PPARγ regulates adipose triglyceride lipase in adipocytes in vitro and in vivo

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ADIPOSE TISSUE PLAYS A CRITICAL ROLE in energy homeostasis in higher organisms. Not only does it serve as the main site for energy storage in the form of triglycerides, but it also contributes to systemic glucose and lipid metabolism via its function as an endocrine organ (24). In the normal physiological state, excess fuel substrate is partitioned to adipose tissue, where it is stored as triglycerides until its subsequent release as nonesterified fatty acids (FAs) in the setting of increased metabolic fuel requirement. Pathophysiological disorders of adipose tissue such as obesity and lipodystrophy are associated with dysregulation of this process. As a result, excess FAs are released into the circulation and accumulate in extra-adipose tissue depots such as muscle and liver, ultimately contributing to dyslipidemia, insulin resistance, and overt diabetes (3).

Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of the steroidal/thyroidic/reinoid receptor superfamily of ligand-activated nuclear transcription factors and is enriched in adipose tissue, where it serves as an essential regulator of adipocyte differentiation and maintenance of the mature adipocyte phenotype (39, 45). PPARγ is the molecular target for thiazolidinedione (TZD) antidiabetic agents that improve insulin sensitivity, glucose tolerance, and lipid homeostasis in vivo (31, 52). A potential mechanism for these beneficial metabolic effects is the net partitioning of FAs from elsewhere in the body to adipose tissue for storage. This mechanism is supported by the finding that, in addition to its role in adipogenesis, PPARγ regulates a variety of adipocyte genes involved in virtually all pathways of lipid metabolism, including local FA release from circulating lipoproteins (40), FA uptake (9, 34), and glycerol/FAME recycling (15), lipid droplet stabilization (1), and lipid FA oxidation (4, 5).

A novel adipocyte triglyceride-specific lipase has recently been reported and is alternatively designated adipocyte triglyceride lipase (ATGL) (54), desnutrin (49), calcium-independent phospholipase A2ζ-c (20), or patatin-like phospholipase domain-containing protein A2 (HUGO Committee on Gene Nomenclature and the Mouse Genome Informatics Committee). ATGL is closely related to other patatin-like phospholipase domain-containing proteins, including adiponutrin, with which it shares the greatest homology. ATGL is induced during adipogenesis and remains highly expressed in mature adipocytes, where it localizes to lipid droplets and promotes the hydrolysis of long-chain FA triglycerides (20, 25, 44, 49, 54). Several studies (25, 42, 54) suggest that ATGL, rather than hormone-sensitive lipase (HSL), is the primary lipase responsible for this initial step of triglyceride hydrolysis and that ATGL may play in a serial fashion with HSL to hydrolyze tri- and diglycerides, respectively. Unlike HSL, ATGL activity is not directly regulated at the posttranslational level via protein kinase A-mediated phosphorylation but rather via association with its colipase CGI-58, a process that also indirectly involves perilipin A (14, 29, 33, 54). In addition to this posttranslational effect on ATGL activity, ATGL mRNA expression is also regulated by a variety of nutritional and hormonal factors, including glucocorticoids, insulin, fasting, and obesity due to
leptin deficiency and resistance (25, 49). Despite these findings, the transcriptional regulation of ATGL expression remains poorly characterized.

Several factors suggest that PPARγ regulates ATGL expression. First, ATGL plays a critical role in lipid metabolism, a process known to be regulated by PPARγ in multiple tissues (20, 49, 54). Even prior to its initial characterization (20, 49, 54), ATGL (then designated by its Riken ID no. 6010039C21) was identified as a gene that was induced sevenfold in liver of PPARγ-null mice overexpressing the PPARγ1 isoform exclusively in liver (53). In addition, the closely related protein adiponutrin, which is frequently reciprocally regulated by similar nutritional and hormonal factors as ATGL (2, 25, 49), is negatively regulated by the TZD PPAR agonist troglitazone in mature adipocytes (36). Although the ability of PPARγ to regulate ATGL mRNA or protein in cultured adipocytes has not yet been evaluated, ATGL mRNA is markedly induced during adipogenesis in parallel with PPARγ and known PPARγ target genes (49) and is reduced in parallel with PPARγ following TNF-α treatment (26). Several recent studies (12, 43) have demonstrated increased ATGL mRNA expression in adipose tissue of rodents treated with rosiglitazone.

In mice (12, 43), ATGL expression in adipose tissue of rodents treated with rosiglitazone cannot be excluded. Hence, the ability of PPARγ to directly regulate ATGL expression in adipose tissue of rodents treated with rosiglitazone, or during adipogenesis in parallel with PPARγ and known PPARγ target genes (49) and is reduced in parallel with PPARγ following TNF-α treatment (26). Several recent studies (12, 43) have demonstrated increased ATGL mRNA expression in adipose tissue of rodents treated with rosiglitazone. Nevertheless, the direct involvement of PPARγ in this process remains unknown. Kim et al. (26) have recently demonstrated transactivation of ATGL promoter-reporter constructs by PPARγ in adipose tissue (BAT) in vivo.

RNA interference. RNA interference by siRNA was performed as previously described (41). Proteins were separated in 10% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Amersham). Membranes were incubated with primary antibodies, antibodies, and small interfering RNAs (siRNA) were obtained from Sigma. Ciglitazone, rosiglitazone, troglitazone, pioglitazone, and glitazone are PPARγ agonists, antagonists, antibodies, and small interfering RNAs (siRNA) were active against both PPARγ1 and PPARγ2 isoforms of PPARγ.

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Materials and Methods

Cell culture experiments. 3T3-L1 cells (American Type Culture Collection) were grown to 2 days postconfluence in DMEM containing 10% calf serum (Invitrogen). Cells were induced to differentiate by changing medium to DMEM containing 10% FBS (Invitrogen), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma), 1 μM dexamethasone (Sigma), and 10 μg/ml bovine insulin (Sigma) (17). After 48 h and every 2 days thereafter, medium was replaced with maintenance medium containing DMEM with 10% FBS. Bovine insulin, cycloheximide, and N-(9-fluorenylmethoxycarbonyl)-t-leucine (FMOC) were obtained from Sigma. Ciglitazone, rosiglitazone, troglitazone, pioglitazone, 15-deoxy-D12,14-prostaglandin J2 (PGJ2), MCC-555, and GW-9662 were obtained from Cayman Chemicals. For in vitro experiments, fully differentiated 3T3-L1 adipocytes from days 12 to 14 of differentiation were treated with the above for the doses and times indicated. Differentiation of preadipocytes to fully differentiated 3T3-L1 adipocytes was >90% and not different among treatment groups as assessed by Oil Red O staining. For all experiments, PPARγ agonists, antagonists, antibodies, and small interfering RNAs (siRNA) were active against both PPARγ1 and PPARγ2 isoforms of PPARγ.

RNA interference. RNA interference by siRNA was performed as described (21, 25). Briefly, 3T3-L1 adipocytes on day 7 of differentiation were detached from culture dishes with 0.25% trypsin (Invitrogen) and 0.5 mg/ml collagenase D (Roche Diagnostics), washed twice, and resuspended in PBS. Control (siControl noninterfering control pool; Dharmacon) or murine PPARγ-specific (5′ CAACAG-GCCTCATGAGAAT; Dharmacon) siRNAs were delivered into adipocytes (2 nmol of each siRNA/1 million cells) by electroporation (NucleofectorII; Amaxa). Adipocytes were then mixed with DMEM containing 10% FBS and seeded onto multiwell plates. Cells were collected 48 h after electroporation (i.e., on day 9 of differentiation) for determination of mRNA and protein expression. Electroporation of 3T3-L1 adipocytes on day 7 and analysis of gene expression on day 9 of differentiation were selected on the basis of prior optimization experiments demonstrating effectiveness of this method for siRNA-mediated gene knockdown in adipocytes at this stage of differentiation (25). The efficiency of electroporation using this method was >95% based on fluorescence microscropy of cells electroporated with Cy3-siRNA (data not shown).

RNA extraction, reverse transcription, and gene expression analysis. Total RNA was extracted from homogenized tissues or cells using RNeasy lipid tissue mini kit with on-column DNase treatment (Qiagen). Reverse transcription (RT) of 1 μg of total RNA was performed using random decamers (RETROscript kit; Ambion). Gene expression was determined by quantitative PCR (qPCR; MX4000 Multiplex qPCR System, Stratagene). Reactions were performed in triplicate. 25 μl containing 2.5 μl of 1:100-diluted cDNA, 1× Taqman Universal PCR Master Mix (Applied Biosystems), and gene-specific primer-probe sets (Taqman Gene Expression Assays; Applied Biosystems). Reactions were run at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Gene expression was determined by the standard curve method and normalized to expression of 18S ribosomal RNA (Taqman Ribosomal RNA Control Reagents; Applied Biosystems) or 36B4 (forward 5′ TCATCCAGCAGTTGTTTGACA, reverse 5′ GGCAAGGAGCAACAGTT, probe 5′ FAM-AGAGAGGCTGCACTCTG-TAMRA) internal control genes. Appropriate analysis was performed to determine that expression of control genes was unchanged under the experimental conditions described. Accuracy of mRNA quantification was optimized by DNase treatment of samples, use of gene-specific primer-probe sets that span intron-exon boundaries, and verification of lack of amplification in no-RT and no-template controls.

Protein analysis. Protein isolation and analysis was performed as previously described (41). Proteins were separated in 10% SDS polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Amersham). Membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Amersham). Detection was performed using an enhanced chemiluminescent substrate kit (Amersham). Specificity of the ATGL antibody was confirmed using protein extracts that span intron-exon boundaries, and verification of lack of amplification in no-RT and no-template controls.

In vivo experiments. Mice were housed individually under standard conditions at 25°C with a 14:10 h light-dark cycle (lights on from 6:00 AM to 8:00 PM), with free access to food and water. Animals were handled in accordance with the guidelines established by the National Institutes of Health. Experimental procedures were approved by the Institutional Animal Care and Use Committee at Beth Israel Deaconess Medical Center. Mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Four-week-old mice were placed on either standard chow (6% fat wt/wt, RD8664; Harlan Teklad) or high-fat diet (HFD; 42% fat wt/wt, TD88137; Harlan Teklad) for 18 wk. Mice were then treated for 10 days with either 0.5% carboxymethyl cellulose vehicle (Fishier Scientific) or vehicle plus rosiglitazone (Avandia; GlaxoSmithKline) at 4 mg·kg−1·day−1 by oral gavage. Body weight and plasma glucose (One Touch FastTAC glucometer; LifeScan) were determined daily. Ad libitum-fed mice were then killed by CO2 inhalation. Trunk blood was collected by cardiac puncture, centrifuged, and stored at −20°C until
analysis. Tissues were collected, frozen in liquid nitrogen, and stored at −80°C until analysis. Serum insulin was determined using the Ultra-Sensitive Rat Insulin ELISA kit (Crystal Chem).

Statistical analysis. Data are expressed as means ± SE. To test the effect of TZD and non-TZD PPARγ agonist treatment on ATGL expression, a one-way ANOVA was conducted with post hoc analysis using Bonferroni’s multiple comparison test. To test time- and dose-response effects of drug treatment on gene expression and to determine the association between mRNA and protein expression, Pearson correlations were conducted. For experiments with only two groups, Student’s t-tests were conducted. For all other analyses, factorial ANOVA with two between-subject factors was conducted, followed by simple effects for pairwise comparisons if relevant. Comparisons were considered significant if the P value was <0.05.

RESULTS

ATGL mRNA and protein expression are induced in 3T3-L1 adipocytes by TZD and non-TZD PPARγ agonists. To evaluate the ability of PPARγ to regulate ATGL expression, fully differentiated 3T3-L1 adipocytes were treated with equimolar concentrations (10 μM) of various TZD and non-TZD PPARγ agonists (Fig. 1). ATGL mRNA expression (Fig. 1A) was significantly increased by the TZD PPARγ agonists rosiglitazone (2.55-fold), troglitazone (2.07-fold), ciglitazone (3.06-fold), pioglitazone (2.55-fold), troglitazone (2.07-fold), ciglitazone (3.06-fold), and MCC-555 (2.09-fold) as well as the structurally distinct non-TZD PPARγ agonists FMOC (2.03-fold) and PGJ2 (2.35-fold). ATGL mRNA was also increased by the non-TZD PPARγ agonists GW-7845 (data not shown). ATGL protein expression (Fig. 1B) was likewise significantly increased by the above TZD and non-TZD PPARγ agonists and positively correlated with mRNA expression (r = 0.862, P < 0.05). In contrast, adiponutrin mRNA, which is negatively regulated by TZD treatment in mature adipocytes (36), was negatively regulated by the above TZD and non-TZD PPARγ agonist (data not shown).

Induction of ATGL mRNA and protein expression by PPARγ agonists is dose and time dependent. To further evaluate the dose response and time course of the induction of ATGL by PPARγ agonists, fully differentiated 3T3-L1 adipocytes were treated with increasing doses of rosiglitazone (1–10,000 nM) for 24 h (n = 6/group for both mRNA and protein). Time course for ATGL mRNA (B), ATGL protein (D), and adiponutrin mRNA (F) fully differentiated 3T3-L1 adipocytes were treated with 100 nM Rosi for increasing time intervals (0–24 h) (n = 6/group). ATGL and adiponutrin mRNA expression were determined by qPCR, normalized to 18S ribosomal RNA, and expressed relative to the control group. P < 0.05 for effect of dose and time on gene expression for both ATGL and adiponutrin. ATGL protein expression was determined by Western blot analysis, with RAN as a loading control (representative blots shown). P < 0.05 for effect of dose and time on ATGL protein expression. ATGL protein expression correlated with mRNA expression (r = 0.894 and 0.864 for dose and time, respectively, P < 0.05).

Fig. 1. Effect of thiazolidinediones (TZD) and non-TZD peroxisome proliferator-activated receptor-γ (PPARγ) agonists on adipose triglyceride lipase (ATGL) mRNA and protein expression in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were treated with DMSO (Cont), rosiglitazone (Rosi), troglitazone (Trog), ciglitazone (Cig), pioglitazone (Pio), MCC-555 (MCC), N-(9-fluorenylmethoxycarbonyl)-L-leucine (FMOC), or 15-deoxyΔ12,14-PGJ2 (PGJ2) at 10 μM for 24 h (n = 6–7/group for both mRNA and protein). A: ATGL mRNA expression was determined by quantitative PCR (qPCR), normalized to 18S ribosomal RNA, and expressed relative to the control group. *P < 0.05 for comparisons between treatment and control groups. B: ATGL protein expression was determined by Western blot analysis using the Ran GTPase (RAN) as a loading control (representative blot shown). ATGL protein expression was highly correlated with mRNA expression (r = 0.862, P < 0.05) and was increased compared with the control group for all treatments (P < 0.05).

Fig. 2. Dose response and time course for ATGL and adiponutrin expression in 3T3-L1 adipocytes treated with rosiglitazone. Dose response for ATGL mRNA (A), ATGL protein (C), and adiponutrin mRNA (E): fully differentiated 3T3-L1 adipocytes were treated with increasing doses of Rosi from 0 to 10,000 nM for 24 h (n = 6/group for both mRNA and protein). Time course for ATGL mRNA (B), ATGL protein (D), and adiponutrin mRNA (F): fully differentiated 3T3-L1 adipocytes were treated with 100 nM Rosi for increasing time intervals from 0 to 24 h (n = 6/group). ATGL and adiponutrin mRNA expression were determined by qPCR, normalized to 18S ribosomal RNA, and expressed relative to the control group. P < 0.05 for effect of dose and time on gene expression for both ATGL and adiponutrin. ATGL protein expression was determined by Western blot analysis, with RAN as a loading control (representative blots shown). P < 0.05 for effect of dose and time on ATGL protein expression. ATGL protein expression correlated with mRNA expression (r = 0.894 and 0.864 for dose and time, respectively, P < 0.05).
expression, \( r = 0.895 \) and 0.864 for dose and time, respectively, \( P < 0.05 \)). The induction of ATGL by rosiglitazone was rapid, with a measurable increase as early as 6 h for mRNA and 6–12 h for protein. The median effective dose (ED\(_{50}\)) for rosiglitazone induction of ATGL expression was between 10 and 100 nM, consistent with the reported in vitro binding affinity of rosiglitazone for PPAR\(\gamma\) (31). ATGL mRNA and protein expression were also increased in a dose-dependent manner by the other TZD and non-TZD PPAR\(\gamma\) agonists noted above, with potencies in agreement with previous reports (data not shown) (19, 28, 31, 37, 38, 51).

Phosphoenolpyruvate carboxykinase, a gene positively regulated by PPAR\(\gamma\) (47), was also increased in a dose- and time-dependent manner (dose response: \( 1.00 \pm 0.02 \) for untreated control \( \rightarrow 5.28 \pm 0.14 \) for 10,000 nM rosiglitazone, \( r = 0.530, P < 0.05 \); time course: \( 1.00 \pm 0.02 \) at \( 0 \), \( 5.41 \pm 0.16 \) at 24 h, \( r = 0.982, P < 0.05 \)), thus confirming the effectiveness of the treatment. In contrast, adiponutrin, which is induced during adipogenesis (2) but negatively regulated by TZD treatment in mature adipocytes (36), was decreased in a dose- (Fig. 2E) and time-dependent (Fig. 2F) manner (dose response: \( r = -0.550, P < 0.05 \); time course: \( r = -0.938, P < 0.05 \)), thus supporting TZD-specific regulation of ATGL in mature adipocytes rather than a nonspecific effect of adipocyte differentiation on ATGL expression.

PPAR\(\gamma\) agonist-mediated induction of ATGL mRNA expression in 3T3-L1 adipocytes does not require protein synthesis. The rapid induction of ATGL by PPAR\(\gamma\) agonists suggested the possibility of direct transcriptional regulation of ATGL without the need for intervening protein synthesis. To evaluate whether this PPAR\(\gamma\) agonist-mediated induction of ATGL requires protein synthesis, fully differentiated 3T3-L1 adipocytes were pretreated with DMSO vehicle or 5 \( \mu \)g/ml cycloheximide for 30 min, followed by addition of DMSO or 100 nM rosiglitazone for an additional 12 h (Fig. 3). Rosiglitazone treatment significantly increased ATGL mRNA expression.

Treatment with cycloheximide decreased basal ATGL mRNA expression but did not inhibit rosiglitazone-mediated induction of ATGL mRNA expression.

PPAR\(\gamma\) agonist-mediated induction of ATGL mRNA and protein expression in 3T3-L1 adipocytes is inhibited by the PPAR\(\gamma\) antagonist GW-9662. To determine whether PPAR\(\gamma\) agonist-mediated induction of ATGL expression is mediated by PPAR\(\gamma\), the ability of rosiglitazone to induce ATGL mRNA and protein expression was determined in the presence of the PPAR\(\gamma\)-specific antagonist GW-9662. GW-9662 acts as a potent full antagonist of PPAR\(\gamma\) (IC\(_{50}\) 7.6 nM) via irreversible covalent modification of a cysteine residue (Cys\(_{285}\)) in PPAR\(\gamma\)'s ligand-binding domain (30). Fully differentiated 3T3-L1 adipocytes were pretreated with increasing doses of GW-9662 (0.1, 1, 10 \( \mu \)M) for 12 h, followed by treatment with 10 \( \mu \)M GW-9662 alone or 100 nM rosiglitazone plus 0, 0.1, or 10 \( \mu \)M GW-9662 for an additional 24 h (Fig. 4). The dose of rosiglitazone (100 nM) was selected on the basis of the proximity of this dose to the reported in vitro binding affinity of rosiglitazone for PPAR\(\gamma\) and the ability of this dose to produce a significant induction of ATGL expression (31). The dose range for GW-9662 (0.1–10 \( \mu \)M) was selected on the basis of previously published concentration-response analysis of GW-9662 antagonism of 100 nM rosiglitazone in a cell-based functional assay for PPAR\(\gamma\) (30). Treatment with GW-9662 inhibited this rosiglitazone-mediated induction of ATGL mRNA expression (Fig. 4A) in a dose-dependent manner (\( r = -0.677, P < 0.05 \)). ATGL protein expression (Fig. 4B) was also significantly inhibited by GW-9662 treatment (\( r = -0.750, P < 0.05 \)) and was positively correlated with mRNA expression (\( r = 0.887, P < 0.05 \)).

siRNA-mediated knockdown of PPAR\(\gamma\) reduces ATGL mRNA and protein expression in 3T3-L1 adipocytes. To further confirm whether PPAR\(\gamma\) is specifically required for ATGL expression in fully differentiated 3T3-L1 adipocytes, 3T3-L1 adipocytes on day 9 of differentiation were evaluated for ATGL expression 48 h after electroporation with PPAR\(\gamma\)-specific siRNA (Fig. 5). Electroporation of 3T3-L1 adipocytes on day 7 and analysis of gene expression on day 9 of differentiation were selected on the basis of prior optimization experiments demonstrating effectiveness of this method for siRNA-mediated gene knockdown in adipocytes at this stage of differentiation (25). Furthermore, previous experiments have shown that ATGL is induced during adipogenesis with maximal expression after day 5 of differentiation, and its expression is maintained at least through day 14 of differentiation (data not shown and Ref. 25). Induction of ATGL by rosiglitazone was observed in fully differentiated adipocytes from as early as day 8 to as late as day 14 of differentiation (data not shown). Thus, rosiglitazone-mediated induction of ATGL does not appear to depend on stage of differentiation. After siRNA treatment, PPAR\(\gamma\) protein (both PPAR\(\gamma\)-1 and PPAR\(\gamma\)-2 isoforms) was significantly reduced in adipocytes treated with PPAR\(\gamma\) siRNA (Fig. 5A). This siRNA-mediated knockdown of PPAR\(\gamma\) resulted in a \( >95\% \) (20-fold) reduction in ATGL mRNA expression (Fig. 5B) and an \( \sim 50\% \) reduction in ATGL protein expression at 48 h posttreatment (Fig. 5A). These results further indicate that the rate of ATGL protein induction by PPAR\(\gamma\) activation (Fig. 2D) is more rapid that its rate of disappearance following removal of PPAR\(\gamma\) activation and that

![Fig. 3](http://ajpendo.physiology.org/)

Effect of cycloheximide (CHX) on Rosi-mediated regulation of ATGL mRNA expression in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were pretreated with either DMSO or 5 \( \mu \)g/ml CHX for 30 min, followed by addition of DMSO or 100 nM Rosi for an additional 12 h (n = 7/group). ATGL mRNA expression was determined by qPCR, normalized to 18S ribosomal RNA, and expressed relative to the control group. \( P < 0.05 \) for overall effect of Rosi treatment on ATGL expression. \( *P < 0.05 \) for the effect of Rosi within the same CHX treatment; \( \dagger P < 0.05 \) for the effect of CHX within the same Rosi treatment. ns, Not significant.
adipose tissue (PGAT; Fig. 6A) and BAT (Fig. 6B) depots. This effect was significant across all three treatment groups, with rosiglitazone treatment increasing ATGL mRNA expression by 1.82-, 1.55-, and 2.83-fold in PGAT and 1.46-, 1.48-, and 2.49-fold in BAT for the chow-fed Lep^{ob/ob}, HFD-fed Lep^{ob/ob}, and chow-fed Lep^{ob/ob} groups, respectively. There was also a significant effect of genotype on ATGL expression with decreased ATGL mRNA expression in both PGAT and BAT of obese leptin-deficient Lep^{ob/ob} mice compared with Lep^{ob/ob} controls, consistent with previous reports of decreased ATGL mRNA expression in WAT of Lep^{ob/ob} mice (49). However, there was no significant effect of diet on ATGL mRNA expression in either PGAT or BAT. Statistical comparisons between groups were similar for ATGL protein expression, which was highly correlated with mRNA expression (r = 0.888 and 0.886 for PGAT (Fig. 6C) and BAT (Fig. 6D), respectively, P < 0.05).

**DISCUSSION**

This study identifies ATGL as a PPARγ target gene in adipocytes. Previous studies (49) have demonstrated induction of ATGL during adipogenesis in parallel with PPARγ and known PPARγ target genes, suggesting a potential role for PPARγ in regulating ATGL expression during adipogenesis. ATGL is also highly expressed in mature adipocytes, where it hydrolyzes the first ester bond of triglycerides (20, 25, 44, 49, 54). This critical role of ATGL in adipocyte lipid metabolism

PPARγ-dependent ATGL protein expression is more stable than mRNA expression.

*Pharmacological activation of PPARγ by rosiglitazone induces ATGL mRNA and protein expression in white and brown adipose tissue in vivo.* To evaluate the effect of pharmacological activation of PPARγ on ATGL expression in vivo, ATGL mRNA and protein expression were determined in white and brown adipose tissue of mice with or without obesity due to either HFD or leptin deficiency (Lep^{ob/ob}) that were treated with 4 mg·kg⁻¹·day⁻¹ oral rosiglitazone (Table 1 and Fig. 6). Phenotypic parameters of mice before and after rosiglitazone treatment are shown in Table 1. As expected, Lep^{ob/ob} mice and HFD-fed mice had higher body weights and serum insulin levels than control mice. Rosiglitazone treatment had a significant overall effect on change in body weight, change in plasma glucose, and serum insulin, with the greatest effect in the Lep^{ob/ob} group. The observed increase in body weight, decrease in plasma glucose, and decrease in serum insulin are consistent with known physiological effects of TZD treatment in rodents, thus confirming the effectiveness of the treatment (7, 8).

There was an overall significant effect of rosiglitazone treatment on ATGL mRNA expression in both perigonadal

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**Fig. 4.** Effect of the PPARγ antagonist GW-9662 (GW) on Rosi-mediated regulation of ATGL mRNA and protein expression in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were treated with DMSO, 10 μM GW alone, 100 nM Rosi alone, or 100 nM Rosi plus either 0.1 or 10 μM GW (n = 3/group for both mRNA and protein). A: ATGL mRNA expression was determined by qPCR, normalized to 18S ribosomal RNA, and expressed relative to the control group. *P < 0.05 for overall effect of Rosi treatment on ATGL expression. †P < 0.05 for the effect of Rosi within the same GW treatment. ‡P < 0.05 for the effect of GW within the same Rosi treatment. P < 0.05 for dose effect of GW on inhibition of Rosi-mediated induction of ATGL expression. B: ATGL protein expression was determined by Western blot analysis, with RAN as a loading control (representative blot shown). ATGL protein expression correlated with mRNA expression (r = 0.887, P < 0.05). Significance of statistical comparisons between groups were the same as stated for mRNA.

**Fig. 5.** Effect of small interfering RNA (siRNA)-mediated knockdown of PPARγ ATGL mRNA and protein expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were electroporated in the presence of either control or PPARγ-specific siRNAs on day 0 of differentiation. Cells were collected 48 h later (day 9 of differentiation) for determination of mRNA (3 experiments with n = 3 each) and protein expression (n = 2). A: PPARγ and ATGL protein expression were determined by Western blot analysis, with RAN as a loading control (representative blot shown). B: ATGL mRNA expression was determined by qPCR, normalized to 36B4 RNA, and expressed relative to ATGL expression in the control group. *P < 0.05.
suggests that PPARγ may also regulate ATGL expression in mature adipocytes as well. This possibility is supported by the finding that the closely related protein adiponutrin is negatively regulated by the PPARγ agonist troglitazone in mature adipocytes in vitro (36). However, direct regulation of ATGL mRNA and protein expression by PPARγ in fully differentiated adipocytes in vitro has not previously been evaluated. In the present study, we demonstrate for the first time that both ATGL mRNA and protein expression are induced by TZD and non-TZD PPARγ agonists in fully differentiated adipocytes and that this regulation directly involves PPARγ. We also demonstrate that ATGL mRNA and protein expression are increased in WAT and BAT of both lean and obese mice following oral treatment with the PPARγ agonist rosiglitazone, consistent with previous reports (12, 43). We further demonstrate for the first time that ATGL protein expression is highly correlated with mRNA expression. Hence, PPARγ positively regulates both ATGL mRNA and protein expression in adipocytes in vitro and in vivo.

Although previous studies (12, 43) have demonstrated TZD-mediated induction of ATGL in rodents, the direct involvement of PPARγ in this process was unclear. A PPARγ-dependent mechanism for induction of ATGL expression is supported by several in vitro findings in our study. First, ATGL mRNA and protein expression were increased in WAT and BAT of both lean and obese mice following oral treatment with the PPARγ agonist rosiglitazone, consistent with previous reports (12, 43).

Table 1. Phenotypic analysis of mice treated with Rosi

<table>
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<th>Group (Treatment)</th>
<th>Weight, g</th>
<th>Glucose, mg/dl</th>
<th>Insulin, ng/ml</th>
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<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Change</td>
</tr>
<tr>
<td>Lepob/+ chow fed</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Vehicle</td>
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<tr>
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<tr>
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<tr>
<td>Lepob/+ chow fed</td>
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<tr>
<td>Vehicle</td>
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<tr>
<td>Rosi</td>
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<td>73.64±1.57</td>
<td>+2.57±0.81</td>
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</table>

Values are means ± SE. Rosi, rosiglitazone; HFD, high-fat diet. Chow-fed Lepob/+ HFD-fed Lepob/+ or chow-fed Lepob/+ mice were treated with either carboxymethyl cellulose vehicle or Rosi at 4 mg·kg⁻¹·day⁻¹ for 10 days by oral gavage (n = 6–8/group). Body weight and plasma glucose were measured before and after treatment. Serum insulin was measured after treatment. P < 0.05 for effect of Rosi treatment on change in weight, change in plasma glucose, and serum insulin.
protein expression are induced by several structurally distinct TZD and non-TZD PPAR-γ agonists. Second, this induction of ATGL mRNA and protein expression is dose and time dependent. With regard to the former, the ED_{50} for induction of ATGL expression by rosiglitazone corresponds to its reported in vitro binding affinity for PPAR-γ (31), and the ED_{50}s for several other TZD and non-TZD PPAR-γ agonists are also consistent with their reported potencies (19, 28, 31, 37, 38, 51). With respect to the latter, the time for induction of ATGL by PPAR-γ agonist is rapid, with a detectable increase as early as 6 h for mRNA and 6–12 h for protein. Third, PPAR-γ agonist-mediated induction of ATGL mRNA expression is not inhibited by the protein synthesis inhibitor cycloheximide, suggesting that the increase in ATGL expression does not require intervening protein synthesis. Fourth, PPAR-γ agonist-mediated induction of ATGL mRNA and protein expression is inhibited by the PPAR-γ-specific antagonist GW-9662, a highly specific potent full antagonist of PPAR-γ (IC_{50} 7.6 nM) that acts via irreversible covalent modification of a cysteine residue (Cys^{285}) in PPAR ligand-binding domain (30), thus suggesting that interaction of ligand with PPAR-γ is required for the induction of ATGL. Finally, siRNA-mediated knockdown of PPAR-γ in fully differentiated 3T3-L1 adipocytes dramatically reduced ATGL mRNA expression by greater than 95% and also significantly reduced ATGL protein expression by approximately 50% at 48 h, thereby providing direct genetic evidence for PPAR-γ’s involvement in ATGL expression. In addition to the above experimental evidence, Yu et al. (53) have shown that ATGL is upregulated in liver of PPAR-γ-null mice over-expressing PPAR-γ1 exclusively in liver. ATGL has also been shown (12, 43) to be increased in rodent adipose tissue. More recently, Kim et al. (26) have demonstrated transactivation of an ATGL promoter-reporter construct by PPAR-γ. Taken together, these data provide strong evidence to support PPAR-γ-dependent regulation of ATGL expression.

PPAR-γ binds to specific DNA response elements, PPAR response elements (PPREs), as heterodimeric with RXR. PPREs most commonly consist of a direct repeat of the hexanucleotide sequence AGGTCA separated by a single nucleotide, also known as direct repeat 1, or DR1 (11). Such PPREs have been identified for several PPAR-γ target genes involved in adipocyte lipid metabolism (11). A recent study experimentally evaluating the upstream ATGL promoter region from position −2,979 to +21 for transactivation by PPAR-γ/RXRα using luciferase reporter constructs identified a 2.5- to 4.8-fold increase in transcriptional activity for promoter constructs containing sequence upstream of position −795 (26). However, indirect effects cannot be excluded. Furthermore, although this region of the ATGL promoter (position −2,979 to −795) contains several putative PPAR-γ half-sites, the specific PPAR-γ-binding elements conferring this PPAR-γ responsiveness have not yet been identified (26). Our study provides evidence to support the direct involvement of PPAR-γ in the regulation of ATGL expression in a metabolically relevant cell type (i.e., mature adipocytes). PPAR-γ may mediate its transcriptional effects via sequences within the region analyzed in the above study or via PPREs outside this region. For example, the PPAR-γ-regulated gene Acyl-CoA-binding protein is activated by a PPRE within its first intron (18). Given the size of ATGL’s first intron, we performed a more extensive computer analysis of a 4,000-base pair (bp) upstream sequence of the murine ATGL start codon as well as the first intron of the ATGL gene using mutiTF (http://mulan.dcode.org). This analysis revealed several additional putative PPAR-γ-binding sites, three of which are highly conserved between mouse and human (position −3,021 to −3,009, +1,151 to +1,171, and +1,731 to +1,747 bp relative to the murine ATGL start codon). However, additional experimental evaluation of these sites is required to define their potential contribution to PPAR-γ-mediated transcriptional regulation of ATGL expression.

PPAR-γ regulates many adipocyte genes involved in lipid metabolism and energy balance (11), and PPREs mediating these effects have been identified for several of these genes (1, 4–6, 9, 13, 15, 18, 22, 27, 34, 40, 47). The genes regulated and directionality of this regulation suggest that PPAR-γ generally promotes FA storage and/or oxidation. This finding has been supported by studies evaluating the effect of PPAR-γ on FA and glycerol uptake and release in white and brown adipocytes (15, 45, 46, 48). Furthermore, previous studies (25, 54) have demonstrated that increasing ATGL expression in adipocytes increases net glycerol and FA release, whereas decreasing ATGL expression has the opposite effect. Although PPAR-γ-mediated induction of ATGL represents a potential mechanism to increase FA supply for oxidation in brown adipocytes, the reason why PPAR-γ would positively regulate a gene critical to lipolysis in white adipocytes is less clear. Other lipolytic enzymes such as HSL and monoglyceride lipase have also been reported to be increased by PPAR-γ activation in differentiated adipocytes and/or adipose tissue in some (32, 45, 50) but not all (10, 43) studies. One possibility is that the increased ATGL expression may reflect a PPAR-γ-mediated increase in preadipocyte differentiation or a general effect of PPAR-γ on maintaining expression of adipocyte-specific genes in mature adipocytes. However, this conclusion is not supported by the observation that adiponutrin, a closely related gene that is also induced during adipogenesis and expressed in mature adipocytes, is decreased by rosiglitazone in a dose- and time-dependent manner in 3T3-L1 adipocytes in our study.

Nevertheless, adipocyte lipid homeostasis may be more complex than previously thought. It is possible that PPAR-γ may simultaneously increases adipose triglyceride hydrolysis and lipogenesis/FA reesterification, with the latter effect predominating under most but not all conditions. For example, in vivo kinetic studies in rats (23, 35) have shown that PPAR-γ activation may paradoxically enhance adipocyte lipolysis in certain physiological states, such as fasting. This finding has been supported by a recent study (12) demonstrating that rosiglitazone may increase basal and stimulate lipolysis in rat WAT explants despite a reduction in plasma nonesterified FAs. PPAR-γ has also been shown to increase expression of adipocyte glycerol kinase, thereby allowing for a “futile cycle” of triglyceride hydrolysis and reesterification (15), and PPAR-γ-mediated induction of ATGL may contribute to this futile cycle. To further complicate the matter, human but not murine ATGL has been shown to have transacylase as well as triacylglycerol hydrolyase activity, the physiological relevance of which remains to be determined. In addition, other functions of ATGL with respect to FA turnover and partitioning may not be known. An ATGL cofactor, CGI-58, that markedly modulates ATGL-mediated triglyceride hydrolyase activity at the post-translation level has recently been described (14, 29, 33, 54), and this CGI-58-mediated modulation of ATGL activity indi-
rectly involves perilipin A. It is possible that PPARγ-mediated regulation of ATGL sets the “lipolytic tone” of the cell, whereas regulation of CGI-58 or some other factor influences acute regulation of ATGL activity. Hence, the precise role of novel lipases such as ATGL and their regulation by PPARγ in adipocyte lipid homeostasis requires further evaluation.

With regard to the in vivo regulation of ATGL by PPARγ, this study demonstrates increased ATGL mRNA and protein expression in WAT and BAT of mice with and without obesity due to HFD or leptin deficiency (Lepob/ob mice). These findings corroborate previous studies (12, 43) demonstrating rosiglitazone-mediated induction of ATGL mRNA expression in chow-fed rodent adipose tissue. However, in contrast to the study by Shen et al. (43), we also demonstrate a significant increase in ATGL mRNA expression in response to rosiglitazone treatment in WAT and BAT of mice with obesity due to HFD feeding. This difference may be due to differences in diet composition, duration of high-fat feeding, dose/mode of rosiglitazone administration, and/or mouse background strain. We also extended the evaluation of the effects of rosiglitazone on ATGL expression to mouse with obesity due to leptin deficiency (Lepob/ob mice) and found induction of ATGL mRNA and protein expression in response to rosiglitazone treatment in these mice as well. Of note, in addition to the above data related to PPARγ-mediated regulation of ATGL, our data are consistent with previous reports of decreased ATGL expression in WAT of animal models with obesity specifically expressed in the adipose lineage. Hence, the precise relationship between adipose ATGL expression/activation increases ATGL mRNA expression in fully differentiated 3T3-L1 adipocytes in vitro and in vivo and provides evidence to support the direct involvement of PPARγ in this process. It also demonstrates that ATGL mRNA and protein expression are highly correlated under several different experimental conditions. PPARγ activation increases ATGL mRNA expression in fully differentiated 3T3-L1 adipocytes in vitro and in both BAT and WAT in vivo. A direct transcriptional mechanism for this effect is supported by in vitro findings demonstrating regulation of ATGL by distinct TZD and non-TZD PPARγ agonists, time and dose dependence of induction, inhibition by the PPARγ-specific antagonist GW-9662, and lack of inhibition by the protein synthesis inhibitor cycloheximide. The dependence of ATGL expression on PPARγ activation is further supported by the significant reduction in ATGL mRNA and protein expression following siRNA-mediated knockdown of PPARγ. Nevertheless, additional studies evaluating transcription per se are required to more clearly define the precise mechanisms by which PPARγ regulates ATGL expression in adipocytes as well as other tissues. In addition, the contribution of PPARγ-mediated regulation of ATGL to adipocyte-specific and whole body lipolysis requires further exploration. Characterization of the function and regulation of ATGL and related patatin-like phospholipase domain-containing proteins such as adiponutrin remain in the early stages. However, the central role of both PPARγ and ATGL in lipid metabolism suggests that regulation of ATGL by PPARγ may have important implications in pathogenesis and treatment of obesity and related disorders of lipid homeostasis.

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REFERENCES

1. Arimura N, Horiba T, Imagawa M, Shimizu M, Sato R. The peroxi-
3. Bays H, Mandarino L, DeFronzo RA. Role of the adipocyte, free fatty acids, and ectopic fat in pathogenesis of type 2 diabetes mellitus: peroxi-
osomal proliferator-activated receptor agonist receptors provide a rational therapeu-
4. Bogacka I, Ukropcova B, McNell M, Gimble JM, Smith SR. Structural and functional consequences of mitochondrial biogenesis in human adi-
6. Castelein H, Gulick T, Declercq PE, Mannaaerts GP, Moore DD, Baes MI. The peroxisome proliferator activated receptor regulates malic en-
10. Deng T, Shan S, Li PP, Shen ZF, Lu XP, Cheng J, Ning ZQ. Peroxisome proliferator-activated receptor-gamma transcriptionally up-
13. Frohme B, Hui TY, Bernlohr DA. Identification of a functional peroxisome proliferator-responsive element in the murine fatty acid trans-


