Adipocyte differentiation-related protein and OXPAT in rat and human skeletal muscle: involvement in lipid accumulation and type 2 diabetes mellitus.

Citation for published version (APA):

Document status and date:
Published: 01/01/2009

DOI:
10.1210/jc.2009-0352

Document Version:
Publisher's PDF, also known as Version of record

Document license:
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Download date: 01 Nov. 2023
Adipocyte Differentiation-Related Protein and OXPAT in Rat and Human Skeletal Muscle: Involvement in Lipid Accumulation and Type 2 Diabetes Mellitus

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Setting: A disordered lipid metabolism is implicated in the development of skeletal muscle insulin resistance. Lipid droplet proteins of the PAT [perilipin, adipocyte differentiation-related protein (ADRP), and TIP47] family have been shown to regulate lipid accumulation and intracellular metabolism in other tissues.

Objective: This study aimed to explore the role of the PAT proteins OXPAT and ADRP in skeletal muscle lipid metabolism and their putative role in modulating insulin sensitivity.

Design: Muscle OXPAT and ADRP protein content was examined during the development of insulin resistance in Zucker diabetic fatty (ZDF) rats and in type 2 diabetes patients and BMI-matched control subjects. Furthermore, we examined the effect of 8 wk of insulin sensitizing by rosiglitazone on muscle OXPAT and ADRP content.

Results: OXPAT and ADRP protein expression is muscle fiber type specific in humans and rats, with highest protein content in fibers containing most intramyocellular lipids (IMCL). Muscle OXPAT and ADRP protein content was 2- to 3-fold higher in ZDF rats during the progression of type 2 diabetes than in lean normoglycemic control rats, which was paralleled by high IMCL levels. Muscle OXPAT and ADRP content, as well as IMCL level, was not different between type 2 diabetes patients and control subjects. ADRP content was negatively associated with insulin-stimulated glucose uptake (r = −0.50; P = 0.017). Interestingly, rosiglitazone treatment decreased muscle OXPAT (−29%) and ADRP (−28%) content in diabetes patients, without affecting IMCL.

Conclusions: These results indicate involvement of OXPAT and ADRP in muscular lipid accumulation and type 2 diabetes. (J Clin Endocrinol Metab 94: 4077–4085, 2009)
Subjects and Methods

ZDF rat study design

Twenty-six male, leptin receptor-deficient ZDF rats (ZDF/Gmi, fa/fa) and 26 heterozygous lean littermates (ZDF/Gmi, fa/+) were purchased from Charles River Laboratories, Inc. (Wilmington, MA), and housed in pairs of one genotype, on a 12-h light, 12-h dark cycle (light from 0700 to 1900 h), at 21–22 °C with ad libitum access to tap water and diet (16.7 energy percentage (En%) fat, 56.4 En% carbohydrates, and 26.8 En% protein; Purina 5008, Altromin, Lage, Germany).

Blood was sampled by orbital puncture after 4 h of fasting. At 6, 12, and 19 wk of age (n = 8, n = 8, and n = 10 per age and genotype, respectively), rats were killed by cervical dislocation, and muscles were dissected, rapidly frozen in melting 2-methyl butane (Fluka, Zwijndrecht, The Netherlands), and stored at −80 °C. Experiments were approved by the Institutional Animal Care and Use Committee.

Human study design

Subjects

Thirteen middle-aged obese men with T2D and nine age- and BMI-matched male control subjects participated in this study (see Table 1 for characteristics). T2D was diagnosed at least 1 yr before the study and was well controlled. Before the study, blood glucose homeostasis was maintained using oral glucose lowering medication in all but one subject; one patient was treated with diet only, six with metformin, five with sulfonylurea, and one with metformin and sulfonylurea. Control subjects had normal glucose tolerance, as measured with an oral glucose tolerance test, and had no family history of diabetes. In addition to their antidiabetic medication, five of 13 patients were taking statins to lower their cholesterol, two were taking angiotensin II receptor blockers, two were taking angiotensin receptor blockers, and two were taking statins and angiotensin II receptor blockers.

<table>
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<tr>
<th>Table 1. Human subject characteristics</th>
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<td><strong>T2D</strong></td>
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<td><strong>Rd-glucose</strong> (μmol/kg⋅min⁻¹)</td>
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VO₂max, Maximal oxygen uptake; HbA1c, glycosylated hemoglobin.

*P < 0.05.

bP < 0.01, T2D patients before rosiglitazone vs. control.

*P < 0.05.

dP < 0.01, T2D patients before vs. after rosiglitazone treatment.
antagonists, and one was taking a β-antagonist to control blood pressure. This medication was maintained throughout the study to avoid differential effects on pre- and posttreatment outcome measures. The Institutional Medical Ethical Review Committee approved the study protocol.

**Design**

Fourteen days before the trial, diabetic patients discontinued their antidiabetic medication. Subjects refrained from any (exhaustive) physical activity 3 d preceding the measurements. After baseline measurements, diabetic subjects received rosiglitazone in the maximum licensed dose for T2D patients (2 × 4 mg/d) for 8 wk. To avoid effects of differences in dosage of rosiglitazone used, we started using identical dosage for all subjects. Fasting blood glucose was measured every 2 wk to allow dose readjustments if required. At the end of this 8-wk period, all measurements were repeated. Despite the variability in antidiabetic medication before the study, all subjects maintained glucose homeostasis well throughout the intervention period, and no readjustments in rosiglitazone dosage had to be made. Control subjects were measured once.

**Hyperinsulinemic euglycemic clamp**

A 3-h hyperinsulinemic euglycemic clamp was performed with parallel infusion of [6,6-²H₁]glucose, as described earlier (23). Before the clamp, after an overnight fast, a muscle biopsy was taken from the vastus lateralis muscle according to Bergstrom et al. (24). Insulin-stimulated plasma glucose rate of disappearance (Rd) was calculated using Steele’s single-pool non-steady-state equations adapted for use with stable isotopes because no isotopic or glycemic steady-state was present. Volume of distribution was assumed to be 0.160 liters/kg for glucose.

**Plasma assays**

Blood was collected in EDTA-containing tubes, and plasma was stored at −80°C. Plasma free fatty acids and glucose were measured in human and rat plasma with enzymatic assays automated on a Cobas Farav/Mira (free fatty acids, Wako Nefa C test kit; Wako Chemicals, Neuss, Germany; glucose, hexokinase method; LaRoche, Basel, Switzerland). Total cholesterol (ABX Diagnostics, Montpelier, France), high-density lipoprotein (LDL) cholesterol (precipitation method; Roche Diagnostics, Montpelier, France), high-density lipoprotein (HDL) cholesterol (precipitation method; LaRoche, Basel, Switzerland). Total cholesterol (ABX Diagnostics, Montpelier, France), high-density lipoprotein (LDL) cholesterol, computed by dividing the area covered by lipid droplets by the cell surface of the measured muscle fibers.

**Histology**

Intramyocellular lipid (IMCL) was quantified in human subjects using Oil Red O (ORO) staining combined with immunofluorescence labeling of the basal membrane marker laminin and myosin heavy chain type 1 (26). IMCL was expressed as area fraction, computed by dividing the area covered by lipid droplets by the cell surface of the measured muscle fibers.

Immunofluorescent staining of OXPAT and ADRP (both 1:50 in PBS) was performed in human muscle biopsies and rat gastrocnemius muscles using the above-mentioned antibodies combined with an Alexa488-conjugated secondary antibody (1:200 in PBS; Molecular Probes, Invitrogen, Breda, The Netherlands). Experiments omitting OXPAT/ADRP antibodies yielded no signal. OXPAT and ADRP staining was performed on cryosections combined with either ORO staining or fiber typing, exactly as described in Ref. 26. Antibodies against myosin heavy chain types 1 and 2a (A4.840 and N2.261, Developmental Studies Hybridoma Bank, both 1:25 in PBS) were used for fiber typing and were combined with Alexa555- and Alexa350-conjugated secondary antibodies, respectively.

**Statistics**

Data are presented as mean ± sd. Substrate fluxes are expressed as micromoles per kilogram per minute. Statistics were performed using SPSS 13.0 for Windows (SPSS, Inc., Chicago, IL). By performing a Kolmogorov-Smirnov test, a normal Gaussian distribution was shown for all data examined. Hence, parametric testing was used to test the measured variables. Differences between groups were compared using an unpaired Student’s t-test; the treatment effect of rosiglitazone was analyzed using a paired Student’s t-test. Correlation analysis was performed using two-sided Pearson’s correlation analysis. Two-way ANOVA was used to examine age, genotype, and age×genotype interaction effects in the rat experiments. A Bonferroni post hoc test was performed to compare time points within genotypes. Statistical significance was set at P < 0.05.
Results

ZDF rat characteristics

Body mass and food intake were significantly higher in the fa/fa than in the fa/+ rats (P < 0.001 at all ages). Body mass of the rats killed at 6 wk of age averaged 194 ± 6 vs. 147 ± 11 g (fa/fa vs. fa/+; P < 0.001); of the 12-wk-old rats, 345 ± 17 vs. 284 ± 32 g (P < 0.001); and of the 19-wk-old rats, 373 ± 16 vs. 335 ± 19 g (P < 0.001). Similar values are reported in literature for the ZDF model (27).

Blood glucose levels were slightly, but significantly, higher in fa/fa rats compared with fa/+ rats at wk 6 (4.6 ± 0.2 vs. 3.9 ± 0.3 mmol/liter; P < 0.05) and rapidly increased in the fa/fa rats toward wk 12 (13.5 ± 1.3 mmol/liter) and wk 19 (16.1 ± 0.8 mmol/liter). Blood glucose levels remained within the normoglycemic range in the fa/+ rats (3.8 ± 0.4 and 4.0 ± 0.7 mmol/liter at wk 12 and 19, respectively; P < 0.05 vs. fa/fa). From wk 6 onward, insulin levels in fa/fa rats were significantly higher than in fa/+ rats (1.6 ± 0.9 vs. 0.2 ± 0.0, 1.6 ± 0.7 vs. 0.4 ± 0.2, and 0.8 ± 0.2 vs. 0.3 ± 0.1 μg/liter at 6, 12, and 19 wk, respectively; P < 0.05 for each comparison). Both genotypes showed similar and stable plasma free fatty acid levels throughout the study (181 ± 48 vs. 166 ± 53, 180 ± 42 vs. 206 ± 61, and 172 ± 36 vs. 193 ± 82 μmol/liter, at 6, 12, and 19 wk for fa/fa vs. fa/+ rats, respectively).

ADRP and OXPAT in ZDF rats

OXPAT and ADRP protein is present in rat gastrocnemius muscle fibers in a punctate staining pattern (Fig. 1, A and C, respectively). In rodents, OXPAT and ADRP expression was highest in type 2a muscle fibers, intermediate in type 1 muscle fibers, and almost absent in type 2b fibers (Fig. 1, B and D, respectively). This pattern corresponds well with the IMCL content of these fibers. OXPAT protein content was not different between genotypes at 6 wk of age [0.84 ± 0.24 arbitrary units (AU) in fa/fa rats vs. 0.77 ± 0.39 AU in fa/+ rats; P = 0.64; Fig. 2A], whereas OXPAT content increased and was higher at 12 wk (2.06 ± 0.33 vs. 0.78 ± 0.38 AU; P < 0.001) and 19 wk (1.36 ± 0.35 vs. 0.43 ± 0.19 AU; P < 0.001) of age in fa/fa compared with fa/+ rats. Interestingly, muscular ADRP content was already higher at 6 wk of age in fa/fa compared with fa/+ rats (1.35 ± 0.35 vs. 0.83 ± 0.22 AU, respectively; P < 0.05; Fig. 2B), and this difference remained at 12 wk (1.42 ± 0.34 vs. 0.61 ± 0.11 AU; P < 0.001) and 19 wk (1.18 ± 0.12 vs. 0.50 ± 0.13 AU; P < 0.001) of age. In contrast with OXPAT, ADRP protein content in fa/fa rats did not change significantly between 6 and 19 wk of age.

Human subjects

Subject characteristics are shown in Table 1. By definition, T2D patients had significantly higher fasting plasma glucose levels (P < 0.01) and a tendency toward higher fasting plasma insulin levels (P = 0.095) compared with control subjects. Plasma HDL cholesterol tended to increase in T2D patients upon rosiglitazone treatment (pretreatment, 1.11 ± 0.2 mmol/liter, vs. posttreatment, 1.20 ± 0.2 mmol/liter; P = 0.06). Other lipoprotein-related parameters (total cholesterol, LDL cholesterol, and circulatory triglycerides) were comparable between T2D and BMI-matched controls and were not significantly affected by rosiglitazone treatment (Table 1).

Insulin sensitivity and substrate oxidation

Insulin-stimulated glucose uptake (Rd) was 27% lower in T2D patients compared with obese controls, but this did not reach significance (14.9 ± 7.5 vs. 20.3 ± 11.2 μmol/kg · min; P = 0.19). Rosiglitazone treatment tended to increase insulin-stimulated glucose uptake almost to control levels in T2D patients (14.9 ± 7.5 to 18.7 ± 7.3 μmol/kg · min; P = 0.089).
Muscle biopsy analyses

In humans, OXPAT and ADRP content was highest in muscle fibers with the most abundant storage of IMCL (Fig. 3, A and C), i.e. type 1 fibers (Fig. 3, B and D). Colocalization of OXPAT with lipid droplets in the muscle fibers is apparent in Fig. 3A. OXPAT and ADRP content were similar in T2D patients and controls (OXPAT, 1.28 ± 0.68 vs. 1.16 ± 0.62 AU, P = 0.67; ADRP, 0.65 ± 0.40 vs. 0.63 ± 0.29 AU, P = 0.88, in T2D patients vs. controls, respectively; Fig. 4). IMCL levels were not different between T2D patients and age- and BMI-matched controls at baseline (7.1 ± 2.9 and 6.7 ± 2.2%, respectively; P = 0.74; Fig. 4E), which matches with the lack of difference in OXPAT and ADRP content. Interestingly, rosiglitazone treatment decreased the muscle content of both OXPAT (1.28 ± 0.68 to 0.91 ± 0.65 AU; P < 0.05; Fig. 4, A and B) and ADRP (0.65 ± 0.40 to 0.47 ± 0.25 AU; P < 0.05; Fig. 4, C and D), whereas IMCL content was not affected by rosiglitazone treatment (7.1 ± 3.1 to 8.3 ± 3.2%; P = 0.22). Moreover, no differences in IMCL content were observed between groups or after rosiglitazone when analyzed for each fiber type separately (Fig. 4E).

Correlations

In humans, skeletal muscle ADRP protein content correlated negatively with insulin-stimulated glucose uptake in all subjects (Fig. 5; r = −0.50; P < 0.02). This correlation approached significance in the separate groups (T2D patients before, r = −0.51, P = 0.08; controls, r = −0.60, P = 0.09). OXPAT protein content did not correlate with insulin sensitivity. Rosiglitazone-induced changes in ADRP and OXPAT protein content did not correlate.

Discussion

We show that OXPAT and ADRP are expressed in human and rat skeletal muscle and that their expression is fiber-type specific, with highest protein content in muscle fibers that contain most IMCL. Moreover, muscular OXPAT and ADRP protein content was much higher in ZDF rats...
during the progression of T2D compared with healthy lean heterozygous control rats. Higher protein levels of OXPAT and ADRP were paralleled by higher IMCL levels in ZDF rats. At 6 wk of age, however, when ZDF rats were insulin-resistant but not diabetic yet, ADRP but not OXPAT protein content was much higher in ZDF rats compared with lean control rats, whereas IMCL levels were only modestly higher in ZDF rats at this age. This may indicate that high ADRP levels facilitate accumulation of IMCL. Muscular OXPAT and ADRP protein content was not different between T2D patients and age- and BMI-matched controls, which corresponds with similar IMCL levels in both groups. Interestingly, in these patients, 8 wk of treatment with the insulin-sensitizer ros-
expression of OXPAT on mRNA (14, 15) and protein (16, 17) level. Moreover, ADRP was shown to be associated with lipid droplets (17). OXPAT expression (both mRNA and protein) was also demonstrated in skeletal muscle (11, 21, 22). In isolated cardiomyocytes, OXPAT and ADRP staining revealed a punctate staining pattern with “dots” lying along the muscle fiber, a pattern typically seen for lipid droplets in muscle tissue. Our data (Figs. 1 and 3) also show that OXPAT and ADRP stains overlap with the lipid staining, demonstrating that both proteins are associated with lipid droplets in human and rat skeletal muscle. From in vitro experiments using multiple cell lines, it is known that OXPAT resides within the cytoplasm in the absence of neutral lipids and moves to the lipid droplet in conditions that promote lipid droplet formation (11, 21). ADRP is degraded by the proteasome if not bound to neutral lipids (28, 29). Our stainings reveal intense punctate, lipid droplet-like staining of OXPAT. Expression of OXPAT on other cytoplasmic sites was less obvious, but might have been overwhelmed by the intense staining associated with the lipid droplet. Our data extend previous data on the muscle expression of OXPAT and ADRP by showing a fiber type-specific expression pattern, with higher levels in fibers that have a higher capacity for lipid storage.

At the age of 12 and 19 wk, when overt diabetes is present in the ZDF rats, both OXPAT and ADRP protein content are much higher than in lean control rats. This matches with the largely increased IMCL content in the tibialis anterior muscle (measured in vivo using 1H-magnetic resonance spectroscopy) of these same rats (30), as well as with the large increases in IMCL content in the soleus muscle of ZDF rats (6, 31). Although IMCL levels in the ZDF rats are very high at these stages of maturation, the drop in IMCL observed from 12 to 19 wk of age is present for OXPAT only, and not for ADRP. At 6 wk, when ZDF rats are in a prediabetic stage (hyperinsulinemic but still normoglycemic), IMCL content in tibialis anterior muscle was slightly higher than in lean controls (30). We show that ADRP, but not OXPAT, is already highly up-regulated at this stage, suggesting that an increased amount of ADRP facilitates the accumulation of lipids. This is supported by data showing that overexpression of ADRP increases long-chain fatty acid uptake (32) and lipid accumulation in several cell types (19, 20). Moreover, down-regulation of ADRP protein in the mouse liver prevented lipid accumulation caused by a high-fat diet or leptin deficiency (33, 34).

We show here that OXPAT and ADRP protein content in skeletal muscle of T2D patients is similar to BMI-matched control subjects, which is in line with similar IMCL levels in both groups (Fig. 4). Our observation of similar baseline levels of OXPAT and ADRP in T2D- and BMI-matched control subjects might have been biased by the use of lipid-lowering drugs, which was higher in the T2D patients. Analysis of baseline OXPAT and ADRP levels in subjects with and without lipid-lowering medication did, however, not hint toward consistently higher or lower content of these proteins in subjects taking lipid-lowering medication. Surprisingly, 8 wk of rosiglitazone treatment did decrease both OXPAT and ADRP protein content in skeletal muscle of T2D patients, without affecting IMCL content. It has been shown that rosiglitazone treatment in humans does not alter IMCL content (35, 36). PPARα and -γ regulate OXPAT and ADRP protein expression in a tissue-specific manner. In mouse skeletal muscle, PPARα is involved in the regulation of OXPAT and ADRP expression (11, 21). Here we show that the PPARγ agonist rosiglitazone does not stimulate ADRP and OXPAT expression in human muscle. Rather, we observe declined OXPAT and ADRP levels. The
present study is the first to present data on myocellular OXPAT protein levels in human skeletal muscle of T2D patients. Previous work in humans on OXPAT in muscle was at the level of gene expression and revealed no change in OXPAT gene expression in impaired glucose-tolerant subjects taking pioglitazone for 10 wk (a PPARγ agonist with PPARα affinity) (11). Comparing the data set in the present study with the data shown in the paper by Wolins et al. (11) is complicated; first and for all, the present study shows data on protein rather than mRNA level. Furthermore, the study by Wolins et al. (11) was performed in a group of impaired glucose-tolerant subjects of unknown gender, age, and BMI. These subjects were selected from a larger population of mixed gender (69 females, 16 males), with wide variety in age (21–66 yr) and BMI (20–55 kg/m²), and hence it is not unlikely that the subgroup that received pioglitazone was heterogeneous as well regarding gender, age, and BMI, all factors likely to affect myocellular lipid metabolism and insulin sensitivity. Our finding of decreased muscle OXPAT and ADRP expression is also interesting in light of the improved insulin sensitivity caused by rosiglitazone treatment. A putative explanation for this may be that the decline in OXPAT and ADRP facilitates a decline in recognized insulin-desensitizing intermediates like diacylglycerol within the lipid droplet. Because both diacylglycerol and triacylglycerol are stained by ORO, interconversion will not be reflected in stainings or in 1H-magnetic resonance spectroscopy-based IMCL analyses. Based on the putative functions of both proteins in the efficient storage of intracellular lipids and the regulation of lipases acting on the lipid droplets, an increase, rather than a decrease, in their expression would be expected after an insulin-sensitizing intervention (9, 37). Indeed, Phillips et al. (16) showed an increase of both muscle ADRP protein and insulin sensitivity in obese nondiabetic subjects after a weight-loss intervention and in obese T2D patients after pharmacological intervention (metformin or troglitazone). Our finding of a negative correlation between insulin-stimulated glucose uptake and ADRP content (Fig. 5) is in contrast with these latter findings (16) and suggests that low rather than high OXPAT and ADRP content would help to improve insulin sensitivity.

In conclusion, our data support a role for OXPAT and ADRP in muscular lipid accumulation. OXPAT and ADRP protein is expressed in human and rat skeletal muscle in a fiber type-specific manner, with higher amounts in fibers that have a higher capacity to store lipids. Moreover, muscle OXPAT and ADRP protein are highly increased in parallel with IMCL content in ZDF rats that progress to insulin resistance. Consistently, in humans no differences in muscle OXPAT and ADRP content were observed between T2D patients and BMI-matched controls that have a similar IMCL content, whereas ADRP expression was inversely related to insulin sensitivity. In contrast, rosiglitazone treatment decreased skeletal muscle OXPAT and ADRP protein content without affecting IMCL content, while improving insulin sensitivity. These data show that in human skeletal muscle, OXPAT and ADRP are not responsive to the PPARγ agonist rosiglitazone in the dosage and duration used. Moreover, our data do not support the hypothesis that an improvement in insulin sensitivity depends on increased levels of PAT proteins that act by improving the efficiency of lipid storage and lipolytic control.

Acknowledgments
We thank J. J. Prompers, K. Nicolay, and H. M. De Feyter (Eindhoven University of Technology, The Netherlands) for their collaboration on the ZDF rat model.

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This work was supported by VIDI Grant 917.66.359 (to M.K.C.H.) for innovative research from The Dutch Organization for Scientific Research.

Disclosure Summary: The authors have nothing to disclose.

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