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SHORT COMMUNICATION

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Fatty acid-induced differential regulation of the genes encoding peroxisome proliferator-activated receptor-γ coactivator-1α and -1β in human skeletal muscle cells that have been differentiated in vitro

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Abstract *Aims/hypothesis:* The transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) enhances metabolically relevant pathways, such as gluconeogenesis, fatty acid oxidation, thermogenesis, oxidative phosphorylation and mitochondrial biogenesis. Since regulation of the expression of the gene encoding PGC-1α (*PPARGC1A*) by nutrients/metabolites has not been assessed in detail, the aim of this study was to determine whether *PPARGC1A* (and *PPARGC1B*) expression is modulated by common plasma fatty acids in human skeletal muscle cells. *Methods:* Human myotubes that had been differentiated in vitro were treated with 0.5 mmol/l myristate (C14:0), palmitate (C16:0), stearate (C18:0), palmitoleate (C16:1ω7), oleate (C18:1ω9) or linoleate (C18:2ω6). *PPARGC1A/B* mRNA was quantified by RT-PCR. Mitochondrial activity was determined by formazan formation. *Results:* Untreated cells expressed 28-fold more *PPARGC1B* mRNA than *PPARGC1A* mRNA (13.33±2.86 vs 0.47± 0.08 fg/μg total RNA, n=5). *PPARGC1A* expression was increased two- to three-fold by all unsaturated fatty acids (UFAs) tested (p<0.05 each, n=5). In contrast, saturated fatty acids (SFAs) did not modulate *PPARGC1A* expression. Furthermore, the effect of linoleate was not blunted by palmitate. *PPARGC1B* mRNA expression was not increased by either the UFAs or the SFAs. SFAs reduced *PPARGC1B* expression (p<0.05 for palmitate and stearate, n=5). Notably, linoleate reversed palmitate's repressive effect on *PPARGC1B*. Myotube mitochondrial activity was increased by all UFAs (p<0.01 each, n=5), but was impaired by the SFA stearate (p<0.001, n=5). *Conclusions/interpretation:* We report here that fatty acids differentially regulated expression of the genes encoding the PGC-1 isoforms. Since these effects were accompanied by significant changes in mitochondrial activity, we suggest that the fatty acid-induced regulation of expression of these genes plays an important role in muscle oxidative metabolism.

Keywords Fatty acids · Mitochondrial activity · PGC-1α · PGC-1β · Skeletal muscle cells

Abbreviations IBMX: 3-isobutyl-1-methylxanthine · MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide · NRF-1: nuclear respiratory factor-1 · PGC-1: PPAR-γ coactivator-1 · PPAR: peroxisome proliferator-activated receptor · SFA: saturated fatty acid · UFA: unsaturated fatty acid

Introduction

Peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator-1α (PGC-1α) is a metabolically important transcriptional coactivator protein that is expressed in adipose tissue, heart, brain, kidney, skeletal muscle, liver and pancreas. In liver, PGC-1α production is induced by glucagon and catecholamines. There, it interacts with hepatocyte nuclear factor-4α, PPAR-α, forkhead box transcription factor O1 and the glucocorticoid receptor to synergistically enhance the expression of genes encoding key gluconeogenic enzymes and to promote glucose production. In brown adipose tissue and skeletal muscle, PGC-1α production is induced by cold and catecholamines. In these tissues, it increases adaptive thermogenesis by coactivation of PPARs and induction of uncoupling proteins.

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Exercise is a potent stimulus for the expression of the gene encoding PGC-1α (*PPARGC1A*) in skeletal muscle, where PGC-1α stimulates oxidative phosphorylation, mitochondrial biogenesis and formation of oxidative type I myofibres. The latter effects are mediated by interactions with nuclear respiratory factor-1 (NRF-1) and, possibly, PPAR-δ. Furthermore, PGC-1α promotes muscular fatty acid oxidation by PPAR-α coactivation and enhanced transcription of genes encoding fatty acid-oxidising enzymes (for review, see [1]). Importantly, obese, insulin-resistant and type 2 diabetic states are associated with decreased mitochondrial oxidative capacity, attenuated expression of respiratory chain components, a lower proportion of type I fibres and diminished *PPARGC1A* expression in skeletal muscle [2, 3].

PGC-1β, the structural homologue closest to PGC-1α, is encoded by a separate gene and displays a similar tissue distribution. The role played by PGC-1β is not well understood, but a significant functional overlap with PGC-1α is suggested by the fact that both proteins are, at least in part, able to coactivate the same transcription factors and to induce the expression of a similar set of target genes. However, novel findings in twins also point to functional differences, with PGC-1β being more important in mitochondrial β-oxidation [4].

Data on the regulation of these two transcriptional coactivators by nutrients and metabolites are scarce. Thus, the aim of this study was to determine whether common plasma NEFAs, which are either derived from the diet or are lipolytically released from triglyceride stores, modulate the expression of *PPARGC1A* and *PPARGC1B* in human skeletal muscle cells in vitro.

Subjects, materials and methods

Cell culture Primary human skeletal muscle cells were obtained from needle biopsies of the vastus lateralis muscle, and grown and differentiated, as previously described [5]. On day 5 of differentiation, cells were treated with either fatty acid-free BSA (Sigma-Aldrich, Taufkirchen, Germany), as a control, or with 0.5 mmol/l NEFA (Sigma-Aldrich) bound to BSA. Stock solutions (8 mmol/l) of myristate (C14:0), palmitate (C16:0), palmitoleate (C16:1ω7), oleate (C18:1ω9) and linoleate (C18:2ω6), and a 4-mmol/l stock solution of stearate (C18:0) were prepared in Krebs–Ringer–HEPES buffer containing 20% BSA by overnight agitation at 37°C under nitrogen. The muscle cell donors were recruited from the Tuebingen Family Study for type 2 diabetes and gave informed written consent to the study. The study was approved by the local ethical committee.

Real-time RT-PCR Cells were treated for 20 h, and then washed and harvested by trypsinisation. RNA was isolated with RNeasy columns (Qiagen, Hilden, Germany). Total RNA treated with RNase-free DNase I was transcribed into cDNA using AMV reverse transcriptase and the First Strand cDNA Synthesis Kit from Roche Diagnostics (Mannheim, Germany). Quantitative PCR was performed in triplicate with SYBR Green on a LightCycler (Roche Diagnostics) using the following primers (Invitrogen, Karlsruhe, Germany): *PPARGC1A*: forward 5′-TGTGCAACTCTCTGGAACTG-3′, reverse 5′-TGAGGACTTGCTGAGTGGTG-3′; *PPARGC1B*: forward 5′-GCTCTCCTCCTTCTTCCTCA-3′, reverse 5′-ATAGAGCGTCTCCACCATCC-3′; 28S rRNA: forward 5′-ACGGCGGGAGTAACTATGACT-3′, reverse 5′-CTTGGCTGTGGTTTCGCT-3′. The PCR conditions were as follows: *PPARGC1A* mRNA: annealing temperature 65°C, 45 cycles; *PPARGC1B* mRNA: annealing temperature 68°C, 45 cycles; 28S rRNA annealing temperature 63°C, 50 cycles ($MgCl_2$ in all reactions 4 mmol/l).

Determination of mitochondrial activity After treatment, cells cultured in duplicate were incubated for 4 h with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) prior to overnight lysis by addition of two volumes of 10% SDS in 0.01 mol/l HCl. The Cell Proliferation Kit I (MTT) from Roche Diagnostics was used. The lysates were transferred into tubes, shaken, and then formazan dye produced by mitochondrial dehydrogenases was photometrically measured at 565 nm.

Results

Untreated in vitro differentiated human myotubes expressed low, but consistently detectable, levels of *PPARGC1A* mRNA (0.47±0.08 fg/μg total RNA, n=5). Levels of *PPARGC1B* mRNA were 28-fold higher in the same cells (13.33±2.86 fg/μg total RNA, n=5).

Treatment with BSA (control), a single NEFA (0.5 mmol/l) or a combination of palmitate and linoleate for 20 h did not alter cellular 28S rRNA content or cell viability (n=5, data not shown); thus, 28S rRNA was used for normalisation. Levels of *PPARGC1A* mRNA were two- to three-fold higher in cells treated with the unsaturated NEFAs (UFAs) palmitoleate (p=0.0282, n=5), oleate (p=0.0412, n=5) or linoleate (p=0.0432, n=5) than in control cells (Fig. 1a). However, treatment with the saturated NEFAs (SFAs) myristate, palmitate or stearate did not modulate *PPARGC1A* mRNA levels (Fig. 1a, n=5). Coincubation of myotubes with palmitate and linoleate revealed that palmitate was not able to block the stimulatory effect of linoleate on *PPARGC1A* expression (Fig. 1a).

In contrast, cellular *PPARGC1B* mRNA content was not elevated by UFA or SFA treatment (Fig. 1b, n=5). However, compared with that in control cells, expression of *PPARGC1B* mRNA was downregulated by the SFAs palmitate (p=0.0356, n=5) and stearate (p=0.0235, n=5) by 35 and 55%, respectively (Fig. 1b). Coincubation of myotubes with palmitate and linoleate resulted in a complete reversal of the inhibitory effect of palmitate on PGC-1β (Fig. 1b; p=0.0340 for palmitate vs. palmitate+linoleate, n=5).

Because modulation of *PPARGC1A* and *PPARGC1B* expression is expected to influence mitochondrial activity,

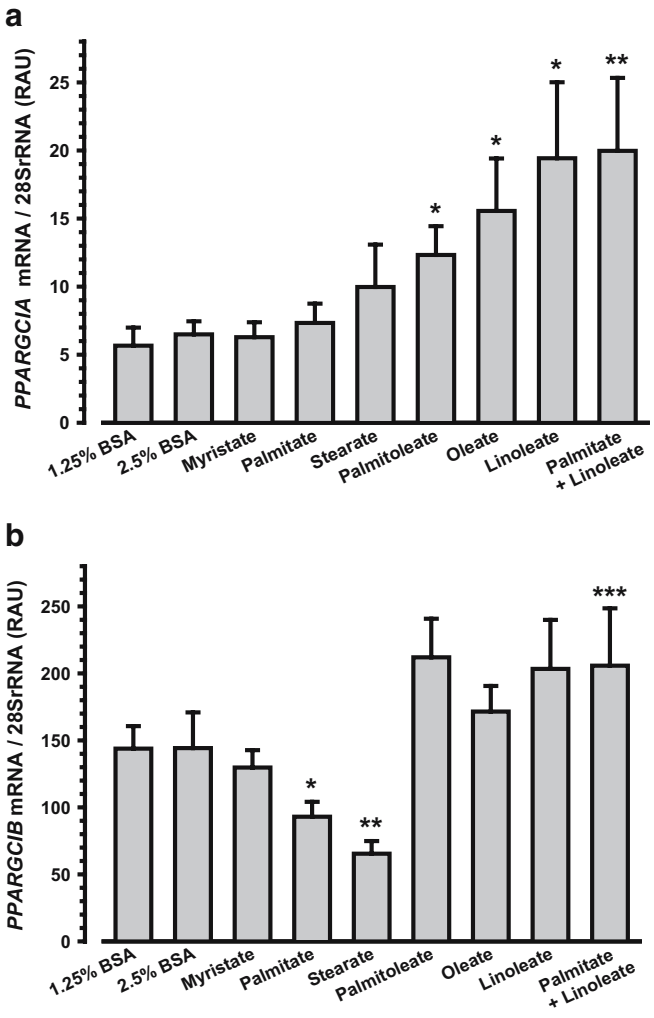

Fig. 1 Regulation of myotube *PPARGC1A* (**a**) and *PPARGC1B* (**b**) mRNA expression by NEFAs. Cells were treated for 20 h with 1.25% BSA (control for myristate, palmitate, palmitoleate, oleate and linoleate), 2.5% BSA (control for stearate and palmitate+linoleate) or 0.5 mmol/l of each NEFA. Levels of *PPARGC1A* and *PPARGC1B* mRNA and 28S rRNA were quantified by real-time RT-PCR. The data are derived from myotube cultures from five donors (n=5) and are presented as means±SE. $^{*}p<0.05$ for difference from 1.25% BSA; $^{**}p<0.05$ for difference from 2.5% BSA; $^{***}p<0.05$ for difference from palmitate (unpaired Student's t-test). RAU, relative arbitrary units

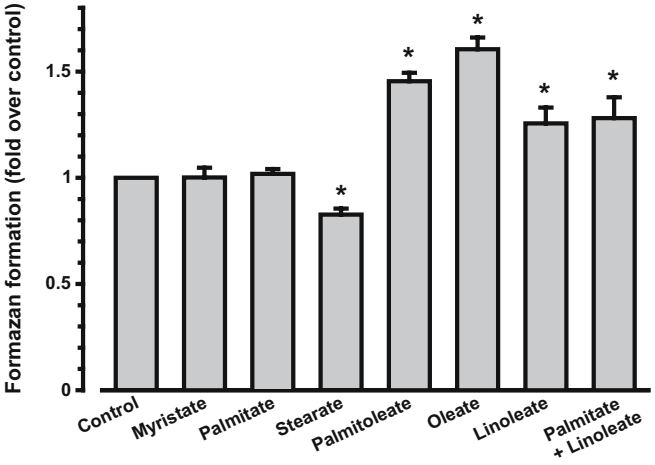

Fig. 2 Mitochondrial activity of myotubes treated with NEFA. Cells were treated for 20 h with BSA (control) or 0.5 mmol/l of each NEFA. Mitochondrial activity was photometrically determined by measuring the formation of formazan from the MTT substrate. The data are derived from myotube cultures from five donors (n=5) and are presented as means±SE. $^{*}p<0.01$ for difference from control (unpaired Student's t-test)

we performed an MTT assay to determine mitochondrial dehydrogenase activity. Incubation with stearate for 20 h significantly reduced formazan formation (Fig. 2; p=0.0003 vs control, n=5), whereas the SFAs myristate and palmitate had no effect. Mitochondrial activity increased in response to treatment with the UFAs palmitoleate ($p<0.0001$, n=5), oleate ($p<0.0001$, n=5) and linoleate (p=0.0090, n=5) relative to treatment with BSA by 45%, 60% and 25%, respectively (Fig. 2). Application of a combination of palmitate and linoleate revealed that palmitate does not block linoleate-enhanced formazan formation (Fig. 2).

Discussion

Recent studies in humans have demonstrated that lipid infusion downregulates muscle *PPARGC1A* expression [6], whereas nicotinic acid intervention suppresses plasma NEFA levels and concomitantly increases muscle *PPARG C1A* mRNA [7]. However, it was not established whether altered *PPARGC1A* expression is directly caused by the changes in plasma NEFA concentrations. We show here that treatment of human myotubes with single NEFA species at high, but physiological, concentrations produced a unique regulation pattern of expression of the genes encoding the PGC-1 isoforms: UFAs increase *PPARGC1A* expression but have no regulatory impact on *PPARGC1B* expression; on the other hand, SFAs downregulate *PPAR GC1B* expression but do not modulate *PPARGC1A* expression. How these NEFA effects on muscle *PPARG C1A/B* expression are mediated is still unknown, but it is conceivable that NEFA-activated transcription factors, such as PPARs, play a role.

Moreover, we treated myotubes with a combination of linoleate and palmitate. The stimulatory effect of the UFA linoleate on *PPARGC1A* expression was not antagonised by the SFA palmitate. This observation is consistent with data obtained in isolated rat islets, where an UFA/SFA (oleate/palmitate) mixture also increased *PPARG C1A* mRNA expression [8]. The reasons why these data do not reflect the results of the intervention studies [6, 7] are unclear, but could be explained by secondary effects of the in vivo manipulations. Notably, palmitate's inhibitory impact on myotube *PPARGC1B* expression was completely reversed by linoleate. Thus, our findings suggest that UFAs not only exert direct beneficial effects on cellular glucose/lipid me-

tabolism via PGC-1α but also protect against the detrimental metabolic effects of SFA at the level of PGC-1β.

A different regulation pattern of expression of the genes encoding the PGC-1 isoform has been reported in primary murine hepatocytes [9]: neither isoform was downregulated by any of the NEFAs tested; *Ppargcla* was induced by stearate only, whereas *Ppargclb* was induced by UFAs and by SFAs. These divergent results might reflect tissue-specific regulation of the genes encoding the two isoforms, as has already been shown in vivo: in type 2 diabetic states, *PPARGC1A* expression is decreased in skeletal muscle [2, 3] and increased in liver [10].

We also assessed whether the changes in myotube *PPARGC1A*/*B* expression were accompanied by alterations in mitochondrial activity, indicating biological relevance. The UFAs stimulated the activity of the mitochondrial dehydrogenases, whereas the SFA stearate decreased their activity. These observations may be explained by enhanced and attenuated transactivation of NRF-1, respectively. In contrast to stearate, palmitate was not inhibitory. This could be due to functional compensation of its weak repressive effect on *PPARGC1B* expression (compared with that of stearate) by its concomitant trend to stimulate *PPARGC1A* expression.

The two major pathways known to promote *PPARGC1A* expression in skeletal muscle are the cAMP-protein kinase A and the calcineurin–calcium–calmodulin-dependent protein kinase IV axes (for review, see [1]). We tested whether UFAs mediate *PPARGC1A* induction in human myotubes via one of these pathways. However, we did not detect cAMP formation after stimulation with any of the NEFAs tested. Furthermore, blockade of protein kinase A and calcineurin (using H-89 and cyclosporin A, respectively) revealed that this pathway is not involved. Thus, other UFA-mediated pathway(s) must exist that promote *PPARGC1A* expression.

In conclusion, in the present study we have demonstrated the differential regulation of *PPARGC1A* and *PPARGC1B* expression by NEFAs: UFAs stimulate *PPARGC1A* expression, whereas SFAs repress *PPARGC1B* expression. Although the absolute PGC-1 isoform mRNA contents of human myotubes do not reflect the expression levels seen in native human muscle biopsies (where *PPARGC1A* is equally [4] or more [3] abundant than *PPARGC1B*) and only mRNA data are presented, we hypothesise that NEFAs play an important role in the regulation of muscle fatty acid oxidation and oxidative phosphorylation.

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