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PPAR γ regulates adipose triglyceride lipase in adipocytes in vitro and in vivo

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Abstract

Peroxisome proliferator-activated receptor- γ (PPAR γ) regulates adipocyte genes involved in adipogenesis and lipid metabolism and is the molecular target for thiazolidinedione (TZD) antidiabetic agents. Adipose triglyceride lipase (ATGL) is a recently described triglyceride-specific lipase that is induced during adipogenesis and remains highly expressed in mature adipocytes. This study evaluates the ability of PPAR γ to directly regulate ATGL expression in adipocytes in vitro and in vivo. In fully differentiated 3T3-L1 adipocytes, ATGL mRNA and protein are increased by TZD and non-TZD PPAR γ agonists in a dose- and time-dependent manner. Rosiglitazone-mediated induction of ATGL mRNA is rapid and is not inhibited by the protein synthesis inhibitor cycloheximide, indicating that intervening protein synthesis is not required for this effect. Rosiglitazone-mediated induction of ATGL mRNA and protein is inhibited by the PPAR γ -specific antagonist GW-9662 and is also significantly reduced following siRNA-mediated knockdown of PPAR γ , supporting the direct transcriptional regulation of ATGL by PPAR γ . In vivo, ATGL mRNA and protein are increased by rosiglitazone treatment in white and brown adipose tissue of mice with and without obesity due to high-fat diet or leptin deficiency. Thus, PPAR γ positively regulates ATGL mRNA and protein expression in mature adipocytes in vitro and in adipose tissue in vivo, suggesting a role for ATGL in mediating PPAR γ 's effects on lipid metabolism.

Keywords

peroxisome proliferator-activated receptor- γ ; thiazolidinediones; desnutrin; patatin-like phospholipase domain-containing protein A2; calcium-independent phospholipase A₂- ζ

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 (PPAR γ) is a member of the steroid/thyroid/retinoid 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). PPAR γ 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, PPAR γ 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), FA and glycerol transport (13,27), intracellular FA binding (18), glyceroneogenesis (47), FA synthesis (6,22), glycerol/FA recycling (15), lipid droplet stabilization (1), and FA oxidation (4,5).

A novel adipocyte triglyceride-specific lipase has recently been reported and is alternatively designated adipose triglyceride lipase (ATGL) (54), desnutrin (49), calcium-independent phospholipase A₂- ζ (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 work 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 PPAR γ regulates ATGL expression. First, ATGL plays a critical role in lipid metabolism, a process known to be regulated by PPAR γ in multiple tissues (20, 49,54). Even prior to its initial characterization (20,49,54), ATGL (then designated by its Riken ID no. 0610039C21) was identified as a gene that was induced sevenfold in liver of PPAR γ -null mice overexpressing the PPAR γ 1 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 PPAR γ to regulate ATGL mRNA or protein in cultured adipocytes has not yet been evaluated, ATGL mRNA is markedly induced during adipogenesis in parallel with PPAR γ and known PPAR γ target genes (49) and is reduced in parallel with PPAR γ 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 PPAR γ in this process remains unknown. Kim et. al. (26) have recently demonstrated transactivation of ATGL promoter-reporter constructs by prostaglandin J₂ in HeLa cells cotransfected with PPAR γ and retinoid X receptor (RXR) α , but the specific elements mediating this effect have not yet been

identified and indirect effects cannot be excluded. Hence, the ability of PPAR γ to directly regulate endogenous ATGL mRNA and protein expression in a metabolically relevant cell type remains unknown. Here, we evaluate the ability of PPAR γ to directly regulate ATGL mRNA and protein expression in fully differentiated 3T3-L1 adipocytes in vitro and in murine white (WAT) and brown adipose tissue (BAT) in vivo.

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 μ M dexamethasone (Sigma), and 10 μ g/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)-L-leucine (Fmoc) were obtained from Sigma. Ciglitazone, rosiglitazone, troglitazone, pioglitazone, 15-deoxy- $\Delta^{12,14}$ -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 adipocytes was >90% and not different among treatment groups as assessed by Oil Red O staining. For all experiments, PPAR γ agonists, antagonists, antibodies, and small interfering RNAs (siRNA) were active against both PPAR γ 1 and PPAR γ 2 isoforms of PPAR γ .

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 PPAR γ -specific (5' CAACAGGCCTCATGAAGAATT; 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 reseeded 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 microscopy 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 μ g 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 in 25 μ l containing 2.5 μ l of 1:100-diluted cDNA, 1 $\times$ Taqman Universal PCR Master Mix (Applied Biosystems), and genespecific 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' TCATCCAGCAGGTGTTTGACA, reverse 5' GGCACCGAGGCAACAGTT, probe 5'

FAM-AGAGCAGGCCCTGCACTCTCG-TAMRA) internal control genes. Appropriate analysis was performed to determine that expression of control genes was unchanged under the experimental conditions described. Accuracy of RNA 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 primary antibody for PPAR γ (PPAR γ E8; Santa Cruz Biotechnology), ATGL (rabbit monoclonal antibody; Cell Signaling Technology), or the Ran GTPase (BD Biosciences) according to the manufacturer's instructions. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (Amersham). Detection was performed using an enhanced chemiluminescent substrate kit (Amersham). Quantification was performed using a Gene Gnome chemiluminescence recording system and GeneTools version 3.04 (SynGene, Cambridge, UK). Specificity of the ATGL antibody was confirmed using protein extracts derived from adipose tissue of mice, with global targeted deletion of ATGL as a negative control (16).

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. *Lep^{ob/ob}* and *Lep^{?ob/+}* mice were obtained 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 (Fisher Scientific) or vehicle plus rosiglitazone (Avandia; GlaxoSmithKline) at 4 mg·kg⁻¹·day⁻¹ by oral gavage. Body weight and plasma glucose (One Touch FastTake 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 $\pm$ SE. To test the effect of TZD and non-TZD PPAR γ 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 PPAR γ agonists

To evaluate the ability of PPAR γ to regulate ATGL expression, fully differentiated 3T3-L1 adipocytes were treated with equimolar concentrations (10 μ M) of various TZD and non-TZD PPAR γ agonists (Fig. 1). ATGL mRNA expression (Fig. 1A) was significantly increased by the TZD PPAR γ agonists rosiglitazone (2.55-fold), troglitazone (2.07-fold), ciglitazone (3.06-fold), pioglitazone (4.21-fold), and MCC-555 (2.09-fold) as well as the structurally distinct non-TZD PPAR γ agonists FMOC (2.03-fold) and PGJ₂ (2.35-fold). ATGL mRNA was also increased by the non-TZD PPAR γ agonists GW-7845 (data not shown). ATGL protein expression (Fig. 1B) was likewise significantly increased by the above TZD and non-TZD PPAR γ 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 PPAR γ agonist (data not shown).

Induction of ATGL mRNA and protein expression by PPAR γ agonists is dose and time dependent

To further evaluate the dose response and time course of the induction of ATGL by PPAR γ agonists, fully differentiated 3T3-L1 adipocytes were treated with increasing doses of rosiglitazone (1–10,000 nM) for 24 h or with 100 nM rosiglitazone for increasing time intervals (0–24 h) (Fig. 2). ATGL mRNA expression was significantly increased in a dose- (Fig. 2A) and time-dependent (Fig. 2B) manner (dose response: $r = 0.598$, $P < 0.05$; time course: $r = 0.896$, $P < 0.05$). ATGL protein expression was likewise significantly increased in a dose- (Fig. 2C) and time-dependent (Fig. 2D) manner and positively correlated with mRNA expression (for correlation between mRNA and protein 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 6 h for mRNA and 6–12 h for protein. The median effective dose (ED₅₀) for rosiglitazone induction of ATGL expression was between 10 and 100 nM, consistent with the reported in vitro binding affinity of rosiglitazone for PPAR γ (31). ATGL mRNA and protein expression were also increased in a dose-dependent manner by the other TZD and non-TZD PPAR γ 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 γ (47), was also increased in a dose- and time-dependent manner (dose response: 1.00 ± 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 ± 0.02 at 0 h $\rightarrow 5.41 \pm 0.16$ 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 γ agonist-mediated induction of ATGL mRNA expression in 3T3-L1 adipocytes does not require protein synthesis

The rapid induction of ATGL by PPAR γ agonists suggested the possibility of direct transcriptional regulation of ATGL without the need for intervening protein synthesis. To evaluate whether this PPAR γ agonist-mediated induction of ATGL requires protein synthesis, fully differentiated 3T3-L1 adipocytes were pretreated with DMSO vehicle or 5 μ 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 γ agonist-mediated induction of ATGL mRNA and protein expression in 3T3-L1 adipocytes is inhibited by the PPAR γ antagonist GW-9662

To determine whether PPAR γ agonist-mediated induction of ATGL expression is mediated by PPAR γ , the ability of rosiglitazone to induce ATGL mRNA and protein expression was determined in the presence of the PPAR γ -specific antagonist GW-9662. GW-9662 acts as a potent full antagonist of PPAR γ (IC₅₀ 7.6 nM) via irreversible covalent modification of a cysteine residue (Cys²⁸⁵) in PPAR γ 's ligand-binding domain (30). Fully differentiated 3T3-L1 adipocytes were pretreated with increasing doses of GW-9662 (0, 0.1, 10 μ M) for 12 h, followed by treatment with 10 μ M GW-9662 alone or 100 nM rosiglitazone plus 0, 0.1, or 10 μ 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 γ and the ability of this dose to produce a significant induction of ATGL expression (31). The dose range for GW-9662 (0.1–10 μ 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 γ (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 γ reduces ATGL mRNA and protein expression in 3T3-L1 adipocytes

To further confirm whether PPAR γ 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 γ -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 γ protein (both PPAR γ 1 and PPAR γ 2 isoforms) was significantly reduced in adipocytes treated with PPAR γ siRNA (Fig. 5A). This siRNA-mediated knockdown of PPAR γ resulted in a >95% (20-fold) reduction in ATGL mRNA expression (Fig. 5B) and an ~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 γ activation (Fig. 2D) is more rapid than its rate of disappearance following removal of PPAR γ activation and that PPAR γ -dependent ATGL protein expression is more stable than mRNA expression.

Pharmacological activation of PPAR γ by rosiglitazone induces ATGL mRNA and protein expression in white and brown adipose tissue in vivo

To evaluate the effect of pharmacological activation of PPAR γ 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 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/+}*, HFD-fed *Lep^{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/+}* 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 PPAR γ target gene in adipocytes. Previous studies (49) have demonstrated induction of ATGL during adipogenesis in parallel with PPAR γ and known PPAR γ target genes, suggesting a potential role for PPAR γ 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 suggests that PPAR γ 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 PPAR γ agonist troglitazone in mature adipocytes in vitro (36). However, direct regulation of ATGL mRNA and protein expression by PPAR γ 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 PPAR γ agonists in fully differentiated adipocytes and that this regulation directly involves PPAR γ . 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 PPAR γ 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, PPAR γ 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 PPAR γ in this process was unclear. A PPAR γ -dependent mechanism for induction of ATGL expression is supported by several in vitro findings in our study. First, ATGL mRNA and 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 ED₅₀ for induction of ATGL expression by rosiglitazone corresponds to its reported in vitro binding affinity for PPAR γ (31), and the ED₅₀s 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 (IC₅₀ 7.6 nM) that acts via irreversible covalent modification of a cysteine residue (Cys²⁸⁵) 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 overexpressing PPAR γ 1 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 heterodimers 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 /RXR α 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 multiTF (<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 increase 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 hydrolase 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 hydrolase activity at the post-translation level has recently been described (14,29,33,54), and this CGI-58-mediated modulation of ATGL activity indirectly involves perilipin A. It is possible that PPAR γ -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 PPAR γ in adipocyte lipid homeostasis requires further evaluation.

With regard to the in vivo regulation of ATGL by PPAR γ , 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 (*Lep^{ob/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 mice with obesity due to leptin deficiency (*Lep^{ob/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 PPAR γ -mediated regulation of ATGL, our data are consistent with previous reports of decreased ATGL expression in WAT of animal models with obesity due to leptin deficiency (26,49). Interestingly, consistent with the finding of Shen et. al. (43), our study found no difference in adipose ATGL expression in mice with obesity due to HFD feeding compared with lean controls, suggesting that dysregulation of ATGL expression in *Lep^{ob/ob}* mice may be due to leptin deficiency rather than to obesity per se. This notion is further supported by a recent report (26) demonstrating no difference in ATGL mRNA expression in WAT of NZB/BINJ mice compared with genetically lean SM/J mice. Hence, the precise relationship between adipose ATGL expression/activity and obesity and/or obesity-related dysmetabolic states such as insulin resistance requires further investigation as well.

In summary, this study demonstrates that PPAR γ positively regulates ATGL mRNA and protein expression in adipocytes in vitro and in vivo and provides evidence to support the direct involvement of PPAR γ in this process. It also demonstrates that ATGL mRNA and protein expression are highly correlated under several different experimental conditions. PPAR γ 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 PPAR γ agonists, time and dose dependence of induction, inhibition by the PPAR γ -specific antagonist GW-9662, and lack of inhibition by the protein synthesis inhibitor cycloheximide. The dependence of ATGL expression on PPAR γ activation is further supported by the significant reduction in ATGL mRNA and protein expression following siRNA-mediated knockdown of PPAR γ . Nevertheless, additional studies evaluating transcription per se are required to more clearly define the precise mechanisms by which PPAR γ regulates ATGL expression in adipocytes as well as other tissues. In addition, the contribution of PPAR γ -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 PPAR γ and ATGL in lipid metabolism suggests that regulation of ATGL by PPAR γ may have important implications in pathogenesis and treatment of obesity and related disorders of lipid homeostasis.

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Fig. 1.

Effect of thiazolidinediones (TZD) and non-TZD peroxisome proliferator-activated receptor- γ (PPAR γ) 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-deoxy- $\Delta^{12,14}$ -PGJ₂ (PGJ₂) at 10 μ M 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, $P < 0.05$).

Fig. 3.

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 μg/ml CHX for 30 min, followed by addition of either 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 AGTL expression. $*P < 0.05$ for the effect of Rosi within the same CHX treatment; $†P < 0.05$ for the effect of CHX within the same Rosi treatment. ns, Not significant.

Fig. 4.

Effect of the PPAR γ 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 μ M GW alone, 100 nM Rosi alone, or 100 nM Rosi plus either 0.1 or 10 μ M 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; $\dagger P < 0.05$ 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 PPAR γ ATGL mRNA and protein expression in 3T3-L1 adipocytes. 3T3-L1 adipocytes were electroporated in the presence of either control or PPAR γ -specific siRNAs on *day 7* 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*: PPAR γ 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. * $P < 0.05$.

Fig. 6.

In vivo effect of Rosi on ATGL mRNA and protein expression in white (WAT) and brown adipose tissue (BAT). *Lep^{ob/+}* and *Lep^{ob/ob}* mice fed chow or high-fat diet (HFD) for 18 wk were treated with either carboxymethyl cellulose vehicle (control) or 4 mg·kg⁻¹·day⁻¹ Rosi for 10 days by oral gavage ($n = 6-8$ /group for mRNA and protein). **A** and **B**: ATGL mRNA expression was determined in perigonadal white adipose tissue (PGAT; **A**) or BAT (**B**) by qPCR, normalized to 18S ribosomal RNA, and expressed relative to the vehicle-treated chow-fed *Lep^{ob/+}* control group. $P < 0.05$ for overall effect of Rosi treatment and genotype on ATGL expression in both PGAT and BAT. * $P < 0.05$ for comparisons between vehicle- and Rosi-treated animals within each genotype/diet group; † $P < 0.05$ for comparisons between *Lep^{ob/+}* vs. *Lep^{ob/ob}* mice or between chow-fed vs. HFD-fed mice within the same Rosi treatment group. **C** and **D**: ATGL protein expression in PGAT (**C**) or BAT (**D**) by Western blot analysis, with RAN as a loading control (representative blots shown). ATGL protein expression correlated with mRNA expression ($r = 0.888$ and 0.886 for PGAT and BAT, respectively, $P < 0.05$). Significance of statistical comparisons between groups was the same as stated for mRNA.

Table 1

Phenotypic analysis of mice treated with Rosi

Group (Treatment)	Weight, g		Glucose, mg/dl		Insulin, ng/ml	
	Before	After	Change	Before	After	Change
<i>Lep^{ob/+}</i> chow fed						
Vehicle	33.72±0.90	33.19±0.87	-0.53±0.26	162±4	162±6	0±4
Rosi	33.83±0.98	33.41±0.42	-0.42±0.16	164±4	152±7	-12±6
<i>Lep^{ob/+}</i> HFD fed						
Vehicle	44.75±0.86	43.43±0.78	-1.31±0.28	152±8	152±5	0±4
Rosi	43.41±0.67	43.52±0.59	+0.12±0.42	156±9	123±3	-33±9
<i>Lep^{ob/ob}</i> chow fed						
Vehicle	71.78±1.06	72.76±0.96	+0.48±0.20	170±25	270±27	+101±29
Rosi	71.07±1.42	73.64±1.57	+2.57±0.81	186±27	94±3	-92±26

Values are means ± SE. Rosi, rosiglitazone; HFD, high-fat diet. Chow-fed *Lep^{ob/+}*, HFD-fed *Lep^{ob/+}*, or chow-fed *Lep^{ob/ob}* 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.