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Time-course Effects of Increased Fatty Acid Supply on the Expression of Genes Involved in Lipid/Glucose Metabolism in Muscle Cells

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Key Words

Skeletal muscle • Free fatty acids • PGC1 • PPAR • Uncoupling Protein(s) • Pyruvate Dehydrogenase Kinase 4

Abstract

Fatty acid (FA) oversupply in skeletal muscle is related with metabolic disorders associated to obesity, and also with normal physiological responses. We studied, *in vivo* and *in vitro*, the chronological response to physiological increases of FA, analyzing the expression of selected genes important for glucose/lipid metabolism. An *in vivo* sequential model of fasting (known to increase circulating FA) and refeeding was used in male Wistar rats to study soleus (more oxidative) and gastrocnemius (more glycolytic) muscles, and a chronological study was made in C2C12 muscle cells under treatment of oleic/linoleic FA mixture, at physiological concentration. Body weight, muscle glycogen and blood parameters (glucose, insulin, free fatty acids -FFA-, triglycerides) were monitored. mRNA levels of muscle carnitine palmitoyl transferase 1 (mCPT1), GLUT 4, insulin receptor (InsR), MyoD1, peroxisome proliferator activated receptor (PPAR) γ coactivator 1α (PGC1α) and β (PGC1β), PPARα, PPARδ, pyruvate dehydrogenase kinase 4 (PDK4) and uncoupling proteins (UCPs) 2 and 3 were analyzed by quantitative RT-PCR. The main results were the quick induction of PGC1α, UCP3 and PDK4 *in vivo* (more marked in gastrocnemius) and of PGC1α, PGC1β, InsR, PDK4, UCP2 and UCP3 *in vitro*. It is concluded that FA are able to rapidly induce the expression in muscle cells of key genes involved in their catabolism and that the oleic/linoleic acid mixture has a positive role increasing the expression of master metabolic regulators and their downstream target genes, facilitating the transition from a more glycolytic to a more lipid-oxidative metabolism.

Introduction

The intramuscular accumulation of fatty acid metabolites is a factor increasingly recognized as having great importance in the obesity related metabolic disorders such as type II diabetes [1]. Skeletal muscle is a relevant organ in the whole body regulation of energy homeostasis and the main site of fatty acid and glucose oxidation [2, 3], and this tissue has a high potential to adjust fuel selection depending on energy supply and de-

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mand [1]. Moreover, during the last years, different genes, apart from the well-known peroxisome proliferator activated receptors (PPARs), have been described to have an increasingly outstanding relevance in muscle metabolism and fuel adjustments in response to physiological conditions (as an increase of the surplus of fatty acids -FA), such as the peroxisome proliferator activated receptor gamma coactivators (PGCs) [1].

Different studies show that prolonged, and even acute, elevation of FA is associated with insulin resistance and impaired function both *in vivo* and *in vitro* [4-9], which prompts us to think that elevated FA is one of the main causes of insulin resistance in muscle cells. However, not only do FA have "adverse" effects in the metabolism, they are also very important for the regulation of metabolic fluxes since normal physiology is characterized by episodes of heavy influx of FA to skeletal muscle [1]. FA can affect the metabolism by interacting with receptors such as PPARs [10].

PPARs are crucial in regulating muscle metabolism, since they can redirect the metabolism when activated by FA and their derivatives [10]. PPARα, important controlling FA oxidation, is predominantly expressed in liver, but also in heart and muscle to a lesser extent, while PPARδ has an ubiquitous expression and is a powerful activator of the genes required for FA catabolism and adaptive thermogenesis [10]. PPARδ strongly associates to PGC1α and it is suggested to up-regulate FA oxidation and energy expenditure in skeletal muscle much more than the lesser expressed PPARα [10]. Moreover, the co-activator PGC1α in muscle is preferentially expressed in oxidative fibers, and increases mitochondrial biogenesis and the expression of the electron transport enzymes and uncoupling proteins (UCPs) [11] (see below). Both PGC1α and PPARδ have been outlined as factors inducing muscle fiber transition from a more glycolytic to a more oxidative metabolism and, in physiological situations, an up-regulation of PGC1α is likely to be involved in the early events leading to muscle fiber shifts [2]. PGC1β, another member of the PGC1 family, also strongly increases total mitochondrial respiration, and can drive the formation of oxidative type IIX muscle fibers [11, 12]. In the regulation of the expression of myotube specific genes, myogenic factors such as MyoD [13], which is the master regulator of terminal differentiation, shown to be involved in UCP3 and PGC1α gene regulation, are also important [14, 15].

With respect to UCPs, UCP2 (ubiquitously expressed) and UCP3 (mainly expressed in brown adipose tissue and muscle) have been proposed to be involved in roles such as the regulation of reactive oxygen species (ROS) production, the regulation of lipids as fuel substrate and the protection of mitochondria against lipid induced oxidative damage [16-18]. In addition, the oxidation of FA in the mitochondria first involves their transport across the mitochondrial membrane, dependent on the activity of the rate-limiting enzyme carnitine palmitoyl transferase 1 (CPT1) [19]. Other main factors regulating the fuel used by muscle are insulin receptor (InsR), the insulin regulated glucose transporter GLUT4 [10, 20], and pyruvate dehydrogenase kinase 4 (PDK4), which inactivates the pyruvate dehydrogenase complex, a crucial step for the inhibition of the whole oxidation of glucose [21].

We aimed to study in skeletal muscle cells the time-course response to physiological (in terms of concentration and time-exposure) increases of FA in the expression of the genes mentioned above (which have stood out as crucial for glucose/lipid fuel control in recent years). In order to reproduce physiological elevations of fatty acid supply to muscle cells, we made an *in vivo* and *in vitro* time-course study: a sequential model of fasting from very short to medium term food-deprivation (since free fatty acids -FFA- are highly increased during fasting) [22, 23] and refeeding in rats, and a model of differentiated C2C12 myotubes in a medium supplemented with the highest concentration of FA achieved *in vivo*. It must be pointed out that this chronological model, both *in vivo* and *in vitro*, has been barely used before (usually longer periods, from 24 hours onwards, are used) and can give as a new insight into the physiological effect of FA as metabolic regulators, also helping us decipher some early and maybe transitory effects that could remain undetected in other models. Considering the metabolic plasticity and variability in fiber composition between skeletal muscles, we used soleus and gastrocnemius as representatives in the *in vivo* model: soleus is a slow-twitch muscle with a high proportion of type I oxidative fibers, and gastrocnemius is a fast-twitch muscle with a greater proportion of type IIA (also mainly oxidative) and IIB (mainly glycolytic) fibers, in general with a more glycolytic metabolism (but with a high capacity to shift to a more oxidative metabolism) [24, 25].

Materials and Methods

Ethical approval

The animal protocol was reviewed and approved by the Bioethical Committee of the University of the Balearic Islands, following its guidelines for the use and care of laboratory animals.

Table 1. Nucleotide sequences of primers and cycling conditions used for PCR amplification. Abbreviations: mCPT1, muscle carnitine palmitoyltransferase 1a; GLUT4, glucose transporter 4; InsR, insulin receptor; MyoD1, myogenic differentiation factor 1; PDK4, pyruvate dehydrogenase kinase 4; PGC1α, peroxisome proliferator activated receptor gamma coactivator 1 alpha; PGC1β, peroxisome proliferator activated receptor gamma coactivator 1 beta; PPARα, peroxisome proliferator activated receptor alpha; PPARδ, peroxisome proliferator activated receptor delta; UCP2, uncoupling protein 2; UCP3; uncoupling protein 3; 18s, ribosomic RNA 18s (reference gene). For PGC1α, primers for rat *in vivo*[1] and murine cells *in vitro*[2] were different.

GENE	FORWARD PRIMER (5' to 3')	REVERSE PRIMER (5' to 3')	CYCLING CONDITIONS	AMPLICON SIZE (bp)
mCPT1	GCTCGCACATTACAAGGACAT	TGGACACCACATAGAGGCAG	95°C for 2s 60°C for 6s 72°C for 10s	180
GLUT4	GGCATGGGTTTCCAGTATGT	GCCCCTCAGTCATTCTCATG	95°C for 2s 60°C for 6s 72°C for 10s	233
InsR	CTCCTGGGATTCATGCTGTT	GTCCGGCGTTCATCAGAG	95°C for 2s 60°C for 6s 72°C for 8s	242
MyoD1	GCTGCCTTCTACGCACCT	CGCACTCTTCCCTGGTCT	95°C for 2s 62°C for 6s 72°C for 8s	207
PDK4	AAAGAGGCGGTCAGTAATCC	TCCTTCACACCTTCACCACA	95°C for 2s 60°C for 6s 72°C for 8s	190
PGC1α[1]	AGGAGGGTCATCGTTTGTGG	GGAGGCAGAAGAGCCGTC	95°C for 2s 60°C for 6s 72°C for 10s	256
PGC1α[2]	CATTTGATGCACTGACAGATGGA	CCGTCAGGCATGGAGGAA	95°C for 2s 60°C for 6s 72°C for 8s	69
PGC1β	ACTGGATGAAGGCGACACAC	GCTTGCTGTTGGGGAGGA	95°C for 2s 60°C for 6s 72°C for 10s	163
PPARα	TGTCGAATATGTGGGGACAA	AAACGGATTGCATTGTGTGA	95°C for 2s 60°C for 6s 72°C for 8s	215
PPARδ	GATCCTCCTGTTGACCCAGA	TCAAAGGAATGGGAGTGGTC	95°C for 2s 60°C for 6s 72°C for 10s	164
UCP2	CCTACAAGACCATTGCACGA	TGTCATGAGGTTGGCTTTCA	95°C for 2s 65°C for 6s 72°C for 11s	149
UCP3	GGAGGAGAGAGGAAATACAGAGG	CCAAAGGCAGAGACAAAGTGA	95°C for 2s 60°C for 6s 72°C for 10s	218
18S	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC	95°C for 2s 60°C for 6s 72°C for 10s	219

	Ad libitum	4 h fasting	8 h fasting	24 h fasting	8 h fasting + 3 h refeeding
Body weight before fasting (g)		366 ± 7	368 ± 2	367 ± 4	366± 3
Body weight at sacrifice (g)	366 ± 3 (a)	359 ± 7 (a,b) *	349 ± 3 (b) *	335 ± 4 (c) *	358 ± 3 #
Muscle glycogen (mg)	1.26 ± 0.12 (a)	1.10 ± 0.14 (a)	0.90 ± 0.08 (a)	0.70 ± 0.13 (b)	1.03 ± 0.12
Glucose (mg/dl)	104 ± 3 (a)	98 ± 2 (a)	88 ± 3 (b)	79 ± 3 (c)	109 ± 3 #
Insulin (µg/l)	1.30 ± 0.15 (a)	0.637 ± 0.066 (b)	0.479 ± 0.083 (b,c)	0.340 ± 0.019 (c)	1.03± 0.18 #
Triglycerides (mg/dl)	150 ± 26 (a)	119± 24 (a,b)	66.1 ± 18 (b)	21.2 ± 30.9 (b)	69.0 ± 16.6
FFA (µmol/l)	413 ± 69 (a)	670 ± 49 (b,c)	714 ± 47 (c)	546 ± 40 (a,b)	240 ± 45 #

Table 2. Body weight, muscle glycogen and blood biochemical parameters. Body weight before fasting and at sacrifice is given, as well as muscle glycogen (in gastrocnemius) and blood glucose, insulin, triglycerides and free fatty acids (FFA) in rats under different feeding conditions: *ad libitum* feeding state, after 4, 8 or 24 h fasting, and after 3 h refeeding following 8 h fasting. Data are means ± SEM (n = 6 to 8). a≠b≠c ($p<0.05$, one-way ANOVA and LSD post-hoc test). # 3 h refeeding *vs* 8 h fasting; * body weight at sacrifice *vs* body weight before fasting ($p<0.05$, repeated measures Student's t test). Mean values after 3 h refeeding, with the exception of triglyceride concentration, were not significantly different to those of animals under *ad libitum* feeding conditions ($p<0.05$, Student's t test).

In vivo study

Three-month-old male Wistar Rats (Charles River Laboratories, Barcelona, Spain) were kept one per cage at 22°C under a 12-h light/12h-dark cycle, fed with standard chow (Panlab, Barcelona, Spain). The experimental groups were (n = 6-8 animals): control group (*ad libitum* access to chow), three fasted groups (for 4, 8 and 24 h), and a 3 h refed group (*ad libitum* access to chow) after 8 h fasting. Animals were killed by decapitation during the first hour after the beginning of the light cycle (thus avoiding possible circadian effects), except those of the refed group, killed 3 h after the beginning of the light cycle. To design the fasting protocol, we considered that rats

Table 3. Gene expression upon fasting and refeeding in soleus and gastrocnemius muscles. Abbreviations are given in the text and in Table 1 legend. Data are means ± SEM (n = 6 to 8) referred to the control group, which was set to 100%. One-way ANOVA analysis: F indicates Fasting effect ($p<0.05$). LSD post-hoc test: a≠b≠c ($p<0.05$; soleus PGC1α, $p=0.061$). * Significant differences between 3 h refeeding and *ad libitum* control groups ($p<0.05$, Student's t test). # Significant differences between 3 h refeeding and 8 h fasting groups ($p<0.05$, Student's t test).

Gene	Muscle	*Ad libitum*	4 h fasting	8 h fasting	24 h fasting	ANOVA	8 h fasting + 3 h refeeding
mCPT1	Soleus	100 ± 7.4	114 ± 9	109 ± 10	104 ± 3		114 ± 8
	Gastrocnemius	100 ± 17	113 ± 19	104 ± 7	127 ± 17		123 ± 14
GLUT4	Soleus	100 ± 10	111 ± 7	87.5 ± 3.6	84.1 ± 4.7		99.0 ± 5.2
	Gastrocnemius	100 ± 17	112 ± 13	86.0 ± 5.0	100 ± 8		111 ± 16
InsR	Soleus	100 ± 12	90.4 ± 9.1	104 ± 18	119 ± 15		123 ± 17
	Gastrocnemius	100 ± 22	152 ± 27	97.3 ± 7.7	131 ± 24		131 ± 15
MyoD1	Soleus	100 ± 16	108 ± 16	110 ± 21.4	139 ± 21		104 ± 22
	Gastrocnemius	100 ± 19	107 ± 19	76.9 ± 3.9	95.3 ± 13		104 ± 14
PDK4	Soleus	100 ± 17 (a)	215 ± 44 (a)	461 ± 81 (b)	415 ± 69 (b)	F	174 ± 24 *#
	Gastrocnemius	100 ± 27 (a)	668 ± 200 (a)	1388 ± 133 (b)	1610 ± 424 (b)	F	582 ± 127 *#
PGC1α	Soleus	100 ± 6.9 (a)	133 ± 9 (b)	105 ± 17 (a,b)	68 ± 4 (c)	F	101 ± 4
	Gastrocnemius	100 ± 14 (a)	161 ± 27 (b)	81.2 ± 14.2 (a)	71.9 ± 6.7 (a)	F	105 ± 28
PGC1β	Soleus	100 ± 7	114 ± 16	116 ± 16	104 ± 5		108 ± 6
	Gastrocnemius	100 ± 19	143 ± 22	95.4 ± 12.6	123 ± 16		140 ± 26
PPARα	Soleus	100 ± 12	106 ± 10	98.3 ± 8.9	107 ± 5		127 ± 11
	Gastrocnemius	100 ± 24	143 ± 42	83.8 ± 11.4	101 ± 22		148 ± 42
PPARδ	Soleus	100 ± 11	97.0 ± 9.5	91.0 ± 10.0	72.1 ± 8.4		94.3 ± 10.2
	Gastrocnemius	100 ± 25	126 ± 22.5	86.3 ± 7.6	106 ± 19		114 ± 18
UCP2	Soleus	100 ± 14	91.6 ± 11.1	121 ± 16	109 ± 12		98.3 ± 9.6
	Gastrocnemius	100 ± 15 (a,b)	116 ± 26 (a,b)	90.3 ± 16.1 (a)	157 ± 25 (b)	F	137 ± 16
UCP3	Soleus	100 ± 23 (a)	222 ± 45 (b)	350 ± 52 (c)	348 ± 28 (c)	F	170 ± 16*#
	Gastrocnemius	100 ± 16 (a)	417 ± 97 (b)	612 ± 57 (c)	696 ± 46 (c)	F	407 ± 60 *#

eat most of their daily food intake at night [26], thus the 4 and 8 h food deprivation were during the dark cycle, and just before the beginning of the light cycle.

Gastrocnemius and soleus muscles were rapidly removed and frozen in liquid nitrogen and stored at -70°C. Blood was collected in heparinized glasses and centrifuged at 700g for 10 minutes to obtain the plasma.

Quantification of circulating parameters and muscle glycogen

Blood glucose was measured by an Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain), and plasma insulin by an ELISA kit (DRG Instruments GmbH, Marburg, Germany). Commercial enzymatic colorimetric kits were used for the determination of free fatty acids (FFA) (Wako Chemicals GmbH, Neuss, Germany) and triglyceride levels (Sigma Diagnostics, St Louis, MO, USA). Muscle glycogen was isolated and measured as previously described [22].

In vitro study

Mouse C2C12 myoblasts (ATCC-LGC Promochem, Barcelona, Spain) were maintained in DMEM supplemented with 10% w/v fetal bovine serum, antibiotics (50 IU penicillin/mL and 50 µg streptomycin/mL) and 3 mM glutamine (growth medium). At confluence, the medium was replaced by a medium containing DMEM and 2% w/v horse serum. After 10 days, the differentiated cells had fused into myotubes. The cells were serum-starved overnight (12 hours), then the medium was replaced with serum-free medium (with the same components as the growth medium except that fetal bovine serum was replaced by 2% of FFA-free bovine serum albumin - BSA). This medium contained the vehicle or the treatment: FFA mixture solution 700 µM, prepared by an equimolar mixture of oleic (C18:1) and linoleic (C18:2) acids dissolved in ethanol and diluted 1:1000 in DMEM [27] (obtained from Sigma-Aldrich, Madrid, Spain). Samples were collected at different times of incubation: 0, 1, 3, 8, 12 and 24 h and immediately stored at -70°C. The FFA concentration of 700 µM is within the maximum levels achieved in the serum of fasted rats as shown here (Table 2) and as reported by other authors [22, 28]; moreover, oleic and linoleic acids were chosen because they are usually used to induce UCP3 expression *in vitro* [29, 30] and fasting for 16 h in rats has been described to cause more increases of unsaturated fatty acids than of saturated ones, with oleic and linoleic acids particularly highly elevated [23].

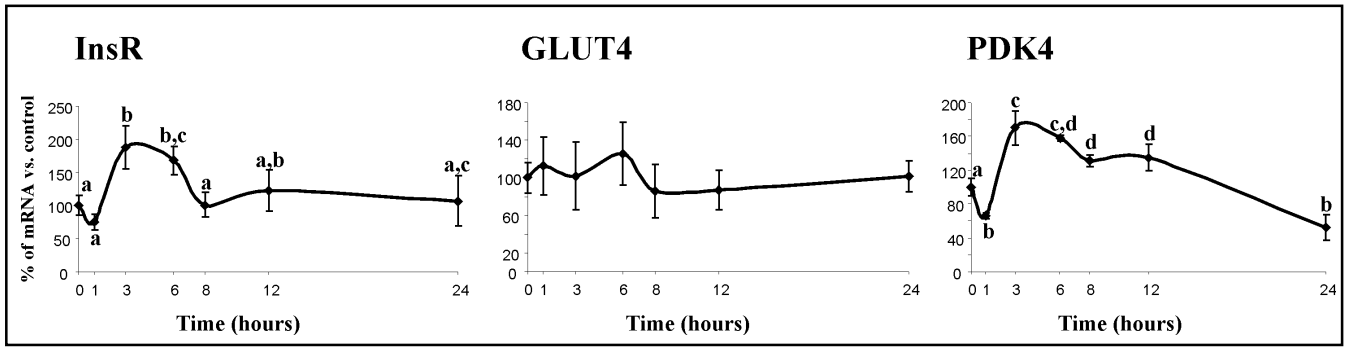

Fig. 1. Time-course mRNA expression of InsR, GLUT4 and PDK4 in response to FFA treatment in C2C12 muscle cells. FFA mixture solution was prepared by equimolar mixture of oleic acid (C18:1) and linoleic acid (C18:2), at a final concentration of 700 μM. mRNA expression is referred to the control (non-treated) group, which was set to 100%. Data represent the mean ± SEM of three separate experiments performed in duplicate (6 samples per hour of treatment). One-way ANOVA, followed by LSD post-hoc test, was performed ($p<0.05$): a≠b≠c≠d.

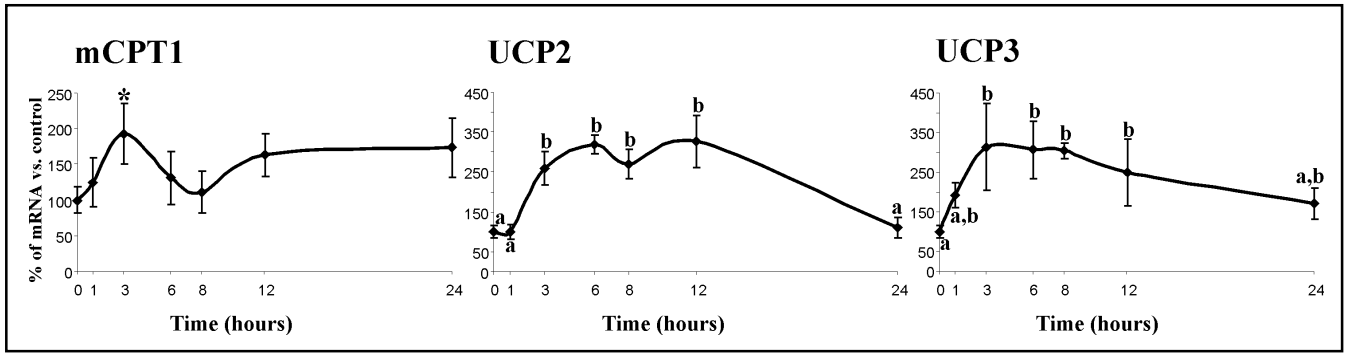

Fig. 2. Time-course mRNA expression of mCPT1, UCP2 and UCP3 in response to FFA treatment in C2C12 muscle cells. The experimental conditions are as explained in Fig. 1 and in the text. One-way ANOVA, followed by LSD post-hoc test, was performed ($p<0.05$): a≠b. * indicates differences *versus* the control (time 0) group assessed by t-test.

RNA isolation

Total RNA was extracted from soleus, gastrocnemius and C2C12 myotubes using Tripure Reagent (Roche, Barcelona, Spain). Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies Inc., Wilmington, Delaware USA) and its integrity confirmed using agarose gel electrophoresis.

mRNA expression analyses by RT-qPCR

Retrotranscription followed by real-time (quantitative) polymerase chain reaction (RT-qPCR) was used to measure mRNA expression levels of muscle CPT1 (mCPT1), GLUT4, InsR, MyoD1, PGC1α, PGC1β, PPARα, PPARδ, PDK4, UCP2 and UCP3 in gastrocnemius and soleus muscles, and in C2C12 muscle cells. Ribosomic RNA 18s was used as reference gene. Total RNA was reversed transcribed to cDNA, and real-time PCR was completed using the LightCycler System with SYBR Green I sequence nonspecific detection (Roche Diagnostic Gmbh, Mannheim, Germany), as previously described [31]. The primers and cycling conditions used are described in Table 1. All primers were obtained from Sigma (Madrid, Spain).

Statistical Analysis

All data are means ± SEM. *In vivo*, differences between groups were assessed by one-way ANOVA followed by least significance differences (LSD) post-hoc comparison. Student's t test was used to assess single differences of the re-fed group *vs.* the 8 h fasting and control groups, and repeated measures Student's t test for differences between body weight before fasting and at sacrifice. *In vitro*, mRNA expression is referred to the control (non-treated) group, set to 100%, and data represent the mean ± SEM of three separate experiments performed in duplicate. Differences between groups *in vitro* were assessed by one-way ANOVA followed by least significance differences (LSD) post-hoc comparison and, when indicated, Student's t test was used to assess single differences between groups of interest. The analyses were performed with SPSS for Windows (SPSS, Chicago, IL). Threshold of significance was defined at the $p< 0.05$ level.

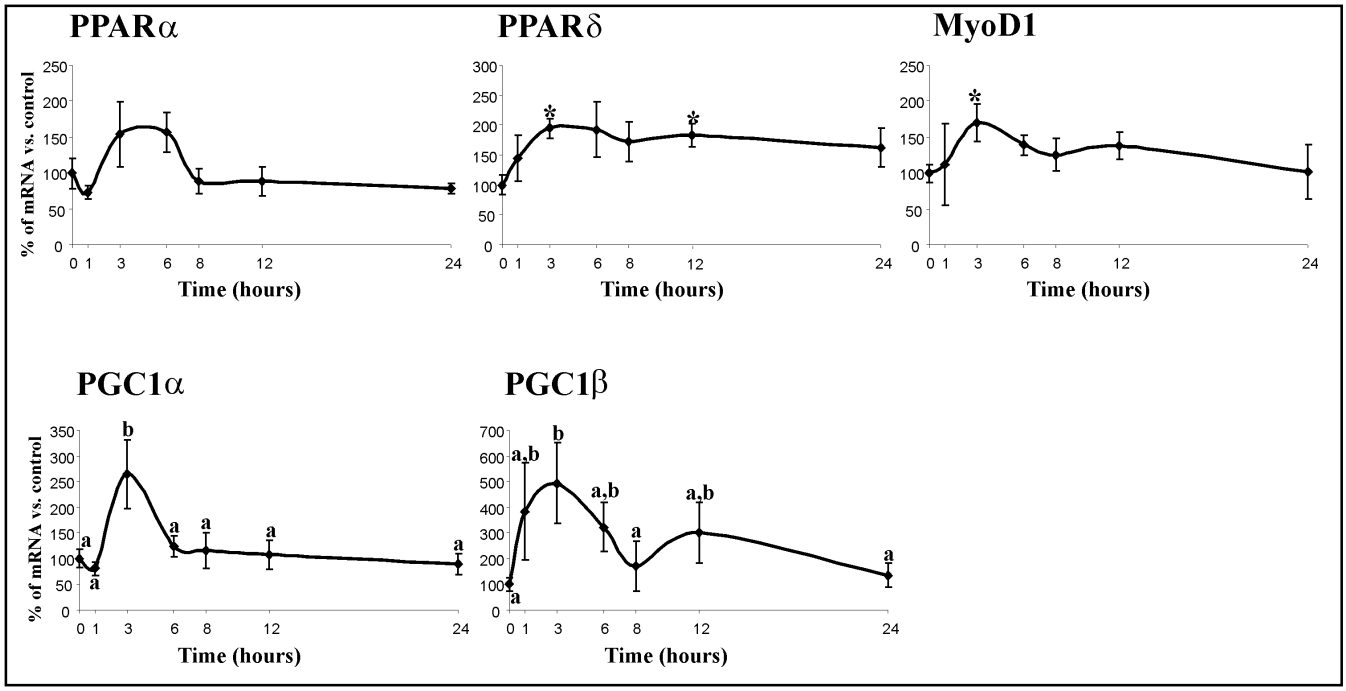

Fig. 3. Time-course mRNA expression of PPARα, PPARδ, MyoD1, PGC1α and PGC1β in response to FFA treatment in C2C12 muscle cells. The experimental conditions are as explained in Figure 1 and in the text. One-way ANOVA, followed by LSD post-hoc test, was performed ($p<0.05$): a≠b. * indicates differences *versus* the control (time 0) group assessed by t-test.

Results

Sequential changes in body weight, muscle glycogen and blood parameters upon fasting

Body weight, gastrocnemius glycogen (soleus glycogen was not measured for reasons of sample size), and circulating levels of glucose, insulin and triglycerides, followed an expected pattern of gradual decrease with the increase of fasting hours (Table 2), as reported [22, 32]. Circulating FFA followed a pattern of sequential increase, reaching maximum values before the maximum drop of the other parameters, i.e. after 8 h fasting. 3 h refeeding after 8 h fasting tended to revert all these changes, significantly increasing (as compared to 8 h fasting values) body weight, blood glucose and insulin, and decreasing circulating FFA. The mean values of these parameters in the 3 h refed group, with the exception of triglycerides (which remained at relatively low levels), were not significantly different to those of animals under *ad libitum* feeding.

Gene expression upon fasting and refeeding in soleus and gastrocnemius muscles

Three genes out of the eleven analyzed showed significant changes in their expression at the mRNA level, in both soleus and gastrocnemius: *pdk4*, *pgc1α* and *ucp3* (see Table 3).

UCP3 and PDK4 mRNA levels presented a gradual increase in both muscles under fasting conditions, showing maximum values at 8 h fasting in soleus, which remained high after 24 h fasting; in gastrocnemius, the highest value for both UCP3 and PDK4 mRNA levels was found at 24 h fasting but was not significantly different to the 8 h fasting value. It must be underlined that the magnitude of response was notably higher in gastrocnemius than in soleus. Moreover, the PDK4 mRNA expression induction was very high (especially in gastrocnemius) although the significant increase in UCP3 mRNA levels occurred earlier (already after 4 h fasting) than for PDK4 mRNA levels (seen after 8 h fasting). With 3 h refeeding after 8 h fasting, the mRNA levels of these two genes were partially restored but still remained higher than control values in both muscles (see Table 3).

PGC1α mRNA levels were significantly increased after 4 h fasting in both gastrocnemius and soleus. This elevation was transient in both muscles, showing restored values after 8 h fasting and even becoming lower than the control ones in soleus after 24 h fasting. Once again, the magnitude of response was higher in gastrocnemius (Table 3). PGC1α mRNA levels in the refed animals were

not statistically different to those of the control and 8 h fasted ones.

UCP2 mRNA levels showed only a slight tendency to respond to fasting in gastrocnemius (see Table 3), although not enough to show significant differences when comparing the highest value reached (after 24 h fasting) with the control one.

Time-course response in gene expression upon FFA treatment in C2C12 muscle cells

The expression of InsR and PDK4 at the mRNA level was increased by the FFA treatment, significantly different with respect to controls after 3 and 6 h of treatment for InsR and after 3, 6, 8 and 12 h for PDK4, which showed a significant drop 24 h after the beginning of the treatment (see Fig. 1). GLUT4 mRNA levels did not show changes under the treatment (Fig. 1).

As shown in Fig. 2, there was an induction of UCP3 and UCP2 mRNAs in C2C12 muscle cells under FFA treatment which was already statistically significant after 3 h in both cases, and was maintained until the 12 h time point but not after 24 h, not statistically different at this time with respect to time 0. For mCPT1 mRNA levels, there was a transitory tendency to increase under the FFA treatment, only significantly elevated at the 3 h time point when compared to the time 0 levels.

Fig. 3 shows that, among the transcription factors and co-activators studied, the most important changes were shown at the PGC1α and PGC1β mRNA levels, with a transitory induction in both cases, significant at the 3 h time point. On the other hand, while PPARα mRNA levels did not show significant changes with the treatment, PPARδ and MyoD1 mRNAs showed a tendency to greater levels, significantly different to the time 0 levels at the 3 and 12 h time points for PPARδ and at the 3 h time point for MyoD1.

Discussion

The *in vivo* model of fasting and refeeding used in this study shows that PGC1α, UCP3 and PDK4 are key genes quickly regulated upon fasting in rat skeletal muscle at the mRNA gene expression level, a fact highlighted by the coincidence of response when comparing two metabolically distinct muscles such as soleus and gastrocnemius. The up-regulation of UCP3 mRNA levels by fasting in both muscles, with a higher magnitude in gastrocnemius, agrees with the suggested role of UCP3 as a regulator of lipids as a fuel substrate [17, 25] (gastrocnemius has to make a more important metabolic shift from glucose to FFA use), and also with other proposed (and related) roles such as the control of ROS production [16] and the protection of mitochondria against lipid induced oxidative mitochondrial damage [18]. In short, UCP3 seems to be related with the mitochondrial handling of FA [18], which are supplied to skeletal muscle in a great quantities during fasting. Our results show that this change in UCP3 mRNA levels is important from the very beginning of fasting (already seen after 4 h) in both muscles; similar to changes described at 6 h fasting in rat gastrocnemius muscle [33]. Moreover, here we report for the first time the fasting time-course mRNA up-regulation of PDK4, comparing two metabolically distinct muscles, showing (as for UCP3) a more marked induction in gastrocnemius *versus* soleus muscle. This makes sense since it would help preserve 3C compounds derived from glycolysis for glucose synthesis in liver and keep glucose availability for dependent tissues [21], and this blocking would be more intense in gastrocnemius (which uses more glucose at the feeding state). Besides, the results show a transient elevation in PGC1α mRNA expression very soon after fasting (at the 4 h time point), once again more marked in gastrocnemius. It must be mentioned that De Lange et al. [33] also described an induction of MyoD, PPARδ and CPT1 mRNAs in rat gastrocnemius after 6 h fasting, while no significant changes were found in our study. These differences may be attributed to the age of the animals (younger in [33]) and the housing conditions (housing at 28°C in [33]). Both the transient induction of PGC1α mRNA levels and the fact that it is higher in gastrocnemius than in soleus suggest that this early induction could be of central importance in the metabolic change to a more oxidative metabolism (especially to burn lipid substrates), as we discuss below.

3 h refeeding reverted the effects of 8 h fasting on UCP3 and PDK4 induction although this was not enough to reach the control levels, especially in gastrocnemius, thus pointing out that these are chronic changes that are not as easily reverted as the acute ones and that they depend on the magnitude of response and the metabolic type of muscle.

The metabolic panorama in fasting is complex, since there are numerous changes in circulating parameters, such as a decrease in glucose levels and in the hormones leptin and insulin and an increase in adrenaline [32, 34], and also an intracellular depletion of ATP and activation of cellular fuel gauges such as AMP-activated protein kinase [2]; but one main factor is the important elevation

of circulating FFA released from the breakdown of triglycerides [34, 35], as shown here (Table 2). Thus, it is interesting to compare the *in vivo* changes observed with the chronological changes also shown *in vitro*, in C1C12 muscle cells, after treatment with FFA in a concentration similar to the one reached *in vivo* and using the type of FFA which undergo the highest increase with fasting (unsaturated oleic and linoleic acids) [23]. This comparison can be useful to see which of the effects observed can be explained, at least in part, by a direct regulation by FFA.

The FFA do not show a direct influence *per se* in the mRNA expression of the GLUT4 receptor, but the treatment is able to induce InsR expression, differently to the response observed *in vivo*. This could be interpreted by the treatment with FFA not reducing, or even slightly increasing, insulin sensitivity. Other authors have related the acute treatment with FFA with the impairment of insulin sensitivity in muscle cells [5, 6] and, particularly, Dey et al. [5] have shown that FFA treatment to rat muscle cells in primary culture reduces InsR mRNA with similar times of exposure and concentration as the ones used here, thus suggesting the involvement of elevated FFA in the induction of insulin resistance. The important difference with respect to our work is that they used palmitate, which is a saturated FA, different to the unsaturated (oleic and linoleoic) ones used here. This points out the possibility that the type of FFA used to treat the cells can be very important and that different types of FA, saturated or unsaturated (or even the identity of the specific FA used), could have opposite effects in the induction or preservation of insulin sensitivity of muscle cells and their metabolism. In fact, it has been recently reported in C2C12 cells that saturated FFA, but not unsaturated FFA, impair mitochondrial function accompanied by decreased insulin response [36]. With respect to PDK4, the *in vitro* induction of its mRNA by FFA suggests that they are probably responsible in part of the induction observed *in vivo*, but it is evident that other fasting related signals or changes (such as less glucose uptake, the fall of insulin and leptin, etc.) would influence the greater induction observed in the *in vivo* model than in the C2C12 cells. With the induction of PDK4 (also observed by Houten et al. [37] with oleate treatment in hepatoma cells and cardiomyocytes *in vitro*) the FA can play a direct role affecting the selection of fuel in order to spare glucose.

Regarding the genes mainly involved in lipid handling, the *in vitro* results show that FFA treatment to the muscle cells mimics the UCP3 induction shown *in vivo* upon fasting, and also stimulates the expression of UCP2 in a similar way, and there is a tendency to induce mCPT1 mRNA levels (facts not shown *in vivo*). The induction curves of UCP3 and UCP2 mRNAs under FFA treatment are similar to what happens *in vivo* with UCP3 in soleus and gastrocnemius (although the induction is not as high as in the gastrocnenius for UCP3), but with a tendency to decrease mRNA levels at 24 h treatment and to return to control levels; it most be pointed out that *in vitro* a single high dose of FFA was delivered to the cells at time 0, while *in vivo* (fasting) the initial concentration of FFA is not so high and there is a continuous increase until 8 h fasting. These results support the role of FFA as physiological stimulators of UCP3 mRNA expression, as previously suggested by both *in vivo* and *in vitro* studies [25, 29, 30, 38], and also of UCP2 (also shown in L6 myotubes with linoleic acid [39]), and, although slightly, of mCPT1 expression. The results are in accordance with the known role of CPT1 in the transport of FA across the mitochondrial membrane for their oxidation and with the suggested roles of the UCPs in the handling of lipids and the control of ROS production [16, 18], as commented above.

Although FFA and their derivatives are probably mainly exerting their effects as ligands of the PPARs, they do not seem to have an important effect on their mRNA expression in the short term, as evidenced by the *in vitro* and *in vivo* results, where only a slight tendency to greater expression under FFA treatment was shown for PPARδ in the C2C12 myotubes. In addition, FFA treatment only resulted in a transient increase of MyoD1 mRNA expression, also in the C2C12 cells. However, it is outstanding that the co-activators PGC1α and PGC1β are importantly induced by FFA during the first hours of treatment, similar to what is observed for PGC1α *in vivo*. These effects, which could have gone undetected if only longer fasting or incubation periods had been used, suggest that FFA can regulate the expression of both PGCs, thus having an important influence in their own metabolism and the induction of the metabolic status from a more glycolytic to a more lipid-oxidative metabolism. Of special interest is PGC1α, induced in both the *in vivo* and *in vitro* experiments, which is considered a master coordinator of oxidative metabolism and, particularly in skeletal muscle, it has been related with the stimulation of mitochondrial biogenesis and the regulation of the genes involved in oxidative phosphorylation and FA catabolism [1], and has been suggested to be an important factor inducing muscle fiber transition from more a glycolytic to more a oxidative metabolism [2]. PGC1α has been con-

sidered fundamental in integrating multiple aspects of the fasting response in other cell types such as hepatocytes [11, 40] but, as shown here, this role may be extended to muscle cells and PGC1α may be a central factor regulating glucose/fat metabolism responding to physiological changes, such as an increase in FFA, in muscle. Due to its described actions [11, 40], PGC1α would act upstream of other events initiating, together with the interaction with PPARs and other transcription factors, the metabolic/structural shift to a more oxidative, FA metabolizing muscle cell by increasing the expression of specific genes such as some of the induced here, like UCP3 and PDK4 [2, 11, 41]. Thus, in physiological conditions of non-chronic but acute exposition to FFA increase, an important effect of FFA can be to help orchestrate their metabolism by the induction of PGC1α expression very soon after their rise. With respect to PGC1β, its functions are less known than PGC1α functions, and our results suggest some differences in their regulation, due to the distinct response observed i*n vivo* when comparing the two PGCs, but also some coincidences, due to the similar induction by FFA *in vitro*, suggesting that PGC1β may also be involved in the metabolic response to increased FFA. It must be mentioned that other authors have described a contrary effect of acute exposure (6 h) to FFA in muscle cells inhibiting PGC1α and β mRNA expression in humans [7] suggesting that this decrease may be a result of lipid-induced insulin resistance. Nevertheless, the type of lipid (20% triacylglycerol emulsion in [7] to increase plasma FFA) and the dose used can be important factors in the response observed suggesting that the specific type of lipids used and their concentration are important, as discussed above.

In conclusion, FFA are important regulators of muscle metabolism able to rapidly induce the expression in muscle cells of key genes involved in their catabolism (and the metabolism of glucose). The type of response may be dependent on the type of FFA, and the unsaturated oleic and linoleic acids seem to have a positive role increasing the expression of master metabolic regulators (such as PGCs, especially PGC1α) and some of their downstream target genes (such as UCPs and PDK4), facilitating the switch in muscle cells from a more glycolytic to a more lipid-oxidative metabolism.

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