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A Prostaglandin J_2 Metabolite Binds Peroxisome Proliferator-Activated Receptor γ and Promotes Adipocyte Differentiation

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Summary

Prostaglandins (PGs) of the J_2 series form in vivo and exert effects on a variety of biological processes. While most PGs mediate their effects through G protein-coupled receptors, the mechanism of action for the J_2 series of PGs remains unclear. Here, we report that PGJ_2 and its derivatives are efficacious activators of peroxisome proliferator-activated receptors α and γ (PPARα and PPARγ, respectively), orphan nuclear receptors implicated in lipid homeostasis and adipocyte differentiation. The PGJ_2 metabolite 15-deoxy-$\Delta^{12,14}$-PGJ_2 binds directly to PPARγ and promotes efficient differentiation of C3H10T1/2 fibroblasts to adipocytes. These data provide strong evidence that a fatty acid metabolite can function as an adipogenic agent through direct interactions with PPARγ and, furthermore, suggest a novel mechanism of action for PGs of the J_2 series.

Introduction

The prostaglandins (PGs) are a family of structurally related molecules that are produced by cells in response to a variety of extrinsic stimuli and regulate cellular growth, differentiation, and homeostasis (reviewed by Smith, 1989, 1992). PGs are derived from fatty acids, primarily arachidonic acid, which are released from membrane phospholipids by the action of phospholipases. Arachidonic acid is first converted to an unstable endoperoxide intermediate by cyclooxygenase, and subsequently converted to one of several related products, including PGD_2, PGE_2, $PGF_2\alpha$, prostacyclin (PGI_2), and thromboxane A_2, through the actions of specific PG synthetases. Each of these products has been shown to affect levels of second messengers through interactions with G protein–coupled receptors with seven transmembrane domains (Hirata et al., 1994; reviewed by Smith, 1992).

PGD_2 is a major cyclooxygenase product in a variety of tissues and cells and has marked effects on a number of biological processes, including platelet aggregation, relaxation of vascular and nonvascular smooth muscle, and nerve cell function (reviewed by Giles and Leff, 1988). PGD_2 has also been shown to be released by mast cells, suggesting a role in immunologic responses (Lewis et al., 1982). While PGD_2 is known to mediate effects through interactions with a cell surface receptor (Hirata et al., 1994), PGD_2 readily undergoes dehydration in vivo and in vitro to yield additional, biologically active PGs of the J_2 series (Fitzpatrick and Wynalda, 1983; Kikawa et al., 1984; Hirata et al., 1988). Members of the PGJ_2 series have been reported to have their own unique spectrum of biological effects, including the inhibition of cell cycle progression, the suppression of viral replication, the induction of heat shock protein expression, and the stimulation of osteogenesis (reviewed by Fukushima, 1992). In contrast with the case of PGD_2, the mechanism of action for the J_2 series of PGs remains unclear. The observation that at least one of the PGJ_2 series, Δ^{12}-PGJ_2, is actively incorporated into the nucleus of cells and associated with proteins (Narumiya et al., 1987) has led to speculation that these PGs may exert their effects through direct interactions with intracellular proteins.

Peroxisome proliferator–activated receptors (PPARs) are members of the nuclear receptor superfamily of ligand-activated transcription factors that includes the steroid, retinoid, and thyroid hormone receptors. To date, three mammalian PPAR subtypes have been isolated and termed PPARα, NUC1 (also known as PPARδ), and PPARγ (reviewed by Keller and Wahli, 1993). Several observations suggest that the PPARs play a physiologic role in the modulation of lipid metabolism. First, PPAR-binding sites have been identified in the regulatory regions of a number of genes encoding proteins involved in lipid metabolism. Furthermore, PPARs have been shown to be activated by high concentrations of a diverse group of lipids and lipid-like compounds, including peroxisome proliferators and long-chain fatty acids. Finally, PPARγ is abundantly expressed in adipocytes and functions as a key regulator of adipocyte differentiation (Tontonoz et al., 1994a, 1994b; Chawla et al., 1994). Thus, PPARs appear to have a broad role in the regulation of lipid homeostasis.

We recently showed that a class of antidiabetic compounds with potent adipogenic properties, termed thiazolidinediones, are high affinity PPARγ ligands (Lehmann et al., 1995). However, naturally occurring ligands for the PPARs have yet to be identified. The observation that PPARs are activated in transient transfection assays by the synthetic arachidonic acid analog 5,8,11,14-eicosatetraynoic acid (ETYA) and high concentrations of arachidonic acid (Göttlicher et al., 1992; Keller et al., 1993; Banner et al., 1993) raised the intriguing possibility that arachidonic acid metabolites might serve as PPAR ligands. Here, we report that the cyclooxygenase product PGD_2 and its PGJ_2 derivatives are efficacious activators of PPARα and PPARγ. Furthermore, the PGJ_2 dehydration product 15-deoxy-$\Delta^{12,14}$-PGJ_2 is shown to bind directly to PPARγ and to promote adipogenesis of cultured fibroblasts. These data suggest that the J_2 series of PGs may

exert their biological effects in part through activation of the PPAR signaling pathways.

Results

PGD_2 and Its PGJ_2 Metabolites Are PPARγ Activators

As part of an effort to identify natural PPAR ligands, a large number of arachidonic acid derivatives were screened in a transient transfection assay. An established chimera system was used, in which the ligand-binding domains of the three murine PPAR subtypes were fused to the DNA-binding domain of the yeast transcription factor GAL4 (Lehmann et al., 1995). Expression plasmids for the GAL4–PPAR chimeras were transfected into CV-1 cells together with a chloramphenicol acetyltransferase (CAT) reporter construct containing five copies of the GAL4-binding site upstream of the minimal thymidine kinase (tk) promoter. As previously shown (Lehmann et al., 1995), all three PPAR chimeras were activated by 1×10^{-4} M of the peroxisome proliferator Wy14,643 (Figure 1). The PPARα and PPARγ chimeras were also activated in the presence of 1×10^{-5} M ETYA, and, to a lesser extent, arachidonic acid (Figure 1). Interestingly, analysis of cyclooxygenase metabolites of arachidonic acid revealed PGD_2 to be an efficacious activator of the PPARα and PPARγ chimeras at a concentration of 1×10^{-5} M (Figure 1). The efficacy of PGD_2 in activating PPARγ was comparable to that of the thiazolidinedione BRL49653, which we recently showed to be a high affinity PPARγ ligand (Lehmann et al., 1995). Little or no activation of the NUC1 chimera was detected in the presence of these PGs (Figure 1).

The potency of PGD_2 in the activation of the PPARα and PPARγ chimeras was examined next. In dose–response analysis, the activities of the PPARα and PPARγ chimeras failed to plateau at PGD_2 concentrations up to 1×10^{-5} M (Figures 2A and 2B, open squares). Thus, while PGD_2 is an efficacious activator of the PPARs, it lacks potency in its induction of these orphan receptors.

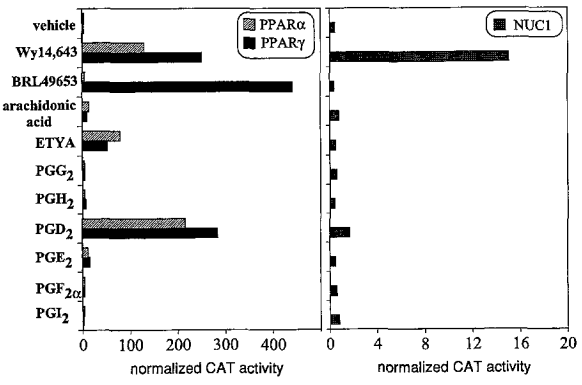

Figure 1. PGD_2 Is an Efficacious Activator of PPARα and PPARγ

CV-1 cells were transiently transfected with expression plasmids for the PPARα–GAL4, NUC1–GAL4, or PPARγ–GAL4 chimeras and the reporter plasmid $(UAS)_5$-tk-CAT. Cells were treated for 24 hr with either vehicle (0.1% DMSO), 1×10^{-4} M Wy14,643, or 1×10^{-5} M BRL49653, arachidonic acid, ETYA, or various PGs. Cell extracts were subsequently assayed for CAT activity.

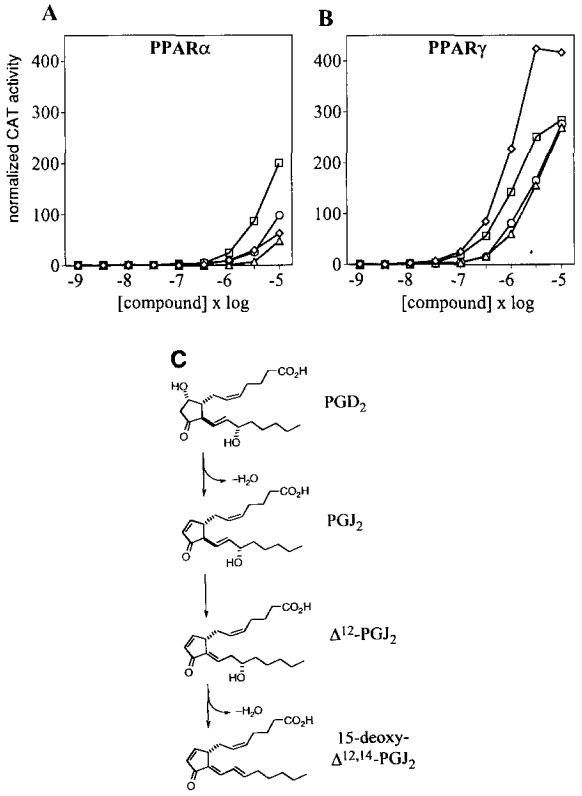

Figure 2. PPARα and PPARγ Dose Response to PGD_2 and Its PGJ_2 Metabolites

CV-1 cells were cotransfected with expression plasmids for the PPARα-GAL4 (A) or PPARγ-GAL4 (B) chimeras and the reporter plasmid $(UAS)_5$-tk-CAT and treated with the indicated concentrations of PGD_2 (squares), PGJ_2 (circles), Δ^{12}-PGJ_2 (triangles), or 15-deoxy-$\Delta^{12,14}$-PGJ_2 (diamonds). Cell extracts were subsequently assayed for CAT activity.

(C) PGD_2 metabolic pathway leading to the formation of PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2.

The efficacy of PGD_2 in activating the PPARα and PPARγ chimeras coupled with its relative lack of potency suggested that PGD_2 might be metabolized in CV-1 cells to more potent PPAR activators. Indeed, PGD_2 is known to be readily metabolized in vitro and in vivo to yield the J_2 series of PGs (Figure 2C). Incubation of PGD_2 in the presence of human plasma or human serum albumin results in the rapid accumulation of several major dehydration and isomerization products, including Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (Fitzpatrick and Wynalda, 1983; Kikawa et al., 1984). Δ^{12}-PGJ_2 has been shown to be a significant PGD_2 metabolite present in human and monkey urine, indicating that the J_2 series of PGs also form in vivo (Hirata et al., 1988).

In cotransfection assays, the three J-ring PGs were efficacious activators of both the PPARα and PPARγ chimeras (Figures 2A and 2B). Interestingly, 15-deoxy-$\Delta^{12,14}$-PGJ_2 was significantly more potent than its precursors in the activation of the PPARγ chimera. The PPARγ chimera responded to 15-deoxy-$\Delta^{12,14}$-PGJ_2 at concentrations as

low as 100 nM and with an EC_{50} of 2 μM (Figure 2B). The results were not dependent upon the use of the chimera system, as similar data were obtained by using the wild-type PPARγ1 and a reporter construct driven by the adipocyte fatty acid–binding protein (*aP2*) enhancer containing two PPAR response elements (data not shown; Tontonoz et al., 1994a). These data are reminiscent of those obtained with the retinoid X receptor (RXR): RXR is activated by high concentrations of all-*trans* retinoic acid through isomerization of a fraction of the compound to the high affinity RXR ligand 9-*cis* retinoic acid (Heyman et al., 1992; Levin et al., 1992).

Dose–response analysis performed with the PPARα chimera in the presence of PGD_2 and the J_2 series of PGs revealed a markedly different activation profile. While the PPARα chimera responded to high concentrations of the J-ring PGs, PGD_2 was the most potent of these compounds (Figure 2A). The differential responsiveness of PPARα and PPARγ to PGD_2 and its PGJ_2 metabolites is consistent with previous studies demonstrating that the PPAR subtypes are pharmacologically distinct (Kliewer et al., 1994; Lehmann et al., 1995).

PGs of the J_2 Series Bind Directly to PPARγ

We previously showed that the thiazolidinedione BRL49653 binds to PPARγ with a K_d of approximately 40 nM (Lehmann et al., 1995). The ability of PGD_2 and its metabolites to interact directly with PPARγ was assessed in a competition binding assay using [^{3}H]BRL49653 and the ligand-binding domain of PPARγ expressed in Escherichia coli. As expected, unlabeled BRL49653 efficiently displaced [^{3}H]BRL49653 from the PPARγ ligand-binding domain (Figure 3A). Notably, 15-deoxy-$\Delta^{12,14}$-PGJ_2 also competed efficiently with [^{3}H]BRL49653, indicating that this PGJ_2 derivative can interact directly with PPARγ. Weaker but reproducible competition was also detected with PGJ_2 and Δ^{12}-PGJ_2 (Figure 3A). No competition was observed with PGD_2, PGE_2, and $PGF_2\alpha$ (Figure 3A). We note that the relative potencies of PGD_2 and PGJ_2 were reversed in the competition binding and PPARγ transactivation assays. A number of potential explanations for this disparity exist, including the possibilities that these compounds are differentially metabolized in the two assays or are transported with different kinetics in the cell-based transfection assay. In control experiments, 15-deoxy-$\Delta^{12,14}$-PGJ_2 at a concentration of 1×10^{-5} M failed to compete with [^{3}H]estradiol for binding to bacterially expressed estrogen receptor ligand-binding domain (data not shown). Thus, 15-deoxy-$\Delta^{12,14}$-PGJ_2 can interact directly and specifically with PPARγ.

A complete dose–response analysis was performed with 15-deoxy-$\Delta^{12,14}$-PGJ_2 in the competition binding assay. 15-Deoxy-$\Delta^{12,14}$-PGJ_2 displaced [^{3}H]BRL49653 binding to the PPARγ ligand-binding domain with a K_i of 2.5 μM (Figure 3B). These data are in good agreement with the EC_{50} of 15-deoxy-$\Delta^{12,14}$-PGJ_2 for PPARγ activation in the transient transfection assay (Figure 2B) and provide evidence that a fatty acid metabolite can activate a PPAR through direct interactions with the ligand-binding domain of the receptor.

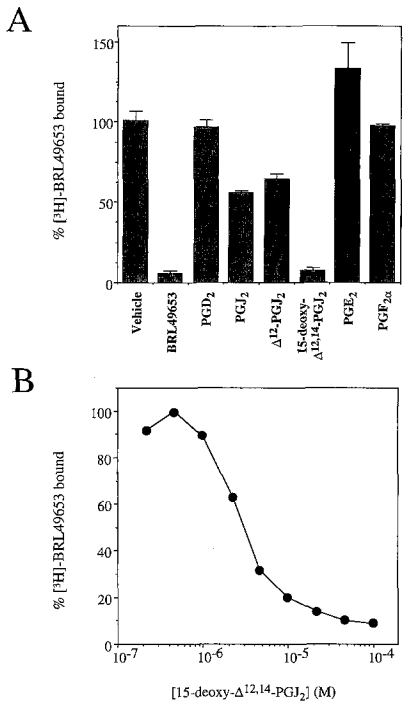

Figure 3. 15-Deoxy-$\Delta^{12,14}$-PGJ_2 Binds PPARγ

(A) Competition binding assays were performed using histidine-tagged PPARγ ligand-binding domain protein and 10 nM [^{3}H]BRL49653 in the presence of vehicle (1% DMSO) or 50 μM unlabeled BRL49653 or PGs. Data shown are the mean of assays performed in triplicate ± standard deviation.

(B) Competition binding assays were performed with histidine-tagged PPARγ ligand-binding domain fusion protein and 10 nM [^{3}H]BRL49653 in the presence of the indicated concentrations of unlabeled 15-deoxy-$\Delta^{12,14}$-PGJ_2 as competitor.

15-Deoxy-$\Delta^{12,14}$-PGJ_2 Promotes Adipocyte Differentiation

PPARγ is abundantly expressed in adipocytes, and its ectopic expression in fibroblasts has been shown to result in adipocyte differentiation (Tontonoz et al., 1994a, 1994b; Chawla et al., 1994). We recently showed that treatment of murine C3H10T1/2 fibroblasts with the high affinity PPARγ ligand BRL49653, an antidiabetic thiazolidinedione, induces their efficient conversion to adipocytes as measured by induction of adipocyte-specific genes and lipid accumulation (Lehmann et al., 1995). Thus, activation of PPARγ is sufficient to initiate the entire adipogenic signaling cascade.

If PGs of the J_2 series can function as signaling molecules in vivo, these compounds might likewise be expected to promote adipocyte differentiation of C3H10T1/2 cells. To test this possibility, C3H10T1/2 cells were treated with 15-deoxy-$\Delta^{12,14}$-PGJ_2 and related PGs for 4 days and assayed for [^{3}H]glucose incorporation into total cellular lipid as a measure of lipogenesis. Lipogenesis is an established measure of adipocyte differentiation (Moody et al., 1974). Lipogenesis was stimulated approximately 11-fold in the presence of 1×10^{-5} M 15-deoxy-$\Delta^{12,14}$-PGJ_2 and with an EC_{50} of 7 μM (Figure 4A; Table 1). Adipocyte differentiation was confirmed by oil red O staining for lipid drop-

let accumulation in the cytoplasm of 15-deoxy-$\Delta^{12,14}$-PGJ_2-treated cells (Figure 4B). For comparative purposes, ciglitazone, a thiazolidinedione with an EC_{50} of 3 μM for PPARγ activation (Lehmann et al., 1995), was tested. Ciglitazone stimulated lipogenesis to a similar degree as 15-deoxy-$\Delta^{12,14}$-PGJ_2 and with an EC_{50} of 4 μM (Figure 4A; Table 1). As expected, the thiazolidinedione BRL49653 was the most potent compound in the assay, inducing lipogenesis 18-fold at 1×10^{-5} M with an EC_{50} of approximately 90 nM (Figure 4A; Table 1). Lipogenesis in C3H10T1/2 cells was stimulated to a lesser degree by treatment with PGD_2, Δ^{12}-PGJ_2, and PGJ_2 (Figure 4A). Taken together, these data provide strong evidence that the arachidonic acid derivative 15-deoxy-$\Delta^{12,14}$-PGJ_2 can function through PPARγ to promote adipocyte differentiation.

Discussion

PGD_2 is known to play an important role in a number of biological processes, presumably through interactions with its cognate G protein–coupled receptor. The finding that PGD_2 readily undergoes dehydration to the J_2 series of PGs both in vitro and in vivo (Fitzpatrick and Wynalda, 1983; Kikawa et al., 1984; Hirata et al., 1988) coupled with the effects of the J-ring PGs on various biological processes in vitro (reviewed by Fukushima, 1992) suggested the existence of a second PGD_2 signaling pathway. Our data demonstrate that the PGJ_2 metabolites of PGD_2 are efficacious activators of PPARα and PPARγ, suggesting that the biological effects of these molecules may be mediated at least in part through activation of nuclear receptor signaling cascades.

Several previous observations have indicated that PGD_2 and its J-ring metabolites might exert effects through interactions with intracellular proteins. First, the two PGD_2 synthetases identified to date, responsible for the conversion of PGH2 to PGD_2, are localized predominantly in the cytosol of cells (Shimuzu et al., 1979; Urade et al., 1989). This contrasts with the other characterized PG synthetases, which are present in the microsomal fraction (reviewed by Giles and Leff, 1988). Second, radiolabeled Δ^{12}-PGJ_2 has been reported to be actively incorporated into cells and transferred to the nucleus, where it is associated with proteins (Narumiya et al., 1987). Finally, PGD_2, PGJ_2, and Δ^{12}-PGJ_2 have been shown to bind with high affinity to liver fatty acid–binding protein (L-FABP), an intracellular protein involved in the uptake, intracellular transport, and metabolism of free fatty acids and their acyl-CoA esters (Khan and Sorof, 1990). L-FABP is a member of a family of lipid carrier proteins that includes the cellular retinol-binding proteins and cellular retinoic acid–binding proteins (Sweetser et al., 1986). This raises the interesting possibility that the FABPs and retinoid binding proteins may play analogous roles in the PPAR and retinoid receptor signaling pathways, respectively.

Table 1. EC_{50} Values of PPARγ Ligands in the C3H10T1/2 Cell Lipogenesis Assay

Compound	EC_{50}(μM)
BRL49653	0.087 ± 0.0010
Ciglitazone	3.9 ± 0.72
15-Deoxy-$\Delta^{12,14}$-PGJ_2	7.0 ± 0.43

C3H10T1/2 cells were treated for 4 days with ten different concentrations of each compound in the range of 0.02–10.00 μM, and [^{3}H]glucose incorporation into total lipid was subsequently measured. The EC_{50} values ± standard deviations are shown.

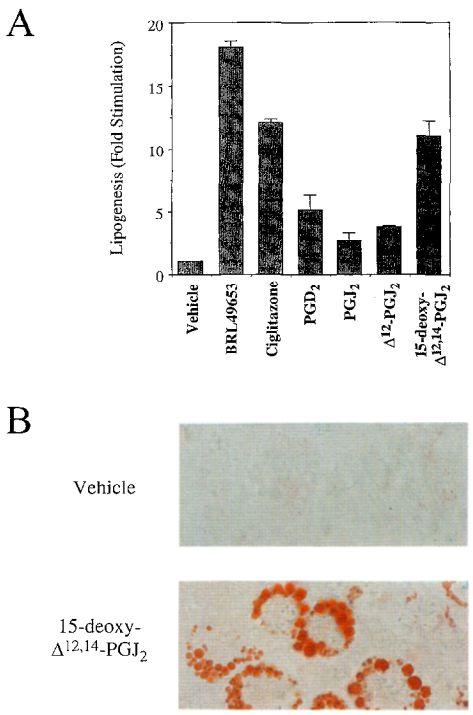

Figure 4. 15-Deoxy-$\Delta^{12,14}$-PGJ_2 Treatment Promotes Adipocyte Differentiation of C3H10T1/2 Cells

(A) [^{3}H]glucose incorporation into total lipid (lipogenesis) was measured in C3H10T1/2 cells treated for 4 days with vehicle alone (0.1% DMSO) or 1×10^{-5} M of the indicated PGs or thiazolidinediones. Data are plotted as fold activation in lipogenesis relative to vehicle treatment.

(B) C3H10T1/2 cells were treated for 5 days with vehicle alone (0.1% DMSO) or 1×10^{-5} M 15-deoxy-$\Delta^{12,14}$-PGJ_2 and subsequently stained for lipid droplet accumulation with oil red O. Magnification is 85×.

Colocalization of PPARs with PGD_2 and PGD_2 Synthetase

We have demonstrated that PPARα and PPARγ are efficiently activated by PGD_2 and its PGJ_2 derivatives at concentrations in the high nanomolar and low micromolar range. These data raise the following question: are PGD_2 and its PGJ_2 metabolites present in vivo at sufficient concentrations to serve as physiologic PPAR activators? While PGD_2 and Δ^{12}-PGJ_2 are known to form in vivo (Nowak and Wennmalm, 1979; Hirata et al., 1988), the rapid metabolism and potential for sequestration of these compounds by binding proteins complicates the quantitation of their levels. Little is known regarding the in vivo levels of the J_2 series of PGs other than that Δ^{12}-PGJ_2 is present

in human and monkey urine in significant quantities (~150 ng/24 hr in human males) (Hirata et al., 1988). Higher amounts of other, uncharacterized Δ^{12}-PGJ_2 immunoreactive compounds have also been detected in human urine (Hirata et al., 1988).

Several groups have examined the levels of PGD_2 formed from endogenous arachidonic acid in homogenates from various tissues. In two studies, PGD_2 was found to be most abundant in extracts prepared from rat spleen, with concentrations of 10–30 μM reported (Chiabrando et al., 1984; Ujihara et al., 1988). PGD_2 synthetase, the enzyme responsible for PGD_2 formation, was likewise found to be most abundant in spleen as determined by both enzymatic activity in tissue homogenates and immunohistochemistry (Ujihara et al., 1988; Urade et al., 1989). Interestingly, PPARγ was recently shown by in situ hybridization analysis to be abundantly expressed in both the red and the white pulp of the rat spleen, indicating a potential role in energy metabolism in the immune system (Braissant et al., 1996). PPARα is also expressed in the red and white pulp of the rat spleen, albeit at lower levels (Braissant et al., 1996). The colocalization of PPARα and PPARγ with PGD_2 synthetase activity in spleen suggests that PGD_2 metabolites may serve as PPAR ligands in this tissue.

PGD_2 synthetase activity has also been demonstrated in intestine, lung, and liver, tissues in which PPARα, PPARγ, or both are expressed (Chiabrando et al., 1984; Ujihara et al., 1988; Urade et al., 1989; Isseman and Green, 1990; Kliewer et al., 1994; Tontonoz et al., 1994a; Braissant et al., 1996). Thus, PGD_2 metabolites may serve as physiologic PPAR ligands in these tissues.

A Potential Role for PGD_2 Metabolites in Adipocyte Differentiation

PPARγ is selectively expressed in adipocytes and was recently shown to play a pivotal role in adipocyte differentiation (Chawla et al., 1994; Tontonoz et al., 1994a, 1994b; Lehmann et al., 1995). Our demonstration that 15-deoxy-$\Delta^{12,\ 14}$-PGJ_2 is a PPARγ ligand and efficiently promotes differentiation of C3H10T1/2 fibroblasts to adipocytes raises the interesting possibility that this PG serves as an adipogenic signaling molecule in vivo. Several PGs, including PGE_2 and prostacyclin, are known to be synthesized by isolated adipocytes as well as adipogenic cell lines and to exert marked effects on adipocyte differentiation (Christ and Nugteren, 1970; Axelrod and Levine, 1981). Furthermore, phospholipase A_2 levels increase during the course of adipocyte differentiation, suggesting the potential for increased production of cyclooxygenase products (Gao and Serrero, 1990). Finally, abnormal PG synthesis has been reported in adipocytes isolated from obese Zucker rats (Gaskins et al., 1989). These results point to an important role for PGs in adipose tissue development and function.

Synthesis of PGD_2 has been reported to be low in homogenates prepared from rat epididymal fat relative to other tissues (Ujihara et al., 1988). It remains possible that PGD_2 and its PGJ_2 metabolites are present at higher concentrations either locally or transiently during the course of adipocyte differentiation in the epididymal depot. However, PGD_2 has been shown to be the major PG synthesized in rat bone marrow homogenates (Kojima et al., 1980; Ujihara et al., 1988). Bone marrow represents a highly active site for the recruitment of stromal cells to the adipocyte lineage as well as a site of abundant PPARγ expression (Greene et al., 1995). Thus, PGD_2 metabolites such as 15-deoxy-$\Delta^{12,\ 14}$-PGJ_2 may serve as adipogenic signaling agents in the context of bone marrow.

Evidence for a Novel PG Signaling Pathway

The previous observations that PPARs are activated by high concentrations of fatty acids, regulate a battery of genes involved in lipid homeostasis, and play a crucial role in adipocyte differentiation suggested that the physiologic ligands for these receptors might be fatty acid metabolites. Our demonstration that the arachidonic acid derivative 15-deoxy-$\Delta^{12,\ 14}$-PGJ_2 binds PPARγ supports this hypothesis. Nonetheless, the finding that a PG can serve as a ligand for a nuclear receptor is surprising in that most PGs are known to exert their effects on gene expression through interactions with cell surface receptors, inducing changes in the levels of second messengers. Our data indicate that the PGJ_2 series of PGs may bypass this second messenger signaling cascade and instead modulate the transcription of target genes directly through interactions with the PPAR family of ligand-activated transcription factors. Our results suggest the existence of a novel PG signaling pathway and, furthermore, raise the intriguing possibility that other eicosanoids may serve as ligands for additional orphan members of the nuclear receptor superfamily.

Experimental Procedures

Chemicals

All PGs were purchased from Cayman Chemical Company (Ann Arbor, MI).

Cotransfection Assay

The pSG5-GAL4-PPARα, pSG5-GAL4-NUC1, and pSG5-GAL4-PPARγ chimeric receptor expression plasmids and $(UAS)_5$-tk-CAT reporter plasmid were previously described (Lehmann et al., 1995). Transient cotransfection assays using the GAL4–PPAR chimeras were performed as previously described (Lehmann et al., 1995).

Ligand Binding Assays

To generate histidine-tagged PPARγ ligand-binding domain protein, cDNA encoding the entire human PPARγ ligand-binding domain (amino acids 176–477) (Greene et al., 1995) was amplified by PCR and inserted into the NdeI–BamHI sites of bacterial expression plasmid PET-16b (Novagen). The His-PPARγ ligand-binding domain protein was expressed in BL21(DE3)plysS cells and bacterial extracts prepared by freeze–thawing of the cells in lysis buffer containing 10 mM Tris (pH 8.0), 50 mM KCl, 10 mM DTT, and 1% Triton X-100 followed by centrifugation at 40,000 × g for 30 min. For competition binding assays, bacterial extracts (approximately 100 μg of protein) were incubated at 4°C for 2–3 hr with 10 nM [^{3}H]BRL49653 (specific activity, 40 Ci/mmol) in the absence or presence of unlabeled competitor in buffer containing 10 mM Tris (pH 8.0), 50 mM KCl, 10 mM DTT. Bound was separated from free radioactivity by elution through 1 ml Sephadex G-25 desalting columns (Boehringer Mannheim). Bound radioactivity eluted in the column void volume and was quantitated by liquid scintillation counting.

Adipocyte Differentiation Assays

C3H10T1/2 clone 8 murine fibroblasts (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (GIBCO,

Grand Island, NY) supplemented with 10% fetal calf serum and 10 μg/ml penicillin and streptomycin. One day after reaching confluence, the cells were treated with BRL49653, ciglitazone, or PGs in the presence of 200 nM insulin. Fresh media and test compounds were added two days postconfluence. Lipogenesis was measured in cells at four days postconfluence as previously described (Moody et al., 1974). Accumulation of lipid droplets in the cytoplasm was determined by oil red O staining (Novikoff et al., 1980).

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