Regulated Production of a Peroxisome Proliferator-activated Receptor-γ Ligand during an Early Phase of Adipocyte Differentiation in 3T3-L1 Adipocytes*

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Peroxisome proliferator-activated receptor-γ (PPARγ) is a nuclear hormone receptor that is critical for adipogenesis and insulin sensitivity. Ligands for PPARγ include some polyunsaturated fatty acids and prostanoids and the synthetic high affinity antidiabetic agents thiazolidinediones. However, the identity of a biologically relevant endogenous PPARγ ligand is unknown, and limited insight exists into the factors that may regulate production of endogenous PPARγ ligands during adipocyte development. To address this question, we created a line of 3T3-L1 preadipocytes that carry a β-galactosidase-based PPARγ ligand-sensing vector system. In this system, induction of adipogenesis resulted in elevated β-galactosidase activity that signifies activation of PPARγ via its ligand-binding domain (LBD) and suggests generation and/or accumulation of a ligand moiety. The putative endogenous ligand appeared early in adipogenesis in response to increases in cAMP, accumulated in the medium, and dissipated later in adipogenesis. Organically extracted and high pressure liquid chromatography-fractionated conditioned media from differentiating cells, but not from mature adipocytes, were enriched in this activity. One or more components within the organic extract activated PPARγ through interaction with its LBD, induced lipid accumulation in 3T3-L1 cells as efficiently as the differentiation mixture, and competed for binding of rosiglitazone to the LBD of PPARγ. The active species appears to be different from other PPARγ ligands identified previously. Our findings suggest that a novel biologically relevant PPARγ ligand is transiently produced in 3T3-L1 cells during adipogenesis.

C-MSV, control monitoring system vector; UAS, upstream activating sequence; TIF, transcription intermediary factor; RID, receptor interaction domain; DMEM, Dulbecco's modified Eagle's medium; aP2, adipocyte fatty acid-binding protein-2; HPLC, high performance liquid chromatography; DIM, differentiation mixture; conditioned medium/media.

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§ The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor-γ; RXR, retinoid X receptor; 15-dPGJ2, 15-deoxy-D12,14-prostaglandin J2; C/EBP, CCAAT enhancer-binding protein; MIX, methylisobutylxanthine; LPA, lysophosphatidic acid; FBS, fetal bovine serum; LBD, ligand-binding domain; GST, glutathione S-transferase; with the retinoid X receptor (RXR) (1) and binds to direct repeat 1-type motifs found in the promoter sites of target genes (2). PPARγ is a major modulator of several aspects of development and homeostasis. It is expressed in breast, colon, prostate, macrophages, and adipose tissue (3–6) and has been shown to play a critical role in glucose and lipid metabolism (7), macrophage function (8), and adipogenesis (9).

As a member of the nuclear receptor superfamily, PPARγ is activated through ligand binding, which results in allosteric changes in receptor conformation, recruitment of coactivators, assembly of a transcriptional complex, and regulated transcription of target genes (10). Among known PPARγ agonists are the synthetic high affinity antidiabetic drugs thiazolidinediones (11) and a number of natural substances. These include the dehydration product of prostaglandin D2, 15-deoxy-D12,14-prostaglandin J2 (12, 13); derivatives of linoleate, 9- and 13-hydroxyoctadecade-9Z,11E-dienoic acids, both found in oxidized low density lipoprotein (14); certain polyunsaturated fatty acids (15, 16); and oxidized alkyl phospholipids such as lysophosphatidic acid (17). Most of these natural ligands bind PPARγ with relatively low affinity compared with the affinity of most bona fide ligands for nuclear receptors. Additionally, they are weak agonists and exist at concentrations in vitro that may be below the levels needed for biological relevance. For these reasons, their physiological significance and role as true endogenous activators for PPARγ have been uncertain.

Adipogenesis is a complex process associated with coordinated changes in gene expression, cell morphology, and hormone sensitivity (18). Several transcription factors influence these processes, among which PPARγ and CCAAT enhancer-binding proteins (C/EBPs) have been extensively studied (19, 20). Early gain-of-function studies showed that forced expression of PPARγ in cultured fibroblasts followed by treatment with exogenous ligands results in their efficient differentiation to mature adipocytes (9). Subsequent loss-of-function studies showed that PPARγ is required for adipogenesis in vivo and in vitro (21–23). Despite conclusive knowledge about the pivotal role that PPARγ plays in adipogenesis, surprisingly little is known about the production, regulation, and identity of endogenous ligands that activate PPARγ in specific cell types. It is possible, of course, that the receptor may respond solely to one or more nutritionally supplied lipid ligands and that no high affinity regulatory ligand is produced during adipogenesis.
Even so, it would be critical to know the net effective concentration of intracellular ligands under a variety of situations and in cells such as adipocytes, where PPARγ activation plays a major role. To address this fundamental question, we have developed an autoregulated inducible reporter system to monitor production of PPARγ ligand activity during adipogenesis in vitro. Here, we present evidence that 3T3-L1 cells produce and release a hydrophobic ligand(s) specific for PPARγ. This activity is most abundant in the early stages of differentiation, is produced in response to a cAMP signal, and does not appear to be a previously described agonist. Our findings suggest that an as yet unidentified pathway for PPARγ ligand generation exists in 3T3-L1 cells. Activation of this pathway results in production of an endogenous PPARγ ligand that, along with the receptor itself, is likely to be a critical mediator of the adipogenic program.

**EXPERIMENTAL PROCEDURES**

**Materials**—Methylisobutylxanthine (MIX), insulin, dexamethasone, oil red O, 8-bromo-cAMP, forskolin, thyroid hormone, 17β-estradiol, trypsin, ethyl acetate, aceton, and a Lichrosorb SI-60 column were all purchased from Sigma. All-trans-retinoic acid, 9-cis-retinoic acid, baicalein, and W64643 were from Biomol Research Labs Inc. (Plymouth Meeting, PA). Acetyl-, oleyl-, myristoyl-, hexanoyl-, and palmitoyl-LPs were from Avanti (Alabaster, AL). The Galacto-light kit was from Tropix Inc. (Foster City, CA); charcoal dextran-separated fetal bovine serum (FBS) was from Hyclone Laboratories (Logan, UT); [35S]methionine (1175 Ci/mmol) was from PerkinElmer Life Sciences; and rosiglitazone was from GlaxoSmithKline.

**Plasmid Construction**—The pCMV-Gal4-PPARγ LB and GST-PPARγ LB plasmids were constructed by inserting the mouse PPARγ LB cDNA (amino acids 163–476) into the pFA-CMV (Stratagene, La Jolla, CA) and pGEX2-TK (Amersham Biosciences) vectors, respectively. To generate the negative control β-galactosidase-monitoring system vector MSVβ, the Gal4 DNA-binding and PPARγ LB Ds from pCMV-Gal4-PPARγ were subcloned downstream of the US6/α67 promoter fragments from pKS-hsp-lacZ (a gift from J. Rossant) were subcloned into pG wager Big Basic (Clontech, Palo Alto, CA). To generate the PPARγ-monitoring system vector MSVγ, the Gal4 DNA-binding and PPARγ LB Ds from pCMV-Gal4-PPARγ were subcloned downstream of the US6/α67 promoter in C-MSV, replacing the lacZ coding region. This plasmid was fused together with the C-MSV reporter plasmid to create the final MSVγ vector. The pG6E1-Luc and pG6E4-TK-Luc reporter plasmids and the pCMX-βGal, pT7-TIF2 R, pG4A-TIF2 R, and pT7-RXR reporters have been described previously (25).

**In Vitro Interactions**—GST protein purification and pull-down assays have been described previously (25, 26). Briefly, for the pull-down assay, GST-PPARγ-LBD was bound to glutathione–Sepharose beads and incubated with [35S]-labeled TIF2 R for 20 min on ice. Then, 1 μM rosiglitazone and increasing amounts (0.25–1 μM) of organically extracted and concentrated control (C100×) or conditioned (CM100×) medium were added, and the reactions were incubated overnight at 4°C. The next day, the beads were washed, and the bound proteins were eluted with 15 mM glutathione, resolved by SDS–PAGE, and visualized by autoradiography. For the protease digestion assay, 2 μl of in vitro translated and [35S]-labeled PPARγ was incubated with 2 μl of in vitro translated RXR. The complex was allowed to dimerize for 10 min on ice before ETOH, 1 μM rosiglitazone, or increasing amounts of C100× or CM100× were added. After a 30-min incubation, 250 ng of trypsin was added to the indicated tubes, and the reaction was performed at room temperature for 7 min. Reactions were analyzed on a 15% SDS gel, after which the gel was dried and exposed to film. For the ligand binding competition assay, 100 μg of GST-PPARγ fusion protein was bound to glutathione–Sepharose beads and incubated at 4°C in buffer containing 10 mM Tris (pH 8.0), 50 mM KCl, 10 mM dithiothreitol, and 1 mM PMSF. The complexes present in supernatant (specific binding activity) were subjected to an RNeasy mini kit (Qiagen Inc., Valencia, CA) and treated with Dnase I (Qiagen Inc.) according to the manufacturer’s instructions. PPARγ, ap2, lipoprotein lipase, and GLUT4 mRNAs were quantitatively measured using a Stratagene MX 4000 Multiplex quantitative PCR system and a Stratagene single step quantitative reverse transcription PCR kit according to the manufacturer’s instructions. Total RNA was diluted in the range of 0.1–100 ng and used to establish standard curves for each individual gene. The quantification of each mRNA was further normalized by the corresponding cyclophilin mRNA measurement.

**Prostaglandin J2 Analysis**—Prostaglandin J2 was extracted from conditioned medium using a Waters HPLC system equipped with a Lichrosorb normal phase column and a binary solvent system. Solvent A consisted of 100% chloroform, and solvent B was a mixture of chloroform/methanol/water.
RESULTS

Generation of a PPARγ Ligand-monitoring System in 3T3-L1 Cells—PPARγ is a ligand-regulated nuclear hormone receptor that is indispensable in the process of adipogenesis both in vivo and in vitro (23). It is therefore reasonable to hypothesize that a specific ligand is produced to activate PPARγ upon conversion of preadipocytes to mature lipid-containing adipocytes. To monitor the presence of such a ligand during adipogenesis, we developed and stably integrated ligand-inducible MSVγ in 3T3-L1 cells. The vector carries the Gal4 DNA-binding domain fused to the PPARγ LBD, located upstream of the β-galactosidase reporter gene. Both the PPARγ fusion protein and the β-galactosidase reporter are under the control of five Gal4-specific binding sites (UASγ) and the hsp68 minimal promoter (MSVγ) (Fig. 1A). The combination of UASγ with the hsp68 minimal promoter and a receptor LBD has been successfully used previously in studies on reporter gene constructs both in vitro and in vivo (24). We also generated a second reporter construct containing just the β-galactosidase gene driven by the UASγ-hsp68 minimal promoter to use independently as a negative control vector (C-MSV) (Fig. 1A). The two vectors were transfected in CV-1 cells to test for their ability to respond to the synthetic PPARγ ligand rosiglitazone. As shown in Fig. 1B, the ligand-monitoring system vector MSVγ responded well to 250 nM rosiglitazone in CV-1 cells, whereas the negative control vector C-MSV did not.

The two vectors were then stably transfected in 3T3-L1 preadipocytes, and two clones were selected for further experiments: the ligand-sensing effector clone 5B2 and the negative control clone UH12. Both cell lines showed normal lipid accumulation after induction of differentiation by standard protocols (Fig. 1C). Line 5B2 expressed PPARγ and C/EBPα at normal levels compared with the parental 3T3-L1 cells, and insulin-stimulated glucose uptake following induction of differentiation was also normal (data not shown). The data suggest that the 3T3-L1-based monitoring system line retains all the biological properties of adipocytes.

PPAR Ligand Production during Adipogenesis—Next, we tested for increases in β-galactosidase activity after induction of differentiation of 5B2 and UH12 cells. Cells were maintained in 10% charcoal dextran-stripped FBS for this and all subsequent experiments. 2 days after confluence (day 0), cells were induced to differentiate using the standard adipogenic mixture of MIX, insulin, and dexamethasone. At the indicated time points, cells were lysed and assayed for β-galactosidase activity. As shown in Fig. 2A, no changes in β-galactosidase activity were observed in the negative control line UH12 up to 12 days after induction of differentiation. On the other hand, a robust increase in activity was observed in line 5B2 as early as 24 h after induction (day 1). The activity remained high for another 24 h, gradually decreased to basal levels by day 4, and remained low up to day 12 after differentiation.
activity by 3.5- and 4-fold, respectively. Interestingly, one of the adipogenic mixture components, MIX, had an effect similar to that of the complete mixture because it increased β-galactosidase activity 4-fold. MIX is generally assumed to exert its proadipogenic effects by inhibiting phosphodiesterases that break down cAMP, but data exist to suggest that it may work through other mechanisms as well (28). We therefore treated cells with forskolin, which increases cAMP via the activation of adenylate cyclase, or with the non-hydrolyzable analog 8-bromo-cAMP. Forskolin and 8-bromo-cAMP both increased β-galactosidase activity to levels similar to those achieved by MIX (Fig. 3B). In addition, transient transfection experiments in CV-1 cells showed that neither the adipogenic mixture nor its components separately or in combination activated the PPARγ-LBD under conditions in which robust activation was observed by rosiglitazone (data not shown). Of note, previous work has demonstrated a similar requirement of cAMP signaling pathways in the adipogenic process (29). It was shown that preadipocytes exposed to dexamethasone and insulin alone fail to differentiate and that addition of exogenous PPARγ ligands bypasses the defect and promotes adipogenesis in the absence of MIX (29). Collectively, these data suggest that production of the PPARγ ligand activity requires cAMP signaling pathways, is specific to preadipocytes and not other cell types, and is independent of pre-existing serum-derived activities.

**Conditioned Medium from 3T3-L1 Cells Contains a Ligand Activity**—Although no molecule has yet been demonstrated to serve as an endogenous PPARγ ligand in vivo, several potential biological activators have been identified. These include lipids such as prostaglandins, leukotrienes, hydroxyeicosatetraenoic acids, and polyunsaturated fatty acids (12–14, 30). It has also been reported that ADD1/SREBP1 promotes the production and secretion of a PPARγ ligand activity in 3T3-L1 cells (31). We therefore tested the supernatants (conditioned media) of differentiating 3T3-L1 cells for the presence of ligand. 3T3-L1 cells plated on 10-cm dishes were induced to differentiate. 2 days later, the conditioned medium (hereafter called CM) was collected, extracted with organic solvents, and evaporated. Negative control medium from 3T3-L1 cells maintained in DMEM with charcoal dextran-stripped FBS, but without DIM, was also collected after 2 days of incubation and treated similarly. Both the evaporated control and conditioned media were reconstituted in DMEM with charcoal dextran-stripped FBS (3× concentrated) before being testing on the 5B2 and UH12 lines. The two cell lines were incubated with control (C3× or conditioned (CM3×) medium for 24 h, after which cells were lysed and assayed for β-galactosidase activity. Fig. 4A shows that incubation of the 5B2 cells with CM3× resulted in an increase in β-galactosidase activity similar to that observed with DIM, whereas incubation with C3× had no effect. In addition, incubation with organically extracted day 8 medium did not increase β-galactosidase activity (data not shown). The β-galactosidase activity of the control line UH12 remained unchanged. Next, CV-1 cells transiently transfected with C-MSV or MSV were incubated with C3× or CM3× for 16 h. Again, CM3× increased β-galactosidase activity by 3-fold, whereas C3× had no effect. This was comparable with the level of activation seen with rosiglitazone, which induced β-galactosidase activity by 4-fold (Fig. 4B).

We then tested the concentrated media for their ability to promote lipid accumulation in 3T3-L1 preadipocytes in the presence or absence of the PPARγ antagonist PD068235. Cells were plated in 12-well dishes and, 2 days post-confluence, were incubated with the adipogenic mixture (DIM), rosiglitazone, C3×, CM3×, day 4 medium (3× concentrated), day 8 medium (3× concentrated), or differentiation medium (3× concen-
treated) as a negative control. The antagonist PD068235 was added as indicated. On day 2, the medium was changed, and insulin was added; on day 4, insulin was withdrawn. Cells were allowed to accumulate lipids for 6 additional days before they were fixed and stained with oil red O. As shown in Fig. 4C, CM3/H11003 induced adipogenesis almost as effectively as the standard mixture or rosiglitazone. Cells were then assayed for β-galactosidase and luciferase activities. Normalized β-galactosidase activity is represented as arbitrary units. C, 3T3-L1 cells were induced to differentiate with DIM, 1 μM rosiglitazone (Rosi), CM3/H11003, or day 4 medium (3× concentrated; Day 4 3X), and day 8 medium (3× concentrated; Day 8 3X). Organically extracted unconditioned medium with DIM (differentiation medium (3× concentrated) (Diff. M 3X)) was used as a negative control. The antagonist PD068235 (PD; 30 μM) was added as indicated for the first 48 h. At day 10 after differentiation, lipids were visualized with oil red O. D, total RNA was collected from 3T3-L1 cells induced to differentiate with DIM, rosiglitazone (Rosi), or CM3/H11003 and was used for the quantitation of PPARγ, aP2, and lipoprotein lipase (LPL).

We also tested the ability of CM3/H11003 to induce PPARγ target genes involved in the process of adipogenesis. For this, 3T3-L1 preadipocytes were induced to differentiate with DIM, rosiglitazone, or CM3/H11003. Total RNA was collected 4 days later and analyzed for the presence of PPARγ and PPARγ target gene mRNAs. As shown in Fig. 4D, rosiglitazone strongly induced PPARγ mRNA, consistent with the hypothesis that activated PPARγ induces expression of C/EBPβ, and C/EBPδ through a positive feedback loop maintains high expression of PPARγ. Interestingly, CM3/H11003 had an effect on PPARγ mRNA similar to that of rosiglitazone, both of which activated much more robustly than DIM. In addition, rosiglitazone and CM3/H11003 activated aP2 and lipoprotein lipase to similar levels. These data indicate that an extractable hydrophobic ligand activity is produced during the early phase of adipocyte differentiation and dissipates later on, once the adipogenic program is fully established. This moiety activates the LBD of PPARγ in both 3T3-L1 and heterologous cell systems and induces adipogenesis of 3T3-L1 cells, a role consistent with that of a natural PPARγ ligand.
FIG. 5. The PPARγ ligand activity is different from previously described ligand activities. A, medium from day 2 or 8 after initiation of differentiation was analyzed for the presence of prostaglandin J₂ (PGJ₂) by TLC. B, 5B2 cells were incubated with DIM 2 days after confluence. At that time, they were also treated with 10, 20, or 40 μM baicalein for 24 h. The cells were then assayed for β-galactosidase activity. C, 5B2 cells were incubated with solvent (−); DIM; or 5 μM acyl-LPA (A), oleoyl-LPA (O), myristoyl-LPA (M), hexanoyl-LPA (H), or palmitoyl-LPA (P). 24 h later, the cells were assayed for β-galactosidase activity.

The Ligand Activity Is Distinct from That of Known PPARγ Ligands—Several naturally found ligands have been shown to activate PPARγ at high concentrations. These include components of oxidized low density lipoprotein (14), LPA (17), the prostaglandin D₁ dehydration product (15-dPGJ₂) (12, 13), and products of the 12/15-lipoxygenase-type enzymes (30). We therefore employed several strategies to examine our CM for the presence of previously known PPARγ ligands.

The enzyme cyclooxygenase-2 converts arachidonic acid to prostaglandins. One of its products is 15-dPGJ₂, a PPARγ ligand that can promote adipocyte differentiation. Using TLC analysis, we did not find any significant amounts of prostaglandin J₂ in the CM or cell extracts at different time points during differentiation (Fig. 5A) (data not shown). This suggests that the ligand activity in the CM is not 15-dPGJ₂.

The 12/15-lipoxygenase enzymes found in platelets, leukocytes, and macrophages produce PPARγ ligands from arachidonic and linoleic acids. It was recently reported that inhibitors of such enzymes block adipogenesis (32). More specifically, high concentrations of the 12-lipoxygenase inhibitor baicalein (20–30 μM) blocked lipid accumulation in 3T3-L1 cells. It was suggested that baicalein may act by inhibiting production of a putative PPARγ ligand (32). We tested the inhibitor for its ability to block activation of the ligand-sensing system in line 5B2 upon incubation with DIM. Not only was baicalein ineffective at 10, 20, and 40 μM, but it also failed to inhibit induction of β-galactosidase activity (Fig. 5B), but it resulted in a modest enhancement of activation. This result, together with the reported absence of 12-lipoxygenase enzymes in 3T3-L1 cells (32), suggests that further examination is required to clarify the effect of lipoxygenase enzyme inhibitors on the adipogenic process.

Oleoyl-LPA was recently shown to activate PPARγ directly in RAW264.7 monocyctic cells (17). This LPA species is also a potent paracrine mediator of adipocyte growth and function (33). We tested five different derivatives of LPA for their ability to activate the PPARγ ligand-monitoring system vector in 5B2 cells or to induce lipid accumulation in 3T3-L1 cells. We failed to see increases in β-galactosidase activity when 5B2 cells were incubated with the LPA derivatives for 24 h (Fig. 5C). In addition, the compounds did not promote lipid accumulation in confluent 3T3-L1 cells, whereas incubation with rosiglitazone during the same time frame resulted in significant lipid accumulation (data not shown). Our results suggest that, unlike the reported agonist-type function of LPA on PPARγ in RAW264.7 cells, a possible role of LPA as a mediator in adipogenesis of 3T3-L1 cells may be independent of PPARγ.

Specificity of the Ligand Activity—To determine the specificity of the CM ligand activity among different members of the nuclear receptor superfamily, CV-1 cells were transfected with plasmids expressing various Gal4 DNA-binding domain-receptor LBD fusion proteins. The receptors tested included the PPARγ heterodimeric partner RXRα, PPARα, estrogen receptor-α (ERα), thyroid receptor-β (TRβ), and retinoic acid receptor-α (RARα). PPARγ is activated by both RXRα and 9-cis-retinoic acid, 100 nm triiodothyronine, 1 μM 17β-estradiol, 500 nm 15-deoxynordihydrotestosterone, and 250 nm rosiglitazone. The next day, cells were assayed for luciferase (Luc) and β-galactosidase activities.

Characterization of the PPARγ Ligand Activity—Since ligand-induced transactivation is achieved by the recruitment of coactivators to nuclear receptors, two sets of experiments were performed to determine the ability of the CM to recruit coactivators to the LBD of PPARγ. Initially, a mammalian two-hybrid assay was performed. For this, CV-1 cells were transfected with the pGal4-TIF2 RID and pVP16-PPARγ LBD expression plasmids in the presence of solvent, rosiglitazone, or the CM. Incubation of the cells with the CM resulted in enhanced interaction between the TIF2 RID and the PPARγ LBD to levels similar to those caused by rosiglitazone (Fig. 6A). Similar results were observed when the RID of the coactivator SRC-1 was used (data not shown). Next, we performed a coactivator-dependent receptor ligand assay. For this, the bacterially expressed PPARγ-LBD was incubated with the in vitro translated and 35S-labeled TIF2 RID in the presence of solvent, rosiglitazone, the control medium, or the CM. Again, incubation with the CM enhanced the interaction between the coactivator and the receptor as effectively as rosiglitazone (Fig. 6B).

We further assessed the direct interaction of the ligand moiety with the LBD of the receptor by testing the ability of the CM to protect the PPARγ LBD from digestion by proteases. As shown in Fig. 6C, incubation of 35S-labeled PPARγ with trypsin...
resulted in almost complete protein degradation (lane 2). Addition of rosiglitazone to the digestion reaction partially protected PPARγ, as shown by the existence of two major protected bands (lane 3). Incubation of the digestion reaction with increasing amounts of concentrated CM resulted in a similar protection pattern (lanes 8–10), whereas incubation with control medium failed to protect from degradation.

We then performed a “ligand depletion assay” using day 2 CM. The GST-PPARγ LBD fusion protein or equimolar amounts of control GST protein, both coupled to GST beads, were incubated with day 2 CM. The next day, the supernatant was organically extracted, evaporated, and resuspended in medium. This was indeed the case. As shown in Fig. 8A, GST-PPARγ LBD effectively depleted the activity from the medium. The resultant ligand-depleted CM failed to induce lipid accumulation in 3T3-L1 cells (Fig. 8A). In contrast, incubation of the CM with GST did not remove the ligand activity, and this CM promoted adipogenesis as effectively as DIM. Similarly, the β-galactosidase activity of 5B2 cells was increased after overnight incubation with the GST-depleted (but not PPARγ LBD-depleted) CM (Fig. 8B). Finally, control medium, day 2 medium, or day 8 medium was tested in a ligand binding competition assay. As shown in Fig. 8C, day 2 medium effectively competed for binding of \([	ext{3}^3\text{H}]\)rosiglitazone (R) to the PPARγ LBD in a ligand binding competition assay (C).

Analysis of HPLC-fractionated Conditioned Media—We next attempted to purify the ligand activity further and to test for its presence in media from preadipocytes (day 0) and mature adipocytes (day 8). For this purpose, 12 ml of CM from day 0, 2, or 8 differentiating 3T3-L1 cells was organically extracted, evaporated, resuspended in 50 μl of chloroform/methanol, and fractionated by HPLC. Fractions were collected and analyzed for their ability to induce adipogenesis in 3T3-L1 cells. Fig. 9A shows that fraction C derived from day 2 CM potently promoted lipid accumulation in 3T3-L1 cells. None of the remaining day 2 CM-derived fractions or any fractions from day 0 or 8 of differentiation were able to induce adipogenesis. Following this observation, we tested for the ability of the day 2 CM-derived fractions to activate the LBD of PPARγ in CV-1 cells.
Indeed, only fraction C activated Gal4-PPARγ LBD by 4-fold (Fig. 9B). These results show that a PPARγ ligand activity appeared early in adipogenesis and accumulated in the media. This activity was organically extractable and could be concentrated following HPLC fractionation. The medium from preadipocytes or, most interestingly, from mature adipocytes did not contain significant PPARγ ligand activities as detected by these assays.

**DISCUSSION**

Since its discovery almost 10 years ago (9), the role of PPARγ in cellular processes such as adipogenesis (34), insulin sensitivity (35), and glucose and lipid metabolism has been the subject of extensive investigation. Although the role of the receptor in the aforementioned cellular and metabolic processes (22, 36–38) has been well established, the identity of its true biological ligands remains elusive. One hypothesis views PPARγ primarily as a sensor for nutritional fatty acids (39). Thus, a feed forward mechanism was proposed in which dietary fatty acids activate PPARγ in preadipocytic cells to promote adipogenesis, which, in turn, results in storage of the diet-derived fat. In this model, which has parallels in other lipid-sensing protein systems (39), unmodified nutritional free fatty acids are the endogenous ligands, and there is no requirement for active synthesis of a specific regulatory ligand for PPARγ. However, free fatty acids bind to PPARγ with affinities in the range of 2–50 μM, well above the published affinities of *bona fide* ligands for most nuclear hormone receptors. Although the effective concentration of free fatty acids in 3T3-L1 preadipocytes in close proximity to nuclear PPARγ is not currently known, we suspect that these concentrations are well below those likely to be required based on prior studies in cell-free systems.

3T3-L1 adipocytes have been the primary model for the study of adipogenesis, the process wherein fibroblastic cells differentiate into fat cells. Several studies have demonstrated the requirement for PPARγ in the initiation of the adipogenic program *in vivo* and *in vitro* (21–23). Since PPARγ gene expression requires the coordinate interaction of receptors ligands, and coactivators, it is surprising that studies examining production and/or accumulation of a putative ligand in these cells during adipogenesis are limited (29, 31). In this study, we have shown that an endogenous PPARγ ligand is produced in a regulated fashion during the course of adipogenesis in 3T3-L1 cells. To accomplish this, we utilized an autoregulated inducible reporter system to show that ligand activity is generated in a time- and cAMP-dependent fashion during an early phase of the adipogenic process in 3T3-L1 cells. Activation of the PPARγ ligand-monitoring system by DIM in our stable cell line could be reproduced by MIX, forskolin, or 8-bromo-cAMP, an observation reinforced by a large number of *in vitro* studies demonstrating a pivotal role for cAMP in adipogenesis (20, 40). For example, addition of plasma membrane-permeable CAMP analogs, forskolin, or MIX to cultured 3T3-L1 preadipocytes or primary rat preadipocytes enhances lipid accumulation and increases expression of several adipogenic markers such as aP2, stearoyl-CoA desaturase-1, glycerol-3-phosphate dehydrogenase, and lipoprotein lipase (41, 42). Our data suggest that CAMP signaling pathways activated by MIX act on as yet unidentified enzymatic pathways that produce one or more specific endogenous PPARγ ligands.

The intracellular ligand activity detected early during differentiation by the ligand-sensing 3T3-L1 line was paralleled by the appearance of a ligand activity in the CM, which was extractable with organic solvents. This observation made possible the use of the organically extracted and concentrated CM in a large array of experiments. We have shown here that the concentrated CM activated the LBD of PPARγ in both the 3T3-L1-based system and a heterologous cell system, whereas it was unable to activate other nuclear receptors. More importantly, the concentrated CM was able to induce adipogenesis, similar to the ability of the established PPARγ ligands (43–45), a process that is completely reversed by the PPARγ-specific antagonist PD068235 (46). Furthermore, the ligand present in the CM activated PPARγ directly rather than indirectly, as demonstrated by both coactivator recruitment and protease digestion assays. In addition, the ability of the PPARγ LBD to specifically deplete both the ligand-sensing and adipogenic activities from day 2 CM and to compete for rosiglitazone binding to the receptor demonstrates the existence of a *bona fide* agonist that binds to the PPARγ LBD. In addition, the activity present in the CM robustly increased the mRNA of PPARγ itself, possibly via a positive feedback loop involving C/EBPα (47), and of PPARγ target genes involved in fatty acid synthesis and storage.

Numerous reports have described a wide variety of structurally diverse molecules that the PPARγ LBD can accommodate in its large hydrophobic pocket (10, 48). The prostaglandin 15-dPGJ₂ was the first molecule to be identified as a possible

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**Fig. 9.** HPLC fractions contain adipogenic and PPARγ ligand-type moieties. Day 0, 2, and 8 media from differentiating 3T3-L1 cells were organically extracted, concentrated, and fractionated by HPLC. A, the three sets of fractions collected (fractions A–H) were tested for their ability to induce adipogenesis in 3T3-L1 cells. Cells were treated with solvent (none), DIM, or the indicated fractions from days 0 to 2. Lipids were stained with oil red O at day 10. B, fractions A–H derived from day 2 medium were tested for their ability to activate the PPARγ LBD in CV-1 cells by transient transfection assay. Luc, luciferase.
endogenous PPARγ ligand (12, 13) and has been widely studied since. We were able to exclude 15-dPGJ$_2$ as the regulated adipogenic ligand in this system because of its almost complete absence in the CM or cells. Our results are in agreement with a recent report (49) that demonstrated only low picomolar levels of 15-dPGJ$_2$ in the medium of 3T3-L1 cells, amounts that do not increase during differentiation despite induction of cyclooxygenase-2. These observations, together with another report (50) that showed involvement of cyclooxygenase-2 enzymatic pathways solely in the clonal expansion state of the differentiation process, suggest that 15-dPGJ$_2$ is not the endogenous adipogenic PPARγ ligand. They also suggest that cyclooxygenase-2 enzymatic pathways may not be involved in production of this ligand in 3T3-L1 cells.

Madsen et al. (32) recently proposed that lipooxygenase enzymatic pathways generate PPARγ ligand(s) in 3T3-L1 cells by showing that baicalein, an inhibitor of 12-lipoxygenase, blocks lipid accumulation in 3T3-L1 cells without acting as a PPARγ agonist. We did not see repression of the ligand-monitoring system when 5B2 cells where incubated with baicalein in the presence of DIM, suggesting that production of a PPARγ ligand may occur in the presence of this inhibitor. In addition, the lack of 12-lipoxygenase expression in 3T3-L1 cells (32) and of an adipocyte phenotype in the 12/15- or 5-lipoxygenase knockout mice (51, 52) suggests that utilization of adipogenesis by baicalein may take place via an alternative mechanism or via a novel yet unidentified enzymatic pathway.

LPA is a potent lipid mediator that controls growth and motility of preadipocytes (33). LPA also exists in monocytes present in atherosclerotic lesions and in aggregating platelets. A report by McIntyre et al. (17) proposed LPA to be a PPARγ agonist. The authors presented evidence for direct interaction between LPA and the LBD of the receptor and for stimulation of transfected PPARγ by medium derived from thrombin-activated platelets in RAW264.7 cells. Our experiments did not point to LPA as the regulated adipogenic ligand. None of the five LPA derivatives we tested activated the ligand-sensing system or promoted lipid accumulation. Hence, the action of LPA in preadipocytes may be different from its action in activated monocytes, possibly because specificity of receptor responses to cognate ligands depends on the coactivator milieu present in the relevant cell types.

Our finding that the ligand-monitoring system activity decreased to basal levels later in adipogenesis was not anticipated. Activity was high only for the first 48 h, a period that coincides with the up-regulation of the PPARγ mRNA and protein (9). This suggests that endogenous PPARγ ligand production initiates just before PPARγ protein levels begin to rise from low, but clearly detectable levels and dissipates once the adipogenic program is more fully established, even though PPARγ mRNA and protein levels remain high throughout the life of the mature adipocyte. This result is supported by the observation that the specific PPARγ antagonist PD068335 effectively blocked differentiation only when added at very early time points, when ligand-sensing activity was still maximal. The antagonist had no inhibitory effect if added at later time points, viz. after day 2, when the cells had already committed to differentiating and when the ligand-sensing activity was minimal. Another line of evidence that supports a decline in ligand production is illustrated in Fig. 8A. Only HPLC fraction C from day 2 CM contained adipogenic activity. The corresponding fraction C from day 0, when the cells were still fibroblastic and from later days when the cells were mature adipocytes, had no adipogenic activity. In agreement with this observation is the ability of fraction C from day 2 medium to activate the LBD of PPARγ in a heterologous system. It may be possible that activation of PPARγ through its LBD by an endogenous ligand is not required once the adipogenic program is fully in place. It is well established that the level of the PPARγ protein peaks soon after differentiation and remains high in mature adipocytes. Along these lines, several investigators have demonstrated that exposure of mature adipocytes to exogenous PPARγ ligands results in reduction of both the mRNA and protein levels of the receptor (53–55). In other words, the continued presence of ligands causes PPARγ down-regulation, a phenomenon common among several hormone nuclear receptors (56, 57). These findings, combined with data presented in this study, support a model in which the presence of the PPARγ ligand detected in our system may not be necessary for maintenance of the adipogenic program. If this is correct, one can hypothesize either that other transcription factors such as C/EBPα may complement the function of PPARγ or, alternatively, that ligand-independent activation functions in the N terminus of the receptor predominate and may contribute to maintaining differentiated function of mature adipocytes (58). Further investigation into the requirements for an endogenous PPARγ ligand in mature adipocytes is needed to clarify this question.

The biological importance of PPARγ has been well demonstrated through genetic gain- and loss-of-function experiments and through the proven efficacy of pharmacological PPARγ agonists, the thiazolidinediones, in the treatment of diabetes. In this context, it is disappointing that so little is known about the identity and levels of the cognate ligand(s) for this receptor in relevant tissues in normal physiology and disease states. It might be predicted that this knowledge, once obtained, would be highly relevant to our understanding of the biology of PPARγ-dependent pathways and their potential dysregulation in diseases, including (but not limited to) those related to adipose function and glucose metabolism. The studies presented here provide compelling evidence that a hydrophobic PPARγ ligand is produced under a cAMP-dependent signal and is released into the medium early and transiently during adipogenesis in 3T3-L1 cells. Our studies with a PPARγ antagonist also strongly suggest that this ligand production is critical to the elaboration of the adipogenic program. Preliminary characterization of the ligand activity revealed that it interacts directly with the LBD of PPARγ and is capable of producing a conformation that promotes transcriptional activation. This partially purified activity promotes adipogenesis in a manner similar to that of known pharmacological and natural ligands. These studies provide important new information on the status of PPARγ ligand production during adipogenesis in 3T3-L1 cells that complements the large body of data on PPARγ itself and on PPARγ-dependent transcriptional events in these cells. Current efforts to identify this ligand, if successful, will likely provide important new insights into the role of PPARγ-dependent pathways in physiology and disease.

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