Research Article

The Myocyte Expression of Adiponectin Receptors and PPARδ Is Highly Coordinated and Reflects Lipid Metabolism of the Human Donors

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Muscle lipid oxidation is stimulated by peroxisome proliferator-activated receptor (PPAR) δ or adiponectin receptor signalling. We studied human myocyte expression of the PPARδ and adiponectin receptor genes and their relationship to lipid parameters of the donors. The mRNA levels of the three adiponectin receptors, AdipoR1, AdipoR2, and T-cadherin, were highly interrelated ($r \geq 0.91$). However, they were not associated with GPBAR1, an unrelated membrane receptor. In addition, the adiponectin receptors were positively associated with PPARδ expression ($r \geq 0.75$). However, they were not associated with PPARα. Using stepwise multiple linear regression analysis, PPARδ was a significant determinant of T-cadherin expression ($P = .0002$). However, pharmacological PPARδ activation did not increase T-cadherin expression. The myocyte expression levels of AdipoR1 and T-cadherin were inversely associated with the donors’ fasting plasma triglycerides ($P < .03$). In conclusion, myocyte expression of PPARδ and the adiponectin receptors are highly coordinated, and this might be of relevance for human lipid metabolism in vivo.

1. Introduction

In states of increased circulating nonesterified fatty acids (NEFA), such as fasting, high dietary fat intake, or obesity, appropriate fatty acid oxidation by skeletal muscle and liver is crucial to maintain insulin sensitivity and glucose tolerance. Impairment of fatty acid oxidation, for example, due to genetics, provokes increments in plasma NEFA levels, ectopic lipid deposition in nonfat tissues, enhanced hepatic VLDL production, and finally hyperlipidaemia [1]. Insulin resistance, glucose intolerance, and type 2 diabetes may arise from enhanced fatty acid signalling in skeletal muscle and liver initiated by the elevated plasma NEFA levels per se or by the accumulation of ectopic lipids [2].

Peroxisome proliferator-activated receptor (PPAR) δ, a nuclear receptor and transcription factor, represents an important regulator of fatty acid oxidation in rodent and human skeletal muscle. PPARδ is activated by long-chain NEFA and, in concert with coactivator proteins, such as PPARγ coactivator 1, induces the expression of genes involved in cellular fatty acid uptake, β-oxidation, and energy uncoupling [3]. Alternatively, muscular fatty acid oxidation is stimulated by adiponectin, an insulin-sensitizing adipokine negatively associated with adipose tissue mass. Adiponectin signalling triggers phosphorylation and inactivation of acetyl-CoA carboxylase promoting a decline in cytosolic malonyl-CoA levels and, thereby, enhancing fatty acyl-CoA import into mitochondria and β-oxidation [4, 5]. The functions of adiponectin are mediated via three different receptors, that is, AdipoR1, AdipoR2, and T-cadherin, which are abundant in skeletal muscle. AdipoR1 and AdipoR2 were shown to bind trimeric full-length adiponectin as well as...
its proteolytically cleaved globular domain, while T-cadherin binds hexameric and high-molecular-weight oligomeric forms of adiponectin [6].

Whether the PPARδ and adiponectin receptor signalling pathways are linked at any cellular level is currently unknown. In this gene expression study, we therefore investigated whether the expression of the genes encoding PPARδ (PPARD) and the adiponectin receptors AdipoR1 (ADIPO1), AdipoR2 (ADIPO2), and T-cadherin (CDH13) is interrelated. To this end, we used in vitro differentiated human skeletal muscle cells (myotubes) from 39 young and healthy human donors. Since basal gene expression of human myotubes was previously shown to reflect in vivo human skeletal muscle cells (myotubes) from 39 young and healthy Caucasian participants of the Tübingen family study for type 2 diabetes, we used muscle biopsies and differentiated them to myotubes, as previously described [10]. Some myotube cultures were treated for 20 h either with 0.1% DMSO for carrier control or with 1 μmol/L of the PPARδ-specific agonist GW501516 (Alexis Biochemicals, Grünberg, Germany).

2.2. Myotube Donors. The 39 donors were young and healthy Caucasian participants of the Tübingen family study for type 2 diabetes and gave informed written consent to the study. All individuals were metabolically characterised by an oral glucose tolerance test, as described earlier [11]. The subject characteristics are given in Table 1. The study was in accordance with the principles of the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Medical Faculty of the University of Tübingen.

2.3. Quantitative RT-PCR (qPCR). Myotubes were washed with PBS and harvested by trypsinisation. RNA was isolated with RNase-free DNase I was transcribed into cDNA using AMV reverse transcriptase and the First Strand cDNA kit from Roche Diagnostics (Mannheim, Germany). Total RNA treated with RNase-free DNase I was transcribed into cDNA using AMV reverse transcriptase and the First Strand cDNA kit from Roche Diagnostics (Mannheim, Germany). The primers were purchased from TIB MOLBIOL (Berlin, Germany): PPARD mRNA forward 5’-ATTGAGGTA-CAGCCAGATG-3’, reverse 5’-GGTTGCTATGACCAT-GTAC-3’, ADIPO1 mRNA forward 5’-ATTGAGTCA-CTGTGTCTGAG-3’, reverse 5’-CTGGAGACTGTAGG-TATCA-3’, CDH13 mRNA forward 5’-TGCTGATAACCCTGAGGAC-3’, reverse 5’-ATGGCCGATGTGGATCTTG-GC-3’, GPBAR1 mRNA forward 5’-GCTGCTTTCTCC-TAGCCTAC-3’, reverse 5’-GGTTGGAGCAAGTAAGAC-GAG-3’, PPARD mRNA forward 5’-AAGAGGATGGCAGGAGGAC-3’, reverse 5’-TGCCACAGCTCTCTCTTCTT-CT-3’, PPARD mRNA forward 5’-CCATCGCCGAGGATAG-TTCT-3’, reverse 5’-CTCCCGTGCACCTGTGCTCA-3’; 28S-rRNA forward 5’-ACGGCGGAGTAACCTATGACT-3’, reverse 5’-CTTTGGCTGTTGTTCTGCT-3’. The annealing temperatures were ADIPO1 mRNA –66°C; ADIPO2 mRNA* –64°C; CDH13 mRNA –66°C; GPBAR1 mRNA –68°C; PPARD mRNA* –67°C; PPARA mRNA* –67°C; 28S-rRNA –63°C. All reactions contained 4 mmol/l MgCl2 (reactions marked with asterisk additionally contained 5% DMSO) and were run for 45 cycles. Cellular mRNA contents are given in fg mRNA (or rRNA)/μg total RNA. The basal expression levels are given in Table 1. Furthermore, we performed probe-based qPCR (LightCycler, Roche Diagnostics) using probes from the Universal ProbeLibrary (Roche Diagnostics) and primers from TIB MOLBIOL to determine the mRNA expression levels of ADIPO1, ADIPO2, CDH13, PPARD, UCP3, TFAM, PPARGC1A, and the housekeeping gene RPS13 (primer sequences and PCR conditions can be provided upon request).

2.4. Oral Glucose Tolerance Test (OGTT) and Hyperinsulinaemic-Euglycaemic Clamp. Both procedures were performed as previously described in detail [11].

2.5. Laboratory Measurements. Glucose was determined using a bedside glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA). Insulin was determined by a microparticle enzyme immunoassay (Abbott Laboratories, Tokyo, Japan). NEFA and glycerol were measured using enzymatic assays from WAKO Chemicals (Neuss, Germany) and Sigma (Deisenhofen, Germany), respectively. Triglycerides, total, HDL, and LDL cholesterol were determined with standard colorimetric methods using a Roche/Hitachi analyzer (Roche Diagnostics, Mannheim, Germany). Adiponectin was determined by a radioimmunoassay (Linco Research, St. Charles, MO, USA).

2.6. Selection and Genotyping of Single Nucleotide Polymorphisms (SNPs). To study the influence of genetic variation on PPARD, ADIPO1, ADIPO2, and CDH13 expression, we selected the unlinked PPARD SNPs rs2267668 A/G (intron 2) and rs1053049 T/C (3’-untranslated region) and the ADIPO1 promoter SNP rs6666089 for which we previously reported in vivo functionality [11–13]. For genotyping, DNA was isolated from whole blood using a commercial DNA isolation kit (NucleoSpin, Macherey & Nagel, Düren, Germany). Genotyping was performed with TaqMan assays (Applied Biosystems, Foster City, CA, USA). All SNPs passed the quality controls. Details on this as well as on minor allele frequencies, genotyping success rates, and Hardy-Weinberg equilibrium are reported in the aforementioned references.

2.7. Statistics. To approximate normal distribution, all data were log transformed prior to statistical analysis. Two-group comparisons were performed using unpaired Student’s t-test. To adjust the dependent variable for confounding variables, multiple linear regression models were used (standard least squares method). Stepwise multiple linear regression analysis was performed to identify the best predictor for the dependent variable. A P value <.05 was considered statistically
significant. The statistical software package JMP 4.0 (SAS Institute, Cary, NC, USA) was used.

3. Results

The basal myotube mRNA expression levels were CDH13 > ADIPOR1 > ADIPOR2 > PPARδ (Table 1). No significant differences were seen in PPARD, ADIPOR1, ADIPOR2, or CDH13 mRNA levels between myotubes from male versus female donors (Table 1).

Using bivariate regression analysis, the mRNA expression levels of all three adiponectin receptors, each normalised for the housekeeping gene 28S-rRNA, were highly interrelated (all $r > 0.91$, all $P < .0001$). Since both the dependent and the independent variable were normalised for 28S-rRNA in these initial analyses, these unusually strong correlations could, theoretically, have reflected the correlation of 28S-rRNA with itself. To avoid this bias, we no longer normalised the mRNA levels for 28S-rRNA but adjusted the mRNA levels of the gene selected as dependent variable for 28S-rRNA using multiple linear regression models. As presented in Figures 1(a)–1(c), the strong correlations between the adiponectin receptors remained unaffected by this procedure. This strengthens the observation that the basal expression of all three adiponectin receptors in human myotubes is highly coordinated. For additional control, we studied the association between the three adiponectin receptors and GPBAR1 encoding the unrelated membrane-type bile acid receptor known to be expressed in skeletal muscle. After adjustment for 28S-rRNA, none of the adiponectin receptors was significantly correlated with GPBAR1 (all $P > .2$).

Then, we analysed the association between the adiponectin receptors and PPARδ which encodes PPARα, the second PPAR family member with pro-oxidative properties and of importance in muscle, was not found (all $P > .2$). This underscores the specificity of the association between the adiponectin receptors and PPARδ at the gene expression level.

To further strengthen the relationship between the adiponectin receptor and PPARδ genes, we analysed these genes’ expression levels according to SNPs in PPARD and ADIPOR1. As depicted in Figure 2, homo- and heterozygous carriers of the minor allele of ADIPOR1 SNP rs6666089 (dominant model) revealed significantly reduced expression levels of ADIPOR1 ($P = .0317$) and CDH13 ($P = .0349$) and tended to associate with reduced PPARD ($P = .06$; $P$ for trend in the additive model = .0242) and ADIPOR2 ($P = .1$; $P$ for trend in the additive model = .0284) expression. Heterozygous minor allele carriers of PPARδ SNP rs1053049 (homozygous carriers of the minor allele were absent in this small group) showed significantly higher expression of PPARδ ($P = .0048$), ADIPOR2 ($P = .0207$), and CDH13 ($P = .0334$) and tended to associate with elevated expression of ADIPOR1 ($P = .06$) as presented in (Figure 3). PPAR SNP rs2267668 had markedly weaker effects and, thus, revealed significant association with PPARδ expression only ($P = .0258$) (data not shown). These results support the idea that the expression of the adiponectin receptor and PPARδ genes is highly coordinated.

Using stepwise multiple linear regression analysis, we observed that PPARD is, independently of the other genes, a significant determinant of CDH13 ($P = .0002$), but not of ADIPOR1 or ADIPOR2 (both $P > .05$). This suggested that CDH13 represents a novel PPARδ target gene. To address this issue, myotubes were treated for 20 h with the PPARδ-specific agonist GW501516 (1 μmol/l) and DMSO (0.1%) for carrier control. However, GW501516 treatment did not significantly increase CDH13 mRNA expression as compared
to DMSO ($P = .8, n = 16$), whereas it increased the expression of the described PPARδ target genes $PDK4$ and $ANGPTL4$ (for data, see [9]).

To assess whether the highly coordinated expression of $PPARD$ and the adiponectin receptor genes—all located on different chromosomes—is caused by a common regulatory miRNA, we screened the miRBase Targets database, Release Version v5 (at http://microrna.sanger.ac.uk/targets/v5/), for common miRNA binding sites in the four genes. In $ADIPOR2$, only one putative binding site was identified, namely, for miRNA-617. The other genes, however, did not reveal any bona fide binding site for miRNA-617. Thus, miRNAs are improbably to represent the unifying link between these genes. To approach whether the genes are under the control of a common transcription factor, we performed in silico analyses of the sequences spanning from 3 kb upstream to 1 kb downstream of the transcription initiation sites. Using AliBaba 2.1 (at http://www.gene-regulation.com/pub/programs/alibaba2/index.html?), PROMO 3.0 (at http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3/, MATCH1.0 (at http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi), and P-MATCH 1.0

Figure 1: Correlations between $ADIPOR1$, $ADIPOR2$, $CDH13$, and $PPARD$ mRNA contents of skeletal muscle cells from 39 human donors ($N = 39$). To approximate normal distribution, all data were log$_e$ transformed prior to statistical analysis. The dependent variable was adjusted for 28S-rRNA using multiple linear regression models.
**Figure 2**: Impact of the *ADIPOR1* promoter SNP rs6666089 on *ADIPOR1*, *ADIPOR2*, *CDH13*, and *PPARD* mRNA contents of human skeletal muscle cells. mRNA data were normalised for 28S-rRNA and are given in arbitrary units (AUs). To approximate normal distribution, mRNA data were log-transformed prior to statistical analysis. The rs6666089 genotype was determined by TaqMan assay. Since there were only two homozygous carriers of the A-allele among the muscle cells, we joined them with the heterozygous subjects to form the “XA” group. Data are presented as means + SE.

Since both adiponectin and PPARδ signalling regulate muscle metabolism and mitochondrial biogenesis, we investigated whether the expression of genes crucially involved in these processes, that is, *PPARGC1A* (encoding PPARγ coactivator 1α), *UCP3* (encoding uncoupling protein 3), and *TFAM* (encoding mitochondrial transcription factor A), is correlated with the expression of *ADIPOR1*, *ADIPOR2*, *CDH13*, and *PPARD*. After adjustment for the housekeeping gene, we found significant associations of *ADIPOR1*, *CDH13*, and *PPARD* expression levels with *PPARGC1A*, *UCP3*, and *TFAM* (all \(P < .045\)). *ADIPOR2* was significantly associated with *TFAM* only (\(P = .007\)). This strengthens the role of these receptors in mitochondrial oxidative metabolism.

Finally, we assessed whether the basal myotube expression levels of *PPARD* and the adiponectin receptor genes are of relevance in vivo and reflect fasting plasma lipid parameters of the myotube donors. After adjustment for gender, age,
and BMI, the fasting plasma triglyceride concentrations were inversely associated with the ADIPOR1 and CDH13 mRNA contents normalised for 28S-rRNA (Figures 4(a) and 4(c)). Moreover, even though not significant, the ADIPOR2 and PPARD mRNA contents tended to inversely associate with plasma triglycerides (Figures 4(b) and 4(d)). No significant associations were detected with fasting plasma NEFA, glycerol, total-, HDL-, or LDL-cholesterol concentrations (all \( P > .1 \)).

In addition, we assessed these genes’ association with in vivo parameters of glucose metabolism. However, we did not detect any significant association of PPARD and adiponectin receptors expression with fasting insulin concentrations (all \( P \geq .3 \)), fasting and 120-min glucose concentrations (all \( P \geq .3 \)), or with insulin sensitivity indices derived from the OGTT and the hyperinsulinaemic-euglycaemic clamp (all \( P > .5 \)), respectively. Serum adiponectin levels did not associate with the expression of PPARD, ADIPOR1, ADIPOR2, or CDH13 either (all \( P \geq .3 \)).

### 4. Discussion

In this study, we could demonstrate, also by assessing SNP effects, that two signalling pathways known to mediate lipolysis and formerly considered independent, that is, the PPAR\( \delta \) and the adiponectin pathway, are closely linked at the gene expression level. The most obvious mechanism possibly underlying this phenomenon, that is, transcriptional regulation of the adiponectin receptor genes by PPAR\( \delta \), could be excluded since PPAR\( \delta \) activation using a specific and well-described pharmacological agonist had no effect on adiponectin receptor expression. Therefore, we assessed other conceivable upstream regulatory factors. Using RNA interference, transcription factors identified as putative candidates for binding to all four promoters, such as signal transducer and activator of transcription 5A, specificity protein 1, and CCAAT/enhancer-binding protein \( \alpha \), turned out not to be involved in this highly coordinated gene regulation, neither did FoxO1, a known transcription factor regulating...
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Figure 4: Association of myotube ADIPOR1, ADIPOR2, CDH13, and PPARD mRNA contents with fasting plasma triglyceride concentrations of the 39 human donors (N = 39). mRNA data were normalised for 28S-rRNA and are given in arbitrary units (AUs). To approximate normal distribution, all data were log transformed prior to statistical analysis. Plasma triglycerides were adjusted for gender, age, and BMI using multiple linear regression models.

the murine Adipor1 and Adipor2 genes. miRNAs targeting all four genes could also not be identified. Thus, the unifying regulatory mechanism responsible for our observation remains to be uncovered in further studies addressing other, for example, epigenetic mechanisms (DNA methylation, histone modification, and nucleosome positioning).

Most importantly, we could demonstrate that myotube expression of PPARD and the adiponectin receptors inversely reflects the fasting plasma triglyceride concentration of the donors. There may be two explanations to this: either circulating triglycerides via their products of lipolytic breakdown, that is, via NEFA, affect the expression of PPARD and the adiponectin receptor genes or increased expression of these genes reduces the plasma triglyceride concentration. At least for PPARD, we were recently able to show that, with the only exception of stearate, no long-chain fatty acid is able to change its expression [9]. Thus, an influence of triglyceride-derived NEFA on these genes’ coordinated expression appears less plausible. Instead, it is well known that the adiponectin and PPARδ pathways stimulate β-oxidation and, in this way, may reduce the plasma triglyceride concentration of the donors. Since plasma triglycerides are a well-known readout of the hepatic lipid load [15], this association is in line with the observation that an impairment of fatty acid oxidation provokes increments in plasma NEFA levels, ectopic lipid deposition in nonfat tissues, and enhanced hepatic VLDL production [1] and underscores the idea of a muscle-liver axis with muscle fatty acid oxidation being an important determinant of hepatic lipid storage. In this context, metabolic signals from β-oxidation (acyl-carnitines) or muscle-derived humoral mediators (myokines) may represent interesting candidate mediators of muscle-liver
crosstalk. Finally, this translational finding provides evidence that our in vitro data are of relevance for humans in vivo and may explain the biological variance between individuals in terms of good versus bad fat burners.

5. Conclusions

Expression of PPARD, ADIPOR1, ADIPOR2, and CDH13 in human skeletal muscle cells is highly coordinated, and this might be of relevance for human lipid metabolism in vivo, as reflected by these genes’ consistent inverse association with plasma triglycerides. Thus, the upstream regulatory factor(s) responsible for this coordinated gene expression could represent promising future targets for the control of circulating lipids and hepatic fat load.

Conflict of Interests

The authors have no conflict of interests related to this paper to declare.

Abbreviations

AdipoR: Adiponectin receptor
NEFA: Nonesterified fatty acids
PPAR: Peroxisome proliferator-activated receptor.

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References


