucose uptake (C) and GLUT4 translocation (D) were measured after stimulation with 25 nmol/l insulin for 15 min in 5 days differentiated L6-GLUT4*myc* myotubes. Data are expressed as percentage of insulin-stimulated glucose uptake or GLUT4 translocation in absence of FA. Basal and insulin-stimulated conditions are represented by the open and black bars, respectively. * $p < 0.05$ versus control. # $p < 0.05$ versus insulin-stimulated control. Data shown are representative examples of 3 independent experiments.

Altogether, our results are in agreement with recent *in vitro* data, showing no effects on insulin signaling at the level of p-Akt, after incubation of C2C12 myotubes with elaidic and vaccenic acid [26]. The same authors showed that diets enriched in *trans* MUFAs of dairy or industrial origin did not impair muscle insulin sensitivity in Wistar rats. Nevertheless, opposing effects of CLA on insulin sensitivity have been described in rodents: many studies in the obese Zucker rat model describe positive effects on insulin sensitivity [27-29], while studies in mice showed that CLA, and in particular the *trans*-10, *cis*-12 isomer, induces insulin resistance [30-33]. Little is known on the effects of CLA on insulin sensitivity and inflammation in humans. In healthy young men, a diet rich in CLA (5.5 g/day, 5 weeks) did not

affect inflammatory and diabetic risk markers [34]. In obese men with metabolic syndrome, supplementation with (3.4 g/day) *trans*-10, *cis*-12 CLA or *cis*-9, *trans*-11 CLA for 12 weeks resulted in an increased insulin resistance [35-36] and supplementation with *trans*-10, *cis*-12 CLA also resulted in increased levels of inflammatory biomarkers in serum [37]. Considering the latter observation and the striking induction of NF-κB by *trans*-10, *cis*-12 CLA in the cultured muscle cells in our study, it appears that *trans*-10, *cis*-12 CLA may initiate systematic inflammatory signaling in multiple tissues, and it remains to be determined if this does have pathological consequences. Finally, we also evaluated the effects of the n-3 FAs eicosapentaenoic acid, docosahexaenoic acid and the n-6 FA arachidonic acid, but none of these FAs affected NF-κB activity or insulin sensitivity (data not shown).

Our results show that the apparent association between SFA-induced NF-κB activation and insulin resistance in skeletal muscle cells cannot be generalized to all long chain FAs. Therefore, the proposed causal role of NF-κB activation in the induction of SFA-induced insulin resistance requires further investigation. It has been proposed that intramuscular accumulation of FA metabolites like diacylglycerol (DAG) [2, 4, 38-40] plays a role in inducing insulin resistance. Increased FA oxidation results in a decreased accumulation of FA metabolites like DAG and a protection against palmitate-induced insulin resistance in skeletal muscle cells [41]. Furthermore, it has been shown that the oxidation rate of FAs in muscle increases proportionally with the number of double bonds [42-43]. This may explain why C16:0 and C18:0 did [16], but the C16-MUFA and C18-MUFA/PUFAs did not induce insulin resistance.

In conclusion, the results reported here reveal that FA-induced NF-κB activation is not sufficient for the induction of insulin resistance in skeletal muscle cells.

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CHAPTER 4

Trans-10, *cis*-12 conjugated linoleic acid inhibits skeletal muscle differentiation and GLUT4 expression independently from NF-κB activation

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ABSTRACT

The capacity of skeletal muscle to contribute to glucose homeostasis depends on muscular insulin sensitivity. The expression of glucose transporter (GLUT)-4 is increased during myoblast differentiation, a process essential in maintenance of adult muscle. Therefore, processes that affect muscle differentiation may influence insulin dependent glucose homeostasis. Conjugated linoleic acids, and in particular *trans*-10, *cis*-12 CLA (*t*10, *c*12-CLA), are potent inducers of NF-κB in cultured skeletal myotubes, and NF-κB activation inhibits muscle differentiation. The aims of this study were to evaluate whether CLAs inhibit myogenic differentiation and lower GLUT4 mRNA expression and to address the involvement of NF-κB activation in potential effects of CLA on these processes.

Incubation of C2C12 cells with *t*10, *c*12-CLA blocked the formation of myotubes, which was accompanied by reduced expression of the muscle specific genes creatine kinase, myogenin, myosin heavy chain perinatal and myosin heavy chain IIB, as well as decreased GLUT4 mRNA levels. However, genetic blockade of NF-κB was not sufficient to restore reduced myosin heavy chain protein expression following *t*10, *c*12-CLA treatment. Surprisingly, in contrast to myotubes, *t*10, *c*12-CLA was not able to activate NF-κB transcriptional activity in myoblasts. In conclusion, *t*10, *c*12-CLA inhibits myogenic differentiation and GLUT4 expression, independently from NF-κB activation.

INTRODUCTION

Myogenic differentiation, a process essential in the formation and maintenance of muscle, can be defined as the irreversible transition from the proliferative myoblast stage into fused multinucleated myotubes [1]. In addition to myoblast fusion, myogenic differentiation is characterized by the expression of muscle specific genes, such as muscle creatine kinase (MCK), the structural protein myosin heavy chain (MyHC), and muscle specific transcription factors including myogenin [2]. Likewise, the expression of the insulin-sensitive glucose transporter GLUT4 in skeletal muscle increases during muscle differentiation [3-4].

The contribution of skeletal muscle to glucose homeostasis is not only determined by insulin-mediated GLUT4 translocation, but is also sensitive to differences in GLUT4 expression [5-8]. This indicates that processes affecting muscle differentiation indirectly influence insulin dependent glucose homeostasis.

It has been reported that cytokines are able to inhibit myogenic differentiation via activation of the NF-κB pathway [9-10]. The intracellular signal transduction of inflammatory cues such as pro-inflammatory cytokines depends to a large extent on the transcriptional regulator nuclear factor kappa B (NF-κB). NF-κB is considered a key regulator of inflammatory responses and is normally present in a latent form in the cytoplasm, bound to its inhibitory protein IκBα. After stimulation, an intracellular signaling cascade is initiated, resulting in the activation of the serine kinase inhibitor κB kinase (IKK). This leads to phosphorylation and subsequent degradation of IκBα. NF-κB is subsequently released from its inhibitory protein and translocates to the nucleus, where it regulates the transcription of many genes involved in inflammation, growth regulation and survival, by binding to its cognate DNA sequence in promoters and enhancers of their corresponding genes [11-12].

In addition to inflammatory cytokines, fatty acids (FAs) have also been identified as strong inducers of NF-κB in skeletal muscle [13-16]. Previously, we demonstrated that conjugated linoleic acids (CLAs), and in particular the *trans*-10, *cis*-12 isoform of CLA (*t*10, *c*12-CLA), are potent inducers of NF-κB in cultured myotubes [17]. Interestingly, no acute effects on insulin sensitivity were observed in myotubes exposed to CLA [17]. However, although CLAs have been studied extensively for their proposed health benefits, including anti-carcinogenic, anti-inflammatory, anti-obesity and anti-diabetic effects [18], unfavorable effects of especially *t*10, *c*12-CLA have previously been reported for example on lipid metabolism, glucose metabolism and insulin sensitivity [19]. Therefore we hypothesize that CLAs may indirectly affect glucose homeostasis via reduced muscle differentiation, provoked by NF-κB activation.

The aims of this study were to evaluate the consequences of CLAs on myogenic differentiation and GLUT4 expression, and address the involvement of NF-κB activation in potential effects of CLA on these processes.

MATERIALS AND METHODS

Cell culture

The C2C12 murine skeletal muscle cell line (ATCC CRL1772. Manassas, VA), stably transfected with the 6κB-TK-luciferase, was used for the assessment of NFκB transcriptional activity. In brief, C2C12 cells were plated (1 x 10^4 cells/cm^2) on Matrigel (Becton-Dickinson Labware, Bedford, MA) coated (1:50 in DMEM) dishes, as described previously [20]. C2C12 myoblasts were cultured in growth medium (GM), composed of low glucose Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; both from Gibco-Invitrogen, Rockville, MD) and 9% (vol/vol) fetal bovine serum (FBS; PAA Laboratories). To induce differentiation, GM was replaced by differentiation medium (DM), containing DMEM with 1% (vol/vol) heat-inactivated FBS and antibiotics. As a positive control for NF-κB transcriptional activity, murine TNFα (Calbiochem, San Diego, CA) was added to the dishes.

The L6 rat skeletal muscle cell line, stably transfected with a construct encoding GLUT4 with an exofacial myc epitope (L6-GLUT4myc) was kindly provided by Dr. Amira Klip from the Hospital for Sick Children, Toronto, ON, Canada. L6 myoblasts were cultured in GM, composed of α-MEM (Gibco-Invitrogen, Rockville, MD) containing 9% (vol/vol) FBS and antibiotics. The plating density used for the experiments was 2 x 10^4/cm^2. After 24h of culturing in growth medium, differentiation was induced by replacing GM with DM, containing α-MEM with 2% (vol/vol) heat-inactivated FBS and antibiotics.

Morphology of myotubes was assessed by bright field microscopy after staining of the myotubes with May Grunwald-Giemsa. Cells were washed twice in PBS, fixed in methanol and stained in May-Grunwald Giemsa (Sigma) according to manufactures' instructions. All experiments described for both cell lines were performed in 5- or 6-day differentiated myotubes unless stated otherwise.

Fatty acid incubations

Fatty acid (FA) stock solutions of 40 mmol/l were prepared in ethanol (linoleic acid from Sigma, St.Louis, MO; c9, t11-CLA and t10, c12-CLA from Bio-connect, Netherlands). Before application to the cells, FAs were conjugated to bovine serum albumin (BSA) by diluting the FA solution with differentiation medium containing 1% (wt/vol) FA-free BSA (Sigma, St.Louis, MO), to obtain final FA concentrations of 5-200 μmol/l. Solutions were filter-sterilized before addition to the cells. Vehicle controls contained 0.125% or 0.5% (vol/vol) ethanol and 1% (wt/vol) BSA.

Transfections and plasmids

Stable cell lines were created by transfection with Nanofectin (PAA, UK) according to manufacturers' recommendations.

To inhibit NF-κB activation, L6 myoblasts (3 x 10^3 cells/cm^2) were stably transfected with nanofectin in the presence of a plasmid encoding IκBα-SR (3 μg), which was constitutively expressed under control of the SFFV-LTR (pSFFV-NEO IκBα-SR), kindly provided by Dr. Rosa Ten (Mayo Clinic, Rochester, MN). A vehicle cell line was created with the same strategy, using 3 μg plasmid DNA containing the neomycin resistance gene (pSV2-Neo, Stratagene, La Jolla, CA). For selection of positive clones, cells were cultured in GM containing the presence of 800 μg/ml G-418 (Calbiochem, San Diego, CA).

NF-κB transcriptional activity

To determine NF-κB transcriptional activity, luciferase activity was measured in the NF-κB sensitive reporter cell line as previously described [20]. After the appropriate incubation time with the various stimuli, cells were washed twice with cold phosphate-buffered saline (PBS) and lysed by adding 100 μl 1x Reporter Lysis Buffer (Promega, Madison, WI). After incubation on ice for 10 min, the cell lysates were centrifuged (13,000 g, 2 min) and stored at -80°C for later analysis. Luciferase activity was measured according to the manufacturers' instructions (Promega) and corrected for total protein content (Bio-Rad, Hercules, CA).

RNA isolation and assessment of mRNA abundance by qPCR

Total RNA from C2C12 cells was isolated using the acid guanidium thiocyanate-phenol-chloroform-isoamylalcohol extraction method (Totally RNA kit, Ambion, Austin, USA). After isolation, RNA was dissolved in RNA storage solution (1 mmol/l Na-citrate, pH 6.4) (Ambion) and stored at -80°C. RNA concentrations were determined using a spectrophotometer (Nanodrop, ND-1000). 400 ng of RNA was reverse transcribed into cDNA according to manufacturer's instructions (Transcriptor first strand cDNA synthesis kit, Roche Diagnostics GmbH, Mannheim, Germany) with anchored oligo-dT primers. The genome databases of NCBI or Ensembl were used as a source for transcript sequences.

qPCR primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and obtained from Sigma Genosys (Haverhill, UK).

Standard curves were made by preparing 5 serial fivefold dilutions of pooled cDNA aliquots. cDNA samples were diluted 1/25. From the cDNA dilutions 5 μl was loaded in a 96-well PCR plate (Thermo Fast plates, semi skirted, transparent, Abgene). Standard dilutions were loaded in duplicate. 96 well plates were covered

with Microseals B adhesive seals (Biorad). Real-time PCR reactions were performed in a MyiQ single-color Real-Time thermal cycler (Bio-Rad Herculus, CA).

qPCR reactions (20 µl total volume) contained absolute qPCR SyBr Green Fluorescein Mix (Abgene), 300 nmol/l primers (Table 1) and cDNA dilution. The PCR reaction was performed by a two-step PCR using the following cycling conditions: 15"95ºC, 40 cycles of (15''95ºC, 45''60ºC), 30''95ºC, 30''60ºC followed by a melting curve (heating from 60ºC to 95 ºC). Ct values were obtained for the standard curve and each sample and the relative DNA starting quantities of the samples were derived from the standard curve by using the MyiQ analysis software (Biorad). The expression of the genes of interest were normalized to three reference genes (β-actin, GAPDH and cyclophilin A) using geNorm software [21].

Table 1: Primer details.

Gene	ID*	QPCR primer sequences Forward 5'-3'	Reverse 5'-3'
MCK	NM_007710	AGGTTTTCCGCCGCTTCT	CGGTGCCCAGGTTGGA
Myogenin	ENSMUST00000027730	CCCATGGTGCCCAGTGAA	GCAGATTGTGGGCGTCTGTA
MyHC peri	M12289	GAGTCCCAGGTCAACAAGC	AACCCAGAGAGGCAAGTGAC
MyHC IIB	(Sartorius *et al.*, 1998)	ACAAGCTGCGGGTGAAGAGC	CAGGACAGTGACAAAGAACG
GLUT4	(Jove *et al.*, 2005)	GATGCCGTCGGGTTTCCAGCA	TGAGGGTGCCTTGTGGGATGG
β-Actin	NM_007393	CTGAATGGCCCAGGTCTGA	CCCTCCCAGGGAGACCAA
Cyclophilin A	BC099478	TTCCTCCTTTCACAGAATTATTCCA	CCGCCAGTGCCATTATGG
GAPDH	BC096590	CAACTCACTCAAGATTGTCAGCAA	TGGCAGTGATGGCATGGA

MCK: muscle creatine kinase; MyHC: myosin heavy chain; glucose transporter 4: GLUT4; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; HPRT: hypoxanthine phosphoribosyltransferase. * ID from http://www.ncbi.nlm.nih.gov/entrez or http://www.ensembl.org

Western blotting

The abundance of the fast twitch isotype of myosin heavy chain (MyHCf) and GAPDH was evaluated by Western blotting. Cells were washed in PBS, and whole cell lysates were prepared by adding lysis buffer (20 mmol/l Tris, 150 mmol/l NaCl, 1% (vol/vol) Nonidet P-40, 1 mmol/l dithiothreitol, 1 mmol/l Na_3VO_4, 1 mmol/l phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 1% (vol/vol) aprotinin). Lysates were incubated on ice for 30 min, followed by 30 min centrifugation at 14,000 x *g*. A fraction of the supernatant was saved for protein determination, and 4x Laemmli sample buffer (0.25 mol/l Tris-HCl, pH 6.8, 8% (wt/vol) SDS, 40% (vol/vol) glycerol, 0.4 mol/l dithiothreitol, and 0.04% (wt/vol) bromophenol blue) was added, followed by boiling of the samples for 5 min and storage at -20 °C. Total

protein was assessed by the Bio-Rad DC protein assay kit (Bio-Rad) according to the manufacturer's instructions, and 15 μg of protein was loaded per lane and separated using 4-12% Bis-TRIS Criterion XT precast gels (Bio-Rad), followed by electroblot transfer to a 0.45-μm nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked for 1 h at room temperature in 5% (wt/vol) nonfat, dried milk. Nitrocellulose blots were washed in PBS-Tween 20 (0.05%), followed by overnight incubation (4 °C) with a monoclonal antibody specific for MyHCf (MY-32, Sigma; 1:2000) or GAPDH (Cell Signaling; 1:5000). After three wash steps of 20 min each, the blots were probed with a peroxidase-conjugated secondary antibody, 1:5000 (Vector Laboratories, Burlingame, CA), and visualized by Supersignal® WestPico chemiluminescent substrate (Pierce) according to the manufacturer's instructions.

Electrophoretic mobility shift analysis

To determine DNA binding activity of NF-κB, complexes binding to an oligonucleotide containing a NF-κB consensus sequence were analyzed by EMSA. Nuclear extracts were isolated to analyze NF-κB DNA binding. To this end, cells were harvested following experimental treatments and lysed on ice in 400 μl buffer containing 20 mmol/l HEPES, pH 7.8, 20 mmol/l KCl, 4 mmol/l $MgCl_2$, 0.2 mmol/l EDTA, 1 mmol/l DTT, 0.2 mmol/l sodiumorthovanadate, 0.4 mmol/l phenylmethyl sulfonylfluoride, 0.3 μg/ml leupeptin and 0.2 mmol/l NaF for 15 min. Subsequently, 25 μL 10% Nonidet P40 was added and samples were vortexed for 15 seconds followed by centrifugation (14,000 rpm for 30 seconds). Supernatants were removed and pelleted nuclei were washed with the previously mentioned buffers and resuspended with a buffer containing 100 mmol/l HEPES, pH 7.8, 100 mmol/l KCl, 600 mmol/l NaCl, 0.2 mmol/l EDTA, 20% glycerol, 1 mmol/l DTT, 0.2 mmol/l sodiumorthovanadate, 0.667 mmol/l phenylmethyl sulfonylfluoride and 0.2 mmol/l NaF. Nuclei were mixed vigorously for 20 minutes at 4 °C using a rotating platform, centrifuged (14,000 rpm for 5 minutes) and samples were stored at -20°C (for protein concentration determination) and -80°C (for DNA binding activity measurements). 7 μg nuclear cell extracts were used per binding reaction, and protein-DNA complexes were resolved on a 5% polyacrylamide gel in 0.25x Tris borate-EDTA buffer at 160 V for 2 h. Gels were dried and exposed to film (X-Omat Blue XB-1, Kodak, Rochester, NY). Shifted complexes were detected by a phosphoimager (Bio-Rad).

Statistical analysis

SPSS (version 16.0) was used for statistical analysis. Data were analyzed by one-way ANOVA, and the various treatment groups were compared by using the post-hoc

Bonferroni test in which a P<0.05 was considered statistically significant. Data are presented as means ± SD.

RESULTS

*t*10, *c*12-CLA induces NF-κB transcriptional activation in a dose- and time-dependent manner

The effect of *c*9, *t*11-CLA and *t*10, *c*12-CLA versus linoleic acid on NF-κB transcriptional activity was investigated in C2C12 myotubes, stably transfected with a NF-κB sensitive reporter construct. As shown in Figure 1 A, incubation with linoleic acid did not result in increased NF-κB transcriptional activity, while *c*9, *t*11-CLA incubation resulted in a maximum 2.6-fold increase at 200 μmol/l. Incubation with only 5 μmol/l *t*10, *c*12-CLA already resulted in an increase in NF-κB transcriptional activity, and there was a peak of ~65- fold increase after incubation with 50 and 100 μmol/l *t*10, *c*12-CLA. NF-κB transcriptional activation by *t*10, *c*12-CLA (50 μmol/l) was induced in a time-dependent manner (Figure 1 B). Remarkably NF-κB activation by 10-12 CLA required 12h of incubation, whereas NF-κB activation by TNF-α, which was used as a positive control (data not shown), was clearly detectable after 2-4h. These data demonstrated that the effects of CLA on NF-κB transcriptional activity are isomer specific with *t*10, *c*12-CLA being the most potent inducer.

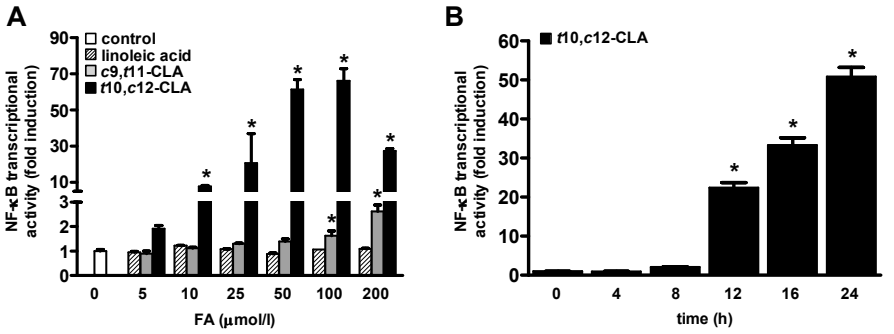

Figure 1: *t*10, *c*12-CLA induces NF-κB transcriptional activation in a dose- and time-dependent manner. C2C12 cells from the NF-κB sensitive reporter cell line were differentiated for 6 days before treatment with linoleic acid, *c*9, *t*11-CLA and *t*10, *c*12-CLA (0-200 μmol/l) for 24h (A) or treatment with 50 μmol/l of *t*10, *c*12-CLA for 0-24h (B). Lysates were prepared for assessment of luciferase activity. Values were corrected for protein content and expressed as fold-induction over control. Statistically significant differences between the indicated treatment and corresponding BSA-treated control (* $p < 0.05$) were determined by one-way ANOVA.

Inhibition of myotube formation by *t*10, *c*12-CLA

Since it is known that activation of NF-κB inhibits myogenic differentiation [9], we evaluated the effects of two CLAs and linoleic acid on myotube formation and muscle specific gene expression during the myogenic differentiation process.

Addition of 50 μmol/l *t*10, *c*12-CLA completely inhibited the formation of myotubes (Figure 2, bottom left). *c*9, *t*11-CLA inhibited myotube formation less potently (Figure 2, top right), while linoleic acid incubation, although not quantively determined, appeared to increase myotube size (Figure 2, bottom right).

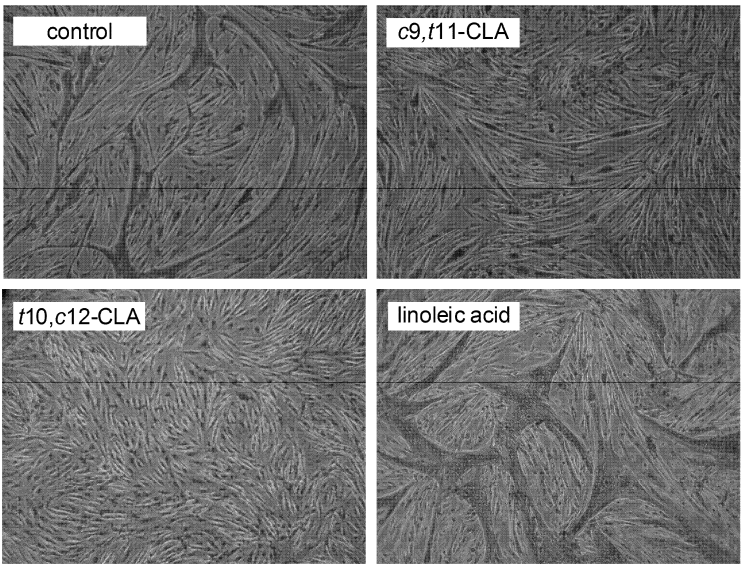

Figure 2: Inhibition of myotube formation by *t*10, *c*12-CLA. C2C12 cells were allowed to differentiate for 5 days in the presence or absence of 50 μmol/l linoleic acid, *c*9, *t*11-CLA or *t*10, *c*12-CLA. Morphology was assessed by bright field microscopy after staining with May-Grunwald Giemsa.

_t_10, _c_12-CLA reduces muscle specific gene- and GLUT4 expression during myogenic differentiation

We next evaluated if the fatty acid-induced changes in morphological differentiation were reflected by changes in the expression of muscle specific genes after induction of differentiation. The expression of muscle creatine kinase (MCK), an enzyme expressed in mature muscle, and myogenin, a muscle-specific transcription factor, was reduced by 64% and 59% respectively, after incubation of *t*10, *c*12-CLA during 3 days after the onset of differentiation (Figure 3 A). The mRNA of the myofibrillar

proteins perinatal myosin heavy chain (MyHC-peri) and myosin heavy chain IIB (MyHC-IIB) genes, which are expressed during later phases of myogenic differentiation, was reduced by 62% and 85%,respectively, after incubation with *t*10, *c*12-CLA in 6 days differentiating myocytes (Figure 3 B). A slight decrease (28%) in MyHC-peri was visible after incubation with *c*9, *t*11-CLA. Remarkably, linoleic acid incubation led to a 70% increase in MCK expression and a doubling of MyHC-IIB expression.

Furthermore, we examined whether the effects of *t*10, *c*12-CLA on myogenic differentiation also affected GLUT4 mRNA levels. As shown in Figure 3 C, GLUT4 mRNA levels were reduced (-50%) after *t*10, *c*12-CLA incubation and induced (+94%) by linoleic acid incubation. No differences on GLUT4 expression were caused by *c*9, *t*11-CLA incubation.

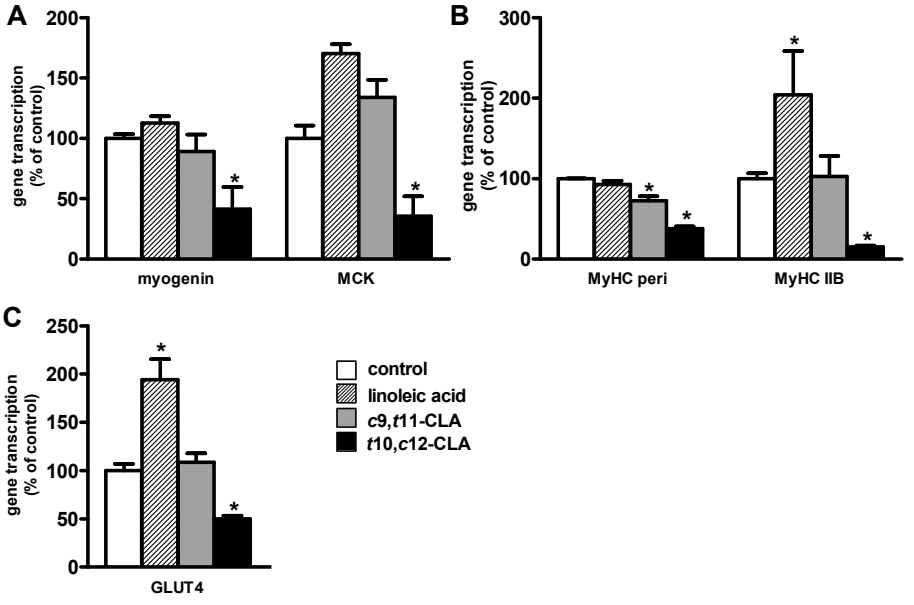

Figure 3: *t*10, *c*12-CLA reduces muscle specific gene- and GLUT4 expression during myogenic differentiation. C2C12 cells were allowed to differentiate for 3 (A) or 6 (B,C) days in the presence of 50 µmol/l of the corresponding fatty acids. Expression levels of myogenin and MCK (A), MyHCperi and MyHC IIB (B), and GLUT4 (C) are depicted as % of control transcript levels. Statistically significant differences between the indicated treatment and corresponding BSA-treated control (* $p < 0.05$) were determined by one-way ANOVA.

Inhibition of NF-κB does not prevent the *t*10, *c*12-CLA-induced reduction of differentiation

To test potential causality of NF-κB activation in the reduction of muscle differentiation by *t*10, *c*12-CLA, we measured protein abundance of fast twitch myosin heavy chain (MyHCf) in L6 cells, stably transfected with the IκBα-super repressor (SR), a non-degradable mutant of IκBα. As expected, induction of maximal NF-κB DNA binding observed in control myotubes was markedly decreased in L6 cells expressing the IκBα-SR (supplementary data).

However, treatment with *t*10, *c*12-CLA during the differentiation process still resulted in a decrease in MyHCf protein expression in the IκBα-SR cell line as well as the vehicle (Figure 4). Thus, genetic blockade of NF-κB was not sufficient for the restoration of the *t*10, *c*12-CLA -induced disturbed differentiation process.

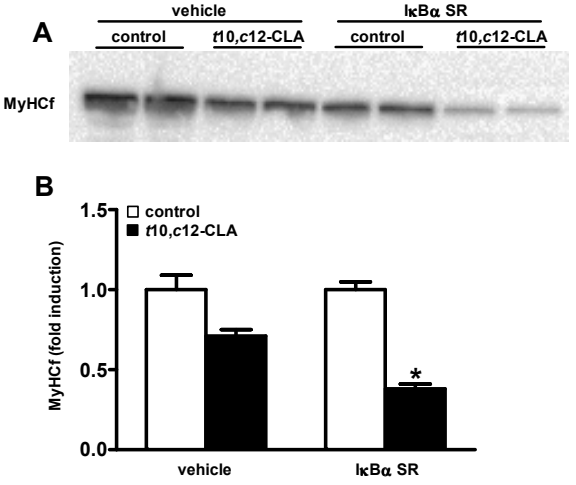

Figure 4: Inhibition of NF-κB does not prevent the *t*10, *c*12-CLA-induced reduction of differentiation. L6 cells were stably transfected with a plasmid encoding a stabilized mutant of the Inhibitor of NF-κB (IκBα-SR) or a vehicle vector (PcDNA 3.1; vehicle). Cells were cultured with differentiation medium for 5 days in the presence of 50 μmol/l *t*10, *c*12-CLA. MyHCf expression was assessed in 15 μg of protein lysate by Western blot analysis. A representative Western blot is shown (A) and values are expression as fold-induction over control (B). Statistically significant differences between the indicated treatment and corresponding BSA-treated control (* $p < 0.05$) were determined by one-way ANOVA.

Differential NF-κB activation by *t*10, *c*12-CLA in myoblasts and myotubes

We next investigated NF-κB transcriptional activity in C2C12 cells that were continuously exposed to *t*10, *c*12-CLA, e.g. for 6 days from the onset of induction of differentiation. No cell death was observed after the different treatments with *t*10, *c*12-CLA. Surprisingly, no sustained NF-κB transcriptional activity was observed

following *t*10, *c*12-CLA treatment (Figure 5A). Moreover, in myoblasts, 24h incubation of *t*10, *c*12-CLA did not induce NF-κB transactivation (Figure 5B). In contrast, in 3-day differentiated myotubes, NF-κB activation was apparent following 24h incubation with *t*10, *c*12-CLA. Maximal transactivation of the NF-κB-sensitive promoter construct was observed in myotubes that were first allowed to differentiate for 6 days prior to the addition of *t*10, *c*12-CLA for 24h (Figure 5B). TNF-α stimulation resulted in a 7-fold increase in NF-κB transcriptional activity in both myoblasts and myotubes (Figure 5B), confirming that myoblasts were capable of activating the NF-κB pathway.

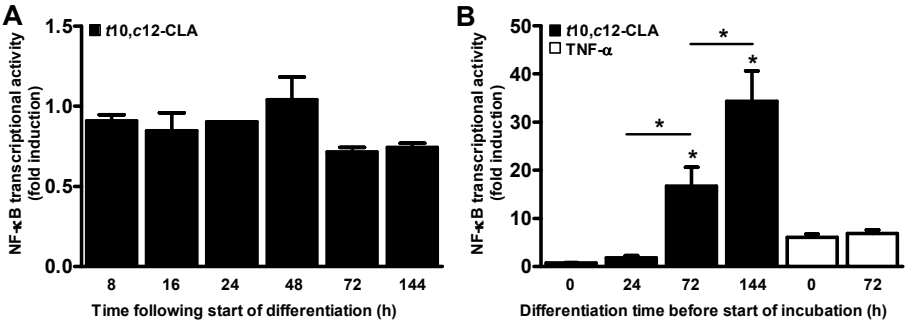

Figure 5: Differential NF-κB activition in myoblasts and myotubes. A: C2C12 cells were cultured with differentiation medium during 6 days in the presence of 50 μmol/l *t*10, *c*12-CLA or vehicle. NF-κB activity was assessed at several time-points and depicted as fold-induction over the corresponding BSA-treated control. B: C2C12 cells were differentiated for 0, 1, 3 and 6 days before treatment with 50 μmol/l *t*10, *c*12-CLA for 24h, or TNF-α (10ng/ml, 4h). Statistically significant differences between the indicated treatment and corresponding BSA-treated control and between treatments (* $p < 0.05$) were determined by one-way ANOVA.

DISCUSSION

The current study reveals that activation of NF-κB transcriptional activity in skeletal muscle and inhibition of myogenic differentiation by *t*10, *c*12-CLA are not causally related. This is in contrast to what has been reported for blockade of myogenesis by the inflammatory cytokines TNF-α and interleukin-1β [9-10]. Our findings that myotube formation and expression of muscle specific genes were blocked upon treatment with *t*10, *c*12-CLA in differentiation medium are in line with other studies using human primary muscle cells and L6 cells [22-23]. Conversely, increased formation of multinucleated myotubes upon treatment with linoleic acid, corresponds with studies that have shown stimulatory effects of linoleic acid on the formation of myotubes from primary rat skeletal muscle satellite cells [23-25].

It is remarkable that 50 μmol/l *t*10, *c*12-CLA evoked a >60-fold increase in NF-κB transcriptional activity, which, in comparison, is a much more potent activator of NF-κB in myotubes than the prototypical inducer TNF-α. Furthermore, CLA-isomeric effects were apparent, since there was a noticeable difference in the ability of both CLA isomers in activating NF-κB and inhibiting skeletal muscle differentiation. The same concentration of *c*9, *t*11-CLA had no effect on NF-κB transcriptional activity and muscle differentiation. In a previous report [26] we showed that the isomeric effects on NF-κB transactivation also applied to NF-κB DNA binding.

Surprisingly, in contrast to myotubes, *t*10, *c*12-CLA was not able to activate NF-κB transcriptional activity in myoblasts, and the magnitude of activation appeared to depend on the maturity of the differentiation program. This is remarkable since NF-κB transcriptional activation by TNFα or any other NF-κB stimuli to our knowledge, is not dependent on the differentiation status of skeletal muscle cells, as identical NF-κB DNA binding and transcriptional activity were observed in response to TNF-α (this study) and interleukin-1β [9] in undifferentiated myoblasts and 5-day differentiated myotubes. Apparently, the signaling constituents required for *t*10, *c*12-CLA -mediated NF-κB activation are not in place in undifferentiated myoblasts. It is known that myoblasts and myotubes display highly differential protein expression patterns [4, 27-28], but the responsible factor(s) for the observed differences in NF-κB inducibility by *t*10, *c*12-CLA remain elusive. Signaling responses triggered by FAs may result from receptor activation as well as intracellular accumulation of metabolites. For example, palmitate has been shown to induce accumulation of lipid metabolites like ceramides and diacylglycerol, which have been implicated in activation of NF-κB [29]. Furthermore, palmitate can activate toll-like receptor (TLR) signaling in skeletal muscle cells, which can induce NF-κB activity [30], but whether TLRs are required for *t*10, *c*12-CLA-mediated NF-κB activation is currently unknown. In fact, undifferentiated myoblasts were previously reported to express functional TLRs [31], suggesting that NF-κB activation by *t*10, *c*12-CLA in skeletal muscle cells likely occurred independent of TLR activation, or at least indicating that TLR expression is not sufficient for NF-κB activation in myoblasts by *t*10, *c*12-CLA. In addition, TLR-mediated transcriptional activation of NF-κB by LPS [32], as well as TNF-induced NF-κB transactivation [9] in skeletal muscle were reported to occur within 2-4h. In contrast to this receptor-mediated signaling, the 8-12h required for transcriptional activation of NF-κB by 10-12 CLA was more in line with the time frame of palmitate-induced NF-κB transactivation [13]. This suggests NF-κB activation by FA may not be receptor mediated but rather depend on intracellular lipid intermediates.

Besides inhibiting myogenic differentiation, CLAs are also able to inhibit adipocyte differentiation [33-34]. Interestingly, analogues to our findings in muscle cells, isomer-specificity of CLA was also observed in adipocytes, since only *t*10, *c*12-

CLA and not *c*9, *t*11-CLA inhibited differentiation and reduced GLUT4 mRNA expression and insulin-induced glucose uptake in differentiating human pre-adipocytes [35]. The authors speculated on the involvement of peroxisome proliferator-activated receptor (PPAR)-γ, since these isomer-specific effects were paralleled by a reduced expression of PPAR-γ and several of its downstream target genes. Furthermore, *t*10, *c*12-CLA was able to antagonize ligand-dependent PPAR-γ activity [36] and it has been shown that PPAR-γ is required for the differentiation of adipose tissue [37]. Interestingly, decreased PPAR-γ expression in C2C12 cells was also reported to inhibit myogenic differentiation [38]. Nevertheless, whether decreased PPAR-γ expression is responsible for *t*10, *c*12-CLA-induced inhibition of myogenic differentiation, remains to be investigated.

Opposing effects of CLA on insulin sensitivity have been described in rodents and humans [39-50]. Many of these studies are performed with mixtures of the two major CLA isomers. In rodent [51-52] and human [53] studies the *t*10, *c*12-CLA isomer was associated with decreased body fat. However, most studies that tested isomer specific properties of CLA, showed that *t*10, *c*12-CLA induced insulin resistance and hepatic steatosis in mice [43, 54-56]. Also in obese men, treatment with *t*10, *c*12-CLA caused isomer-specific insulin resistance [47]. In human adipocytes, *t*10, *c*12-CLA increased NF-κB activation and reduced GLUT4 expression and insulin-stimulated glucose uptake [57-58], which is in line with our findings on GLUT4 mRNA expression and NF-κB transactivation in skeletal muscle cells. Despite the association between *t*10, *c*12-CLA inflammation and insulin resistance that emerges from these studies, in previous work we failed to detect acute effects of CLAs on insulin-induced glucose uptake in skeletal muscle myotubes, despite a potent induction of NF-κB [13]. Our data presented in this work suggest that *t*10, *c*12-CLA may affect glucose homeostasis indirectly by inhibiting muscle differentiation and myogenesis-associated GLUT4 expression.

Overall, these data reveal *t*10, *c*12-CLA as a very potent activator of the NF-κB pathway and inhibitor of myogenic differentiation, but demonstrate that these effects of *t*10, *c*12-CLA are not causally related, as genetic blockade of NF-κB signaling failed to restore myogenic differentiation and *t*10, *c*12-CLA did not induce NF-κB activation in undifferentiated myoblasts. The results of numerous studies that described anti-inflammatory [59] effects of mixtures of CLA suggest a potential for CLA as a novel dietary supplement for patients suffering from muscle wasting in chronic inflammatory related diseases. However, the results of this study regarding the isomer-specific inhibition of muscle differentiation and induction of muscle NF-κB signaling by *t*10, *c*12-CLA imply that this CLA isomer may even be detrimental in inflammatory muscle wasting conditions.

In conclusion, we found that the inhibitory effects of *t*10, *c*12-CLA on myogenic differentiation did not require NF-κB activation and coincided with a

marked reduction in GLUT4 expression, suggesting that long-term administration of *t*10, *c*12-CLA may reduce insulin sensitivity in skeletal muscle.

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CHAPTER 5

Palmitate-induced skeletal muscle insulin resistance does not require NF-κB activation

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ABSTRACT

Palmitate activates the NF-κB pathway, and induces accumulation of lipid metabolites and insulin resistance in skeletal muscle cells. Little information is available whether and how these processes are causally related. Therefore, the objectives were to investigate whether intra-cellular lipid metabolites are involved in FA-induced NF-κB activation and/or insulin resistance in skeletal muscle and to investigate whether FA-induced insulin resistance and NF-κB activation are causally related. Inhibiting DGAT or CPT-1 by using respectively amidepsine or etomoxir increased DAG accumulation and sensitized myotubes to palmitate-induced insulin resistance. While co-incubation of palmitate with etomoxir increased NF-κB transactivation, co-incubation with amidepsine did not, indicating that DAG accumulation is associated with insulin resistance but not with NF-κB activation. Furthermore, pharmacological or genetic inhibition of the NF-κB pathway could not prevent palmitate-induced insulin resistance.

In conclusion, we have demonstrated that activation of the NF-κB pathway is not required for palmitate-induced insulin resistance in skeletal muscle cells.

INTRODUCTION

Insulin resistance is recognized as an important risk factor in the development of type 2 diabetes of which the prevalence is rapidly increasing [1]. Although it is generally accepted that this may relate to a Western-type diet high in saturated fatty acids [2], the detailed pathogenesis underlying the initiation of insulin resistance is still poorly understood. Since skeletal muscle is responsible for 70-80% of insulin-stimulated glucose uptake, skeletal muscle insulin resistance is most likely a major determinant of type 2 diabetes [3].

Type 2 diabetic patients are characterized by an elevation in plasma free fatty acids (FFA) [4]. These FFA play a major role in the pathogenesis of insulin resistance, since lowering FFA-levels in the plasma significantly improves insulin sensitivity [5-6], whereas lipid infusion leading to increased plasma FFA induces skeletal muscle insulin resistance in rodents and humans [7-9]. It is thought that circulating FFA interfere with insulin signaling as a consequence of cellular FA oversupply or impaired mitochondrial FA oxidation, leading to intra-muscular accumulation of FA metabolites. Increased levels of the lipid metabolite diacylglycerol (DAG) in skeletal muscle indeed accompanies diet-induced insulin resistance in rodents [10] and lipid infusion-induced insulin resistance in humans [8]. Whether DAG is the crucial trigger and via which cellular processes DAG accumulation leads to insulin resistance is still unclear. However, since DAG accumulation during lipid infusion coincides with enuclear factor kappa B (NF-κB) activation in insulin resistant muscle, NF-κB has been implicated in the mechanism underlying FA-induced DAG-mediated muscular insulin resistance [8]. Although several *in vitro* studies in skeletal muscle provide evidence that long-chain saturated FA-induced insulin resistance coincides with DAG accumulation [11] and activation of the NF-κB pathway [12-14], little information is available if and how these processes are causally related. Therefore, the first objective of this study was to investigate whether DAG accumulation is involved in NF-κB activation and/or FA-induced insulin resistance in skeletal muscle by interfering with β-oxidation and DAG to TAG conversion.

Several studies in rodents and humans showed that insulin resistance induced by hyperlipidemia (lipid infusion) or an obesity-inducing high fat diet was associated with decreased IκBα protein levels in skeletal muscle, which is suggestive for NF-κB activation [8, 15-16]. Furthermore, whole body inhibition of NF-κB activity by pharmacological means or by heterozygous deletion of the NF-κB activating kinase IKK2, prevented lipid infusion-, obesity- and diet-induced insulin resistance [17-18], illustrating an important role for NF-κB in whole body glucose homeostasis.

However, the specific contribution of NF-κB activation in skeletal muscle was not assessed in these studies. Mice with a muscle-specific inhibition of NF-κB by transgenic expression of the IκBα super-repressor (MISR mice) were not protected

against the development of diet-induced whole body insulin resistance [19] and muscle-specific IKK-2 deficient mice showed no differences in whole body glucose homeostasis after gold thioglucose-induced obesity [20]. Since insulin resistance of other tissues could potentially mask a protective effect of NF-κB inhibition on muscle glucose homeostasis, in this respect studies using cultured muscle cells are of great advantage. In L6 myotubes it has been shown that palmitate-induced insulin resistance could be prevented by pharmacological inhibitors of IKK and NF-κB nuclear translocation in L6 myotubes [21-22]. In contrast, non-saturated FA-induced NF-κB activation in absence of insulin resistance has been described in skeletal muscle [23]. Moreover, several studies showed that stimulation of muscle cells with TNF-α, a well-known and potent NF-κB activator, does not induce insulin resistance [24-26], while others showed that muscle-specific transgenic expression of activated IKK in mice (MIKK mice) [19] and over-expression of IKK-2 and p65 via *in vivo* electrotransfer in rat muscles does not induce insulin resistance [27]. From the latter studies it appears that muscular NF-κB activation per se is not sufficient to induce insulin resistance.

Considering that these conflicting results regarding the requirement of muscular NF-κB activation in FA-induced insulin resistance may be attributable to differences in strategies to modulate NF-κB activity, the second objective of this study was to address the contribution of NF-κB activation to palmitate-induced insulin resistance in cultured skeletal muscle using both pharmacological and genetic approaches to inhibit NF-κB activity.

MATERIALS AND METHODS

Cell culture

The C2C12 murine skeletal muscle cell line (ATCC CRL1772. Manassas, VA), stably transfected with the 6κB-TK-luciferase, was used for the assessment of NFκB transcriptional activity. In brief, C2C12 cells were plated (1 x 10^4 cells/cm^2) on growth factor reduced Matrigel (Becton-Dickinson Labware, Bedford, MA) coated (1:50 in DMEM) dishes, as described previously [28]. C2C12 myoblasts were cultured in growth medium (GM), composed of low glucose Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; both from Gibco-Invitrogen, Rockville, MD) and 9% (vol/vol) fetal bovine serum (FBS; PAA Laboratories, UK). To induce differentiation, GM was replaced by differentiation medium (DM), containing DMEM with 1% (vol/vol) heat-inactivated FBS and antibiotics. As a positive control for NF-κB transcriptional activity, murine TNFα (Calbiochem, San Diego, CA) was added to the dishes.

Myoblasts from the L6 rat skeletal muscle cell line were cultured in GM, composed of α-MEM (Gibco-Invitrogen) containing 9% (vol/vol) FBS and antibiotics. The plating density used for the experiments was 2 x $10^4/cm^2$. After 24h of culturing in growth medium, differentiation was induced by replacing GM with DM, containing α-MEM with 2% (vol/vol) heat-inactivated FBS and antibiotics.

Amidepsine A (Alexis, San Diego, CA), etomoxir and parthenolide (both from Sigma, St. Louis, MO) were added 30 min. before palmitate incubations.

All experiments described for both cell lines were performed in 5- or 6-day differentiated myotubes unless stated otherwise.

Palmitate incubations

Palmitate (C16:0; Sigma) stock solutions of 40 mmol/l were prepared in ethanol. Before application to the cells, palmitate was conjugated to bovine serum albumin (BSA) by diluting the palmitate solution with differentiation medium containing 1% (wt/vol) palmitate-free BSA (Sigma). Solutions were filter-sterilized before addition to the cells. Because of different sensitivities of both cell lines towards FA-induced NF-κB activation and insulin resistance [12], maximal palmitate concentrations used are 200 µmol/l for L6 myotubes and 400 µmol/l for C2C12 myotubes.

Vehicle controls contained equal amounts of ethanol (max 1% in C2C12 or 0.5% in L6) (vol/vol) and BSA (1% (wt/vol)).

Diacylglycerol analysis

Freeze-dried L6 myotubes were extracted overnight at 4ºC with chloroform-methanol (2:1 by volume), containing 10 mg/l of butylated hydroxytoluene. After centrifugation the supernatant was evaporated under nitrogen gas. Lipids were reconstituted in chloroform-methanol (1:1), containing 10 mg/l of butylated hydroxytoluene, spotted onto TLC plates and developed in a solvent containing petroleumbenzin-diethyl ether-acetic acid (120:25:1,5 by volume). The DAG fractions were identified against a DAG standard (Sigma) and scraped into a vial. From the DAG extracts, methyl esters were prepared by transmethylation using a mixture of toluene-methanol-(BF_3-methanol 14%) (20%-55%-25% by volume) at 100ºC for 30 min. The fatty acid methyl esters were separated by capillary gas liquid chromatography using a 50 m X 0.25 mm CP-sil 88 silica column (Varian) with helium as carrier gas at a flow of 130 kPa. Fatty acids were identified by comparison with a standard mixture of fatty acid methyl esters.

Alternatively, cells were pre-incubated for 16 hours with [1-^{14}C] palmitate (0.5 µCi/ml; PerkinElmer, Boston, MA) and respective non-labeled (cold) palmitate (100 µmol/l). Following incubation, cells were washed twice with 1X PBS and harvested into 0.25 ml of 0.05% SDS for subsequent protein measurement and total

lipid extraction with 1 ml of chloroform/methanol (2 vol/1 vol). Lipids were washed with 70% ethanol and re-dissolved for thin layer chromatography (TLC) (Anal Tech TLC plates, Whatman Ltd., Kent, ME) and run in a mobile phase containing hexane/diethyl ether/acetic acid, vol/vol/vol, 80:20:1). Bands corresponding diacylglycerol (DAG) were scraped and transferred into scintillation vials, and radioactivity was measured on a multipurpose scintillation counter (LS 6500; Beckman Coulter, Brea, CA). All assays were performed in triplicates, and data were normalized to protein content.

Transfections and plasmids

Stable cell lines were created by transfection with Nanofectin (PAA) according to manufacturers' recommendations.

To inhibit NF-κB activation, L6 myoblasts (3 x 10^3 cells/cm^2) were stably transfected with nanofectin in the presence of a plasmid encoding IκBα-SR (3 μg), which was constitutively expressed under control of the SFFV-LTR (pSFFV-NEO IκBα-SR), kindly provided by Dr. Rosa Ten (Mayo Clinic, Rochester, MN). A control cell line was created using the same strategy, with plasmid DNA containing the neomycin resistance gene (pSV2-Neo, Stratagene, La Jolla, CA). For selection of positive clones, cells were cultured in GM containing the presence of 800 μg/ml G-418 (Calbiochem).

NF-κB transcriptional activity

To determine NF-κB transcriptional activity, luciferase activity was measured in a NF-κB sensitive reporter cell line as previously described [28]. After the appropriate incubation time with the various stimuli, cells were washed twice with cold phosphate-buffered salin (PBS) and lysed by adding 100 μl 1x Reporter Lysis Buffer (Promega, Madison, WI). After incubation on ice for 10 min, cell lysates were centrifuged (13,000 g, 2 min) and stored at -80°C until analysis. Luciferase activity was measured according to the manufacturers' instructions (Promega) and corrected for total protein content (Bio-Rad, Hercules, CA).

Electrophoretic mobility shift analysis

To determine DNA binding activity of NF-κB, complexes binding to an oligonucleotide containing a NF-κB consensus sequence were analyzed by EMSA. Nuclear extracts were isolated to analyze NF-κB DNA binding. To this end, cells were harvested following experimental treatments and lysed on ice in 400 μl buffer containing 20 mmol/l HEPES, pH 7.8, 20 mmol/l KCl, 4 mmol/l $MgCl_2$, 0.2 mmol/l EDTA, 1 mmol/l DTT, 0.2 mmol/l sodiumorthovanadate, 0.4 mmol/l phenylmethyl

sulfonylfluoride, 0.3 μg/ml leupeptin and 0.2 mmol/l NaF for 15 min. Subsequently, 25 μL 10% Nonidet P40 was added and samples were vortexed for 15 seconds followed by centrifugation (14,000 rpm for 30 seconds). Supernatants were removed and pelleted nuclei were washed with the previously mentioned buffers and resuspended with a buffer containing 100 mmol/l HEPES, pH 7.8, 100 mmol/l KCl, 600 mmol/l NaCl, 0.2 mmol/l EDTA, 20% glycerol, 1 mmol/l DTT, 0.2 mmol/l sodiumorthovanadate, 0.667 mmol/l phenylmethyl sulfonylfluoride and 0.2 mmol/l NaF. Nuclei were mixed vigorously for 20 minutes at 4 °C using a rotating platform, centrifuged (14,000 rpm for 5 minutes) and samples were stored at -20°C (for protein concentration determination) and -80°C (for DNA binding activity measurements). 7 μg nuclear cell extracts were used per binding reaction, and protein-DNA complexes were resolved on a 5% polyacrylamide gel in 0.25x Tris borate-EDTA buffer at 160 V for 2 h. Gels were dried and exposed to film (X-Omat Blue XB-1, Kodak, Rochester, NY). Shifted complexes were quantified by phosphoimager analysis (Bio-Rad). To determine the presence of RelA (p65) by supershift analysis, nuclear extracts were pre-incubated with an antibody specific to the RelA subunit of NF-κB (Santa Cruz Biotecchnology).

[^{3}H]deoxyglucose uptake

Basal and insulin-dependent deoxyglucose uptake was determined as described previously [12]. In brief, L6 myotubes with or without FA preincubation were serum deprived for 3 h prior to incubation with 25 nmol/l insulin (Sigma) for 15 min at 37°C in glucose-free medium. Label (0.2 μCi/ml [^{3}H]deoxyglucose) was added (final concentration of 20 μmol/l) and incubated for 20 minutes at 37°C. Glucose transport was terminated by washing three times with an ice-cold stop-solution containing 0.2 mmol/l phloretin and cells were harvested in 0.5 ml 0.05 mol/l NaOH. Cell-associated radioactivity was determined by scintillation counting.

Statistical analysis

SPSS (version 16.0) was used for statistical analysis. Data were analyzed by one-way ANOVA, and the various treatment groups were compared by using the post-hoc Bonferroni test in which a $P<0.05$ was considered statistically significant. Data is presented as means ± SD. Data shown are representative examples of 3 independent experiments.

RESULTS

Palmitate induces insulin resistance, DAG accumulation and NF-κB activation in myotubes

Verifying previous findings considering palmitate-induced insulin resistance, L6 myotubes were treated for 16 hours with 200 μmol/l palmitate. As expected, incubation with palmitate suppressed insulin-stimulated deoxyglucose uptake compared with untreated cells (Figure 1A). This palmitate-induced insulin resistance coincided with a 5-fold increase in diacylglycerol (DAG) accumulation (Figure 1B). To determine the effects of palmitate incubation on NF-κB activation, NF-κB DNA binding was investigated in L6 myotubes. Palmitate treatment resulted in a ~3.2-fold increase in DNA binding (Figure 1C). To test whether the increased DNA binding led to an increased NF-κB transactivation, we measured transcriptional activity in C2C12 myotubes, stably transfected with a NF-κB sensitive reporter construct. Palmitate increased NF-κB dependent luciferase activity (figure 1D), which confirmed the DNA binding results in L6 myotubes.

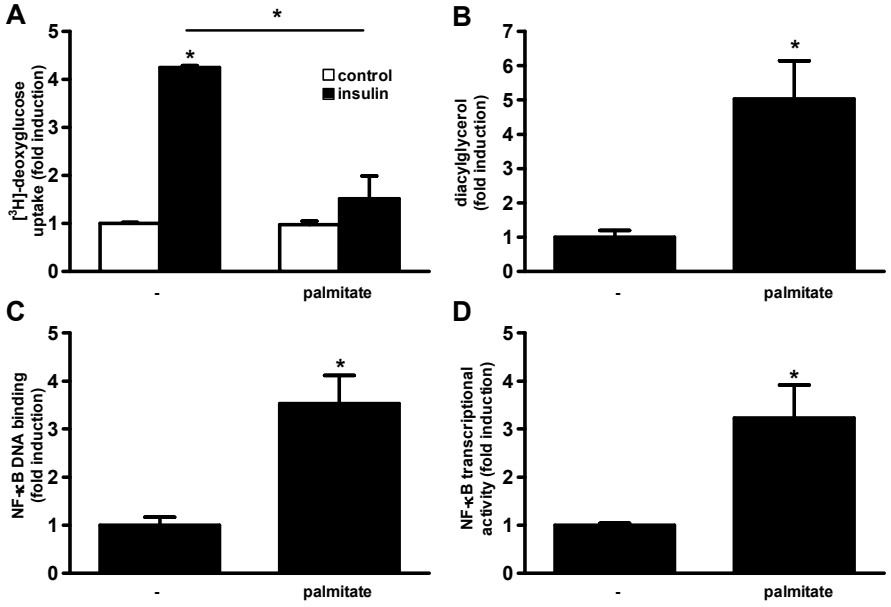

Figure 1: Palmitate induces insulin resistance, DAG accumulation and NF-κB transactivation in myotubes. A: L6 myoblasts were differentiated for 5 days and subsequently incubated for 16 hours with and without 200 µmol/l palmitate prior to stimulation with 25 nmol/l insulin for 15 min (white bars: no insulin stimulation; black bars: insulin stimulation). 2-deoxyglucose uptake was measured as described in the 'materials and methods' section, and insulin-stimulated glucose uptake was expressed as fold induction over basal (non-insulin stimulated) glucose uptake. *$p < 0.05$.
Alternatively, L6 myotubes were incubated with 200 µmol/l palmitate for 16 hours, after which B: DAG accumulation was determined by thin layer chromatography, corrected for protein content and expressed as fold change to control, or C: NF-κB DNA binding was determined by EMSA, as described in the 'materials and methods' section and expressed as fold induction over control. *$p < 0.05$.
D: Alternatively, C2C12 myoblasts stably transfected with the 6κB-TK-luciferase construct were differentiated for 5 days and incubated with 400 µmol/l of palmitate for 24 hours. Lysates were prepared for assessment of luciferase activity. Values were corrected for protein content and expressed as fold-induction over control. *$p < 0.05$.

Blocking β-oxidation sensitizes myotubes to palmitate-induced insulin resistance and NF-κB transactivation

Impaired FA oxidation causes lipid accumulation in skeletal muscle [29]. To assess the effects of intramyocellular lipid (IMCL) accumulation on insulin sensitivity and NF-κB activity, FA-oxidation was inhibited by blocking carnitine palmitoyltransferase-1 (CPT-1), an enzyme mediating the transport of long chain fatty acids across the mitochondrial membrane. Myotubes were incubated with 80 µmol/l etomoxir, an irreversible inhibitor of CPT-1 [30]. This concentration of

etomoxir resulted in a ~2-fold increase in DAG accumulation in presence of 100 μmol/l palmitate (supplementary figure).

Treatment of etomoxir without additional FA had no effects on insulin-induced glucose uptake (Figure 2A). Also treatment of myotubes with the low concentration of 100 μmol/l palmitate alone had no effects on insulin sensitivity, while increasing the palmitate concentrations resulted as expected in a reduced insulin-induced glucose uptake. In contrast, co-incubation of palmitate and etomoxir already reduced insulin-stimulated glucose uptake at 100 μmol/l palmitate. Moreover, inhibition of β-oxidation, inducing DAG accumulation, further enhanced the insulin resistance induced by increasing palmitate concentrations.

To investigate if sensitization to palmitate-induced insulin resistance by etomoxir coincided with enhanced NF-κB activation, we determined the effect of co-incubation of etomoxir and palmitate on NF-κB transcriptional activity (Figure 2B). Again, etomoxir and 100 μmol/l palmitate alone had no effect compared to control, while co-incubation with these two agents simultaneously resulted in an increased NF-κB transcriptional activity. Palmitate increased NF-κB transcriptional activity in a dose-responsive manner, whereas etomoxir had a synergistic effect, as it further enhanced (an additional 1.4-1.8 fold) palmitate-induced NF-κB activation. These data reveal that inhibition of β-oxidation sensitizes myotubes to palmitate-induced NF-κB activation and insulin resistance, and suggest that NF-κB activation and insulin resistance are coupled under conditions of DAG accumulation.

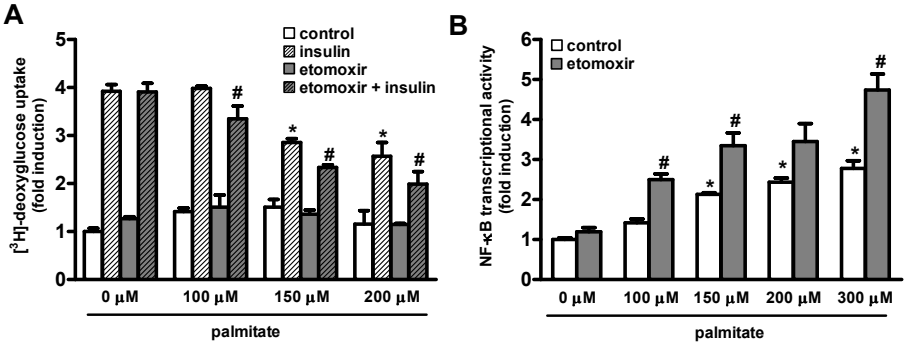

Figure 2: Blocking β-oxidation sensitizes myotubes to palmitate-induced insulin resistance and NF-κB transactivation. A: L6 myotubes were treated for 16 hours with the indicated palmitate concentrations in presence (grey bars) or absence (white bars) of etomoxir prior to insulin stimulation (open bars: no insulin stimulation; hatched bars: insulin stimulation). 2-deoxyglucose uptake was measured as described in the 'materials and methods' section, and insulin-stimulated glucose uptake was expressed as fold induction over basal (non-insulin stimulated) glucose uptake. * $p < 0.05$ versus insulin-stimulated cells without palmitate treatment. # $p < 0.05$ versus insulin-stimulated cells with the same palmitate treatment. **B**: C2C12 myoblasts stably transfected with the 6κB-TK-luciferase reporter construct were differentiated for 5 days and incubated with indicated palmitate concentrations for 24 hours. Cells were co-incubated with (grey bars) and without (white bars) etomoxir. Lysates were prepared for assessment of luciferase activity. Values were corrected for protein content and expressed as fold-induction over control. * $p < 0.05$ versus control cells without palmitate treatment. # $p < 0.05$ versus non-etomoxir treated cells with the same palmitate treatment.

Segregation of palmitate-induced NF-κB transactivation and insulin resistance following inhibition of DAG conversion

Diacylglycerol acyltransferase (DGAT) is an enzyme involved in the formation of triglycerides from diacylglycerol (DAG) and acyl-coA in insulin-sensitive tissues like skeletal muscle. In a second approach to study the effects of DAG accumulation on NF-κB activation and insulin resistance, we used the DGAT inhibitor amidepsine [31]. Amidepsine significantly increased DAG accumulation (supplementary figure) in L6 myotubes. Treatment of myotubes with 10 μg/ml (~8.8 μmol/l) amidepsine in absence of palmitate did not affect insulin-induced glucose uptake (Figure 3A). However, in line with the effects of etomoxir treatment, amidepsine sensitized myotubes to palmitate-induced insulin resistance, and co-incubation of 150 μmol/l palmitate with amidepsine already completely abolished insulin-mediated glucose uptake.

In contrast to the effects of etomoxir, amidepsine treatment did not further increase, but even slightly reduced palmitate-induced NF-κB transcriptional activity (Figure 3B), despite a significant increase (38% compared to palmitate without amidepsine, p=0.004) in C2C12 myotubes. These findings reveal segregation of

palmitate-induced DAG accumulation, insulin resistance and NF-κB activation following inhibition of DAG conversion, and suggest that NF-κB may not be required for palmitate-induced insulin resistance.

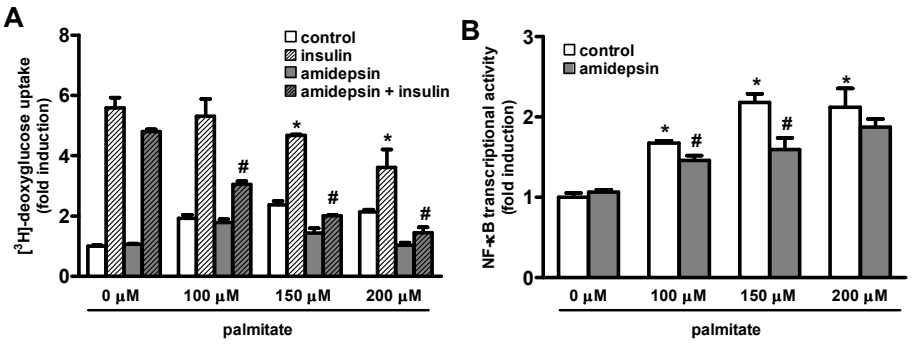

Figure 3: Segregation of palmitate-induced NF-κB transactivation and insulin resistance following inhibition of DAG conversion. A: L6 myotubes were treated for 16 hours with the indicated palmitate concentrations in presence (grey bars) or absence (white bars) of amidepsine A prior to insulin stimulation (open bars: no insulin stimulation; hatched bars: insulin stimulation). 2-deoxyglucose uptake was measured as described in the 'materials and methods' section, and insulin-stimulated glucose uptake was expressed as fold induction over basal (non-insulin stimulated) glucose uptake. * $p < 0.05$ versus insulin-stimulated cells without palmitate treatment. # $p < 0.05$ versus insulin-stimulated cells with the same palmitate treatment. **B**: C2C12 myoblasts stably transfected with the 6κB-TK-luciferase reporter construct were differentiated for 5 days and incubated with indicated palmitate concentrations for 24 hours. Cells were co-incubated with (grey bars) and without (white bars) amidepsine A. Lysates were prepared for assessment of luciferase activity. Values were corrected for protein content and expressed as fold-induction over control. * $p < 0.05$ versus control cells without palmitate treatment. # $p < 0.05$ versus non- amidepsine A treated cells with the same palmitate treatment.

Pharmacological or genetic inhibition of NF-κB does not prevent palmitate-induced insulin resistance in myotubes

To test whether the NF-κB pathway is causally linked to palmitate-induced insulin resistance, pharmacological and genetic approaches were employed to inhibit NF-κB. First, C2C12 myotubes were incubated with 20 µmol/l parthenolide, an agent that inhibits NF-κB activation by inhibiting IKK activity [32]. The NF-κB inhibitory capacity of parthenolide was confirmed as it blocked TNFα-induced NF-κB activity in myotubes (data not shown). Parthenolide resulted in a complete abolishment of palmitate-induced NF-κB transcriptional activity (Figure 4A). In spite of this potent NF-κB inhibitory capacity, the palmitate-induced reduction of glucose uptake could not be prevented by parthenolide (Figure 4B), which – in line with the amidepsine findings - suggests that palmitate-induced NF-κB activation and insulin resistance are not causally related.

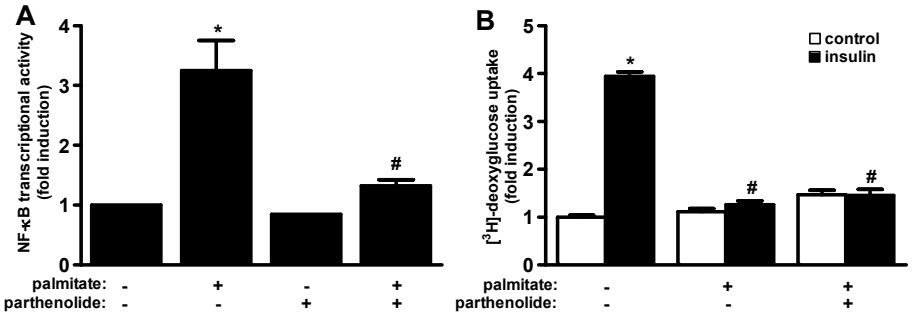

Figure 4: Pharmacological inhibition of NF-κB does not prevent palmitate-induced insulin resistance in myotubes. **A**: C2C12 myoblasts stably transfected with the 6κB-TK-luciferase construct were differentiated for 5 days and incubated with 400 μmol/l palmitate and 20 μmol/l parthenolide for 24 hours as indicated. Lysates were prepared for assessment of luciferase activity. Values were corrected for protein content and expressed as fold-induction over control. * $p < 0.05$ versus control. # $p < 0.05$ versus non-parthenolide palmitate-treated cells. **B**: L6 myotubes were treated for 16 hours with 200 μmol/l palmitate in presence or absence of 20 μmol/l parthenolide prior to insulin stimulation (open bars: no insulin stimulation; black bars: insulin stimulation). 2-deoxyglucose uptake was measured as described in the 'materials and methods' section, and insulin-stimulated glucose uptake was expressed as fold induction over basal (non-insulin stimulated) glucose uptake. * $p < 0.05$ versus control. # $p < 0.05$ versus insulin-stimulated control.

To further confirm these findings, we also inhibited the NF-κB pathway by genetic modification, using IκBα-SR, a non-degradable mutant of IκBα. As expected, increased NF-κB DNA binding after treatment with palmitate (Figure 5A) or TNFα (data not shown) was not observed in L6 cells expressing the IκBα-super repressor (SR), in contrast to control myotubes. In complete accordance with the parthenolide results, there was no improvement in insulin-induced glucose uptake in IκBα-SR myotubes treated with palmitate (Figure 5B). Thus, both pharmacological and genetic blockade of NF-κB could not prevent palmitate-induced insulin resistance in skeletal muscle cells, illustrating NF-κB activity in skeletal muscle is not required for palmitate-induced insulin resistance.

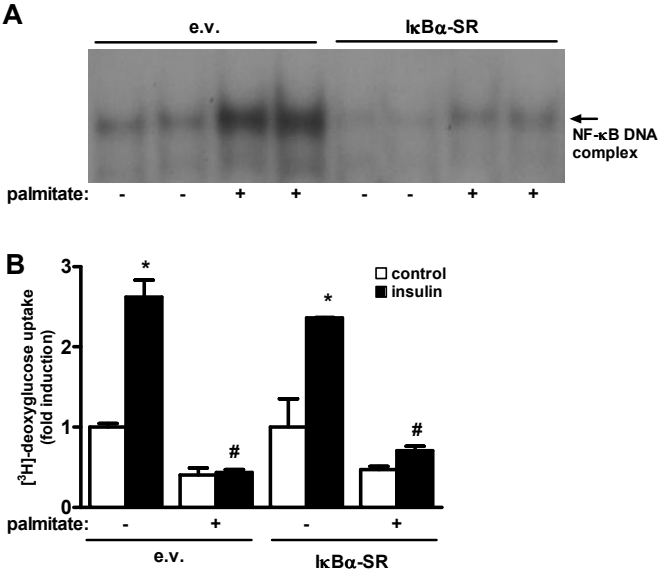

Figure 5: Genetic inhibition of NF-κB does not prevent palmitate-induced insulin resistance in myotubes. Stably transfected L6 cells, carrying a plasmid encoding a stabilized mutant of the Inhibitor of NF-κB (IκBα-SR) or an empty vector (e.v.), were differentiated for 5 days and cultured for 16 hours in presence or absence of 200 μmol/l palmitate. A: Nuclear extracts were prepared and assessed for DNA binding activity to a consensus NF-κB oligonucleotide by EMSA. B: Control- or IκBα-SR-myotubes were treated for 16 hours with 200 μmol/l palmitate prior to insulin stimulation (open bars: no insulin stimulation; black bars: insulin stimulation). 2-deoxyglucose uptake was measured as described in the 'materials and methods' section, and insulin-stimulated glucose uptake was expressed as fold induction over own basal (non-insulin stimulated) glucose uptake. * $p < 0.05$ versus control.

DISCUSSION

Lipid infusion-induced insulin resistance in human skeletal muscle is asscociated with an increase in diacylglycerol (DAG) mass and a reduction in IκBα protein levels [8]. This led to the suggestion that DAG accumulation, NF-κB activation and insulin resistance are causally related. Here, we show that palmitate-induced insulin resistance in skeletal muscle cells indeed coincides with increased NF-κB activation and DAG accumulation.

However, our intramyocellular lipid metabolite modulating experiments uncoupled the effects of palmitate-induced DAG accumulation and insulin resistance from NF-κB activation. Furthermore, pharmacological and genetic inhibition of the NF-κB pathway could not prevent the palmitate-induced reduction in insulin-stimulated glucose uptake. Altogether, this implies that NF-κB activation is not a causal factor in palmitate-induced insulin resistance in skeletal muscle.

FA oversupply results in increased intra-myocellular lipid (IMCL) and consequent lipid metabolite accumulation, and this was reproduced by pharmacological inhibition of carnitine palmitoyltransferase 1 (CPT-1) using etomoxir. By irreversible inhibition of CPT-1, etomoxir is able to block the entry of long-chain fatty acyl-CoA into mitochondria [30, 33-34]. Consequently, CPT-1 inhibition results in intramyocellular lipid (IMCL) accumulation [29] and may therefore lead to cytoplasmatic accumulation of lipid metabolites, like palmitoyl-CoA, DAG and ceramide, which all have been implicated in the pathogenesis of insulin resistance in skeletal muscle [8, 11, 35-39]. Indeed, treatment with etomoxir sensitized myotubes to palmitate-induced DAG accumulation and insulin resistance, causing decreased insulin-stimulated glucose uptake even with non-insulin resistance-inducing palmitate concentrations. These results are in agreement with a study in rats, where inhibition of CPT-1 by prolonged etomoxir administration resulted in increased IMCL accumulation, associated with insulin resistance [29]. Furthermore, CPT-1 over-expression in L6E9 muscle cells prevented DAG and ceramide accumulation and activation of protein kinase C (PKC)-θ and -ζ after palmitate incubation and resulted in protection from palmitate-induced insulin resistance [40].

Another approach to manipulate accumulation of lipid metabolites is by inhibiting diacylglycerol acyltransferase (DGAT), the enzyme facilitating binding of DAG to long-chain acyl-CoAs to form triacylglycerol (TAG), using amidepsine A [31, 41]. In accordance with the etomoxir results, we found that inhibiting DGAT by amidepsine A resulted in DAG accumulation and sensitized myotubes to palmitate-induced insulin resistance. In line with this, over-expression of DGAT1 in skeletal muscle of mice resulted in protection against high fat diet-induced insulin resistance, which coincided with decreased DAG and ceramide levels and increased TAG levels [42]. This was associated with attenuated fat-induced activation of JNK and DAG-responsive PKCs. Furthermore, the same authors showed that in isolated soleus muscle from DGAT knockout mice, the insulin-induced reduction in glucose uptake after administration of a palmitate/oleate mixture is exacerbated compared to wild type muscle, which coincided with increased DAG and ceramide levels. Conversely, muscles over-expressing DGAT1 showed enhanced insulin sensitivity, associated with decreased DAG and ceramide levels (comparable to muscle from wild type mice) [42]. Whether DAG or ceramides are responsible for palmitate-induced insulin resistance cannot be concluded from our experiments, since accumulation of palmitoyl-CoA might not only lead to the formation of DAG, but also to ceramides. Since palmitoyl-CoA is a precursor in sphingolipid synthesis [43], etomoxir or amidepsine treatment may lead to enhanced ceramide synthesis, as has been reported for etomoxir in hematopoietic cell lines [44].

While etomoxir alone did not induce NF-κB activation, it did increase NF-κB transactivation when co-incubated with 100 umol/l palmitate. However, in contrast

to etomoxir, direct interference with DAG metabolism by inhibiting the conversion from DAG to TAG with amidepsine, did not increase palmitate-induced NF-κB transactivation. Based on these results, it is unlikely that DAG or DAG-induced PKC activation is responsible for NF-κB activation as DAG accumulation was observed in response to both etomoxir and amidepsine. Ceramides are known activators of NF-κB [45-46] and are therefore a likely candidate for the observed NF-κB activation in response to palmitate.

Moreover, these differential effects of lipid metabolite modulation on insulin resistance and NF-κB activation suggest that FA-induced insulin resistance and NF-κB activation in skeletal muscle are not related. This dissociation between FA-induced NF-κB activity and insulin resistance was also postulated in a recent study from our group [23], in which we showed that several unsaturated fatty acids were able to increase NF-κB DNA binding and transcriptional activity in myotubes without inducing insulin resistance, which further supports that fatty acid-induced insulin resistance and NF-κB activation in muscle may occur independently.

Several studies in rodents and humans show that fat-induced insulin resistance is associated with NF-κB activation in skeletal muscle [8, 15-16]. Although previous associations between palmitate-induced insulin resistance and NF-κB activation in skeletal muscle cells also have been reported [12, 14], we now show by pharmacological and by genetic inhibition, that a causal relationship between both processes can be ruled out.

Previous studies investigating this potential association, used inhibition of IKK by high doses of salicylate or by heterozygous deletion, which prevented lipid infusion-, obesity- and diet-induced insulin resistance in skeletal muscle [17-18]. However, it has been shown that salicylate may reverse TNF-α-induced insulin resistance independently of IKK-2/NF-κB inhibition [47]. In this study, salicylate inhibited JNK activation, and the latter has also implicated in obesity-induced insulin resistance [48]. Furthermore, there is evidence from several *in vivo* studies that the NF-κB pathway in skeletal muscle is not required for high-fat diet-induced insulin resistance. Muscle-specific inhibition of NF-κB by transgenic expression of the IκBα super-repressor (MISR mice) did not protect against the development of obesity-induced insulin resistance, following a high fat diet for 3 months [19]. Muscle-specific IKK-2 deficient mice were not protected against whole body or muscle insulin resistance induced by gold thioglucose or high-fat [20]. Taken together, these *in vivo* studies suggest that activation of the NF-κB pathway in muscle does not seem to play a causal role in high fat-induced insulin resistance.

In fact, the evidence causally relating FA-induced insulin resistance in muscle to NF-κB activation is entirely derived from *in vitro* studies using L6 skeletal muscle cells [21] in which it was shown that pharmacological inhibition of IKK (using parthenolide and acetylsalicylate) or blockade of nuclear translocation of NF-κB prevented palmitate-induced reductions in insulin-stimulated glucose uptake. The

acetylsalicylate-induced effects on insulin resistance may be IKK independent, since it is known that acetylsalicylate blocks several kinases, including JNK, [49], which as pointed out above has been implicated in insulin resistance. In addition, SN50, a peptide used to block nuclear import of NF-κB [50], was used as proof that nuclear translocation of NF-κB is required for the insulin desensitizing effects of palmitate [21]. However, these data should be interpreted with care as SN50 displays inhibitory activity towards the nuclear translocation of a range of transcription factors other than NF-κB [51]. Importantly, SN50 also blocks nuclear import of activator protein (AP)-1, which is a JNK responsive transcription factor, suggesting reversal of insulin resistance by SN50 can also be explained by inhibition of the JNK/AP-1 pathway which has been implicated in insulin resistance [48].

In contrast to the observations concerning reversal of insulin sensitivity following NF-κB inhibition by parthenolide [21], in our hands parthenolide, despite complete ablation of NF-κB activation, displayed no protection against palmitate-induced insulin resistance. Although it is difficult to reconcile these opposing findings, we are confident of our data as a second, genetic approach to inhibit NF-κB yielded identical results. Here we showed that complete ablation of NF-κB activation by IκBα-SR in L6 cells did not prevent palmitate-induced insulin resistance. A similar strategy to inhibit muscular NF-κB activation also ruled out a role of muscle NF-κB in high fat-induced insulin resistance [19]. Although insulin resistance of other tissues could potentially have masked a protective effect of NF-κB inhibition in muscle as whole body insulin sensitivity was measured in that study, our findings further extend the evidence against an important role for NF-κB in skeletal muscle insulin resistance as insulin sensitivity was measured directly in cultured muscle. Finally, in line with previous reports [24-26], LPS and TNF-α were not able to induce insulin resistance in myotubes despite potent NF-κB activation (data not shown). This further confirms findings that muscle-specific NF-κB activation is not sufficient to induce insulin resistance [19, 52], and underlines the notion that NF-κB activation and insulin resistance in skeletal muscle are not causally related.

In conclusion, we demonstrate that manipulation of DAG accumulation by inhibiting DGAT or β-oxidation resulted in segregation of palmitate-induced insulin resistance and NF-κB activation, suggesting these processes are not related. By pharmacological and genetic inhibition of the NF-κB pathway we reveal that activation of the NF-κB pathway is not required for palmitate-induced insulin resistance in skeletal muscle cells.

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CHAPTER 6

Inflammatory signaling in skeletal muscle insulin resistance: green signal for nutritional intervention?

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ABSTRACT

Purpose of review

To review the evidence implying a role of inflammatory signaling pathways, specifically nuclear factor-κB and c-Jun NH_2-terminal kinase, in fatty acid-induced skeletal muscle insulin resistance, and to discuss the potential of dietary interventions to interfere with these processes.

Recent findings

Fatty acids can induce skeletal muscle insulin resistance via inflammatory signaling after binding Toll-like receptors at the cell membrane of muscle cells or after accumulating as intramyocellular lipid metabolites. In both processes, activation of intracellular inflammatory signaling is involved. The majority of literature addressing the causality of muscle nuclear factor-κB activation in skeletal muscle insulin resistance suggests that insulin resistance does not require muscle nuclear factor-κB activation. Recently, strong evidence was given that c-Jun NH_2-terminal kinase signaling is an important inflammatory pathway involved in skeletal muscle insulin resistance. Furthermore, it is well established that pro-inflammatory cytokines originating from the enlarged adipose tissue or from activated adipose tissue macrophages can cause muscle insulin resistance. Recently, also macrophages resided in the muscle have been proposed to play an important role in muscle insulin resistance.

Because of their anti-inflammatory characteristics, several dietary components like polyphenols may be interesting candidates for manipulating skeletal muscle insulin resistance.

Summary

Several dietary components, like polyphenols, have been reported to interfere with inflammatory signaling. To test whether these compounds can be used to prevent or reverse insulin resistance, well controlled human intervention studies have to be designed.

INTRODUCTION

Understanding the etiology of insulin resistance is required to halt and reverse the increasing prevalence of diabetes mellitus type 2 (T2D). Although insulin resistance is often associated with obesity, it also accompanies cachexia, a condition characterized by loss of muscle with or without loss of fat mass. As excess adipose tissue is an unlikely cause of cachexia-associated insulin resistance, a low-grade inflammatory response, a common denominator of type 2 diabetes and cachexia, may underlie muscle insulin resistance.

Here we will review inflammatory signaling mechanisms by which fatty acids (FAs) can induce skeletal muscle insulin resistance, and some novel dietary interventions to interfere with these processes will be discussed.

INFLAMMATORY SIGNALS AND SIGNALING PATHWAYS INVOLVED IN INSULIN RESISTANCE

Although a chronic inflammatory status has been observed in both T2D and cachexia, including increased inflammatory signaling, in skeletal muscle, the source and nature of the inflammatory signal likely differs between these conditions. Increased circulating cytokines, either derived from the diseased organ or host response in cachexia, or from excess adipose tissue or macrophages in T2D, may trigger insulin resistance upon receptor activation in skeletal muscle. In contrast, increased plasma FA levels derived from excess adipose tissue may induce inflammatory signaling and muscle insulin resistance by intracellular accumulation of lipid metabolites or activation of Toll-like receptors (TLRs) as will be discussed below, but is likely only relevant to T2D. Ultimately, these various inflammatory signals may converge in common signaling routes, for example, the nuclear factor-κB (NF-κB) or Jun NH_2-terminal kinase (JNK) signaling pathways, resulting in muscle insulin resistance.

TOLL-LIKE RECEPTORS AND MUSCLE INSULIN RESISTANCE

TLRs play an important role in the innate immune system, as they activate pro-inflammatory signaling pathways in response to pathogen-associated molecular pattern recognition [1-2]. Nowadays, TLR signaling is considered to be a potential cause of insulin resistance and other aspects of the metabolic syndrome [3]. Although the main site of TLR expression is on cells of the immune system like macrophages, functional TLRs, and in particular TLR2 and TLR4, are also expressed in skeletal muscle [4-6].

TLR4, the best-characterized TLR, binds the ligand lipopolysaccharide (LPS). It was shown that addition of saturated fatty acids (SFAs), the main constituent of the lipid part of LPS, Lipid A, was sufficient to trigger TLR4 activation in a macrophage cell line [7]. A recent study presents TLR4 as the link between innate immunity, FAs and insulin resistance [8]. Furthermore, in contrast to wild-type mice, lipid infusion in TLR4-knockout mice did not result in insulin resistance in skeletal muscle, which pointed to an important role of TLR4 in obesity-induced insulin resistance *in vivo* [8-9]. Moreover, mice with a loss-of-function mutation in TLR4 showed a reduction in the development of diet-induced insulin resistance and isolated muscles from these mice were protected from SFA-induced insulin resistance [10]. In L6 myotubes, blocking TLR4 reduced palmitate-induced inhibition of glucose uptake [9]. Recently, activation of TLR2 in skeletal muscle was postulated as a potential mechanism for palmitate-induced insulin resistance in cultured skeletal muscle cells. Specifically, a TLR2 antagonist antibody or RNAi-induced inhibition of TLR2 expression both inhibited palmitate-induced insulin resistance, as indicated by reduced Akt phosphorylation in C2C12 cells [11]. These studies demonstrate that SFA-induced TLR activation causes skeletal muscle insulin resistance. The underlying inflammatory signaling will be discussed below.

INTRAMYOCELLULAR LIPID METABOLITES AS INDUCERS OF SKELETAL MUSCLE INSULIN RESISTANCE

Many high-fat diet (HFD) studies on animals [12-13] and nuclear magnetic resonance spectroscopy studies on humans [14-16] provided evidence for an association between intramyocellular triglycerides (IMTGs) and insulin resistance that is now well established. In spite of these and numerous other studies, continuous observations [17-19] postulate that IMTG accumulation itself is not the responsible factor for defects in muscle insulin signaling. Currently it is believed that IMTG may represent a storage form for FAs, whereas the intramuscular accumulation of lipid metabolites like diacylglycerol (DAG) and ceramides [16, 20-25] is more directly responsible for the observed FA-induced decreased insulin action in skeletal muscle. An increased IMTG may even provide a protective effect by limiting lipid metabolite levels, thereby maintaining insulin sensitivity [26-28].

In muscle of animals with HFD-induced insulin resistance and obese humans, DAG and ceramides accumulate [29]. DAG can activate conventional and novel protein kinase C (PKCs): serine kinases that are able to inhibit insulin action by phosphorylation of defined serine residues of insulin receptor substrate (IRS)-1[14, 23, 30]. In several studies it was postulated that PKCs are involved in skeletal muscle insulin resistance [31-33]. Furthermore, PKC-θ knockout mice are protected against fat-induced insulin resistance [34]. Besides direct interference with insulin signaling,

PKCs also feed into inflammatory signaling by activation of IκBα kinase β (IKK) and JNK. Increased catalytic activity of these kinases may negatively influence insulin signalling by serine phosphorylation of IRS-1 [35-36].

ROLE OF INTRAMUSCULAR NUCLEAR FACTOR-κB ACTIVATION

Activation of cytokine receptors, TLR receptors, and the intracellular accumulation of lipid metabolites can all lead to the activation of the NF-κB pathway. NF-κB is under normal conditions retained in the cytoplasm, bound to its inhibitory protein IκBα. Stimulation of the pathway results in activation of IKK, which leads to phosphorylation and subsequent ubiquitination of IκBα. As a consequence, IκBα is degraded and NF-κB is liberated from its inhibitory protein and translocates to the nucleus, resulting in transcription of inflammatory genes.

Muscle NF-κB activation has been associated with insulin resistance. An acute elevation in plasma free fatty acids (FFAs) in humans induced insulin resistance and this coincided with accumulation of DAG, an increase in PKC activity, and a reduction in IκBα in skeletal muscle [20]. The latter is a sign of increased NF-κB activation and suggested that accumulation of DAG could lead to insulin resistance via activation of the NF-κB pathway. Moreover, several studies in rodents demonstrated that obesity-inducing HFD or acute hyperlipidemia (lipid infusion) resulted in a reduction of IκBα levels in skeletal muscle, which was associated with reduced insulin signaling [37-38]. In line with this, increased IKK-2 activity in skeletal muscle was observed after high-fat feeding, which coincided with a reduced insulin-stimulated glucose uptake in rat muscle [39]. Moreover, also several *in vitro* studies demonstrated associations between long-chain SFA-induced insulin resistance and activation of the NF-κB pathway [40-41]. Recently, we demonstrated that these associations are not observed for all FAs. More specifically, several unsaturated FAs are potent inducers of the NF-κB pathway in muscle cells but fail to induce insulin resistance in these cells [42]. For example, although *trans*-10, *cis*-12 conjugated linoleic acid (CLA) was approximately four-times stronger NF-κB activator than palmitic acid, cells incubated with this CLA-isomer remained insulin-sensitive. These studies indicate that FA-induced NF-κB activation *per se* is not sufficient for insulin resistance.

Initial studies addressing the causality of NF-κB signaling in insulin resistance by inhibiting IKK-2/NF-κB signaling by heterozygous knockout in rodents [43] or high doses salicylate in rodents [43-44] and humans [45] suggested an important role for the NF-κB pathway in skeletal muscle insulin resistance after lipid infusion or a HFD. Moreover, from a study on L6 skeletal muscle cells, it was concluded that NF-κB activation was causally related to FA-induced insulin resistance as palmitate-induced insulin resistance was prevented by pharmacological blockade of IKK (using

parthenolide and acetylsalicylate). Furthermore, a pharmacological inhibitor of NF-κB nuclear translocation prevented palmitate-induced insulin resistance in L6 myotubes [46], suggesting nuclear translocation and subsequent NF-κB-dependent gene expression are required for FA-induced insulin resistance in skeletal muscle. However, this notion was not supported by an *in vivo* study in which muscle-specific inhibition of NF-κB-dependent gene expression by transgenic expression of the IκBα superrepressor (MISR mice) did not protect against the development of HFD-induced insulin resistance [47].

Besides IKK-2 mediated IκBα degradation and subsequent NF-κB-dependent gene expression, IKK-2 has also been suggested to be directly responsible for interference with insulin signaling via IRS-1 serine phosphorylation [48]. However, muscle-specific expression of a constitutive activate IKK-2 mutant protein in mice did not result in muscle insulin resistance, indicating that IKK-2 activation is not sufficient to induce insulin resistance in muscle [47]. Moreover, muscle-specific IKK-2-deficient mice were not protected against insulin resistance after gold thioglucose-induced or HFD-induced obesity [49], illustrating muscle IKK-2 activation is not required for diet-induced insulin resistance.

Altogether, the majority of studies addressing the causality of muscle NF-κB activation in skeletal muscle insulin resistance suggest that insulin resistance does not require muscle NF-κB activation.

Interestingly, some of the pharmacological approaches to investigate the involvement of NF-κB in insulin resistance may have prevented or reversed insulin resistance independently of NF-κB. For instance, the prevention of insulin resistance by acetylsalicylate [44-46] may be IKK independent, as it is known that salicylates block the activity of JNK, Erk and S6K [50-52], serine kinases that are all able to interfere with insulin signaling by inducing serine phosphorylation of IRS-1. It has been shown that salicylate may reverse TNF-α-induced insulin resistance independently of IKK-2 inhibition [53]. In this study, salicylate inhibited JNK activation. In addition, the inhibitory activity of SN50, the peptide used to block nuclear import of NF-κB [46], appears not restricted to NF-κB, as other transcription factors like the JNK responsive transcription factor activator protein (AP)-1 are inhibited by SN50 [54].

ROLE OF C-JUN TERMINAL KINASE ACTIVATION IN SKELETAL MUSCLE INSULIN RESISTANCE

Ceramides, DAG-induced PKC activation, TLR, and cytokine receptor activation are all known activators of JNK. In insulin resistant skeletal muscle of rats and humans, JNK activity was increased [39, 55]. Furthermore, JNK activity is elevated in HFD-induced insulin resistance or in obese mice, and disruption of the JNK signaling

pathway in mice has been shown to reduce or prevent insulin resistance [56-57]. In several *in vitro* studies, it was demonstrated that palmitate-induced insulin resistance is associated with increased JNK activity [11, 30]. Recently, it has been shown that shRNA-mediated knock-down of JNK in C2C12 myotubes reduced palmitate-induced insulin resistance [58]. Moreover, a study with mice containing a muscle-specific *Jnk1* deficiency provided evidence that JNK1 in muscle is required for HFD-induced muscle insulin resistance, whereas fat tissue and liver were not protected against insulin resistance in these mice [59-60]. The authors proposed a role for muscle lipoprotein lipase (LPL), as muscle-specific JNK deficiency resulted in reduced muscle LPL expression, leading to a redistribution of triglycerides to non muscle tissues and in increased triglycerides in the circulation and hepatocytes [59] with a subsequent increase in muscle sensitivity and reduced insulin sensitivity in other organs.

ROLE OF ADIPOSE TISSUE AND MACROPHAGES IN SKELETAL MUSCLE INSULIN RESISTANCE

Besides potential direct, muscle cell autonomous effects of FAs resulting in inflammatory signaling and insulin resistance in skeletal muscle, another line of evidence proposes circulating inflammatory cytokines as a causative factor for skeletal muscle insulin resistance. In T2D, these cytokines may originate from adipose tissue or macrophages infiltrated in adipose tissue or skeletal muscle.

Adipose tissue

Currently, it is well established that adipose tissue is not a passive fat storage organ, but an endocrine organ secreting many factors, including cytokines [61]. A large number of cytokines and chemokines, such as tumor-necrosis factor α (TNFα), IL-6, plasminogen activator inhibitor 1 (PAI-1), and monocyte chemotactic protein-1 (MCP-1), are released from the adipose tissue of obese insulin-resistant individuals [62]. Thus, skeletal muscle becomes not only a target of FAs, as described in the previous sections, but also a target of cytokines [63]. Direct evidence for a negative cross-talk between fat and skeletal muscle was first demonstrated by coculturing primary human adipocytes and skeletal muscle cells *in vitro* [64]. Coculturing resulted in impaired insulin signaling of muscle IRS-1 and Akt phosphorylation. Reduced insulin signaling was also shown in a similar model, in which skeletal muscle cells were incubated with conditioned medium from adipose tissue [65-66].

Macrophages

There is accumulating evidence that besides adipocytes, also macrophages are also an important source for pro-inflammatory cytokine secretion. Adipose tissue from obese persons is characterized by increased macrophage infiltration and the presence of these so-called adipose tissue macrophages (ATMs) correlates with insulin resistance [67-68]. These resident macrophages are a source of pro-inflammatory cytokines such as TNF-α [67, 69], IL-6, and MCP-1. The expansion of adipose tissue in diet-induced obesity is associated with an increased infiltration of the so-called "classically" activated, or M1 macrophages. These macrophages are in a pro-inflammatory state, as opposed to the M2 or "alternatively" activated macrophages, which are residing in lean adipose tissue [70]. The chemokine MCP-1, can be secreted by adipocytes to recruit macrophages to the adipose tissue [71]. Macrophages from obese individuals appeared to be in a pro-inflammatory state, as they showed increased NF-κB activity and TNF-α gene expression [72].

It has been demonstrated that besides adipose tissue, skeletal muscle can also contain macrophages. An increase in macrophages was observed in muscle from obese mice [67] and increased M1-macrophage infiltration has been described in muscle from mice fed a HFD [73-74]. Importantly, increased macrophage infiltration has also been demonstrated in skeletal muscle of insulin resistant humans [75]. Infiltrated macrophages may contribute to muscle insulin resistance based on observations that conditioned medium from macrophages with deleted PPAR-γ, known to be necessary in M2 skewing of macrophages, caused the production of insulin resistance-inducing factors and skeletal muscle insulin resistance [76]. Recent *in vitro* studies demonstrated that palmitate induced M1 skewing of macrophages [77]. Conditioned medium of these macrophages induced insulin resistance in L6 myotubes. Also coculturing experiments with human primary myotubes and macrophages demonstrated a palmitate-induced increase in expression of several cytokines and chemokines in the myotubes, which coincided with decreased IκBα levels, increased JNK activation, and reduced Akt phosphorylation [75]. The latter studies demonstrated the potential of FA-induced activation of macrophages, subsequently affecting muscle insulin sensitivity. A crucial role for macrophages in the development of insulin resistance was demonstrated by showing that myeloid-specific deletion of IKK-2 protected against high-fat-induced and obesity-induced skeletal muscle and systemic insulin resistance in mice [78].

Altogether, cytokines from adipose tissue or macrophages residing in adipose tissue or muscle could activate JNK or the NF-κB pathway in muscle in an endocrine or autocrine manner [79]. Although skeletal muscle JNK activation may play a more important role than NF-κB, the latter pathway seems to be involved in macrophage activation that may lead to skeletal muscle insulin resistance.

The underlying inflammatory pathways proposed to play a role in skeletal muscle insulin resistance are summarized in Figure 1.

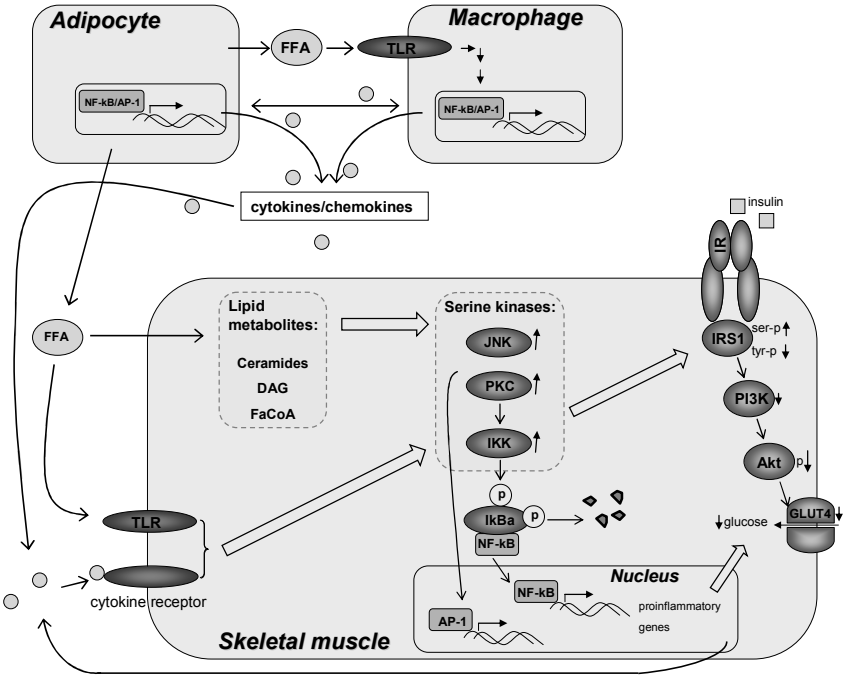

Figure 1*:* Summary of inflammatory mechanisms that may contribute to skeletal muscle insulin resistance. Increased circulating cytokines produced by adipose tissue and/or macrophages may trigger insulin resistance upon receptor activation in skeletal muscle. In contrast, increased plasma FA levels derived from the enlarged adipose tissue may induce inflammatory signaling and muscle insulin resistance by intracellular accumulation of lipid metabolites or activation of toll-like receptors. Ultimately, these various inflammatory signals may converge in common signaling routes, e.g. the NF-κB or JNK sigaling pathways, resulting in muscle insulin resistance.

DIETARY INTERVENTION STRATEGIES AIMED AT IMPROVING SKELETAL MUSCLE INSULIN SENSITIVITY VIA INTERFERING WITH INFLAMMATORY SIGNALING

Dietary interventions directed at inflammatory pathways may be an attractive strategy to prevent or improve skeletal muscle insulin resistance. Theoretically, there are two lines of argumentation for this (Figure 2).

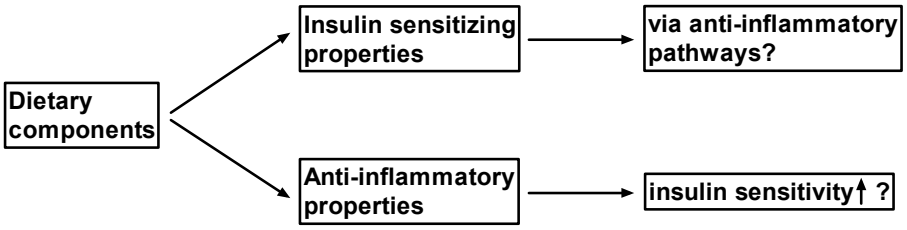

Figure 2: Dietary components that are known to affect insulin sensitivity may work via anti-inflammatory mechanisms. Conversely, dietary compounds with anti-inflammatory characteristics may be interesting candidates for manipulating skeletal muscle insulin resistance.

Dietary components that are known to affect insulin sensitivity may work via anti-inflammatory mechanisms. Conversely, dietary compounds with anti-inflammatory characteristics may be interesting candidates for manipulating (skeletal muscle) insulin resistance. These latter components are often not yet or incompletely evaluated in terms of insulin-sensitizing characteristics, but are candidates to improve insulin resistance based on their potential ability to suppress the elevated inflammatory status, which may be underlying insulin resistance as discussed above. A careful screen of the literature resulted in a list of potential interesting nutritional components that could interfere with inflammatory signalling hereby being part of the above indicated line two (Table 1) [80-101]. Here we will focus on the polyphenols as a group of dietary components that could improve insulin sensitivity via interfering with inflammatory signaling.

Table 1. Reported inflammatory targets of dietary compounds

Component:	Target:	References:
Guggelsterone	IKK/NF-κB/JNK	[80, 81]
Ursolic acid	IKK/NF-κB	[82]
Gingerol	NF- κB/macrophage	[83, 84]
Capsaicin	NF-κB/AP-1/macropage/adipocyte	[85-87]
Betulinic acid	NF-κB/macrophage	[88, 89]
Quercetin	NFκB-JNK/AP-1/macrophage	[90, 91]
Indole-3-carbinol	IKK/NF-κB/JNK/AP-1/macrophage	[92, 93]
Lycopene	NF-κB/macrophage	[94, 95]
Garlic extracts	NF-κB/macrophages	[96, 97]
Curcumin	NF-κB/JNK/AP-1/adipocyte/macrophage	[85, 98, 99]
Resveratrol	IKK/NF-κB/JNK/AP-1/adipocyte/macrophage	[100, 101]

Polyphenols

Polyphenols like curcumin (diferuloylmethane), the active component of the yellow powder that comes from rhizomes of the Asian plant turmeric *(Curcuma longa)*, green tea polyphenols like quercetin, and resveratrol, a compound from red grapes, are known inhibitors of the IKK/NF-κB and JNK/AP-1 pathways [100, 102-105]. *In vitro* studies with 3T3-L1 adipocytes demonstrated that curcumin was able to improve palmitate-induced reductions in insulin-stimulated glucose uptake and elevations in NF-κB, JNK, ERK and p38MAPK activity, and expression and secretion of cytokines TNF-α and IL-6 [98]. Furthermore, treatment of 3T3-L1 adipocytes with the polyphenols resveratrol or curcumin resulted in an inhibition of NF-κB activation and a reduction in the gene expression and secretion of several cytokines [106]. A recent study demonstrated that resveratrol prevented adipokine expression and insulin resistance in 3T3-L1 adipocytes treated with conditioned medium of LPS-stimulated macrophages [107]. A study with adipose tissue conditioned medium from obese mice fed a HFD showed that several spice-derived components, including curcumin, inhibited Raw 264.7 macrophages migration and activation (which was estimated by measuring TNF-α and nitric oxide production and inhibited MCP-1 release from 3T3-L1 adipocytes) [99]. Pretreatment of RAW 264.7 macrophages with quercetin, a polyphenol present in green tea, prevented LPS-induced TNF-α transcription. It was shown that the effect of quercetin was mediated via inhibition of JNK phosphorylation and suppressed AP-1 DNA binding [90]. Similarly, it was demonstrated that resveratrol suppressed nitric oxide synthase and down-regulated NF-κB activation in LPS-stimulated RAW 264.7 macrophages [101]. Altogether, these *in vitro* studies suggest that the anti-inflammatory potency of resveratrol may reduce the inflammatory properties of adipose tissue and/or macrophages, thereby indirectly improving peripheral (skeletal muscle) insulin resistance.

In vivo studies in mice fed a high calorie diet (60% fat) showed increased insulin sensitivity after supplementation with resveratrol [108]. In line with this, resveratrol treatment protected mice against HFD-induced insulin resistance [109]. Direct effects of polyphenols on muscle have recently been found. Curcumin has been reported to improve skeletal muscle of diabetic rats and L6 myotubes [110], whereas quercetin enhanced basal glucose uptake in C2C12 cells, although the authors proposed AMPK activation as the mechanism behind the insulin sensitizing effects [111].

There are only limited data available describing the effects of polyphenol supplementation on insulin sensitivity in humans. In healthy subjects, a 4-week intervention study with turmeric supplementation did not provoke differences in plasma blood glucose levels [112]. A randomized trial with diabetic patients demonstrated that consuming approxametely 750 mg green tea polyphenols for 2

months resulted in lower blood glucose and insulin levels and lower HbA1c and homeostasis model assessment index values. However, comparison with the control group did not show significant differences, which can be explained by the high daily consumption of tea by the control group [113].

More well-controlled human intervention studies specifically designed to investigate glucose homeostasis in insulin-resistant individuals are required to address the potential positive effects of polyphenols on insulin resistance in humans and whether these are related to their postulated anti-inflammatory properties.

CONCLUSION

FAs can activate intramyocellular inflammatory signaling pathways via activation of the TLRs or after accumulating as intramyocellular lipid metabolites. Controversial data has been found on the role of the NF-κB pathway in mediating skeletal muscle insulin resistance. Altogether, the majority of the literature suggests that insulin resistance does not require muscle NF-κB activation. Muscle JNK1 activation has recently been proposed as an important factor in inducing impaired skeletal muscle insulin signaling. Besides, many studies provided direct evidence that adipose tissue-derived cytokines could induce skeletal muscle insulin resistance. Furthermore, a crucial role for M1-macrophages residing in adipose tissue and muscle in fat-induced insulin resistance has been demonstrated.

Many dietary components have been reported to interfere with inflammatory signalling. Among these, polyphenols are interesting candidates to prevent or reverse insulin resistance because of abovementioned ant-inflamamtory properties. Well controlled human intervention studies have to be designed to test the insulin-sensitizing effects of these dietary compounds. In theory, these agents could be the insulin sensitizers of the future, opening possibilities to develop functional foods to improve insulin sensitivity for the general population.

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CHAPTER 7

General Discussion

INTRODUCTION

Insulin resistance plays an important role in the development of type 2 diabetes mellitus. Currently, around 250 million people are having diabetes worldwide, and the number of people suffering from this disease is rapidly increasing [1].

Skeletal muscle plays a crucial role in maintaining glucose metabolism, accounting for more than 80% of total insulin-stimulated glucose uptake, and skeletal muscle insulin resistance precedes the clinical diagnosis of type 2 diabetes mellitus [2]. It has become clear that most insulin resistant subjects have increased plasma free fatty acids (FFA) concentrations. Moreover, acute elevations of FFAs by lipid infusion results in skeletal muscle insulin resistance within hours in rodents [3, 4] and humans [5-7], whereas the induction of insulin resistance in skeletal muscle after feeding high-fat diets (HFD) to rats was induced within weeks [8, 9]. More evidence about the role of lipids in muscle insulin resistance was obtained by NMR spectroscopy studies showing a strong relationship between the accumulation of intramyocellular triglyceride (IMTG) content and insulin resistance in humans [10, 11]. Also high-fat diet intervention studies in rodents showed associations between the development of insulin resistance and the accumulation of IMTG [12]. However, several observations [13-16] led to the conclusion that it is unlikely that increased IMTG itself is responsible for the defects in insulin signaling and currently it is believed that IMTG may represent a storage form for FAs, while the accumulation of lipid metabolites like diacylglycerol (DAG) and ceramides [5, 11, 17-21] are more directly responsible for the observed FA-induced decreased insulin action in skeletal muscle.

To enlarge our understanding how dietary composition induces, prevents or could reverse insulin resistance, it is important to increase our knowledge on how specific dietary FAs affect insulin sensitivity in peripheral tissues like skeletal muscle, and to unravel the mechanisms underlying these effects. For fats, diets always consist of a mix of different fatty acids and therefore it is difficult to draw conclusions about the effects of individual FA types. Furthermore, dietary compositions frequently differ between studies, which makes it difficult to compare their effects. Therefore, while acknowledging limitations of extrapolating findings from studies in cultured cells to intact tissue in humans, experiments in cultured cells are useful, since conditions of the experiment, including the type and concentration of the FA can be tightly controlled. Another advantage of not only cell, but also of animal studies is the possibility to investigate possible causal relationships by pharmacological and genetic approaches. Furthermore, studying isolated cells makes it possible to study direct effects of FAs without interference of effects from other organs. Therefore, the work described in this thesis was based on the use of two well-established skeletal muscle cell lines - the rat L6 skeletal muscle cells and the mouse C2C12 skeletal muscle cells. In a series of experiments, we

determined the effects of different FAs and the role of lipid metabolite accumulation and activation of the NF-κB pathway on insulin resistance.

FATTY ACIDS AND SKELETAL MUSCLE INSULIN RESISTANCE

The role of fatty acid chain length and saturation on skeletal muscle insulin resistance

It is generally accepted that the saturated fatty acid (SFA) palmitate is able to induce insulin resistance in skeletal muscle cells. However, a systematic evaluation of other fatty acids was lacking. In **chapter 2** we therefore tested the effects of SFAs classified according to chain length, on insulin sensitivity in L6 myotubes. After incubation of myotubes with the medium-chain length FAs caprylic acid (C8:0) and lauric acid (C12:0), the muscle cells remained insulin sensitive. However, incubation with the long-chain SFAs palmitic acid (C16:0) and stearic acid (C18:0) reduced insulin-stimulated GLUT4 translocation and deoxyglucose uptake. In **chapter 3** we compared FAs from the same backbone (C16 and C18), with their mono-unsaturated [palmitoleic acid (C16:1), oleic acid (C18:1), elaidic acid (*trans*-9 C18:1), and vaccenic acid (*trans*-11 C18:1)] and poly-unsaturated [linoleic acid (C18:2), linolenic acid (C18:3), *cis*-9, *trans*-11 conjugated linoleic acid (CLA) and *trans*-10, *cis*-12 CLA] counterparts. None of the tested unsaturated FAs reduced insulin-stimulated GLUT4 translocation or deoxyglucose uptake, as compared to non-FA incubated myotubes.

Although we demonstrated that unsaturated FAs did not induce insulin resistance in the muscle cells, we did not test whether the tested unsaturated FAs protected against insulin resistance induced by SFAs. Recently, it has been described that an addition of oleate was able to reverse palmitate-induced insulin resistance in skeletal muscle cells [22]. Whether the other unsaturated FAs we have tested, also protect against insulin resistance induced by C16:0 or C18:0 remains to be investigated. In this light, it is noteworthy that over-expression of Stearoyl CoA desaturase 1 (SCD1), the enzyme that converts SFAs to their monounsaturated counterparts, resulted in TG esterification, but attenuated ceramide and DAG accumulation and protected myotubes from fatty acid-induced insulin resistance [23].

Lipid metabolite accumulation and insulin resistance

Concerning the mechanism underlying the effects of palmitate, we demonstrated in **chapter 5** that pharmacological inhibition of diacylglycerol acyltransferase (DGAT), the enzyme that converts DAG and acyl-CoAs into TAG, with amidepsine A resulted

in DAG accumulation and in a stronger reduction of insulin-stimulated deoxyglucose uptake in L6 myotubes with increasing palmitate concentrations. Similar effects were found after inhibition of carnitine palmitoyltransferase (CPT)-1 by etomoxir, thereby preventing β-oxidation and stimulating DAG accumulation. These results support the concept that lipid metabolites like DAG play an important role in FA-induced insulin resistance in skeletal muscle cells and are in line with studies in DGAT1 knockout mice. These mice have lower IMTG, but higher DAG levels and are more susceptible to HFD-induced insulin resistance [24]. Furthermore, muscle-specific over-expression of DGAT1 lowers DAG and ceramide levels and increases insulin sensitivity [25].

Altogether, it seems that especially long-chain SFAs, leading to the accumulation of lipid metabolites like DAG, induce insulin resistance in skeletal muscle cells. In this respect, it is interesting to note that especially diets high in SFAs may induce insulin resistance in humans [26]. Also animals fed diets rich in SFAs are more prone to induce insulin resistance, coinciding with DAG accumulation, compared to animals fed diets rich in unsaturated FAs [27].

The differential effects of FAs on inducing insulin resistance, as we (**chapters 2 and 3**) and others [18] have found, suggest that individual FAs may be handled differently by the myotube, resulting in differences in intramuscular lipid metabolite accumulation and subsequent effects on insulin sensitivity.

A possible explanation for the differential effects for medium-chain length and long-chain length SFAs on insulin resistance (**chapter 2**) might relate to the fact that medium-chain FAs (MCFAs) do not require the carnitine palmitoyltransferase (CPT) system, which is necessary for long-chain FA (LCFA) transport over the mitochondrial membrane. This means that MCFAs are preferentially oxidized by the mitochondria [28]. Measurements of FA oxidation in rats [29] and humans [30] demonstrated that oxidation of SFAs decreases with increasing carbon length. Therefore, LCFAs may be more available for incorporation into lipid metabolites like DAG and ceramides, thereby potentially activating PKC and the NF-κB pathway as will be discussed later. Indeed, we (data not shown) and others [18] have demonstrated that only the long-chain SFAs were able to induce an accumulation of DAG.

Also the differential effects of saturated versus unsaturated FAs on insulin resistance (**chapter 3**) may relate to a differential utilization of these FAs. In C2C12 [18], L6 [27] and primary human myotubes [31], it has been demonstrated that especially SFAs accumulated into DAG. Analysis in human myotubes of the incorporation of FAs in different lipid pools, demonstrated that palmitic acid, stearic acid, oleic acid and linoleic acid were similarly incorporated into phospholipids. However, while the highest increase in incorporation of the saturated FAs was associated with the DAG fraction, oleic acid and linoleic acid were readily diverted to the TAG pool and induced minor increases in the DAG pool [31]. Another study in

cultured human myocytes demonstrated that palmitate was directed to the DAG pool, whereas oleate accumulated as intracellular FFA [32]. In L6 myotubes, linoleate was mainly directed towards the TAG pool, whereas no DAG accumulation was detected, while palmitate incubation led to a two-fold increase in ceramide levels and a fivefold increase in DAG accumulation [27], which was in line with our observations on palmitate-induced DAG accumulation as described in **chapter 5**.

A possible explanation for accumulation of SFAs in intramuscular DAG and the preferential usage of unsaturated fatty acids for TAG, could be the different affinity of DGAT for DAG molecules varying in fatty-acid composition. Isolated DGAT demonstrated maximal saturable activity with diolein (DAG type containing two oleic acid molecules), whereas the activity was minimal when dipalmitin (DAG type containing two palmitic acid molecules) was used as a substrate [33]. In line with this, in L6 myotubes it has been demonstrated that DGAT has an optimal affinity when long-chain unsaturated acyl CoAs were used as substrates [34].

Increased FA oxidation, induced by CPT-I over-expression, also results in a decreased accumulation of FA metabolites like DAG and a protection against palmitate-induced insulin resistance in skeletal muscle cells [35]. Furthermore, it has been shown that the oxidation rate of FAs in muscle increases proportionally with the number of double bonds [30, 36]. This may also explain why C16:0 and C18:0 did, but the C16-MUFA and C18-MUFA/PUFAs did not induce insulin resistance in our cell model (**chapter 3**).

Interestingly, co-incubation of human muscle cells with a mixture of palmitic acid and oleic acid diverted palmitic acid into the TAG pool as compared to incubations with palmitic acid only. Increasing the concentration of oleic acid on top of a constant palmitic acid concentration, decreased the incorporation of palmitic acid into DAG, whereas the amount of palmitic acid in the TAG pool was increased [31]. In line with this, in C2C12 myotubes, addition of oleate prevented the palmitate-induced increase in DAG accumulation, PKCθ/NF-κB activation and insulin resistance [22]. Furthermore, the authors found that oleate was mainly incorporated into TAG and palmitate into DAG: the TAG/DAG ratio was 17 times higher for oleate. When oleate was added to palmitate, the TAG/DAG ratio increased ~5-fold compared to palmitate alone. Interestingly, palmitate incubation also led to a decrease in the expression of Dgat2 and the expression of genes involved in fatty acid oxidation, Cpt-1 and Pgc-1α, effects that were prevented after co-incubation with oleate. As previously mentioned, whether the other unsaturated FAs we tested, which by themselves did not induce insulin resistance, also protect against DAG accumulation and insulin resistance induced by C16:0 or C18:0, needs to be further investigated.

Altogether, we have shown that fatty acid-induced skeletal muscle insulin resistance, reflected by reduced insulin-stimulated GLUT4 translocation and deoxyglucose uptake, only occurs in response to saturated long-chain but not to

unsaturated or medium-chain fatty acids. Mechanisms underlying these differential effects of FAs on insulin resistance may be differences in oxidation characteristics or incorporation into triglycerides, ultimately resulting in differences in intracellular accumulation of lipid metabolites like DAG.

INDIRECT IMPAIRMENT OF MUSCLE GLUCOSE HOMEOSTASIS VIA REDUCED MUSCLE DIFFERENTIATION BY FATTY ACIDS

Changes in muscle mass induce changes in total glucose disposal. For example, resistance training leading to an increased muscle mass, results in an increased total glucose disposal [37]. Therefore, loss of muscle mass - a process that is known to occur in sepsis, cancer and COPD, but also in type 2 diabetes [38] - may impair insulin sensitivity and glucose homeostasis through the reduction of total glucose uptake. Maintenance of muscle mass requires a balance between protein synthesis and protein breakdown, as well as equilibrium between the loss and gain of myonuclei. For the latter, myonuclear accretion, the process of myogenic differentiation is essential. Inhibition of myogenic differentiation has been implicated in muscle atrophy and can be induced by pro-inflammatory cytokines, like TNF-α or IL-1 and are dependent on NF-κB activation [39-41].

Since we and others have shown that FAs are strong inducers of NF-κB, we hypothesized in **chapter 4** that FAs may reduce myogenic differentiation. Since *trans*-10, *cis*-12 CLA appeared to be the strongest inducer of NF-κB (50 µmol/l *t*10, *c*12-CLA was able to induce a >60-fold induction in NF-κB transactivation in myotubes), we chose to investigate the effects of this FA on muscle differentiation. Indeed, *t*10, *c*12-CLA was able to block the formation of myotubes, which was accompanied by reduced expression of the muscle specific genes creatine kinase, myogenin, myosin heavy chain perinatal, and myosin heavy chain IIB. Furthermore, this abolishment of differentiation coincided with a reduced GLUT4 mRNA expression. This is not surprising, because it is known that the expression of the insulin-sensitive glucose transporter GLUT4 in skeletal muscle increases during muscle differentiation [42, 43]. Since the contribution of skeletal muscle to glucose homeostasis is not only determined by insulin-mediated GLUT4 translocation, but is also sensitive to differences in GLUT4 expression [44-47], decreased muscle differentiation induced by *t*10, *c*12-CLA may indirectly influence insulin dependent glucose homeostasis via a reduced muscle mass and via a reduction in GLUT4 expression. Although no acute effects on insulin sensitivity were observed in myotubes, it will be interesting to investigate if the inhibitory effects of *t*10, *c*12 CLA on muscle differentiation and GLUT4 expression coincide with a reduced insulin-stimulated glucose uptake. Whether other FAs are also able to reduce muscle differentiation and GLUT4 mRNA levels, even if they do not directly induce insulin

resistance in differentiated myotubes, will be important to investigate. Interestingly, a recent study demonstrated that mice fed a HFD, leading to an elevation of circulating FAs, showed impaired regeneration of injured muscles [48]. Also, in cultured primary myotubes, the authors showed that palmitate inhibited muscle cell growth. Unexpectedly, the inhibitory effects of *t*10, *c*12-CLA on muscle differentiation appeared to be independent from activation of the NF-κB pathway, since the *t*10, *c*12-CLA induced inhibition of differentiation did not coincide with an increase in NF-κB activation. Moreover, genetic blockade of NF-κB was not sufficient to restore reduced myosin heavy chain protein expression following *t*10, *c*12-CLA treatment.

NF-κB ACTIVATION IN INSULIN RESISTANCE

A role for NF-κB activation in FA-induced skeletal muscle insulin resistance?

Intramyocellular accumulation of DAG in obese individuals or in rodent models of HFD-induced insulin resistance can result in activation of conventional and novel protein kinase C (PKC) [49, 50]. Especially the increased activity of the novel PKC-θ has been described to lead to reduced insulin signaling via serine phosphorylation of insulin receptor substrate 1 (IRS-1) [19, 51]. Also in cultured C2C12 myotubes, PKC-θ activation was able to inhibit insulin signaling [52]. Furthermore, PKC-θ knockout mice are protected for fat-induced skeletal muscle insulin resistance [53]. Besides direct interference with insulin signaling, another possibility may be that PKC acts upstream of other serine/stress kinases, like IκBα kinase (IKK)-2, as has been described for T-cell activation [54, 55]. IRS-1 is a direct *in vitro* substrate of IKK-2.

It has been demonstrated that an acute elevation in plasma FFA concentrations in humans induced insulin resistance and that this was associated with an accumulation of DAG, an increase in PKC activity, and a reduction in IκBα in skeletal muscle [5]. The latter is a sign of increased NF-κB activation and suggested that accumulation of DAG could lead to insulin resistance via activation of the NF-κB pathway. Moreover, several studies in rodents and humans indeed show that fat-induced insulin resistance is associated with NF-κB activation in skeletal muscle [56, 57]. We tested the importance of this DAG-PKC-NF-κB pathway in muscle cells by investigating whether the ability of several FAs to induce skeletal muscle insulin resistance coincided with their potency to induce activation of the NF-κB pathway.

In **chapter 2** we investigated the role of FA chain length on NF-κB activity in L6 and C2C12 myotubes. Besides increasing NF-κB DNA binding activity in both cell lines, C16:0 and C18:0 also induced NF-κB transcriptional activity, whereas the two medium-chain length SFAs (C8:0 and C12:0) that did not result in insulin resistance, could not activate NF-κB activation. These results demonstrated that SFA-induced

NF-κB activation coincides with insulin resistance and DAG accumulation and depends on chain-length.

To investigate if the association between long-chain SFA-induced NF-κB activation and insulin resistance in skeletal muscle cells could be generalized to all long-chain FAs (≥16 carbon atoms), we investigated in **chapter 3** the effect of saturation and configuration on long chain FA-induced NF-κB activity in relation to insulin sensitivity in cultured skeletal muscle cells. Elaidic acid and *c*9, *t*11-CLA induced a 3-fold NF-κB transactivation, similar to the effects of C16:0 and C18:0, while *t*10, *c*12-CLA incubation even resulted in a 13-fold increase. However, as mentioned before, none of these FAs induced skeletal muscle insulin resistance. These results show that the apparent association between SFA-induced NF-κB activation and insulin resistance in skeletal muscle cells can not be generalized to all long-chain FAs and that FA-induced NF-κB activation is not sufficient for the induction of insulin resistance in skeletal muscle cells (Table 1). Therefore, in **chapter 5** we investigated the proposed causal role of NF-κB activation in the induction of SFA-induced insulin resistance. Both inhibiting DGAT as well as inhibiting CPT-I sensitized myotubes to palmitate-induced insulin resistance. However, while etomoxir co-incubation with palmitate increased NF-κB transactivation, inhibiting DAG conversion did not increase palmitate-induced NF-κB transactivation. Based on these results, it is unlikely that DAG is responsible for NF-κB activation. However, ceramides are also known activators of NF-κB [58, 59] and are therefore a likely candidate for the observed NF-κB activation in response to palmitate. Furthermore, the demonstrated seggregation of palmitate-induced NF-κB transactivation and insulin resistance suggested that NF-κB activation and induction of insulin resistance by palmitate were not causally linked (Table 1).

Table 1: Dissociation of NF-κB and insulin resistance in skeletal muscle cells

Fatty acids:		**Insulin resistance:**	**NF-kappaB:**	**Other:**
MC-SFA	**Caprylic acid**	-	-	
	Lauric acid	-	-	
LC-SFA	**Palmitic acid**	+	+	
	Stearic acid	+	+	
LC-MUFA/PUFA	**Oleic acid**	-	-	
Cis	**Palmitoleic acid**	-	-	
	Linoleic acid	-	-	
	Linolenic acid	-	-	
LC-MUFA/PUFA	**Elaidic acid**	-	+	
Trans	**Vaccenic acid**	-	-	
	***c*9, *t*11-CLA**	-	+	
	***t*10, *c*12-CLA**	-	+++	GLUT4 mRNA ↓
Lipid metabolite manipulation:				
Palmitic acid + amidepsine A		++	-	
Palmitic acid + etomoxir		++	++	

Definitive experiments to address the causality of NF-κB in palmitate-induced insulin resistance were based on pharmacological and genetic approaches to inhibit NF-κB. Incubating myotubes with parthenolide, an agent inhibiting NF-κB activation by inhibiting IKK activity, could not prevent the palmitate-induced reduction of glucose uptake. In line with this, inhibiting the NF-κB pathway by genetic modification, using the IκBα super repressor (SR), a non-degradable mutant of IκBα, could also not improve insulin-stimulated glucose uptake after palmitate treatment. In contrast to parthenolide treatment, this strategy does not rule out a role for IKK, it proves that inhibiting NF-κB activation does not prevent insulin resistance, implying that palmitate-induced NF-κB activation and insulin resistance are not causally related in skeletal muscle cells.

Although initial studies inhibiting IKK-2/NF-κB signaling by heterozygous knockout in rodents [60] or by high doses salicylate in rodents [60, 61] and humans [62] suggested an important role for the NF-κB pathway in skeletal muscle insulin resistance after lipid infusion or a high fat diet, opposite findings have been published arguing against a substantial role for muscular NF-κB signaling in HFD-induced insulin resistance. Muscle-specific inhibition of NF-κB by transgenic expression of the IκBα super-repressor (MISR mice) did not protect against the development of obesity-induced insulin resistance, following a high fat diet for 3 months [63] and muscle-specific IKK-2 deficient mice were not protected against whole body or muscle insulin resistance, induced by gold thioglucose or a HFD [64].

Furthermore, it has to be emphasized that the insulin sensitizing effects of salicylates may work independently from IKK-2/NF-κB inhibition, since aspirin or salicylate is also able to alter the activity of JNK, ERK and S6K [65-67], all serine kinases that are all able to interfere with insulin signaling by inducing serine phosphorylation of IRS-1. In HEK293 cells, reversal of TNF-α-induced inhibition of insulin signaling by salicylates occurred independently of NF-κB inhibition, but coincided with reduced JNK activation [68]. JNK activation has also been implicated in obesity-induced insulin resistance [69]. In insulin resistant skeletal muscle of rats and humans, JNK activity was increased [70, 71]. Furthermore, JNK activity is elevated in obese mice and disruption of the JNK signaling pathway in mice has been shown to reduce insulin resistance [69, 72]. In several *in vitro* studies it was demonstrated that palmitate-induced insulin resistance is associated with increased JNK activity [73, 74]. DAG-induced PKC activation, ceramides or TLRs could all play a role upstream of JNK, since they are known activators of JNK. Recently, it has been shown that shRNA-mediated knock-down of JNK in C2C12 myotubes reduced palmitate-induced insulin resistance [75]. Finally, a recent study with mice containing a muscle-specific JNK1-deficiency provided evidence that JNK1 in muscle is required for HFD-induced muscle insulin resistance, whereas fat tissue and liver were not protected against insulin resistance in these mice [76]. The authors proposed a role for muscle lipoprotein lipase (LPL), since muscle-specific JNK deficiency resulted in reduced muscle LPL expression, leading to a redistribution of triglycerides to non-muscle tissues, resulting in increased TGs in the circulation and hepatocytes [76] with a coherent increase in muscle insulin sensitivity and decrease in insulin sensitivity in other organs.

The role of NF-κB activation in whole body insulin resistance

As discussed above, muscular NF-κB signaling is not essential in muscular insulin resistance and whole body insulin resistance. However, since whole body heterozygous deletion of IKK-2 protected against the development of insulin resistance during high-fat feeding and in ob/ob mice [60], it is clear that NF-κB mediated inflammatory processes still have to be considered as important players in the pathogenesis of whole body insulin resistance.

Indeed, liver cells and myeloid cells have been implicated as potential cells where NF-κB activation may result in generating whole body insulin resistance and muscle insulin resistance in an indirect way. It has been demonstrated that HFDs or obesity result in the activation of NF-κB in the liver and insulin resistance, and over-expression of a constitutive active IKK-2 in the liver (LIKK) of mice resulted in insulin resistance in both liver and muscle [77].

Liver-specific ablation of IKK-2 in mice resulted in protection from HFD-induced hepatic insulin resistance [78]. However, these mice still developed skeletal

muscle and adipose insulin resistance. In contrast, IKK-2 deletion in myeloid cells protected against insulin resistance in all tissues. Because the circulating cytokines were not greatly affected, the authors suggested that the primary mediator of the inflammatory response to elevated lipids may be adipose tissue macrophages and Kupffer cells.

Altogether, it appears that FA-induced activation of the IKK-2/NF-κB pathway in muscle does not contribute to muscle insulin resistance, whereas HFD-induced NF-κB activation in other tissues (liver and macrophages) induces insulin resistance including in skeletal muscle. In vitro experiments investigating muscle cell insulin sensitivity following treatment with conditioned medium from WT and IKK-2 -/- macrophages or -hepatocytes pre-incubated with FAs could give more insight in if and which secreted mediators may play a role in inducing insulin resistance in muscle cells.

INFLAMMATORY MEDIATORS ORIGINATING FROM ADIPOSE TISSUE AND MACROPHAGES

Over the last 15 years, evidence has accumulated that adipose tissue cannot longer be considered as being only a site for energy storage. In contrast, adipose tissue should be seen as a secretory site for a wide variety of lipid and peptide molecules that can regulate energy metabolism and insulin sensitivity [79].

Adipose tissue from obese subjects is characterized by hypertrophied and insulin-resistant adipocytes, having a distinct secretion pattern compared to the small more insulin-sensitive adipocytes [80, 81]. First of all, FAs are released into the circulation after lipolysis, a process that is increased in insulin-resistant adipose tissue [82]. Second, it is now well established that a large number of cytokines and chemokines, the so-called adipokines, can be released by adipose tissue [83]. Thus, skeletal muscle becomes a target of both FAs and cytokines.

The crosstalk between fat and skeletal muscle that may be responsible for skeletal muscle insulin resistance was first demonstrated by co-culturing primary human adipocytes and skeletal muscle cells *in vitro* [84]. Co-culturing resulted in an impairment of muscle IRS-1 and Akt phosphorylation. Reduced insulin-induced Akt phosphorylation and GLUT4 translocation was also shown in a similar model, in which skeletal muscle cells were incubated with conditioned medium from adipose tissue [85, 86]. Altogether, these experiments provided direct evidence that adipose tissue derived factors could induce skeletal muscle insulin resistance.

Adipokines

In 1993, Hotamisligil and colleagues were the first to reveal a link between inflammation and obesity-associated insulin resistance. They found that the expression of the pro-inflammatory cytokine tumor necrosis factor (TNF)-α was increased in adipose tissue in obese and diabetic rodents [87, 88]. Furthermore, it was shown that TNF-α directly interfered with insulin signaling by suppressing insulin-induced tyrosine phosphorylation of the insulin receptor and IRS-1 in adipocytes and hepatocytes [89-91]. Many studies have shown that there is a relationship between elevated serum levels of TNF-α and insulin resistance in type 2 diabetes in humans [92-94]. Studies in C2C12 skeletal muscle cells [95] and neonatal rat myocytes [96] showed that TNF-α could interfere with insulin signaling. In contrast, other *in vitro* studies reported no insulin resistance in skeletal muscle cells after stimulation with TNF-α [97-99]. *In vivo* infusion of TNF-α in rats resulted however in a reduced insulin-induced glucose uptake in skeletal muscle [100]. Direct evidence for the role of TNF-α in causing skeletal muscle insulin resistance in healthy subjects was provided by showing that infusion of TNF-α inhibited insulin-induced glucose disposal, IRS-1 tyrosine phosphorylation, and AS160 phosphorylation in skeletal muscle [101]. Several mechanisms have been attributed to TNF-α induced insulin resistance in muscle, like activation of the catalytic activity of the serine kinases extracellular signal-regulated kinase-1/2 (ERK1/2) [102], IKK [3, 96] and JNK [102, 103], although as outlined before, IKK seems to be the less likely a factor responsible for insulin desensitizing effects. Besides, activation of sphingomyelinase, which produces ceramides may also be responsible for the negative effects of TNF-α on insulin sensitivity [104].

It is known that interleukin-6 (IL-6) is released by adipose tissue of obese people [105] and a relationship between elevated IL-6 levels in the circulation and insulin resistance in humans has been reported [106]. However, the role of IL-6 in (skeletal muscle) insulin resistance remains controversial [107, 108], since both positive [109] and negative effects [110] of IL-6 on skeletal muscle insulin sensitivity have been reported. It has been argued that these differences may lie in acutely (positive) versus prolonged exposure to IL-6 (negative) [111]. The negative effects were attributed to activation of JNK and SOCS-3 expression, whereas the positive effects were ascribed to AMPK-induced AS160 phosphorylation.

The monocyte chemoattractant protein-1 (MCP-1) is secreted by various cells, including adipocytes. Adipose tissue expression of MCP-1 increases in proportion to adiposity and is increased in diet-induced insulin resistance [112-115]. Besides playing a role in the recruitment of immune cells, MCP-1 was shown to have direct insulin-resistance inducing effects in adipocytes [113]. A role for MCP-1 in the negative cross-talk between adipose tissue and skeletal muscle has been proposed, since it was shown that MCP-1 directly induced insulin resistance in skeletal muscle

cells [116]. Recently, it has been shown that the adipokine chemerin can induce insulin resistance in skeletal muscle cells, which suggests that adipocyte-derived secretion of chemerin is involved in the negative cross talk between adipose tissue and skeletal muscle [117].

Besides above mentioned adipokines, many other compounds involved in inflammation are released by adipose tissue and are increased in obese and/or diabetic humans and rodents, like resistin [118], interleukin-8 [119, 120], and plasminogen activator inhibitor 1 [121, 122] ([83] and [123] for an overview). Whether these factors are able to change insulin resistance in skeletal muscle remains to be elucidated.

Macrophage infiltration in adipose tissue and skeletal muscle

Adipose tissue from obese subjects is characterized by increased macrophage infiltration and by the presence of these so-called adipose tissue macrophages (ATM) correlates with insulin resistance [124, 125]. Not only adipocytes, but also resident macrophages are a source of pro-inflammatory cytokines like TNF-α [124, 126], IL-6 and MCP-1. Since these ATMs and adipocytes produce many of the same substances, it is difficult to determine the relative production of cytokines of each cell type. MCP-1, a chemokine secreted by adipocytes, may recruit macrophages to the adipose tissue. Mice lacking a receptor for MCP-1 showed reduced macrophage recruitment in adipose tissue and are partly protected from developing high fat-induced insulin resistance [127]. Macrophages from obese individuals appeared to be in a pro-inflammatory state, since they showed increased NF-κB activity and TNF-α gene expression [128]. As described above, an important role for macrophages in the development of insulin resistance was demonstrated by showing that myeloid-specific deletion of IKK-2 attenuated high-fat, and obesity-induced systemic insulin resistance in mice [78].

It has been demonstrated that besides adipose tissue, skeletal muscle can also contain macrophages. A threefold increase in macrophages in muscle from obese mice [124] and increased infiltration of M1-activated (pro-inflammatory) macrophages in muscle from mice fed a HFD [129, 130] have been found. Recently, an increased infiltration of macrophages has been demonstrated in skeletal muscle of insulin resistant humans [131]. Macrophages infiltrated in skeletal muscle may contribute to muscle insulin resistance based on observations that conditioned medium from macrophages with deleted PPAR-γ, known to be necessary in M2 skewing (anti-inflammatory) of macrophages, caused the production of insulin resistance inducing factors and skeletal muscle insulin resistance [132]. Recent in vitro studies demonstrated that palmitate induced an M1 skewing of macrophages [133]. Conditioned medium of these macrophages induced insulin resistance in L6 myotubes. The latter study demonstrated the potential of FA-induced activation of

macrophages, secondarily affecting muscle insulin sensitivity. Altogether, there is substantial evidence for an inter-organ inflammatory signaling in inducing skeletal muscle insulin resistance.

Figure 1 summarizes all inflammatory mechanisms that may contribute to skeletal muscle insulin resistance.

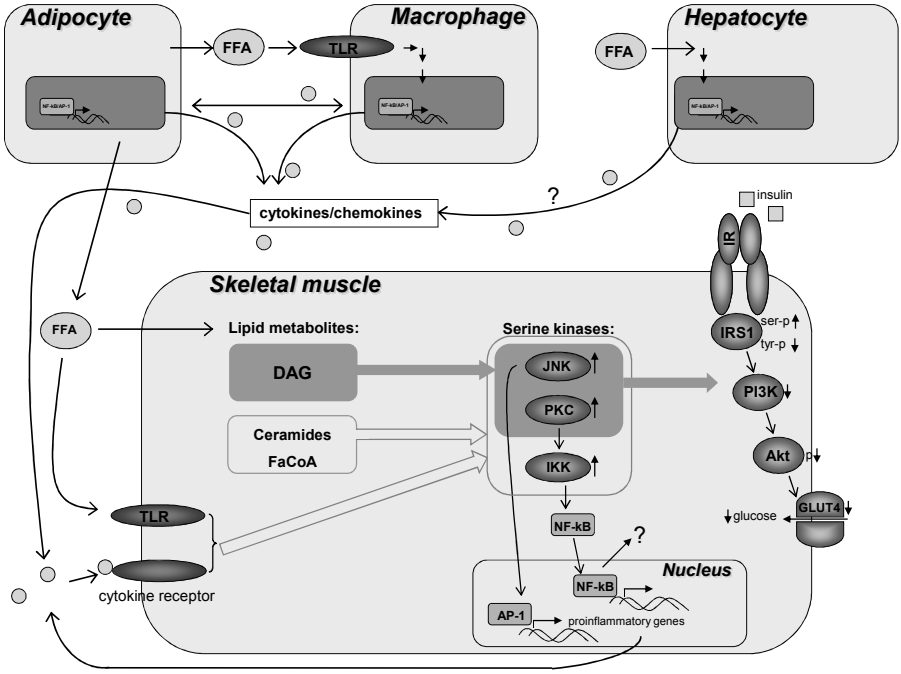

Figure 1: Summary of inflammatory mechanisms that may contribute to skeletal muscle insulin resistance. An increase in serum free fatty acids (FFA) can result in increased FA uptake in skeletal muscle, leading to intramyocellular accumulation of triglycerides. Subsequently, lipid metabolites like diacylglycerol, long-chain acyl-coenzyme A and ceramides may activate PKC and/or JNK. Subsequent serine phosphorylation of IRS proteins results in a reduced tyrosine phosphorylation and an impairment of insulin signaling, ultimately leading to a reduced insulin-stimulated glucose uptake. FA may also be able to induce insulin resistance via the toll-like receptor-mediated serine kinase activation. FA also induce activation of the IKK/NF-κB pathway in skeletal muscle, either via TLR activation or accumulation of lipid metabolites (but not DAG). The consequences of FA-mediated NF-κB activation in skeletal muscle remain unresolved, as NF-κB activation is not involved in FA-induced insulin resistance. Another way in which inflammation (including the NF-κB patyhway) may be involved in FA-induced insulin resistance is via the production of adipokines by the increased adipose tissue mass or by infiltrated macrophages. Some evidence exists that also the hepatocyte may be involved in skeletal muscle insulin resistance via the secretion of cytokines, like IL-6. Furthermore, skeletal muscle insulin resistance may also be induced in an autocrine manner via the production of myokines.

CONCLUDING REMARKS

The results described in this thesis indicate that activation of the NF-κB pathway in muscle does not play a causal role in high fat-induced skeletal muscle insulin resistance. Although SFA-induced NF-κB activation, which was dependent on chain-length, coincided with insulin resistance, several unsaturated FAs increased the activation of the NF-κB pathway without inducing insulin resistance. Furthermore, pharmacological and genetic inhibition of the NF-κB pathway could not prevent palmitate-induced insulin resistance. Since fat-induced NF-κB activation in other organs (hepatocytes, macrophages) may play an important role in muscle insulin resistance, it is important to elucidate the nature of the factors that mediate these effects. Incubation of muscle cells with conditioned medium from macrophages or hepatocytes could be very helpful to answer this question.

Diets containing FAs that are preferentially oxidized or metabolized into TG, thereby preventing the accumulation of harmful lipid metabolites, may be beneficial. Furthermore, it will be interesting to investigate if co-incubation with mono- or polyunsaturated FAs that do not lead to insulin resistance, protects against the insulin desensitizing effects of palmitic or stearic acid.

The knowledge of the direct and indirect (via other tissues) effects of individual FAs on insulin resistance in skeletal muscle cells can ultimately be used in developing dietary intervention strategies to prevent and combat insulin resistance.

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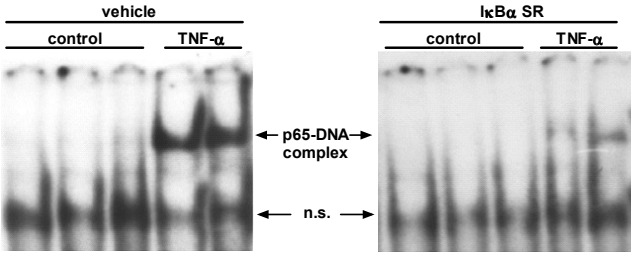
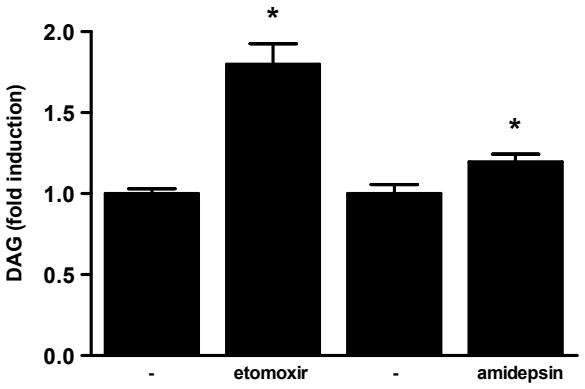
Supplemental data

SUPLEMENTAL FIGURE FROM CHAPTER 4



Supplementary figure: Inhibition of p65 translocation in IκBα-SR L6 myotubes. L6 cells were stably transfected with a plasmid encoding a stabilized mutant of the inhibitor of NF-κB (IκBα-SR) or a vehicle vector (PcDNA 3.1; vehicle). Cells were differentiated for 5 days in differentiation medium. Myotubes were stimulated with 0.1% BSA (control) or 10 ng/ml TNF-α for 1h to induce maximal NF-κB translocation and DNA binding to assess the inhibitory capacity of the IκBα-SR in the corresponding cell line. Nuclear extracts were prepared to assess NF-κB DNA binding capacity by electrophoretic mobility shift assay. n.s.: non-specific band.

SUPLEMENTAL FIGURE FROM CHAPTER 5



Supplementary figure: Etomoxir and amidepsin increase palmitate-induced DAG accumulation. L6 myotubes were differentiated for 5 days in differentiation medium. Myotubes were coincubated with 100 μmol/l palmitate and 80 μmol/l etomoxir or 100 μmol/l palmitate and 10 μg/ml amidepsin. DAG levels were analyzed as described in the materials and methods section.

Summary

SUMMARY

The skeletal muscle plays an important role in maintaining glucose metabolism, accounting for the majority of insulin-stimulated glucose uptake. Skeletal muscle insulin resistance, which can be defined as the reduced ability of muscle cells to respond to physiological levels of insulin, precedes the clinical diagnosis of type 2 diabetes mellitus. Currently, around 250 million people are having diabetes worldwide, a number that is rapidly increasing. In the last decades, it has become evident that increased free fatty acid (FFA) levels in plasma are associated with skeletal muscle insulin resistance. The molecular basis of skeletal muscle insulin resistance is still not elucidated, but substantial evidence supports a role for inflammatory signaling and the accumulation of lipid metabolites in insulin resistance in response to increased FA levels. The work described in this thesis is based on the use of two well-established skeletal muscle cell lines: the rat L6 skeletal muscle cells and the mouse C2C12 skeletal muscle cells. In a series of experiments, we determined the effects of different FAs and the role of lipid metabolite accumulation on insulin resistance. In addition, we investigated the role of the NF-κB pathway (an important inflammatory signaling pathway) in skeletal muscle insulin resistance.

Although previous research revealed that the long-chain saturated fatty acid (SFA) palmitic acid induces insulin resistance and NF-κB activation in muscle cells, a systematic evaluation of the effects of other FAs, classified according to chain length was lacking. Therefore, we evaluated the effects of SFAs differing in chain length on their ability to affect insulin sensitivity and activation of the NF-κB pathway in muscle cells (chapter 2). L6 and C2C12 myoblasts were differentiated into multinucleated myotubes and incubated with four different fatty acids: caprylic acid (C8:0), lauric acid (C12:0), palmitic acid (C16:0), and stearic acid (C18:0). Incubation of myotubes with the long-chain FAs C16:0 and C18:0 induced insulin resistance, as insulin-stimulated GLUT4 translocation and deoxyglucose uptake were decreased. In contrast, myotubes incubated with the medium-chain SFAs C8:0 and C12:0 remained insulin sensitive. Besides increasing NF-κB DNA binding activity in muscle cells, C16:0 also induced NF-κB transcriptional activity. C18:0 showed comparable effects, whereas the SFAs with shorter chain lengths did not elevate NF-κB transcriptional activity. Altogether, this demonstrated that SFA-induced NF-κB activation in muscle cells coincides with insulin resistance and depends on the chain length of the fatty acid.

Next, we investigated if the association between long-chain SFA-induced NF-κB activation and insulin resistance applied to long-chain FAs (≥16 carbon atoms) in general. For this, we studied the effects of saturation and *cis*/*trans* configuration of long-chain FA on NF-κB activity in relation to insulin sensitivity in cultured skeletal muscle cells (chapter 3). Saturated FAs with a certain backbone (C16 or C18) were

compared to their mono-unsaturated [palmitoleic acid (C16:1), oleic acid (C18:1), elaidic acid (*trans*-9 C18:1), and vaccenic acid (*trans*-11 C18:1)] and poly-unsaturated [linoleic acid (C18:2), linolenic acid (C18:3), *cis*-9, *trans*-11 conjugated linoleic acid (CLA), and *trans*-10, *cis*-12 CLA] counterparts. Elaidic acid and *c*9, *t*11-CLA induced a 3-fold NF-κB transactivation, similar to the effects of palmitic acid and stearic acid, while *t*10, *c*12-CLA incubation even resulted in a 13-fold increase of NF-κB transactivation. Remarkably, none of these FAs induced skeletal muscle insulin resistance at the level of GLUT4 translocation or deoxyglucose uptake. These results demonstrated that the apparent association between SFA-induced NF-κB activation and insulin resistance in skeletal muscle cells is not applicable to long-chain FAs in general. Furthermore, this implied that FA-induced NF-κB activation is not sufficient for the induction of insulin resistance in skeletal muscle cells.

The capacity of skeletal muscle to contribute to glucose homeostasis depends on muscular insulin sensitivity. As the expression of glucose transporter (GLUT)-4 increases during myoblast differentiation, a process essential in the maintenance of the adult muscle, processes that affect muscle differentiation may influence insulin dependent glucose homeostasis. Inhibition of myogenic differentiation has been implicated in muscle atrophy and can be induced by pro-inflammatory cytokines, like TNF-α or IL-1 and is dependent on NF-κB activation. As we showed that FAs are strong inducers of NF-κB, we hypothesized in chapter 4 that *t*10, *c*12CLA (> 60-fold induction of NF-κB) reduces myogenic differentiation and consequently GLUT4 expression. The formation of myotubes was blocked after incubation of C2C12 myoblasts with *t*10, *c*12-CLA. This effect was accompanied by a reduced expression of muscle specific genes creatine kinase, myogenin, myosin heavy chain perinatal and myosin heavy chain IIB, as well as decreased GLUT4 mRNA levels. Genetic blockade of NF-κB was not sufficient to restore reduced myosin heavy chain protein expression following *t*10, *c*12-CLA treatment. Surprisingly, in contrast to myotubes, *t*10, *c*12-CLA was not able to activate NF-κB transcriptional activity in myoblasts. Thus, *t*10, *c*12-CLA inhibited myogenic differentiation and GLUT4 expression, independently from NF-κB activation.

Concerning the mechanism underlying the insulin resistance-inducing effects of palmitate, we demonstrated in chapter 5 that pharmacological inhibition of diacylglycerol acyltransferase (DGAT), the enzyme that converts DAG and acyl-CoAs into TAG, with amidepsine A resulted in DAG accumulation. Furthermore, DGAT inhibition resulted in a stronger reduction of insulin-stimulated deoxyglucose uptake in L6 myotubes with increasing palmitate concentrations. Similar effects were found after inhibition of carnitine palmitoyltransferase (CPT)-1 by etomoxir, thereby preventing β-oxidation and stimulating DAG accumulation. These results support the concept that lipid metabolites like DAG play an important role in FA-induced insulin resistance in skeletal muscle cells. Although both inhibiting DGAT as well as inhibiting CPT-1 sensitized myotubes to palmitate-induced insulin resistance,

etomoxir co-incubation with palmitate increased NF-κB transactivation, whereas inhibiting DAG conversion did not increase palmitate-induced NF-κB transactivation. Based on these results, we concluded that it is unlikely that DAG is responsible for the NF-κB activation. Moreover, the apparent segregation of palmitate-induced NF-κB transactivation and insulin resistance suggested that NF-κB activation and induction of insulin resistance by palmitate were not causally linked. Furthermore, in chapter 5 we employed pharmacological and genetic approaches to inhibit NF-κB in order to definitively address the causality of NF-κB in palmitic acid-induced insulin resistance. Incubating myotubes with parthenolide, an agent preventing NF-κB activation by inhibiting IKK activity, could not prevent the palmitate-induced reduction of insulin-stimulated glucose uptake. In line with this, inhibiting the NF-κB pathway by genetic modification, using the IκBα super repressor, a non-degradable mutant of IκBα, did not improve insulin-stimulated glucose uptake after palmitate treatment. Altogether, this implies that palmitate-induced NF-κB activation and insulin resistance are not causally related in skeletal muscle cells.

In chapter 6, an overview of the current literature on the role of inflammatory signaling in skeletal muscle insulin resistance is given. Both intracellular inflammatory signaling in muscle cells as well as inflammatory processes originating from other tissues and their effects on skeletal muscle insulin sensitivity are discussed. Furthermore, potential dietary interventions to improve or prevent insulin resistance in muscle via interfering with inflammatory signaling are described.

In conclusion, the results described in this thesis indicate that activation of the NF-κB pathway in muscle does not play a causal role in fatty acid-induced skeletal muscle insulin resistance.

Samenvatting

SAMENVATTING

Insuline resistentie, dat gedefinieerd kan worden als een verminderd vermogen van cellen om te reageren op een fysiologische concentratie van insuline, speelt een belangrijke rol in de ontwikkeling van type 2 diabetes mellitus. Op dit moment lijden wereldwijd ongeveer 250 miljoen mensen aan diabetes en dit aantal blijft snel stijgen. De skeletspier speelt een cruciale rol bij het glucose metabolisme, aangezien 80% van de totale insuline-gestimuleerde glucose opname in de skeletspier plaatsvindt. Daarnaast ontstaat insuline resistentie in de spier voorafgaand aan de ontwikkeling van diabetes. De laatste decennia is duidelijk geworden dat verhoogde concentraties vrije vetzuren in het bloed geassocieerd zijn met insuline resistentie in de skeletspier. De moleculaire basis van skeletspier insuline resistentie is nog steeds niet volledig ontrafeld. Ondanks dat, zijn er aanwijzingen dat inflammatoire signaleringsroutes en de ophoping van lipide metabolieten in de cel een belangrijke rol spelen. Het onderzoek dat in dit proefschrift is beschreven is gebaseerd op twee *in vitro* modellen voor skeletspier: de L6 rat skeletspier cellijn en de C2C12 muis skeletspier cellijn. In een serie experimenten werden de effecten van verschillende klasse vetzuren en de rol van lipide metaboliet accumulatie op insuline resistentie bepaald. Daarnaast hebben we de rol van NF-κB activatie (een belangrijke inflammatoire signaleringsroute) bij het ontstaan van insuline resistentie onderzocht.

Eerder onderzoek heeft laten zien dat het verzadigde lange keten vetzuur palmitinezuur insuline resistentie en NF-κB activatie induceert in spiercellen. Echter, een systematische evaluatie van de effecten van andere vetzuren, geclassificeerd naar ketenlengte, was afwezig. Daarom hebben we de effecten van verzadigde vetzuren van verschillende ketenlengte op insuline gevoeligheid en activatie van de NF-κB signaleringsroute onderzocht (hoofdstuk 2). Hiervoor hebben we gebruik gemaakt van L 6 en C2C12 cellen. Myoblasten werden gedifferentieerd in meerkernige myotubes, waarna deze geïncubeerd werden met vier verschillende vetzuren: caprylzuur (C8:0), laurinezuur (C12:0), palmitinezuur (C16:0) en stearinezuur (C18:0). De lange keten vetzuren C16:0 en C18:0 induceerden insuline resistentie, aangezien zowel de insuline-gestimuleerde GLUT4 translocatie als de insuline-gestimuleerde glucose opname afnamen. Echter, de myotubes die geïncubeerd werden met de middenlange keten vetzuren C8:0 en C12:0 bleven gevoelig voor insuline. Incubatie met C16:0 en C18:0 resulteerde daarnaast in een verhoging van de NF-κB activiteit in de spiercellen, terwijl de middenlange keten vetzuren deze effecten niet lieten zien. Deze resultaten laten zien dat NF-κB activatie door verzadigde vetzuren samengaat met insuline resistentie en dat deze twee processen afhankelijk zijn van de ketenlengte van het vetzuur.

Vervolgens onderzochten we of de associatie tussen NF-κB activatie en insuline resistentie na incubatie met verzadigde lange keten vetzuren,

doorgetrokken kon worden naar andere lange keten vetzuren. Daarom hebben we de effecten van verzadiging en *cis*/*trans* configuratie van lange keten vetzuren onderzocht. Vetzuren van dezelfde ketenlengte werden vergeleken met hun enkelvoudig en meervoudig onverzadigde equivalenten. Alleen de trans-isomeer van oliezuur, elaïdinezuur, en twee geometrische en positionele isomeren van linolzuur, *c*9, *t*11-CLA en *t*10, *c*12-CLA, lieten een verhoging in NF-κB transactivatie zien. Ondanks dit, veroorzaakten geen van deze vetzuren insuline resistentie in de skeletspiercel op het niveau van GLUT4 translocatie of deoxyglucose opname. Deze resultaten demonstreerden dat de duidelijke associatie tussen NF-κB activatie en insuline resistentie na incubatie met verzadigde vetzuren, niet doorgetrokken kan worden naar lange keten vetzuren in het algemeen. Bovendien impliceerde dit dat vetzuur-geïnduceerde NF-κB activatie niet voldoende is voor het ontstaan van insuline resistentie in spiercellen.

De capaciteit van de skeletspier om bij te dragen aan de glucose homeostase hangt af van de insuline gevoeligheid van de spier. Aangezien de expressie van de glucose transporter (GLUT)-4 omhoog gaat gedurende spiercel differentiatie, een essentieel proces in het onderhoud van de volwassen spier, zouden processen die spier differentiatie beïnvloeden ook invloed kunnen hebben op insuline afhankelijke glucose homeostase. Inhibitie van spier differentiatie is beschreven betrokken te zijn bij spier atrofie en kan geïnduceerd worden door pro-inflammatoire cytokines zoals TNF-α of IL-1 en is afhankelijk van NF-κB activatie. Aangezien we aangetoond hebben dat vetzuren sterke activatoren zijn van NF-κB, hypothetiseerden we in hoofdstuk 4 dat *t*10, *c*12 CLA (> 60-voudige NF-κB activatie) myogene differentiatie en GLUT4 expressie verlaagt. De vorming van myotubes werd geremd na incubatie van C2C12 myoblasten met *t*10, *c*12 CLA. Dit effect werd vergezeld door een verminderde expressie van spierspecifieke genen en verlaagde GLUT4 mRNA niveaus. Genetische blokkade van NF-κB bleek niet voldoende om de gereduceerde myosin heavy chain eiwit expressie na *t*10, *c*12-CLA behandeling te herstellen. Het was verrassend dat *t*10, *c*12-CLA niet in staat was transcriptionele NF-κB activiteit in myoblasten te activeren, dit in tegenstelling tot myotubes. Concluderend inhibeerde *t*10, *c*12-CLA de spierdifferentiatie en GLUT4 expressie onafhankelijk van NF-κB activatie.

Hoofdstuk 5 beschrijft het verdere onderzoek dat we hebben verricht naar de onderliggende mechanismen van de insuline resistentie inducerende effecten van palmitinezuur . Farmacologische remming van diacylglyceroltransferase (DGAT), het enzym dat DAG en acyl-CoA omzet naar TAG, met amidepsine A resulteerde in DAG ophoping. Bovendien resulteerde DGAT remming in een sterkere afname van insuline gestimuleerde deoxyglucose opname in L6 myotubes bij oplopende palmitinezuur concentraties. Vergelijkbare effecten werden gevonden na inhibitie van carnitine palmitoyltransferase (CPT)-1 met etomoxir, waardoor β-oxidatie werd voorkomen en DAG ophoping werd gestimuleerd. Deze resultaten ondersteunen de

opvatting dat lipide metabolieten zoals DAG een belangrijke rol spelen in vetzuur geïnduceerde insuline resistentie in skeletspiercellen. Zowel remming van DGAT als remming van CPT-1 sensibiliseerde myotubes naar palmitinezuur-geïnduceerde insuline resistentie. Echter, co-incubatie van palmitinezuur met etomoxir verhoogde NF-κB transactivatie, terwijl inhibitie van DAG conversie palmitinezuur-geïnduceerde NF-κB transactivatie niet verhoogde. Gebaseerd op deze resultaten concludeerden we dat het onwaarschijnlijk is dat DAG verantwoordelijk is voor de NF-κB activatie. Bovendien suggereerde de gedemonstreerde segregatie van NF-κB transactivatie en insuline resistentie dat activatie van de NF-κB cascade en de inductie van insuline resistentie door palmitinezuur niet causaal verbonden zijn. Vervolgens werd zowel een farmacologische als een genetische aanpak gebruikt om een definitieve uitspraak te kunnen doen over eventuele causaliteit van NF-κB activatie in door palmitaat veroorzaakte insulineresistentie. Parthenolide, een IKK remmer, kon insuline resistentie na toediening van palmitaat niet voorkomen. In overeenstemming met dit resultaat, liet remming van de NF-κB cascade met behulp van de IκBα super repressor, een niet afbreekbare mutant van IκBα, ook geen herstel van palmitaat-geïnduceerde insuline resistentie zien. Deze resultaten impliceren dat NF-κB activatie en insulineresistentie in skeletspiercellen na palmitaat toediening niet met elkaar verbonden zijn.

Hoofdstuk 6 geeft een overzicht van de huidige literatuur over de rol van inflammatie in skeletspier insuline resistentie. Zowel inflammatoire processen in de spiercel als inflammatoire processen die hun oorsprong hebben in andere organen en hun effect uitoefenen op insuline gevoeligheid in de spier, worden bediscussieerd. Daarnaast worden potentiële voedingsinterventies die insuline resistentie in de spier kunnen voorkomen of verminderen besproken.

Samengevat laten de resultaten zoals beschreven in dit proefschrift zien dat activatie van de NF- κB cascade in spier geen causale rol speelt in vet geïnduceerde insuline resistentie in de skeletspier.

Dankwoord

DANKWOORD

Yes, het proefschrift is af! Dan nu het laatste hoofdstuk, het dankwoord. Had me voorgenomen die kort te houden, maar realiseer me nu dat dit wel niet zal gaan lukken. Er zijn namelijk veel mensen die op wat voor wijze dan ook hun bijdrage aan dit proefschrift hebben geleverd en aan wie ik dank verschuldigd ben. Hopelijk vergeet ik niemand…

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List of publications

LIST OF PUBLICATIONS

1. **Hommelberg PPH**, Plat J, Sparks LM, Schols AMWJ, van Essen ALM, Kelders MCJM, van Beurden D, Mensink RP, Langen RCJ. Palmitate-induced skeletal muscle insulin resistance does not require NF-κB activation. *Cell Mol Life Sci, accepted for publication.*

2. **Hommelberg PPH**, Plat J, Remels AHV, van Essen ALM, Kelders MCJM, Mensink RP, Schols AMWJ, Langen .RCJ. *Trans*-10, c*is*-12 conjugated linoleic acid inhibits skeletal differentiation and GLUT4 expression independently from NF-κB activation. *Mol Nutr Food Res*. 2010; 54(12): 1763-72.

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5. **Hommelberg PPH**, Langen RCJ, Schols AMWJ, van Essen AL, Snepvangers FJ, Mensink RP, Plat J. Trans fatty-induced NF-kappaB activation does not induce insulin resistance in cultured murine skeletal muscle cells. *Lipids* 2010; 45(3):285-90.

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Curriculum vitae

CURRICULUM VITAE

Pascal Hommelberg was born on the 17th of July 1978 in Roermond, The Netherlands. He completed secondary school in 1996 at the Stedelijk Lyceum Roermond. After this, he studied Biology and Medical Laboratory Research at the Fontys University of Applied Science and Technology Eindhoven, where he received his BSc degree in 2000. In the same year, he started to study Biology at Nijmegen University. During his first internship at the department of Cell Biology at Nijmegen University, he participated in the characterization of optimized recombinant neuregulin-1β produced by the yeast *Pichia pastoris*. His second internship was done at Maastricht University at the department of Molecular Cell Biology, where he studied the correlation between integration of the human papillomavirus and genomic aberrations in cervical cancer. After receiving his MSc degree in 2004, he started working as a research assistant at the departments of Human Biology and Respiratory Medicine at Maastricht University. He became a PhD fellow at the same departments. His research, which was part of the TI Food and Nutrition project entitled 'Diet, insulin resistance and chronic inflammation,' was aimed on elucidation of the role of the NF-κB pathway in fatty acid-induced insulin resistance in skeletal muscle cells. Since April 2010 he is working as a post-doctoral fellow in the field of inflammation and diabetes at the department of Molecular Genetics at the University Medical Center Groningen.