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

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Review

Title page

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

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Running title: Activation of MAPK by NEFA during exercise

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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 NH₂-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.

41

Key words: p38 MAPK, ERK1/2, JNK, NF- κ B, acipimox, cycling, TLR4, type 2 diabetes

43

44 Introduction

45 The mitogen-activated protein kinases (MAPK) are a group of intracellular signaling proteins
46 activated by multiple signals and are critically involved in the transmission of information
47 from outside the cell to the nucleus, where they regulate transcription. Extracellular signal-
48 regulated kinases (ERK) 1 and 2 (ERK1/2), p38 MAPK and c-Jun NH₂-terminal kinases
49 (JNK) are three widely expressed MAPK that regulate different cellular functions (Johnson &
50 Lapadat, 2002). Endurance exercise is a well-known activator of MAPK but the mechanisms
51 by which ERK1/2, p38 MAPK and JNK are activated during exercise remain incompletely
52 resolved (Kramer & Goodyear, 2007). Increase in cytosolic calcium and subsequent
53 calcium/calmodulin-dependent protein kinase II activation, as well as production of reactive
54 oxygen species (ROS), acidification, increase in catecholamines, growth hormones or
55 cannabinoids, glycogen depletion or mechanical stretch have been proposed to participate to
56 the activation of p38 MAPK during endurance exercise (Blair, Hajdуч, Litherland, &
57 Hundal, 1999; Chambers, Moylan, Smith, Goodyear, & Reid, 2009; Chan, McGee, Watt,
58 Hargreaves, & Febbraio, 2004; Clerk, Fuller, Michael, & Sugden, 1998; Galbo, 1986; Li et
59 al., 2005; Tedesco et al., 2010; Wright, Geiger, Han, Jones, & Holloszy, 2007; Wretman et
60 al., 2001). In addition to those general mechanisms, MAPK undergo member-specific
61 regulations. ERK1/2 and p38 have been shown to be involved in exercise-induced signalling
62 in human skeletal muscle but the picture is less clear for JNK (Widegren et al., 1998; Long,
63 Widegren, & Zierath, 2004; Yu, Blomstrand, Chibalin, Krook, & Zierath, 2001). Cycling
64 markedly increased ERK1/2 phosphorylation, albeit only transiently, whereas similar exercise
65 has been demonstrated to lead to a smaller but more persistent increase in p38 activation
66 (Widegren et al., 1998). MAPK signalling seems to be regulated differentially according to
67 the mode of contraction (concentric vs eccentric), the intensity of the exercise and the training
68 status of the subjects (Widegren, Wretman, Lionikas, Hedin, & Henriksson, 2000).

69

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

80

81 Beside their role in energy supply for skeletal muscle, NEFA have been suggested to also play
82 a role in activating signaling pathways which regulate gene expression (Tunstall, McAinch,
83 Hargreaves, van Loon, & Cameron-Smith, 2007). It has been proposed that NEFA could
84 stimulate a family of membrane receptors called the toll-like receptors (TLR) (Senn, 2006;
85 Shi et al., 2006), the most expressed in skeletal muscle being TLR2 and TLR4 (Reyna et al.,
86 2008). Upon stimulation, TLR2 and TLR4 induce complex intracellular signaling leading to
87 the activation of the MAPK family and transcription factors including nuclear factor- κ B (NF-
88 κ B) (Akira, Yamamoto, & Takeda, 2003). Using TLR2 and TLR4 KO mice, our group
89 recently found that during endurance exercise, TLR2 and TLR4 mediated a signal linking the
90 elevated plasma NEFA concentration to the activation of p38 MAPK and JNK in mice
91 skeletal muscle (Zbinden-Foncea, Raymackers, Deldicque, Renard, & Francaux, 2012). For
92 the first time, a link was established between circulating NEFA and stimulation of MAPK via
93 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.m²⁻¹, 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 ($VO_{2\max}$) 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.m²⁻¹, fat content 28.8 ± 1.8 %, $W_{\max}$ 200 ± 15 W, 50% $W_{\max}$

100 $\pm$ 6 W, and VO_2max 32 $\pm$ 2 ml.kg $\text{bw}^{-1}.\text{min}^{-1}$], 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. 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 ($t=180$).

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, I κ B α 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 $\pm$ 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. 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 ($\sim 0.1\text{mM}$, $P<0.05$; Fig. 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. 2B), 71 ($P<0.01$, Fig. 2C) and 3 ($P<0.05$, Fig. 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. 2B) and ERK1/2 ($P<0.01$, Fig. 2C) and partially repressed for JNK (Fig. 2D). I κ B α expression was not modified by exercise alone although the combination of acipimox and exercise increased I κ B α expression by about 50% compared to exercise in the placebo conditions ($P<0.05$, Fig. 2E).

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. 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 ($0.70 \pm$

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 ($\sim 0.15\text{mM}$, $P<0.05$; Fig. 3A) compared to placebo conditions.

Phosphorylation of p38 MAPK, ERK1/2 and JNK were 5 ($P<0.05$, Fig. 3B), 14 ($P<0.01$, Fig. 3C) and 2 ($P<0.05$, Fig. 3D) fold higher immediately after exercise compared to resting values. The exercise-induced increases in p38 MAPK ($P<0.05$, Fig. 3B) and ERK1/2 ($P<0.01$, Fig. 3C) were totally abolished after acipimox administration. The increase in JNK phosphorylation after exercise was not repressed in acipimox conditions (Fig. 3D). I κ B α expression decreased after exercise in placebo conditions ($P<0.05$, Fig. 3E) but remained unchanged when acipimox was administered.

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.

269

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

287

288 No muscle biopsy was taken between the first ingestion of acipimox and the beginning of the
289 exercise, therefore making it difficult to draw conclusions on the effect of acipimox itself on
290 the MAPK pathways. The effect of acipimox on MAPK has never been studied before but
291 nicotinic acid has been shown to increase p38 MAPK and to reduce ERK1/2 without altering
292 JNK at rest (Watt, Southgate, Holmes, & Febbraio, 2004). Although acipimox is an analog of
293 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 $\text{I}\kappa\text{B}\alpha$, based on our recently published study (Zbinden-Foncea et al., 2012), which reported a possible NEFA/TLR/MAPK signaling. $\text{I}\kappa\text{B}\alpha$ is a well-known downstream target of TLR2/4 signaling (Akira et al., 2003) and regulator of the NF- κB pathway. However, we could not find any consistent regulation of $\text{I}\kappa\text{B}\alpha$ by either exercise or acipimox. These results discard any role of the NF- κB in the present study but it is still possible that NEFA activate MAPK through TLR, independently of NF- κB . 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.

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Figure captions

Figure 1

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

Figure 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.*

Figure 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.*

Title page

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

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Running title: Activation of MAPK by NEFA during exercise

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24 **Abstract**

25 Mitogen-activated protein kinase (MAPK) pathways are activated in skeletal muscle during
26 endurance exercise, but the upstream molecular events are incompletely resolved. As an
27 increase in plasma non-esterified fatty acids (NEFA) is a common feature of long-lasting
28 exercise, we tested the hypothesis that NEFA contribute to the activation of MAPK during
29 endurance exercise. Acipimox was used prior and during endurance exercise to prevent the
30 elevation of plasma NEFA levels in healthy subjects and diabetics. In two separate studies,
31 healthy subjects cycled for 2h and diabetics for 1h at 50% W_{max} . In control conditions, plasma
32 NEFA concentrations increased from 0.35mM to 0.90mM during exercise in healthy subjects
33 and from 0.55mM to 0.70mM in diabetics ($P<0.05$). Phosphorylation state of extracellular-
34 regulated kinase 1 and 2 (ERK1/2), p38 and, c-Jun NH₂-terminal kinases (JNK) were
35 significantly increased after exercise in the vastus lateralis in both groups. Acipimox blocked
36 the increase in plasma NEFA concentrations and almost completely repressed any rise in
37 ERK1/2 and p38 but not in JNK. In conclusion, our data support a role for plasma NEFA in
38 the activation of p38 and ERK1/2 in skeletal muscle tissue of healthy and diabetic subjects
39 during endurance exercise. Further investigation will be required to determine the molecular
40 link between NEFA and MAPK activation during exercise in human skeletal muscle.

41

42 **Key words:** p38 MAPK, ERK1/2, JNK, NF- κ B, acipimox, cycling, TLR4, type 2 diabetes

43

44 Introduction

45 The mitogen-activated protein kinases (MAPK) are a group of intracellular signaling proteins
46 activated by multiple signals and are critically involved in the transmission of information
47 from outside the cell to the nucleus, where they regulate transcription. Extracellular signal-
48 regulated kinases (ERK) 1 and 2 (ERK1/2), p38 MAPK and c-Jun NH₂-terminal kinases
49 (JNK) are three widely expressed MAPK that regulate different cellular functions (Johnson &
50 Lapadat, 2002). Endurance exercise is a well-known activator of MAPK but the mechanisms
51 by which ERK1/2, p38 MAPK and JNK are activated during exercise remain incompletely
52 resolved (Kramer & Goodyear, 2007). Increase in cytosolic calcium and subsequent
53 calcium/calmodulin-dependent protein kinase II activation, as well as production of reactive
54 oxygen species (ROS), acidification, increase in catecholamines, growth hormones or
55 cannabinoids, glycogen depletion or mechanical stretch have been proposed to participate to
56 the activation of p38 MAPK during endurance exercise (Blair, Hajdуч, Litherland, &
57 Hundal, 1999; Chambers, Moylan, Smith, Goodyear, & Reid, 2009; Chan, McGee, Watt,
58 Hargreaves, & Febbraio, 2004; Clerk, Fuller, Michael, & Sugden, 1998; Galbo, 1986; Li et
59 al., 2005; Tedesco et al., 2010; Wright, Geiger, Han, Jones, & Holloszy, 2007; Wretman et
60 al., 2001). In addition to those general mechanisms, MAPK undergo member-specific
61 regulations. ERK1/2 and p38 have been shown to be involved in exercise-induced signalling
62 in human skeletal muscle but the picture is less clear for JNK (Widegren et al., 1998; Long,
63 Widegren, & Zierath, 2004; Yu, Blomstrand, Chibalin, Krook, & Zierath, 2001). Cycling
64 markedly increased ERK1/2 phosphorylation, albeit only transiently, whereas similar exercise
65 has been demonstrated to lead to a smaller but more persistent increase in p38 activation
66 (Widegren et al., 1998). MAPK signalling seems to be regulated differentially according to
67 the mode of contraction (concentric vs eccentric), the intensity of the exercise and the training
68 status of the subjects (Widegren, Wretman, Lionikas, Hedin, & Henriksson, 2000).

69

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

80

81 Beside their role in energy supply for skeletal muscle, NEFA have been suggested to also play
82 a role in activating signaling pathways which regulate gene expression (Tunstall, McAinch,
83 Hargreaves, van Loon, & Cameron-Smith, 2007). It has been proposed that NEFA could
84 stimulate a family of membrane receptors called the toll-like receptors (TLR) (Senn, 2006;
85 Shi et al., 2006), the most expressed in skeletal muscle being TLR2 and TLR4 (Reyna et al.,
86 2008). Upon stimulation, TLR2 and TLR4 induce complex intracellular signaling leading to
87 the activation of the MAPK family and transcription factors including nuclear factor- κ B (NF-
88 κ B) (Akira, Yamamoto, & Takeda, 2003). Using TLR2 and TLR4 KO mice, our group
89 recently found that during endurance exercise, TLR2 and TLR4 mediated a signal linking the
90 elevated plasma NEFA concentration to the activation of p38 MAPK and JNK in mice
91 skeletal muscle (Zbinden-Foncea, Raymackers, Deldicque, Renard, & Francaux, 2012). For
92 the first time, a link was established between circulating NEFA and stimulation of MAPK via
93 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.m²⁻¹, 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 ($VO_{2\max}$) 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.m²⁻¹, fat content 28.8 ± 1.8 %, $W_{\max}$ 200 ± 15 W, 50% $W_{\max}$

100 $\pm$ 6 W, and VO_2max 32 $\pm$ 2 ml.kg $\text{bw}^{-1}.\text{min}^{-1}$], 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. 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 ($t=180$).

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, I κ B α 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 $\pm$ 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. 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 ($\sim 0.1\text{mM}$, $P<0.05$; Fig. 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. 2B), 71 ($P<0.01$, Fig. 2C) and 3 ($P<0.05$, Fig. 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. 2B) and ERK1/2 ($P<0.01$, Fig. 2C) and partially repressed for JNK (Fig. 2D). I κ B α expression was not modified by exercise alone although the combination of acipimox and exercise increased I κ B α expression by about 50% compared to exercise in the placebo conditions ($P<0.05$, Fig. 2E).

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. 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 ($0.70 \pm$

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 ($\sim 0.15\text{mM}$, $P<0.05$; Fig. 3A) compared to placebo conditions.

Phosphorylation of p38 MAPK, ERK1/2 and JNK were 5 ($P<0.05$, Fig. 3B), 14 ($P<0.01$, Fig. 3C) and 2 ($P<0.05$, Fig. 3D) fold higher immediately after exercise compared to resting values. The exercise-induced increases in p38 MAPK ($P<0.05$, Fig. 3B) and ERK1/2 ($P<0.01$, Fig. 3C) were totally abolished after acipimox administration. The increase in JNK phosphorylation after exercise was not repressed in acipimox conditions (Fig. 3D). I κ B α expression decreased after exercise in placebo conditions ($P<0.05$, Fig. 3E) but remained unchanged when acipimox was administered.

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.

269

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

287

288 No muscle biopsy was taken between the first ingestion of acipimox and the beginning of the
289 exercise, therefore making it difficult to draw conclusions on the effect of acipimox itself on
290 the MAPK pathways. The effect of acipimox on MAPK has never been studied before but
291 nicotinic acid has been shown to increase p38 MAPK and to reduce ERK1/2 without altering
292 JNK at rest (Watt, Southgate, Holmes, & Febbraio, 2004). Although acipimox is an analog of
293 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 $\text{I}\kappa\text{B}\alpha$, based on our recently published study (Zbinden-Foncea et al., 2012), which reported a possible NEFA/TLR/MAPK signaling. $\text{I}\kappa\text{B}\alpha$ is a well-known downstream target of TLR2/4 signaling (Akira et al., 2003) and regulator of the NF- κB pathway. However, we could not find any consistent regulation of $\text{I}\kappa\text{B}\alpha$ by either exercise or acipimox. These results discard any role of the NF- κB in the present study but it is still possible that NEFA activate MAPK through TLR, independently of NF- κB . 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.

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For Peer Review

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Figure captions**Figure 1**

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

Figure 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) I κ B α expression in skeletal muscle before and immediately after exercise in placebo and acipimox conditions. Values are expressed as means $\pm$ SEM (n=5). *P<0.05, **P<0.01 vs Pre-ex same treatment; §P<0.05, §§P<0.01 vs Placebo same condition.*

Figure 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) I κ B α expression in skeletal muscle before and immediately after exercise in placebo and acipimox conditions. Values are expressed as means $\pm$ SEM (n=10). *P<0.05, **P<0.01 vs Pre-ex same treatment; §P<0.05, §§P<0.01 vs Placebo same condition.*

Experimental protocols of study 1 in healthy subjects and study 2 in diabetic patients.
616x448mm (300 x 300 DPI)

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 $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs Pre-ex same treatment; § $P < 0.05$, §§ $P < 0.01$ vs Placebo same condition.
145x200mm (300 x 300 DPI)

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.
146x205mm (300 x 300 DPI)