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***PPARGC1A* mRNA levels of in vitro differentiated human skeletal muscle cells are negatively associated with the plasma oleate concentrations of the donors**

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Abbreviations ISI: insulin sensitivity index · MUFA: monounsaturated fatty acid · PGC-1: PPAR- γ coactivator-1 · PPAR: peroxisome proliferator-activated receptor · PUFA: polyunsaturated fatty acid · SNP: single-nucleotide polymorphism

To the Editor: Peroxisome proliferator-activated receptor (PPAR)- γ coactivator-1 α (PGC-1 α) is a metabolically relevant transcriptional coactivator protein. Through PPARs and nuclear respiratory factor-1, it enhances adaptive thermogenesis, oxidative phosphorylation, mitochondrial biogenesis and β -oxidation in adipose tissue and skeletal muscle [1]. Obese, insulin-resistant and type 2 diabetic states reveal low muscle mitochondrial oxidative capacity, reduced expression of respiratory chain components, and decreased expression of the gene encoding PGC-1 α (*PPARGC1A*) [2, 3]. We have recently shown that *PPARGC1A* expression in in vitro differentiated human skeletal muscle cells (myotubes) is induced by common unsaturated plasma NEFAs, such as palmitoleate, oleate and linoleate, but is not influenced by common saturated NEFAs, such as myristate, palmitate and stearate [4]. In the present study, we investigated (1) whether the basal expression of *PPARGC1A* and the gene encoding its close homologue, PGC-1 β (*PPARGC1B*) in myotubes derived from metabolically characterised white donors display marked individual variations, and, if so, (2) whether these

variations are linked to in vivo metabolic features of the donors, such as adiposity, insulin resistance, total NEFA concentration, or concentration of individual plasma NEFAs.

We measured levels of *PPARGC1A* and *PPARGC1B* mRNAs in myotubes from 25 healthy subjects and performed linear regression analysis with the subjects' anthropometric and metabolic parameters. The donors (11 women, 14 men, age 26.6 ± 4.9 years, body fat content $21.0 \pm 8.1\%$, WHR 0.81 ± 0.07 [all means $\pm$ SD]) were participants in the Tübingen family study for type 2 diabetes and gave written informed consent to be included in the study. The study was approved by the local ethics committee.

Myoblasts were grown from satellite cells obtained from percutaneous needle biopsies performed on the lateral portion of musculus quadriceps femoris and were differentiated into myotubes as described previously [4]. First-pass cells were used. RNA isolation was performed using RNeasy silica-gel 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). Levels of *PPARGC1A* and *PPARGC1B* mRNAs were quantified using SYBR Green I dye and a LightCycler (Roche Diagnostics). The following primers were used: *PPARGC1A* forward 5'-TGTGCAA CTCTCTGGAAGT-3', reverse 5'-TGAGGACTTGCT GAGTGGTG-3'; *PPARGC1B* forward 5'-GCTCTCCTCC TTCTTCCTCA-3', reverse 5'-ATAGAGCGTCTCCAC CATCC-3'. PCR conditions were as follows: *PPARGC1A*: -65°C annealing, 45 cycles, 4 mmol/l MgCl_2 ; *PPARGC1B*: -68°C annealing, 45 cycles, 4 mmol/l MgCl_2 . Percentage of body fat was measured by bioimpedance. The insulin sensitivity index (ISI) was calculated as glucose infusion rate divided by the steady-state insulin concentration during the hyperinsulinaemic-euglycaemic clamp protocol described previously [5]. Fasting blood parameters were determined after a 10-h overnight fast. Total plasma NEFA concentration was measured enzymatically (NEFAC kit; Waco Chemicals, Neuss, Germany). The fatty acid composition of the plasma NEFA fraction was determined as previously described in

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detail [6]. The data were logarithmically transformed to approximate normal distribution and were analysed by linear regression analysis and ANOVA. The statistical software package JMP, version 4.0 (SAS Institute, Cary, NC, USA) was used. Generally, a p value of less than 0.05 was considered statistically significant. With Bonferroni's correction for multiple comparison, a p value of less than 0.0028 was considered statistically significant.

There was wide variation in the myotube *PPARGC1A* mRNA content, with values ranging from 0.20 to 3.15 fg mRNA/ μ g total RNA (mean $\pm$ SD: 1.06 $\pm$ 0.73 fg mRNA/ μ g total RNA). The *PPARGC1B* mRNA levels ranged from 0.68 to 2.68 fg mRNA/ μ g total RNA (mean $\pm$ SD: 1.83 $\pm$ 0.61 fg mRNA/ μ g total RNA). Notably, the cells underwent an extended cell culture protocol (including isolation from biopsies, growth for 2 weeks, trypsinisation and subculture, freezing, storage in liquid nitrogen and thawing, growth for another week, and, finally, in vitro differentiation for 5 days). Therefore, we suggest that (1) the detected variations in basal *PPARGC1A/B* mRNA expression are determined by individual genetics, and (2) simultaneous influences of environmental/humoral factors on both *PPARGC1A/B* expression and metabolic parameters are a priori excluded.

By simple linear regression analysis, neither *PPARGC1A* nor *PPARGC1B* mRNAs were correlated with the anthropometric parameters sex, age, percentage of body fat, or WHR (data not shown). Before and after adjustment for sex, age, body fat and WHR, neither ISI nor the total plasma NEFA concentration were correlated with myotube

levels of *PPARGC1A* mRNA (ISI unadjusted: $r < -0.01$, $p = 1.0$; ISI adjusted: $r = 0.03$, $p = 0.9$; NEFA unadjusted: $r = -0.12$, $p = 0.6$; NEFA adjusted: $r = -0.11$, $p = 0.6$) or *PPARGC1B* mRNA (ISI unadjusted: $r = 0.26$, $p = 0.21$; ISI adjusted: $r = 0.36$, $p = 0.07$; NEFA unadjusted: $r = -0.09$, $p = 0.7$; NEFA adjusted: $r = -0.07$, $p = 0.7$). However, *PPARGC1A* mRNA expression was negatively correlated with the oleate content of the plasma NEFA fraction and positively correlated with the long-chain polyunsaturated fatty acid (PUFA) fraction (C20+C22 PUFAs of the plasma NEFA fraction), as presented in Table 1. The positive correlation with long-chain PUFAs was due to the individual contributions of dihomo- γ -linolenate, arachidonate, eicosapentaenoate and docosahexaenoate (Table 1). Even though the cellular *PPARGC1B* mRNA content was positively correlated with the *PPARGC1A* mRNA content (unadjusted: $r = 0.46$, $p = 0.0193$; adjusted for sex and age: $r = 0.50$, $p = 0.0114$), no correlation between *PPARGC1B* mRNA levels and any fatty acid of the plasma NEFA fraction was detected that retained significance after Bonferroni's correction for multiple testing (data not shown).

In the present study we show that individual variations in levels of human myotube *PPARGC1A* and *PPARGC1B* mRNAs, which are most probably determined by genetics, do not predict the adiposity, insulin sensitivity or total plasma NEFA concentration of the donors. However, the myotube content of *PPARGC1A* mRNA, but not *PPARGC1B* mRNA, predicts a characteristic donor NEFA pattern. Firstly, *PPARGC1A* mRNA is negatively associated with oleate, the preferred storage fatty

Table 1 Association of myotube *PPARGC1A* mRNA content with fatty acids of the plasma NEFA fraction of samples from healthy subjects ($N=25$) before and after adjustment for sex, age, body fat and WHR

Fatty acid	Simple correlations		Correlations after adjustment	
	r value	p value	r value	p value
Myristate (C14:0)	0.25	0.23	0.25	0.22
Pentadecanoate (C15:0)	0.13	0.5	0.12	0.6
Palmitate (C16:0)	0.33	0.11	0.33	0.10
Stearate (C18:0)	-0.19	0.4	-0.34	0.10
Arachidate (C20:0)	0.03	0.9	-0.05	0.8
Behenate (C22:0)	-0.09	0.7	-0.09	0.7
Palmitoleate (C16:1 ω 7)	-0.17	0.4	-0.08	0.7
Oleate (C18:1 ω 9)	-0.65 ^a	0.0004 ^a	-0.66 ^a	0.0003 ^a
Vaccenate (C18:1 ω 7)	0.07	0.7	0.12	0.6
Linoleate (C18:2 ω 6)	0.29	0.16	0.34	0.09
α -Linolenate (C18:3 ω 3)	0.25	0.23	0.27	0.20
γ -Linolenate (C18:3 ω 6)	0.38	0.06	0.39	0.06
Dihomo- γ -linolenate (C20:3 ω 6)	0.63 ^a	0.0009 ^a	0.66 ^a	0.0005 ^a
Arachidonate (C20:4 ω 6)	0.58 ^a	0.0025 ^a	0.63 ^a	0.0007 ^a
Eicosapentaenoate (C20:5 ω 3)	0.64 ^a	0.0005 ^a	0.65 ^a	0.0005 ^a
Docosatetraenoate (C22:4 ω 6)	0.24	0.24	0.32	0.12
Docosapentaenoate (C22:5 ω 3)	0.44	0.0297	0.44	0.0295
Docosahexaenoate (C22:6 ω 3)	0.53	0.0065	0.67 ^a	0.0003 ^a
Σ SFA	0.17	0.4	0.12	0.6
Σ UFA	-0.24	0.25	-0.20	0.3
Σ MUFA	-0.60 ^a	0.0014 ^a	-0.61 ^a	0.0011 ^a
Σ PUFA	0.39	0.06	0.46	0.0216
Σ Long-chain PUFA ($\geq$ C20)	0.62 ^a	0.0011 ^a	0.65 ^a	0.0004 ^a

^aAssociations that retained significance after correction for multiple comparisons ($\alpha < 0.0028$)
SFA saturated fatty acids, UFA unsaturated fatty acids

acid in the anabolic state. Since we performed our measurements in the fasting (lipolytic) state, low plasma oleate concentrations may reflect enhanced oleate oxidation, which is in agreement with the reported stimulatory effect of PGC-1 α on β -oxidation [7]. Secondly, we detected a positive correlation between myotube *PPARGC1A* mRNA and plasma long-chain PUFAs. To analyse whether this correlation is due to genetic variation within the *PPARGC1A* promoter, we genotyped the donors for the four recently described major *PPARGC1A* promoter single-nucleotide polymorphisms (SNPs) [8], namely, -1422C/T, -1679A/G, -3818T/G and -3959C/T. The -3818T/G SNP was rare (frequency of the G allele=0.02) and therefore was not further analysed. The other more common promoter SNPs (-1422C/T, frequency of the T allele=0.26; -1679A/G, frequency of the G allele=0.43; -3959C/T, frequency of the T allele=0.29) were not associated with *PPARGC1A* mRNA or with PUFA (data not shown). Thus, sequence variation in the *PPARGC1A* promoter, at least in this small population, does not seem to represent a genetic determinant of myotube *PPARGC1A* expression or plasma PUFA levels. Therefore, upstream regulators of *PPARGC1A* expression (e.g. cAMP response element-binding protein, calcineurin) represent more promising candidate genes.

Mechanistically, the relationship between genetically determined myotube *PPARGC1A* mRNA levels and plasma PUFAs can be explained by PGC-1 α -dependent stimulation of PUFA synthesis via activation of fatty acid desaturases or by PGC-1 α -mediated inhibition of PUFA degradation. However, using established product : educt ratios, we were not able to detect significant correlations between *PPARGC1A* expression and Δ 5-desaturase (arachidonate/dihomo- γ -linolenate) or Δ 6-desaturase (γ -linolenate/linoleate) activity. An effect of PGC-1 α on selective PUFA degradation remains to be investigated.

In conclusion, we provide evidence that myotube *PPARGC1A* expression may play a major role in oleate oxidation in human subjects in vivo. Furthermore, we have shown that *PPARGC1A* expression is correlated with long-chain PUFA metabolism.

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