Université catholique de Louvain
Institute of Neuroscience

Activation of Toll Like Receptors 2 and 4 by fatty acids in skeletal muscle

Hermann Zbinden Foncea

Thèse présentée en vue de l'obtention du grade
de Docteur en Sciences de la Motricité

Promoteur : Prof. Marc Francaux
Co-Promoteur : Dr. Jean-Marc Raymackers

Louvain-la-Neuve
2013

Remerciements

Cette partie de la thèse est une des plus difficiles à écrire mais c'est pourtant avec le plus grand plaisir que j'adresse mes remerciements sincères à tous ceux qui ont été impliqués dans ces longues années de travail. J'aimerais remercier mon promoteur Marc Francaux et mon co-promoteur Jean-Marc Raymackers.

Je remercie Marc de m'avoir donné l'envie de faire de la recherche. Merci de m'avoir transmis la passion de relever de grands défis qui nous permettront, entre autres, d'exporter la science du sport aussi loin qu'au Chili. Merci pour ton soutien dans les moments difficiles, sans oublier les bons moments passés ensemble, en famille, que ce soit au Chili ou en Belgique.

Je voudrais remercier Jean-Marc pour son expertise dans différents domaines de la science, son aide précieuse au labo, son accompagnement. Tes idées m'ont été d'une grande utilité pour avancer dans mes recherches. Je te remercie pour ta générosité dans le partage de tes connaissances et ta simplicité qui resteront pour moi une source d'inspiration.

Ensuite, je tiens à remercier mon comité d'accompagnement les professeurs Léon Plaghki, Sonia Brichard et Michel Hermans. Leurs expertises dans les différents domaines m'ont permis d'avancer dans ma recherche. Je les remercie également pour leurs commentaires lors de la défense privée. Merci aux professeurs Patrice Cani et Xavier Bigard d'avoir accepté d'être membre de mon jury ainsi que pour vos commentaires sur le manuscrit de la thèse.

Mes remerciements s'adressent aussi à l'Université catholique de Louvain, pour l'obtention de la Bourse de la Coopération au Dévelopement qui m'a permis de passer des années de recherches fructueuses et passionnées.

Je voudrais remercier toutes les personnes du labo avec qui j'ai partagé la réussite mais aussi la déception et la persévérance : Louise, Cécile, Laurent, Nico P, Nico B, Gang-Li…les moments passés autour d'un verre le vendredi soir resteront de très bons souvenirs.

Je souhaiterais dire quelques mots à Daniel Theisen. Je te remercie pour ton accueil au début de mon séjour en Belgique mais surtout pour ton amitié pendant toutes ces longues années. Ton soutien dans les moments difficiles, tes encouragements et aussi ton aide pour l'anglais à certains moments… Gracias Dan !
Ensuite, je remercie tous les membres au sein de la FSM qui m'ont très bien accueilli, plus particulièrement les professeurs Thierry Zintz , Patrick Willems et Thierry Marique que m'ont aussi soutenu dans mes projets au Chili.

Je voudrais aussi remercier tous ceux qui ont contribué à faire en sorte que je considère la Belgique comme mon deuxième pays. Merci à tous mes amis, et à toutes les personnes qui de près ou de loin ont participé à mes aventures dans le pays de la bière… Un tout grand merci à la Famille Foncea-Laduron, la Famille Collige, la Famille Serey et la famille Bastin, pour les moments partagés pendant mes années en Belgique.

Merci à mes parents, mes frères et soeurs, mes amis au Chili qui m'ont toujours soutenu. Merci pour m'avoir donné le courage nécessaire à accomplir ce défit.

Finalement, mes plus grands remerciements vont vers ma famille. Merci Christine de ton accompagnement et soutien tout au long de ces années et aussi pour m'avoir fait confiance, ce qui a permis la réussite de ce projet. Esteban et Yann, vous m'avez donné la force dans les moments de faiblesse, le courage dans les moments de doute mais aussi mes plus grandes de joies pendant ces années. Ustedes son y seguirán siendo mi cable a tierra!

A mis padres Cristián y Viviana,
cuyo esfuerzo se ve reflejado en estas páginas….

Table of contents

List of abbreviations

ACC, acetyl-CoA carboxylase

ACS, acyl-CoA synthetase

ADP, adenosine diphosphate

AMPK, adenosine monophosphate-activated protein kinase

AS160, Akt substrate 160

ATM, telangiectasia mutated kinase

ATP, adenosine triphosphate

CACT, acyl-L-carnitine translocase

CaMK II , Ca^{2+} / Calmodulin-dependent protein kinase

cAMP, cyclic adenosine monophosphate

COX-2, inducible cyclooxygenase

CPT, carnitine-palmitoytransferasa

CS, citrate synthetase

DAG, diacylglycerol

DNA, desoxyribonucleic acid

DNA-PK, DNA-dependent protein kinase

eIF4E, eukaryotic initiation factor 4E

ERRα, estrogen-related receptor-α

ERK1/2, extracellular signal-regulated kinase 1/2

FABP, fatty acid binding protein

FATP, fatty acid transport protein

FA, fatty acids

FetA, Fetuin-A

FOXO, forkhead box gene, group O

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

GDP, guanosine diphosphate

GCN5, general control of amino-acid synthesis 5

GEF, guanosine exchange factor

GH, growth hormone

GS, glycogen synthesis

GSK3, glycogen synthase kinase 3

GTP, guanosine triphosphate

GLUT4, glucose transporter type 4

HAD, β-Hydroxy acyl CoA dehydrogenase

hsCRP, serum high-sensitivity C-reactive protein

HSP70, heat schock protein 70

HSL, sensitive hormone lipase

IMCL, intramyocellular lipid

IGF, insulin-like growth factor

IL, interleukin

IL6, interleukin 6

IL-1R, interleukin-1 receptor

IRAK, Interleukin 1 receptor

IRS, insulin receptor substrate

JNK, c-Jun NH_2 terminal

kDa, kilodalton

LAL, limulus amebocyte lysate

LCFA, long-chain fatty acids

LPS, lipopolysaccaharide

LRR, leucine-rich repeats

MAPK, mitogen-activated protein kinase

MCD, malonyl CoA decarboxylase

MEF2, myocyte enhancer factor 2

MEK, extracellular signal-regulated kinase kinase

MGF, mechano growth factor

MKK, mitogen-activated protein kinase kinase

MKKK, mitogen-activated protein kinase kinase kinase

Mnks, mitogen-activated protein kinase signal-integrating kinases

mTOR, mammalian target of rapamycin

mTORC1, mammalian target of rapamycin complex 1

mTORC2, mammalian target of rapamycin complex 2

MyD88, Myeloid differentiation primary response gene 88

NEAA, non-essential amino acid

NFκB, nuclear factor kappa B

NRF-1, nuclear respiratory factor-1

PA, palmitic acid

PAL, peptidoglycan-associated lipoprotein

PAMP, pathogen-associated molecular patterns

PDK1, 3-phosphoinosotidine –dependent protein kinase-1

PGC1α, proliferator-activated receptor-γ coactivator-1α

PPARγ coactivator 1 alpha

PI3K, phosphatidylinositol 3 kinase

PKB, protein kinase B

PKC, protein kinase C

PIP2, phosphatidylinositol-4,5-biphosphate

PPARs, peroxisome proliferator-activated receptor family

PP2A, protein phosphatase 2A

PRR, pattern recognition receptors

$p70^{s6k}$, 70kDa ribosomal protein S6 kinase

Rac1, *Ras-related C3 botulinum toxin substrate 1*

Rheb, Ras homolog enriched in brain

Raptor, regulatory associated protein to mTOR

RER, respiratory exchange ratio

Rictor, rapamycin-insensitive companion of mTOR

RNA, ribonucleic acid

RNA-BP, RNA-binding protein

SGK1, glucocorticoid-induced protein kinase 1

S6, ribosomal protein S6

S6K1, ribosomal protein S6 Kinase 1

siRNA, small interfering RNA

TAK1 kinase, transforming growth factor (TGF)-p-activating kinase 1

TG, triglycerides

TGFβ, transforming growth factor beta

TIR, Toll/IL1R domain

TNFα, tumor necrosis factor alpha

TLR, toll-like receptor

TRAF6, TNF receptor-associated factor 6

TSC1/2, tumor sclerosis complex ½

TSH, thyroid-stimulating hormone

TSC1, tuberous sclerosis complex complex-1

TSC1, tuberous sclerosis complex complex-1

VLC-FA, very-long-chain fatty acids

VLDL, very low density lipoprotein

4E-BP1, eukaryotic initiation factor 4E-binding protein

Chapter 1

Lipolysis and fatty acid consumption at rest and during

1.1. Introduction

Non-esterified fatty acids (NEFA) derived from adipose tissue are a major source of fat for the working muscles during exercise. In this chapter, we will explain the mechanisms that regulate fat metabolism in skeletal muscle during endurance exercise, from the release of NEFA from adipose tissue into the plasma to the regulation of the β-oxidation pathways. We will emphasize the importance of fat transport across the plasma and mitochondrial membranes as major sites of control that regulate fat metabolism and oxidation during exercise. In addition, we will try to explain the complex mechanisms involved in fat oxidation during intense aerobic exercise. Finally, we will describe how high plasma fatty acid concentration induces insulin resistance, affecting the insulin signaling pathways.

1.2. Hormonal regulation of lipolysis

The main energy storage in the human body is constituted of triglycerides in adipose tissue. During exercise, triglycerides are hydrolyzed resulting in the release of NEFA into the plasma, which are then transported to the mitochondria to be oxidized. The lipolysis of adipose tissue is an important part of this mechanism.

The activation of hormone sensitive lipase (HSL) caused by a signaling cascade leading to its phosphorylation and the conversion of one triglyceride molecule into three fatty acids and a glycerol; this happens inside the adipose tissue cells, specifically on the surface of the lipid droplets (Egan et al., 1992). There are other proteins called perilipins, which are mandatory for enabling HSL to catalyze lipolysis (Sztalryd et al.,

2003).

The hormones implicated in lipid metabolism are the catecholamines (epinephrine and norepinephrine), cortisol, growth hormone and thyroid hormones. Nevertheless, other hormones are also involved in specific conditions such as insulin, glucagon, androgens and estrogens.

1.2.1. Regulation of lipolysis by catecholamines

When facing a stressful stimulus such as exercise, the adrenal glands are activated provoking the release of epinephrine, which in turn, unleashes the activation of HSL and increases the lipolysis in adipose tissue. The adrenergic effects of the hormones depend on the kind of receptor implicated (α- or β-receptor). Subcutaneous adipose tissue contains mainly α-$_2$ receptors, which, when activated, have the ability to inhibit lipolysis. On the contrary, intra- abdominal tissue contains more β-receptors, which increase lipolysis (Astrup et al., 1995). The difference of the aforementioned mechanisms comes from the activation of the guanosine triphosphate-binding regulatory protein (G protein). It seems that the β-receptors are capable of activating G protein, which in turn can activate the adenylate cyclase known for converting the adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). The cAMP could act on the cAMP-dependent protein kinase as a second messenger, triggering the phosphorylation of HSL and the perilipins proteins (Sztalryd et al., 2003). On the other hand, the α-receptors cannot activate those mechanisms and even lead to the inhibition of the G protein.

Catecholamines must bind to β-receptors in order to induce lipolysis activation. There are three types of these β-receptors: $\beta_1, \beta_2, \beta_3$. The specific role of each one is unknown, but the affinity of catecholamines differs depending on the type of receptor (Galitzky et al., 1993).

Considering that intra-abdominal adipose tissue contains more β-receptors, the activation of the catecholamines may reduce abdominal fat while subcutaneous adipose tissue, which contains the majority of α-receptors is less sensitive to catecholamines (Arner, 1999). The effect of catecholamines on lipolysis occurs mainly during stressful situations since, in a normal state, these hormones appear to account for only 2-3% of 24-hour energy expenditure (Astrup, 1995).

1.2.2. Regulation of lipolysis by insulin

Lipolysis in adipose tissue is very sensitive to the presence of plasma insulin (Campbell et al., 1992). A decreased in lipolysis close to 50% of the basal values has been observed in response to a small increase of plasma insulin (Bonadonna et al., 1990; Campbell et al., 1992). Others studies have shown that a decrease in insulin, among others during exercise, can induce an increased lipolysis (Wasserman et al., 1989). This phenomenon is explained by the presence of α- adrenergic receptors in the β cells of pancreas. When these receptors are activated, they inhibit insulin production. On the contrary, β- adrenergic receptors are located in the α-cells of the pancreas and, when activated, release glucagon. Therefore, both the decrease of insulin and the glucagon production, work in parallel to increase lipolysis. In healthy subjects, this mechanism is triggered in stress situations as well as during a thermal stress, hypoglycemia or exercise (McMurray and Hackney, 2005).

1.2.3. Other hormones regulating lipolysis

The thyroid hormones, in particular triiodothyronine (T3), can participate to activation of adipose tissue lipolysis by playing a permissive role for

catecholamines. The thyroid hormone, together with catecholamines, activates HSL in adipose tissue and increases the lipoprotein-lipase activity (Pucci et al., 2000; McMurray and Hackney, 2005).

Physiological and psychological stresses lead to an increase of plasma cortisol, which is known for having a role in the lipolysis and an important function in glucogenesis (Bjorntorp and Rosmond, 2000). Cortisol could regulate lipolysis together, or in parallel, with growth hormone (GH) (Djurhuus et al., 2004). The effect of cortisol on lipolysis is controversial: some studies have shown an increase (Djurhuus et al., 2002; Djurhuus et al., 2004; Samra et al., 1996) whilst another has shown a decrease in lipolysis activity (Ottosson et al., 2000). Ottosson et al. used an incubation technique to show that preincubation for 3 days in a control medium containing insulin, followed by exposure to cortisol for 3 days induced a reduction of the basal lipolysis activity in human adipose tissue *in vitro* (Ottosson et al., 2000). The difference in these results may be based on the different models studied and the dose-response of cortisol concentration used.

1.3. Fatty acid transport and oxidation

1.3.1 Transport from circulation to plasma membrane

The plasma NEFA need to cross the endothelium, the interstitial space, the plasma membrane, the cytosol and the mitochondrial membrane before being oxidized.

Circulating NEFA are bound to albumin. Each albumin molecule has a high affinity to bind NEFA and, as the plasma NEFA levels increase during exercise, the bound albumin-NEFA also increases (Curry et al., 1999).

There are various ways for NEFA to cross the endothelium from the vascular space towards the interstitial space: 1) after dissociation from albumin, NEFA are able to cross the endothelium by a simple diffusion or 2) via a fatty acid translocase, CD36; 3) the complex "albumin bound to NEFA" is also able to cross the endothelium. However, considering the three ways, CD36 has been the most involved in the binding/transport of NEFA towards the interstitial space (Kiens, 2006).

1.3.2. Transsarcolemmal transport

NEFA can dissociate from the albumin binding sites and cross the membrane by simple diffusion. Proteins of the myocyte membrane are also known to play a role in this process. In human muscle, two lipid binding proteins are implicated, either acting alone or together, as NEFA acceptors. The first one is the 43kDa membrane bound fatty acid binding protein ($FABP_{pm}$). The second one is the 88kDa fatty acid translocase CD36 (FAT/CD36). Once at the inner side of the cell, NEFA are bound by cytoplasmic $FABP_c$ before entering metabolic or signaling pathways (Fig. 1.1) (for detailed review see (Glatz et al., 2010)). Studies have shown that another family of carrier proteins called fatty acid transport proteins (FATP) work as membrane-bound fatty acid transporters or as acyl-CoA synthetases. These proteins can both import NEFA and activate very long-chain fatty acids (VLC-FA). Within the FATPs family, it seems that mainly FATP1, 2 and 4 are involved in this mechanism (DiRusso et al., 2005). For example, VLC-FA can be directly transported by FATP1 and can be converted by the synthetase activity of this carrier in VLC-acyl-CoA esters (uptake by vectorial acylation) (Fig. 1.1).

Although many studies in various models have been contradictory regarding the FATPs function, it seems that their role is conditioned by both the tissue studied (Lobo et al., 2007; Milger et al., 2006; Pei et al.,

2004) and the affinity of carrier for a substrate, for example, a specific fatty acid (Gimeno et al., 2003).

Current studies show that FATPs share homologous sequences and domain organization with acyl-CoA synthetase (ACS), suggesting that FATPs are members of the superfamily of adenylate-forming acyl-CoA synthetases, particularly long-chain acyl-CoA synthetases (FACS1) (Watkins et al., 2000). This latter is essential as there is a coordinated action between CD36 and FACS1. This mechanism facilitates a greater rate of fatty acid activation in response to a greater rate of fatty acid transport, either among different types of muscles or in muscles in which capacity for fatty acid metabolism has been enhanced (Luiken et al., 2001).

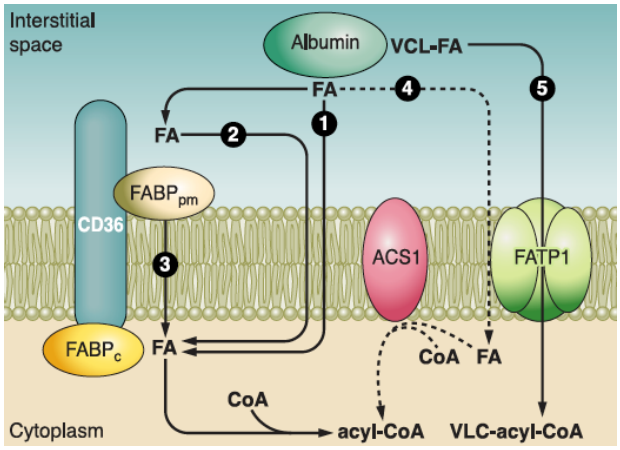


Fig. 1.1. Putative molecular mechanism(s) for the celular uptake of long-chain fatty acids (FA) and of very-long-chain fatty acids (VLC-FA). 1) Fatty acids could cross the plasma membrane by simple diffusion. 2) $FABP_{pm}$ or protein CD36 could act, either alone or together and thus enhance the diffusion of fatty acids. 3) CD36 itself may also facilitate the transport of fatty acids. Once at the inner side of the cell membrane, are bound by cytoplasmic $FABP_c$ before entering metabolic or signaling pathways. 4) A minority of fatty acids could be transport by FATP1 and activated by plasma membrane ACS1 to form acyl-Co esters. 5) VLC-FA are transported by FATP1 before converting into VLC-acyl-CoA esters (Glatz et al., 2010).

1.3.3. Mitochondrial metabolism

After entering the muscle cell, NEFA are activated by reacting with coenzyme A (CoA) to produce fatty acyl-CoA. This process is regulated by acyl-CoA synthetase (ACS) and prepares their import into mitochondria, where they will be oxidized and will provide ATP for different cellular processes.

The carnitine-palmitoytransferase (CPT) is essential to allow the entrance of these fatty acyl-CoAs through the mitochondrial membrane. This mechanism occurs because the acyl-CoA cannot cross the internal membrane of mitochondria. CPT1 catalyzes the transesterification of fatty acyl-CoA to acyl-L-carnitine. The acyl-L-carnitine can be translocated to the inner membrane of mitochondria by carnitine: acyl-L-carnitine translocase (CACT), and finally acyl-CoA is regenerated from acyl-L-carnitine by the latent CPT2 within the mitochondrial matrix (Kerner and Hoppel, 2000). This mechanism can be inhibited by an increase of malonyl CoA, known as CPT1 inhibitor (McGarry et al., 1983). In humans, an inverse relationship has been detected between the malonyl CoA concentration and the fatty acid oxidation at rest (Bavenholm et al., 2000).

To date, there are three ways to control malonyl-CoA formation, the first one by controlling the enzyme acetyl-CoA carboxylase (ACC), which transforms acetyl CoA to malonyl CoA. This enzyme is activated by an increased citrate concentration originating from glucose metabolism (Bavenholm et al., 2000; Rasmussen et al., 2002). Conversly, ACC is negatively regulated by 5`-AMP-activated protein kinase (AMPK) (Rasmussen and Winder, 1997).

In addition, AMPK activity can positively regulate malonyl CoA decarboxylase (MCD), an enzyme able to transform malonyl CoA into acetyl CoA. This results in a decrease in malonyl CoA in muscle and provides substrates for tricarboxylic acid cycle and consequently for β-

oxidation. The third way is related to excess of NEFA and a subsequent increase in citrate contration, which leads to a higher Acetyl CoA availability for malonyl CoA synthesis. See Fig. 1.2.

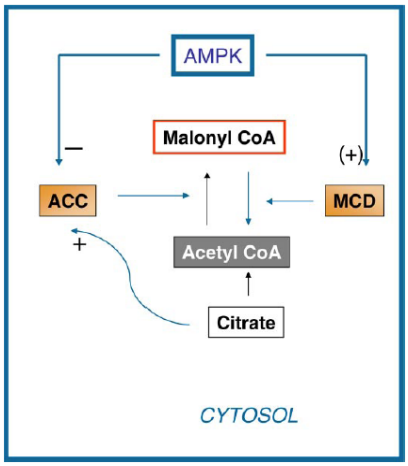


Fig. 1. 2. Regulation of malonyl-CoA content in skeletal muscle. See details in the text. AMP-activated protein kinase (AMPK), malonyl CoA decarboxylase (MCD), acetyl-CoA carboxylase (ACC) (Kiens, 2006).

On the other hand, another way to increase the β-oxidation pathways is to increase the mitochondrial content. Interestingly, an elevated peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) has been shown to increase mitochondrial protein encoded both by nuclear and mitochondrial DNA, indicating a coordinated biogenesis in skeletal muscle (Wu et al., 1999). PGC-1α is a transcriptional coactivator, which interacts with nuclear receptors and transcription factors, including estrogen-related receptor-α (ERRα) (Huss et al., 2004), nuclear respiratory factor-1 (NRF-1) (Wu et al., 1999) and, myocyte enhancer factor 2 (MEF2) (Handschin et al., 2003). To date, PGC-1α transcription is regulated by 1) calcium/calmodulin-dependent protein kinase IV (CaMKIV) and calcineurin A (CnA) activity (Handschin et al., 2003), 2) metabolic stress (Pogozelski et al., 2009) and 3) reactive oxygen species (ROS) (Irrcher et

al., 2009). PGC-1α activity is regulated by posttranslational modifications through phosphorylation (Jager et al., 2007; Puigserver et al., 2001) and deacetylation (Canto et al., 2009). For example, Puigserver et al. showed that p38 MAPK, following activation by its upstream kinase MKK6, phosphorylates PGC-1α at Thr262 and 298 and Ser265 (Puigserver et al., 2001). Another study showed that AMPK cannot induce mitochondrial biogenesis in PGC-1α knockout cells (Jager et al., 2007). On the other hand, cells knockdown for Sirtuin 1 (SIRT1) decreased both *Pgc-1α* mRNA levels and representative enzymes in the β-oxidation pathways (Gerhart-Hines et al., 2007). However, a recent study using SIRT1 knockout mice showed that this protein is not required for mitochondrial biogenesis, suggesting that the acetyltransferase, general control of amino-acid synthesis 5 (GCN5) is an important regulator of PGC-1α (Philp et al., 2011b). The relationship with exercise will be discussed below.

It is known that PGC-1α interacts with the peroxisome proliferator-activated receptors (PPARs) family (Barroso et al., 2011; Duncan, 2011). A recent study showed that PGC-1α/PPAR/NRF-1 signaling was impaired upon classical nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation in myotubes from mouse and human cell lines (Remels et al., 2013). PPARs have been shown to make the link between lipids, metabolic diseases, and innate immunity since they are activated by fatty acids and their derivatives (Wahli and Michalik, 2012). The family of PPARs is composed of PPARα, PPARβ/δ and PPARγ. High levels of PPARα are found in tissues in which the catabolism of fatty acids is high, such as brown adipose tissue, liver, heart, kidney, and intestine (Michalik et al., 2006). PPARβ/δ plays an important role in adipose tissue, skeletal and heart muscles, among others (Michalik et al., 2006). PPARγ is found in two isoforms. PPARγ1 has important functions in the gut, brain, vascular cells, and immune and inflammatory cells, whereas high levels of PPARγ2

are found in adipose tissue (Michalik et al., 2006). Several natural compounds have been identified as PPAR agonist, including unsaturated and saturated fatty acids (Wahli and Michalik, 2012). In addition to these natural ligands, a wide range of synthetic ligands have been developed, some of which are used in dyslipidemia and diabetes (thiazolidine-2,4-diones or TZDs) (Varga et al., 2011). When PPARs are activated by their ligands, they may regulate metabolic activities leading to fatty acid catabolism, lipid storage, and/or other effects, such as those affecting inflammation (Varga et al., 2011). Signaling pathways induced by fatty acids resulting in PPARs activation/inhibition will be discussed below.

1.4. Fatty acids utilization during exercise

1.4.1. Fatty acids released from adipose tissue during exercise

Endurance exercise increases the release of NEFA by adipose tissue and the uptake and oxidation by skeletal muscle. It is generally accepted that the participation of NEFA in energy supply depends on the duration and intensity of exercise. NEFA contribute highly to energy supply during moderate-intensity exercise whereas their contribution decreases during high-intensity exercise. Long duration exercise at a low intensity induces also a decrease of the respiratory exchange ratio (RER), reflecting an increase in lipid utilization and, concomitantly, a decrease in carbohydrate utilization as energetic substrates.

Current research focuses on the changes occuring in lipid metabolism during exercise of different intensities. Romijn et al. (Romijn et al., 1993) studied glycerol R_a (rate of appearance), a known lipolysis marker. There was no change in glycerol between exercise performed at 85%$VO2_{max}$ and 65%$VO2_{max}$. The authors concluded that lipolysis did not decrease during high-intensity exercise. Studies have shown that glycerol R_a reflects the

lipolysis rate of adipose tissue and skeletal muscle, as well as the hydrolysis of very low density lipoprotein (VLDL) binding to triglycerides. It is relevant to mention a major difficulty for measuring lipolysis, by reference to glycerol concentration. Indeed, glycerol can be released but also reabsorbed by skeletal muscle during exercise. This results in reduced net balance of glycerol during the exercise, which leads to an underestimation of the lipolysis rate (Stallknecht et al., 2004; van Hall et al., 2002; Wallis et al., 2007). In the study of Stallnecht et al. the authors compared three intensities: a low one (25% W_{max}), a moderate one (65% W_{max}) and a high one (85% W_{max}). They found that at low intensity there was an increase in glycerol concentration from the skeletal muscle, which did not occur at a moderate and high intensity. In the same study, they observed that the interstitial glycerol concentration was ≈ 10 times higher in the subcutaneous adipose tissue than in the skeletal muscle. Both increased with the intensity of exercise. The authors concluded that glycerol can be reabsorbed by muscle during moderate and high intensity exercise and that at arterial level the skeletal muscle contributes less to the glycerol concentration than the adipose tissue (Stallknecht et al., 2004). High intensity exercise induces an increase of catecholamines in plasma, triggering an inhibition of the lipolysis in the adipose tissue whilst lipolysis increased during a low or moderate exercise intensity (Frayn, 2010). This explains the results observed by other studies that found a decrease of the NEFA release in high intensities when compared to low and moderate intensity (Romijn et al., 1993; van Loon et al., 2001).

1.4.2. Regulation and transport of fatty acids during exercise

During low to moderate intensity exercise, lipids constitute the main energy source in the skeletal muscle metabolism. Plasma NEFA and muscle TG constitute the main lipid energy sources. We will see below

how exercise can modulate NEFA carrier, depending on its duration and intensity.

Training induces an increase in NEFA utilization during exercise (Kiens et al., 1993). This could be due to an increase in the number of the lipid-binding proteins activity facilitating the transport of NEFA from the plasma to the muscle cell.

Bonen et al. showed that in female Harlan Sprague-Dawley rats subjected to muscle contractions, FAT/CD36 promotes an increase of NEFA uptake. This increase was related to both an increase of FAT/CD36 in the plasma membrane and a marked FAT/CD36 reduction in the cytosol. This study also showed that FAT/CD36 acts in a very similar way to glucose uptake regulation by the carrier GLUT4 (Bonen et al., 1999).

An increase in NEFA oxidation was observed in transgenic mice over-expressing FAT/CD36 submitted to the same protocol of muscle contraction than WT controls. However, at rest there was no difference in NEFA oxidation between these two types of mice (Ibrahimi et al., 1999). On the other hand, NEFA oxidation was decreased in FAT/CD36 null mice (Febbraio et al., 1999). In well-trained athletes, a fat-rich diet caused an increase of FAT/CD36 in the skeletal muscle showing that feeding can also modify the NEFA transport and the oxidative metabolism (Cameron-Smith et al., 2003).

Two studies of Keins et al. (1997 and 2004) in muscle of men have shown an increase in the $FABP_{pm}$ proteins with endurance exercise. Three weeks of intense one-legged endurance training increased the content of FABPpm by 49% whereas in the untrained control muscle no change was observed. In the same study the activity of citrate synthase was increased by 20% (Kiens et al., 1997). Another study compared protein and mRNA $FABP_{pm}$ levels between untrained vs. endurance trained men and women. This study showed that $FABP_{pm}$ content was higher in endurance -trained men

compared with all other groups. They also found that *Fabp$_{pm}$* mRNA was higher in untrained women than in endurance-trained women and untrained men. These results were accompanied by a FAT/CD36 protein level 49% higher in women than in men, irrespective of training status. Untrained women, therefore, had higher muscle mRNA levels of several proteins related to muscle lipid metabolism compared with men (Kiens et al., 2004). Finally, an acute effect of exercise (90min) caused a 25% increase of FAT/CD36 and a 15% increase of FABPpm regardless of gender and training level (Kiens et al., 2004). This difference of 10% in the increase of FAT/CD36 in comparison with $FABP_{pm}$ could be caused by the fact that the expression of FAT/CD36 induced by exercise is an early adaptation of the proteins linked to muscle lipid metabolism.

Once inside the cell, NEFA are bound by cytoplasmic $FABP_c$ carriers. They were initially studied to assess the effect of diet manipulation (Clarke et al., 2004; Clavel et al., 2002; Storch and Thumser, 2000), but they have also been studied in relation to exercise.

In the study of Kiens et al., the amount of $FABP_c$ was compared in eight conditions: non-trained men and women v/s trained, before and after exercise. No significant effects of training status were observed on *Fabp$_c$* mRNA. They showed also that the *Fabp$_c$* mRNA level was 101% higher in sedentary women compared to senderaty men. However, no difference was observed between genders in trained subjects. (Kiens et al., 2004).

A recent study of Jeppesen et al. analysed the effect of exercise on the proteins FATP1 and FATP4, both found in skeletal muscle. Eight weeks of aerobic training in young healthy subjects showed an increase in $VO2_{peak}$ and CS activity. The content of FATP4 increased by 33%, while a decrease of 20% was observed in FATP1. In the same study a relationship between the increase in FATP4 and the level of NEFA oxidation was found (Jeppesen et al., 2012). These results indicate that the FATP expression can increase due to training. It also seems that FATP4 is an important

oxidation regulator and NEFA uptake during exercise.

1.4.3. Mitochondrial metabolism and exercise

It has been proposed that an increase in mitochondrial enzymatic activity induced by exercise training is important for the increased ability of trained muscle to consume fatty acids during exercise. β-hydroxy acyl CoA dehydrogenase (HAD) is a key enzyme of β-oxidation. During exercise, an strong correlation has been detected between NEFA uptake, or NEFA oxidation, and the activity of HAD (Kiens, 1997). Nevertheless, the mechanisms by which exercise is able to increase HAD activity are still uncompletely resolved.

Studies have shown that both exercise training (Jong-Yeon et al., 2002; Niu et al., 2010; Spina et al., 1996) and acute exercise (Wallace et al., 2011) are capable of increasing CPT1 activity. Howerver, this effect was not observed in other studies (Huang et al., 2006; Jorgensen et al., 2005; Lennon et al., 1983). Carnitine is a substrate for CPT1, but several studies have shown that carnitine does not limit β-oxidation. Nevertheless, studies have been focused on the effect of exercise on muscle carnitine concentration. These studies have concluded that this concentration increases with the intensity of exercise, decreasing the availability of free carnitine. Thus, a decrease of free carnitine may induce CPT1 activity reduction (Constantin-Teodosiu et al., 1991; van Loon et al., 2001).

Malonyl-CoA formation from the acetyl-CoA in skeletal muscle is catalyzed by ACC. Current studies have shown involvement of AMPK in the ACC inhibition during exercise (Richter and Ruderman, 2009). However, AMPK$\alpha 2^{-/-}$ transgenic mice show similar results of NEFA oxidation compared with WT mice at rest and during muscle contractions (Dzamko et al., 2008). These results together with another more recent study (O'Neill et al., 2011) suggest that AMPK is not the main regulator of

NEFA oxidation during exercise in the skeletal muscle.

According to already mentioned data, malonyl-CoA would not be the main regulator of the NEFA oxidation during exercise. Roepstorff et al. compared two conditions, a high-carbohydrate diet (H-CHO) versus a low-carbohydrate diet (L-CHO), before exercise to determine the effect on muscle glycogen content and whether this leads to high or low level of NEFA oxidation during 60 minutes exercise at 65% of $VO2_{peak}$. Interestingly, they showed a marked increase in NEFA oxidation in the L-CHO condition (+122%) compared to H-CHO condition, whilst the decrease of malonyl-CoA concentration was similar (-13%) (Roepstorff et al., 2005).

In the same study they found that AMPKα2 was more activated in the L-CHO condition (+100%) in comparison to H-CHO condition, whereas the ACC phosphorylation (inhibition) was similar in both conditions (Roepstorff et al., 2005), suggesting that malonyl-CoA concentration does not limit β-oxidation.

At rest and during exercise, several processes can regulate NEFA uptake and oxidation, all of which are important. However, it seems that the concentration of free carnitine is crucial for allowing CPT1 to work properly. This has been proved in exercise protocol at high intensities as a decrease of free carnitine has been observed together with a decreased in β-oxidation.

In the late 1960s, J.O Holloszy was the first to show that 12 weeks of run training increases oxygen uptake while maintaining constant the number of ATP generated per mole of oxygen. Also, he was able to demonstrate that run training doubles mitochondrial enzymes and, concomitantly, a 6-fold increase in time to exhaustion was observed in male rat (Holloszy, 1967). Subsequent studies have confirmed these findings in human skeletal

muscle (Gollnick et al., 1972; Hoppeler et al., 1973).

Exercise can increase the activation of the cAMP response element-binding protein (CREB) (Widegren et al., 1998). One of the targets of CREB is PGC-1α (Louet et al., 2002). Current works in this field are oriented to identify the impact of different types of training and how exercise can increase transcriptional activation of PGC-1α. Endurance exercise increased *Pgc-1α* mRNA and PGC-1α protein 18h after exercise (Baar et al., 2002). Also, a single bout of exercise increased the DNA-binding activity of MEF2 (McGee et al., 2006). This later, can bind to the PGC-1α promoter and increase the expression of *Pgc-1α* mRNA (Handschin et al., 2003).

Another study has shown that PGC-1α was deacetylated after exercise even without active SIRT1 in mice, suggesting that the acetyltransferase GNC5 does not associate with PGC-1α following exercise (Philp et al., 2011b). They also found a low association between GCN5 and PGC-1α. Therefore, phosphorylation of PGC-1α causes its translocation into the nucleus where it does not interact with GCN5 and can bind SIRT1. Consequently PGC-1α becomes deaceteylated and activated (Philp et al., 2011b).

A recent study have examined the time course of responses of mitochondrial mRNA and protein in human muscle to seven sessions of intense interval training (Perry et al., 2010). They showed that *Pgc-1α* mRNA was increased +10-fold 4h after the first session and returned to control within 24h. These effects continued until the seventh bout. However, PGC-1α protein was increased 24h after the first session (23%) and reached +40% between bouts 3 and 7 (Perry et al., 2010). Also, an increase was observed in PPARα and PPARγ protein from the first until the seventh session, whereas that *Pparβ/δ* mRNA was increased 4h after

the first session and returned to control within 24h. These effects continued until the seventh bout. However, PPARβ/δ protein increased only after the fifth session (Perry et al., 2010). The increase of plasma NEFA during exercise results in an increase in fatty acids oxidition and an increase in the activity of the PPARs that are activated by fatty acids (Philp et al., 2012). It seems that PPARα and PPARγ, together with PGC-1α, bind to the promoters of the key enzymes of fat metabolism including CPT1, CD36/FAT and HSL (Philp et al., 2011a).

The paper by Narkar et al. (Narkar et al., 2008) showed that trained rats receiving GW1516 (GW), a drug that activates PPARγ, had an increased capacity to use fat as fuel. Likewise, they observed that CPT1, CD36/FAT and LPL were increased in trained rats receiving GW, in comparison with rats only trained. In addition, they showed that when GW was given together with AICAR, a drug that activates AMPK, an increase in enzymes of fat metabolism was observed in male rats (Narkar et al., 2008). These data suggest that PPARγ interact with AMPK and PGC-1α to increase enzymes of mitochondrial biogenesis resulting an elevated oxidative phenotype in skeletal muscle.

1.5. Signalling pathways induced by fatty acids

Insulin is a major controller of glucose homeostasis. Insulin signalling is initiated by the binding of insulin to the extracellular α-subunits of the heterotetrameric insulin receptor (IR). This linkage induces conformational changes and facilitates autophosphorylation of tyrosine residues on the intracellular part of membrane-spanning β-subunits (Schultze et al., 2012). Insulin receptor substrate (IRS) proteins are attracted by these phosphotyrosines, which are mediated by the tyrosine kinase activity of the IR and possibly via other kinase. When phosphorylated, IRS proteins

activate downstream signalling molecules, thus connecting the IR to various downstream signalling pathways (White, 2002).

One of the downstream signalling pathways is composed of phosphatidylinositol 3-kinase / protein kinase B (PI3K/PKB). PKB, also known as Akt, is necessary for insulin-dependent regulation of systemic and cellular metabolism (Holst et al., 2009). Insulin and other agents (e.g. growth factors, cytokines) can activate PI3K/PKB, playing a role in cell proliferation, motility, differentiation and survival (Parcellier et al., 2008). Therefore, PI3K/PKB are susceptible to being activated by various stimuli due to the recruitment of different isoforms including p85α, p110α, p110β and PKB1, PKB2, PKB3 for PI3K and PKB respectively. These different isoforms provoke downstream activation due to various stimuli (Manning and Cantley, 2007; Schultze et al., 2011; Vanhaesebroeck et al., 2010). Among the PKB isoforms, PKB2 is the main isoform implicated in metabolic insulin action.

Binding an SH2 domain within the regulatory subunit PI3K (p85) activates the PI3K/PKB pathway to phosphotyrosines in IRS1/2. This mechanism allows the recruitment and activation of the catalytic subunit of PI3K (p110). Once activated, PI3K converts phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3) at the plasma membrane. PKB binds through its PH domain (N-terminal pleckstrin homology) to PIP3 allowing PKB activation by upstream kinase. Another protein, 3-phosphoinosotidine –dependent protein kinase-1 (PDK1) induces about 10% of activation by phosphorylating Thr308 in the catalic domain of PKB (Schultze et al., 2012). On the other hand, a full kinase activation of PKB could be achieved by a mechanism involving DNA-dependent protein kinase (DNA-PK) and ataxia telangiectasia mutated kinase (ATM) through in Ser473 phosphorylation in the regulatory domain (Schultze et al., 2012).

Activated PKB is known to target glycogen synthase kinase-3 (GSK-3).

The resulting phosphorylation of GSK-3 leads to its inhibition, a step considered crucial for the stimulation of glycogen synthesis (Cohen et al., 1997). PKB can also play a role in the regulation of the gluconeogeonesis and glucose uptake through forkhead box protein O1 (FOXO1) and Akt substrate 160 (AS160) activation, respectively (Bruss et al., 2005; Nakae et al., 2001). AS160 allows translocation of glucose transporter type 4 (GLUT4) towards muscle membrane.

Mammalian target of rapamycyn complex (mTOR) is another protein related to PKB. mTOR exists in the form of 2 functionally and structurally distinct complexes, rapamycin-sensitive mTOR complex 1 (mTORC1) and rapamycin-insensitve mTOR complex 2 (mTORC2) (Loewith et al., 2002). PKB activates mTORC1 through inhibition of tuberous sclerosis complex (TSC1-TSC2 complex) which inhibits Rheb activating mTORC1.mTORC1 phosphorylates the ribosomal protein S6 (S6K), called also p70S6K and, eIF4-binding protein 1 (4E-BP1) resulting in the regulation of translation, autophagy, growth, lipid biosynthesis, mitochondria biogenesis and ribose biogenesis. On the other hand, mTORC2, phosphorylates serum-and glucocorticoid-induced protein kinase 1 (SGK1), PKB Ser473 (as a marker of mTORC2 activation), Ras-related C3 botulinum toxin substrate 1 (Rac1), a member of the Rho family of GTPases and protein kinase C - α isoform (PKCα) and regulates survival, metabolism, proliferation, and cytoskeletal organization (Ma and Blenis, 2009; Wullschleger et al., 2006). Raptor, a specific component of mTORC1, functions as a scaffold protein to recruit S6K and 4E-BP1 in order to stimulate protein synthesis (Inoki and Guan, 2009).On the other hand, mTORC2 has a specific component called Rictor that competes with Raptor in binding to mTOR through the HEAT domain. Once bound to Rictor, mTORC2 is able to activate PKB and consequently mTORC1 and its downstream target S6K1. The latter may in turn inhibit Rictor as well as IRS resulting in a disruption of mTOR-Raptor interaction (Fig. 1.3) (Watanabe et al., 2011).

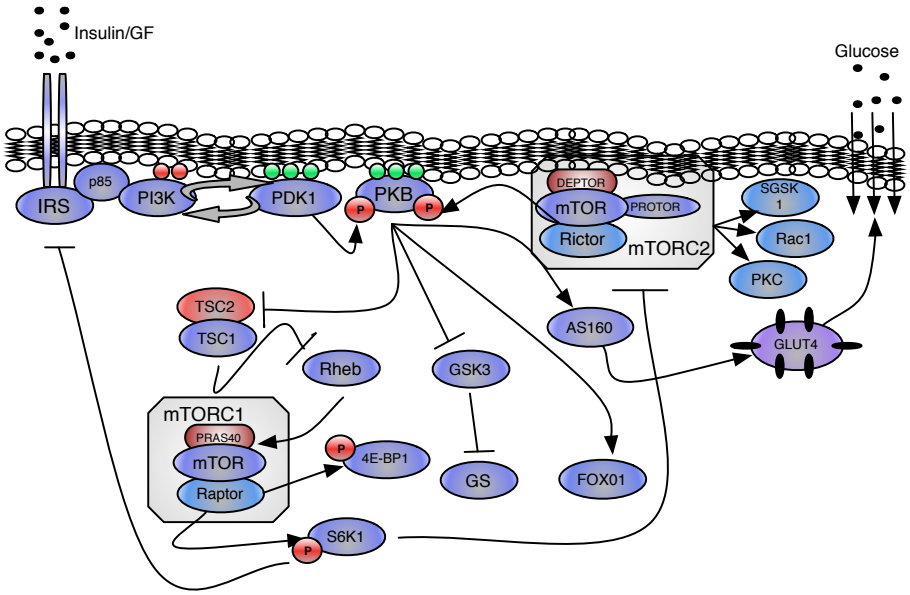


Fig. 1.3. Simplified schema of insulin-stimulated PI3K/PKB/mTOR signalling pathways. See details in the text. Insulin receptor substrate (IRS), phosphatidylinositol 3-kinase (PI3K), 3-phosphoinosotidine –dependent protein kinase-1 (PDK1), protein kinase B (PKB), glycogen synthase kinase-3 (GSK3), glycogen synthesis (GS), forkhead box protein O1 (FOXO1), Akt substrate 160 (AS160), tuberous sclerosis 1 and 2 (TSC1-2), mammalian target of rapamycyn (mTOR), rapamycin-sensitive mTOR complex 1 (mTORC1), rapamycin-insensitve mTOR complex 2 (mTORC2), ribosomal protein S6-1 (S6K1), eIF4-binding protein 1 (4E-BP1), glucocorticoid-induced protein kinase 1 (SGK1), Ras-related C3 botulinum toxin substrate 1 (Rac1), protein kinase C (PKC), glucose transporter type 4 (GLUT4). Small red circles are distinct phosphorylation sites.

Some bioactive lipid metabolites may interfere in the abovementioned signalling pathways. Results from recent studies indicate that intramyocellular lipid (IMCL) content may correlate with insulin resistance only in untrained individuals (Aguer et al., 2010; Moro et al., 2009). While it is true that diet-induced reduction in body weight has been shown to decrease IMCL content (Anastasiou et al., 2010; Lara-Castro et al., 2008; Toledo et al., 2008), exercise has been shown to even increase IMCL content (Goodpaster et al., 2001; Meex et al., 2010). The latter leads us to believe that lipid droplets *per se* may not be damaging. However, IMCL generates bioactive lipid metabolism such as diacylglycerol (DAG) and

ceramides that may intervene with insulin signalling pathways (Eckardt et al., 2011).

Studies in obese insulin-resistant subjects have shown an association between high IMCL content and elevated levels of DAG and ceramides (Adams et al., 2004; Moro et al., 2009). Yu et al. demonstrated that rats infused with a lipid emulsion provoked DAG accumulation resulting in protein kinase C (PKC)-θ activation and a reduction in both insulin-stimulated IRS-1 tyrosine phosphorylation and IRS-1 associated PI3-kinase activity causing decreased insulin-stimulated glucose transport activity (Yu et al., 2002).

GLUT-4, a known transporter of glucose toward the muscle through translocation to the membrane compartment, is impaired when PKB is attenuated, leading to insulin resistance. Studies from Shulman G. laboratory (Dresner et al., 1999; Griffin et al., 1999; Roden et al., 1996) have demonstrated that high levels of plasma fatty acids cause a 50% reduction in insulin-stimulated rates of muscle glycogen synthesis and whole-body glucose oxidation compared to controls due to a fall in intramuscular glucose-6-phosphate. This data suggests that high plasma fatty acid concentration induces insulin resistance by reducing glucose transport and phosphorylation activity, triggering both a reduction in muscle glycogen synthesis and glucose oxidation. Insulin resistance in normal subjects when exposed to high plasma fatty acid levels was similar to that of obese individuals (Petersen et al., 1998). Also, offspring of parents with type 2 diabetes have elevated levels of plasma NEFA. Insulin resistance in these subjects has been found to correlate with elevated plasma NEFA, but not with other indicators of metabolic status (Liang et al., 2013; Perseghin et al., 1997).

NEFA have been shown to affect insulin sensitivity in adipocytes, muscle,

hepatocytes, pancreatic islets (Evans et al., 2002). Actually, the most well known mechanism is a decreased activation of the IRS-PI3K-PKB pathways, but the molecular link between NEFA and IRS-PI3K-PKB pathways is not clear. While this decrease was suspected to lower downstream S6K1 activation, two studies have shown, on the contrary, an increase after palmitic acid incubation in muscle (Ragheb et al., 2009; Wang et al., 2010).

Fatty acid exposure induces the activation of intracellular signalling via stress-related kinases such as p38 MAPK, JNK (MAP Kinase family members) and atypical PKC's (Senn, 2006). Elevated NEFA concentration, may also lead to an elevation in reactive oxygen species (Tripathy et al., 2003), activation of proinflammatory pathways such as the transcriptional factor NF-κB (Senn, 2006; Tripathy et al., 2003), the production of cytokines (Bunn et al., 2010; Maloney et al., 2009; Shi et al., 2006) and mitochondrial dysfunction (Chavez et al., 2010). Recent studies have shown that both PPARα and PPARβ/δ activation by the monounsaturated fatty acid oleate reversed both inflammation and impairment of insulin signaling pathways by channeling palmitate into triglycerides and by up-regulating the expression of genes involved in mitochondrial β-oxidation (Coll et al., 2008; Henique et al., 2010). In addition, a study in human skeletal muscle showed that GW501516, a known PPARβ/δ-agonist, increases NEFA oxidation via PPARβ/δ and AMPK activation (Kramer et al., 2007). On the other hand, an anti-inflammatory agent pioglitazone (PIO), a known PPARγ-agonist, was able to decrease NF-κB activation induced by both peptidoglycan-associated lipoprotein (Pal) and lipopolysaccharide (LPS) (Dasu et al., 2009). PAL and LPS are known agonists of Toll-like receptor 2 and 4 (TLR2 and TLR4), respectively. Also, they observed that PIO inhibits TLR2 and TLR4 expression (Dasu et al., 2009).

While the adverse effects of NEFA in the various mechanisms mentioned above are well known, both the molecular mechanism by which plasma NEFA can affect the muscle cell and the protective effect of exercise are poorly understood.

Various studies have shown an increase in glucose transport induced by exercise (Kristiansen et al., 1996; Richter et al., 1984; Zierath, 2002). There are two well-known mechanisms capable of demonstrating the effect of exercise on metabolism. The first mechanism implicates AMPK protein since exercise/muscle contraction leads to a reduction of ATP relative to AMP levels, resulting in AMPK activation (Long and Zierath, 2006). The second potential mechanism is via a release of Ca^{2+} from the sarcoplasmic reticulum (Youn et al., 1991). This mechanism induces activation of Ca^{2+} / (CaMK)II in skeletal muscles (Wright et al., 2005). Independent of the two phenomena described above, a moderate-intensity exercise of long duration is known to increase lipolysis triggering an increase in plasma NEFA. Few studies have been interested in the acute effect of exercise (or acute increase of NEFA) in different muscle cell signalling and proinflammatory pathways.

We believe that an acute increase in plasma NEFA may trigger a cellular signalling pathway inside the skeletal muscle. In order to support our hypothesis, Shi et al. showed that NEFA induces insulin resistance and inflammation in adipose cell and macrophages through TLR4 activation (Shi et al., 2006). Furthermore, in C57BL/6 mice they showed that 5-hour lipid infusion induces insulin resistance by IRS1 Ser307 phosphorylation triggering a decrease of glucose uptake in skeletal muscle and adipose tissue. These effects do not occur in mice lacking TLR4 (Shi et al., 2006). Later, palmitic acid was found to be an agonist of TLR2 to induce insulin

resistance and to activate proinflammatory pathways in C2C12 cells (Senn, 2006). Consequently, Toll-Like Receptor could participate as a modulator between elevated plasma NEFA and a signal pathway in the skeletal muscle.

References

Adams JM, 2nd, Pratipanawatr T, Berria R, Wang E, DeFronzo RA, Sullards MC, Mandarino LJ. Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. Diabetes. 2004. 53:25-31.

Aguer C, Mercier J, Man CY, Metz L, Bordenave S, Lambert K, Jean E, Lantier L, Bounoua L, Brun JF, Raynaud de Mauverger E, Andreelli F, Foretz M, Kitzmann M. Intramyocellular lipid accumulation is associated with permanent relocation ex vivo and in vitro of fatty acid translocase (FAT)/CD36 in obese patients. Diabetologia. 2010. 53:1151-63.

Anastasiou CA, Kavouras SA, Lentzas Y, Gova A, Sidossis LS, Melidonis A. Moderate weight loss depletes intramyocellular triglycerides but has no effect on diglycerides in type II diabetes. European journal of clinical nutrition. 2010. 64:328-30.

Arner P. Catecholamine-induced lipolysis in obesity. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 1999. 23 Suppl 1:10-3.

Astrup A. The sympathetic nervous system as a target for intervention in obesity. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 1995. 19 Suppl 7:S24-S8.

Astrup A, Buemann B, Gluud C, Bennett P, Tjur T, Christensen N. Prognostic markers for diet-induced weight loss in obese women. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 1995. 19:275-8.

Baar K, Wende AR, Jones TE, Marison M, Nolte LA, Chen M, Kelly DP, Holloszy JO. Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2002. 16:1879-86.

Barroso E, Rodriguez-Calvo R, Serrano-Marco L, Astudillo AM, Balsinde J, Palomer X, Vazquez-Carrera M. The PPARbeta/delta activator GW501516 prevents the down-regulation of AMPK caused by a high-fat diet in liver and amplifies the PGC-1alpha-Lipin 1-PPARalpha pathway leading to increased fatty acid oxidation. Endocrinology. 2011. 152:1848-59.

Bavenholm PN, Pigon J, Saha AK, Ruderman NB, Efendic S. Fatty acid oxidation and the regulation of malonyl-CoA in human muscle. Diabetes. 2000. 49:1078-83.

Bjorntorp P, Rosmond R. Obesity and cortisol. Nutrition. 2000. 16:924-36.

Bonadonna RC, Groop LC, Zych K, Shank M, DeFronzo RA. Dose-dependent effect of insulin on plasma free fatty acid turnover and oxidation in humans. Am J Physiol. 1990. 259:E736-50.

Bonen A, Dyck DJ, Ibrahimi A, Abumrad NA. Muscle contractile activity increases fatty acid metabolism and transport and FAT/CD36. Am J Physiol. 1999. 276:E642-9.

Bruss MD, Arias EB, Lienhard GE, Cartee GD. Increased phosphorylation of Akt substrate of 160 kDa (AS160) in rat skeletal muscle in response to insulin or contractile activity. Diabetes. 2005. 54:41-50.

Bunn RC, Cockrell GE, Ou Y, Thrailkill KM, Lumpkin CK, Jr., Fowlkes JL. Palmitate and insulin synergistically induce IL-6 expression in human monocytes. Cardiovascular diabetology. 2010. 9:73.

Cameron-Smith D, Burke LM, Angus DJ, Tunstall RJ, Cox GR, Bonen A, Hawley JA, Hargreaves M. A short-term, high-fat diet up-regulates lipid metabolism and gene expression in human skeletal muscle. The American journal of clinical nutrition. 2003. 77:313-8.

Campbell PJ, Carlson MG, Hill JO, Nurjhan N. Regulation of free fatty acid metabolism by insulin in humans: role of lipolysis and reesterification. Am J Physiol. 1992. 263:E1063-9.

Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P, Auwerx J. AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity. Nature. 2009. 458:1056-60.

Chavez AO, Kamath S, Jani R, Sharma LK, Monroy A, Abdul-Ghani MA, Centonze VE, Sathyanarayana P, Coletta DK, Jenkinson CP, Bai Y, Folli F, Defronzo RA, Tripathy D. Effect of short-term free Fatty acids elevation on mitochondrial function in skeletal muscle of healthy individuals. The Journal of clinical endocrinology and metabolism. 2010. 95:422-9.

Clarke DC, Miskovic D, Han XX, Calles-Escandon J, Glatz JF, Luiken JJ, Heikkila JJ, Bonen A. Overexpression of membrane-associated fatty acid binding protein (FABPpm) in vivo increases fatty acid sarcolemmal transport and metabolism. Physiological genomics. 2004. 17:31-7.

Clavel S, Farout L, Briand M, Briand Y, Jouanel P. Effect of endurance training and/or fish oil supplemented diet on cytoplasmic fatty acid binding protein in rat skeletal muscles and heart. Eur J Appl Physiol. 2002. 87:193-201.

Cohen P, Alessi DR, Cross DA. PDK1, one of the missing links in insulin signal transduction? FEBS Lett. 1997. 410:3-10.

Coll T, Eyre E, Rodriguez-Calvo R, Palomer X, Sanchez RM, Merlos M, Laguna JC, Vazquez-Carrera M. Oleate reverses palmitate-induced insulin resistance and inflammation in skeletal muscle cells. The Journal of biological chemistry. 2008. 283:11107-16.

Constantin-Teodosiu D, Carlin JI, Cederblad G, Harris RC, Hultman E. Acetyl group accumulation and pyruvate dehydrogenase activity in human muscle during incremental exercise. Acta Physiol Scand. 1991. 143:367-72.

Curry S, Brick P, Franks NP. Fatty acid binding to human serum albumin: new insights from crystallographic studies. Biochim Biophys Acta. 1999. 1441:131-40.

Dasu MR, Park S, Devaraj S, Jialal I. Pioglitazone inhibits Toll-like receptor expression and activity in human monocytes and db/db mice. Endocrinology. 2009. 150:3457-64.

DiRusso CC, Li H, Darwis D, Watkins PA, Berger J, Black PN. Comparative biochemical studies of the murine fatty acid transport proteins (FATP) expressed in yeast. The Journal of biological chemistry. 2005. 280:16829-37.

Djurhuus CB, Gravholt CH, Nielsen S, Mengel A, Christiansen JS, Schmitz OE, Moller N. Effects of cortisol on lipolysis and regional interstitial glycerol levels in humans. Am J Physiol Endocrinol Metab. 2002. 283:E172-7.

Djurhuus CB, Gravholt CH, Nielsen S, Pedersen SB, Moller N, Schmitz O. Additive effects of cortisol and growth hormone on regional and systemic lipolysis in humans. Am J Physiol Endocrinol Metab. 2004. 286:E488-94.

Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, Shulman GI. Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. J Clin Invest. 1999. 103:253-9.

Duncan JG. Peroxisome proliferator activated receptor-alpha (PPARalpha) and PPAR gamma coactivator-1alpha (PGC-1alpha) regulation of cardiac metabolism in diabetes. Pediatric cardiology. 2011. 32:323-8.

Dzamko N, Schertzer JD, Ryall JG, Steel R, Macaulay SL, Wee S, Chen ZP, Michell BJ, Oakhill JS, Watt MJ, Jorgensen SB, Lynch GS, Kemp BE, Steinberg GR. AMPK-independent pathways regulate skeletal muscle fatty acid oxidation. J Physiol. 2008. 586:5819-31.

Eckardt K, Taube A, Eckel J. Obesity-associated insulin resistance in skeletal muscle: role of lipid accumulation and physical inactivity. Reviews in endocrine & metabolic disorders. 2011. 12:163-72.

Egan JJ, Greenberg AS, Chang MK, Wek SA, Moos MC, Jr., Londos C. Mechanism of hormone-stimulated lipolysis in adipocytes: translocation of hormone-sensitive lipase to the lipid storage droplet. Proc Natl Acad Sci U S A. 1992. 89:8537-41.

Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. Endocrine reviews. 2002. 23:599-622.

Febbraio M, Abumrad NA, Hajjar DP, Sharma K, Cheng W, Pearce SF, Silverstein RL. A null mutation in murine CD36 reveals an important role in fatty acid and lipoprotein metabolism. The Journal of biological chemistry. 1999. 274:19055-62.

Frayn KN. Fat as a fuel: emerging understanding of the adipose tissue-skeletal muscle axis. Acta physiologica. 2010. 199:509-18.

Galitzky J, Reverte M, Portillo M, Carpene C, Lafontan M, Berlan M. Coexistence of beta 1-, beta 2-, and beta 3-adrenoceptors in dog fat cells and their differential activation by catecholamines. Am J Physiol. 1993. 264:E403-12.

Gerhart-Hines Z, Rodgers JT, Bare O, Lerin C, Kim SH, Mostoslavsky R, Alt FW, Wu Z, Puigserver P. Metabolic control of muscle mitochondrial function

and fatty acid oxidation through SIRT1/PGC-1alpha. The EMBO journal. 2007. 26:1913-23.

Gimeno RE, Ortegon AM, Patel S, Punreddy S, Ge P, Sun Y, Lodish HF, Stahl A. Characterization of a heart-specific fatty acid transport protein. The Journal of biological chemistry. 2003. 278:16039-44.

Glatz JF, Luiken JJ, Bonen A. Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. Physiological reviews. 2010. 90:367-417.

Gollnick PD, Armstrong RB, Saubert CWt, Piehl K, Saltin B. Enzyme activity and fiber composition in skeletal muscle of untrained and trained men. Journal of applied physiology. 1972. 33:312-9.

Goodpaster BH, He J, Watkins S, Kelley DE. Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. The Journal of clinical endocrinology and metabolism. 2001. 86:5755-61.

Griffin ME, Marcucci MJ, Cline GW, Bell K, Barucci N, Lee D, Goodyear LJ, Kraegen EW, White MF, Shulman GI. Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. Diabetes. 1999. 48:1270-4.

Handschin C, Rhee J, Lin J, Tarr PT, Spiegelman BM. An autoregulatory loop controls peroxisome proliferator-activated receptor gamma coactivator 1alpha expression in muscle. Proc Natl Acad Sci U S A. 2003. 100:7111-6.

Henique C, Mansouri A, Fumey G, Lenoir V, Girard J, Bouillaud F, Prip-Buus C, Cohen I. Increased mitochondrial fatty acid oxidation is sufficient to protect skeletal muscle cells from palmitate-induced apoptosis. The Journal of biological chemistry. 2010. 285:36818-27.

Holloszy JO. Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. The Journal of biological chemistry. 1967. 242:2278-82.

Holst JJ, Vilsboll T, Deacon CF. The incretin system and its role in type 2 diabetes mellitus. Molecular and cellular endocrinology. 2009. 297:127-36.

Hoppeler H, Luthi P, Claassen H, Weibel ER, Howald H. The ultrastructure of the normal human skeletal muscle. A morphometric analysis on untrained

men, women and well-trained orienteers. Pflugers Archiv : European journal of physiology. 1973. 344:217-32.

Huang H, Iida KT, Sone H, Yokoo T, Yamada N, Ajisaka R. The effect of exercise training on adiponectin receptor expression in KKAy obese/diabetic mice. J Endocrinol. 2006. 189:643-53.

Huss JM, Torra IP, Staels B, Giguere V, Kelly DP. Estrogen-related receptor alpha directs peroxisome proliferator-activated receptor alpha signaling in the transcriptional control of energy metabolism in cardiac and skeletal muscle. Mol Cell Biol. 2004. 24:9079-91.

Ibrahimi A, Bonen A, Blinn WD, Hajri T, Li X, Zhong K, Cameron R, Abumrad NA. Muscle-specific overexpression of FAT/CD36 enhances fatty acid oxidation by contracting muscle, reduces plasma triglycerides and fatty acids, and increases plasma glucose and insulin. The Journal of biological chemistry. 1999. 274:26761-6.

Inoki K, Guan KL. Tuberous sclerosis complex, implication from a rare genetic disease to common cancer treatment. Human molecular genetics. 2009. 18:R94-100.

Irrcher I, Ljubicic V, Hood DA. Interactions between ROS and AMP kinase activity in the regulation of PGC-1alpha transcription in skeletal muscle cells. Am J Physiol Cell Physiol. 2009. 296:C116-23.

Jager S, Handschin C, St-Pierre J, Spiegelman BM. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proc Natl Acad Sci U S A. 2007. 104:12017-22.

Jeppesen J, Jordy AB, Sjoberg KA, Fullekrug J, Stahl A, Nybo L, Kiens B. Enhanced fatty acid oxidation and FATP4 protein expression after endurance exercise training in human skeletal muscle. PLoS One. 2012. 7:e29391.

Jong-Yeon K, Hickner RC, Dohm GL, Houmard JA. Long- and medium-chain fatty acid oxidation is increased in exercise-trained human skeletal muscle. Metabolism. 2002. 51:460-4.

Jorgensen SB, Wojtaszewski JF, Viollet B, Andreelli F, Birk JB, Hellsten Y, Schjerling P, Vaulont S, Neufer PD, Richter EA, Pilegaard H. Effects of alpha-AMPK knockout on exercise-induced gene activation in mouse

skeletal muscle. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2005. 19:1146-8.

Kerner J, Hoppel C. Fatty acid import into mitochondria. Biochim Biophys Acta. 2000. 1486:1-17.

Kiens B. Effect of endurance training on fatty acid metabolism: local adaptations. Med Sci Sports Exerc. 1997. 29:640-5.

Kiens B. Skeletal muscle lipid metabolism in exercise and insulin resistance. Physiological reviews. 2006. 86:205-43.

Kiens B, Essen-Gustavsson B, Christensen NJ, Saltin B. Skeletal muscle substrate utilization during submaximal exercise in man: effect of endurance training. J Physiol. 1993. 469:459-78.

Kiens B, Kristiansen S, Jensen P, Richter EA, Turcotte LP. Membrane associated fatty acid binding protein (FABPpm) in human skeletal muscle is increased by endurance training. Biochem Biophys Res Commun. 1997. 231:463-5.

Kiens B, Roepstorff C, Glatz JF, Bonen A, Schjerling P, Knudsen J, Nielsen JN. Lipid-binding proteins and lipoprotein lipase activity in human skeletal muscle: influence of physical activity and gender. Journal of applied physiology. 2004. 97:1209-18.

Kramer DK, Al-Khalili L, Guigas B, Leng Y, Garcia-Roves PM, Krook A. Role of AMP kinase and PPARdelta in the regulation of lipid and glucose metabolism in human skeletal muscle. The Journal of biological chemistry. 2007. 282:19313-20.

Kristiansen S, Hargreaves M, Richter EA. Exercise-induced increase in glucose transport, GLUT-4, and VAMP-2 in plasma membrane from human muscle. Am J Physiol. 1996. 270:E197-201.

Lara-Castro C, Newcomer BR, Rowell J, Wallace P, Shaughnessy SM, Munoz AJ, Shiflett AM, Rigsby DY, Lawrence JC, Bohning DE, Buchthal S, Garvey WT. Effects of short-term very low-calorie diet on intramyocellular lipid and insulin sensitivity in nondiabetic and type 2 diabetic subjects. Metabolism. 2008. 57:1-8.

Lennon DL, Stratman FW, Shrago E, Nagle FJ, Madden M, Hanson P, Carter AL. Effects of acute moderate-intensity exercise on carnitine metabolism in men and women. Journal of applied physiology. 1983. 55:489-95.

Liang H, Tantiwong P, Sriwijitkamol A, Shanmugasundaram K, Mohan S, Espinoza S, Dube JJ, Defronzo RA, Musi N. Effect of a Sustained Reduction in Plasma Free Fatty Acid Concentration onInsulin Signaling and Inflammation in Skeletal Muscle from Human Subjects. J Physiol. 2013.

Lobo S, Wiczer BM, Smith AJ, Hall AM, Bernlohr DA. Fatty acid metabolism in adipocytes: functional analysis of fatty acid transport proteins 1 and 4. Journal of lipid research. 2007. 48:609-20.

Loewith R, Jacinto E, Wullschleger S, Lorberg A, Crespo JL, Bonenfant D, Oppliger W, Jenoe P, Hall MN. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. Mol Cell. 2002. 10:457-68.

Long YC, Zierath JR. AMP-activated protein kinase signaling in metabolic regulation. J Clin Invest. 2006. 116:1776-83.

Louet JF, Hayhurst G, Gonzalez FJ, Girard J, Decaux JF. The coactivator PGC-1 is involved in the regulation of the liver carnitine palmitoyltransferase I gene expression by cAMP in combination with HNF4 alpha and cAMP-response element-binding protein (CREB). The Journal of biological chemistry. 2002. 277:37991-8000.

Luiken JJ, Han XX, Dyck DJ, Bonen A. Coordinately regulated expression of FAT/CD36 and FACS1 in rat skeletal muscle. Molecular and cellular biochemistry. 2001. 223:61-9.

Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. Nature reviews. Molecular cell biology. 2009. 10:307-18.

Maloney E, Sweet IR, Hockenbery DM, Pham M, Rizzo NO, Tateya S, Handa P, Schwartz MW, Kim F. Activation of NF-kappaB by palmitate in endothelial cells: a key role for NADPH oxidase-derived superoxide in response to TLR4 activation. Arterioscler Thromb Vasc Biol. 2009. 29:1370-5.

Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell. 2007. 129:1261-74.

McGarry JD, Mills SE, Long CS, Foster DW. Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. Demonstration of the presence of malonyl-CoA in non-hepatic tissues of the rat. Biochem J. 1983. 214:21-8.

McGee SL, Sparling D, Olson AL, Hargreaves M. Exercise increases MEF2- and GEF DNA-binding activity in human skeletal muscle. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2006. 20:348-9.

McMurray RG, Hackney AC. Interactions of metabolic hormones, adipose tissue and exercise. Sports Med. 2005. 35:393-412.

Meex RC, Schrauwen-Hinderling VB, Moonen-Kornips E, Schaart G, Mensink M, Phielix E, van de Weijer T, Sels JP, Schrauwen P, Hesselink MK. Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity. Diabetes. 2010. 59:572-9.

Michalik L, Auwerx J, Berger JP, Chatterjee VK, Glass CK, Gonzalez FJ, Grimaldi PA, Kadowaki T, Lazar MA, O'Rahilly S, Palmer CN, Plutzky J, Reddy JK, Spiegelman BM, Staels B, Wahli W. International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. Pharmacological reviews. 2006. 58:726-41.

Milger K, Herrmann T, Becker C, Gotthardt D, Zickwolf J, Ehehalt R, Watkins PA, Stremmel W, Fullekrug J. Cellular uptake of fatty acids driven by the ER-localized acyl-CoA synthetase FATP4. Journal of cell science. 2006. 119:4678-88.

Moro C, Galgani JE, Luu L, Pasarica M, Mairal A, Bajpeyi S, Schmitz G, Langin D, Liebisch G, Smith SR. Influence of gender, obesity, and muscle lipase activity on intramyocellular lipids in sedentary individuals. The Journal of clinical endocrinology and metabolism. 2009. 94:3440-7.

Nakae J, Kitamura T, Ogawa W, Kasuga M, Accili D. Insulin regulation of gene expression through the forkhead transcription factor Foxo1 (Fkhr) requires kinases distinct from Akt. Biochemistry. 2001. 40:11768-76.

Narkar VA, Downes M, Yu RT, Embler E, Wang YX, Banayo E, Mihaylova MM, Nelson MC, Zou Y, Juguilon H, Kang H, Shaw RJ, Evans RM. AMPK and PPARdelta agonists are exercise mimetics. Cell. 2008. 134:405-15.

Niu Y, Yuan H, Fu L. Aerobic exercise's reversal of insulin resistance by activating AMPKalpha-ACC-CPT1 signaling in the skeletal muscle of C57BL/6 mice. International journal of sport nutrition and exercise metabolism. 2010. 20:370-80.

O'Neill HM, Maarbjerg SJ, Crane JD, Jeppesen J, Jorgensen SB, Schertzer JD, Shyroka O, Kiens B, van Denderen BJ, Tarnopolsky MA, Kemp BE, Richter EA, Steinberg GR. AMP-activated protein kinase (AMPK) beta1beta2 muscle null mice reveal an essential role for AMPK in maintaining mitochondrial content and glucose uptake during exercise. Proc Natl Acad Sci U S A. 2011. 108:16092-7.

Ottosson M, Lonnroth P, Bjorntorp P, Eden S. Effects of cortisol and growth hormone on lipolysis in human adipose tissue. The Journal of clinical endocrinology and metabolism. 2000. 85:799-803.

Parcellier A, Tintignac LA, Zhuravleva E, Hemmings BA. PKB and the mitochondria: AKTing on apoptosis. Cell Signal. 2008. 20:21-30.

Pei Z, Fraisl P, Berger J, Jia Z, Forss-Petter S, Watkins PA. Mouse very long-chain Acyl-CoA synthetase 3/fatty acid transport protein 3 catalyzes fatty acid activation but not fatty acid transport in MA-10 cells. The Journal of biological chemistry. 2004. 279:54454-62.

Perry CG, Lally J, Holloway GP, Heigenhauser GJ, Bonen A, Spriet LL. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. J Physiol. 2010. 588:4795-810.

Perseghin G, Ghosh S, Gerow K, Shulman GI. Metabolic defects in lean nondiabetic offspring of NIDDM parents: a cross-sectional study. Diabetes. 1997. 46:1001-9.

Petersen KF, Hendler R, Price T, Perseghin G, Rothman DL, Held N, Amatruda JM, Shulman GI. 13C/31P NMR studies on the mechanism of insulin resistance in obesity. Diabetes. 1998. 47:381-6.

Philp A, Burke LM, Baar K. Altering endogenous carbohydrate availability to support training adaptations. Nestle Nutrition Institute workshop series. 2011a. 69:19-31; discussion -7.

Philp A, Chen A, Lan D, Meyer GA, Murphy AN, Knapp AE, Olfert IM, McCurdy CE, Marcotte GR, Hogan MC, Baar K, Schenk S. Sirtuin 1

(SIRT1) deacetylase activity is not required for mitochondrial biogenesis or peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) deacetylation following endurance exercise. The Journal of biological chemistry. 2011b. 286:30561-70.

Philp A, Hargreaves M, Baar K. More than a store: regulatory roles for glycogen in skeletal muscle adaptation to exercise. Am J Physiol Endocrinol Metab. 2012. 302:E1343-51.

Pogozelski AR, Geng T, Li P, Yin X, Lira VA, Zhang M, Chi JT, Yan Z. p38gamma mitogen-activated protein kinase is a key regulator in skeletal muscle metabolic adaptation in mice. PLoS One. 2009. 4:e7934.

Pucci E, Chiovato L, Pinchera A. Thyroid and lipid metabolism. International journal of obesity and related metabolic disorders : journal of the International Association for the Study of Obesity. 2000. 24 Suppl 2:S109-12.

Puigserver P, Rhee J, Lin J, Wu Z, Yoon JC, Zhang CY, Krauss S, Mootha VK, Lowell BB, Spiegelman BM. Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1. Mol Cell. 2001. 8:971-82.

Ragheb R, Shanab GM, Medhat AM, Seoudi DM, Adeli K, Fantus IG. Free fatty acid-induced muscle insulin resistance and glucose uptake dysfunction: evidence for PKC activation and oxidative stress-activated signaling pathways. Biochem Biophys Res Commun. 2009. 389:211-6.

Rasmussen BB, Holmback UC, Volpi E, Morio-Liondore B, Paddon-Jones D, Wolfe RR. Malonyl coenzyme A and the regulation of functional carnitine palmitoyltransferase-1 activity and fat oxidation in human skeletal muscle. J Clin Invest. 2002. 110:1687-93.

Rasmussen BB, Winder WW. Effect of exercise intensity on skeletal muscle malonyl-CoA and acetyl-CoA carboxylase. Journal of applied physiology. 1997. 83:1104-9.

Remels AH, Gosker HR, Bakker J, Guttridge DC, Schols AM, Langen RC. Regulation of skeletal muscle oxidative phenotype by classical NF-kappaB signalling. Biochim Biophys Acta. 2013. 1832:1313-25.

Richter EA, Garetto LP, Goodman MN, Ruderman NB. Enhanced muscle glucose metabolism after exercise: modulation by local factors. Am J Physiol. 1984. 246:E476-82.

Richter EA, Ruderman NB. AMPK and the biochemistry of exercise: implications for human health and disease. Biochem J. 2009. 418:261-75.

Roden M, Price TB, Perseghin G, Petersen KF, Rothman DL, Cline GW, Shulman GI. Mechanism of free fatty acid-induced insulin resistance in humans. J Clin Invest. 1996. 97:2859-65.

Roepstorff C, Halberg N, Hillig T, Saha AK, Ruderman NB, Wojtaszewski JF, Richter EA, Kiens B. Malonyl-CoA and carnitine in regulation of fat oxidation in human skeletal muscle during exercise. Am J Physiol Endocrinol Metab. 2005. 288:E133-42.

Romijn JA, Coyle EF, Sidossis LS, Gastaldelli A, Horowitz JF, Endert E, Wolfe RR. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. Am J Physiol. 1993. 265:E380-91.

Samra JS, Clark ML, Humphreys SM, Macdonald IA, Matthews DR, Frayn KN. Effects of morning rise in cortisol concentration on regulation of lipolysis in subcutaneous adipose tissue. Am J Physiol. 1996. 271:E996-1002.

Schultze SM, Hemmings BA, Niessen M, Tschopp O. PI3K/AKT, MAPK and AMPK signalling: protein kinases in glucose homeostasis. Expert reviews in molecular medicine. 2012. 14:e1.

Schultze SM, Jensen J, Hemmings BA, Tschopp O, Niessen M. Promiscuous affairs of PKB/AKT isoforms in metabolism. Archives of physiology and biochemistry. 2011. 117:70-7.

Senn JJ. Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes. The Journal of biological chemistry. 2006. 281:26865-75.

Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest. 2006. 116:3015-25.

Spina RJ, Chi MM, Hopkins MG, Nemeth PM, Lowry OH, Holloszy JO. Mitochondrial enzymes increase in muscle in response to 7-10 days of cycle exercise. Journal of applied physiology. 1996. 80:2250-4.

Stallknecht B, Kiens B, Helge JW, Richter EA, Galbo H. Interstitial glycerol concentrations in human skeletal muscle and adipose tissue during graded exercise. Acta Physiol Scand. 2004. 180:367-77.

Storch J, Thumser AE. The fatty acid transport function of fatty acid-binding proteins. Biochim Biophys Acta. 2000. 1486:28-44.

Sztalryd C, Xu G, Dorward H, Tansey JT, Contreras JA, Kimmel AR, Londos C. Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. The Journal of cell biology. 2003. 161:1093-103.

Toledo FG, Menshikova EV, Azuma K, Radikova Z, Kelley CA, Ritov VB, Kelley DE. Mitochondrial capacity in skeletal muscle is not stimulated by weight loss despite increases in insulin action and decreases in intramyocellular lipid content. Diabetes. 2008. 57:987-94.

Tripathy D, Mohanty P, Dhindsa S, Syed T, Ghanim H, Aljada A, Dandona P. Elevation of free fatty acids induces inflammation and impairs vascular reactivity in healthy subjects. Diabetes. 2003. 52:2882-7.

van Hall G, Sacchetti M, Radegran G, Saltin B. Human skeletal muscle fatty acid and glycerol metabolism during rest, exercise and recovery. J Physiol. 2002. 543:1047-58.

van Loon LJ, Greenhaff PL, Constantin-Teodosiu D, Saris WH, Wagenmakers AJ. The effects of increasing exercise intensity on muscle fuel utilisation in humans. J Physiol. 2001. 536:295-304.

Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B. The emerging mechanisms of isoform-specific PI3K signalling. Nature reviews. Molecular cell biology. 2010. 11:329-41.

Varga T, Czimmerer Z, Nagy L. PPARs are a unique set of fatty acid regulated transcription factors controlling both lipid metabolism and inflammation. Biochim Biophys Acta. 2011. 1812:1007-22.

Wahli W, Michalik L. PPARs at the crossroads of lipid signaling and inflammation. Trends in endocrinology and metabolism: TEM. 2012. 23:351-63.

Wallace MA, Hock MB, Hazen BC, Kralli A, Snow RJ, Russell AP. Striated muscle activator of Rho signalling (STARS) is a PGC-1alpha/oestrogen-related receptor-alpha target gene and is upregulated in human skeletal muscle after endurance exercise. J Physiol. 2011. 589:2027-39.

Wallis GA, Friedlander AL, Jacobs KA, Horning MA, Fattor JA, Wolfel EE, Lopaschuk GD, Brooks GA. Substantial working muscle glycerol turnover during two-legged cycle ergometry. Am J Physiol Endocrinol Metab. 2007. 293:E950-7.

Wang X, Yu W, Nawaz A, Guan F, Sun S, Wang C. Palmitate induced insulin resistance by PKCtheta-dependent activation of mTOR/S6K pathway in C2C12 myotubes. Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association. 2010. 118:657-61.

Wasserman DH, Lacy DB, Goldstein RE, Williams PE, Cherrington AD. Exercise-induced fall in insulin and increase in fat metabolism during prolonged muscular work. Diabetes. 1989. 38:484-90.

Watanabe R, Wei L, Huang J. mTOR signaling, function, novel inhibitors, and therapeutic targets. Journal of nuclear medicine : official publication, Society of Nuclear Medicine. 2011. 52:497-500.

Watkins PA, Lu JF, Braiterman LT, Steinberg SJ, Smith KD. Disruption of a yeast very-long-chain acyl-CoA synthetase gene simulates the cellular phenotype of X-linked adrenoleukodystrophy. Cell biochemistry and biophysics. 2000. 32 Spring:333-7.

White MF. IRS proteins and the common path to diabetes. Am J Physiol Endocrinol Metab. 2002. 283:E413-22.

Widegren U, Jiang XJ, Krook A, Chibalin AV, Bjornholm M, Tally M, Roth RA, Henriksson J, Wallberg-henriksson H, Zierath JR. Divergent effects of exercise on metabolic and mitogenic signaling pathways in human skeletal muscle. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1998. 12:1379-89.

Wright DC, Geiger PC, Holloszy JO, Han DH. Contraction- and hypoxia-stimulated glucose transport is mediated by a Ca2+-dependent

mechanism in slow-twitch rat soleus muscle. Am J Physiol Endocrinol Metab. 2005. 288:E1062-6.

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell. 1999. 98:115-24.

Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006. 124:471-84.

Youn JH, Gulve EA, Holloszy JO. Calcium stimulates glucose transport in skeletal muscle by a pathway independent of contraction. Am J Physiol. 1991. 260:C555-61.

Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI. Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. The Journal of biological chemistry. 2002. 277:50230-6.

Zierath JR. Invited review: Exercise training-induced changes in insulin signaling in skeletal muscle. Journal of applied physiology. 2002. 93:773-81.

Chapter 2

Interaction between fatty acids and Toll-like receptors

2.1. Introduction

Toll-like receptors (TLR) are transmembrane proteins implicated in the regulation of innate immunity in various patho-physiological states like sepsis and cardiovascular disease (Agnese et al., 2002; Kiechl et al., 2002). TLR are highly expressed in the cells of the innate immune system but are also found in other tissues. For example, TLR2 and TLR4 are also expressed in myocytes (Reyna et al., 2008). In this chapter, we will more deeply summarize the knowledge regarding the role of TLR2 and TLR4 in skeletal muscle. Their different ligands will also be addressed and the link between TLR activation and insulin resistance will be discussed. The signalling pathways downstream TLR will be described in order to present the possible relationships between these receptors and proteins known to be activated during endurance exercise.

2.2. Toll-like receptors

2.2.1. Discovery of Toll-like receptors.

In 1985, Christiane Nüsslein-Volhard of the Max Planck Institute in Tübingen discovered that a mutation of the Toll gene caused abnormal development of the ventral portion of fly larvae (Anderson et al., 1985). A decade later, the laboratory of Jules Hoffman in Strasbourg reported that the protein coded by that gene not only controls dorsoventral polarity but is also essential for the immunity of Drosophila (Lemaitre et al., 1996). Without Toll, flies did not survive fungal infection. With Hoffmann's discovery, for the first time Toll was associated with host defense. In other words, flies with a mutant Toll gene are highly susceptible to fungal infection. Interestingly, Toll activation induced the NF-κB cascade, which was known to be implicated in the defense against fungi. Ruslan Medzhitov and Charles Janeway at Yale University were the first to clone a mammalian homologue called the Toll-like receptor, today known as TLR4 (Medzhitov et al., 1997). At that time, the TLR ligand remained unknown, but by constructing a constitutively active mutant, they were able to show that TLR was implicated in the activation of NF-κB in a similar way as in the ligation of interleukin-1 receptor 1 (IL1R1). In addition, TLR4 is a receptor for lipopolysacharride (LPS), the active component of endotoxin from Gram-negative bacteria (Poltorak et al., 1998). With this finding, we know that TLRs constitute a family of pattern recognition receptors (PRR). The existence of such receptors had been predicted by Charles Janeway in 1992, but their nature had remained enigmatic (Janeway, 1992).

2.2.2. Function and structure

Toll-like receptors had been studied mainly in the immune system. As

mentioned before, in mammals, the TLR family serves as key PRRs playing central roles in the induction of innate immune responses as well as the subsequent development of adaptive immune responses (Akira et al., 2006).

TLRs are transmembrane proteins belonging to the interleukin-1 receptor (IL-1R), which identifies PAPMs by means of its extracellular domain containing leucine-rich repeats (LRR) located at the amino-terminal portion. In the carboxyl terminal end, TLRs contain a domain with homology to IL-1R; the Toll/IL1R domain (TIR) (Takeda and Akira, 2004).

Toll-like receptors are expressed in various tissues (Muzio et al., 2000) and are highly present in cells of the innate immune system (Muzio and Mantovani, 2000). TLR2 and TLR4 are also found in various other cell types including adipocytes, hepatocytes and myocytes (Lang et al., 2003; Lin et al., 2000).

To date, 13 TLRs have been reported: 10 in humans and 13 in mice (Beutler, 2004). TLR1 to TLR9 are highly conserved between humans and mice, but TLR10 is found only in humans and TLR11 is functional only in mice (West et al., 2006).

TLRs may form homo or heterodimers and thus detect a larger variety of pathogens with ligands, possibly because of a higher distribution variability in its LRR (West et al., 2006) (Fig.1).

The recognition of a PAMP by the TLRs leads to recruitment of molecular adapters to the TIR domain, and the subsequent activation of an adequate response to eradicate the intruder. From the intracellular signals activated by TLRs characterized to date, the one depending on the molecular adapter MyD88 (Myeloid differentiation primary response gene 88) is the main signaling pathway for these receptors (Imler and Zheng, 2004; Muzio et al.,

1997). However, other alternate ways may also regulate the defensive response against pathogenic stimuli.

The association of the TIR domain with MyD88 induces the recruiting of kinases associated to the Interleukin 1 receptor (IRAKs) (Burns et al., 2003; Suzuki et al., 2002). IRAK1 and IRAK4 are sequentially phosphorylated and activated, dissociating from the complex and, in turn, associating with TRAF6 (TNF receptor-associated factor 6) (Xiong et al., 2011). TRAF6 forms a complex with ubiquitin-conjugated enzymes to activate the TAK1 kinase (transforming growth factor (TGF)-p-activating kinase 1), which in turn activates the NF-κB transcription factors and AP-1 (activator protein 1) through the IκB kinase complex (IKK) and through kinase proteins activated by mitogen (MAPKs), respectively (Bradley and Pober, 2001; Takaesu et al., 2001).

2.2.3. Types of Toll-like receptors

As mentioned before, TLRs can recognize distinct ligands and are divided into two categories, one that recognizes bacterial PAMPs (TLR2, 4 and 5), mostly located at the cell surface (Akira and Takeda, 2004), and another composed of TLR3, 7/8 and 9, which recognize viruses (Akira and Takeda, 2004) and requires their internalization to endosomal compartments (Rutz et al., 2004).

TLR2, recognizes gram-positive bacterial PAMPs, such as peptidoglycan, lipoproteins, lipopeptides and lipoteichoic acid; it may also respond to PAMPs of diverse origins, such as mycobacteria lipoarabinomannan, zymosan from fungi and glycosylphosphatidylinositol from *Tripanosoma cruzi* (Campos et al., 2001).

The TLR2/TLR1 heterodimer recognizes a variety of triacylated lipoproteins including mycobacteria and meningococci lipoproteins,

whereas the TLR2/TLR6 complex binds with diacylated ones such as mycoplasma lipoproteins (Takeuchi and Akira, 2001). In addition, human TLR10 forms heterodimers with TLR2 and TLR1, although a ligand for these heterodimers remains unknown (Akira et al., 2006).

LPS is the most studied ligand of the TLR4 homodimer. Resistance to endotoxic shock among C3H/HeJ and C57BL/10ScCr mice is the consequence of a natural mutation of gene *tlr4*, which decreases the response to LPS (Poltorak et al., 1998). However, TLR4 alone is not sufficient to induce a response to LPS and other proteins of the LPS recognition complex; CD14 and MD-2 are required (da Silva Correia et al., 2001). The critical component of LPS for the stimulation of TLR4 is the lipid A-subunit, which is almost entirely composed of fatty acids (Lien et al., 2001). Taken together, these data suggest that circulating NEFA are potential ligands for TLR and could mediate intracellular events induced by changes in extracellular lipid concentrations. The fact that palmitic acid have been shown to activate TLR2 in C2C12 myogenic cells (Senn, 2006) and TLR4 in the RAW264.7 macrophage cell line and in 3T3-L1 preadipocytes (Shi et al., 2006) corroborates this hypothesis.

TLR5 recognize protein ligand as well as bacterial flagellin (Uematsu et al., 2006) a protein comprising the bacterial flagellum that is absent in eukaryotes (Smith et al., 2003). The flagellum is one of the most complex structures found in bacteria and is involved in the movement towards an optimal environment for growth and survival.
Another TLR able to recognize protein is TLR11, which is abundant in the kidney and bladder and senses uropathogenic bacteria (Zhang et al., 2004). Another subfamily includes TLR3, 7, 8, and 9 which are localized intracellularly where they detect nucleic acids derived from viruses and bacteria. TLR3 recognize double-stranded (ds) RNA that is generated in

the lifecycle of RNA viruses during infection (Alexopoulou et al., 2001). The TLR7 and TLR8 genes show high homology to each other, and are both located on the X chromosome. Mouse TLR7 and human TLR8 recognize synthetic antiviral imidazoquinoline components and some guanine nucleotide analogs as well as uride-rich ssRNA from both viral and host origins (Diebold et al., 2004; Heil et al., 2004). DNA viruses contain genomes that are rich in CpG-DNA, they can activate inflammatory cytokines and interferon type I (IFN) secretion-mediated TLR9 regulation (Hochrein et al., 2004; Tabeta et al., 2004).

2.3. Toll-like receptor signaling pathways

Activation in almost all TLRs, with the exception of TLR3, stimulates a signaling pathway involving the initial recruitment of the adaptor protein MyD88. There are alternate pathways for transduction of exclusive signals for each TLR that regulate the classic signaling pathway and usually do not depend on MyD88 participation (Fig.2.1).

2.3.1. MyD88 dependent signaling pathway

The TIR domain of TLRs begins the signaling pathway by recruiting the adaptor molecule MyD88 and then protein kinases. MyD88 is a 45-kDa protein presenting three functional domains: a TIR domain at the carboxyl terminal, by which it associates to TLR (Janssens and Beyaert, 2002; O'Neill et al., 2003); a death domain (DD) in the amino terminal, which mediates the interaction with DD present in other proteins (Boldin et al., 1995); and one intermediate domain (DI), which, downstream, activates the protein kinases associated to the receptor of IL-1 (IRAKs). Likewise, a study using mice MyD88$^{-/-}$ have demonstrated that MyD88 is a critical component in signaling cascade that is mediated by IL-1 receptor as well as IL18 receptor (Adachi et al., 1998). The IRAKs family is has four

components: IRAK-1, -2, -4 and –M, all of which have a DD in the amino terminal and a domain with kinase activity in its central portion. However, only IRAK-1 and -4 present kinase activity, since IRAK-2 and –M inhibit the IL-1/TLR signaling pathway. The phosphorylated form of IRAK-4 mediates IRAK-1 phosphorylation, allowing IRAK-1 to separate from the TIR/MyD88/IRAK-4/-1 complex, and thus, subsequently, interact with TRAF6 (Li et al., 2010; Li et al., 2002).

TRAF6 is an adaptor molecule found in cytoplasm, which is activated by IRAK in the TLRs signal cascade. TRAF6 also activates the inhibitor kinases (IKKs) of the nuclear-κB factor (IκB) and those activated by mitogen (MAPKs) (Bradley and Pober, 2001; Dadgostar and Cheng, 1998; Takaesu et al., 2001) (Fig.2.1). Recently, a zebrafish MyD88$^{-/-}$ mutant has been presented as a valuable model for determining the role of TLR and IL1R signaling in numerous important processes (van der Vaart et al., 2013).

2.3.2. MyD88 independent intracellular signaling pathway

Some TLR family components do not need the participation of MyD88 to activate innate immune responses. TLR3 and TLR4 activation involves an alternative signaling pathway that has been intervened by the TRIF adaptor protein (a TIR domain-containing adaptor inducing IFN-β) and TRAM (TRIF-related adaptor molecule), apart from interferon 3 and 7 regulatory factors, which induce the expression of type I IFN and co-stimulating molecules (Lund et al., 2003; Takeda, 2005).

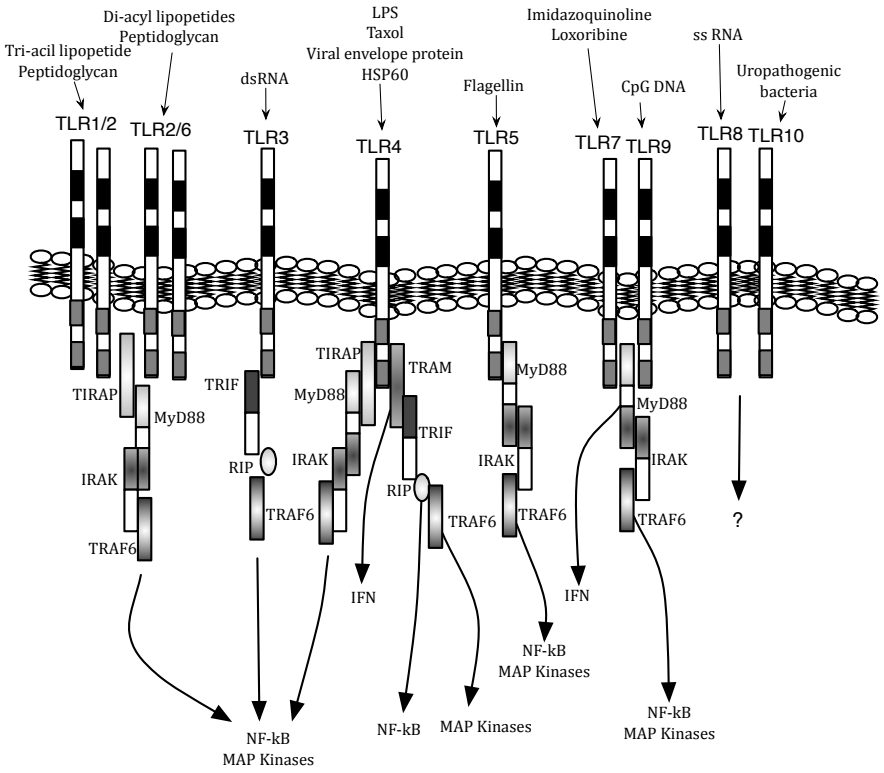


Fig. 2.1. Main ligands and signaling pathways of TLRs. (Adapted from (Kawai and Akira, 2005). See details in the text. Myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adapter protein (TIRAP), protein kinases associated to the receptor of IL-1 (IRAK), TNF receptor associated factor (TRAF), TIR-domain-containing adapter-inducing interferon-β (TRIF), receptor interacting protein (RIP), (Trif)-related adaptor molecule (TRAM), nuclear factor kappa-light-chain-enhancer of activated B cells, mitogen-activated protein kinase (MAP kinase), interferón (IFN), lipopolysaccharides (LPS), double-stranded RNA (dsRNA), heat shock protein 60 (HSP60), single-strandes RNA (ss RNA), short stretch of DNA (CpG DNA).

2.3.3. Toll-like receptor activation, insulin signaling and inflammation

As mentioned above, NEFA can activate TLR2 and TL4 in cellular and animal models, demonstrating that palmitic acid, the main saturated fatty acid, increase the risk of insulin resistance through of a proinflammatory

signaling activation (Davis et al., 2008; Ehses et al., 2010; Himes and Smith, 2010; Kim et al., 2007; Kleinridders et al., 2009; Nguyen et al., 2007; Shi et al., 2006; Suganami et al., 2007). Recently, Holland et al. showed that insulin resistance mediated by TLR4 activation requires saturated fatty acid-induced ceramides biosynthesis in mice (Holland et al., 2011). Nonetheless, studies published in 2009 cast doubt on the relation between NEFA and TLR activation. Erridge et al. suggested that NEFA-induced TLR activation was due to possible BSA contamination used for solubilizing fatty acids in cell culture system (Erridge and Samani, 2009) but a recent study, without user BSA, has confirmed that TLR activation is induced by the presence of NEFA (Huang et al., 2012). On the other hand, NEFA were shown not to bind directly to TLR4/MD-2 in adipose tissue (Schaeffler et al., 2009), but Fetuin-A (FetA), a liver secretory glycoprotein that acts as a major carrier protein of NEFA in the blood circulation (Cayatte et al., 1990) could be an endogenous ligand of TLR4 promoting lipids-induce insulin resistance and inflammation (Pal et al., 2012).

2.4. Mitogen-activated kinases pathway

The intracellular signaling pathway involving mitogen-activated kinases (MAPKs) participates in a variety of cellular processes. The MAPK family is composed of four distinct signaling modulates in skeletal muscle (Kramer and Goodyear, 2007).

- Extracellular-signal-regulated kinases (ERK) 1 and 2 (ERK 1/2)
- c-Jun amino-terminal kinases (JNK)
- p38 MAPK
- ERK5 or big MAPK

Each MAPK family is formed by a group of three progressively conserved

kinases, sequentially activated: one MAPK, one MAPK kinase (MAPKK) and one MAPKK kinase (MAPKKK). MAPKKK are serine/threonine kinases activated through phosphorylation or as a result of their interaction with small G proteins (from the Ras/Rho family), in response to extracellular stimuli (Kolch et al., 2005).

This activation leads to MAPKK phosphorylation and activation that stimulates MAPK through dual phosphorylation in its threonine/tyrosine residues located in the activation site of sub-domain VIII (Seger et al., 1994).

The functions of MAPKs are linked to phosphorylation of different substrates including phospholipases, transcription factors, and cytoskeletal proteins, among others (Kramer and Goodyear, 2007). Recently, a study demonstrated that a novel mitogen-activated protein 4 kinase 4 (Map4k4) is a suppressor of skeletal muscle differentiation in C2C12 myoblast (Wang et al., 2013).

However, the most-studied and better-characterized groups in vertebrates are ERK1/2, JNK and p38 MAPK. These MAPKs are activated by a great range of different stimuli, but in general, ERK1 and ERK2 are induced preferably in response to stressing stimuli, such as osmotic shock, ultraviolet radiation (UV) or cytokines (Pearson et al., 2001). Despite that ERK5 is abundantly expressed in skeletal muscle (Zhou et al., 1995) has not been sufficiently studied following exercise.

The ERK 1/2 family, made up of classic mitogen kinases, activates mostly due to growth factors, serum and phorbol ester, as well as to receptors ligands coupled to G proteins, osmotic stress, cytokines and microtubules disorganization (Roux and Blenis, 2004). In basal conditions, ERK is

distributed in the whole cell, accumulating in the nucleus in response to specific stimuli, to regulate important processes such as cellular proliferation (Chen et al., 1992). Once ERK1 and ERK2 are activated, numerous substrates phosphorylate in several cell compartments including membrane proteins, nuclear proteins and cytoskeletal components.

The JNK family is strongly activated in reaction to cytokines, UV irradiation, growth factor deprivation, DNA damaging-agents, or by G-protein coupled receptors, serum and growth factors (Kyriakis and Avruch, 2001). Once activated, JNK changes its subcellular distribution from the cytoplasm to the nucleus allowing the phosphorylation of transcription factors such as c-Jun (Khurana and Dey, 2004; Mizukami et al., 1997).

p38 MAPK presents four isoforms (α, β, γ and δ) which activate mostly in reaction to stimuli such as environmental stress and inflammatory cytokines, for example, oxidative stress, UV radiation, hypoxia, ischemia and various cytokine, including interleukin-1 (IL-1) and alpha tumor necrosis (TNF-α) (Roux and Blenis, 2004). p38 has been identified both in the cytoplasm (Ben-Levy et al., 1998) and in the cell nucleus (Raingeaud et al., 1995).

There is considerable evidence that p38 MAPK activity is essential both for normal immune response and for inflammatory status. For example, p38 MAPK is activated in macrophages stimulated with cytokines, chemokines and bacterial products in general, and it participates in exocytosis, adherence and apoptosis (Karahashi et al., 2000). p38 MAPK also regulates, at the mRNA level, the expression of cytokines involved in inflammatory processes, as well as the expression of surface localization receptors (Meusel and Imani, 2003; Zarubin and Han, 2005).

Activated p38 MAPK, has been shown to regulate several cellular targets, including ATF1 transcription factors (activating transcription factor 1) and AP-1, which are involved in pro-inflammatory cytokines-expression regulation and innate immunity molecules (Kyriakis and Avruch, 2001).

2.5. Nuclear Factor -κB (NF-kB)

The NF-κB transcription factor is an important gene regulator that alters the production of over 150 genes, including those encoding cytokines, immune, and antigen-presenting receptors, and regulators of redox status, acute phase response, apoptosis, cachexia and disuse atrophy, and host defense (Petersen and Pedersen, 2005), contributing to the pathogenesis of chronic inflammatory processes.

In neutrophils, macrophages, lymphocytes, endothelial cells, epithelial cells, and mesenchymal cells, NF-κB may be activated by a range of pathogen stimuli, including bacterial products and viral proteins besides cytokines, growth factors, ischemia/reperfusion, and oxidative stress (Salminen et al., 2008). The coordinated activation occurring in almost all cell types involves an inflammatory response, which is an integral part of the defensive response to pathogens and stress (Makarov, 2000).

NF-κB is a transcription factor that includes the Rel protein family RelA (p65), c-Real, RelB, NF-κB 1 (p50), and NF-κB2 (p52) (Delhalle et al., 2004). The most abundant form in stimulated cells is the RelA/NF-κB (p65/p50) heterodimer. In cells without stimuli, the NF-κB is found, in a latent form, in the cytoplasm and has to be translocated towards the nucleus in order to function. NF-κB cytoplasmic retention is supplied by the interaction of an inhibiting protein known as IκB (Gilmore, 2006).

Activation of NF-κB complexes is mediated by degradation of IκB. The

IκB family is integrated by IκBα, IκBβ, IκBε, NF-κB p100, NF-κB p100 (Hayden and Ghosh, 2008). Actually, various approaches have been employed to inhibit NF-κB using in vitro and in vivo experimental models (Gilmore and Garbati, 2011).

The signaling process induced by IκB involves successive phosphorylation, ubiquitination and proteasomal degradation steps, which are controlled by three long multiprotein complexes, called IκB kinase (IKK) (Gilmore, 2006).

It has also been shown that NF-κB stimulates the enzymes expression, the products of which contribute to the inflammatory process pathogenesis, including the iNOS inducible form that generates nitric oxide and the inducible cyclooxygenase COX-2 enzyme (Yamamoto and Gaynor, 2001).

2.6. Toll-like receptors and exercise.

The first studies onto relationship between exercise and TLRs date back to 2003 when Kyle Timmerman and his team became interested in a possible reduction in *Tlr4/CD14* mRNA expressed on monocyte in older women after to resistive exercise training (Flynn et al., 2003). One year later, the same team found that trained elderly women showed a cell-surface TLR4 reduction. Nervertheless, the inflammatory response to LPS stimulation was not reduced (McFarlin et al., 2004). However, another study demonstrated that regular physical activity, regardless of age, may have an anti-inflammatory effect caused by cell-surface TLR4 reduction, lower LPS-stimulation of cytokines and lower serum high-sensitivity C-reactive protein (hsCRP) (McFarlin et al., 2006). Furthermore, moderate endurance exercise (~65%VO2max) at a high temperature (34 degrees C) for 1.5 hours decreased TLR1, TLR2 and TLR4 expression on $CD14^+$ monocytes

from peripheral venous blood samples from healthy subjects (Lancaster et al., 2005).

Exhaustive exercise is known to increase the production of cytokines in adipose tissue (Rosa Neto et al., 2009). This mechanism could be mediated by TLR4-signaling resulting in a DNA-binding of NF-κB activation in rat adipose tissue. This provides fuel not only during exhaustive exercise, but also during the recovery period by increasing adipose tissue lipid recruitment (Rosa et al., 2011).

Two recent studies in humans have shown that TLR4 signaling after exercise may play a role in proinflammatory response. Twenty males were divided into a control group and a training group. Both groups were subjected to two acute eccentric exercise protocols separated by a 9-week interval. The training group performed a 6 week long eccentric training program. Acute eccentric exercise increased *Tlr4* mRNA, TLR4 protein, cytokines proinflammatory, IKK and ERK phosphorylation. In addition, a 6 week of eccentric training reduced TLR4-mediated activation of the proinflammatory response without showing an increase in phospho-IKK, phospho-ERK and *Tnf-α* mRNA after the second acute eccentric in peripheral blood mononuclear cells (Fernandez-Gonzalo et al., 2012). However, a 12 week long resistance training program in obese women was not capable of inducing a *Tlr4* mRNA reduction in whole blood (Phillips et al., 2012). Unfortunately, however this study did not measure the cell surface expression of TLR4.

To date, few studies have focused on the effect of exercise on TLR signaling pathways in the muscle (Fig.2), even though TLR2 and TLR4 are also present in myocytes (Reyna et al., 2008). Lambert et al. demonstrated that exercise but not diet-induced weight loss decreases inflammatory gene expression in obese elderly individuals (Lambert et al., 2008). They have shown that 12 week exercise program (a mix of aerobic and anaerobic exercise) was capable of decreasing *Il-6* and *Tnf-α* mRNA expression but

without any change in body weight. Conversely, caloric restriction induced weight loss but without affecting *Il-6* and *Tnf-α*. The reduction in muscle inflammation markers can be due to decreases in *Tlr4* mRNA expression induced by exercise. Weight loss by diet did not caused TLR4 reduction in skeletal muscle, possibly because of a low abundance of CD68+ cell (macrophages) in this tissue (Lambert et al., 2008).

In another study (Zanchi et al., 2010), 12 week of resistance training predominantly composed of concentric stimuli, in healthy rats showed a decreased expression of *Tnf-α* (-40%) and *Tlr4* mRNA (-60%). The importance of this study was the protocol used; a low frequency/low volume program known as "non-damaging" resistance training capable to preventing muscle inflammation (important as a form of therapy in inflammatory disease). Furthermore, acute and chronic exercise could suppress TLR4 signaling pathways in the liver, muscle, and adipose tissue, reduces LPS serum levels, and improved insulin signaling and sensitivity in DIO rats. These phenomena were accompanied by a reduction in JNK and IKKβ phosphorylation and IRS-1 serine 307 phosphorylation (Oliveira et al., 2011).

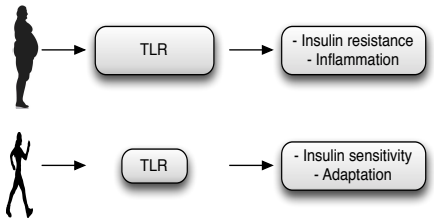


Fig. 2.2. Hypothetical role of Toll-like receptors in skeletal muscle. Chronic activation of TLR may induce insulin resistance and inflammation. However, an acute TLR activation could protect against these diseases and trigger adpatations in skeletal muscle.

In conclusion, few studies have examined the effect of exercise mediated by TLR on metabolic pathways such as protein synthesis, fatty acids

synthesis and glycogen synthesis in skeletal muscle. Currently, studies are mainly focused on glucose uptake and inflammation. However, TLR are found in non-immune cells making them an important target in distinct metabolic pathways.

2.7. Crosstalk between p38 MAPK and PKB-mTOR-S6K1 signaling pathways

As previously described p38 MAPK functions in a number of cellular processes including cell growth, cell differentiation, cell cycle and apoptosis. Many extracellular stimuli, including growth factors and hormones, could activate the p38 pathways. Studies indicate that p38 participates in intracellular processes affecting the myogenesis (Zarubin and Han, 2005). One approach is that the role of p38 during differentiation involves MyoD and the MEF family. MyoD and MEF are transcription factors affected by p38 MAPK isoforms (p38α and β), using a known inhibitor of these isoforms (SB203580), have been involved in myoblast differentiation (Cuenda and Cohen, 1999a; Wu et al., 2000). Beside p38, there are other important signaling pathways involved in myogenesis processes. The relationship of these pathways to the p38 pathways is interesting. For example, a major signaling pathway in differentiation processes includes PI3-K impacting on PKB-mTOR-S6K1 pathways (Fig.2.3).

A study by Tamir and Bengal (Tamir and Bengal, 2000) shows that PI3-K affects skeletal muscle differentiation by inducing phosphorylation and transcriptional activity of MEF2 proteins in a parallel but distinct route from p38 MAPK (Tamir and Bengal, 2000). In addition, they have shown that p38 is not affected by PI3-K in skeletal muscle.

With respect to a possible crosstalk between p38-PKB, two studies have

been able to identify this relationship. Once p38 is activated, it may induce PKB activation in C2C12 cells during myogenic differentiation. The same study has shown that this mechanism was not reciprocal since PKB did not affect p38 activation (Cabane et al., 2004). This crosstalk would happen 24 hours following p38 activation through a mechanism implicating PKB2 by Ser473 phosphorylation (Gonzalez et al., 2004) (Fig.2.3). In addition, to p38-induced PKB activation, the same group has shown that the inhibition of PI3-K regulates p38 activation. This demonstrates that reciprocal communication between both pathways is implicated in myogenic regulation. The latter is still, however, under debate. The use of different inhibitors and their concentrations may explain the differences observed between these studies.

An interesting crosstalk between p38 and S6K1 was observed for the first time by Cuenda and Cohen (Cuenda and Cohen, 1999b). They showed the differentiation of myoblast to myotubes happened together with a strong S6K1 and of p38 activation, without affecting ERK MAPK protein and with only a slight activation on JNK MAPK and PKB. With the aid of SB 203580, a known inhibitor of p38, the authors were able to demonstrate a slow activation of S6K1, which had no effect on myotube formation in C2C12. Deldicque et al. demonstrated that creatine enhances differentiation in C2C12 by activating both p38 MAPK and PKB pathways (Deldicque et al., 2007). Another study by Deldicque et al. on humans showed that, immediately after resistance exercise, an increase of S6K1 on Thr421/Ser424 phosphorylation occurred. This was accompanied by an elevated p38 MAPK and ERK phosphorylation in the skeletal muscles. Furthermore, SB 202190 and PD 098059 (an ERK inhibitor) were able to decrease S6K1 on Thr421/Ser424 phosphorylation in C2C12 cells (Deldicque et al., 2008) (Fig. 2.3).

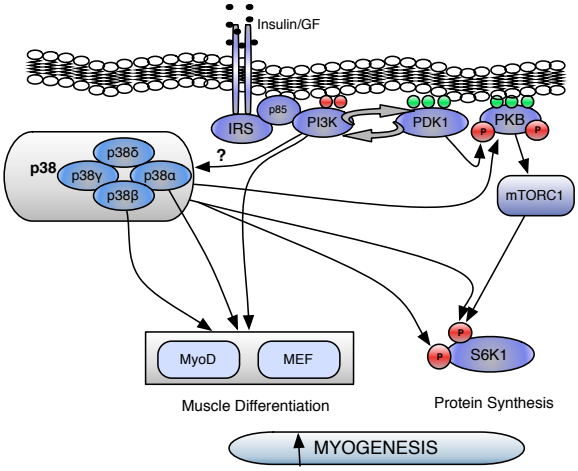


Fig. 2.3. Hypothetical crosstalk between p38 MAPK and insulin signaling pathways. See details in the text. Briefly, p38 MAPK may induce myogenesis through both MyoD and MEF transcription and S6K1 activation.

In conclusion, the relationship between p38 MAPK and PI3-K/PKB is still controversial. On the other hand, for the abovementioned reasons, the crosstalk between p38 MAPK and S6K1 proteins could be an important subject in studying cell differentiation processes.

References

Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity. 1998. 9:143-50.

Agnese DM, Calvano JE, Hahm SJ, Coyle SM, Corbett SA, Calvano SE, Lowry SF. Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. The Journal of infectious diseases. 2002. 186:1522-5.

Akira S, Takeda K. Functions of toll-like receptors: lessons from KO mice. Comptes rendus biologies. 2004. 327:581-9.

Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006. 124:783-801.

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001. 413:732-8.

Anderson KV, Bokla L, Nusslein-Volhard C. Establishment of dorsal-ventral polarity in the Drosophila embryo: the induction of polarity by the Toll gene product. Cell. 1985. 42:791-8.

Ben-Levy R, Hooper S, Wilson R, Paterson HF, Marshall CJ. Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2. Current biology : CB. 1998. 8:1049-57.

Beutler B. Inferences, questions and possibilities in Toll-like receptor signalling. Nature. 2004. 430:257-63.

Boldin MP, Varfolomeev EE, Pancer Z, Mett IL, Camonis JH, Wallach D. A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. The Journal of biological chemistry. 1995. 270:7795-8.

Bradley JR, Pober JS. Tumor necrosis factor receptor-associated factors (TRAFs). Oncogene. 2001. 20:6482-91.

Burns K, Janssens S, Brissoni B, Olivos N, Beyaert R, Tschopp J. Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the

alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. The Journal of experimental medicine. 2003. 197:263-8.

Cabane C, Coldefy AS, Yeow K, Derijard B. The p38 pathway regulates Akt both at the protein and transcriptional activation levels during myogenesis. Cell Signal. 2004. 16:1405-15.

Campos MA, Almeida IC, Takeuchi O, Akira S, Valente EP, Procopio DO, Travassos LR, Smith JA, Golenbock DT, Gazzinelli RT. Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J Immunol. 2001. 167:416-23.

Cayatte AJ, Kumbla L, Subbiah MT. Marked acceleration of exogenous fatty acid incorporation into cellular triglycerides by fetuin. The Journal of biological chemistry. 1990. 265:5883-8.

Chen RH, Sarnecki C, Blenis J. Nuclear localization and regulation of erk- and rsk-encoded protein kinases. Mol Cell Biol. 1992. 12:915-27.

Cuenda A, Cohen P. Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. The Journal of biological chemistry. 1999a. 274:4341-6.

Cuenda A, Cohen P. Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. The Journal of biological chemistry. 1999b. 274:4341-6.

da Silva Correia J, Soldau K, Christen U, Tobias PS, Ulevitch RJ. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. The Journal of biological chemistry. 2001. 276:21129-35.

Dadgostar H, Cheng G. An intact zinc ring finger is required for tumor necrosis factor receptor-associated factor-mediated nuclear factor-kappaB activation but is dispensable for c-Jun N-terminal kinase signaling. The Journal of biological chemistry. 1998. 273:24775-80.

Davis JE, Gabler NK, Walker-Daniels J, Spurlock ME. Tlr-4 deficiency selectively protects against obesity induced by diets high in saturated fat. Obesity. 2008. 16:1248-55.

Deldicque L, Atherton P, Patel R, Theisen D, Nielens H, Rennie MJ, Francaux M. Decrease in Akt/PKB signalling in human skeletal muscle by resistance exercise. Eur J Appl Physiol. 2008. 104:57-65.

Deldicque L, Theisen D, Bertrand L, Hespel P, Hue L, Francaux M. Creatine enhances differentiation of myogenic C2C12 cells by activating both p38 and Akt/PKB pathways. Am J Physiol Cell Physiol. 2007. 293:C1263-71.

Delhalle S, Blasius R, Dicato M, Diederich M. A beginner's guide to NF-kappaB signaling pathways. Annals of the New York Academy of Sciences. 2004. 1030:1-13.

Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science. 2004. 303:1529-31.

Ehses JA, Meier DT, Wueest S, Rytka J, Boller S, Wielinga PY, Schraenen A, Lemaire K, Debray S, Van Lommel L, Pospisilik JA, Tschopp O, Schultze SM, Malipiero U, Esterbauer H, Ellingsgaard H, Rutti S, Schuit FC, Lutz TA, Boni-Schnetzler M, Konrad D, Donath MY. Toll-like receptor 2-deficient mice are protected from insulin resistance and beta cell dysfunction induced by a high-fat diet. Diabetologia. 2010. 53:1795-806.

Erridge C, Samani NJ. Saturated fatty acids do not directly stimulate Toll-like receptor signaling. Arterioscler Thromb Vasc Biol. 2009. 29:1944-9.

Fernandez-Gonzalo R, De Paz JA, Rodriguez-Miguelez P, Cuevas MJ, Gonzalez-Gallego J. Effects of eccentric exercise on toll-like receptor 4 signaling pathway in peripheral blood mononuclear cells. Journal of applied physiology. 2012. 112:2011-8.

Flynn MG, McFarlin BK, Phillips MD, Stewart LK, Timmerman KL. Toll-like receptor 4 and CD14 mRNA expression are lower in resistive exercise-trained elderly women. Journal of applied physiology. 2003. 95:1833-42.

Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. Oncogene. 2006. 25:6680-4.

Gilmore TD, Garbati MR. Inhibition of NF-kappaB signaling as a strategy in disease therapy. Current topics in microbiology and immunology. 2011. 349:245-63.

Gonzalez I, Tripathi G, Carter EJ, Cobb LJ, Salih DA, Lovett FA, Holding C, Pell JM. Akt2, a novel functional link between p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways in myogenesis. Mol Cell Biol. 2004. 24:3607-22.

Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. Cell. 2008. 132:344-62.

Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science. 2004. 303:1526-9.

Himes RW, Smith CW. Tlr2 is critical for diet-induced metabolic syndrome in a murine model. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2010. 24:731-9.

Hochrein H, Schlatter B, O'Keeffe M, Wagner C, Schmitz F, Schiemann M, Bauer S, Suter M, Wagner H. Herpes simplex virus type-1 induces IFN-alpha production via Toll-like receptor 9-dependent and -independent pathways. Proc Natl Acad Sci U S A. 2004. 101:11416-21.

Holland WL, Bikman BT, Wang LP, Yuguang G, Sargent KM, Bulchand S, Knotts TA, Shui G, Clegg DJ, Wenk MR, Pagliassotti MJ, Scherer PE, Summers SA. Lipid-induced insulin resistance mediated by the proinflammatory receptor TLR4 requires saturated fatty acid-induced ceramide biosynthesis in mice. J Clin Invest. 2011. 121:1858-70.

Huang S, Rutkowsky JM, Snodgrass RG, Ono-Moore KD, Schneider DA, Newman JW, Adams SH, Hwang DH. Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. Journal of lipid research. 2012. 53:2002-13.

Imler JL, Zheng L. Biology of Toll receptors: lessons from insects and mammals. Journal of leukocyte biology. 2004. 75:18-26.

Janeway CA, Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol Today. 1992. 13:11-6.

Janssens S, Beyaert R. A universal role for MyD88 in TLR/IL-1R-mediated signaling. Trends in biochemical sciences. 2002. 27:474-82.

Karahashi H, Nagata K, Ishii K, Amano F. A selective inhibitor of p38 MAP kinase, SB202190, induced apoptotic cell death of a lipopolysaccharide-

treated macrophage-like cell line, J774.1. Biochim Biophys Acta. 2000. 1502:207-23.

Kawai T, Akira S. Toll-like receptor downstream signaling. Arthritis research & therapy. 2005. 7:12-9.

Khurana A, Dey CS. Involvement of c-Jun N-terminal kinase activities in skeletal muscle differentiation. Journal of muscle research and cell motility. 2004. 25:645-55.

Kiechl S, Lorenz E, Reindl M, Wiedermann CJ, Oberhollenzer F, Bonora E, Willeit J, Schwartz DA. Toll-like receptor 4 polymorphisms and atherogenesis. N Engl J Med. 2002. 347:185-92.

Kim F, Pham M, Luttrell I, Bannerman DD, Tupper J, Thaler J, Hawn TR, Raines EW, Schwartz MW. Toll-like receptor-4 mediates vascular inflammation and insulin resistance in diet-induced obesity. Circulation research. 2007. 100:1589-96.

Kleinridders A, Schenten D, Konner AC, Belgardt BF, Mauer J, Okamura T, Wunderlich FT, Medzhitov R, Bruning JC. MyD88 signaling in the CNS is required for development of fatty acid-induced leptin resistance and diet-induced obesity. Cell Metab. 2009. 10:249-59.

Kolch W, Calder M, Gilbert D. When kinases meet mathematics: the systems biology of MAPK signalling. FEBS Lett. 2005. 579:1891-5.

Kramer HF, Goodyear LJ. Exercise, MAPK, and NF-kappaB signaling in skeletal muscle. Journal of applied physiology. 2007. 103:388-95.

Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiological reviews. 2001. 81:807-69.

Lambert CP, Wright NR, Finck BN, Villareal DT. Exercise but not diet-induced weight loss decreases skeletal muscle inflammatory gene expression in frail obese elderly persons. Journal of applied physiology. 2008. 105:473-8.

Lancaster GI, Khan Q, Drysdale P, Wallace F, Jeukendrup AE, Drayson MT, Gleeson M. The physiological regulation of toll-like receptor expression and function in humans. J Physiol. 2005. 563:945-55.

Lang CH, Silvis C, Deshpande N, Nystrom G, Frost RA. Endotoxin stimulates in vivo expression of inflammatory cytokines tumor necrosis factor alpha, interleukin-1beta, -6, and high-mobility-group protein-1 in skeletal muscle. Shock. 2003. 19:538-46.

Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell. 1996. 86:973-83.

Li J, Lin S, Chen Q, Peng L, Zhai J, Liu Y, Yuan Z. Inhibition of hepatitis B virus replication by MyD88 involves accelerated degradation of pregenomic RNA and nuclear retention of pre-S/S RNAs. Journal of virology. 2010. 84:6387-99.

Li S, Strelow A, Fontana EJ, Wesche H. IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. Proc Natl Acad Sci U S A. 2002. 99:5567-72.

Lien E, Chow JC, Hawkins LD, McGuinness PD, Miyake K, Espevik T, Gusovsky F, Golenbock DT. A novel synthetic acyclic lipid A-like agonist activates cells via the lipopolysaccharide/toll-like receptor 4 signaling pathway. The Journal of biological chemistry. 2001. 276:1873-80.

Lin Y, Lee H, Berg AH, Lisanti MP, Shapiro L, Scherer PE. The lipopolysaccharide-activated toll-like receptor (TLR)-4 induces synthesis of the closely related receptor TLR-2 in adipocytes. The Journal of biological chemistry. 2000. 275:24255-63.

Lund J, Sato A, Akira S, Medzhitov R, Iwasaki A. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. The Journal of experimental medicine. 2003. 198:513-20.

Makarov SS. NF-kappaB as a therapeutic target in chronic inflammation: recent advances. Molecular medicine today. 2000. 6:441-8.

McFarlin BK, Flynn MG, Campbell WW, Craig BA, Robinson JP, Stewart LK, Timmerman KL, Coen PM. Physical activity status, but not age, influences inflammatory biomarkers and toll-like receptor 4. The journals of gerontology. Series A, Biological sciences and medical sciences. 2006. 61:388-93.

McFarlin BK, Flynn MG, Campbell WW, Stewart LK, Timmerman KL. TLR4 is lower in resistance-trained older women and related to inflammatory cytokines. Med Sci Sports Exerc. 2004. 36:1876-83.

Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 1997. 388:394-7.

Meusel TR, Imani F. Viral induction of inflammatory cytokines in human epithelial cells follows a p38 mitogen-activated protein kinase-dependent but NF-kappa B-independent pathway. J Immunol. 2003. 171:3768-74.

Mizukami Y, Yoshioka K, Morimoto S, Yoshida K. A novel mechanism of JNK1 activation. Nuclear translocation and activation of JNK1 during ischemia and reperfusion. The Journal of biological chemistry. 1997. 272:16657-62.

Muzio M, Mantovani A. Toll-like receptors. Microbes Infect. 2000. 2:251-5.

Muzio M, Ni J, Feng P, Dixit VM. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science. 1997. 278:1612-5.

Muzio M, Polentarutti N, Bosisio D, Prahladan MK, Mantovani A. Toll-like receptors: a growing family of immune receptors that are differentially expressed and regulated by different leukocytes. Journal of leukocyte biology. 2000. 67:450-6.

Nguyen MT, Favelyukis S, Nguyen AK, Reichart D, Scott PA, Jenn A, Liu-Bryan R, Glass CK, Neels JG, Olefsky JM. A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. The Journal of biological chemistry. 2007. 282:35279-92.

O'Neill LA, Fitzgerald KA, Bowie AG. The Toll-IL-1 receptor adaptor family grows to five members. Trends in immunology. 2003. 24:286-90.

Oliveira AG, Carvalho BM, Tobar N, Ropelle ER, Pauli JR, Bagarolli RA, Guadagnini D, Carvalheira JB, Saad MJ. Physical exercise reduces circulating lipopolysaccharide and TLR4 activation and improves insulin signaling in tissues of DIO rats. Diabetes. 2011. 60:784-96.

Pal D, Dasgupta S, Kundu R, Maitra S, Das G, Mukhopadhyay S, Ray S, Majumdar SS, Bhattacharya S. Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. Nat Med. 2012.

Pearson G, English JM, White MA, Cobb MH. ERK5 and ERK2 cooperate to regulate NF-kappaB and cell transformation. The Journal of biological chemistry. 2001. 276:7927-31.

Petersen AM, Pedersen BK. The anti-inflammatory effect of exercise. Journal of applied physiology. 2005. 98:1154-62.

Phillips MD, Patrizi RM, Cheek DJ, Wooten JS, Barbee JJ, Mitchell JB. Resistance training reduces subclinical inflammation in obese, postmenopausal women. Med Sci Sports Exerc. 2012. 44:2099-110.

Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998. 282:2085-8.

Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, Davis RJ. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. The Journal of biological chemistry. 1995. 270:7420-6.

Reyna SM, Ghosh S, Tantiwong P, Meka CS, Eagan P, Jenkinson CP, Cersosimo E, Defronzo RA, Coletta DK, Sriwijitkamol A, Musi N. Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. Diabetes. 2008. 57:2595-602.

Rosa JC, Lira FS, Eguchi R, Pimentel GD, Venancio DP, Cunha CA, Oyama LM, De Mello MT, Seelaender M, do Nascimento CM. Exhaustive exercise increases inflammatory response via Toll like receptor-4 and NF-kappaBp65 pathway in rat adipose tissue. Journal of cellular physiology. 2011. 226:1604-7.

Rosa Neto JC, Lira FS, Oyama LM, Zanchi NE, Yamashita AS, Batista ML, Jr., Oller do Nascimento CM, Seelaender M. Exhaustive exercise causes an anti-inflammatory effect in skeletal muscle and a pro-inflammatory effect in adipose tissue in rats. Eur J Appl Physiol. 2009. 106:697-704.

Roux PP, Blenis J. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiology and molecular biology reviews : MMBR. 2004. 68:320-44.

Rutz M, Metzger J, Gellert T, Luppa P, Lipford GB, Wagner H, Bauer S. Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. European journal of immunology. 2004. 34:2541-50.

Salminen A, Huuskonen J, Ojala J, Kauppinen A, Kaarniranta K, Suuronen T. Activation of innate immunity system during aging: NF-kB signaling is the molecular culprit of inflamm-aging. Ageing research reviews. 2008. 7:83-105.

Schaeffler A, Gross P, Buettner R, Bollheimer C, Buechler C, Neumeier M, Kopp A, Schoelmerich J, Falk W. Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-kappaB pathway in adipocytes links nutritional signalling with innate immunity. Immunology. 2009. 126:233-45.

Seger R, Seger D, Reszka AA, Munar ES, Eldar-Finkelman H, Dobrowolska G, Jensen AM, Campbell JS, Fischer EH, Krebs EG. Overexpression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH 3T3 cells. Evidence that MAPKK involvement in cellular proliferation is regulated by phosphorylation of serine residues in its kinase subdomains VII and VIII. The Journal of biological chemistry. 1994. 269:25699-709.

Senn JJ. Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes. The Journal of biological chemistry. 2006. 281:26865-75.

Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest. 2006. 116:3015-25.

Smith MF, Jr., Mitchell A, Li G, Ding S, Fitzmaurice AM, Ryan K, Crowe S, Goldberg JB. Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for Helicobacter pylori-induced NF-kappa B activation and chemokine expression by epithelial cells. The Journal of biological chemistry. 2003. 278:32552-60.

Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S, Kotani H, Yamaoka S, Miyake K, Aoe S, Kamei Y, Ogawa Y. Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced

inflammatory changes in the interaction between adipocytes and macrophages. Arterioscler Thromb Vasc Biol. 2007. 27:84-91.

Suzuki N, Suzuki S, Duncan GS, Millar DG, Wada T, Mirtsos C, Takada H, Wakeham A, Itie A, Li S, Penninger JM, Wesche H, Ohashi PS, Mak TW, Yeh WC. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. Nature. 2002. 416:750-6.

Tabeta K, Georgel P, Janssen E, Du X, Hoebe K, Crozat K, Mudd S, Shamel L, Sovath S, Goode J, Alexopoulou L, Flavell RA, Beutler B. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. Proc Natl Acad Sci U S A. 2004. 101:3516-21.

Takaesu G, Ninomiya-Tsuji J, Kishida S, Li X, Stark GR, Matsumoto K. Interleukin-1 (IL-1) receptor-associated kinase leads to activation of TAK1 by inducing TAB2 translocation in the IL-1 signaling pathway. Mol Cell Biol. 2001. 21:2475-84.

Takeda K. Evolution and integration of innate immune recognition systems: the Toll-like receptors. Journal of endotoxin research. 2005. 11:51-5.

Takeda K, Akira S. TLR signaling pathways. Semin Immunol. 2004. 16:3-9.

Takeuchi O, Akira S. Toll-like receptors; their physiological role and signal transduction system. International immunopharmacology. 2001. 1:625-35.

Tamir Y, Bengal E. Phosphoinositide 3-kinase induces the transcriptional activity of MEF2 proteins during muscle differentiation. The Journal of biological chemistry. 2000. 275:34424-32.

Uematsu S, Jang MH, Chevrier N, Guo Z, Kumagai Y, Yamamoto M, Kato H, Sougawa N, Matsui H, Kuwata H, Hemmi H, Coban C, Kawai T, Ishii KJ, Takeuchi O, Miyasaka M, Takeda K, Akira S. Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c+ lamina propria cells. Nature immunology. 2006. 7:868-74.

van der Vaart M, van Soest JJ, Spaink HP, Meijer AH. Functional analysis of a zebrafish myd88 mutant identifies key transcriptional components of the innate immune system. Disease models & mechanisms. 2013. 6:841-54.

Wang M, Amano SU, Flach RJ, Chawla A, Aouadi M, Czech MP. Identification of Map4k4 as a novel suppressor of skeletal muscle differentiation. Mol Cell Biol. 2013. 33:678-87.

West AP, Koblansky AA, Ghosh S. Recognition and signaling by toll-like receptors. Annu Rev Cell Dev Biol. 2006. 22:409-37.

Wu Z, Woodring PJ, Bhakta KS, Tamura K, Wen F, Feramisco JR, Karin M, Wang JY, Puri PL. p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps. Mol Cell Biol. 2000. 20:3951-64.

Xiong Y, Qiu F, Piao W, Song C, Wahl LM, Medvedev AE. Endotoxin tolerance impairs IL-1 receptor-associated kinase (IRAK) 4 and TGF-beta-activated kinase 1 activation, K63-linked polyubiquitination and assembly of IRAK1, TNF receptor-associated factor 6, and IkappaB kinase gamma and increases A20 expression. The Journal of biological chemistry. 2011. 286:7905-16.

Yamamoto Y, Gaynor RB. Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. J Clin Invest. 2001. 107:135-42.

Zanchi NE, Lira FS, de Siqueira Filho MA, Rosa JC, de Oliveira Carvalho CR, Seelaender M, Santos RV, Lancha AH, Jr. Chronic low frequency/low volume resistance training reduces pro-inflammatory cytokine protein levels and TLR4 mRNA in rat skeletal muscle. Eur J Appl Physiol. 2010. 109:1095-102.

Zarubin T, Han J. Activation and signaling of the p38 MAP kinase pathway. Cell research. 2005. 15:11-8.

Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, Flavell RA, Ghosh S. A toll-like receptor that prevents infection by uropathogenic bacteria. Science. 2004. 303:1522-6.

Zhou G, Bao ZQ, Dixon JE. Components of a new human protein kinase signal transduction pathway. The Journal of biological chemistry. 1995. 270:12665-9.

Chapter 3

Aims of the thesis

The mechanisms by which the MAPK family is activated during exercise are not fully understood. Local activation mechanisms could be implicated, such as reactive oxygen species, cell acidification, and mechanical perturbations (Kramer and Goodyear, 2007). TLR are transmembrane proteins that detect a variety of molecular components, including NEFA. Endurance exercise provokes an elevated plasma NEFA release from adipose tissue in order to contribute to the energy supply of skeletal muscle.

Current knowledge about the potential control points that regulate fat metabolism from the release of plasma NEFA to the metabolism of the β-oxidation were described in the first chapter. TLR are highly expressed in the cells of the immune system, but TLR2 and TLR4 are also found in various other cells types, including myocytes (Reyna et al., 2008). Their functions, structure and the downstream signaling pathways including the MAPK family and NF-κB have been addressed in this chapter. In addition, we addressed the potential interaction between p38 MAPK and S6K1 protein.

In the next chapter, we will present results showing that TLR2 and TLR4 activation induces p38 MAPK phosphorylation and a concomitant S6K1 activation in C2C12. Data acquired using pharmacological and genetic tools support the idea that palmitic acid (PA) can activate TLR2 and TLR4, which trigger a signalling pathway leading to an increase of p38 MAPK and S6K1 activation through a crosstalk between these two proteins.

Endurance exercise results in an increased plasma NEFA. In the fifth chapter, we will present evidence suggesting that the activation of MAPK observed after endurance exercise might be initiated by circulating NEFA via a signal transduced by TLR2 and TLR4.

Thanks to data obtained from muscle biopsies taken in the vastus lateralis of

healthy subjects and diabetics, in Chapter 6, we will present human results supporting the same hypothesis. Acipimox administration prevents the elevation of plasma NEFA during exercise. We will show that blocking a plasma NEFA increase with acipimox blunts the MAPK activation during endurance exercise, both in healthy subjects and diabetic patients.

The results of each experimental protocol will be discussed at the end of the relevant chapter. In Chapter 7, we will discuss the data as a whole and we will develop general perspectives regarding the interaction of plasma NEFA released from adipose tissue during endurance exercise and the activation of TLR2 and TLR4. This mechanism will provide new insights into the MAPK activation induced by endurance exercise.

References

Kramer HF, Goodyear LJ. Exercise, MAPK, and NF-kappaB signaling in skeletal muscle. Journal of applied physiology. 2007. 103:388-95.

Reyna SM, Ghosh S, Tantiwong P, Meka CS, Eagan P, Jenkinson CP, Cersosimo E, Defronzo RA, Coletta DK, Sriwijitkamol A, Musi N. Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. Diabetes. 2008. 57:2595-602.

Chapter 4

TLR2 and TLR4 activation induces p38 MAPK-dependent phosphorylation of S6 kinase 1 in C2C12 myotubes

Hermann Zbinden-Foncea[1,2], Louise Deldicque[1,3], Nicolas Pierre[1], Marc Francaux[1] and Jean-Marc Raymackers[1]

[1]Institute of Neuroscience, Université catholique de Louvain, Louvain-la-Neuve, Belgium

[2]School of Kinesiology and Health Research Center, Faculty of Medicine, Universidad Finis Terrae, Santiago, Chile

[3]Research Centre for Exercise and Health, Department of Kinesiology, KU Leuven, B-3001 Leuven, Belgium

Cell Biol. Int. (2012) 36, 1107-1113

Abstract

Toll-like receptors 2 (TLR2) and 4 (TLR4) are present in the plasma membrane of skeletal muscle cells where their functions remain incompletely resolved. They are able to bind various extra-cellular ligands, such as FSL-1, lipopolysaccharide (LPS) and/or palmitic acid (PA). The purpose of this study was to investigate the link between PA, TLR2/4, and ribosomal S6 kinase 1 (S6K1) in C2C12 myotubes. Incubation with agonists of either TLR2 or TLR4, as well as incubation with high concentration of PA, led to an increase in S6K1 phosphorylation level. Canonical upstream kinases of S6K1, protein kinase B (PKB) and mammalian target of rapamycin complex 1 (mTORC1), were regulated in the opposite way by PA, indicating that those kinases were probably not involved. By using the SB202190 inhibitor, we evidenced that p38 MAPK was a key mediator of PA-induced phosphorylation of S6K1. Down-regulation of either *tlr2* or *tlr4* gene expression by small interfering RNAs prevented the activation of both p38 MAPK and S6K1 by FSL-1, LPS or PA. In summary, our results showed that TLR2 and TLR4 agonists are able to increase the level of S6K1 phosphorylation in a p38 MAPK dependent way in C2C12 myotubes. As PA induced the same intracellular signaling, we evidenced for the first time an atypical pathway for PA that is induced at the cellular membrane level and results in a higher phosphorylation state of S6K1.

Keywords

Palmitic acid, muscle, lipopolysaccharide, PKB, mTOR, cell signalling

Introduction

Toll-like receptors (TLRs) play a major role in the immune response and are implicated in septic shock and in chronic diseases, amongst other. TLRs are expressed in various tissues. In white blood cells, they are known to induce the production of cytokines such as tumor necrosis factor-a (TNF-a), interleukin-1a (IL1a) and interleukin-6 (IL6), via nuclear factor-kappa B (NF-kB) and mitogen-activated protein kinases (MAPK) signaling (Akira et al., 2003, West et al., 2006). More than 70% of the TLR family is expressed in C2C12 myoblasts and myotubes, but their roles are still under investigation (Frost et al., 2006). The most frequently expressed, TLR2 and TLR4, have been shown to play a role in insulin resistance and atherosclerosis induced by a high-fat diet in animal models (Caricilli et al. , 2008, Madan and Amar, 2008, Radin et al. , 2008).

Palmitic acid (PA), a long chain saturated fatty acid, is acutely and highly increased during endurance exercise. while it is chronically and moderately elevated in obesity and type 2 diabetes. These two conditions are associated with both insulin resistance and proinflammatory cytokines production, the first situation being transitory whereas the second one being chronic (Boden, 2003). PA is capable of activating TLR2 pathway in C2C12 myogenic cells (Senn, 2006) and TLR4 in the RAW264.7 macrophage cell line and in 3T3-L1 preadipocytes (Shi et al. 2006). Therefore, besides its role as energy substrate, extra-cellular PA is suggested to activate intra-cellular signaling pathways (Clarke, 2004).

Insulin activates the PKB (protein kinase B) pathway leading to an increased activity of mTORC1 (mammalian target of rapamycin complex 1) and S6K1 (ribosomal S6 kinase 1). The latter is a Ser/Thr protein kinase that catalyses

the phosphorylation of S6 protein, a component of the eukaryotic ribosomal 40S subunit which is essential for increasing protein synthesis (Baar and Esser, 1999, Pullen and Thomas, 1997). Activity of S6K1 changes in response to growth factors and nutrients signaling, and besides its role in protein synthesis, S6K1 may play a role in various pathologies, including obesity, diabetes, ageing and cancer. For a detailed review see (Fenton and Gout, 2011).

PA is often considered a negative regulator of insulin sensitivity through decreased activation of the PKB pathway but the molecular links between PA and PKB are not clear. As TLR are likely candidates, the initial purpose of the present study was to explore a potential relationship between PA, TLR2/4 and the PKB/mTOR/S6K1 pathway. Contrary to our hypothesis, the intermediates of this pathway were not regulated in the same way by PA: the phosphorylation state of S6K1 was increased whereas PKB and mTOR were dephosphorylated. Therefore, we decided to explore a potential regulation of PA on S6K1 which we hypothesized to be transduced by TLR and p38 MAPK.

2. Material and Methods

2.1. Cell culture

C2C12 cells were purchased from the ATCC (Manassas, VA) and incubated at 37°C in a humidified atmosphere of 5% CO_2. Myoblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 µM non essential amino acids (NEAA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 4.5 g/l glucose until 70% confluence and differentiated for 72-96h in a medium in which FBS was replaced by 1% horse serum.

FSL-1 (Pam2Cys-GDPKHPKSF), Ultrapure lipopolysacharride from 0111:B4 E.coli (LPS) and PA were added to culture medium at a final concentration of 0.75 μM, 1 μg/ml, and 0.75 mM for 30 min, 3 hours and 3 hours, respectively. SB202190 was added to culture medium 15 minutes prior addition of either TLR activator. LPS and FSL-1 were dissolved in water, SB202190 in DMSO, and PA in ethanol; control vehicle contained the same solvant.

FSL-1 and LPS were obtained from InvivoGen (Toulouse, France), palmitic acid (PA, P5585) was purchased from Sigma-Aldrich (Bornem, Belgium) and p38 MAPK inhibitor SB202190 was obtained from Alexis Biochemicals (Zandhoven, Belgium).

2.2 Protein extraction

Cells were extracted using the method described by Deldicque et al. (2007). Briefly, cells were rinsed with phosphate-buffered saline (PBS) and homogenised in an ice-cold lysis buffer containing 20mM Tris, pH 7.0, 270mM sucrose, 5mM EGTA, 1mM EDTA, 0.1% Triton X-100, 1mM sodium orthovanadate, 50mM sodium β-glycerophosphate, 5mM sodium pyrophosphate, 50mM sodium fluoride, 1mM 1,4-dithiothreitol (DTT), and a protease inhibitor cocktail (Roche Applied Science, Vilvoorde, Belgium). Cells homogenates were centrifuged at 10,000g for 10min at 4°C. Protein concentration was determined using a DC protein assay kit (Bio-Rad Laboratories, Nazareth, Belgium) with bovine serum albumin as a standard. Cells lysates were stored at -80°C until subsequent analyses.

2.3 Western blotting.

Protein lysates (10-20μg) were combined with Laemmli and separated by SDS-PAGE (10-12%). After electrophoretic separation (40mA), the proteins were transferred to PVDF membranes at 80V for 2h. Membranes were blocked for 60min in Tris-buffered saline with 0.1% Tween 20 (TBST) and

5% milk. Then, the membranes were incubated overnight at 4°C in TBST containing 1% bovine serum albumin and one of the following antibodies: anti-phospho-p38 Thr180/Tyr182 (1:1,000), anti-total p38 (1:1,000), anti-phospho-PKB Ser473 (1:1,000), anti-total PKB (1:1,000), anti-phospho-mTOR Ser2448 (1:1,000), anti-total mTOR (1:1,000) and anti-TLR2 (1:1,000) were obtained from Cell Signaling Technology (Leiden, The Netherlands). Anti-phospho-S6K1 Ser 389 (1:1,000), anti-total S6K1 (1:1,000) and anti-TLR4 (1:1,000) were from Santa Cruz Biotechnology. Anti-GAPDH (1:20,000) was obtained from Abcam (Cambridge, UK). Anti-actin (1: 20000) was from BD Transduction laboratories (Erembodegen, Belgium). Membranes were washed three times with TBST and were incubated with a secondary antibody at room temperature for 60min [anti-rabbit (1:10,000) or anti-mouse (1:20,000) from Sigma (Bornem, Belgium)]. After three additional washes, chemiluminescent detection was carried out using an ECL Western blotting kit (Amersham ECL Plus, GE Healthcare, Diegem, Belgium). Bands were visualized on film, scanned and quantified by densitometry using the ImageMaster 1D image analysis software (Amersham-GE Healthcare, Diegem, Belgium).

2.4 Gene silencing

Ambion Silencer® Select Pre-designed siRNAs against mouse *tlr2* and *tlr4* and Silencer® Validated siRNA against *gapdh* were purchased from Ambion International/Applied Biosystems (Huntingdon, UK). Transfection of C2C12 cells was performed with Lipofectamine 2000 (Invitrogen, Belgium), according to manufacturer's instructions. Briefly, cells were differentiated for 72 hours, then transfected with 50 nM of siRNA in the presence of 4 µl Lipofectamine 2000. Transfection durations were 17 and 6 hours for siRNA against *tlr2* and *tlr4*, respectively. Transfection with siRNA against *gapdh* was included for both durations. Since transfection, antibiotics

were omitted from the medium for a 40-hour period, and then re-added. Cell harvesting was done 72 hours after transfection.

2.5 Treatment with palmitic acid.

PA was dissolved in 100% ethanol to a final stock solution of 75mM. The final concentration used in all experiments was 0.75mM, diluted in cell DMEM media supplemented with 100 µM nonessential amino acids. Ethanol was added in all control samples as a vehicle. After 4 days of differentiation, matures myotubes were exposed to PA for 3h.

2.6 LAL test

Limulus Amebocyte Lysate (LAL) concentration was determined using a disposal kit (PYROGENT® Ultra – LONZA). LAL test is a qualitative test from Gram-negative bacterial endotoxin. It was performed according the instructions of the manufacturer. Briefly, Gram-negative bacterial endotoxin catalyzes the activation of a proenzyme in the LAL. The initial rate of activation is determined by the concentration of endotoxin present. A lysate provided (Limulus polyphemus) was used in order to determinate the labeled concentration (EU/ml) as Standard Endotoxin. LAL reagent water was used as a negative control. 100µl of standard, sample (DMEM with ethanol and DMEM with ethanol and palmitic acid) or water was diluted with 100µl of reconstituted lysate into the appropriate 10 x 75 mm tube. After being mixed, the tubes were placed in a 37ºC hot water for a 60-minute incubation. After exactly 60 min, the absorbance was measured at 405nm using a microplate reader FLUOstar OPTIMA (BMG Labtech).

2.7 Statistical analysis

All results are shown as means ± SEM from 4 independent experiments. Student's unpaired t-test was used for comparing groups, with the exception of figure 2 after incubation with palmitic acid, FSL and LPS with or without

p38 MAPK inhibitor, where a two-way ANOVA and Bonferroni post-hoc tests were used. Statistical threshold was set at P<0.05.

3. Results

3.1 Incubation of C2C12 myotubes with palmitic acid results in a PKB-independent p38 MAPK-dependent increase in the phosphorylation state of S6K1

In C2C12 myotubes, a 3 hour incubation with 0.75 mM PA resulted in a 40% decrease in PKB phosphorylation, as compared to incubation with the vehicle alone (*P*<0.05; Fig. 4.1A). Likewise, phosphorylation level of mTOR, a kinase downstream of PKB, was reduced by ~50% after C2C12 myotube incubation with PA (*P*<0.05; Fig. 4.1B). However, in the same conditions, incubation with PA resulted in a 1.7-fold increase in p38 MAPK and in a 1.6-fold increase in S6K1 phosphorylation levels (*P*<0.05 in both case; Fig. 4.1C & 4.1D).

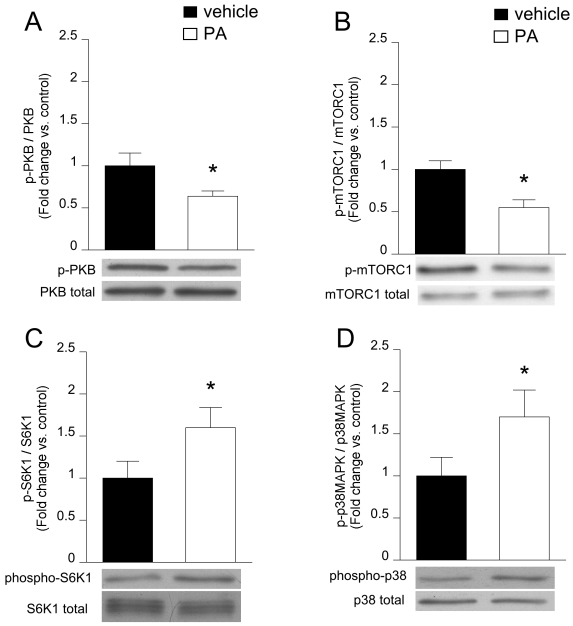


Fig. 4.1. Effect of a 3 hour 0.75mM palmitic acid incubation on the phosphorylation state of PKB (A), mTORC1 (B), p38 MAPK (C) and S6K1 (D) in C2C12 cells. Results were normalized to the untreated (control) groups. n=4 / group. *: *P* < 0.05 versus control. Representative western blots are shown

A 30 minute incubation with 0.75 μM FSL-1 (a synthetic diacylated lipoprotein TLR2 agonist) resulted in a 2-fold increase in S6K1 phosphorylation (P<0.001; Fig. 4.2). This effect was not observed when the cultures were pre-treated with SB202190, suggesting that p38 MAPK is controlling S6K1 phosphorylation. Likewise, TLR4 activation induced by 3 hour incubation process with 1 μg/ml LPS (lipopolysaccharide, a TLR4 agonist) resulted in a 1.5-fold increase in S6K1 phosphorylation (P=0.002), which was repressed by the inhibitor of p38 MAPK. The same effect was observed after incubation with 0.75 mM PA for 3 hours (1.7-fold increase, P =0.002).

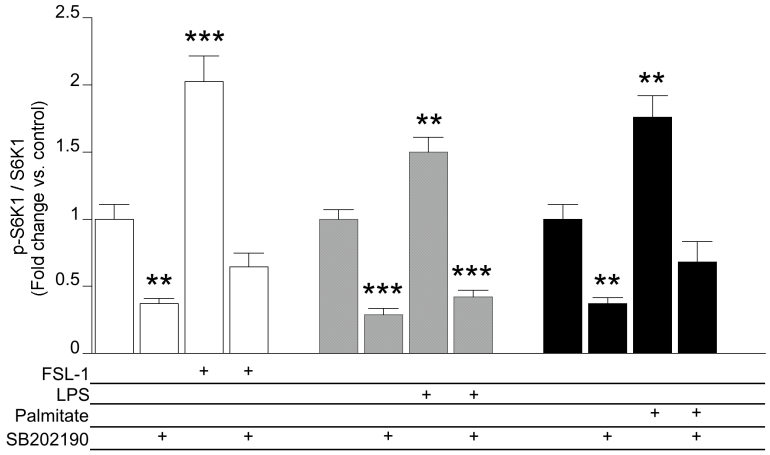


Fig. 4.2. Phosphorylation of S6K1 is induced by FSL-1, LPS and palmitic acid in C2C12 myotubes. SB202190 is a p38 MAPK antagonist. Results were normalized to the untreated (control) groups. **: $P < 0.01$ and ***: $P < 0.001$ versus controls (n = 4-5 / group).

3.2 Downregulation of TLR2 or TLR4 protein expression prevents palmitic acid-induced phosphorylation of p38 MAPK and S6K1 in C2C12 myotubes

To further explore the link between PA, p38 MAPK and S6K1 in muscle cells, we transfected C2C12 myotubes by either an anti-*tlr2* or an anti-*tlr4* siRNA.

First we made sure that downregulation of TLR expression using small interfering oligonucleotides was significant and specific. As compared to transfection with an anti-*gapdh* siRNA, the levels of TLR2 and TLR4 proteins were decreased by 46% (P <0.05) and 35% (P <0.001), respectively (Figs 4A & 4B). No significant off-target effect was observed, since neither TLR2 protein content was change after anti-*tlr4* transfection (Fig. 4.3A), nor TLR4 protein content was modified after anti-*tlr2* transfection (Fig. 4.3B). As compared to non-transfected myotubes, transfection of anti-*gapdh* siRNA resulted in a reduction of GAPDH protein expression of 58% (P <0.001) and 61% (P <0.001), using the protocol of anti-*tlr2* or anti-*tlr4* siRNAs, respectively (see Materials and Methods section). Furthermore, transfection with anti-*gapdh* siRNA had no effect on TLR2 and TLR4 protein expression, with actin protein being used as gel loading control (not shown).

In C2C12 cells transfected with anti-*tlr2* siRNA, no increase in the phosphorylation levels of p38 MAPK and S6K1 was observed upon incubation with PA, while it was the case when C2C12 myotubes were transfected with anti-*gapdh* siRNA (+100% for p38 MAPK, P <0.05, Fig. 4.3C; +78% for S6K1, P <0.05, Fig. 4.3E). Similar results were observed when comparing the effect of PA on C2C12 myotubes after either anti-*gadph* or anti-*tlr4*: a significant increase in the phosphorylation levels of p38 MAPK (+100%, P =0.002) and S6K1 (+89%, P=0.009) was seen only in control anti-*gapdh* transfected cells (Figs. 4.3D & 4.3F).

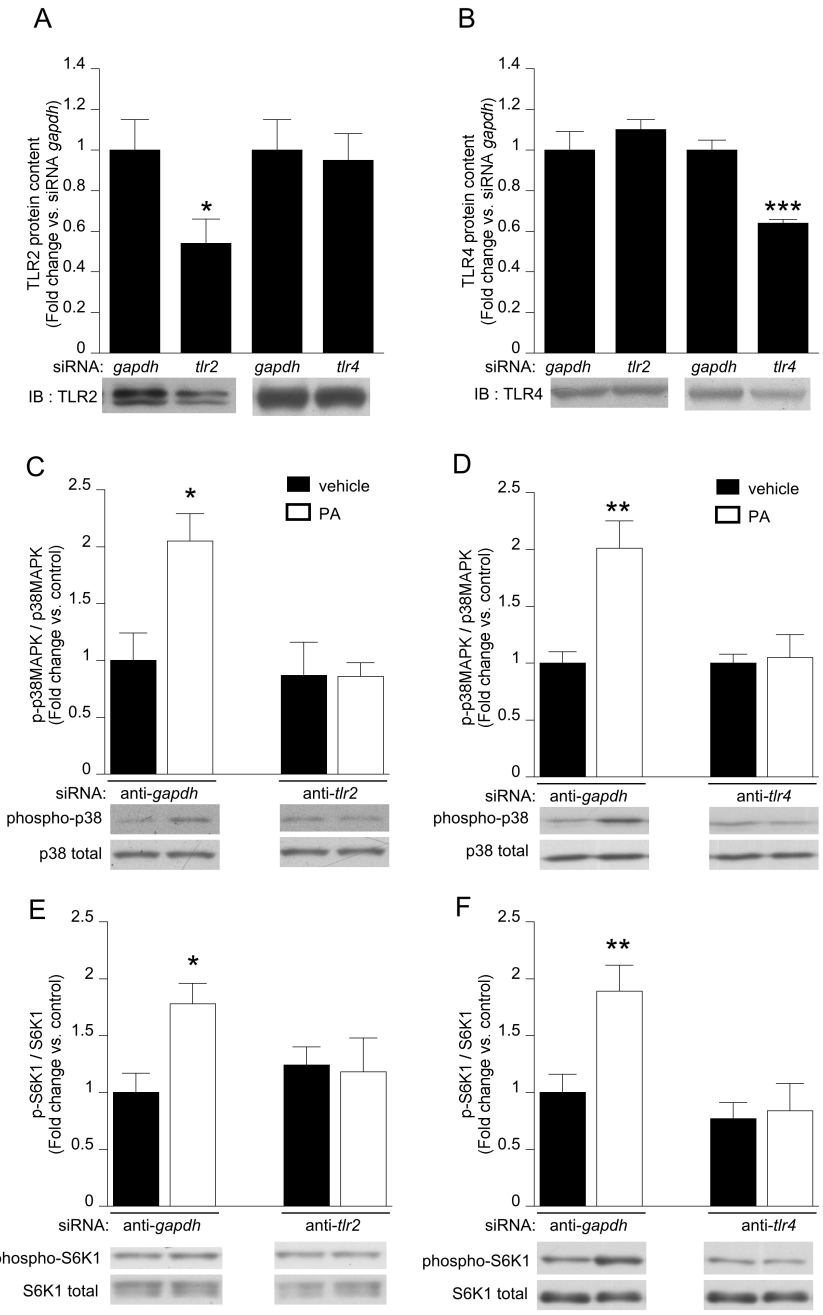


Fig. 4.3. TLR-mediated activation of p38 MAPK and S6K1 by palmitic acid. TLR2 and TLR4 protein expression are specifically downregulated after transfection with anti-tlr2 (A) or anti-tlr4 (B), respectively. The effect of 0.75mM palmitic acid on p38 MAPK and S6K1 phosphorylation was assessed in C2C12 cells transfected with

anti-gapdh (control) anti-tlr2 (C & E) or anti-tlr4 (D & F) siRNA. n=4 / group. Values are reported to cells transfected with anti-gapdh and exposed with a vehicle. *: P < 0.05 and **: P < 0.01, versus control (n=4 / group). Representative western blots are shown.

3.3 In C2C12 myotubes, S6K1 phosphorylation is increased by stimulation of either TLR2 or TLR4 receptors

To ascertain that TLR stimulation results in an increase in S6K1 phosphorylation as seen after PA incubation, C2C12 myotubes were submitted to either a 30-min 0.75μM FSL-1 or a 3-h 1μg/ml LPS incubation. In control myotubes transfected with anti-*gapdh* siRNA, FSL-1 and LPS induced a 1.8-fold (P <0.05, Fig. 4.4A) and a 1.7-fold increase in S6K1 phosphorylation (P <0.05*,* Fig. 4.4B), respectively. As expected, FSL-1 did not produce any change in S6K1 in C2C12 myotubes transfected with anti-*tlr2* siRNA (Fig. 4.4A). Similarly, transfection with anti-*tlr4* siRNA prevented the LPS-induced change in S6K1 phosphorylation (Fig. 4.4B).

To further investigate the activation of S6K1 induced by TLR2 and TLR4 agonists in muscle cells, we first measured the levels of phosphorylation of p38 MAPK. Both FSL-1 and LPS induced significant increases in control myotubes transfected with anti-*gapdh* siRNA [FSL-1: 2.2-fold (P <0.05*)* and LPS: 1.9-fold (P <0.05), Fig. 4.4C & 4.4D]. When transfected with the appropriate siRNA, this effect was not detected (Fig. 4.4C & 4.4D). Additionally, as previously mentioned, pre-incubation with p38 MAPK inhibitor SB202190 prevented both FSL1- and LPS-induced phosphorylation of S6K1 (Fig. 4.2) suggesting that p38 MAPK is essential to induce the TLR related S6K1 phosphorylation in cultured myotubes.

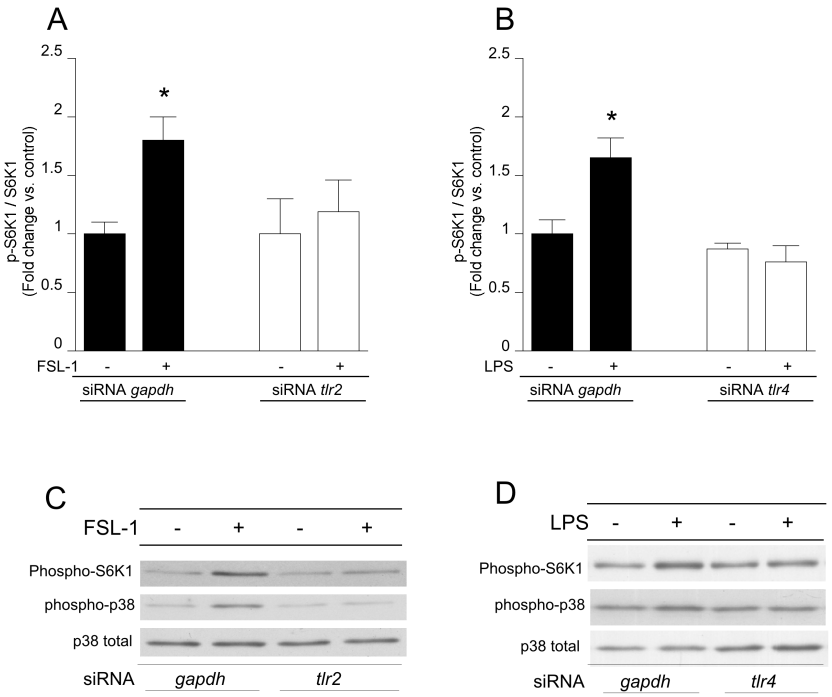


Fig. 4.4. Activation of p38 MAPK and S6K1 by agonist of TLR2 and TLR4 receptor. Phosphorylation levels of S6K1 and p38 MAPK protein induced by incubation with FSL-1 or LPS were measured in myotubes transfected with anti-g*apdh* siRNA (control) anti-*tlr2* (A & C) or anti-*tlr4* (B & D). S6K1 phosphorylation was analyzed without (-) and with (+) FSL-1 (A) or LPS (B) after transfection with anti-*tlr2* or anti-*tlr4*, respectively. Under the same conditions, representative western-blot reflecting p38 MAPK are shown to demonstrate a similar increase in levels of S6K1 and p38 MAPK after incubation with both FSL-1 (C) and LPS (D). *: $P < 0.05$ versus control (n=4 / group).

4. **Discussion**

In this study, we clearly established a link between TLR2/4, p38 MAPK and S6K1 in C2C12 myotubes. Incubation with either FSL-1, LPS or PA led to rapid increase of S6K1 phosphorylation level, in a p38 MAPK dependent way, beyond a known inhibition of the PKB-mTORC1 pathway.

4.1. Palmitic acid as a signaling molecule for myotubes

In several studies in 3T3-L1 adipocytes and C2C12 myotubes, incubating the cells with 0.75 mM free PA diminished the phosphorylation levels of PKB

(Chavez and Summers, 2003, Schmitz-Peiffer et al., 1999). While this decrease was suspected to lower downstream S6K1 activation, we found, to the contrary, that PA application for 3 hours resulted in increased S6K1 phosphorylation. This observation indicates that another kinase is able to activate S6K1, reversing the effect of PKB inhibition on S6K1. Only two studies reported an increase in S6K1 phosphorylation after PA incubation in muscle (Ragheb et al., 2009, Wang et al., 2010). Both authors found concomitant decrease in the phosphorylation state of PKB.

The relationship between activation of TLR2/4 and insulin resistance was demonstrated in cell (Senn 2006), animal (Shi et al. 2006) and human (Lambert et al. 2008) models. Our results confirm that a decreased activity of the IRS/PKB pathway is implicated in this phenomenon. However, the clear relationship between the activation of TLR2/4 by various ligands amongst which PA and the dephosphorylation of PKB have still to be elucidated. Considering the results reported in the present paper, the most direct hypothesis is the negative feedback mechanism exerted by S6K1 on PKB (Tremblay and Marette, 2001). However another mechanism implicating the activation of the IKK/NF-kB pathway by TLR2/4 might not be ruled out. Indeed, TLR2/4 activate the pro-inflammatory program of the cell via the IKK/NF-kB pathway. Infusion of PA in WT mice resulted in an increased expression of IL6 and TNF-a whereas the same response was not observed in $TLR4^{-/-}$ mice (Shi et al. 2006). Therefore, the dephosphorylation of PKB reported in the present paper may potentially be related to an inhibition of IRS initiated by a pro-inflammatory process induced by TLR2/4 activation.

4.2. Crosstalk between S6K1 and p38 MAPK

We explored p38 MAPK as an upstream activator of S6K1 because we and others previously reported such crosstalk in C2C12 (Cuenda and Cohen, 1999, Deldicque et al., 2008) and because it has been showed to be

phosphorylated in a TLR2 dependent manner in the same cell line (Senn, 2006). We confirmed that PA induced an increase in p38 MAPK phosphorylation in our conditions. When cells were pre-treated with SB202190, a widely used p38 MAPK inhibitor, PA-induced S6K1 phosphorylation was totally repressed, indicating the presence of a crosstalk between p38 MAPK and S6K1 during PA incubation. Since pre-treatment with SB202190 decreased the basal levels of S6K1 phosphorylation by ~60%, it is probable that the crosstalk also exists at a basal state without PA stimulation. Although the role of S6K1 in protein synthesis is well known, the role of the crosstalk between p38 MAPK and S6K1 is still unresolved (Cuenda and Cohen, 1999, Deldicque et al., 2007).

Noteworthy, long-term administration of PA to L6 muscle cells was showed to reduce the phosphorylation state of S6K1 observed after insulin administration (Dimopoulos et al., 2006), Hence, brief elevation of extracellular PA concentration, as what was reproduced in our *in vitro* conditions, could activate S6K1 via a MAPK-dependent PKB/mTORC1-independent pathway, while longer stimulation could induce PKB/mTORC1/S6K1 inhibition. This may explain how muscle adapts differentially to a steep elevation of plasma free fatty acid, as observed during exercise (van Loon et al., 2005), or to a prolonged mild elevation of PA, as observed in diabetes and obesity. S6K1 phosphorylation was correlated with increased skeletal muscle mass in both animals (Baar and Esser, 1999, Nader et al., 2005) and humans (Koopman et al., 2006, Terzis et al., 2008). However, we had no opportunity for measuring incorporation of a labelled amino acid. Therefore, the correlation between S6K1 and protein synthesis relies on previous findings in the same systems. Further confirmation that short-lasting elevation of PA is able to activate protein synthesis will bring valuable information on the *in vivo* relevance of our findings *in vitro*.

4.3. Pharmacological and genetic controls

PA can induce cellular signaling via either intra- or extracellular action (Aas et al., 2005). We investigated the second way of action, since PA was reported to activate TLR in different cell types (Caricilli, Nascimento, 2008, Reyna et al. , 2008, Senn, 2006, Shi et al., 2006, Varma et al., 2009). We further investigated TLR2 and TLR4 pathways using two techniques, namely agonist assay and gene silencing. Either FSL-1 or LPS induced an activation of S6K1 mediated by p38 MAPK, thus indicating that both TLR2 and TLR4 receptors are functional in the myotube model that we used. While p38 MAPK is one of the canonical targets of TLR2 and TLR4, further increased phosphorylation of S6K1 after incubation with TLR agonist was only reported once in macrophages (Esser et al., 2011).

Small interfering RNA against *tlr2* was efficient in reducing TLR2 protein content by 46%. Although the direct repression of TLR2 was not mentioned in his paper, Senn reported a similar ~50% decrease of interleukin 6 expression, a downstream product of TLR activation (Senn, 2006). Interestingly, a ~50% decrease in TLR2 expression was sufficient to prevent further signaling induced by FSL-1. This was also true for TLR4: LPS did not produce any downstream phosphorylation of p38 MAPK or S6K1 when the receptor was knocked down by ~50%. Therefore, we hypothesize that a TLR activation threshold (depending on the number of receptors) exists below which no activation of p38 MAPK would be possible. Furthermore, when knocking down either *tlr2* or *tlr4* gene expression, we observed a dramatic decrease in the activation of p38 MAPK and S6K1 phosphorylation by PA, thus strongly suggesting that these effects were related to the extracellular binding of PA to TLR2 or TLR4. This also suggests some kind of synergy between TLR2 and TLR4 to mediate PA-induced signaling. Although no direct interaction has been found between the receptors, cooperation between TLR2 and TLR4 has been reported in brain microglia (Laflamme et al., 2003).

4.4. Potential contaminants

To avoid any effects due to endotoxin contamination of commercially available preparations of bovine serum albumin (BSA), we added PA to culture medium, without the adjunction of BSA. Contrary to us, in the same BSA-free condition, Errigde and Samani (2009) did not report any change in p38 MAPK in C2C12 cells exposed to 0.1mM PA. The different results between both studies could be explained by the different PA concentration used and the differentiation state of the cells. To further discard a role of endotoxins, we performed a LAL test on PA-containing media, as well as media containing PA vehicle, i.e. ethanol (data not shown).

5. CONCLUSION

In conclusion, we showed that TLR2 and TLR4 agonists are able to increase the level of S6K1 phosphorylation in a p38 MAPK dependent way in C2C12 myotubes. Furthermore PA, an endogenous ligand of TLR, induced the same intracellular signaling. Our results evidenced for the first time an atypical pathway for PA that is induced at the cellular membrane level and results in a higher phosphorylation state of S6K1 via activation of TLR and phosphorylation of p38 MAPK.

References

Aas V, Rokling-Andersen M, Wensaas AJ, Thoresen GH, Kase ET, Rustan AC. Lipid metabolism in human skeletal muscle cells: effects of palmitate and chronic hyperglycaemia. Acta Physiol Scand. 2005;183:31-41.

Akira S, Yamamoto M, Takeda K. Role of adapters in Toll-like receptor signalling. Biochemical Society transactions. 2003;31:637-42.

Baar K, Esser K. Phosphorylation of p70(S6k) correlates with increased skeletal muscle mass following resistance exercise. The American journal of physiology. 1999;276:C120-7.

Boden G. Effects of free fatty acids (FFA) on glucose metabolism: significance for insulin resistance and type 2 diabetes. Exp Clin Endocrinol Diabetes. 2003;111:121-4.

Caricilli AM, Nascimento PH, Pauli JR, Tsukumo DM, Velloso LA, Carvalheira JB, et al. Inhibition of toll-like receptor 2 expression improves insulin sensitivity and signaling in muscle and white adipose tissue of mice fed a high-fat diet. The Journal of endocrinology. 2008;199:399-406.

Chavez JA, Summers SA. Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes. Archives of biochemistry and biophysics. 2003;419:101-9.

Clarke SD. The multi-dimensional regulation of gene expression by fatty acids: polyunsaturated fats as nutrient sensors. Curr Opin Lipidol. 2004;15:13-8.

Cuenda A, Cohen P. Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. The Journal of biological chemistry. 1999;274:4341-6.

Deldicque L, Atherton P, Patel R, Theisen D, Nielens H, Rennie MJ, et al. Decrease in Akt/PKB signalling in human skeletal muscle by resistance exercise. Eur J Appl Physiol. 2008;104:57-65.

Deldicque L, Theisen D, Bertrand L, Hespel P, Hue L, Francaux M. Creatine enhances differentiation of myogenic C2C12 cells by activating both p38 and Akt/PKB pathways. Am J Physiol Cell Physiol. 2007;293:C1263-71.

Dimopoulos N, Watson M, Sakamoto K, Hundal HS. Differential effects of palmitate and palmitoleate on insulin action and glucose utilization in rat L6 skeletal muscle cells. The Biochemical journal. 2006;399:473-81.

Esser J, Gehrmann U, Salvado MD, Wetterholm A, Haeggstrom JZ, Samuelsson B, et al. Zymosan suppresses leukotriene C synthase activity in differentiating monocytes: antagonism by aspirin and protein kinase inhibitors. The

FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2011;25:1417-27.

Fenton TR, Gout IT. Functions and regulation of the 70kDa ribosomal S6 kinases. Int J Biochem Cell Biol. 2011;43:47-59.

Frost RA, Nystrom GJ, Lang CH. Multiple Toll-like receptor ligands induce an IL-6 transcriptional response in skeletal myocytes. American journal of physiology Regulatory, integrative and comparative physiology. 2006;290:R773-84.

Koopman R, Zorenc AH, Gransier RJ, Cameron-Smith D, van Loon LJ. Increase in S6K1 phosphorylation in human skeletal muscle following resistance exercise occurs mainly in type II muscle fibers. Am J Physiol Endocrinol Metab. 2006;290:E1245-52.

Laflamme N, Echchannaoui H, Landmann R, Rivest S. Cooperation between toll-like receptor 2 and 4 in the brain of mice challenged with cell wall components derived from gram-negative and gram-positive bacteria. European journal of immunology. 2003;33:1127-38.

Madan M, Amar S. Toll-like receptor-2 mediates diet and/or pathogen associated atherosclerosis: proteomic findings. PloS one. 2008;3:e3204.

Nader GA, McLoughlin TJ, Esser KA. mTOR function in skeletal muscle hypertrophy: increased ribosomal RNA via cell cycle regulators. Am J Physiol Cell Physiol. 2005;289:C1457-65.

Pullen N, Thomas G. The modular phosphorylation and activation of p70s6k. FEBS letters. 1997;410:78-82.

Radin MS, Sinha S, Bhatt BA, Dedousis N, O'Doherty RM. Inhibition or deletion of the lipopolysaccharide receptor Toll-like receptor-4 confers partial protection against lipid-induced insulin resistance in rodent skeletal muscle. Diabetologia. 2008;51:336-46.

Ragheb R, Shanab GM, Medhat AM, Seoudi DM, Adeli K, Fantus IG. Free fatty acid-induced muscle insulin resistance and glucose uptake dysfunction: evidence for PKC activation and oxidative stress-activated signaling pathways. Biochem Biophys Res Commun. 2009;389:211-6.

Reyna SM, Ghosh S, Tantiwong P, Meka CS, Eagan P, Jenkinson CP, et al. Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. Diabetes. 2008;57:2595-602.

Schmitz-Peiffer C, Craig DL, Biden TJ. Ceramide generation is sufficient to account for the inhibition of the insulin-stimulated PKB pathway in C2C12 skeletal muscle cells pretreated with palmitate. The Journal of biological chemistry. 1999;274:24202-10.

Senn JJ. Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes. The Journal of biological chemistry. 2006;281:26865-75.

Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. The Journal of clinical investigation. 2006;116:3015-25.

Terzis G, Georgiadis G, Stratakos G, Vogiatzis I, Kavouras S, Manta P, et al. Resistance exercise-induced increase in muscle mass correlates with p70S6 kinase phosphorylation in human subjects. Eur J Appl Physiol. 2008;102:145-52.

van Loon LJ, Thomason-Hughes M, Constantin-Teodosiu D, Koopman R, Greenhaff PL, Hardie DG, et al. Inhibition of adipose tissue lipolysis increases intramuscular lipid and glycogen use in vivo in humans. Am J Physiol Endocrinol Metab. 2005;289:E482-93.

Varma V, Yao-Borengasser A, Rasouli N, Nolen GT, Phanavanh B, Starks T, et al. Muscle inflammatory response and insulin resistance: synergistic interaction between macrophages and fatty acids leads to impaired insulin action. Am J Physiol Endocrinol Metab. 2009;296:E1300-10.

Wang X, Yu W, Nawaz A, Guan F, Sun S, Wang C. Palmitate induced insulin resistance by PKCtheta-dependent activation of mTOR/S6K pathway in C2C12 myotubes. Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association. 2010;118:657-61.

West AP, Koblansky AA, Ghosh S. Recognition and signaling by toll-like receptors. Annual review of cell and developmental biology. 2006;22:409-37.

Chapter 5

TLR2 and TLR4 activate p38 MAPK and JNK during endurance exercise in skeletal muscle

Hermann Zbinden Foncea[1,2], Jean-Marc Raymackers[1], Louise Deldicque[1,3], Patricia Renard[4], and Marc Francaux[1]

[1]Institute of Neuroscience, UCLouvain, Louvain-la-Neuve, Belgium

[2]School of Kinesiology and Health Research Center, Faculty of Medicine, Finis Terrae University, Santiago, Chile

[3]Research Centre for Exercise and Health, Department of Biomedical Kinesiology, K.U. Leuven, B-3001 Leuven, Belgium

[4]Research Unit of Cell Biology, University of Namur, Namur, Belgium

Med Sci Sports Exerc. 2012 Aug;44(8):1463-72.

Abstract

Purpose: Toll-like receptors 2 and 4 (TLR2, TLR4) are found in the membrane of skeletal muscle cells. It is known that a variety of molecular components amongst which the long-chain fatty acids can activate TLR2 and TLR4 and that the activation of these receptors induces downstream signalling leading to the activation of MAPK and NF-kB pathways. Therefore, the purpose of this study was to test whether the elevated level of extracellular non-esterified fatty acids (NEFA) observed during endurance exercise may trigger the activation of MAPK and NF-kB pathways via TLR2 and TLR4. **Methods:** *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice and wild-type C57BL/6J animals (WT) were submitted to an endurance exercise. **Results:** Immediately after exercise, the phosphorylation state of p38 MAPK, JNK and c-Jun was increased in tibialis anterior (TA) and soleus (SOL) muscles of WT ($P<0.05$). The phosphorylation state of ERK1/2 and IKKα/β and the DNA-binding of NF-kB remained unchanged. The activation of p38 MAPK, JNK and c-Jun was completely blunted in TA of *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice whereas in SOL, the repression was ~75%. Mice were injected with heparin for ensuring the causal relationship between NEFA concentration and MAPK activation. Heparin induced an increase in plasma NEFA similar to the one observed after endurance exercise. JNK and p38 MAPK were also activated in TA and SOL of WT ($P<0.05$), but not in muscles of *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice. **Conclusions:** The present study support the idea that during endurance exercise, TLR2 and TLR4 mediate a signal linking the elevated plasma NEFA concentration to the activation of p38 MAPK and JNK.

Keywords

NF-kB, fatty acids, HSP70, LPS

Introduction

Endurance exercise results in an increased extracellular level of non-esterified fatty acids (NEFA), which contributes to energy expenditure depending on relative power output and duration of exercise. A number of reports have suggested that beside their role in energy supply for skeletal muscle, NEFA could also play a role in activating signalling pathways which regulate gene expression (7, 34).

Toll-like receptors (TLR) are transmembrane proteins that detect a variety of molecular components. To date, 11 members of the TLR family have been identified in humans and 13 in mice (28). TLR are highly expressed in cells of the innate immune system, but TLR2 and TLR4 are also found in various other cell types including myocytes (19). TLR2 and TLR4 bind specific ligands, the best described of which are FSL-1 (Pam2Cys-GDPKHPKSF) and peptidoglycan for TLR2 and lipopolysaccharide (LPS) for TLR4. Other ligands such as heat shock protein 70 kDa (HSP70) can bind both TLR2 and TLR4 (2). NEFA are also able to activate them although the direct binding between NEFA and TLR2 and TLR4 remains questioned (12). NEFA have been shown to activate TLR2 in C2C12 myogenic cells (31) and TLR4 in the RAW264.7 macrophage cell line and in 3T3-L1 preadipocytes (32). This suggests that the elevated level of NEFA in the extracellular space could trigger the TLR-mediated signalling pathways. On this basis, we hypothesized that the increased plasma NEFA concentration observed during endurance exercise might activate intracellular signalling cascades through TLR2 and/or TLR4.

Upon stimulation, TLR2 and TLR4 recruit IL1R1-associated protein kinases (IRAK) via adaptors such as myeloid differentiation primary response gene 88 (MyD88) and TIR domain-containing adapter protein (TIRAP) (1). TLR4 is also capable of inducing a MyD88-independent pathway in which TRIF (toll interleukin-1 receptor-domain-containing adapter-inducing interferon-β)

and TRAM (TRIF-related adaptor molecule) play the role of adaptors (1). Both signalling pathways lead to the activation of the mitogen-activated protein kinase (MAPK) family and transcription factors including nuclear factor-kB (NF-kB) (1).

The results available in the literature regarding the activation of NF-kB pathways during exercise are confusing. On the one hand, an increased phosphorylation state of IKK (IkB kinase), a kinase upstream NF-kB, has been observed during a 60min submaximal exercise in rats whereas NF-kB activity increased in skeletal muscle only 1-3h after the completion of exercise (16). On the other hand, fatiguing muscle contractions have been shown to diminish NF-kB activity in both humans and mice (11). Another study showed no changes in NF-kB protein level one hour after a 2h run at 65% of VO_{2max} in rats (23).

The mitogen-activated protein kinase (MAPK) family is composed of four distinct governing members: *1*) extracellular signal-regulated kinases (ERK) 1 and 2 (ERK1/2); *2*) p38 MAPK; *3*) c-Jun NH_2-terminal kinases (JNK); and *4*) ERK5 or big MAPK. With the exception of ERK5, all MAPK are known to be activated by exercise [for review see (18)]. They participate to the regulation of metabolism, cell proliferation, differentiation and growth by regulating transcription factors and coactivators (18). The mechanisms by which p38 MAPK, JNK and ERK1/2 are activated during exercise remain incompletely resolved. Mechanisms of activation initiated locally amongst which production of ROS, acidification and mechanical perturbations seem implicated (18). In addition to those general mechanisms, MAPK undergo member-specific regulations as, for example, the phosphorylation state of p38 MAPK and JNK increases during endurance exercise whereas the activation of ERK1/2 is not affected in an animal model (35). The nature and the intensity of the exercise seem to be determinant in the fine regulation of the different members of the MAPK family.

In this study, we tested the hypothesis that the elevated plasma NEFA concentration observed during endurance exercise may activate the MAPK and the NF-kB pathways via a signal transduced by TLR2 and TLR4. The results provide evidence that plasma NEFA participate to the activation of p38 MAPK and JNK observed after treadmill exercise in mice via a mechanism implicatingTLR2 and TLR4.

Material and Methods

Animals. Male C57BL/6J mice (12-14 weeks) were housed under standard laboratory conditions (12:12h light-dark cycle) and provided with water and food ad libitum. *tlr2*$^{-/-}$ (25) and *tlr4*$^{-/-}$ mice (17) were purchased from Transgenose Institute, CNRS, Orleans-France. Animals were systematically genotyped for verifying deletion of genes coding for TLR2 or TLR4. All protocols were approved by the ethical committee for animal use of the Université catholique de Louvain (Belgium) and the housing conditions were as specified by the Belgian Law of November 14, 1993 on the protection of laboratory animals (agreement n° LA 1220548). The animals were housed in accordance with the ACSM standards for animal care.

Exercise protocol. Thirty-six mice, including wild-type (WT, n=12), *tlr2*$^{-/-}$ (n=12) and *tlr4*$^{-/-}$ (n=12) mice, were familiarized with treadmill exercise by running 20min (8-12m·min^{-1}) on two successive days. The third day, mice were submitted to an incremental exercise test. The starting velocity was 8m·min^{-1} and was increased by 2m·min^{-1} every 2min until exhaustion. The maximal velocity (V_{max}) was defined as the velocity of the last step completed by the animals. No interventions were programmed on the next day. Afterwards, the animals were kept fasted 12h overnight. Eighteen animals (WT: n=6, *tlr2*$^{-/-}$: n=6 and *tlr4*$^{-/-}$: n=6) were kept at rest (non runners) whereas the others (runners) were submitted to an endurance exercise

protocol which consisted in two bouts of 60min running at 70% of V_{max}. The two bouts were separated by a 30min recovery period. This exercise protocol was chosen because it was reported to enhance adipose tissue lipolysis (14, 33). Immediately after exercise completion, the animals were anesthetized by an intraperitoneal injection of a solution ($4ml \cdot kg^{-1}$) containing ketamine ($40mg \cdot ml^{-1}$) and xylazine ($4mg \cdot kg^{-1}$), in order to preserve muscle perfusion during dissection. The depth of anaesthesia was assessed by the disappearance of the eyelid, corneal and pedal withdrawal reflexes. Soleus (SOL) and tibialis anterior (TA) muscles were rapidly removed, frozen in liquid nitrogen and stored at -80°C until subsequent analyses. Anesthesia took effect in about 2min in every strain; this short delay should not have affected the state of phosphorylation of the studied proteins. Blood samples were collected through heart puncture by using syringes containing EDTA and immediately centrifuged for 10min at 1,500g. Plasma was kept frozen at -80°C. After dissection, the animals were killed by rapid neck dislocation.

		Body mass (g)	V_{max} ($m.min^{-1}$)
Wild-type	Non runners	24.4±0.83	19.0±1.34
	Runners	26.1±0.57	18.0±1.15
$TLR2^{-/-}$	Non runners	24.4±0.27	19.3±1.60
	Runners	23.2±0.66	20.0±1.00
$TLR4^{-/-}$	Non runners	25.5±0.70	17.0±1.10
	Runners	23.7±0.48	16.3±0.60

Table 1 - Body mass and maximal running velocity (Vmax) in wild type and transgenic $TLR2^{-/-}$ or $TLR4^{-/-}$ mice.

Lipolysis stimulation by heparin. After an overnight fast, 18 mice (WT, *tlr2*$^{-/-}$ or *tlr4*$^{-/-}$, n=6 in each group) were injected with 2,000U·kg^{-1} heparin (Heparin LEO®, Wilrijk, Belgium) intraperitoneally. The same number of animals was injected with a saline solution. After 150min, the animals were anesthetized and muscles and blood were collected following the same procedure as the one described above.

Lipopolysaccharide injection. Eighteen mice were injected with 8 mg·kg^{-1} Ultrapure LPS (Invivogen, Toulouse, France) (WT, *tlr2*$^{-/-}$, *tlr4*$^{-/-}$, n=3 in each strain) or with a saline solution (WT, *tlr2*$^{-/-}$, *tlr4*$^{-/-}$, n=3 in each strain). Blood and muscles were collected after 120min.

Analysis of blood samples. Plasma NEFA concentration was determined using a kit coupling enzymatic reaction and spectrophotometric detection (550nm) of reaction end-product (Wako, Neuss, Germany). Plasma HSP70 concentration were determined by an ELISA-kit (Biocompare, Brussels, Belgium) according to the instructions of the manufacturer.

Analysis of muscle samples. Preparation of muscle lysates. Muscles were ground by using a pestle (Bel-Art Products, Pequannock, NJ, USA) and homogenised in an ice-cold lysis buffer containing 20mM Tris, pH 7.0, 270mM sucrose, 5mM EGTA, 1mM EDTA, 1% Triton X-100, 1mM sodium orthovanadate, 50mM sodium β-glycerophosphate, 5mM sodium pyrophosphate, 50mM sodium fluoride, 1mM 1,4-dithiothreitol (DTT), and a protease inhibitor cocktail (Roche Applied Science, Vilvoorde, Belgium). Muscle homogenates were centrifuged at 10,000g for 15min at 4°C. Protein concentration was determined using a DC protein assay kit (Bio-Rad

Laboratories, Nazareth, Belgium) with bovine serum albumin as a standard. Muscle lysates were stored at -80°C until subsequent analyses.

Nuclear proteins were extracted using the method described by Deldicque et al.(10). Muscles were ground and then harvested in a hypotonic buffer containing 20mM HEPES, 5mM sodium fluoride, 1mM sodium molybdate, 0.1mM EDTA and 0.5% Igepal CA-630 (Nonidet P-40). Homogenates were centrifuged for 30s at 10,000g. The pellet was resuspended in a buffer containing 20mM HEPES, 5mM sodium fluoride, 1mM sodium molybdate, 0.1mM EDTA, 20% glycerol and a protease inhibitor cocktail (Roche Applied Science, Vilvoorde, Belgium), to which the same volume of a saline buffer containing 20mM HEPES, 5mM sodium fluoride, 1mM sodium molybdate, 0.1mM EDTA, 20% glycerol, 0.8M NaCl and a protease inhibitor cocktail (Roche Applied Science, Vilvoorde, Belgium) was added. The solution was mixed for 30min at 4°C and centrifuged for 10min at 10,000g. The supernatant was removed and stored at -80°C. Protein concentration was determined as described above.

Western blotting. Protein lysates (25-50µg) were combined with Laemmli and separated by SDS-PAGE (10-12%). After electrophoretic separation (40mA), the proteins were transferred to PVDF membranes at 80V for 2h. Membranes were blocked for 60min in Tris-buffered saline with 0.1% Tween 20 (TBST) and 5% non-fat dried milk. Then, the membranes were incubated overnight at 4°C in TBST containing 1% bovine serum albumin and one of the following antibodies: phospho-p38 MAPK Thr180/Tyr182 (1:1,000), phospho-SAPK/JNK Thr183/Tyr185 (1:750), phospho-ERK1/2 Thr202/Tyr204 (1:1,000), phospho-IKKα/β Ser176/180 (1:1,000), phospho-c-Jun Ser63 (1:1,000), IκBα (1:1,000) and GAPDH (1:10,000). All antibodies were obtained from Cell Signaling Technology (Leiden, The Netherlands) except GAPDH from Abcam (Cambridge, UK). Membranes were washed three times with TBST and were incubated with a secondary

antibody at room temperature for 60min [anti-rabbit (1:10,000) or anti-mouse (1:20,000) from Sigma (Bornem, Belgium)]. After three additional washes, chemiluminescent detection was carried out using an ECL Western blotting kit (Amersham ECL Plus, GE Healthcare, Diegem, Belgium). Bands were visualized on film, scanned and quantified by densitometry using the ImageMaster 1D image analysis software (Amersham-GE Healthcare, Diegem, Belgium). Results are reported as the ratio of the signal induced by the protein of interest divided by the signal induced by GAPDH.

DNA binding of NF-κB. DNA binding of NF-κB was measured as described by Renard et al. (29). 96-well plates were coated with double-stranded oligonucleotidic probe containing the consensus binding for NF-κB. Muscle lysate containing nuclear proteins (35µg) were placed in the wells and incubated 1h at room temperature. Then, wells were washed three times with a phosphate-buffer saline (PBS) solution (phosphate=10mM, NaCl=50mM, pH=7.5) containing 0.1% Tween. Rabbit anti-NF-κBp65 (100µl, Santa Cruz, Boechout, Belgium) diluted 1,000 times in a PBS-10 solution (phosphate=10mM, NaCl=10mM, 1% non-fat dried milk, pH=7.5) was added in each well for 1h at room temperature. The wells were washed three times. Subsequently, 100µl of a peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz, Boechout, Belgium) diluted 1,000 times in a PBS-10 solution were added in each well for 1h at room temperature. After three additional washes, tetramethyldbenzidine (100µl, Biosource, Nivelles, Belgium) was added in the wells and incubated 10min at room temperature before adding 100µl of stopping solution (Biosource, Nivelles, Belgium). Optical density was read at 405nm using a 655nm reference wavelength with a microplate reader Benchmark (Bio-Rad Laboratories, Nazareth, Belgium).

Statistical analysis. All results are presented as means ± standard errors of the mean. The significance of differences observed between means was inferred by a two-way ANOVA design wherein mice strains and treatment were the independent factors. When appropriate, Bonferroni post hoc tests were applied. Testing for outliers was done with Grubbs's test (21). No more than one sample was rejected in each series. The signification threshold was set to $P<0.05$.

Results

Body weight and exercise performance. Baseline characteristics such as body weight and V_{max} were similar between WT and transgenic *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice. Likewise, body weight and V_{max} were not different between runners and non runners mice, prior to training (Table 1).

Plasma NEFA concentration. Plasma NEFA concentration was similar in all mice kept at rest whatever the strain (Fig. 5.1A,B). Higher plasma NEFA concentration was observed after exercise ($P<0.001$) (Fig. 5.1A). NEFA level was more than doubled after exercise in WT and increased even more in *tlr2*$^{-/-}$ ($P=0.007$) and in *tlr4*$^{-/-}$ ($P=0.019$) in comparison with WT mice (Fig.5.1A).

Heparin (2,000U.kg^{-1}, IP) was injected to WT, transgenic *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice, while a control group was injected with an equal volume of sterile saline. Heparin injection induced an increase in plasma NEFA concentration by about 290% (Fig. 5.1B) whatever the strain. To avoid any confounding variable due to changes in extracellular HSP70 le vel during exercise (a TLR ligand that can be increased during exercise (27)), plasma HSP70 concentrations were measured (Fig. 5.1C,D). None of the experimental strategies produced any increase in circulating HSP70, either in WT, or in *tlr2*$^{-/-}$ or *tlr4*$^{-/-}$.

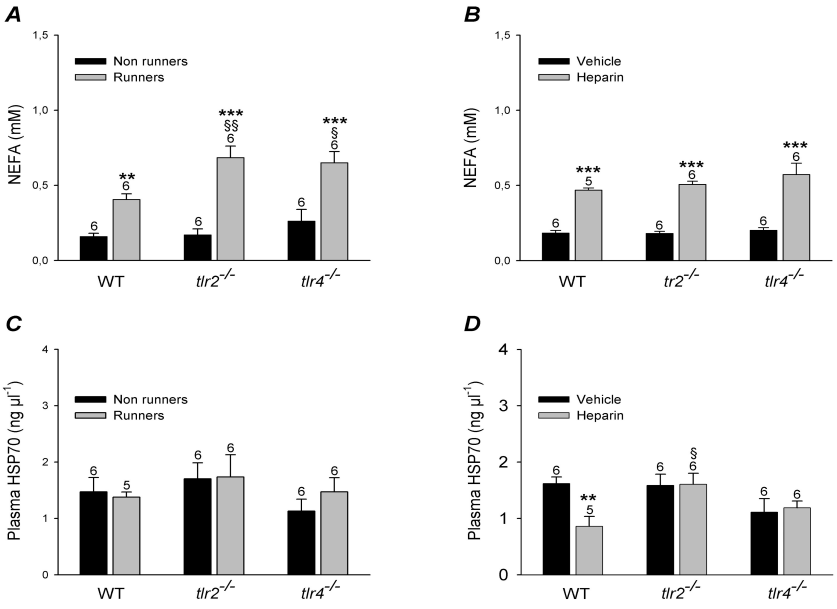


Fig. 5.1. NEFA and HSP70 concentrations in WT (n=5) and tlr2$^{-/-}$ (n=6) or tlr4$^{-/-}$ (n=6) mice after exercise or heparin-induced lipolysis. Significant differences between runners and non runners mice (A&C) or between heparin and vehicle treated mice (B&D) from the same strain are indicated by **, P<0.01, ***, P<0.001. Significant differences between WT and *tlr2*$^{-/-}$ or *tlr4*$^{-/-}$ mice are indicated by §, P<0.05; §§, P<0.01. The number of animals used is indicated above each histogram.

Implication of TLR2 and TLR4 in p38 MAPK, JNK and c-Jun activation by endurance exercise. At rest, the phosphorylation states of p38 MAPK, JNK and ERK1/2 were similar between strains both in TA and in SOL (Fig. 5.2)

In WT, endurance exercise resulted in a higher phosphorylation state of p38 MAPK (+2.3±0.40 fold, P=0.001; Fig.5.2A) and JNK (+2.0±0.1 fold, P<0.001; Fig. 5.2C) in TA. JNK and p38 MAPK activations elicited by endurance exercise were repressed in *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ transgenic mice (Fig. 5.2A,C). In WT SOL, endurance exercise increased the phosphorylation state of p38 MAPK (+2.1±0.4 fold, P=0.006; Fig. 5.2B) and JNK (+2.5±0.3 fold, P=0.008; Fig. 5.2D), whereas in *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ transgenic mice the

activation was largely repressed without reaching the statistical threshold (Fig. 5.2B and D). Both in TA and in SOL, the phosphorylation state of ERK1/2 was not affected by the exercise protocol whatever the strain (Fig. 5.2E and F). In WT mice, c-Jun, a target of JNK, was more phosphorylated after exercise in both TA (2.3 ± 0.5 fold; P=0.023) and SOL (3.2 ± 1.0 fold; P<0.001) muscles (Fig. 5.2G,H). No significant increase in phosphorylation was seen neither in *tlr2*$^{-/-}$, nor in *tlr4*$^{-/-}$ muscles at rest, while in *tlr4*$^{-/-}$ SOL, a 3.7-fold raise in c-Jun phosphorylation was observed after exercise (P<0.05; Fig. 5.2H).

Activation of p38 MAPK and JNK by heparin through TLR2 and TLR4 receptors. Heparin is known to stimulate NEFA release in plasma (9). To assess whether a higher level of circulating NEFA may initiate the activation of p38 MAPK and JNK, heparin (2,000U.kg^{-1}, IP) was injected to WT, transgenic *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice.

In TA of WT mice, heparin increased the phosphorylation states of p38 MAPK (+2.6±0.5 fold, P<0.001; Fig. 5.3A) and JNK (+2.2±0.4 fold, P=0.001; Fig. 5.3C). In transgenic *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ these activations were not detectable. Similar results were found in SOL muscle. In the WT group the phosphorylation state of p38 MAPK increased by 3.2±0.8 fold (P=0.01; Fig. 5.3B) and JNK phosphorylation was significantly increased by 2.1±0.5 fold (P=0.022; Fig. 5.3D) above basal levels. As observed in TA, transgenic *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice did not show any change in the phosphorylation state of p38 MAPK and JNK in SOL muscle.

Heparin injection did not modify either the phosphorylation state of IKKα/β, or the phosphorylation state of ERK1/2 (data not shown).

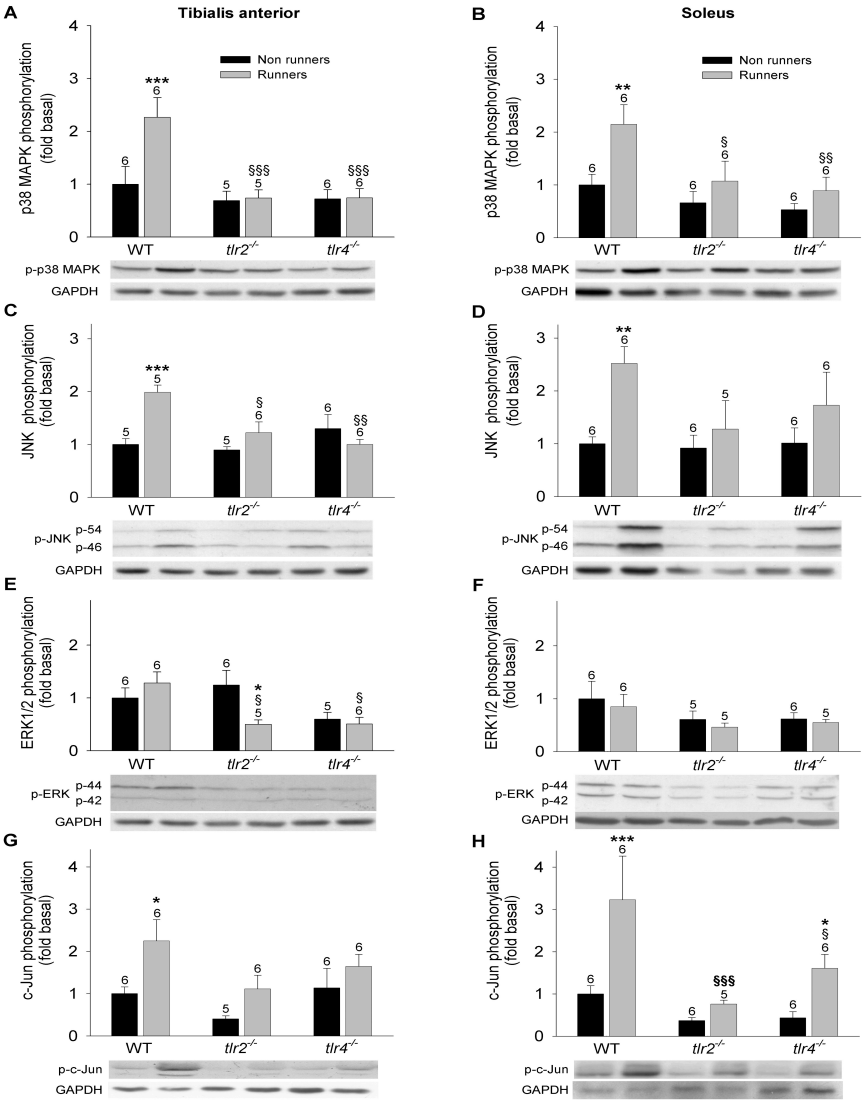


Fig. 5.2 . Phosphorylation states of p38 MAPK (A&B), JNK (C&D), ERK1/2 (E&F) and c-Jun (G&H) at rest (non runners) or after an endurance running exercise (runners) in tibialis anterior (A,C, E&G) and soleus (B, D, F&H) muscles of WT and tlr2$^{-/-}$ or tlr4$^{-/-}$ mice. Significant differences between mice from the same strain are indicated by *, P<0.05, **, P<0.01, ***, P<0.001 Significant differences between WT and *tlr2*$^{-/-}$ or *tlr4*$^{-/-}$ mice are indicated by §, P<0.05; §§, P<0.01; §§§, P<0.001. The number of animals used is indicated above each histogram.

No effect of exercise on NF-κB pathway. Several techniques were used to assess the activation of the NF-κB pathway in skeletal muscle exposed to high levels of NEFA in mouse. In resting conditions, the phosphorylation state of IKKα/β was similar in WT and in *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ transgenic mice in TA (Fig. 5.4A) and SOL (Fig. 5.4C). Whatever the strain and the muscle analysed, the exercise protocol did not modify the phosphorylation state of IKKα/β.

To further ascertain that the NF-κB pathway was not activated by exercise, the DNA binding of NF-κB was measured. In TA muscle, the DNA binding of NF-κB was not different between strains and was not affected by the exercise protocol (Fig. 5.4B). WT mice treated with 8mg.kg^{-1} LPS for 120 minutes were used as a positive control. In TA, the phosphorylation state of IKKa/b was more than doubled (Fig. 5.4A) and the DNA binding of NF-κB increased by ~5-fold compared to control conditions where mice were injected with vehicle (Fig. 5.4B).

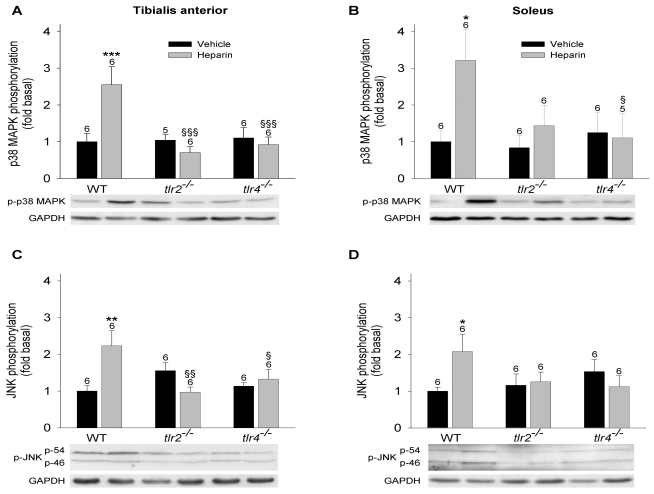


Fig. 5.3. Phosphorylation state of p38 MAPK (A&B) and JNK (C&D) in tibialis anterior (A&C) and soleus (B&D) muscles of WT and tlr2$^{-/-}$ or tlr4$^{-/-}$ mice injected either with 2,000U·kg^{-1} heparin or with a saline vehicle.

Significant differences between saline- and heparin-injected animals from the same strain are indicated by *, P<0.05; **P<0.01, and ***, P<0.001. Significant differences between WT and *tlr2*$^{-/-}$ or *tlr4*$^{-/-}$ heparin injected mice are indicated by §, P<0.05; §§, P<0.01 and §§§, P<0.001. The number of animals used is indicated above each histogram.

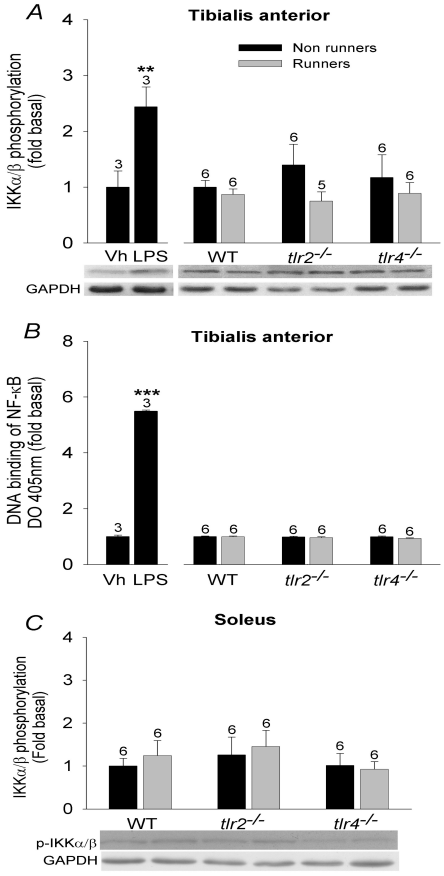


Fig. 5.4. Phosphorylation state of IKKa/b (Ser176/180) in tibialis anterior (A) and soleus (C) and DNA binding of NF-kB in tibialis anterior (B) at rest (non runners) or after an endurance running exercise (runners) in wild type and transgenic tlr2$^{-/-}$ or tlr4$^{-/-}$ mice. Significant differences with vehicle are indicated by ** (P<0.01) and *** (P<0.001). The number of animals used is indicated above each histogram.

Discussion

This study is the first to unravel a role of TLR2 and TLR4 in the activation of p38 MAPK and JNK during endurance exercise in mice. TLR are receptors capable of recognising multiple ligands among which lipids (31, 32). Although a direct activation of TLR by NEFA remains controversial (12, 32), our data support the idea that during exercise, p38 MAPK and JNK activation might be related to circulating NEFA. In this view, TLR2 and TLR4 could play a role of signal transducer between extracellular NEFA and p38 MAPK and JNK activation.

Activation of p38 MAPK. Evidence from studies on isolated muscle preparations show that p38 MAPK activation may be triggered by CaMKII,

suggesting that the increase in cytosolic calcium concentration in skeletal muscle during exercise might mediate the exercise-induced p38 MAPK activation (39). Stretch, production of reactive oxygen species (ROS) and glycogen depletion are other factors participating potentially to the activation of p38 MAPK during endurance exercise (4, 5, 8). Previous experiments made on cell cultures and *ex vivo* muscle preparations reported that MAPK could be activated by electrical, mechanical or chemical conditions without the need of NEFA (15). However, Widegren *et al.* showed that a 30-min one-legged exercise induced a 2.2-fold increase in p38 MAPK phosphorylation in muscles from both exercised and unexercised legs (37), indicating that p38 MAPK is sensitive to systemic agents. This was not the case for ERK1/2 which presented an increased phosphorylation in the exercised leg only.

Activation of JNK. Activation of JNK seems particularly sensitive to stretch since eccentric contractions induce a larger activation of JNK than concentric contractions (3). The results of the present study reveal that the signalling cascades initiated by TLR2 and TLR4 mediate the major mechanisms of p38 MAPK and JNK activation during running endurance exercise in mice. The transgenic animals showed an almost complete repression of the increase in p38 MAPK and JNK phosphorylation in TA and at least a 75% repression in SOL. Definitely, SOL is more recruited than TA during running exercise in rodents. Therefore, TLR-independent mechanisms of p38 MAPK and JNK activation should be more visible in SOL than in TA. However, they remained quantitatively lower than the TLR-dependent mechanisms which seem to be the main regulators of p38 MAPK and JNK activation during endurance running exercise in mice.

For verifying that increased phosphorylation of MAPK reflects higher kinase activity, we also measured c-Jun, a protein downstream of JNK, in SOL and TA muscles from exercised mice. In WT animals, c-Jun was more

phosphorylated after exercise, confirming the higher activity of JNK. In the SOL of *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice, JNK tended to be more phosphorylated after running and this was reflected by a higher phosphorylation state of c-Jun in those mice, reaching the statistical threshold in *tlr4*$^{-/-}$ mice. This might suggest that small non-significant increases in phospho-JNK are able to activate c-Jun although we may not rule out that other kinases, such as cyclin-dependent kinase-3 (6) participated to the increased phospho-c-Jun in *tlr4*$^{-/-}$ mice after exercise.

Activation of p38 and JNK by NEFA. To rule out any confounding variables related to exercise, we injected animals with heparin a well-known activator of lipolysis (9) with the goal of increasing NEFA concentration at rest. After heparin injection, the phosphorylation state of p38 MAPK and JNK were increased in WT animals within the same range as during exercise, i.e. a 2 to 3 fold increase. But, in this case changes in the phosphorylation state of p38 MAPK and JNK were completely blunted in *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice whatever the muscle considered. This reinforced the suggestion that the incomplete repression of p38 MAPK and JNK activation observed after exercise in *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ SOL is probably due to TLR-independent mechanisms. This also supports the idea that transitory changes in extracellular NEFA concentrations such as those observed during endurance exercise are sufficient to elicit activation of p38 MAPK and JNK and that this activation is mediated by TLR2 and TLR4.

In separate experiments we attempted to blocked lipolysis during exercise by injecting various doses of acipimox or nicotinic acid (13) (data not shown). We did not succeed in reducing the increase in NEFA plasma concentration without affecting significantly the exercise capacity of animals. This is a limitation of our study.

Cooperation between TLR2 and TLR4. Cooperation between TLR2 and TLR4 has been demonstrated in immune cells (24). Recognition of *Mycobacterium bovis* BCG by TLR activates MAPK. This activation is completely blocked by anti-TLR2 MAb or with anti-TLR4 MAb indicating that simultaneous recognition of *Mycobacterium bovis* BCG by TLR2 and TLR4 is required for activating MAPK (24). To further evaluate such cooperation in skeletal muscle, we injected LPS intraperitoneally into WT, *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice. This yielded a +731% ($P<0.001$), a +309% ($P=0.03$) and a +82% ($P=0.109$) increase in phosphorylated p38 MAPK in TA, respectively (not shown). Therefore, it is possible that either our *tlr2*$^{-/-}$ strain has a slight TLR4 impairment too or, LPS signal transduction in muscle requires both TLRs to be efficient.

The results of the present study extend our knowledge of the activation of p38 MAPK and JNK by extracellular NEFA in skeletal muscle. Whatever the mechanism by which extracellular NEFA were increased, i.e. endurance exercise or heparin injection, the phosphorylation state of p38 MAPK and JNK were largely repressed in *tlr2*$^{-/-}$ and in *tlr4*$^{-/-}$ animals, indicating that recognition of NEFA by both TLR2 and TLR4 is necessary to activate p38 MAPK and JNK.

Exercise is known to activate ERK1/2 pathway in an intensity-dependent manner (38). In the present study, the endurance exercise protocol was designed with the goal of increasing plasma NEFA concentrations (33). It did not change the phosphorylation state of ERK1/2 either in wild type or in *tlr2*$^{-/-}$ or in *tlr4*$^{-/-}$ animals suggesting that the intensity of our exercise protocol was likely too low to stimulate ERK1/2. Moreover, ERK1/2 phosphorylation was not changed by heparin injection. So, our results do not support the hypothesis of an activation of ERK1/2 initiated by extracellular NEFA during endurance exercise.

Lack of NF-kB pathway activation. TLR4 is known to mediate the activation of NF-kB pathway by long-chain unsaturated fatty acids in pre-adipocytes in culture and by lipid infusion in mice (32). It has also been shown that the activation of NF-kB pathway by palmitate is repressed when TLR2 is blocked by anti-TLR2 MAb or by siRNA (31). We were unable to depict changes in the phosphorylation state of IKKa/b after endurance exercise and after heparin injection whereas plasma NEFA concentration was increased. To ensure that we did not miss any activation of the NF-kB pathway, we measured the DNA binding of the NF-kB. We also injected animals with LPS as a positive control. These results convince us that our experimental running protocols did not induce any activation of the NF-kB pathway possibly because extracellular lipid levels were not high enough and/or because the stimulation was too short. Our results are in agreement with those reported by McKenzie et al. who did not observe any activation of the NF-kB pathway during endurance exercise in rats (23).

Metabolism regulation. Transgenic animals used in this study (17, 25) were not muscle conditional knock-out mice. The higher plasma NEFA concentrations observed after exercise in *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ animals suggest that TLR2 and TLR4 could also regulate metabolic functions in organs such as adipose tissue or could modify the repartition of energy substrates consumed during exercise. This does not challenge the main findings of our study since circulating NEFA concentrations were higher in TLR deficient than in WT mice after exercise. These observations open new research perspectives regarding the role of TLR2 and TLR4 in various tissues during exercise.

HSP70 is another ligand recognized by both TLR2 and TLR4 (2). Plasma HSP70 concentrations were not changed during our exercise protocol indicating that HSP70 does not interfere with the proposed mechanisms of activation of p38 MAPK and JNK. Increase of HSP70 after exercise is controversial. An increase in HSP70 after exercise has been observed in both

animals (22, 30) and human (36) but it is not always the case (20). These controversial results may be explained by the fact that HSP70 production after exercise is intensity-dependent (26). Our exercise protocol could be intense enough to promote an increment in plasma NEFA concentrations but not to increase HSP70 production.

Conclusions. This study provides for the first time evidence that during endurance exercise TLR2 and TLR4 mediate a signal linking the elevated plasma NEFA concentration to the activation of p38 MAPK, JNK and c-Jun. Further research will be warranted to elucidate the molecular intermediates between TLR2/TLR4 and MAPK in skeletal muscle.

References

Akira S, and Sato S. Toll-like receptors and their signaling mechanisms. Scand J Infect Dis. 2003;35(9):555-62.

Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, and Calderwood SK. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem. 2002;277(17):15028-34.

Boppart MD, Aronson D, Gibson L, Roubenoff R, Abad LW, Bean J, Goodyear LJ, and Fielding RA. Eccentric exercise markedly increases c-Jun NH(2)-terminal kinase activity in human skeletal muscle. J Appl Physiol. 1999;87(5):1668-73.

Chambers MA, Moylan JS, Smith JD, Goodyear LJ, and Reid MB. Stretch-stimulated glucose uptake in skeletal muscle is mediated by reactive oxygen species and p38 MAP-kinase. J Physiol. 2009;587(Pt 13):3363-73.

Chan MH, McGee SL, Watt MJ, Hargreaves M, and Febbraio MA. Altering dietary nutrient intake that reduces glycogen content leads to phosphorylation of nuclear p38 MAP kinase in human skeletal muscle: association with IL-6 gene transcription during contraction. Faseb J. 2004;18(14):1785-7.

Cho YY, Tang F, Yao K, Lu C, Zhu F, Zheng D, Pugliese A, Bode AM, and Dong Z. Cyclin-dependent kinase-3-mediated c-Jun phosphorylation at Ser63 and Ser73 enhances cell transformation. Cancer Res. 2009;69(1):272-81.

Civitarese AE, Hesselink MK, Russell AP, Ravussin E, and Schrauwen P. Glucose ingestion during exercise blunts exercise-induced gene expression of skeletal muscle fat oxidative genes. Am J Physiol Endocrinol Metab. 2005;289(6):E1023-9.

Clerk A, Fuller SJ, Michael A, and Sugden PH. Stimulation of "stress-regulated" mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and p38-mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. J Biol Chem. 1998;273(13):7228-34.

Connor WE, and Armstrong ML. Plasma lipoprotein lipase after subcutaneous heparin. Circulation. 1961;24:87-93.

Deldicque L, Atherton P, Patel R, Theisen D, Nielens H, Rennie MJ, and Francaux M. Decrease in Akt/PKB signalling in human skeletal muscle by resistance exercise. Eur J Appl Physiol. 2008;104(1):57-65.

Durham WJ, Li YP, Gerken E, Farid M, Arbogast S, Wolfe RR, and Reid MB. Fatiguing exercise reduces DNA binding activity of NF-kappaB in skeletal muscle nuclei. J Appl Physiol. 2004;97(5):1740-5.

Erridge C, and Samani NJ. Saturated Fatty Acids Do Not Directly Stimulate Toll-Like Receptor Signaling. Arterioscler Thromb Vasc Biol. 2009;29(11):1944-9.

Fuccella LM, Goldaniga G, Lovisolo P, Maggi E, Musatti L, Mandelli V, and Sirtori CR. Inhibition of lipolysis by nicotinic acid and by acipimox. Clin Pharmacol Ther. 1980;28(6):790-5.

Goto K, Ishii N, Mizuno A, and Takamatsu K. Enhancement of fat metabolism by repeated bouts of moderate endurance exercise. J Appl Physiol. 2007;102(6):2158-64.

Hayashi T, Hirshman MF, Dufresne SD, and Goodyear LJ. Skeletal muscle contractile activity in vitro stimulates mitogen-activated protein kinase signaling. The American journal of physiology. 1999;277(4 Pt 1):C701-7.

Ho RC, Hirshman MF, Li Y, Cai D, Farmer JR, Aschenbach WG, Witczak CA, Shoelson SE, and Goodyear LJ. Regulation of IkappaB kinase and NF-kappaB in contracting adult rat skeletal muscle. Am J Physiol Cell Physiol. 2005;289(4):C794-801.

Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, and Akira S. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol. 1999;162(7):3749-52.

18. Kramer HF, and Goodyear LJ. Exercise, MAPK, and NF-kappaB signaling in skeletal muscle. J Appl Physiol. 2007;103(1):388-95.

Lang CH, Silvis C, Deshpande N, Nystrom G, and Frost RA. Endotoxin stimulates in vivo expression of inflammatory cytokines tumor necrosis factor alpha, interleukin-1beta, -6, and high-mobility-group protein-1 in skeletal muscle. Shock. 2003;19(6):538-46.

Liu Y, Lormes W, Wang L, Reissnecker S, and Steinacker JM. Different skeletal muscle HSP70 responses to high-intensity strength training and low-intensity endurance training. Eur J Appl Physiol. 2004;91(2-3):330-5.

Livesey JH. Kurtosis provides a good omnibus test for outliers in small samples. Clinical biochemistry. 2007;40(13-14):1032-6.

Locke M, Noble EG, and Atkinson BG. Exercising mammals synthesize stress proteins. The American journal of physiology. 1990;258(4 Pt 1):C723-9.

McKenzie MJ, and Goldfarb AH. Aerobic exercise bout effects on gene transcription in the rat soleus. Med Sci Sports Exerc. 2007;39(9):1515-21.

Mendez-Samperio P, Belmont L, and Miranda E. Mycobacterium bovis BCG Toll-like receptors 2 and 4 cooperation increases the innate epithelial immune response. Arch Med Res. 2008;39(1):33-9.

Michelsen KS, Aicher A, Mohaupt M, Hartung T, Dimmeler S, Kirschning CJ, and Schumann RR. The role of toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DCS). Peptidoglycan and lipoteichoic acid are inducers of DC maturation and require TLR2. J Biol Chem. 2001;276(28):25680-6.

Milne KJ, and Noble EG. Exercise-induced elevation of HSP70 is intensity dependent. Journal of applied physiology. 2002;93(2):561-8.

Morton JP, Holloway K, Woods P, Cable NT, Burniston J, Evans L, Kayani AC, and McArdle A. Exercise training-induced gender-specific heat shock protein adaptations in human skeletal muscle. Muscle & nerve. 2009;39(2):230-3.

Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, and Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998;282(5396):2085-8.

Renard P, Ernest I, Houbion A, Art M, Le Calvez H, Raes M, and Remacle J. Development of a sensitive multi-well colorimetric assay for active NFkappaB. Nucleic Acids Res. 2001;29(4):E21.

Samelman TR. Heat shock protein expression is increased in cardiac and skeletal muscles of Fischer 344 rats after endurance training. Experimental physiology. 2000;85(1):92-102.

Senn JJ. Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes. J Biol Chem. 2006;281(37):26865-75.

Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, and Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest. 2006;116(11):3015-25.

Stich V, de Glisezinski I, Berlan M, Bulow J, Galitzky J, Harant I, Suljkovicova H, Lafontan M, Riviere D, and Crampes F. Adipose tissue lipolysis is increased during a repeated bout of aerobic exercise. J Appl Physiol. 2000;88(4):1277-83.

Tunstall RJ, McAinch AJ, Hargreaves M, van Loon LJ, and Cameron-Smith D. Reduced plasma free fatty acid availability during exercise: effect on gene expression. Eur J Appl Physiol. 2007;99(5):485-93.

van Ginneken MM, de Graaf-Roelfsema E, Keizer HA, van Dam KG, Wijnberg ID, van der Kolk JH, and van Breda E. Effect of exercise on activation of the p38 mitogen-activated protein kinase pathway, c-Jun NH2 terminal kinase, and heat shock protein 27 in equine skeletal muscle. Am J Vet Res. 2006;67(5):837-44.

Walsh RC, Koukoulas I, Garnham A, Moseley PL, Hargreaves M, and Febbraio MA. Exercise increases serum Hsp72 in humans. Cell stress & chaperones. 2001;6(4):386-93.

Widegren U, Jiang XJ, Krook A, Chibalin AV, Bjornholm M, Tally M, Roth RA, Henriksson J, Wallberg-henriksson H, and Zierath JR. Divergent effects of exercise on metabolic and mitogenic signaling pathways in human skeletal muscle. Faseb J. 1998;12(13):1379-89.

Widegren U, Wretman C, Lionikas A, Hedin G, and Henriksson J. Influence of exercise intensity on ERK/MAP kinase signalling in human skeletal muscle. Pflugers Arch. 2000;441(2-3):317-22.

Wright DC, Geiger PC, Han DH, Jones TE, and Holloszy JO. Calcium induces increases in peroxisome proliferator-activated receptor gamma coactivator-1alpha and mitochondrial biogenesis by a pathway leading to p38 mitogen-activated protein kinase activation. J Biol Chem. 2007;282(26):18793-9.

Chapter 6

Contribution of non-esterified fatty acids to mitogen-activated protein kinases activation in human skeletal muscle during endurance exercise

Hermann Zbinden-Foncea[1,2], Luc J.C. van Loon[3], Jean-Marc Raymackers[1], Marc Francaux[1,*], Louise Deldicque[4]

[1]Institute of Neuroscience, Université catholique de Louvain, Place Pierre de Coubertin 1, 1348 Louvain-la-Neuve, Belgium

[2]School of Kinesiology and Health Research Center, Faculty of Medicine, Universidad Finis Terrae, Avenuda Pedro De Valdivia 1509, Santiago, Chile

[3]Department of Human Movement Sciences, Maastricht University Medical Center, PO Box 616, 6200 MD Maastricht, The Netherlands

[4]Exercise Physiology Research Group, Department of Kinesiology, KU Leuven, Tervuursevest 101, 3001 Leuven, Belgium

Int. J Sport Nutr Exer Metab. 2012 Oct 30. [Epub ahead of print].

Abstract

Mitogen-activated protein kinase (MAPK) pathways are activated in skeletal muscle during endurance exercise, but the upstream molecular events are incompletely resolved. As an increase in plasma non-esterified fatty acids (NEFA) is a common feature of long-lasting exercise, we tested the hypothesis that NEFA contribute to the activation of MAPK during endurance exercise. Acipimox was used prior and during endurance exercise to prevent the elevation of plasma NEFA levels in healthy subjects and diabetics. In two separate studies, healthy subjects cycled for 2h and diabetics for 1h at 50% W_{max}. In control conditions, plasma NEFA concentrations increased from 0.35mM to 0.90mM during exercise in healthy subjects and from 0.55mM to 0.70mM in diabetics ($P<0.05$). Phosphorylation state of extracellular-regulated kinase 1 and 2 (ERK1/2), p38 and, c-Jun NH_2-terminal kinases (JNK) were significantly increased after exercise in the vastus lateralis in both groups. Acipimox blocked the increase in plasma NEFA concentrations and almost completely repressed any rise in ERK1/2 and p38 but not in JNK. In conclusion, our data support a role for plasma NEFA in the activation of p38 and ERK1/2 in skeletal muscle tissue of healthy and diabetic subjects during endurance exercise. Further investigation will be required to determine the molecular link between NEFA and MAPK activation during exercise in human skeletal muscle.

Keywords

p38 MAPK, ERK1/2, JNK, NF-kB, acipimox, cycling, TLR4, type 2 diabetes

Introduction

The mitogen-activated protein kinases (MAPK) are a group of intracellular signaling proteins activated by multiple signals and are critically involved in the transmission of information from outside the cell to the nucleus, where they regulate transcription. Extracellular signal-regulated kinases (ERK) 1 and 2 (ERK1/2), p38 MAPK and c-Jun NH_2-terminal kinases (JNK) are three widely expressed MAPK that regulate different cellular functions (Johnson & Lapadat, 2002). Endurance exercise is a well-known activator of MAPK but the mechanisms by which ERK1/2, p38 MAPK and JNK are activated during exercise remain incompletely resolved (Kramer & Goodyear, 2007). Increase in cytosolic calcium and subsequent calcium/calmodulin-dependent protein kinase II activation, as well as production of reactive oxygen species (ROS), acidification, increase in catecholamines, growth hormones or cannabinoids, glycogen depletion or mechanical stretch have been proposed to participate to the activation of p38 MAPK during endurance exercise (Blair, Hajduch, Litherland, & Hundal, 1999; Chambers, Moylan, Smith, Goodyear, & Reid, 2009; Chan, McGee, Watt, Hargreaves, & Febbraio, 2004; Clerk, Fuller, Michael, & Sugden, 1998; Galbo, 1986; Li et al., 2005; Tedesco et al., 2010; Wright, Geiger, Han, Jones, & Holloszy, 2007; Wretman et al., 2001). In addition to those general mechanisms, MAPK undergo member-specific regulations. ERK1/2 and p38 have been shown to be involved in exercise-induced signalling in human skeletal muscle but the picture is less clear for JNK (Widegren et al., 1998; Long, Widegren, & Zierath, 2004; Yu, Blomstrand, Chibalin, Krook, & Zierath, 2001). Cycling markedly increased ERK1/2 phosphorylation, albeit only transiently, whereas similar exercise has been demonstrated to lead to a smaller but more persistent increase in p38 activation (Widegren et al., 1998). MAPK signalling seems to be regulated differentially according to the mode of contraction (concentric *vs* eccentric), the intensity of the

exercise and the training status of the subjects (Widegren, Wretman, Lionikas, Hedin, & Henriksson, 2000).

Moderate to strenuous endurance exercise results in an increased extracellular level of non-esterified fatty acids (NEFA) (Stich et al., 2000), which contributes to energy expenditure depending on relative power output and duration of exercise (Kiens, 2006). This increment in NEFA availability is known to be less pronounced in obese and diabetic people mainly due to a lower sensitivity to catecholamines (Kanaley, Cryer, & Jensen, 1993). The contribution of NEFA to energy production has been studied by using acipimox, a nicotinic acid analogue able to prevent adipose tissue lipolysis at rest and during exercise (O'Kane, Trinick, Tynan, Trimble, & Nicholls, 1992). A reduction in NEFA use during exercise following nicotinic acid administration increased the use of endogenous glycogen stores and intramuscular lipid in healthy trained (van Loon et al., 2005b) and type 2 diabetic males (van Loon et al., 2005a).

Beside their role in energy supply for skeletal muscle, NEFA have been suggested to also play a role in activating signaling pathways which regulate gene expression (Tunstall, McAinch, Hargreaves, van Loon, & Cameron-Smith, 2007). It has been proposed that NEFA could stimulate a family of membrane receptors called the toll-like receptors (TLR) (Senn, 2006; Shi et al., 2006), the most expressed in skeletal muscle being TLR2 and TLR4 (Reyna et al., 2008). Upon stimulation, TLR2 and TLR4 induce complex intracellular signaling leading to the activation of the MAPK family and transcription factors including nuclear factor-kB (NF-kB) (Akira, Yamamoto, & Takeda, 2003). Using TLR2 and TLR4 KO mice, our group recently found that during endurance exercise, TLR2 and TLR4 mediated a signal linking the elevated plasma NEFA concentration to the activation of

p38 MAPK and JNK in mice skeletal muscle (Zbinden-Foncea, Raymackers, Deldicque, Renard, & Francaux, 2012). For the first time, a link was established between circulating NEFA and stimulation of MAPK via activation of TLR2/4 during endurance exercise in mice but this remains to be confirmed in human. We have recently got the opportunity to analyze the activation of the MAPK pathways in remaining human muscle biopsies from previously published studies (van Loon et al., 2005a; van Loon et al., 2005b), where healthy subjects or diabetic patients received a placebo or acipimox, thereby blocking the rise in plasma NEFA during exercise. Diabetic patients contributed to better understand the role of NEFA in MAPK activation during exercise as the increment in NEFA availability is known to be less in this population (Kanaley et al., 1993), although the resting concentration is higher. In the present study, we hypothesise that NEFA contribute to MAPK activation in human skeletal muscle during endurance exercise.

Material and Methods

Subjects

Blood samples and muscle biopsies from five healthy subjects (van Loon et al., 2005b) and ten diabetic patients (van Loon et al., 2005a) who participated in a previous experiment were further analyzed. Subjects were informed about the nature and risks of the experimental procedures before their written informed consent was obtained. The two studies were approved by the Medical Ethical Committee of the Academic Hospital Maastricht and all procedures were carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association.

Study 1. Five healthy active male subjects [age 23 ± 1 y, height 1.79 ± 0.04 m, body weight 70 ± 3 kg, BMI 22.0 ± 0.9 $kg.m^{2\,-1}$, fat content 12.3 ± 0.8 %, maximal power output (W_{max}) 354 ± 11 W, 50% W_{max} 177 ± 6 W, and

maximal oxygen uptake capacity (VO_2max) 61 ± 3 ml.kg $bw^{-1}.min^{-1}$] participated in this study.

Study 2. Ten sedentary, overweight subjects [age 60 ± 2 y, height 1.79 ± 0.02 m, body weight 91 ± 3 kg, BMI 28.4 ± 1.0 $kg.m^{2\ -1}$, fat content 28.8 ± 1.8 %, W_{max} 200 ± 15 W, 50% W_{max} 100 ± 6 W, and VO_2max 32 ± 2 ml.kg $bw^{-1}.min^{-1}$], who had been diagnosed with type 2 diabetes for over 5 years, participated in this study. All subjects were using oral blood-glucose-lowering medication (metformin with or without a sulphonylurea derivative). Type 2 diabetic status was verified with an OGTT according to WHO criteria (Alberti & Zimmet, 1998). In addition, insulin resistance was estimated using the homeostasis model assessment for insulin resistance index (HOMA-IR) (Hosker et al., 1985). Medication was withheld for 24h prior to the experimental trials.

Protocol

All subjects maintained normal dietary and physical activity patterns throughout the experimental period. In addition, they filled out a food intake diary for 2 days before the first exercise trial to keep their dietary intake as identical as possible before the other trials. The 2 trials were separated by a one-week interval. The evening before each trial, subjects received the same standardized meal. The 2 experimental protocols are summarized in Fig. 6.1 and have been detailed elsewhere (van Loon et al., 2005a; van Loon et al., 2005b).

Study 1. Briefly, a muscle biopsy was taken from the *vastus lateralis* muscle at rest (t=0), after an overnight fast. Thereafter, a resting blood sample was taken and subsequent blood samples were collected every 15 or 30min. Subjects performed 120min of exercise (t=90 to t=210) on a cycle ergometer at a workload of 50% W_{max}, as determined one week before the experimental trial during an incremental exhaustive exercise test. A capsule containing

250mg acipimox (Nedios, Byk, Zwanenburg, The Netherlands) or a placebo was randomly orally administered prior (t=0) to and 75min into the exercise session (t=165). Immediately after cessation of exercise, a second muscle biopsy was taken (t=210).

Study 2. After an overnight fast and 30 min of supine rest, a percutaneous muscle biopsy was taken from the vastus lateralis muscle (t=0). A catheter was inserted into an antecubital vein for blood sampling and an oral dose of 250mg acipimox or a placebo was randomly administered (t=0). At t=120 min, subjects started to exercise at 50% W_{max} for a 60-min period. At t=150, another dose of 250mg Acipimox or a placebo was administered. Immediately after cessation of exercise, a second muscle biopsy was taken (t=180).

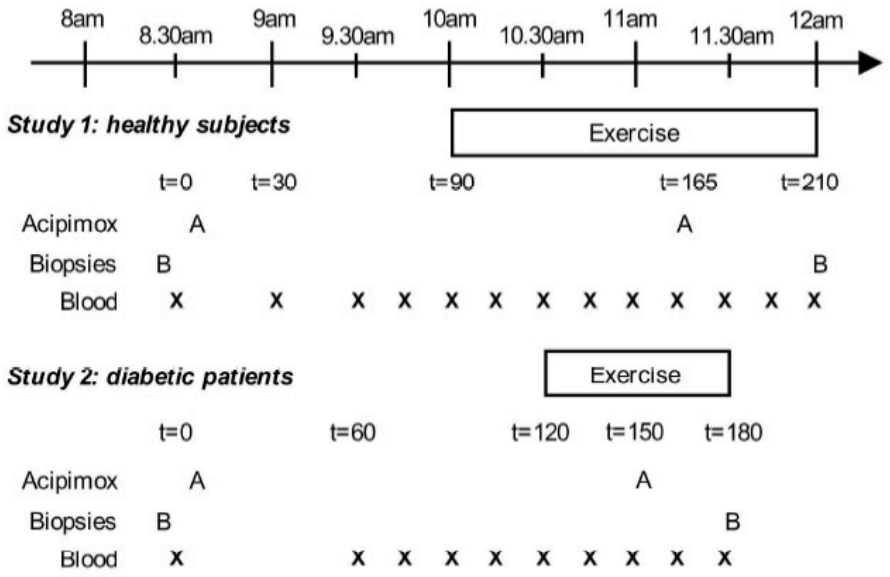


Fig. 6.1. Experimental protocols of study 1 in healthy subjects and study 2 in diabetic patients.

Analysis of muscle samples

Preparation of muscle lysates. Muscles were ground by using a pestle and homogenized in an ice-cold lysis buffer containing 20mM Tris, pH 7.0, 270mM sucrose, 5mM EGTA, 1mM EDTA, 1% Triton X-100, 1mM sodium orthovanadate, 50mM sodium β-glycerophosphate, 5mM sodium pyrophosphate, 50mM sodium fluoride, 1mM 1,4-dithiothreitol (DTT), and a protease inhibitor cocktail (Roche Applied Science, Vilvoorde, Belgium). Muscle homogenates were centrifuged at 10,000g for 15min at 4°C. Protein concentration was determined using a DC protein assay kit (Bio-Rad Laboratories, Nazareth, Belgium) with bovine serum albumin as a standard. Muscle lysates were stored at -80°C until subsequent analyses.

Western blotting.

The detailed procedure has been described in Deldicque et al. (2008). Briefly, protein lysates were combined with Laemmli and separated by SDS-PAGE. After electrophoretic separation at 40mA, the proteins were transferred to PVDF membranes. Membranes were blocked for 60min in TBST and 5% non-fat dried milk. Then, the membranes were incubated overnight at 4°C in TBST containing 1% bovine serum albumin and one of the following antibodies: phospho-p38 Thr180/Tyr182, phospho-SAPK/JNK Thr183/Tyr185, phospho-ERK1/2 Thr202/Tyr204, IκBα and GAPDH. All antibodies were obtained from Cell Signaling Technology (Leiden, The Netherlands) except GAPDH from Abcam (Cambridge, UK). Membranes were washed with TBST and were incubated with the appropriate secondary antibody at room temperature for 60min (Sigma, Bornem, Belgium). After additional washes, chemiluminescent detection was carried out using an ECL Western blotting kit (Amersham ECL Plus, GE Healthcare, Belgium). Bands were visualized on film, scanned and quantified by densitometry. When several blots were needed for obtaining values of the whole

experiments, an internal control was used to minimize inter blot variations due to incubation or exposure times. The internal control was a pool of remaining muscle biopsies from previous human studies of the laboratory. Results are reported as the ratio of the signal induced by the protein of interest divided by the signal induced by GAPDH. A value of 1.0 was assigned to the mean value of the pre-exercise samples to which all other values from the respective condition were reported.

Analysis of blood samples

Plasma NEFA concentration was determined using a kit coupling enzymatic reaction and spectrophotometric detection (550nm) of reaction end-product (Wako, Neuss, Germany). The results presented below constitute a subset of data already published (van Loon et al., 2005a; van Loon et al., 2005b). Means, SEM and statistics have been re-calculated according to the new number of samples.

Statistical analysis

A two-way ANOVA for repeated measures was used to test the interaction between time (NEFA) or exercise (MAPK) and treatment. Student-Newman-Keuls tests were used as post-hoc tests. A student t-test was used to test differences in plasma NEFA concentrations between healthy subjects and diabetic patients at rest and at the end of the exercise. The significant threshold was set to $P<0.05$. All results are presented as means ± standard errors of the mean.

Results

Acipimox prevents exercise-induced increase in plasma NEFA and reduces MAPK activation following exercise in skeletal muscle of healthy subjects

A 2-hour cycling exercise induced a rise in plasma NEFA concentration from 0.35mM to 0.90mM immediately at the end of the exercise ($P<0.001$) and to 1.35mM 15min after the end ($P<0.001$) in subjects having received a placebo (Fig. 6.2A). After 1h15 of exercise, plasma NEFA levels became significantly higher than pre-exercise values ($P<0.05$). Acipimox administration before and during exercise prevented the rise in NEFA levels, resulting in lower values during exercise in the acipimox conditions (~0.1mM, $P<0.05$; Fig. 6.2A) compared to placebo conditions (0.35-0.9mM).

Under placebo, phosphorylation of p38 MAPK, ERK1/2 and JNK were 26 ($P<0.05$, Fig. 6.2B), 71 ($P<0.01$, Fig. 6.2C) and 3 ($P<0.05$, Fig. 6.2D) fold higher immediately after exercise compared to resting values. The exercise-induced increases in MAPK were totally abolished after acipimox administration for p38 MAPK ($P<0.05$, Fig. 6.2B) and ERK1/2 ($P<0.01$, Fig. 6.2C) and partially repressed for JNK (Fig. 6.2D). IκBα expression was not modified by exercise alone although the combination of acipimox and exercise increased IκBα expression by about 50% compared to exercise in the placebo conditions ($P<0.05$, Fig. 6.2E).

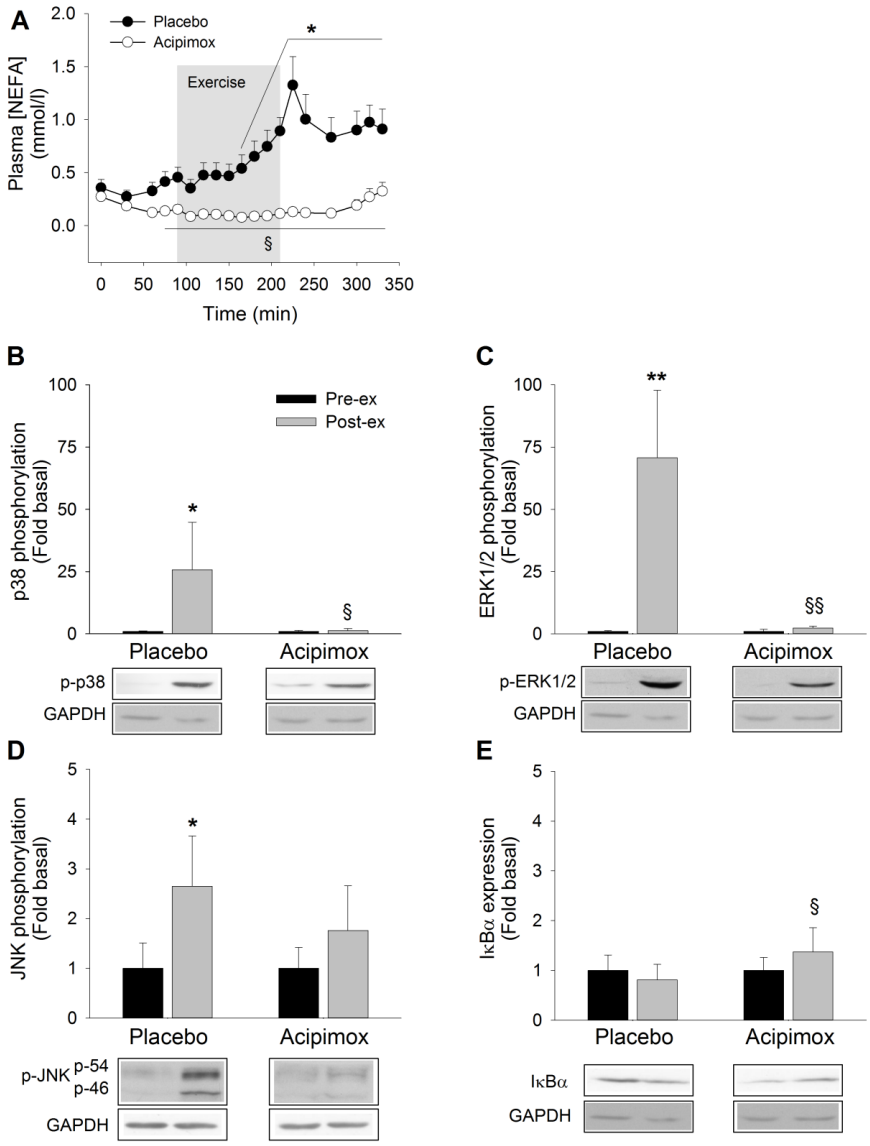


Fig. 6.2. Plasma NEFA concentrations and MAPK activation in healthy subjects. (A) Plasma NEFA concentrations (mmol/l) in placebo and acipimox conditions during the whole trial. (B) p38 MAPK, (C) ERK1/2, (D) JNK phosphorylation and (E) IκBα expression in skeletal muscle before and immediately after exercise in placebo and acipimox conditions. Values are expressed as means ± SEM (n=5). *P<0.05, **P<0.01 vs Pre-ex same treatment; §P<0.05, §§P<0.01 vs Placebo same condition.

Acipimox prevents exercise-induced increase in plasma NEFA and reduces MAPK activation following exercise in skeletal muscle of diabetic patients

The ANOVA revealed a time effect and a time x treatment interaction for plasma NEFA concentrations of diabetic patients ($P<0.001$). Resting plasma NEFA concentrations of diabetic patients (Fig. 6.3A) were higher than those of healthy subjects (0.55 ± 0.05 vs 0.35 ± 0.08, $P<0.05$). At the end of the exercise, plasma NEFA concentrations were similar (0.70 ± 0.09 in diabetic patients vs 0.90 ± 0.13 in healthy subjects, $P=0.247$) despite a shorter exercise duration for diabetic patients (1h vs 2h in healthy subjects). Compared to healthy subjects, the rise in NEFA concentrations was less pronounced and did not reach statistical significance when comparing immediately pre- and post-exercise values. However, when taking the lowest pre-exercise value (second one) as basal value, both the immediately post-exercise and the 30min post-exercise values were significantly higher ($P<0.05$). Acipimox administration before and during exercise prevented the rise in NEFA concentrations, which remained lower throughout the trial in the acipimox (~0.15mM, $P<0.05$; Fig. 6.3A) compared to placebo conditions.

Phosphorylation of p38 MAPK, ERK1/2 and JNK were 5 ($P<0.05$, Fig. 6.3B), 14 ($P<0.01$, Fig. 6.3C) and 2 ($P<0.05$, Fig. 6.3D) fold higher immediately after exercise compared to resting values. The exercise-induced increases in p38 MAPK ($P<0.05$, Fig. 6.3B) and ERK1/2 ($P<0.01$, Fig. 6.3C) were totally abolished after acipimox administration. The increase in JNK phosphorylation after exercise was not repressed in acipimox conditions (Fig. 6.3D). IκBα expression decreased after exercise in placebo conditions ($P<0.05$, Fig. 6.3E) but remained unchanged when acipimox was administered.

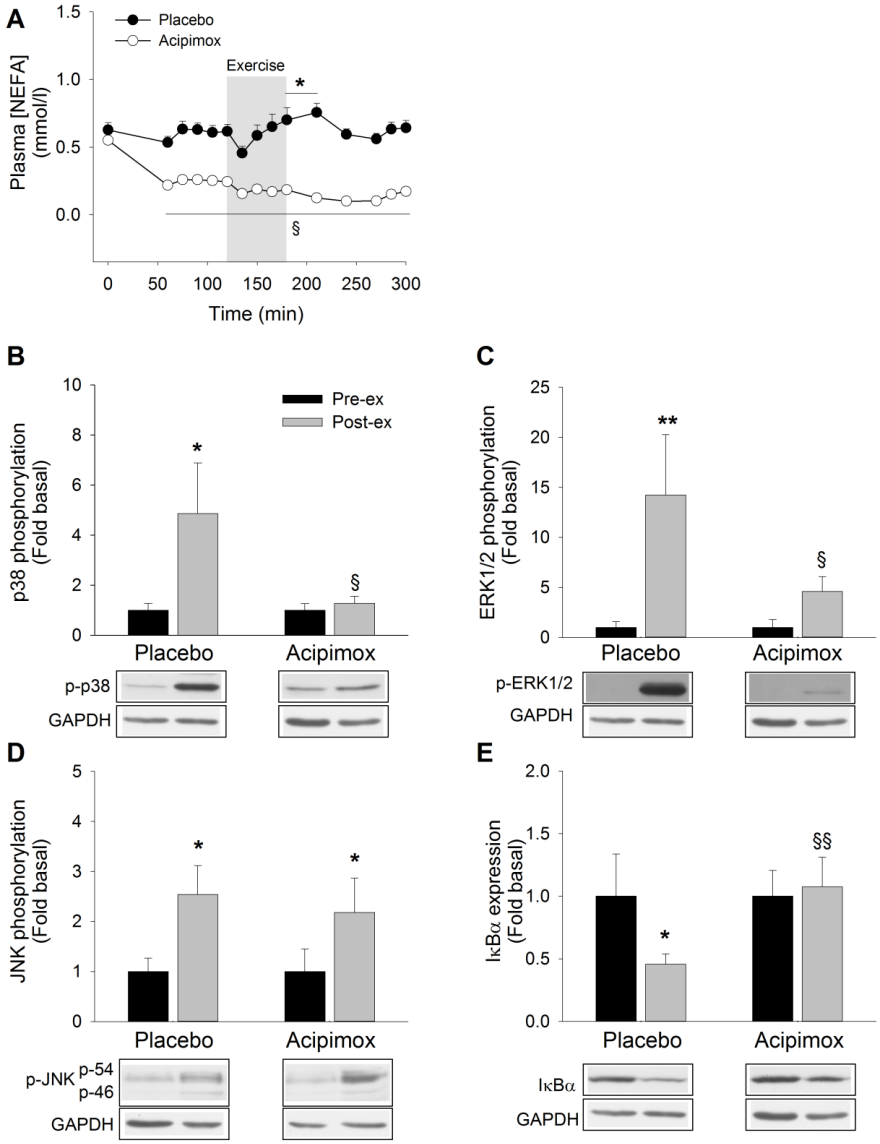


Fig. 6.3. Plasma NEFA concentrations and MAPK activation in diabetic patients. (A) Plasma NEFA concentrations (mmol/l) in placebo and acipimox conditions during the whole trial. (B) p38 MAPK, (C) ERK1/2, (D) JNK phosphorylation and (E) IκBα expression in skeletal muscle before and immediately after exercise in placebo and acipimox conditions. Values are expressed as means ± SEM (n=10). *P<0.05, **P<0.01 vs Pre-ex same treatment; §P<0.05, §§P<0.01 vs Placebo same condition.

Discussion

Exercise has been linked to the activation of the MAPK pathway in skeletal muscle, although the mechanisms responsible for this activation are as yet unsolved. Increases in cytosolic calcium and subsequent calcium/calmodulin-dependent protein kinase II activation, as well as production of reactive oxygen species, increases in catecholamines, growth hormones or cannabinoids, glycogen depletion or mechanical stretch have been proposed to participate in the activation of p38 MAPK during endurance exercise (Blair et al., 1999; Chambers et al., 2009; Chan et al., 2004; Clerk et al., 1998; Galbo, 1986; Li et al., 2005; Tedesco et al., 2010; Wright et al., 2007). The present study identifies NEFA as an additional trigger for MAPK phosphorylation in skeletal muscle during exercise.

Increases in NEFA levels following moderate to intense exercise have been regularly reported (Kiens, 2006; Watt et al., 2004; Wolfe, Klein, Carraro, & Weber, 1990), as well as the inhibitory effect of the nicotinic acid analog acipimox (Gautier et al., 1994). The results from the present study show, for the first time, that p38 MAPK and ERK1/2 activation after exercise is severely repressed in subjects having received acipimox. Compared to p38 MAPK and ERK1/2, JNK phosphorylation was less affected by exercise or by acipimox. The lower response of JNK to cycling exercise may be related to the nature of the contraction as JNK phosphorylation was shown to have a larger increase after eccentric rather than concentric contractions (Boppart et al., 1999).

Under acipimox, some subjects showed higher phosphorylation levels of ERK1/2 or p38 MAPK after exercise vs basal, but the extent of such activation was ~10 fold smaller than with a placebo. Therefore, the increase

in NEFA should be considered as an enhancer of MAPK phosphorylation in skeletal muscle rather than a mandatory event. Previous experiments performed on cell cultures and ex vivo muscle preparations reported that MAPK can be activated by electrical, mechanical or chemical conditions without the need of NEFA (Hayashi, Hirshman, Dufresne, & Goodyear, 1999; Sherwood et al., 1999). It has also been shown that a 30-min one-legged exercise induced a 2.2-fold increase in p38 MAPK phosphorylation in muscles from both exercised and non-exercised legs (Widegren et al., 1998). This was not the case for ERK1/2 which presented an increased phosphorylation in the exercised leg only. Our results suggest that circulating molecules such as NEFA may help to maintain high levels of MAPK phosphorylation during exercise of a long-term duration.

Although we could not establish any correlation between individual values of plasma NEFA and MAPK activation, there was a general trend to a less pronounced MAPK activation in the second study, in which the exercise-induced increase in NEFA concentrations was lower probably due to a shorter cycling protocol, the age and/or the pathological state of the subjects, i.e. diabetics. In the present study, subjects within the diabetic group were older than that of the healthy group (60 vs 23 years old in average). The age could have contributed to the difference in NEFA concentrations and MAPK activation. People with insulin resistance generally have higher resting NEFA levels and mobilize the same amount or slightly less fatty acids during exercise. The magnitude of the increase in plasma NEFA levels during exercise is thus less in these patients compared to healthy people . The reduced lipolytic activity during exercise in diabetics has been explained by a decreased sensitivity to catecholamines (Kanaley et al., 1993). Whereas no clear correlation could be established between NEFA levels and MAPK activation during exercise, the previous observation reinforces the idea that

increased plasma NEFA levels contribute to the stimulation of MAPK during exercise. Whether the higher basal NEFA concentration in diabetic patients leads to a higher basal MAPK phosphorylation was beyond the scope of the present study, i.e. exercise-induced activation of MAPK, and therefore was not investigated.

No muscle biopsy was taken between the first ingestion of acipimox and the beginning of the exercise, therefore making it difficult to draw conclusions on the effect of acipimox itself on the MAPK pathways. The effect of acipimox on MAPK has never been studied before but nicotinic acid has been shown to increase p38 MAPK and to reduce ERK1/2 without altering JNK at rest (Watt, Southgate, Holmes, & Febbraio, 2004). Although acipimox is an analog of nicotinic acid (O'Kane et al., 1992), it is possible that its molecular mechanisms of action are not exactly the same, hence the regulation of MAPK may differ. Nevertheless, the effect of acipimox alone was not the purpose of the present study as we were mainly interested in the MAPK response to exercise in combination with acipimox ingestion. Only one study (Watt et al., 2004) has previously measured the phosphorylation state of p38, ERK1/2 and JNK after nicotinic acid ingestion and cycling. The results of that study do not argue about a regulation of MAPK by NEFA during exercise. Exercise, in combination with or without nicotinic acid, induced a MAPK member-specific regulation rather than an overall increase in phosphorylation, suggesting that a general factor such as circulating NEFA was not involved in MAPK regulation by exercise. The discrepancies between our results and those of Watt et al. (2004) may potentially be found in the following methodological issues. The protocol we used in the present study differed in the duration of exercise, 1 or 2h in the present study vs 3h. As MAPK activation is dependent on the duration of exercise, it is not unexpected to see different patterns of activation. After 3h of cycling, JNK

phosphorylation was increased whereas ERK1/2 was decreased and p38 remained unchanged (Watt et al., 2004). In another study of the same group (Watt et al., 2004), ERK1/2 increased after 90min of cycling, indicating that MAPK are very sensitive to the duration of exercise and the time of biopsy sampling. At the same time, exercise-induced increase in ERK1/2 was repressed by nicotinic acid ingestion (Watt et al., 2004). Interestingly, the increase in JNK found after 1 or 2h of exercise in our study or after 3h by Watt et al. (2004) was not repressed by either acipimox or by nicotinic acid, respectively. In summary, acipimox and nicotinic acid are able to repress exercise-induced increase in p38 MAPK and ERK1/2. As their most important common effect is to reduce plasma NEFA concentrations, it is highly possible that NEFA contribute to the increase in p38 MAPK and ERK1/2 (but not JNK) during exercise. The specificity to p38 and ERK1/2 needs to be investigated further. It should be mentioned that nicotinic acid and acipimox are known to increase plasma epinephrine concentration as well (O'Neill, Watt, Heigenhauser, & Spriet, 2004). However, MAPK regulation was shown not to follow the increase in plasma epinephrine concentrations due to nicotinic acid whether at rest or after exercise (Watt et al., 2004).

To further study how NEFA could regulate MAPK we measured the expression of IkBa, based on our recently published study (Zbinden-Foncea et al., 2012), which reported a possible NEFA/TLR/MAPK signaling. IkBa is a well-known downstream target of TLR2/4 signaling (Akira et al., 2003) and regulator of the NF-kB pathway. However, we could not find any consistent regulation of IkBa by either exercise or acipimox. These results discard any role of the NF-kB in the present study but it is still possible that NEFA activate MAPK through TLR, independently of NF-kB. It is also possible that, contrary to what we found in mice, TLR do not mediate the

effect of NEFA on MAPK but this clearly needs further investigation. Another potential candidate for mediating the effect of NEFA on MAPK is the newly discovered hydroxy-carboxylic acid receptor 2 (Hanson et al., 2010) as the currently known ligands for this receptor are nicotinic acid, fumaric acid esters and 3-hydroxy-butyric acid, all being structurally close to fatty acids. The hydroxy-carboxylic acid receptor 2 is expressed in white and brown adipose tissue, keratinocytes and various immune cells (Hanson, Gille, & Offermanns, 2012). Activation of hydroxy-carboxylic acid receptor 3 by 3-hydroxy-octanoate leads to activation of ERK1/2 in Chinese hamster ovary and human epidermoid cell lines (Zhou et al., 2012) but whether the activation of hydroxy-carboxylic acid receptor 2 leads to the activation of the MAPK pathway has not been reported yet. The possible activation of hydroxy-carboxylic acid receptor 2 by NEFA and the downstream activation of MAPK in skeletal muscle would be worth to be investigated.

In conclusion, our data support a role for plasma NEFA in the activation of p38 MAPK and ERK1/2 in skeletal muscle tissue of healthy and diabetic subjects during endurance exercise. Further investigation will now be required to determine the molecular link between NEFA and MAPK activation during exercise in human skeletal muscle.

References

Akira, S., Yamamoto, M., & Takeda, K. (2003). Role of adapters in Toll-like receptor signalling. Biochem.Soc.Trans., 31, 637-642.

Alberti, K. G. & Zimmet, P. Z. (1998). New diagnostic criteria and classification of diabetes--again? Diabet.Med., 15, 535-536.

Blair, A. S., Hajduch, E., Litherland, G. J., & Hundal, H. S. (1999). Regulation of glucose transport and glycogen synthesis in L6 muscle cells during oxidative stress. Evidence for cross-talk between the insulin and SAPK2/p38 mitogen-activated protein kinase signaling pathways. J.Biol.Chem., 274, 36293-36299.

Boppart, M. D., Aronson, D., Gibson, L., Roubenoff, R., Abad, L. W., Bean, J. et al. (1999). Eccentric exercise markedly increases c-Jun NH(2)-terminal kinase activity in human skeletal muscle. J.Appl.Physiol, 87, 1668-1673.

Chambers, M. A., Moylan, J. S., Smith, J. D., Goodyear, L. J., & Reid, M. B. (2009). Stretch-stimulated glucose uptake in skeletal muscle is mediated by reactive oxygen species and p38 MAP-kinase. J.Physiol, 587, 3363-3373.

Chan, M. H., McGee, S. L., Watt, M. J., Hargreaves, M., & Febbraio, M. A. (2004). Altering dietary nutrient intake that reduces glycogen content leads to phosphorylation of nuclear p38 MAP kinase in human skeletal muscle: association with IL-6 gene transcription during contraction. FASEB J., 18, 1785-1787.

Clerk, A., Fuller, S. J., Michael, A., & Sugden, P. H. (1998). Stimulation of "stress-regulated" mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and p38-mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. J.Biol.Chem., 273, 7228-7234.

Deldicque, L., Atherton, P., Patel, R., Theisen, D., Nielens, H., Rennie, M. J. et al. (2008). Decrease in Akt/PKB signalling in human skeletal muscle by resistance exercise. Eur.J.Appl.Physiol, 104, 57-65.

Galbo, H. (1986). The hormonal response to exercise. Diabetes Metab Rev., 1, 385-408.

Gautier, J. F., Pirnay, F., Jandrain, B., Lacroix, M., Mosora, F., Scheen, A. J. et al. (1994). Availability of glucose ingested during muscle exercise performed under acipimox-induced lipolysis blockade. Eur.J.Appl.Physiol Occup.Physiol, 68, 406-412.

Hanson, J., Gille, A., & Offermanns, S. (2012). Role of HCA(2) (GPR109A) in nicotinic acid and fumaric acid ester-induced effects on the skin. Pharmacol.Ther..

Hanson, J., Gille, A., Zwykiel, S., Lukasova, M., Clausen, B. E., Ahmed, K. et al. (2010). Nicotinic acid- and monomethyl fumarate-induced flushing involves GPR109A expressed by keratinocytes and COX-2-dependent prostanoid formation in mice. J.Clin.Invest, 120, 2910-2919.

Hayashi, T., Hirshman, M. F., Dufresne, S. D., & Goodyear, L. J. (1999). Skeletal muscle contractile activity in vitro stimulates mitogen-activated protein kinase signaling. Am.J.Physiol, 277, C701-C707.

Hosker, J. P., Matthews, D. R., Rudenski, A. S., Burnett, M. A., Darling, P., Bown, E. G. et al. (1985). Continuous infusion of glucose with model assessment: measurement of insulin resistance and beta-cell function in man. Diabetologia, 28, 401-411.

Johnson, G. L. & Lapadat, R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science, 298, 1911-1912.

Kanaley, J. A., Cryer, P. E., & Jensen, M. D. (1993). Fatty acid kinetic responses to exercise. Effects of obesity, body fat distribution, and energy-restricted diet. J.Clin.Invest, 92, 255-261.

Kiens, B. (2006). Skeletal muscle lipid metabolism in exercise and insulin resistance. Physiol Rev., 86, 205-243.

Kramer, H. F. & Goodyear, L. J. (2007). Exercise, MAPK, and NF-kappaB signaling in skeletal muscle. J.Appl.Physiol, 103, 388-395.

Li, Y. P., Chen, Y., John, J., Moylan, J., Jin, B., Mann, D. L. et al. (2005). TNF-alpha acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. FASEB J., 19, 362-370.

Long, Y. C., Widegren, U., & Zierath, J. R. (2004). Exercise-induced mitogen-activated protein kinase signalling in skeletal muscle. Proc.Nutr.Soc., 63, 227-232.

O'Kane, M. J., Trinick, T. R., Tynan, M. B., Trimble, E. R., & Nicholls, D. P. (1992). A comparison of acipimox and nicotinic acid in type 2b hyperlipidaemia. Br.J.Clin.Pharmacol., 33, 451-453.

O'Neill, M., Watt, M. J., Heigenhauser, G. J., & Spriet, L. L. (2004). Effects of reduced free fatty acid availability on hormone-sensitive lipase activity in human skeletal muscle during aerobic exercise. J.Appl.Physiol, 97, 1938-1945.

Reyna, S. M., Ghosh, S., Tantiwong, P., Meka, C. S., Eagan, P., Jenkinson, C. P. et al. (2008). Elevated toll-like receptor 4 expression and signaling in muscle from insulin-resistant subjects. Diabetes, 57, 2595-2602.

Senn, J. J. (2006). Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes. J.Biol.Chem., 281, 26865-26875.

Sherwood, D. J., Dufresne, S. D., Markuns, J. F., Cheatham, B., Moller, D. E., Aronson, D. et al. (1999). Differential regulation of MAP kinase, p70(S6K), and Akt by contraction and insulin in rat skeletal muscle. Am.J.Physiol, 276, E870-E878.

Shi, H., Kokoeva, M. V., Inouye, K., Tzameli, I., Yin, H., & Flier, J. S. (2006). TLR4 links innate immunity and fatty acid-induced insulin resistance. J.Clin.Invest, 116, 3015-3025.

Stich, V., De, G., I, Berlan, M., Bulow, J., Galitzky, J., Harant, I. et al. (2000). Adipose tissue lipolysis is increased during a repeated bout of aerobic exercise. J.Appl.Physiol, 88, 1277-1283.

Tedesco, L., Valerio, A., Dossena, M., Cardile, A., Ragni, M., Pagano, C. et al. (2010). Cannabinoid receptor stimulation impairs mitochondrial biogenesis in mouse white adipose tissue, muscle, and liver: the role of eNOS, p38 MAPK, and AMPK pathways. Diabetes, 59, 2826-2836.

Tunstall, R. J., McAinch, A. J., Hargreaves, M., van Loon, L. J., & Cameron-Smith, D. (2007). Reduced plasma free fatty acid availability during exercise: effect on gene expression. Eur J Appl Physiol, 99, 485-93.

van Loon, L. J., Manders, R. J., Koopman, R., Kaastra, B., Stegen, J. H., Gijsen, A. P. et al. (2005a). Inhibition of adipose tissue lipolysis increases intramuscular lipid use in type 2 diabetic patients. Diabetologia, 48, 2097-107.

van Loon, L. J., Thomason-Hughes, M., Constantin-Teodosiu, D., Koopman, R., Greenhaff, P. L., Hardie, D. G. et al. (2005b). Inhibition of adipose tissue lipolysis increases intramuscular lipid and glycogen use in vivo in humans. Am J Physiol Endocrinol Metab, 289, 482-93.

Watt, M. J., Holmes, A. G., Steinberg, G. R., Mesa, J. L., Kemp, B. E., & Febbraio, M. A. (2004). Reduced plasma FFA availability increases net triacylglycerol degradation, but not GPAT or HSL activity, in human skeletal muscle. Am J Physiol Endocrinol Metab, 287, 120-7.

Watt, M. J., Southgate, R. J., Holmes, A. G., & Febbraio, M. A. (2004). Suppression of plasma free fatty acids upregulates peroxisome proliferator-activated receptor (PPAR) alpha and delta and PPAR coactivator 1alpha in human

skeletal muscle, but not lipid regulatory genes. J Mol Endocrinol, 33, 533-44.

Widegren, U., Jiang, X. J., Krook, A., Chibalin, A. V., Bjornholm, M., Tally, M. et al. (1998). Divergent effects of exercise on metabolic and mitogenic signaling pathways in human skeletal muscle. FASEB J., 12, 1379-1389.

Widegren, U., Wretman, C., Lionikas, A., Hedin, G., & Henriksson, J. (2000). Influence of exercise intensity on ERK/MAP kinase signalling in human skeletal muscle. Pflugers Arch., 441, 317-322.

Wolfe, R. R., Klein, S., Carraro, F., & Weber, J. M. (1990). Role of triglyceride-fatty acid cycle in controlling fat metabolism in humans during and after exercise. Am.J.Physiol, 258, E382-E389.

Wretman, C., Lionikas, A., Widegren, U., Lannergren, J., Westerblad, H., & Henriksson, J. (2001). Effects of concentric and eccentric contractions on phosphorylation of MAPK(erk1/2) and MAPK(p38) in isolated rat skeletal muscle. J.Physiol, 535, 155-164.

Wright, D. C., Geiger, P. C., Han, D. H., Jones, T. E., & Holloszy, J. O. (2007). Calcium induces increases in peroxisome proliferator-activated receptor gamma coactivator-1alpha and mitochondrial biogenesis by a pathway leading to p38 mitogen-activated protein kinase activation. J.Biol.Chem., 282, 18793-18799.

Yu, M., Blomstrand, E., Chibalin, A. V., Krook, A., & Zierath, J. R. (2001). Marathon running increases ERK1/2 and p38 MAP kinase signalling to downstream targets in human skeletal muscle. J.Physiol, 536, 273-282.

Zbinden-Foncea, H., Raymackers, J. M., Deldicque, L., Renard, P., & Francaux, M. (2012). TLR2 and TLR4 Activate p38 MAPK and JNK during Endurance Exercise in Skeletal Muscle. Med.Sci.Sports Exerc., 44, 1463-1472.

Zhou, Q., Li, G., Deng, X., He, X., Chen, L., Wu, C. et al. (2012). Activated human hydroxy-carboxylic acid receptor-3 signals to MAP kinase cascades via the PLC-dependent PKC and MMP-mediated EGFR pathways. Br.J.Pharmacol.,166,1756-1773.

Chapter 7

Discussion, perspectives and conclusion

Discussion and perspectives

1. Effect of fatty acid in skeletal muscle cells

A link has clearly been established between NEFA and insulin resistance in skeletal muscle (Boden, 2003; Dey et al., 2006; Senn, 2006). Despite the fact that much has been written about the pathogenesis of insulin resistance, its molecular mechanism of action is still not fully understood. Although intracellular triglyceride accumulation has been correlated to lipid-induced insulin resistance in T2DM subjects, this association has been contested with the athlete's paradox, a phenomenon where endurance athletes have enhanced muscle triglycerides but are insulin sensitive (Goodpaster et al., 2001). To elucidate the role of different fatty acids on insulin signalling, we measured the effect of short- medium- and long-chain fatty acid incubation in C2C12 cells. We found that during 24h of incubation, a medium chain fatty acid, 3mmol/l caprilic acid (C8:0, also called octanoic acid), increased PKB phosphorylation Ser473 and S6K1 phosphorylation Thr389 levels by 18-fold ($p<0.05$) and 16.6 fold ($p<0.05$), respectively (data not shown). Likewise, S6K1 phosphorylation levels were increased 9.8-fold ($p<0.05$) when the cells were exposed to 3mmol/l during 24h with butyric acid, a short-chain fatty acid (data not shown). Little research has been carried out on the contribution of short- and medium-chain fatty acids; therefore, further research is warranted to elucidate the role of short-and medium-chain fatty acids in insulin signalling pathways with in vitro and in vivo models.

Accumulation of lipid metabolites (e.g. diacylglycerols, ceramides) may mediate intracellular signalling, and they are known to function as second messengers in insulin signalling pathways. We found that 100nmol/l of insulin incubation during 30min increased the PKB phosphorylation state on Ser473 by 5.7-fold ($p<0.001$) compared to control values in C2C12 cells. This effect was not detected when the cells were pre-incubated with

100μmol/l ceramide for 2h and was unaffected in cells treated with the inactive ceramide analogue (dihydroceramide) (data not shown). These results agree with previous studies, which found similar findings (Hajduch et al., 2001; Stratford et al., 2004). Important studies have proposed mechanisms whereby ceramides may impair PKB phosphorylation with ceramides activating protein phosphatase 2A, for example (a known inhibitor of PKB) (Teruel et al., 2001). Another possible mechanism implicates PKCζ phosphorylation in the prevention of PKB activation (Blouin et al., 2010; Powell et al., 2003). In addition, we measured phosphorylation of S6K1 Thr389 and the insulin increased by 2.0-fold S6K1 phosphorylation levels. This increase was not found when the cells were pre-incubated with ceramides for 2h (data not shown).

With in vivo models, ceramides levels were enhanced in skeletal muscle from insulin-resistant humans (Adams et al., 2004). In recent published studies on obese and T2DM subjects, the authors have shown that acipimox decreased all NEFA species. Inflammatory gene expression was also reduced, resulting in improvement in muscle insulin signalling from insulin resistance subjects, but surprisingly the ceramides content did not change (Liang et al., 2013). In addition, exercise could reduce the total content and change the composition of ceramides in each muscle type in rats. These changes in the content of individual ceramides depended mostly on fatty acid residue. The reduction in the concentration of ceramides after exercise could be caused by either the reduced formation or increased breakdown of the compound (Dobrzyn and Gorski, 2002). The ceramides influence on the insulin cellular mechanism has been highly studied using cell systems, however, more studies are required utilizing in vivo and human models of insulin resistance.

It seems that a reduction of plasma NEFA provokes an improvement of insulin sensitivity in skeletal muscle. Palmitic acid (PA) is considered a negative regulator of insulin sensitivity through decreased activation of the

PKB pathways. We have shown that incubation for 3h with 0.75mM PA decreased by 40% PKB phosphorylation in C2C12 cells (chapter 4), concomitant with various studies that have shown different concentrations of free PA reduced PKB phosphorylation levels (Chavez and Summers, 2003; Senn, 2006). However, these findings contradict others studies (Liu et al., 2012; Rivas et al., 2009). It seems that concentration, time of incubation and state of cellular differentiation could intervene in the PA effect of PKB phosphorylation in skeletal muscle cells. In addition to an inhibition of PKB phosphorylation, we have shown that free PA incubated for 3h increased S6K1 phosphorylation in C2C12. These findings are in line with other studies that found a reduction of PKB phosphorylation levels with a concomitant increase of S6K1 phosphorylation levels (Ragheb et al., 2009; Wang et al., 2010). Results of the present investigation may support the hypothesis that S6K1 activation decreases serine phosphorylation of IRS leading to PKB inhibition and insulin desensitization in cultured cells (Um et al., 2004).

However, long-term incubation with PA in L6 muscle cells reduces the phosphorylation state of S6K1 following insulin administration (Dimopoulos et al., 2006). Hence, we could suggest that brief elevation of plasma NEFA concentration described in chapter 4, could activate S6K1 via PKB/mTORC1 independent pathways. In contrast, a chronic and elevated plasma PA concentration could inhibit S6K1 via a PKB/mTORC1 dependent manner.

A recent study has shown that PA incubation (with adjunction of 1% BSA) for 4h allowed PKB activation associated with an increase in the S6K1 phosphorylation level in C2C12 myotubes. These findings are explained through AMPK inhibition (Liu et al., 2012). Discrepancies with our results may be explained by both a different state of cell differentiation and/or utilization of BSA in the preparation with PA. The latter has been shown to be a possible endotoxin contaminant (Erridge and Samani, 2009).

Despite the substantial evidence supporting the implication of plasma NEFA concentrations in the damage of the insulin signalling pathways, the molecular mechanism by which this phenomenon occurs is not well defined. It has been postulated that NEFA induces an inflammatory response via activation of TLR2/4 in C2C12 cell (Senn, 2006), animal (Shi et al., 2006) and human (Lambert et al., 2008; Liang et al., 2013). We were able to show that activation of TLR2 and TLR4 in the muscle could be due to an increased PA concentration (chapter 4). Likewise, by utilizing small interfering RNA against *tlr2* and another against *tlr4*, we were the first to demonstrate that the activation of TLR2 and TLR4 by known ligands (FSL for TLR2 and LPS for TLR4) besides PA provoked a decrease in PKB phosphorylation, accompanied with both elevated p38 MAPK and S6K1 phosphorylation. We found a similar increase of p38 MAPK phosphorylation when the cells were incubated with PA compared to Senn (2006), who found in the same cell line an elevated p38 MAPK phosphorylation induced by PA in a TLR2-dependent manner (Senn, 2006). In addition, by using an antagonist TLR2 antibody called TLR2.5 we were able to prevent an increase of p38 MAPK phosphorylation induced by PA exposure (data not shown). Furthermore, when the cells were pre-treated with SB202190 (a known p38 inhibitor) PA-induced S6K1 phosphorylation was totally repressed, suggesting a possible interaction between p38MAPK and S6K1 activation.

Hence, our results confirmed that both TLR2 and TLR4 are mediators between extracellular signal and insulin pathways. In addition, our results confirmed a possible crosstalk between S6K1 and p38 MAPK induced by PA concentration.

2. Role of TLR2 and TLR4 during exercise

As described in Chapter 5, our findings are the first to provide evidence that during endurance exercise, TLR2 and TLR4 transduce a signal induced by an elevated plasma NEFA concentration triggering p38 MAPK, JNK and c-Jun activation. This latter is a transcription factor downstream of JNK. Exercise is known to activate MAPK, however, the molecular mechanisms are still not clear. We have shown that immediately after an endurance exercise in C57BL/6J wild-type mice (WT) an increase of plasma NEFA was induced resulting in an elevated p38 MAPK, JNK and c-Jun phosphorylation state in the tibialis anterior (TA) and soleus (SOL) muscles. These effects were not observed in *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice despite the fact that they were submitted to the same exercise protocol accompanied by a similar increase of plasma NEFA (Chapter 5). Despite the results obtained in this study, where plasma NEFA released during endurance exercise may activate MAPK via TLR2 and TLR4, various studies have put forth evidence of other factors implicated in exercise-induced p38 MAPK activation. For example, during exercise there is an increase in cytosolic calcium concentration in skeletal muscle, triggering a calcium/calmodulin-dependent protein kinase II activation resulting in elevated p38 MAPK activation (Wright et al., 2007). Other factors such as stretch, production of reactive oxygen species, and glycogen depletion are also involved in p38 MAPK activation (Chambers et al., 2009; Chan et al., 2004; Clerk et al., 1998). Furthermore, a study indicated that MAPK could be activated by electrical stimulation, mechanical, or chemical conditions without the need of NEFA release (Hayashi et al., 1999). However, our hypothesis is supported by the study of Widegrem et al. (Widegren et al., 1998), which showed that p38 MAPK is sensitive to systemic agents since a 30-min one-legged exercise induced an increase in p38 MAPK phosphorylation in muscle from both the exercised and unexercised leg. In addition, they were not able to increase ERK in the

unexercised leg muscle. In line with our findings, they showed that systemic agents do not cause ERK phosphorylation during exercise and/or our exercise protocol was low intensity resulting in non-activation of ERK 1/2.
Intense exercise protocols and/or exercise causing muscular damage could stimulate the JNK pathways (Aronson et al., 1997; Aronson et al., 1998b; Fujii et al., 2004). We have shown that endurance exercise might activate JNK pathways through the binding of extracellular NEFA with TLR2 and TLR4 in mouse skeletal muscle (Chapter 5). We observed a complete repression of JNK phosphorylation in transgenic mice in TA muscle immediately after our exercise protocol. However, this repression was not totally seen in SOL muscle, but represented only 25% of the increase observed in WT muscle. This latter, could be explained by a potential fiber type-specific response, but our findings suggest that during endurance exercise both the SOL and TA muscle in mice activates p38 MAPK and JNK through a TLR-dependent mechanism. Moreover, we also measured c-Jun, a transcription factor downstream of JNK, in SOL and TA muscle from exercised mice. Our results agree with the study of Aronson et al. in 1998, which was the first to demonstrated that after 60 min of cycle ergometer exercise increased JNK activity and its downstream nuclear target *c-Jun* mRNA in human muscle (Aronson et al., 1998a). We were able to shown an increase of c-Jun after exercise in WT mice accompanied by a higher activity of JNK. Nonetheless, in SOL muscle of *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice, JNK tended to be more phosphorylated after running, which is in line with a higher phosphorylation state of c-Jun in those mice, reaching the statistical threshold in *tlr4*$^{-/-}$ mice (Chapter 5).
Hence, our findings provide strong evidence that TLR2 and TLR4 mediate between an extracellular signal (e.g. plasma NEFA release) and MAPK activation during endurance exercise.

Furthermore, in order to rule out any confounding effect of other exercise-

induced factors, we injected with heparin to WT mice, such as *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ transgenic mice. We observed an elevated plasma NEFA concentration induced by heparin reflecting a lipolysis increase similar to our endurance exercise. After the heparin injection in WT mice, the phosphorylation state of p38 MAPK and JNK was increased in both TA and SOL muscles. This activation was completely suppressed in *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ transgenic mice regardless of the type of muscle. These findings are very similar to those obtained from our endurance exercise, and they have in common an elevated plasma NEFA in both experiments. In summation, our data strongly suggest that extracellular NEFA plays a role in MAPK activation mediated by TLR2 and TLR4 in skeletal mice muscle.

Moreover, we tried to reveal the presence of crosstalk between p38 MAPK and S6K1 in mice submitted to endurance exercise. We tested the hypothesis that an elevated plasma FA during exercise in mice might activate TLR2 and TLR4, similar to what we have shown with PA incubation in C2C12. This activation could induce activation of p38 MAPK and, subsequently, a S6K1 phosphorylation. However, the phosphorylation state of S6K1 was not detected in mouse muscle. We believed that the fasting state of the mice did not allow the detection of S6K1 phosphorylation. However, Deldicque et al. 2010 showed that incubation of PA (1mM) for 17h decreased S6K1 phosphorylation in C2C12 cells (Deldicque et al., 2010). These findings support our hypothesis that a crosstalk between p38 and S6K1 is possible after a brief elevation of PA, on the contrary, a longer stimulation causes a complete insulin signalling inhibition.

Moreover, it seems that the relationship between endurance exercise and S6K1, as a marker of anabolic process, is more complex than a simple crosstalk between p38 MAPK and S6K1 activation. For example, when AMPK is activated due to high intensity endurance exercise, it can directly phosphorylate raptor resulting in mTORC1 inhibition (Gwinn et al., 2008)

and a concomitant decrease in S6K1 phosphorylation. In addition, α1 AMPK knockout mice were submitted to mechanical overloading. The α1 AMPK$^{-/-}$ mice showed more hypertrophy compared to WT mice (Mounier et al., 2009). Nonetheless, a study has shown that 10 weeks of endurance training consisting of single leg cycling for 45 min at 75% V_{o2peak} increased AMPK phosphorylation together with mTORC1 and S6K1 activation in both untrained and trained subjects (Wilkinson et al., 2008). Another more recent study with subjects submitted to aerobic and resistance exercise (AE+RE) showed that phosphorylation levels of S6K1 were more elevated than in subjects submitted only to resistance exercise (RE) (Lundberg et al., 2012). Furthermore, the same authors suggest that the increased aerobic capacity induced by AE+RE was accompanied by a more robust increase in muscle size compared with RE (Lundberg et al., 2013). Unfortunately, none of the abovementioned studies measured the phosphorylation state of p38 MAPK in order to investigate a possible crosstalk between p38 MAPK and S6K1. Further investigations will be required to determine the molecular link between NEFA and p38 MAPK/S6K1 activation during endurance exercise in human skeletal muscle.

The TLR2 and TLR4 signaling pathways lead to the activation of diverse transcription factors including nuclear factor-κB (NF-κB) (Akira and Sato, 2003). We tested the hypothesis that an elevation of extracellular NEFA observed during endurance exercise may activate NF-κB pathways. Surprisingly, our endurance exercise protocol was not able to activate NF-κB pathways, in either WT mice or *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ transgenic mice (Chapter 5). In order to check our NF-κB DNA binding measure, we injected WT mice with 8mg·kg^{-1} of LPS for 120 min as a positive control. In TA, the phosphorylation state of IKKα/β was increased and the DNA binding of NF-κB was elevated compared with control mice. These results convince us that

our experimental running protocol did not induce any activation of the NF-κB pathways. This contradictory finding could be due to the fact that extracellular NEFA after endurance exercise was not high enough and/or because the stimulation was too short. Our results are in agreement with those observed by McKenzie and Goldfarb, who did not report any activation of the NF-κB pathways during endurance exercise in rats (McKenzie and Goldfarb, 2007). Also, it seems that the nature of exercise (intensity, duration, and frequency) might explain the strong and contradictory differences found between NF-κB induced by exercise (Kramer and Goodyear, 2007).

Furthermore, we observed that increased of plasma NEFA induced by heparin injection in WT mice and *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ transgenic mice was also not able to activate NF-κB pathways.

These are the first studies to provide evidence that TLR2 and TLR4 transduce signals linking the elevated extracellular NEFA concentration to the activation of p38 MAPK, JNK and c-Jun during endurance exercise in mouse muscle.

3. Effect of plasma NEFA release during endurance exercise on intracellular signalling pathways in human skeletal muscle.

As we have mentioned in the previous chapter, an elevated extracellular NEFA might activate MAPK in cellular models and in vivo mice models. We tested the hypothesis that plasma FA contributes to the activation of MAPK during endurance exercise in human model. In order to establish the relationship between plasma NEFA and MAPK, we used acipimox prior to and during the endurance exercise (120 min on a cycle ergometer at 50%W_{max}) to prevent the elevation of plasma NEFA in healthy subjects. Our study showed, for the first time, that p38 and ERK1/2 activation is

suppressed in healthy subjects after having received acipimox. However, in contrast to what we have shown in animal models, JNK phosphorylation was less affected by exercise. This lower JNK response might be explained by the nature of the contraction, as JNK phosphorylation is more elevated after eccentric rather than concentric contractions (Boppart et al., 1999). Considering p38 MAPK, our findings found in animal models were very similar to those results obtained in human skeletal muscle. An elevated plasma NEFA induced by endurance exercise increased p38 MAPK phosphorylation levels in both animal and human models. Also, this activation was found when C2C12 cells were incubated with an acute palmitic acid concentration. Hence, it suggests that p38 MAPK activation during endurance exercise is plasma NEFA-dependent. However, we could not establish any correlation between individual values of plasma NEFA and MAPK activation.

Furthermore, we measured the phosphorylation levels of MAPK in diabetic subjects that used acipimox prior and during endurance exercise (60 min on a cycle ergometer at 50%W_{max}). We found similar findings on MAPK compared to healthy subjects. An elevated plasma NEFA was observed in the placebo group of diabetic subjects after endurance exercise. In diabetic subjects with acipimox administration, the plasma NEFA decreased even before of the beginning of exercise, and it was maintained at low levels during exercise. Phosphorylation levels of p38, ERK1/2 and JNK were increased after endurance exercise in subjects with placebo. Activation of p38 and ERK were supressed in diabetic subjects having received acipimox. However, some subjects who received acipimox showed higher phosphorylation levels of ERK1/2 or p38 MAPK after exercise vs basal rate, but the extent of such activation was ~ 10 fold smaller than that with placebo. Hence, as mentioned previously in Chapter 6, the increase in plasma NEFA should be considered as an enhancer of MAPK

phosphorylation in skeletal muscle rather than a mandatory event. Further, JNK phosphorylation levels were not changed with acipimox administration maintaining increased levels after the exercise session.

Unfortunately, a muscle biopsy was not taken between the first ingestion of acipimox and the beginning of the exercise, making it difficult to draw conclusions on the effect of acipimox itself on the MAPK pathways. Nonetheless, a recent study has shown that acipimox administration for seven day (250mg each six hours) decreased plasma NEFA resulting in improvement in insulin signalling pathways, but it did not affect JNK phosphorylation in obese and diabetic subjects. However, other MAPK families were not measured in this study (Liang et al., 2013).

An anti-lipolytic drug called nicotinic acid (NA) used in another study has been shown to increase p38 MAPK and to reduce ERK1/2 without any change in JNK phosphorylation at rest (Watt et al., 2004). In the same study, they showed that NA prevented an increase of plasma NEFA induced by exercise in recreationally active subjects. After exercise cessation p38 MAPK and JNK increased without altering ERK1/2 phosphorylation. These discrepancies with our results might be explained by 1) the duration of exercise; our exercise protocol was 1 or 2h, while the protocol used by Watt et al. was 3h. 2) Despite the fact that acipimox is an analog of nicotinic acid (O'Kane et al., 1992), it is possible that its molecular mechanisms of action are not exactly the same.

Furthermore, we measured IκBα in order to establish one plausible interaction between plasma NEFA and NF-κB pathways in human muscle. IκBα content was not changed by either exercise or acipimox (Chapter 6). Considering that our endurance exercise in mice did not alter NF-κB pathways, it was also not activated in healthy subjects submitted to cycle ergometer for 2h. The hypothesis given to this paradoxical finding was

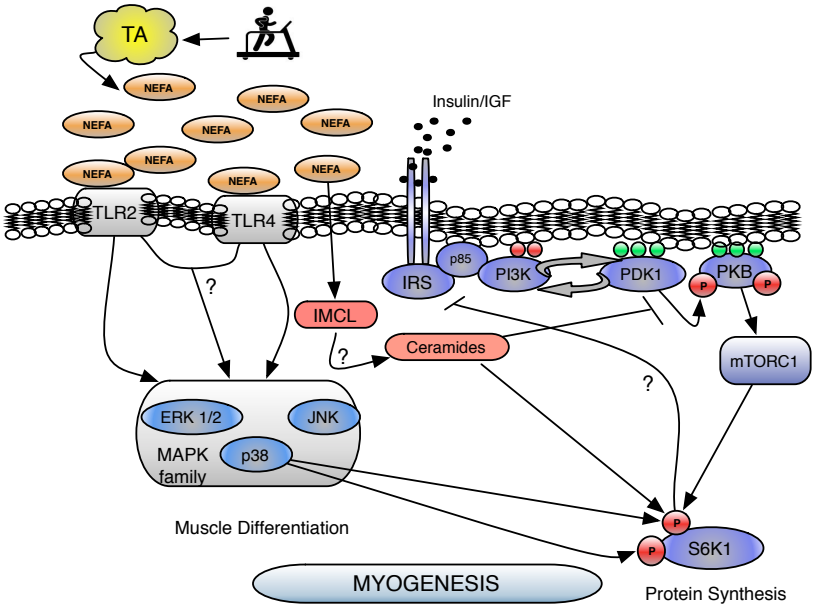
already analyzed previously. However, we could speculate that the elevated plasma NEFA, which occurred during endurance exercise, might activate MAPK through TLR, independently of NF-κB in healthy subjects. On the other hand, in diabetic subjects we have shown that endurance exercise decreased IκBα content, reflecting a plausible NF-κB activation. These effects were countered with acipimox administration, suggesting that plasma NEFA are necessary in the activation of NF-κB induced by exercise in diabetic subjects. NF-κB activation may, among others, induce a brief but important pro-inflammatory response critical for post-exercise muscle regeneration (Kramer and Goodyear, 2007). A recent study elaborated by Liang et al. (2013) has shown that diabetic subjects have less IκBα content compared to lean and obese subjects at rest. Also, they showed that acipimox administration for 7 days might revert the decline of IκBα content observed in diabetic subjects (Liang et al., 2013). Therefore, we cannot exclude the possibility that in our study the increases showed after exercise and acipimox administration in diabetic subjects were induced only by acipimox. Nevertheless, our protocol of acute administration was completely different from a chronic administration of acipimox studied by Liang et al. (2013). Further investigations considering single doses of acipimox without exercise might determine the role of plasma NEFA in the IκBα content and subsequent NF-κB activation.

In summary, our data supports a role for plasma NEFA in the activation of p38 MAPK and ERK1/2 in skeletal muscle tissue of healthy and diabetic subjects during endurance exercise. Further research is warranted to elucidate the molecular intermediates between plasma NEFA and MAPK activation during exercise in human skeletal muscle.

Conclusion

The present work aims to identify the molecular mechanism by which endurance exercise regulates MAPK activation in skeletal muscle. Our findings provide evidence that increased NEFA (palmitic acid) might initiate a TLR signaling pathway resulting in PKB inhibition but the activation of p38 MAPK and JNK in C2C12 cells. We clearly showed a crosstalk between p38 MAPK and S6K1 induced by elevated NEFA concentration.

An increase in plasma NEFA during endurance exercise could activate MAPK protein via a signal transduced by TLR2 and TLR4. Fig. 7.1 summarizes how NEFA release from adipose tissue may initiate a signaling pathway activating MAPK and S6K1 protein mediated by TLR.



7.1. Schema hypothetic of the signal induced by plasma NEFA/TLR/MAPK and insulin signalling.

Summary

Toll-like receptors 2 (TLR2) and 4 (TLR4) are present in the plasma membrane of skeletal muscles, where their functions are only partially understood. A variety of molecular components could activate TLR2 and TLR4, including non-esterified fatty acids (NEFA). NEFA are also known to be released during endurance exercise. Likewise, endurance exercise may activate known downstream targets of TLR2 and TLR4 such as mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB).

The purpose of this work was to investigate the possible interaction between the elevated plasma NEFA observed during endurance exercise and the MAPK activation mediated by TLR2 and TLR4. From a methodological point of view, three experimental models were used: myotubes cultured from C2C12 cells, soleus and tibialis muscle from C57BL/6J animals wild-type (WT) and transgenic *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice, and finally, muscle biopsies taken from the vastus lateralis of healthy and diabetic subjects.

Palmitic acid (PA) was able to increase p38 MAPK and S6K1 phosphorylation accompanied by PKB inhibition in C2C12. TLR2 and TLR4 seem to be implicated in this phenomenon. In addition, our results showed evidence of a possible crosstalk between S6K1 and p38 MAPK, induced by PA elevation. When mice were submitted to an endurance exercise, plasma NEFA levels increased. Likewise, we observed an increase of p38 MAPK, JNK and c-Jun phosphorylation in the muscles of WT mice. These effects were not observed in *tlr2*$^{-/-}$ and *tlr4*$^{-/-}$ mice, which suggests that TLR2 and TLR4 are necessary to activate the above-mentioned proteins. Acipimox was used prior to and during endurance exercise to prevent the elevation of plasma NEFA levels in healthy and diabetic subjects. Our findings have shown an increase in the phosphorylation levels of ERK1/2,

p38 MAPK and JNK after exercise in both groups. Acipimox was able to suppress the increase of plasma NEFA, resulting in a concomitant repression of p38 MAPK and ERK1/2 but not in the JNK phosphorylation induced by endurance exercise. On the other hand, the NF-κB pathways were unaffected by our endurance protocols in both mouse and human models.

Our data support a role for plasma FA in the activation of MAPK during endurance exercise. This activation may be mediated by TLR2 and TLR4 in skeletal muscle.

References

Adams JM, 2nd, Pratipanawatr T, Berria R, Wang E, DeFronzo RA, Sullards MC, Mandarino LJ. Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. Diabetes. 2004. 53:25-31.

Akira S, Sato S. Toll-like receptors and their signaling mechanisms. Scandinavian journal of infectious diseases. 2003. 35:555-62.

Aronson D, Boppart MD, Dufresne SD, Fielding RA, Goodyear LJ. Exercise stimulates c-Jun NH2 kinase activity and c-Jun transcriptional activity in human skeletal muscle. Biochem Biophys Res Commun. 1998a. 251:106-10.

Aronson D, Dufresne SD, Goodyear LJ. Contractile activity stimulates the c-Jun NH2-terminal kinase pathway in rat skeletal muscle. The Journal of biological chemistry. 1997. 272:25636-40.

Aronson D, Wojtaszewski JF, Thorell A, Nygren J, Zangen D, Richter EA, Ljungqvist O, Fielding RA, Goodyear LJ. Extracellular-regulated protein kinase cascades are activated in response to injury in human skeletal muscle. Am J Physiol. 1998b. 275:C555-61.

Blouin CM, Prado C, Takane KK, Lasnier F, Garcia-Ocana A, Ferre P, Dugail I, Hajduch E. Plasma membrane subdomain compartmentalization contributes to distinct mechanisms of ceramide action on insulin signaling. Diabetes. 2010. 59:600-10.

Boden G. Effects of free fatty acids (FFA) on glucose metabolism: significance for insulin resistance and type 2 diabetes. Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association. 2003. 111:121-4.

Boppart MD, Aronson D, Gibson L, Roubenoff R, Abad LW, Bean J, Goodyear LJ, Fielding RA. Eccentric exercise markedly increases c-Jun NH(2)-terminal kinase activity in human skeletal muscle. Journal of applied physiology. 1999. 87:1668-73.

Chambers MA, Moylan JS, Smith JD, Goodyear LJ, Reid MB. Stretch-stimulated glucose uptake in skeletal muscle is mediated by reactive oxygen species and p38 MAP-kinase. J Physiol. 2009. 587:3363-73.

Chan MH, McGee SL, Watt MJ, Hargreaves M, Febbraio MA. Altering dietary nutrient intake that reduces glycogen content leads to phosphorylation of nuclear p38 MAP kinase in human skeletal muscle: association with IL-6 gene transcription during contraction. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2004. 18:1785-7.

Chavez JA, Summers SA. Characterizing the effects of saturated fatty acids on insulin signaling and ceramide and diacylglycerol accumulation in 3T3-L1 adipocytes and C2C12 myotubes. Arch Biochem Biophys. 2003. 419:101-9.

Clerk A, Fuller SJ, Michael A, Sugden PH. Stimulation of "stress-regulated" mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and p38-mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. The Journal of biological chemistry. 1998. 273:7228-34.

Deldicque L, Cani PD, Philp A, Raymackers JM, Meakin PJ, Ashford ML, Delzenne NM, Francaux M, Baar K. The unfolded protein response is activated in skeletal muscle by high-fat feeding: potential role in the downregulation of protein synthesis. Am J Physiol Endocrinol Metab. 2010. 299:E695-705.

Dey D, Basu D, Roy SS, Bandyopadhyay A, Bhattacharya S. Involvement of novel PKC isoforms in FFA induced defects in insulin signaling. Molecular and cellular endocrinology. 2006. 246:60-4.

Dimopoulos N, Watson M, Sakamoto K, Hundal HS. Differential effects of palmitate and palmitoleate on insulin action and glucose utilization in rat L6 skeletal muscle cells. Biochem J. 2006. 399:473-81.

Dobrzyn A, Gorski J. Ceramides and sphingomyelins in skeletal muscles of the rat: content and composition. Effect of prolonged exercise. Am J Physiol Endocrinol Metab. 2002. 282:E277-85.

Erridge C, Samani NJ. Saturated fatty acids do not directly stimulate Toll-like receptor signaling. Arterioscler Thromb Vasc Biol. 2009. 29:1944-9.

Fujii N, Boppart MD, Dufresne SD, Crowley PF, Jozsi AC, Sakamoto K, Yu H, Aschenbach WG, Kim S, Miyazaki H, Rui L, White MF, Hirshman MF, Goodyear LJ. Overexpression or ablation of JNK in skeletal muscle has no effect on glycogen synthase activity. Am J Physiol Cell Physiol. 2004. 287:C200-8.

Goodpaster BH, He J, Watkins S, Kelley DE. Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. The Journal of clinical endocrinology and metabolism. 2001. 86:5755-61.

Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, Turk BE, Shaw RJ. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell. 2008. 30:214-26.

Hajduch E, Balendran A, Batty IH, Litherland GJ, Blair AS, Downes CP, Hundal HS. Ceramide impairs the insulin-dependent membrane recruitment of protein kinase B leading to a loss in downstream signalling in L6 skeletal muscle cells. Diabetologia. 2001. 44:173-83.

Hayashi T, Hirshman MF, Dufresne SD, Goodyear LJ. Skeletal muscle contractile activity in vitro stimulates mitogen-activated protein kinase signaling. Am J Physiol. 1999. 277:C701-7.

Kramer HF, Goodyear LJ. Exercise, MAPK, and NF-kappaB signaling in skeletal muscle. Journal of applied physiology. 2007. 103:388-95.

Lambert CP, Wright NR, Finck BN, Villareal DT. Exercise but not diet-induced weight loss decreases skeletal muscle inflammatory gene expression in frail obese elderly persons. Journal of applied physiology. 2008. 105:473-8.

Liang H, Tantiwong P, Sriwijitkamol A, Shanmugasundaram K, Mohan S, Espinoza S, Dube JJ, Defronzo RA, Musi N. Effect of a Sustained Reduction in Plasma Free Fatty Acid Concentration onInsulin Signaling and Inflammation in Skeletal Muscle from Human Subjects. J Physiol. 2013.

Liu X, Yuan H, Niu Y, Niu W, Fu L. The role of AMPK/mTOR/S6K1 signaling axis in mediating the physiological process of exercise-induced insulin sensitization in skeletal muscle of C57BL/6 mice. Biochim Biophys Acta. 2012. 1822:1716-26.

Lundberg TR, Fernandez-Gonzalo R, Gustafsson T, Tesch PA. Aerobic exercise alters skeletal muscle molecular responses to resistance exercise. Med Sci Sports Exerc. 2012. 44:1680-8.

Lundberg TR, Fernandez-Gonzalo R, Gustafsson T, Tesch PA. Aerobic exercise does not compromise muscle hypertrophy response to short-term resistance training. Journal of applied physiology. 2013. 114:81-9.

McKenzie MJ, Goldfarb AH. Aerobic exercise bout effects on gene transcription in the rat soleus. Med Sci Sports Exerc. 2007. 39:1515-21.

Mounier R, Lantier L, Leclerc J, Sotiropoulos A, Pende M, Daegelen D, Sakamoto K, Foretz M, Viollet B. Important role for AMPKalpha1 in limiting skeletal muscle cell hypertrophy. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2009. 23:2264-73.

O'Kane MJ, Trinick TR, Tynan MB, Trimble ER, Nicholls DP. A comparison of acipimox and nicotinic acid in type 2b hyperlipidaemia. British journal of clinical pharmacology. 1992. 33:451-3.

Powell DJ, Hajduch E, Kular G, Hundal HS. Ceramide disables 3-phosphoinositide binding to the pleckstrin homology domain of protein kinase B (PKB)/Akt by a PKCzeta-dependent mechanism. Mol Cell Biol. 2003. 23:7794-808.

Ragheb R, Shanab GM, Medhat AM, Seoudi DM, Adeli K, Fantus IG. Free fatty acid-induced muscle insulin resistance and glucose uptake dysfunction: evidence for PKC activation and oxidative stress-activated signaling pathways. Biochem Biophys Res Commun. 2009. 389:211-6.

Rivas DA, Yaspelkis BB, 3rd, Hawley JA, Lessard SJ. Lipid-induced mTOR activation in rat skeletal muscle reversed by exercise and 5'-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside. J Endocrinol. 2009. 202:441-51.

Senn JJ. Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes. The Journal of biological chemistry. 2006. 281:26865-75.

Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest. 2006. 116:3015-25.

Stratford S, Hoehn KL, Liu F, Summers SA. Regulation of insulin action by ceramide: dual mechanisms linking ceramide accumulation to the inhibition of Akt/protein kinase B. The Journal of biological chemistry. 2004. 279:36608-15.

Teruel T, Hernandez R, Lorenzo M. Ceramide mediates insulin resistance by tumor necrosis factor-alpha in brown adipocytes by maintaining Akt in an inactive dephosphorylated state. Diabetes. 2001. 50:2563-71.

Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, Fumagalli S, Allegrini PR, Kozma SC, Auwerx J, Thomas G. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature. 2004. 431:200-5.

Wang X, Yu W, Nawaz A, Guan F, Sun S, Wang C. Palmitate induced insulin resistance by PKCtheta-dependent activation of mTOR/S6K pathway in C2C12 myotubes. Experimental and clinical endocrinology & diabetes : official journal, German Society of Endocrinology [and] German Diabetes Association. 2010. 118:657-61.

Watt MJ, Southgate RJ, Holmes AG, Febbraio MA. Suppression of plasma free fatty acids upregulates peroxisome proliferator-activated receptor (PPAR) alpha and delta and PPAR coactivator 1alpha in human skeletal muscle, but not lipid regulatory genes. Journal of molecular endocrinology. 2004. 33:533-44.

Widegren U, Jiang XJ, Krook A, Chibalin AV, Bjornholm M, Tally M, Roth RA, Henriksson J, Wallberg-henriksson H, Zierath JR. Divergent effects of exercise on metabolic and mitogenic signaling pathways in human skeletal muscle. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1998. 12:1379-89.

Wilkinson SB, Phillips SM, Atherton PJ, Patel R, Yarasheski KE, Tarnopolsky MA, Rennie MJ. Differential effects of resistance and endurance exercise in the fed state on signalling molecule phosphorylation and protein synthesis in human muscle. J Physiol. 2008. 586:3701-17.

Wright DC, Geiger PC, Han DH, Jones TE, Holloszy JO. Calcium induces increases in peroxisome proliferator-activated receptor gamma coactivator-1alpha and mitochondrial biogenesis by a pathway leading to p38 mitogen-activated protein kinase activation. The Journal of biological chemistry. 2007. 282:18793-9.