

# Suppression of plasma free fatty acids upregulates peroxisome proliferator-activated receptor (PPAR) $\alpha$ and $\delta$ and PPAR coactivator 1 $\alpha$ in human skeletal muscle, but not lipid regulatory genes

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## Abstract

Fatty acids are an important ligand for peroxisome proliferator-activated receptor (PPAR) activation and transcriptional regulation of metabolic genes. To examine whether reduced plasma free fatty acid (FFA) availability affects the mRNA content of proteins involved in fuel metabolism *in vivo*, the skeletal muscle mRNA content of various transcription factors, transcriptional coactivators and genes encoding for lipid regulatory proteins were examined before and after 3 h of cycle exercise with (NA) and without (CON) pre-exercise ingestion of nicotinic acid (NA). NA resulted in a marked (3- to 6-fold) increase ( $P < 0.05$ ) in PPAR $\alpha$ , PPAR $\delta$  and PPAR coactivator 1 $\alpha$  (PGC1 $\alpha$ ) mRNA, but was without effect on nuclear respiratory factor-1 and Forkhead transcription factor, fatty acid translocase/CD36, carnitine palmitoyl transferase 1, hormone sensitive lipase (HSL) and pyruvate dehydrogenase kinase 4. Exercise in CON was associated with increased ( $P < 0.05$ ) PPAR $\alpha$ , PPAR $\delta$  and PGC1 $\alpha$  mRNA, which was similar in magnitude to levels observed with NA at rest. Exercise was generally without effect on the mRNA content of lipid regulatory proteins in CON and did not affect the mRNA content of the measured subset of transcription factors, transcriptional co-activators and lipid regulatory proteins during NA. To determine the possible mechanisms by which NA might affect PGC1 $\alpha$  expression, we measured p38 MAP kinase (MAPK) and plasma epinephrine. Phosphorylation of p38 MAPK was increased ( $P < 0.05$ ) by NA treatment at rest, and this correlated ( $r^2 = 0.84$ ,  $P < 0.01$ ) with increased PGC1 $\alpha$ . Despite this close relationship, increasing p38 MAPK in human primary myotubes was without effect on PGC1 $\alpha$  mRNA content. Plasma epinephrine was elevated ( $P < 0.05$ ) by NA at rest (CON:  $0.27 \pm 0.06$ , NA:  $0.72 \pm 0.11$  nM) and throughout exercise. Incubating human primary myotubes with epinephrine increased PGC1 $\alpha$  independently of changes in p38 MAPK phosphorylation. Hence, despite the fact that NA ingestion decreased FFA availability, it promoted the induction of PPAR $\alpha/\delta$  and PGC1 $\alpha$  gene expression to a similar degree as prolonged exercise. We suggest that the increase in PGC1 $\alpha$  may be due to the elevated plasma epinephrine levels. Despite these changes in transcription factors/coactivators, the mRNA content of lipid regulatory proteins was generally unaffected by plasma FFA availability.

*Journal of Molecular Endocrinology* (2004) **33**, 533–544

## Introduction

Skeletal muscle is a major site of fatty acid uptake and oxidation at rest and during contraction (for review see van der Vusse & Reneman 1996) and aberrant control of fatty acid metabolism is related to obesity and type 2 diabetes (Kelley *et al.* 2002). Chronic exercise improves the capacity to utilize fatty acids by a co-ordinated upregulation of

proteins involved in the sarcolemmal uptake (fatty acid translocase; FAT/CD36), mitochondrial transport (carnitine palmitoyl transferase 1; CPT1) and  $\beta$ -oxidation ( $\beta$ -hydroxyacyl-coenzyme-A) of fatty acids (Spina *et al.* 1996, Berthon *et al.* 1998, Bonen *et al.* 1999). The upregulation of these proteins is rapid and due, at least partially, to increased gene expression (Hood 2001). In this regard, increased transcription and expression of several metabolic

regulatory genes have been demonstrated immediately after a single prolonged exercise bout (Cortright *et al.* 1999, Pilegaard *et al.* 2000, 2002).

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear hormone receptors that mediate adaptive metabolic responses by ligand-dependent transcriptional activation of target genes (Kliwer *et al.* 2001). PPAR $\alpha$  and  $\delta$  are considered the most important subtypes in skeletal muscle by virtue of their abundant expression (Braissant *et al.* 1996). PPAR $\alpha$  mediates transcriptional control of nuclear genes encoding for proteins involved in fuel oxidation, including CPT1, pyruvate dehydrogenase kinase 4 (PDK4) and uncoupling protein 3 (UCP3). Consistent with a central role in fatty acid oxidation, PPAR $\alpha$  null mice exhibit decreased expression of lipid regulatory genes, decreased  $\beta$ -oxidation and greater lipid accumulation in hepatic and cardiac tissue (Djouadi *et al.* 1998). Although much less is known regarding PPAR $\delta$ , recent evidence has demonstrated increased expression of genes involved in lipid catabolism in skeletal muscle cells following acute PPAR $\delta$  activation (Dressel *et al.* 2003, Wang *et al.* 2003). The PPARs are activated by naturally occurring fatty acids and fatty acid metabolites, indicating that they act as fatty acid sensors. Since fatty acids and lipid metabolites (e.g. fatty acyl CoA) are ligands of the PPARs and their availability is increased during prolonged exercise (Watt *et al.* 2003), the marked increase in fatty acid availability during prolonged exercise may provide a stimulus for increased PPAR $\alpha$ / $\delta$  activity and subsequent induction of numerous lipid regulatory genes.

Emerging evidence demonstrates the importance of coactivator molecules in the transcriptional activation of nuclear receptor target genes. Fatty acid induction of gene expression through PPAR $\alpha$  and  $\delta$  is enhanced by the PPAR coactivator 1 $\alpha$  (PGC1 $\alpha$ ), which is expressed in oxidative tissues such as skeletal and cardiac muscle (Dressel *et al.* 2003, for review see Puigserver & Spiegelman 2003). Overexpression of PGC1 $\alpha$  and PPAR $\alpha$  in 3T3-L1 adipocytes resulted in the induction of PPAR $\alpha$  target genes and concomitant increases in palmitate oxidation (Vega *et al.* 2000). Of note, PGC1 $\alpha$  expression is induced during metabolic stresses such as cold exposure (Puigserver *et al.* 1998), severe caloric restriction (Larrouy *et al.* 1999) and prolonged exercise (Terada *et al.* 2002, Pilegaard *et al.* 2003). In these situations, plasma

free fatty acid (FFA) availability is increased dramatically, which provides a clue to a potential mechanism through which PGC1 $\alpha$  gene expression is enhanced.

In the present study, we chose to examine the effect of the anti-lipolytic drug nicotinic acid (NA) on the expression of the nuclear transcription factors, PPAR $\alpha$  and  $\delta$ , the transcriptional coactivator, PGC1 $\alpha$  and various PPAR target genes encoding for proteins involved in lipid metabolism. We hypothesized that decreased plasma FFA availability would suppress the exercise-induced increase in the lipid regulatory genes, PGC1 $\alpha$ , PPAR $\alpha$  and PPAR $\delta$ .

## Materials and methods

### Experimental subjects

The experimental protocol was approved by the Royal Melbourne Institute of Technology human research ethics committee, and written informed consent was obtained prior to testing. All testing was in accordance with the Declaration of Helsinki. Subjects were recreationally active males ( $24 \pm 3$  years;  $80 \pm 3$  kg) who participated in two to four aerobic exercise bouts per week.

### Pre-experimental protocol

Subjects performed an incremental exercise bout to volitional exhaustion to determine peak pulmonary oxygen uptake ( $\text{VO}_2$  peak) which averaged  $4.33 \pm 0.31$  litres/min. Expired contents of oxygen and carbon dioxide and ventilation data were collected and analysed on-line (Quark b<sup>2</sup>; COSMED, Rome, Italy). At least 2 days later, subjects completed a practice trial, the purpose of which was to familiarize the subjects with the effects of NA and confirm their ability to perform the exercise bout. Subjects ingested 10 mg/kg NA (Aspen Pharmacare, St Leonards, NSW, Australia) and rested for 60 min, after which they cycled for 120 min at 60%  $\text{VO}_2$  peak. Further NA doses (5 mg/kg) were ingested at 30-min intervals at rest ( $-30$  and 0 min) and throughout exercise. For the day preceding each trial, subjects were provided with a food parcel (14 MJ, 80% carbohydrate) and were required to abstain from exercise, caffeine and alcohol. All trials were performed at an ambient temperature of 19–21 °C.

## Experimental protocol

Subjects performed 180 min of cycle exercise at 60%  $\text{VO}_2$  peak on two occasions; either with (NA) or without (CON) NA ingestion before and during exercise. The trials were randomly assigned and counter-balanced. Subjects visited the laboratory at 0800 h after an overnight fast. A Teflon catheter was inserted into a forearm vein and was kept patent by saline infusion. A resting blood sample was obtained and the vastus lateralis was prepared for needle biopsy. Three incisions were made through the skin and fascia under local anaesthesia (lidocaine, no epinephrine). During NA, subjects followed the NA ingestion regime as described above. A placebo was not administered in CON subjects because subjects can easily determine the effects of NA ingestion, which include peripheral vasodilation, itchiness and in some instances mild nausea. One blood and one muscle sample were obtained immediately prior to exercise and at 90 and 180 min during exercise. The exercise biopsy samples were obtained while the subjects remained on the cycle ergometer and were rapidly frozen in liquid nitrogen for later analysis. An extra biopsy was not obtained prior to NA ingestion to minimize subject stress, which may have confounded the gene expression measurements. Subjects were not permitted to eat during the trial but were provided with water (1 litre/h) to ensure euhydration.

## Blood analysis

Whole blood was mixed in a sodium–heparin collection tube and the plasma was obtained after centrifugation at 5000 *g* for 2 min. Plasma samples were frozen at  $-80^\circ\text{C}$  for later analysis of FFA by an enzymatic colorimetric method (NEFA C; Wako Chemicals, Richmond, VA, USA), insulin (Coat-a-Count; DPC, Los Angeles, CA, USA) and epinephrine (LDN, Nordhorn, Germany) by radioimmunoassay.

## Muscle analysis

Muscle tissue was homogenized and extracted for total RNA using the acid guanidium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi 1987) and modified according to methods described elsewhere (Febbraio

& Koukoulas 2000). RNA samples were reverse transcribed using a thermal cycler (Perkin Elmer GeneAmp PCR 2400 thermal cycler; Perkin Elmer, Rowville, Victoria, Australia) with Taqman Reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) in 40  $\mu\text{l}$  reaction mixtures containing 1 $\times$  Taqman RT buffer, 5.5 mM  $\text{MgCl}_2$ , 500  $\mu\text{M}$  2'-deoxynucleoside 5'-triphosphate, 2.5  $\mu\text{M}$  random hexamers, 0.4 U/ $\mu\text{l}$  RNase inhibitor and 1.25 U/ $\mu\text{l}$  multiscribe reverse transcriptase. Control (reverse transcriptase negative) samples were also made and included in all future PCR analyses to check for genomic DNA contamination.

## Real-time PCR analysis

Real-time PCR was employed to quantify the genes of interest. All reactions were performed according to the multiplex comparative critical threshold ( $C_T$ ) method. This method allows for the detection of the reference gene (ribosomal 18S) and the gene of interest in the same well or tube. Preliminary experiments were first performed to determine the efficiency of amplification of 18S vs the gene of interest.

PCR reactions were performed in 96-well plates on a BioRad i-CYCLER iQ real-time PCR detection system in 25  $\mu\text{l}$  reaction volumes consisting of: BioRad iQ Supermix PCR mix (2 $\times$ ; BioRad, Applied Biosystems, Scoresby, Victoria, Australia), Applied Biosystems pre-developed assay reagent for 18S (Applied Biosystems), the forward and reverse primers and probes of the genes of interest (see Table 1 for sequences) and sterile  $\text{H}_2\text{O}$ . A  $\Delta C_T$  value was obtained by subtracting 18S  $C_T$  values from the  $C_T$  value of the gene of interest. For each gene, the  $\Delta C_T$  value for CON 0 was then subtracted from the  $\Delta C_T$  values of the other time-points for each subject to derive a  $\Delta\Delta C_T$  value. The expression was then evaluated by  $2^{-\Delta\Delta C_T}$  with all CON 0 values for each subject being 1.

## Protein analysis

Muscle was homogenized (Polytron; Brinkman Instruments, New York, NY, USA) in ice-cold buffer containing 50 mM HEPES, 150 mM NaCl, 10 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , 5 mM EDTA, 0.5% Triton X-100, 10% glycerol (v/v), 2  $\mu\text{g}/\text{ml}$  leupeptin, 100  $\mu\text{g}/\text{ml}$  phenylmethylsulfonyl fluoride

**Table 1** Primer and probe sequences for real-time PCR

Gene	Forward primer	Reverse primer	TaqMan probe
CPT1	5'-CTGCAGTGGGACATTCCAAA-3'	5'-CAACGCCCTTGGCCACCT-3'	5'-ACTCTCGATGACCGCCTGGCACTG-3'
FAT/36	5'-AGTCACTGCGACTGATTAATGGT-3'	5'-CTGCAATACCTGGCTTTTCTCA-3'	5'-ACAGATGCAGCCTCATTTCCACCTTTTG-3'
FKHR	5'-CGTGCCCTACTTCAAGCATAAGG-3'	5'-CGACTATGCAGTGACAGGTTGTG-3'	5'-TCTGCCGGCTGGAAGAACTCCATC-3'
HSL	5'-ACGCTGCATAAGGGATGCTT-3'	5'-CCTGTCTCGTTGCGTTTGTAGT-3'	5'-AGTTACAGCCTGCCATCCGGC-3'
NRF-1	5'-GGCACTGTCTCACTATCCAGGTT-3'	5'-CAGCCACGGCAGATAATTCA-3'	5'-ACCACGGTCAACCGTTGCCAA-3'
PGC1 $\alpha$	5'-CAAGCCAAACCAACTTATCTCT-3'	5'-CACACTAAGGTGCGTTCAATAGTC-3'	5'-AGTACCACAAATGACCCCAAGGGTTCC-3'
PPAR $\alpha$	5'-AGCTTTGGCTTACGGAATACCA-3'	5'-CCACAGATAAGTACCGAGGA-3'	5'-TCACGGACACGCTTCCACGCTTC-3'
PPAR $\delta$	5'-CACTGAGTTCGCCAAGAGCAT-3'	5'-CACGCCATACTTGAGAAGGGTAA-3'	5'-TCAGCAGCCTTCCCTCAACGACCA-3'
PDK4	5'-TCCACTGCACCAACCGCCT-3'	5'-TGGCAAGCCGTAACCAAAA-3'	5'-ATAATCCCAGGAATGCTCCCTTTGGCTG-3'
UCP3	5'-TGACTCCGTC AAGCAGGTGTAC-3'	5'-CAAAAATCCGGGTAGTGAGGCT-3'	5'-CCCCCAAAAGCGGGACAAC-3'

CPT1, carnitine palmitoyl transferase 1; FAT/CD36, fatty acid translocase; FKHR, forkhead transcription factor; HSL, hormone sensitive lipase; NRF-1, nuclear respiratory factor-1; PGC1 $\alpha$ , PPAR coactivator 1 $\alpha$ ; PPAR $\alpha$ , PPAR $\delta$ , peroxisome proliferator activated receptor  $\alpha$ ; PPAR $\delta$ , peroxisome proliferator activated receptor  $\delta$ ; PDK4, pyruvate dehydrogenase kinase 4; UCP3, uncoupling protein 3.

and 2 µg/ml aprotinin. Homogenates were centrifuged and the supernatant was removed and rapidly frozen in liquid nitrogen. Protein concentration of the muscle lysates was subsequently determined (Pierce, Rockford, IL, USA). Muscle lysates were solubilized in Laemmli sample buffer and boiled for 5 min, resolved by SDS-PAGE on 10% polyacrylamide gels, transferred to a nitrocellulose membrane, blocked with 5% bovine serum albumin and immunoblotted with the anti-phospho-p38 MAP kinase (MAPK), anti-phospho-extracellular regulated kinase (ERK), anti-phospho-c-Jun NH<sub>2</sub>-terminal kinase (JNK) (1:1000; Cell Signaling, Beverly, MA, USA) or anti-PGC1 antibody (Chemicon International, Boronia, Victoria, Australia). After incubation with horseradish peroxidase-conjugated secondary antibody (1:2000; Amersham Biosciences, Castle Hill, NSW, Australia), the immunoreactive proteins were detected with enhanced chemiluminescence (Perkin Elmer) and quantified by densitometry.

### Primary cell culture

Muscle tissue from the vastus lateralis was obtained via percutaneous needle biopsy and immediately transferred to ice-cold Ham's F-10 media (Gibco, Mt Waverley, Victoria, Australia). The tissue was washed three times in ice-cold Ham's-F10 media to remove any traces of blood, and placed over ice in a sterile Petri dish. The tissue was covered with ~3 ml 0.05% Trypsin/EDTA and minced. The homogenate was collected, diluted in 15 ml 0.05% Trypsin/EDTA, agitated for 20 min and the supernatant was collected and stored on ice. This procedure was repeated three times and the supernatant was filtered and then spun for 7 min at 1600 *g* at room temperature. The cell pellet was re-suspended in growth media ( $\alpha$ -minimum essential medium with 10% fetal calf serum, 0.5% antibiotics and 0.5% amphotericin B) and placed in an uncoated 75 cm flask and incubated for 30 min at 37 °C (95% O<sub>2</sub>/5% CO<sub>2</sub>) to allow attachment of fibroblasts. The supernatant was collected and placed in another flask pre-coated with extracellular matrix (ECM) and incubated as above for 24 h. Satellite cells appeared after ~7 days and were allowed to reach 60–70% confluence, at which point they were sub-cultured into separate dishes. When the myoblasts reached 70% confluence, the growth medium was

replaced with differentiation medium (2% horse serum (HS), 0.5% antibiotics and 0.5% antifungal). Myoblasts were maintained in differentiation media until fully fused myotube cultures were obtained.

Experiments were conducted in overnight serum-starved cells. Myotubes ( $n=12$  plates for each condition) were incubated for 6 h at 37 °C with vehicle (phosphate-buffered saline), 10 µM NA, 500 nM A23187 (calcium ionophore) and 1 µM epinephrine alone or in combination with the specific p38 MAPK inhibitor, SB203580 (10 µM). In one experiment, cells ( $n=6$ ) were rapidly lysed with the ice-cold buffer used for protein homogenization (see above) and rapidly frozen in liquid nitrogen for later analysis of p38 MAPK phosphorylation. In a second experiment, cells ( $n=6$ ) were homogenized and extracted for total RNA as described above. RT-PCR was employed to assess PGC1 $\alpha$  mRNA content.

### Statistical analysis

All values are expressed as means  $\pm$  S.E.M. ( $n=7$ ). Statistical analysis was performed by two-way ANOVA with repeated measures followed by a Student–Newman–Kuels post hoc analysis to determine specific differences where appropriate. Correlations were examined using a Pearson correlation analysis. SigmaStat version 2.03 (SPSS Incorporated, Chicago, IL, USA) was used to compute these statistics. Statistical significance was set at  $P<0.05$ .

## Results

### Physiological variables

NA ingestion suppressed ( $P<0.05$ ) plasma FFA levels at rest ( $0.41 \pm 0.04$  to  $0.10 \pm 0.01$  mM) and throughout exercise whereas plasma FFA was increased ( $P<0.05$ ) from rest at the conclusion of exercise in CON (Table 2). Plasma epinephrine concentration was greater ( $P<0.05$ ) before and after exercise in NA compared with CON (Table 2). Plasma insulin concentration decreased ( $P<0.05$ ) from rest by the cessation of exercise in both trials and there were no differences between trials (Table 2).

**Table 2** Plasma FFA, epinephrine and insulin responses before and after 3 h of exercise with (NA) or without (CON) prior NA ingestion. Values (means $\pm$ s.e.m.) are expressed as fold change from CON at 0 min ( $n=7$ )

	Trial	Pre	Post
FFA (mM)	CON	0.32 $\pm$ 0.03	1.42 $\pm$ 0.07
	NA	0.10 $\pm$ 0.01	0.10 $\pm$ 0.01
Epinephrine (mM)	CON	0.27 $\pm$ 0.06	1.70 $\pm$ 0.35
	NA	0.72 $\pm$ 0.11*	3.09 $\pm$ 0.70*
Insulin (pM)	CON	51.9 $\pm$ 4.8	6.9 $\pm$ 1.5
	NA	54.7 $\pm$ 4.3	6.6 $\pm$ 1.7

\* $P<0.05$  compared with corresponding time-point for CON.

### Skeletal muscle mRNA content

#### Transcription factors

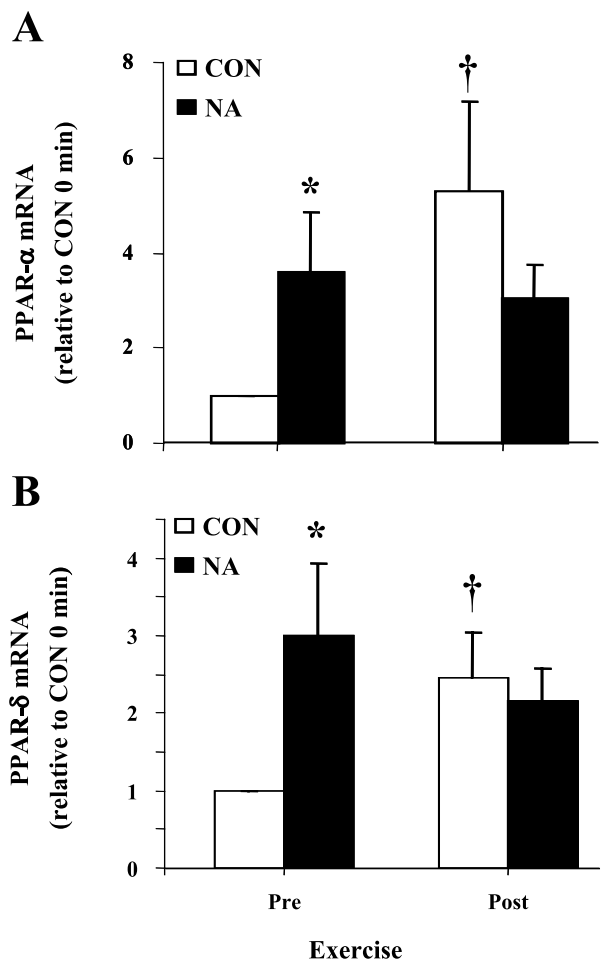
PPAR $\alpha$  (3.8-fold) and PPAR $\delta$  (3-fold) were elevated ( $P<0.05$ ) at rest in NA compared with CON (Fig. 1). PPAR $\alpha$  and PPAR $\delta$  mRNA were increased ( $P<0.05$ ) from rest after exercise in CON, but no such changes were observed in NA. There were no differences between trials after exercise. NRF-1 is a key transcriptional activator of nuclear genes that is transcriptionally activated by PGC1 $\alpha$  and coactivates with PGC1 $\alpha$  to induce mitochondrial biogenesis (Baar *et al.* 2003). Muscle content of NRF-1 was unaffected by NA ingestion at rest and exercise in both trials (Fig. 2). Although it is well known that the phosphorylation of the FKHR protein is via Akt phosphorylation (Brunet *et al.* 1999), relatively less is known with respect to the transcriptional regulation of FKHR. Skeletal muscle FKHR mRNA content was not affected by NA ingestion or exercise in NA. FKHR mRNA tended to increase (2-fold,  $P=0.08$ ) following exercise in CON.

#### Transcriptional coactivators

PGC1 $\alpha$  was markedly ( $\sim 6$  fold) increased ( $P<0.05$ ) at rest in NA compared with CON (Fig. 3A). After exercise, muscle PGC1 $\alpha$  mRNA content was increased ( $P<0.05$ ) in CON, whereas no such change was observed in NA. There was no difference in PGC1 $\alpha$  mRNA content after exercise.

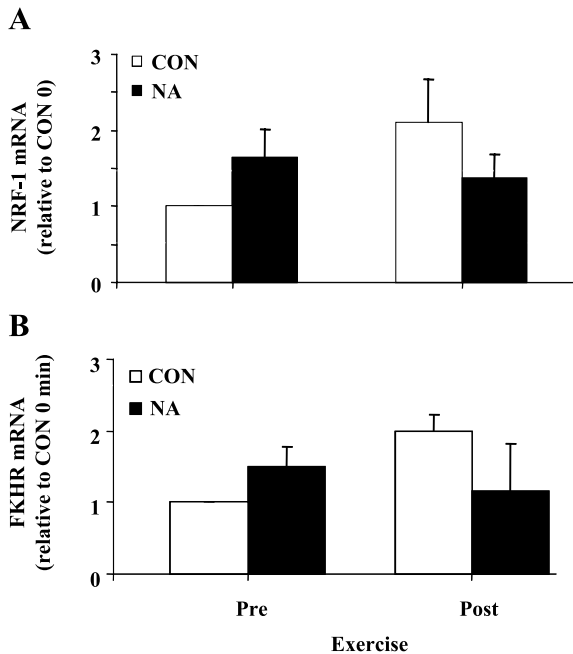
#### Lipid regulatory genes

FAT/CD36, HSL, CPT1 and PDK4 mRNA content were not different between trials at rest,



**Figure 1** Real-time RT-PCR analysis of (A) PPAR $\alpha$  and (B) PPAR $\delta$  mRNA measured before and after 3 h of moderate exercise with (NA) or without (CON) prior NA administration. Values represent the means $\pm$ s.e.m. ( $n=7$ ). \* $P<0.05$ , different from corresponding value for CON; † $P<0.05$ , different from 0 min of the same trial.

whereas UCP3 was 3-fold greater ( $P<0.05$ ) in NA compared with CON (Table 3). Exercise was without effect on CPT1 and HSL mRNA content in both trials, whereas PDK4 was increased ( $P<0.05$ , main effect) after exercise (Table 2). Exercise induced a small ( $\sim 2.5$  fold) increase ( $P<0.05$ ) in FAT/CD36 during NA only. In contrast, UCP3 mRNA was increased ( $P<0.05$ ) from rest values after exercise in CON, whereas it was decreased post-exercise in NA. There were no differences between trials for UCP3 after exercise.



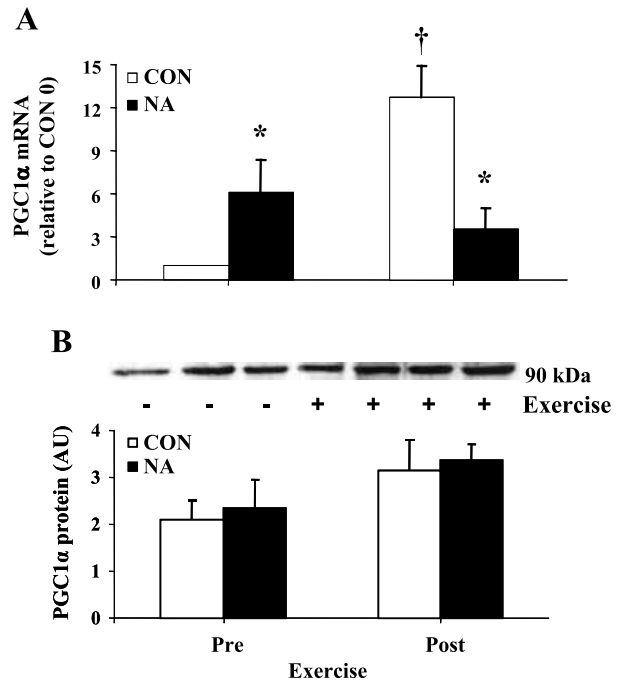
**Figure 2** Real-time RT-PCR analysis of (A) NRF-1 and (B) FKHR mRNA before and after 3 h of moderate exercise with (NA) or without (CON) prior NA administration. Values represent the means  $\pm$  S.E.M. ( $n=7$ ).

### Protein expression in skeletal muscle

PGC1 $\alpha$  protein expression was not affected by NA at rest or after exercise (Fig. 3B). PGC1 $\alpha$  protein tended ( $P=0.09$ , main effect for time) to increase after exercise with increased protein content observed in four of five subjects. Phosphorylation of p38 MAPK was 3-fold higher ( $P<0.05$ ) at rest in NA compared with CON (Fig. 4A). Although exercise did not induce any effects on p38 MAPK phosphorylation in either trial, protein levels remained higher ( $P<0.05$ ) in NA. ERK1/2 phosphorylation was lower ( $P<0.05$ ) in NA at rest compared with CON (Fig. 4B). ERK1/2 phosphorylation was decreased ( $P<0.05$ ) at 180 min, whereas ERK1/2 phosphorylation was unaffected by exercise in NA. JNK phosphorylation was not different between trials at rest and was increased ( $P<0.05$ ) after exercise in both trials (Fig. 4C).

### Relationships between PGC1 $\alpha$ mRNA, phosphorylated p38 MAPK and plasma epinephrine

PGC1 $\alpha$  gene expression is regulated by signals that relay metabolic requirements of the cell, such



**Figure 3** (A) Real-time RT-PCR analysis of PGC1 $\alpha$  mRNA and (B) Western blot analysis of PGC1 $\alpha$  protein content measured before and after 3 h of moderate exercise with (NA) or without (CON) prior NA administration. Values represent the means  $\pm$  S.E.M. ( $n=7$  for mRNA;  $n=5$  for protein). \* $P<0.05$ , different from corresponding value for CON; † $P<0.05$ , different from 0 min of the same trial.

examples including p38 MAPK (Knutti *et al.* 2001) and cAMP (Ichida *et al.* 2002). The increased PGC1 $\alpha$  mRNA content and p38 MAPK phosphorylation correlated significantly at rest ( $r^2=0.84$ ,  $P<0.01$ ), whereas phospho-ERK, phospho-JNK and plasma epinephrine and PGC1 $\alpha$  mRNA were not related.

### Cell culture experiments

To directly examine the effect of p38 MAPK and epinephrine on PGC1 $\alpha$  expression, we incubated human primary myotubes in the presence of A23187, which is a calcium ionophore known to stimulate p38 MAPK phosphorylation (MHS Chan, MJ Watt & MA Febbraio, unpublished observations), epinephrine, which is known to elevate cellular cAMP/protein kinase A (PKA) content and NA. Unexpectedly, increasing p38 MAPK phosphorylation (>20-fold) did not affect PGC1 $\alpha$  mRNA content following A23187 treatment (Fig. 5). Incubation with NA also increased

**Table 3** Expression of lipid regulatory genes before and after 3 h of exercise with (NA) or without (CON) prior NA ingestion. Values (means±S.E.M.) are expressed as fold change from CON at 0 min (*n*=7)

	CON		NA	
	Pre	Post	Pre	Post
CPT1	1	2.09±0.67	1.14±0.18	2.82±1.04
FAT/CD36	1	0.75±0.20	0.75±0.17	2.61±0.74*†
HSL	1	2.11±1.07	2.61±1.91	3.18±2.06
PDK4‡	1	4.33±1.24	1.16±0.43	3.14±1.09
UCP3	1	3.21±0.70†	3.56±0.58*	2.04±0.29†

\**P*<0.05 compared with corresponding time-point for CON; †*P*<0.05 compared with 0 min of the same trial; ‡*P*<0.05 compared with 0 min (main effect).

p38 MAPK phosphorylation (17-fold) and did not affect PGC1 $\alpha$ . In contrast, 6 h of epinephrine treatment increased PGC1 $\alpha$  content by 250% (*P*<0.05) and co-incubation with the specific p38 MAPK inhibitor, SB203580, did not prevent this increase.

## Discussion

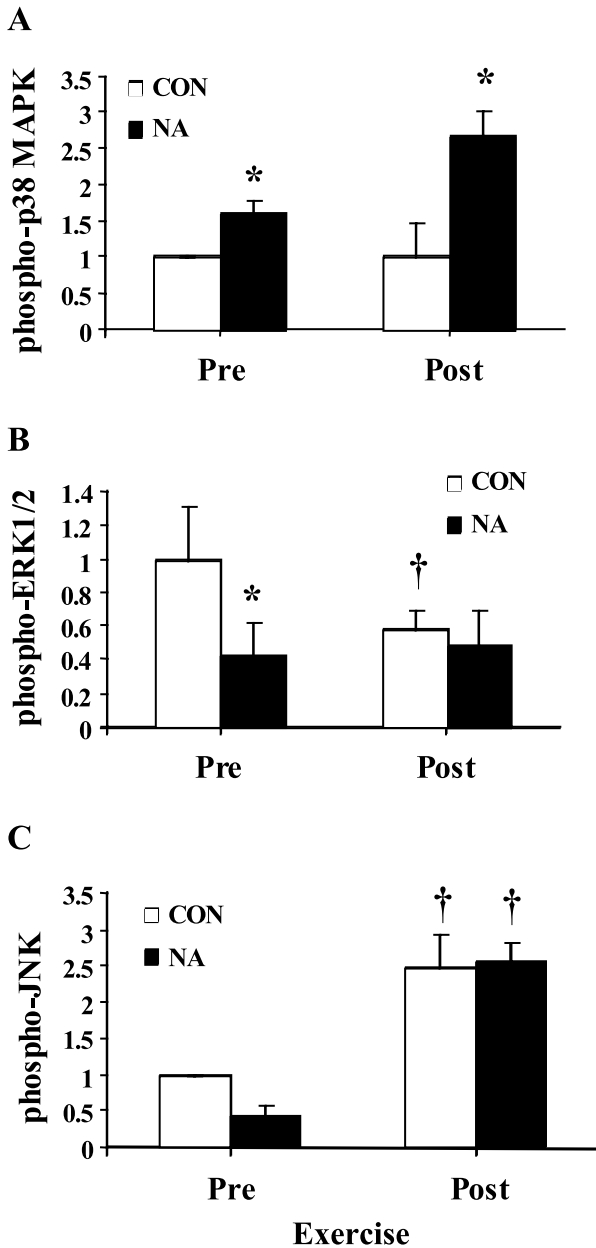
Fatty acids are endogenous ligands for the PPARs (Forman *et al.* 1997) and PPARs stimulate gene expression of proteins central to substrate metabolism. Because plasma FFA and intramuscular long chain fatty acyl CoA increase dramatically during prolonged exercise (Watt *et al.* 2003), we hypothesized that decreasing plasma FFA availability would suppress the exercise-induced activation of metabolic genes. The main finding from the present study was that, despite complete suppression of plasma FFA with NA, PGC1 $\alpha$ , PPAR $\alpha$  and PPAR $\delta$  mRNA content were increased at rest; however, these effects were not associated with increased mRNA of proteins central to lipid metabolism. The increased PGC1 $\alpha$  mRNA coincided with an increase in plasma epinephrine and cell culture studies revealed that epinephrine elevated PGC1 $\alpha$  mRNA. Thus, the measured molecular effects of NA were analogous to those induced with exercise and may be related to increased plasma epinephrine.

PPAR $\alpha$  and  $\delta$  increase gene transcription of numerous proteins involved in fatty acid metabolism including CPT1, FAT/CD36, PDK4 and UCP3 (Gulick *et al.* 1994, Wu *et al.* 1999, Huang *et al.* 2002, Muoio *et al.* 2002, Dressel *et al.* 2003).

PPAR $\alpha$  agonists prevent diabetes in OLETF rats (Koh *et al.* 2003) whereas PPAR $\delta$  overexpression increases fatty acid  $\beta$ -oxidation in adipocytes and causes resistance to high fat diet-induced obesity (Wang *et al.* 2003). In contrast to a previous human study (Tunstall *et al.* 2002), we report increased PPAR $\alpha$  and, for the first time, increased PPAR $\delta$  mRNA immediately following an acute exercise bout. This is consistent with endurance training studies which have demonstrated increased PPAR $\alpha$  mRNA (Russell *et al.* 2003) and protein content (Horowitz *et al.* 2000, Russell *et al.* 2003). The difference between the present study and that of Tunstall *et al.* (2002) is likely to be due to the duration (60 vs 180 min) of exercise. Thus, PPAR $\alpha$  and PPAR $\delta$  mRNA are rapidly upregulated following a single prolonged exercise bout and may contribute, at least partially, to the tighter metabolic control and improved fatty acid handling observed after short-term endurance exercise training (Phillips *et al.* 1996, Spina *et al.* 1996). Reducing plasma FFA availability via NA ingestion also resulted in a rapid and pronounced increase in PPAR $\alpha$  and  $\delta$ . The transcriptional response may be a rapid adaptation designed to increase the potential for increased fat oxidative proteins in the event of reduced fatty acid availability. In this regard, rapid transcriptional responses also occur in response to reduced muscle glycogen availability (Pilegaard *et al.* 2002), suggesting that the availability of metabolic substrates is important for the transcriptional regulation of several transcription factors and metabolic genes.

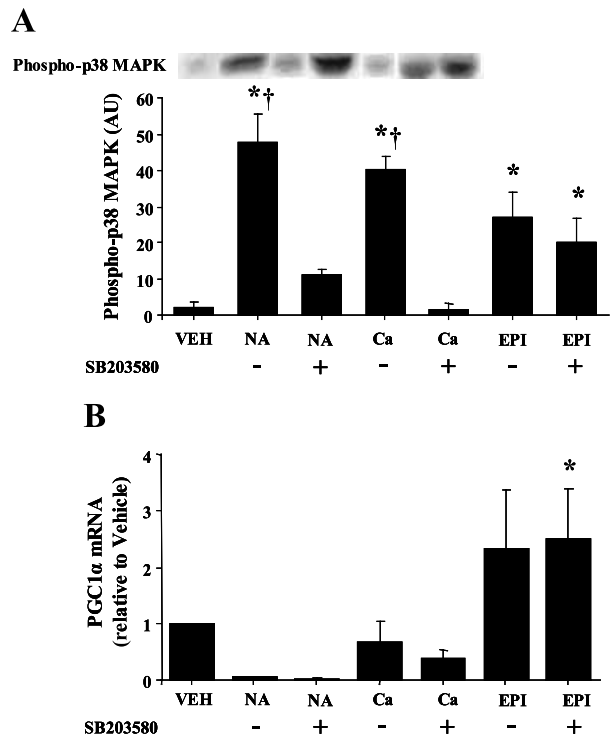
Despite the rapid changes in PPAR $\alpha$ / $\delta$ , the mRNA responses of the measured downstream target genes were limited. Consistent with previous





**Figure 4** (A) Phospho-p38 MAPK, (B) phospho-ERK1/2 and (C) phospho-JNK protein expression measured before and after 3 h of moderate exercise with (NA) or without (CON) prior NA administration. Values represent the means  $\pm$  S.E.M. ( $n=7$ ). \* $P < 0.05$ , different from corresponding value for CON; † $P < 0.05$ , different from 0 min of the same trial.

studies (Pilegaard *et al.* 2000, 2002), we report exercise-induced increases in UCP3 and PDK4 mRNA, whereas other proteins involved with fatty acid uptake (FAT/CD36), intramyocellular



**Figure 5** Epinephrine increases PGC1 $\alpha$  mRNA content. Primary human myotubes were incubated for 6 h with PBS (VEH), NA, a calcium ionophore (Ca) or epinephrine (EPI) in the presence or absence of the specific p38 MAPK inhibitor, SB203580. (A) Cells were lysed and assessed for p38 MAPK phosphorylation by Western blot. The immunoblots (80  $\mu$ g protein) represent the order of treatments as listed on the Figure below. (B) In another experiment, RNA was extracted after treatment and PGC1 $\alpha$  mRNA was assessed by RT-PCR. Values represent the means  $\pm$  S.E.M. ( $n=6$ ). \* $P < 0.05$ , different from VEH, † $P < 0.05$ , different from +SB203580 within the same treatment.

triacylglycerol hydrolysis (HSL) and mitochondrial fatty acid transport (CPT1) were not altered by exercise, and were generally unaffected by reduced fatty acid availability. These data suggested that changes in the mRNA content of some, but not all, transcription factors and coactivators precede changes in the gene expression of proteins involved in lipid metabolism. Although we observed modest or no increases in the mRNA content of numerous lipid-regulatory genes, we cannot discount the possibility that mRNA changes would occur later in recovery from exercise.

There is currently considerable interest in the role of PGC1 $\alpha$  in numerous cellular functions,

including mitochondrial biogenesis and lipid metabolism, and evidence is emerging linking muscle insulin resistance with mitochondrial dysfunction (Petersen *et al.* 2003). Both acute (Baar *et al.* 2002, Terada *et al.* 2002, Pilegaard *et al.* 2003, Norrbom *et al.* 2004) and chronic (Goto *et al.* 2000, Russell *et al.* 2003, Short *et al.* 2003, Terada & Tabata 2004) exercise increase PGC1 $\alpha$  mRNA. In the present study we observed an  $\sim$ 12-fold increase in PGC1 $\alpha$  mRNA immediately following 3 h of cycle exercise in CON and have extended previous studies by demonstrating an  $\sim$ 70% increase ( $P=0.09$ ,  $n=5$ ) of PGC1 $\alpha$  protein content immediately after acute exercise. On balance, these findings suggest that acute exercise increases PGC1 $\alpha$  mRNA and protein content, which is presumably a rapid response to metabolic stress designed to co-ordinate changes in skeletal muscle gene expression.

The regulation of PGC1 $\alpha$  is complex and is activated by factors that increase cAMP, and is repressed by estrogen-related receptor (ERR)- $\alpha$  (Ichida *et al.* 2002), which dissociates from PGC1 $\alpha$  when p38 MAPK is activated (Knutti *et al.* 2001). PGC1 $\alpha$  is likely to be regulated by upstream kinases because its protein sequence includes putative phosphorylation targets for PKA, PKC and mitogen-activated protein kinases. NA was unlikely to exert direct effects because the receptor for NA (HM74) is not expressed in skeletal muscle (Tunaru *et al.* 2003). Instead, the effects are likely to be mediated by secondary changes in the internal metabolic and external hormonal milieu. It is unlikely that the decreased plasma FFA *per se* affected gene expression, because similar transcriptional responses were observed after exercise when plasma FFA was elevated from rest (0.3–1.4 mM). Plasma epinephrine increased 3-fold with NA at rest (Table 2) and during exercise, and direct examination in skeletal muscle myotubes demonstrated increased PGC1 $\alpha$  after epinephrine treatment. Thus, the increased PGC1 $\alpha$  observed *in vivo* is consistent with the stimulatory cAMP/PKA effects.

Another possible mediator was p38 MAPK, which was phosphorylated to a greater extent in NA compared with CON both prior to and following exercise (Fig. 4A). Activation of p38 MAPK increases PGC1 $\alpha$  transcription (Knutti *et al.* 2001) and removes the inhibitory action of p160 myb resulting in increased stability and half-life of

PGC1 $\alpha$  (Puigserver *et al.* 2001, Fan *et al.* 2004). Despite the close temporal association between p38 MAPK phosphorylation and PGC1 $\alpha$  mRNA *in vivo*, induction of p38 MAPK phosphorylation *in vitro* did not increase PGC1 $\alpha$  mRNA (Fig. 5). The disparity between our *in vitro* and *in vivo* data with regard to PGC1 $\alpha$  mRNA expression with NA treatment suggests that the major function of p38 MAPKs in skeletal muscle is to enhance PGC1 $\alpha$  protein stability, rather than acting as a transcriptional regulator. This finding is consistent with previous work in C2C12 myotubes which demonstrated no increase in PGC1 $\alpha$  mRNA despite p38 MAPK activation (Fan *et al.* 2003). A stimulatory role for the other MAPKs on PGC1 $\alpha$  cannot be completely excluded, although ERK1/2 phosphorylation decreased and c-jun N-terminal kinase increased modestly and changes in either of these kinases were not related to the increased PGC1 $\alpha$ . Previous studies have suggested that 5-AMP-activated protein kinase (AMPK) and a Ca<sup>2+</sup>-mediated mechanism (possibly calcineurin) increased PGC1 $\alpha$  expression (Terada *et al.* 2004, Zong *et al.* 2002). However, AMPK activity was not elevated in human skeletal muscle with NA ingestion at rest (Watt *et al.* 2004), and it is unlikely that intracellular Ca<sup>2+</sup> increases with NA at rest. Thus, our data suggested that the increased PGC1 $\alpha$  mRNA content observed with NA is most likely due to a secondary effect of increased epinephrine.

It has been postulated that changes in the PGC1 $\alpha$  coactivation of PPAR and NRF-dependent transcription may underlie the aberrant fatty acid metabolism in disease states such as type 2 diabetes (Mootha *et al.* 2003, Patti *et al.* 2003). In this study, we have observed that the exercise-induced increase in mRNA content of the PPARs, PGC1 $\alpha$  and NRF-1 does not occur following NA ingestion, suggesting that the signalling mechanism/s underlying the NA stimulatory effect and exercise are analogous. Although the mechanism(s) of action of NA remains to be elucidated, and no differences in the gene expression of various lipid regulatory proteins were observed after 1.5 h (data not shown), the rapid and pronounced increase in PGC1 $\alpha$  and PPAR $\alpha/\delta$  may promote NA as a potential therapeutic target for improved fatty acid metabolism. Indeed, NA is administered to treat hypertriglyceridemia and lower cholesterol; however, prolonged treatment (>2 weeks) with NA leads to a modest decrease in insulin sensitivity

as measured by euglycemic-hyperinsulinemic clamp (Alvarsson & Grill 1996). Thus, the efficacy of NA as a treatment for metabolic disorders is questionable and requires further investigation.

In summary, the results from this study have demonstrated that the mRNA of proteins central to the control of lipid metabolism were generally unaffected following prolonged exercise when FFA availability was attenuated, suggesting that mechanisms other than FFA are important for their gene expression. Despite total suppression of lipolysis, NA ingestion at rest increased the mRNA content of PGC1 $\alpha$  and PPAR $\alpha/\delta$ , which was associated with elevated plasma epinephrine. The magnitude of these changes was analogous to those induced by prolonged exercise. The absence of further increases in the mRNA content of PPAR $\alpha/\delta$  and PGC1 $\alpha$  following exercise in NA suggests that the signaling mechanism(s) underlying the NA stimulatory effect are homologous to those induced by exercise.

## Acknowledgements

This study was supported by grants from the National Health and Medical Research Council (NHMRC) of Australia (M A F) and a RMIT Faculty of Life Sciences Research Grant (M J W). M J W is a Peter Doherty postdoctoral fellow and M A F is a senior research fellow of the NHMRC.

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Received in final form 18 June 2004

Accepted 12 July 2004