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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)

Skeletal muscle insulin resistance plays a major role in the pathogenesis of type 2 diabetes (T2D). Disordered lipid metabolism is considered a major factor in causing skeletal muscle insulin resistance. From studies in nontrained humans (1–4) and insulin-resistant rodent models (5–7), it is known that levels of intramyocellular triacylglycerols correlate negatively with insulin sensitivity. A growing body of evidence, however, supports the notion that not

the triacylglycerols (TAGs) *per se*, but the accompanying increase in lipid metabolites (like diacylglycerol, ceramides, and fatty-acyl coenzyme A) may cause skeletal muscle insulin resistance (8).

TAGs are mainly stored in neutral lipid droplets, coated by lipid droplet-associating proteins referred to as the PAT family, named after perilipin, adipocyte differentiation-related protein (ADRP), and tail-interacting protein of 47 kDa

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Abbreviations: ADRP, Adipocyte differentiation-related protein; AU, arbitrary units; BMI, body mass index; En%, energy percentage; HDL, high-density lipoprotein; IMCL, intramyocellular lipid; LDL, low-density lipoprotein; ORO, Oil Red O; PPAR, peroxisome proliferator-activated receptor; Rd, rate of disappearance; TAG, triacylglycerol; T2D, type 2 diabetes; ZDF, Zucker diabetic fatty (rat).

(9). Two other proteins of this PAT family have recently been identified, *i.e.* OXPAT (also known as myocardial lipid droplet protein or lipid storage droplet protein 5) and S3–12 (10–12). Perilipin is the most extensively studied member of this family, and it exerts primary control over adipose tissue TAG stores by regulating both TAG storage and lipolysis. Perilipin is only expressed in adipose tissue, whereas the expression levels of the other four PAT proteins vary widely across tissues. Although originally identified as an early marker of adipocyte differentiation (13), ADRP is now known to be expressed in a variety of tissues, including muscle (14–17). Its expression is related to the total neutral lipid mass in the cell (14, 18). Indeed, ADRP overexpression stimulates fatty acid uptake and triacylglycerol formation in various cell types (19, 20). OXPAT is another PAT-family protein, which is mainly expressed in tissues with high fat oxidative capacity, like heart, skeletal muscle, and brown adipose tissue (11, 21, 22), and is recruited to lipid droplets in conditions that promote lipid droplet formation (11). In addition, a role for OXPAT in the regulation of fatty acid oxidation was observed *in vitro* (11).

Given their tissue distribution, particularly OXPAT and ADRP are interesting candidates to be involved in the modulation of muscular lipid storage and degradation. Little is known, however, about their exact role in skeletal muscle lipid metabolism. OXPAT and ADRP have been implicated in the regulation of both TAG storage and hydrolysis in the lipid droplet, two processes affecting the level and type of lipid intermediates present. Because a dysregulation of lipid metabolism, and especially the presence of these lipid intermediates, is implicated in the development of skeletal muscle insulin resistance and T2D, we here aimed to explore the role of OXPAT and ADRP in the development of insulin resistance. For this purpose, the muscle content of both proteins was determined during the progression of insulin resistance in the Zucker diabetic fatty (ZDF) rat model, as well as in T2D patients and body mass index (BMI)-matched control subjects. Because muscle is comprised of muscle fibers of distinct subtypes that vary in fat oxidative and storage capacity, we also examined muscle fiber type-specific expression of OXPAT and ADRP. Finally, to examine whether OXPAT and ADRP protein content are related to muscular insulin sensitivity, we studied the effect of the thiazolidinedione rosiglitazone—known to improve insulin sensitivity—on muscular OXPAT and ADRP content in T2D patients.

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

TABLE 1. Human subject characteristics

	Controls	T2D	
		Before rosiglitazone	After rosiglitazone
Age (yr)	58.2 ± 7.5	62.8 ± 4.3	
Body weight (kg)	92.8 ± 14.1	91.3 ± 13.1	92.6 ± 13.3
BMI (kg/m ²)	30.6 ± 4.25	30.2 ± 3.8	30.6 ± 3.7
VO ₂ max (ml/kg · min)	33.0 ± 5.1	29.7 ± 6.2 ^a	
Total cholesterol (mmol/liter)	5.3 ± 1.1	5.1 ± 0.5	5.1 ± 0.8
LDL cholesterol (mmol/liter)	3.4 ± 1.0	3.4 ± 0.4	3.4 ± 0.7
HDL cholesterol (mmol/liter)	1.1 ± 0.4	1.1 ± 0.2	1.2 ± 0.2
Triglycerides (mmol/liter)	1.1 ± 0.4	1.3 ± 0.5	1.2 ± 0.5
Fasting glucose (mmol/liter)	5.9 ± 0.5	8.5 ± 2.1 ^b	7.9 ± 1.9
Fasting insulin (mU/liter)	12.8 ± 2.2	18.6 ± 11.2	14.3 ± 5.3 ^d
Free fatty acids (μmol/liter)	518 ± 152	425 ± 111	391 ± 115
HbA1c (%)		7.0 ± 0.7	7.3 ± 1.3
M-value (μmol/kg · min)	24.3 ± 8.0	20.5 ± 8.2	25.3 ± 7.3 ^c
Rd-glucose (μmol/kg · min)	20.3 ± 11.2	14.9 ± 7.5	18.7 ± 7.3

VO₂max, Maximal oxygen uptake; HbA1c, glycosylated hemoglobin.

^a $P < 0.05$.

^b $P < 0.01$, T2D patients before rosiglitazone vs. control.

^c $P < 0.05$.

^d $P < 0.01$, 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 [$6,6\text{-}^2\text{H}_2$]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 Fara/Mira (free fatty acids, Wako Nefa C test kit; Wako Chemicals, Neuss, Germany; glucose, hexokinase method; LaRoche, Basel, Switzerland). Total cholesterol (ABX Diagnostics, Montpellier, France), high-density lipoprotein (HDL) cholesterol (precipitation method; Roche Diagnostics Corporation, Indianapolis, IN), and triglycerides corrected for free glycerol (Sigma-Aldrich, Zwijndrecht, The Netherlands) were analyzed enzymatically. Serum low-density lipoprotein (LDL) cholesterol concentrations were calculated by using the formula of Friedewald *et al.* (25). Plasma insulin concentration was determined in humans using a RIA (Linco Research, St. Charles, MO) and in rats using an ultrasensitive solid phase two-site ELISA kit (Mercodia, Uppsala, Sweden). Isotopic enrichment of human plasma glucose was determined by electron ionization gas chromatography-mass spectrometry and expressed as tracer-tracee ratio.

Muscle analyses

Muscle samples were homogenized in ice-cold PBS containing 1% Nonidet-P40, 0.5% sodium dodecyl sulfate, 0.1 mM phenylmethylsulfonyl fluoride, complete inhibitor (Roche, Almere, The Netherlands) and processed for standard SDS-PAGE and Western blotting. Protein concentration was as-

essed, and equal amounts of protein were loaded per lane. Membranes were incubated with antibodies against OXPAT (no. GP31, guinea pig polyclonal; Progen, Heidelberg, Germany) and ADRP (no. RDI-PROGP40, guinea pig polyclonal; Fitzgerald Industries RDI division, Concord, MA). The antibodies used were tested on a variety of tissues known to vary in OXPAT and ADRP content [white adipose tissue, cardiac muscle, glycolytic skeletal muscle (extensor digitorum longus), oxidative skeletal muscle (soleus), liver tissue, and testis]. For both antibodies, bands corresponding to the anticipated molecular weight of the protein were identified. These bands responded according to the expression profile previously described by others. Finally, we chose to load on each blot a ZDF rat heart sample as a positive control for the OXPAT antibody and an oleate-incubated ($800 \mu\text{M}$, 24 h) C2C12 muscle cell sample as a positive control for the ADRP antibody. Blots were probed with IRDye800-conjugated secondary antibodies (Rockland, Gilbertsville, PA), and bands at a molecular weight corresponding to the control samples were quantified using the Odyssey infrared imaging system (LICOR Biosciences, Lincoln, NE).

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 \pm 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 μ g/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 μ mol/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; $P < 0.01$; 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.

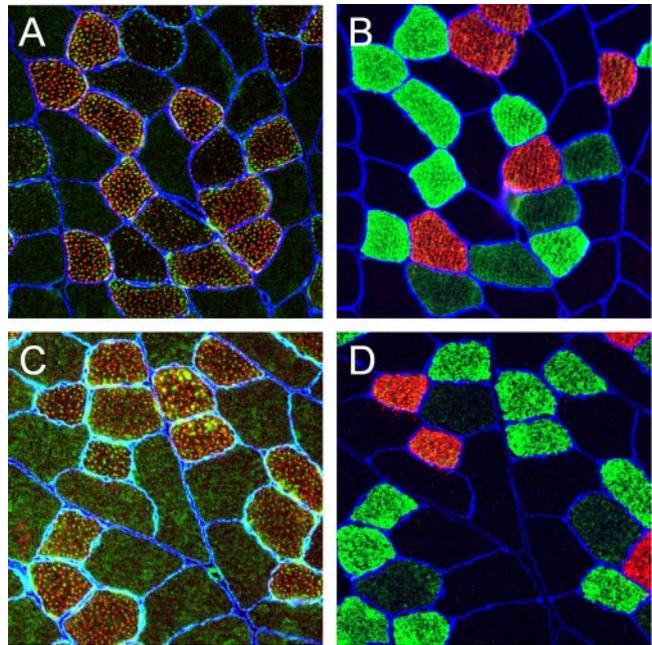


FIG. 1. OXPAT and ADRP immunofluorescent staining on ZDF rat gastrocnemius muscle sections. A, Combined OXPAT (green) and ORO (red) staining showing higher OXPAT levels in fibers containing much lipid. The basal membrane marker laminin is stained blue. B, Fiber-typing performed on serial sections (corresponding to panel A). Type 1 fibers are red, type 2a fibers are green, and type 2b fibers are unstained. C, Combined ADRP (green) and ORO (red) staining showing high ADRP levels in fibers containing much lipid. D, Fiber-typing on serial sections (corresponding to panel C). Images are shown for a ZDF rat aged 12 wk, but stainings performed on two ZDF and two control rats of each age group show similar staining patterns.

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 μ mol/kg \cdot 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 μ mol/kg \cdot min; $P = 0.089$).

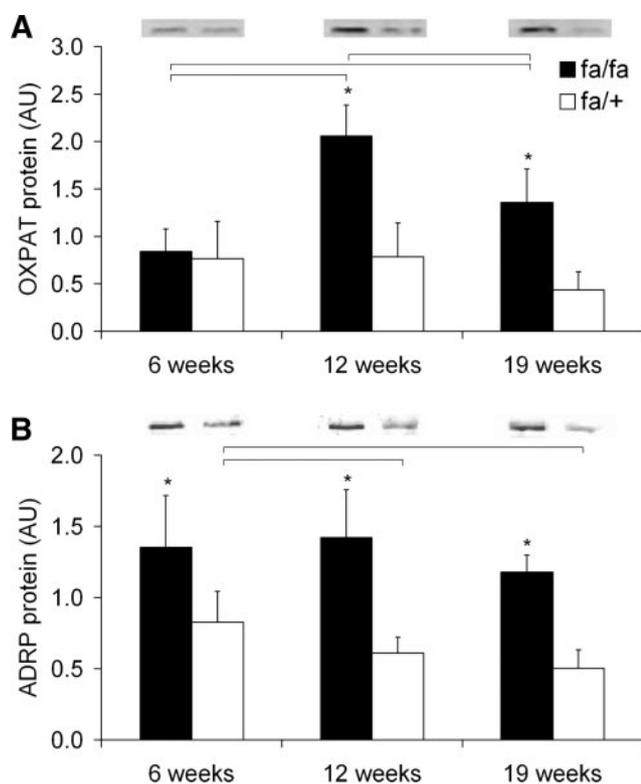


FIG. 2. OXPAT (A) and ADRP (B) protein content in gastrocnemius muscle of 6-, 12-, and 19-wk-old ZDF rats (*fa/fa*) and lean control rats (*fa/+*). *, $P < 0.05$ *fa/fa* vs. *fa/+*. Brackets indicate significant differences between age groups within the genotypes.

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 $6.7 \pm 2.2\%$, 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 $8.3 \pm 3.2\%$; $P = 0.22$). 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

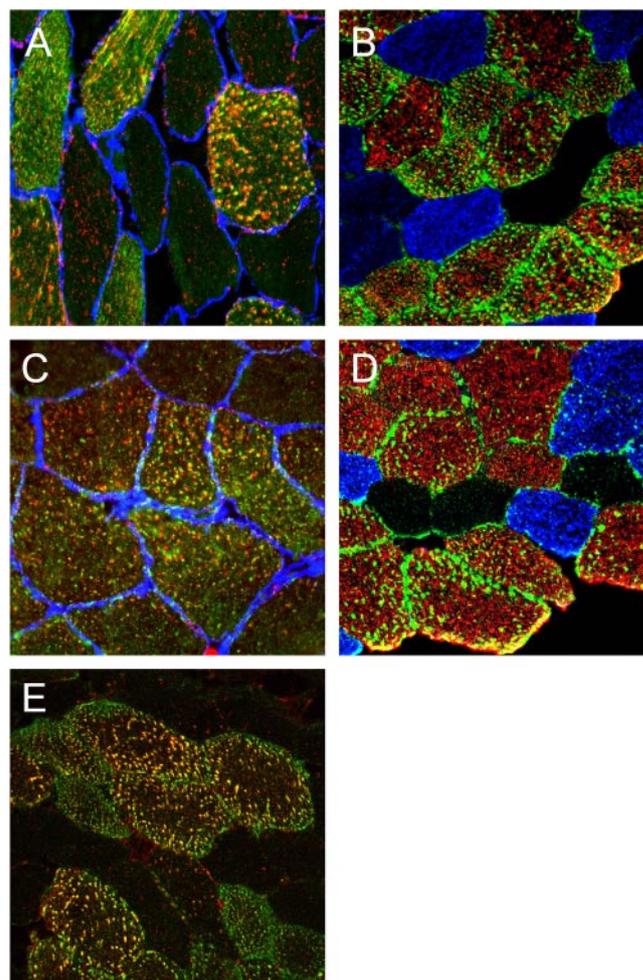


FIG. 3. OXPAT and ADRP immunofluorescent staining on muscle sections from a T2D patient. A, Combined OXPAT (green) and ORO (red) staining showing higher OXPAT levels in fibers containing much lipid. B, OXPAT staining combined with fiber-typing. Type 1 fibers are red, type 2a fibers blue, and type 2b fibers unstained. C, Combined ADRP (green) and ORO (red) staining showing high ADRP levels in fibers containing much lipid. D, ADRP staining combined with fiber-typing. E, Combined OXPAT (green) and ADRP (red) staining showing that both proteins colocalize to a large degree in muscle fibers.

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

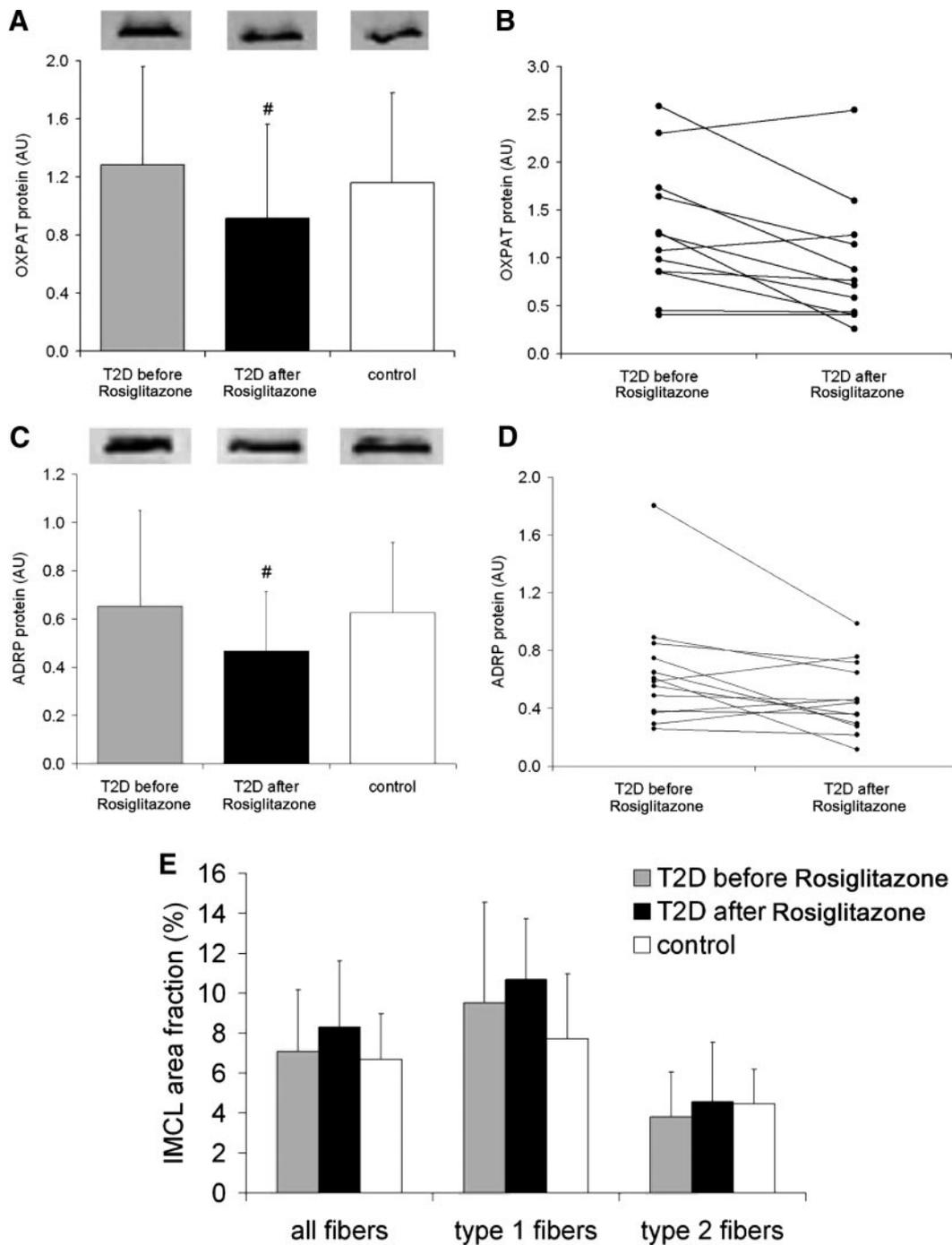


FIG. 4. A–D, OXPAT (A) and ADRP (C) content in muscle biopsies of T2D patients, before and after rosiglitazone treatment, and BMI-matched control subjects. Individual values for OXPAT and ADRP in T2D patients before and after rosiglitazone treatment are shown in B and D, respectively. #, $P < 0.05$ in T2D patients before vs. after rosiglitazone treatment. E, IMCL content (expressed as area fraction) in muscle biopsies of T2D patients, before and after rosiglitazone treatment, and BMI-matched control subjects. The IMCL content is shown for all muscle fibers, as well as for type 1 and 2 fibers separately. No significant differences were present between T2D patients before rosiglitazone treatment and controls, and no significant effect of rosiglitazone treatment was present.

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-

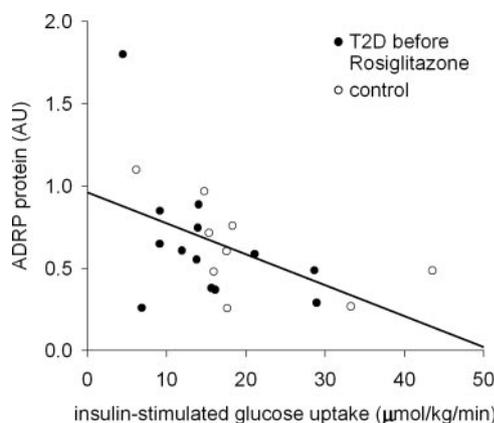


FIG. 5. Muscular ADRP protein content correlates negatively with insulin-stimulated glucose uptake in a combined group of untreated T2D patients before rosiglitazone treatment and healthy, BMI-matched controls ($r = -0.50$, $P = 0.017$).

rosiglitazone [a peroxisome proliferator-activated receptor (PPAR)- γ agonist] decreased both OXPAT and ADRP protein content, without a (detectable) effect on IMCL level. We conclude that OXPAT and ADRP are implicated in myocellular lipid accumulation in humans and rodents. Whether OXPAT and ADRP play a causal role in the development of insulin resistance remains to be established. Interestingly, muscle ADRP protein content correlated negatively with insulin-stimulated glucose uptake, suggesting that less ADRP is beneficial for insulin sensitivity, consistent with the TZD-induced down-regulation of ADRP/OXPAT. These data may therefore indicate that ADRP and OXPAT are early markers of changes in triacylglycerol accumulation and possibly of lipid intermediates in muscle.

Our results confirm the presence of OXPAT and ADRP in human and rat skeletal muscle. ADRP was previously shown to be expressed in skeletal muscle at both the 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 before that rosiglitazone treatment in humans does not alter IMCL content (35, 36). PPAR α and γ regulate OXPAT and ADRP protein expression in a tissue-specific manner. In mouse skeletal muscle, PPAR α is involved in the regulation of OXPAT and ADRP expression (11, 21). Here we show that the PPAR γ 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 PPAR γ agonist with PPAR α 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 PPAR γ 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.

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References

1. Goodpaster BH, He J, Watkins S, Kelley DE 2001 Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. *J Clin Endocrinol Metab* 86:5755–5761
2. Krssak M, Falk Petersen K, Dresner A, DiPietro L, Vogel SM, Rothman DL, Roden M, Shulman GI 1999 Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a ¹H NMR spectroscopy study. *Diabetologia* 42:113–116
3. Pan DA, Lillioja S, Kriketos AD, Milner MR, Baur LA, Bogardus C, Jenkins AB, Storlien LH 1997 Skeletal muscle triglyceride levels are inversely related to insulin action. *Diabetes* 46:983–988
4. Perseghin G, Scifo P, De Cobelli F, Pagliato E, Battezzati A, Arcelloni C, Vanzulli A, Testolin G, Pozza G, Del Maschio A, Luzi L 1999 Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a ¹H–¹³C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. *Diabetes* 48:1600–1606
5. Kuhlmann J, Neumann-Haefelin C, Belz U, Kramer W, Juretschke HP, Herling AW 2005 Correlation between insulin resistance and intramyocellular lipid levels in rats. *Magn Reson Med* 53:1275–1282
6. Kuhlmann J, Neumann-Haefelin C, Belz U, Kalisch J, Juretschke HP, Stein M, Kleinschmidt E, Kramer W, Herling AW 2003 Intramyocellular lipid and insulin resistance: a longitudinal in vivo ¹H-spectroscopic study in Zucker diabetic fatty rats. *Diabetes* 52:138–144

7. Dobbins RL, Szczepaniak LS, Bentley B, Esser V, Myhill J, McGarry JD 2001 Prolonged inhibition of muscle carnitine palmitoyltransferase-1 promotes intramyocellular lipid accumulation and insulin resistance in rats. *Diabetes* 50:123–130
8. Petersen KF, Shulman GI 2006 New insights into the pathogenesis of insulin resistance in humans using magnetic resonance spectroscopy. *Obesity (Silver Spring)* 14(Suppl 1):34S–40S
9. Brasaemle DL 2007 Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *J Lipid Res* 48:2547–2559
10. Scherer PE, Bickel PE, Kotler M, Lodish HF 1998 Cloning of cell-specific secreted and surface proteins by subtractive antibody screening. *Nat Biotechnol* 16:581–586
11. Wolins NE, Quaynor BK, Skinner JR, Tzekov A, Croce MA, Gropler MC, Varma V, Yao-Borengasser A, Rasouli N, Kern PA, Finck BN, Bickel PE 2006 OXPAT/PAT-1 is a PPAR-induced lipid droplet protein that promotes fatty acid utilization. *Diabetes* 55:3418–3428
12. Wolins NE, Skinner JR, Schoenfish MJ, Tzekov A, Bensch KG, Bickel PE 2003 Adipocyte protein S3–12 coats nascent lipid droplets. *J Biol Chem* 278:37713–37721
13. Jiang HP, Serrero G 1992 Isolation and characterization of a full-length cDNA coding for an adipose differentiation-related protein. *Proc Natl Acad Sci USA* 89:7856–7860
14. Brasaemle DL, Barber T, Wolins NE, Serrero G, Blanchette-Mackie EJ, Londos C 1997 Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *J Lipid Res* 38:2249–2263
15. Dalen KT, Schoonjans K, Ulven SM, Weedon-Fekjaer MS, Bentzen TG, Koutnikova H, Auwerx J, Nebb HI 2004 Adipose tissue expression of the lipid droplet-associated proteins S3–12 and perilipin is controlled by peroxisome proliferator-activated receptor- γ . *Diabetes* 53:1243–1252
16. Phillips SA, Choe CC, Ciaraldi TP, Greenberg AS, Kong AP, Baxi SC, Christiansen L, Mudaliar SR, Henry RR 2005 Adipocyte differentiation-related protein in human skeletal muscle: relationship to insulin sensitivity. *Obes Res* 13:1321–1329
17. Prats C, Donsmark M, Qvortrup K, Londos C, Sztalryd C, Holm C, Galbo H, Ploug T 2006 Decrease in intramuscular lipid droplets and translocation of HSL in response to muscle contraction and epinephrine. *J Lipid Res* 47:2392–2399
18. Steiner S, Wahl D, Mangold BL, Robison R, Raymackers J, Meheus L, Anderson NL, Cordier A 1996 Induction of the adipose differentiation-related protein in liver of etomoxir-treated rats. *Biochem Biophys Res Commun* 218:777–782
19. Imamura M, Inoguchi T, Ikuyama S, Taniguchi S, Kobayashi K, Nakashima N, Nawata H 2002 ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. *Am J Physiol Endocrinol Metab* 283:E775–E783
20. Listenberger LL, Ostermeyer-Fay AG, Goldberg EB, Brown WJ, Brown DA 2007 Adipocyte differentiation-related protein reduces the lipid droplet association of adipose triglyceride lipase and slows triacylglycerol turnover. *J Lipid Res* 48:2751–2761
21. Yamaguchi T, Matsushita S, Motojima K, Hirose F, Osumi T 2006 MLDP, a novel PAT family protein localized to lipid droplets and enriched in the heart, is regulated by peroxisome proliferator-activated receptor α . *J Biol Chem* 281:14232–14240
22. Dalen KT, Dahl T, Holter E, Arntsen B, Londos C, Sztalryd C, Nebb HI 2007 LSDP5 is a PAT protein specifically expressed in fatty acid oxidizing tissues. *Biochim Biophys Acta* 1771:210–227
23. Mensink M, Hesselink MK, Russell AP, Schaart G, Sels JP, Schrauwen P 2007 Improved skeletal muscle oxidative enzyme activity and restoration of PGC-1 α and PPAR β/δ gene expression upon rosiglitazone treatment in obese patients with type 2 diabetes mellitus. *Int J Obes (Lond)* 31:1302–1310
24. Bergström J, Hermansen L, Hultman E, Saltin B 1967 Diet, muscle glycogen and physical performance. *Acta Physiol Scand* 71:140–150
25. Friedewald WT, Levy RI, Fredrickson DS 1972 Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18:499–502
26. Koopman R, Schaart G, Hesselink MK 2001 Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochem Cell Biol* 116:63–68
27. Etgen GJ, Oldham BA 2000 Profiling of Zucker diabetic fatty rats in their progression to the overt diabetic state. *Metabolism* 49:684–688
28. Masuda Y, Itabe H, Odaki M, Hama K, Fujimoto Y, Mori M, Sasabe N, Aoki J, Arai H, Takano T 2006 ADRP/adipophilin is degraded through the proteasome-dependent pathway during regression of lipid-storing cells. *J Lipid Res* 47:87–98
29. Xu G, Sztalryd C, Lu X, Tansey JT, Gan J, Dorward H, Kimmel AR, Londos C 2005 Post-translational regulation of adipose differentiation-related protein by the ubiquitin/proteasome pathway. *J Biol Chem* 280:42841–42847
30. De Feyter HM, Lenaers E, Houten SM, Schrauwen P, Hesselink MK, Wanders RJ, Nicolay K, Prompers JJ 2008 Increased intramyocellular lipid content but normal skeletal muscle mitochondrial oxidative capacity throughout the pathogenesis of type 2 diabetes. *FASEB J* 22:3947–3955
31. Korach-André M, Gounarides J, Deacon R, Beil M, Sun D, Gao J, Laurent D 2005 Age and muscle-type modulated role of intramyocellular lipids in the progression of insulin resistance in nondiabetic Zucker rats. *Metabolism* 54:522–528
32. Gao J, Serrero G 1999 Adipose differentiation related protein (ADRP) expressed in transfected COS-7 cells selectively stimulates long chain fatty acid uptake. *J Biol Chem* 274:16825–16830
33. Imai Y, Varela GM, Jackson MB, Graham MJ, Crooke RM, Ahima RS 2007 Reduction of hepatosteatosis and lipid levels by an adipose differentiation-related protein antisense oligonucleotide. *Gastroenterology* 132:1947–1954
34. Varela GM, Antwi DA, Dhir R, Yin X, Singhal NS, Graham MJ, Crooke RM, Ahima RS 2008 Inhibition of ADRP prevents diet-induced insulin resistance. *Am J Physiol Gastrointest Liver Physiol* 295:G621–G628
35. Mayerson AB, Hundal RS, Dufour S, Lebon V, Befroy D, Cline GW, Enocksson S, Inzucchi SE, Shulman GI, Petersen KF 2002 The effects of rosiglitazone on insulin sensitivity, lipolysis, and hepatic and skeletal muscle triglyceride content in patients with type 2 diabetes. *Diabetes* 51:797–802
36. Schrauwen-Hinderling VB, Mensink M, Hesselink MK, Sels JP, Kooi ME, Schrauwen P 2008 The insulin-sensitizing effect of rosiglitazone in type 2 diabetes mellitus patients does not require improved in vivo muscle mitochondrial function. *J Clin Endocrinol Metab* 93:2917–2921
37. Moro C, Bajpeyi S, Smith SR 2008 Determinants of intramyocellular triglyceride turnover: implications for insulin sensitivity. *Am J Physiol Endocrinol Metab* 294:E203–E213