The Peroxisome Proliferator-Activated Receptor γ (PPARγ) Ligands 15-Deoxy-Δ12,14-Prostaglandin J2 and Ciglitazone Induce Human B Lymphocyte and B Cell Lymphoma Apoptosis by PPARγ-Independent Mechanisms

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The Peroxisome Proliferator-Activated Receptor γ (PPARγ) Ligands 15-Deoxy-Δ^{12,14}-Prostaglandin J₂ and Ciglitazone Induce Human B Lymphocyte and B Cell Lymphoma Apoptosis by PPARγ-Independent Mechanisms

Denise M. Ray,* Filiz Akbiyik,† and Richard P. Phipps‡*

Peroxisome proliferator-activated receptor γ (PPARγ) is a transcription factor important for adipogenesis and more recently has been shown to be an anticancer target. PPARγ ligands, including the endogenous ligand 15-deoxy-Δ^{12,14}-PGJ₂ (15d-PGJ₂) and synthetic ligands like ciglitazone and troglitazone, all induce apoptosis in normal and malignant human B lymphocytes, but the dependency of PPARγ for apoptosis induction is unknown. In this study, we used a PPARγ dominant-negative approach and a small molecule irreversible PPARγ antagonist and found that these inhibitors prevented PPARγ activation but did not prevent B cell apoptosis induced by 15d-PGJ₂ or ciglitazone. In addition, a PPARγ agonist that is a structural analog of 15d-PGJ₂, and lacks the electrophilic carbon of the 15d-PGJ₂ cyclopentenone ring, activated PPARγ but did not kill B lymphocytes, further supporting a non-PPARγ-mediated mechanism. To further investigate the apoptotic mechanism, the effects of 15d-PGJ₂ and ciglitazone on reactive oxygen species were investigated. 15d-PGJ₂, but not ciglitazone, potently induced reactive oxygen species, implicating the reactive nature of the 15d-PGJ₂ structure in the apoptosis mechanism. In addition, 15d-PGJ₂ caused an almost complete depletion of intracellular glutathione. Moreover, incubation with glutathione reduced ethyl ester, an antioxidant, prevented apoptosis induced by 15d-PGJ₂, but not by ciglitazone. These findings indicate that the expression of PPARγ may not be predictive of whether a normal or malignant B lineage cell is sensitive to PPARγ agonists. Furthermore, these new findings support continued investigation into the use of PPARγ agonists as agents to attenuate normal B cell responses and as anti-B cell lymphoma agents. The Journal of Immunology, 2006, 177: 5068–5076.

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3 Abbreviations used in this paper: PPAR, peroxisome proliferator-activated receptor; 15d-PGJ₂, 15-deoxy-Δ^{12,14}-PGJ₂; GSH, glutathione; GSH-E, glutathione-reduced ethyl ester; DN, dominant negative; PPRE, PPAR response element; ROS, reactive oxygen species; DiOC₅(3), 3,3’-dihexyloxacarbocyanide iodide; carboxy-H₂DCFDA, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, β-gal, β-galactosidase.

PPARγ is expressed in diverse cell types including endothelial cells, fibroblasts, macrophages, dendritic cells, and T and B lymphocytes (6–12). PPARγ has been implicated in several disease conditions including diabetes, atherosclerosis, and inflammation (13–15). Agonists of PPARγ include the natural ligands, 15-deoxy-Δ^{12,14}-PGJ₂ (15d-PGJ₂) (16, 17), lysophosphatidic acid (18), nitrolinoleic acid (19), as well as the synthetic thiazolidinedione class of anti diabetic drugs such as ciglitazone (14). PPARγ agonists are potent inducers of adipogenesis in fibroblasts through PPARγ-dependent activation of adipocyte differentiation gene transcription (20).

The cyclopentenone PG 15d-PGJ₂ is a product of the cyclooxygenase pathway and is the final metabolite of PGD₂ (21). PGD₂ is synthesized from the common precursor PGH₂ by the action of PGD synthetases (22, 23). 15d-PGJ₂ is formed through the spontaneous dehydration of PGD₂ (21). PGD₂ is produced by mast cells, APCs, and certain T cell subsets (24–26). Although the existence of 15d-PGJ₂ in vivo is unclear, there are reports of 15d-PGJ₂ production in macrophages of atherosclerotic lesions, by normal prostate stromal cells, and during the resolution phase of inflammation (27–29). Cyclopentenone PGs have highly reactive structures that contain an α,β-unsaturated ketone that are susceptible to nucleophilic addition reactions. For example, the cyclopentenone ring of 15d-PGJ₂ covalently modifies cellular proteins such as the p65 and p50 subunits of NF-kB (30, 31). It is this reactivity that is attributed to the potent anti proliferative and antiviral effects of cyclopentenone PGs (32).

PPARγ agonists have both PPARγ-dependent and -independent effects. PPARγ agonists are reported to induce apoptosis of several types of cancer cells and normal cells independently of PPARγ activation including breast cancer cells, dendritic cells, and hepatic...
myofibroblasts (9, 33, 34). For example, hepatic myofibroblasts do not express PPARγ, but still undergo apoptosis when exposed to 15d-PGJ2 (34). Many of the anti-inflammatory effects of PPARγ agonists have been attributed to PPARγ-independent mechanisms although some effects require PPARγ. PPARγ agonists inhibit monocyte and macrophage production of inflammatory mediators (8, 35). However, in a macrophage PPARγ knockout model, the lack of PPARγ had no effect on the ability of PPARγ agonists to block proinflammatory cytokine production, but was required for the up-regulation of the scavenger receptor CD36 (36). In fact, PPARγ can indirectly affect transcription. For example, PPARγ physically interacts with NF-κB resulting in transrepression of NF-κB, which could contribute to the anti-inflammatory effects of PPARγ agonists (37, 38). Unfortunately, knocking out PPARγ in mice is embryonic lethal due to placental dysfunction (39), but PPARγ heterozygous mice (PPARγ+/−) have been used to study the contribution of PPARγ in development and in disease. B cells from PPARγ+/− mice have an enhanced proliferative and Ab response to LPS, but only a slight difference in apoptosis induction by PPARγ agonists as compared with wild-type mice (40). There is little data on the PPARγ dependency of exposure of human B lineage cells to natural and synthetic PPARγ ligands. Therefore, we sought to define the contribution of PPARγ to PPARγ ligand-induced apoptosis of human B lymphocytes.

We previously reported that both mouse and human normal and malignant B lymphocytes abundantly express PPARγ and undergo apoptosis after exposure to both natural and synthetic PPARγ agonists (11, 12, 41). However, it is not known if PPARγ is required for apoptosis induction of human B lymphocytes. In this study, we investigate whether the PPARγ agonists, 15d-PGJ2 and ciglitazone, induce apoptosis independently of PPARγ in normal and malignant human B lymphocytes. These findings are important for the potential use of PPARγ agonists as therapies for B cell malignancies and B cell proliferative disorders.

Materials and Methods

Reagents and Abs

15d-PGJ2, and ciglitazone were purchased from Biomol; glutathione (GSH) reduced ethyl ester (GSH-EH), β-NADPH, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), sulfosalicylic acid, GSH reductase, diptösum protein, hydroperoxide oxidase (K,HPO4), anti-Flag M2 mAb peroxidase conjugate, PG,F2, MTI, Oil-red-O, and DMSO were purchased from Sigma-Aldrich; GW9662, CAY10410, and T0070907 were obtained from Cayman Chemical; 5-(and-6)-carboxy-2',7-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA), 5,6-dihexyloxacarbocyanine iodide (DiOC6(3)), and MitoSOX Red were purchased from Molecular Probes.

Cells and culture conditions

Ramos B cell lymphoma is a human Burkitt’s B cell lymphoma. Ramos cells were cultured in RPMI 1640 tissue culture medium (Invitrogen Life Technologies) supplemented with 10% FBS, 5 × 10−5 M 2-ME (Eastman Kodak), 10 mM HEPES (U.S. Biochemical), 2 mM l-glutamine (Invitrogen Life Technologies), and 50 μg/ml gentamicin (Invitrogen Life Technologies).

Peripheral human blood B cell isolation

One unit of blood was obtained from healthy donors between the ages of 21 and 45, as approved by the University of Rochester Institutional Review Board and Office for Human Subject Protection. Buffy coat was obtained by centrifugation at 933 × g for 8 min. The buffy coat was separated over a Ficoll gradient to obtain PBMCs. The PBMCs were washed four times in PBS, and the B cells were selected with CD19 magnetic beads (Dynal). The CD19-positive cells were selected with a magnet, washed, and the CD19 magnetic beads were detached using CD19 Detach beads (Dynal). After detachment, the B cells were washed and stained for flow cytometry for CD19 and CD3 to check the purity of the isolation. The B cells were purified to >98% CD19-positive cells with <1% CD3-positive cells. The peripheral blood B cells were cultured as described for Ramos cells.

Transfection of PPARγ dominant-negative (DN) and PPARγ response element (PPRE-LUC) reporter constructs

The pcDNAFlag-γ L466A/E469A DN human PPARγ plasmid was a gift from Dr. V. K. K. Chatterjee (University of Cambridge, Cambridge, U.K.). The DN contains two amino acid substitutions at leucine 468 and glutamic acid 471 at the C-terminal end and serves as a powerful inhibitor of wild-type PPARγ (42). A total of 8 × 105 Ramos B lymphoma cells per well of a 24-well plate were transfected with 1 μg of DN plasmid DNA or empty PCDNA3 plasmid (referred to as empty vector (EV)) using Lipofectamine 2000 (Invitrogen Life Technologies). Twenty-four hours after transfection, cells were treated with PPARγ agonists in an MTT assay for 48 h. To test for expression of the Flag-tagged DN PPARγ, 8 × 103 cells were lysed in Nonidet P-40 lysis buffer containing a protease inhibitor mixture (4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, aprotinin) (Sigma-Aldrich), and total protein was quantified using the bichinchoninic acid protein assay (BCA assay kit; Pierce). Briefly, 20 μg of total protein was electrophoresed on a 10% polyacrylamide-stacking gel and transferred to nitrocellulose membrane. The membrane was blocked for 2 h with 5% BLOTTO (PBS/0.1% Tween 20, and 0.1% milk), then incubated for 1 h with a monoclonal anti-Flag peroxidase-conjugated Ab. The membrane was developed by chemiluminescence using a Western Lightning kit (PerkinElmer). To test whether the expressed DN PPARγ was functional in the B cells, the cells were cotransfected with a PPRE-luciferase reporter plasmid containing three copies of the ACO-PPRE (PPAR response element) from rat acylCoA oxidase (a gift from Dr. B. Seeley, Massachusetts General Hospital, Boston, MA) (35) and a β-galactosidase (β-gal) expression plasmid (Promega) to normalize the transfections, exposed to PPARγ agonists for 8 h at which time luciferase activity was assayed using the Promega Luciferase Assay System. Relative light units were determined with a Lumicount Microplate Luminometer (Packard Instrument). β-gal activity was measured using a β-gal enzyme assay system (Promega) and the relative light units were normalized to β-gal activity for the experiment with GW9662, the cells were cotransfected with the PPRE-LUC and β-gal constructs, and then 24 h later were pre-treated with the PPARγ antagonist GW9662 for 3 h. The cells were then exposed to the PPARγ ligands for 8 h and luciferase and β-gal assays were performed. PPARγ activation studies with the PPRE-LUC construct were also performed to determine the concentration of PPARγ agonists required for maximal PPARγ activation in human B lymphocytes. 15d-PGJ2, ciglitazone, and CAY10410 all induced maximal PPARγ activation at 1 μM (data not shown), therefore this concentration was used for the transfection experiments.

Viability assays

A total of 6 × 105 Ramos cells or 8 × 105 human peripheral blood B cells per well were incubated with the PPARγ agonists, CAY10410, or DMSO as a control for 48 h in a 96-well flat-bottom microplate. A solution of 0.5 mg/ml MTT in PBS was added to the last 4 h of incubation. After 4 h, the plate was centrifuged, the medium was removed, and DMSO was added to each well to dissolve the precipitate. The plate was read at 510 nm on a Benchmark microplate reader (Bio-Rad). The results are presented as the percent of the DMSO-treated control. For the GW9662 PPARγ antagonist studies, cells were first pretreated with an optimal dose of 100 nM GW9662 for 3 h and then exposed to PPARγ agonists in the presence of 100 nM GW9662. Dose-response studies with GW9662 determined that increasing the dose over 100 nM did not significantly enhance cell death rescue with PPARγ agonists and some cell toxicity was observed at doses higher than 1 μM (data not shown). The T0070907 PPARγ antagonist was used at 1 μM with a 3 h pretreatment. For the antioxidant treatments, cells were preincubated with 2 mM GSH-EH for 1 h and then exposed to the PPARγ agonists.

Mitochondrial membrane potential

A total of 5 × 106 cells were treated with PPARγ agonists, CAY10410, or DMSO (solvent) for 12 h. The cells were then incubated with 40 nM DiOC6(3) (Molecular Probes) for the last 15 min of culture. The cells were harvested, washed in PBS, and immediately analyzed on a BD Biosciences FACSCalibur flow cytometer. Cells with intact mitochondrial membrane potential incorporate DiOC6(3) into the mitochondria.

Reactive oxygen species (ROS) production

ROS production was measured using the probe carbboxy-H2DCFDA (43). When ROS are present in the cell, the carbboxy-H2DCFDA is modified and becomes fluorescent. A total of 5 × 106 cells were treated with PPARγ agonists or DMSO for 12 h. Ten micromoles of carbboxy-H2DCFDA was added to each well.
added for the last 30 min of culture. The cells were washed and immediately analyzed on a BD Biosciences FACSCalibur flow cytometer. MitoSOX Red dye was used as a specific indicator of superoxide production in the mitochondria. After treatment with PPARγ agonists, cells were washed in HBSS containing Mg²⁺ and Ca²⁺, resuspended in a 5 mM solution of MitoSOX Red in HBSS, and incubated for 10 min at 37°C. The cells were washed in HBSS and immediately analyzed on a flow cytometer using FlowJo software (Tree Star).

Detection of total intracellular GSH
A total of 3 × 10⁶ cells were exposed to DMSO, 15d-PGJ₂, or ciglitazone over a 12-h time course analysis. The cells were lysed by sonication for 30 s in extraction buffer (0.1% Triton X-100, 0.6% sulfosalicylic acid in 0.1 M phosphate buffer with 5 mM EDTA) using a Vibra Cell low volume high intensity Ultrasonic Processor (Sonics and Materials). The samples were incubated with 1 U/ml GSH reductase and 0.5 mM DTNB for 30 s followed by addition of 0.24 mM β-NADPH. The oxidation of GSH was detected at 415 nm on a Benchmark microplate reader and the concentration of GSH (nanomoles per milliliter) was calculated based on a GSH standard curve. The protein concentration of the cell lysates was determined by BCA assay and the nanomoles of GSH per milligram of protein was calculated.

Statistical analysis
For all experiments shown, error bars represent the SD of triplicate samples from the mean. The ANOVA test for statistical significance was performed and p values <0.05 were considered significant. All experiments were repeated at least three times.

Results
A DN PPARγ does not prevent PPARγ agonist-induced cell death of Ramos B lymphoma cells
Human B lymphocytes abundantly express PPARγ and are killed by a variety of structurally dissimilar small molecule PPARγ agonists (12, 41). According to our previous studies and the data reported herein, the EC₅₀ for cell death induction by PPARγ agonists in human B lymphocytes is 2 μM for 15d-PGJ₂ and 7 μM for ciglitazone (12). In the following set of experiments, we wanted to determine whether selected PPARγ agonists killed human B cells by a PPARγ-dependent or -independent mechanism. We first used a FLAG tagged DN human PPARγ construct (DN PPARγ). This DN contains two amino acid substitutions, which reduces coactivator recruitment and enhances corepressor recruitment and is a potent inhibitor of wild-type PPARγ (42). Ramos B lymphoma cells were transfected with EV or the DN PPARγ construct using Lipofectamine reagent. Twenty-four hours after transfection, expression of the Flag-tagged DN PPARγ was confirmed by Western blot using an anti-Flag Ab (Fig. 1A). Next, the functional ability of the DN PPARγ was tested in the Ramos cells using cotransfection with a luciferase reporter construct containing three PPRE elements (PPRE-LUC) (35). The cotransfected cells were treated with 1 μM 15d-PGJ₂ or 1 μM ciglitazone for 8 h and Fig. 1B shows the results of a luciferase assay. There was a low level of luciferase activity with the EV and the PPRE-LUC cotransfection, and the luciferase activity was greatly enhanced with the addition of 15d-PGJ₂ and ciglitazone. Cotransfection of the DN PPARγ completely inhibited PPRE-LUC transcription by both 15d-PGJ₂ and ciglitazone as evidenced by the reduction of luciferase activity back to the level of the control (EV). This confirms that the DN PPARγ is indeed functional in the Ramos cells. Transfected Ramos cells were next exposed to PPARγ agonists for up to 48 h and an MTT assay was performed to evaluate whether the DN PPARγ was able to inhibit cell death induced by the PPARγ agonists. Interestingly, at most doses of 15d-PGJ₂, the DN PPARγ
failed to prevent cell death, although there was a small but statistically significant difference at 2.5 μM 15d-PGJ2 (Fig. 1C). With exposure to the PPARγ agonist ciglitazone, shown in Fig. 1D, there were no significant differences seen with the DN PPARγ compared with ciglitazone alone. These findings support that PPARγ is not a major contributor to B cell death induced by PPARγ agonists.

GW9662, a small molecule irreversible PPARγ antagonist, does not prevent PPARγ agonist-induced cell death of B lymphocytes

To confirm the DN PPARγ results, we next used the PPARγ irreversible antagonist GW9662. GW9662 covalently modifies the PPARγ ligand-binding domain and acts as an irreversible antagonist at concentrations of 100 nM or less (44). In addition to Ramos B lymphoma cells, purified normal human B cells from peripheral blood were pretreated with 100 nM GW9662 for 3 h and then exposed to PPARγ agonists. As shown in Fig. 2A, GW9662 significantly inhibited cell death at only a single concentration of 15d-PGJ2 (2.5 μM), and GW9662 did not significantly inhibit cell death by ciglitazone in Ramos cells. For the normal human B cells, there was no significant inhibition of cell death with the GW9662 (Fig. 2B). The results for the Ramos cells confirm the DN PPARγ findings in Fig. 1. Overall, these observations suggest that for the Ramos B lymphoma cells, there may be a small PPARγ contribution to apoptosis. Normal human B cells do not appear to require PPARγ for apoptosis induction by these PPARγ agonists. An additional PPARγ antagonist, T0070907 (45), also resulted in a slight inhibition of 15d-PGJ2-induced cell death at the 2.5 μM concentration as shown in Fig. 2C, but overall did not change the cell death response to 15d-PGJ2. Because GW9662 did not prevent B cell death by PPARγ ligands, we confirmed that GW9662 inhibited PPARγ activity using the PPRE-LUC construct as shown in Fig. 3A. The GW9662 alone did not induce the PPRE-LUC activity. In fact, GW9662 completely inhibited the induction of PPRE-LUC activity by both 15d-PGJ2 and ciglitazone showing that the inhibitor was effective in human B cells. As an additional confirmation, we tested the effectiveness of GW9662 as a PPARγ antagonist in an adipogenesis assay and 100 nM GW9662 prevented 15d-PGJ2 induced-adipogenesis in human orbital fibroblasts (data not shown).

The PPARγ agonist CAY10410, a 15d-PGJ2 analog, does not kill B lymphocytes

To further dissect the involvement of PPARγ in 15d-PGJ2 induced-apoptosis of B lymphocytes, we used a 15d-PGJ2 analog, CAY10410. CAY10410 is a potent PPARγ agonist, but lacks the reactive electrophilic carbon in the cyclopentenone ring (Fig. 4A). To be certain of the PPARγ agonist ability of CAY10410, we tested this drug for the ability to activate the PPRE-LUC construct in B cells and the ability of the DN-PPARγ to inhibit the PPRE-LUC activity induced by CAY10410. The luciferase results in Fig. 3B clearly show that 1 μM CAY10410 activates PPRE-LUC activity and this activation is inhibited by the PPARγ DN demonstrating that CAY10410 activates PPARγ in B cells. Additionally, CAY10410 induced adipogenesis of human orbital fibroblasts (data not shown); further evidence supporting CAY10410 is a PPARγ agonist. Exposure to CAY10410, as shown in Fig. 4, B and C, did not kill either Ramos cells or normal human B cells, even
at doses up to 25 μM, whereas 15d-PGJ2 kills almost 100% of cells at doses <5 μM. In addition, in an apoptosis assay measuring loss of mitochondrial membrane potential, 15d-PGJ2 induced a loss of mitochondrial membrane potential in both the Ramos and normal human B cells, whereas CAY10410 did not (Fig. 4D). These results suggest that 15d-PGJ2 induces apoptosis independently of PPARγ, perhaps as a result of its reactive electrophilic properties.

15d-PGJ2, but not ciglitazone, induces ROS production in B lymphocytes

Because PPARγ does not significantly contribute to apoptosis induction, we further investigated the apoptotic mechanisms of 15d-PGJ2 and ciglitazone in B lymphocytes. The reactive nature of 15d-PGJ2 has been shown to induce harmful ROS (46). To determine whether human B cells produce ROS after exposure to 15d-PGJ2 or ciglitazone, we used a ROS detecting probe carboxy-H2DCFDA. This cell permeable indicator is nonfluorescent and contains acetate groups that are hydrolyzed by intracellular esterases which enables the probe to react with oxidants to generate fluorescence detectable by flow cytometry (43). Carboxy-H2DCFDA detects a broad range of oxidants that are induced during intracellular oxidant stress, including hydrogen peroxide, superoxide, peroxynitrate, and NO (43). Ramos B lymphoma cells (Fig. 5) and normal human B cells (Table I) were exposed to increasing doses of 15d-PGJ2 and ciglitazone for 12 h at which time the cells were incubated with 10 μM carboxy-H2DCFDA for 30 min. The flow cytometry analysis shown in Fig. 5A and Table I demonstrates a dose-dependent increase in ROS with 15d-PGJ2 exposure with almost 100% of cells positive for ROS at 10 μM 15d-PGJ2 for both Ramos and normal human B cells. In a time-course experiment, shown in Fig. 5B, ROS were detectable in Ramos cells as early as 2 h after 15d-PGJ2 exposure. Interestingly, ciglitazone did not induce ROS in either Ramos or normal human B cells (Fig. 5A and Table I), suggesting that it induces apoptosis by a different mechanism. 15d-PGJ2, when incubated with carboxy-H2DCFDA, did not increase fluorescence of the dye indicating that 15d-PGJ2 does not react with the carboxy-H2DCFDA (data not shown).

In addition to total intracellular ROS, we investigated the ability of 15d-PGJ2 and ciglitazone to induce an increase in mitochondrial superoxide using the mitochondrial specific superoxide indicator MitoSOX Red. Ramos cells and normal human B cells exposed to 15d-PGJ2 showed an induction in mitochondrial superoxide production as shown in Fig. 6A. A time-course analysis revealed that the Ramos cells become positive for mitochondrial superoxide after 6 h of 15d-PGJ2 exposure, suggesting the intracellular ROS detected by carboxy-H2DCFDA occurs before mitochondrial ROS induction. Ciglitazone did not induce an increase in mitochondrial superoxide (Fig. 6A).
15d-PGJ$_2$ causes a decrease in total intracellular GSH

We next investigated whether 15d-PGJ$_2$ or ciglitazone had any effect on the intracellular GSH levels in B cells. Total intracellular GSH (both reduced and oxidized forms) was measured at 3, 6, 8, and 12 h after exposure to 10$\mu$M 15d-PGJ$_2$ or 20$\mu$M ciglitazone. As shown in Fig. 7A, 15d-PGJ$_2$ decreased GSH in Ramos B lymphoma cells with levels reduced by 85% of the untreated cells at 12 h. In contrast, ciglitazone caused a small (~20%), but sustained decrease in GSH seen as early as 3 h. Unlike the rapid decrease in GSH observed with 15d-PGJ$_2$ over time, ciglitazone exposure did not cause a further decrease in GSH levels over time.

15d-PGJ$_2$-, but not ciglitazone-, induced cell death is prevented by an antioxidant

Because 15d-PGJ$_2$ induced ROS and significantly reduced intracellular GSH levels, we tested the ability of the antioxidant GSH-EE to block B cell death induced by PPAR$\gamma$ agonists. Ramos and normal human B cells were first pretreated with 2 mM GSH-EE and then exposed to doses up to 20$\mu$M 15d-PGJ$_2$ or 25$\mu$M ciglitazone for 24 h and an MTT assay was performed. GSH-EE significantly inhibited 15d-PGJ$_2$-induced cell death in both the Ramos (Fig. 7B) and normal human B cells (data not shown). In agreement with the inability of ciglitazone to induce ROS production or to deplete intracellular GSH levels, GSH-EE was unable to prevent ciglitazone-induced cell death (data not shown). Addition of GSH-EE prevented the decrease in intracellular GSH observed with 15d-PGJ$_2$ treatment (95% for Ramos and 95% for normal human B cells). Results from one representative experiment are shown.

### Table I. ROS are induced by 15d-PGJ$_2$ but not ciglitazone in freshly purified human B cells

<table>
<thead>
<tr>
<th>PPAR$\gamma$ Agonist</th>
<th>% ROS$^a$</th>
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<tbody>
<tr>
<td>15d-PGJ$_2$</td>
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</tr>
<tr>
<td>1</td>
<td>66 ± 4$^b$</td>
</tr>
<tr>
<td>5</td>
<td>83 ± 5$^b$</td>
</tr>
<tr>
<td>10</td>
<td>94 ± 3$^b$</td>
</tr>
<tr>
<td>Ciglitazone</td>
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</tr>
<tr>
<td>1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>5</td>
<td>9 ± 2</td>
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$^a$ The percent of ROS-positive B cells after a 12-h exposure to PPAR$\gamma$ agonists.

$^b$ A value of $p < 0.01$ for induction of ROS with 15d-PGJ$_2$ treatment.
FIGURE 6. 15d-PGJ2, but not ciglitazone, induces mitochondrial superoxide accumulation in B lymphocytes. A, Ramos B lymphoma cells (left panel) and purified normal human B cells (right panel) were exposed to 15d-PGJ2 or ciglitazone for 12 h. The fluorescence of the MitoSOX Red dye was detected by flow cytometry. Results from one representative experiment are shown. B, Ramos B lymphoma cells were exposed to DMSO vehicle (untreated), 5 μM or 10 μM 15d-PGJ2 for 2, 4, 6, 8, and 12 h and analyzed for MitoSOX Red fluorescence. *, p < 0.05 as compared with the untreated control.

Discussion
Understanding the mechanism of PPARγ agonist-induced apoptosis of B lymphocytes will be critical for the use of these small molecules as anti-B cell and anti-inflammatory agents and in the treatment of B cell malignancies. Herein we demonstrate, using a PPARγ DN and the irreversible small molecule PPARγ antagonist GW9662, both of which inhibit PPARγ activation in human B cells, that both 15d-PGJ2 and ciglitazone induced apoptosis of both normal and malignant human B lymphocytes independently of PPARγ activation. In addition, these new findings show that 15d-PGJ2 and ciglitazone induced apoptosis through different mechanisms. The electrophilic properties of 15d-PGJ2 are required for apoptosis, as a nonelectrophilic 15d-PGJ2 analog, CAY10410, which retains its ability to activate PPARγ in human B cells, does not kill B lymphocytes. 15d-PGJ2 induced apoptosis by a ROS-dependent mechanism, whereas ciglitazone did not induce ROS and ciglitazone-induced apoptosis was not prevented with an antioxidant. From these findings, we conclude that 15d-PGJ2 and ciglitazone, while both activate PPARγ in B cells, have distinct apoptotic mechanisms in human B lymphocytes that are independent of PPARγ.

This is the first report to examine the PPARγ dependency of PPARγ agonists in human B cell apoptosis. An interesting finding that our laboratory previously described was that 15d-PGJ2 was always more potent at killing B cells than synthetic PPARγ agonists. In fact, the thiazolidinediones, which are reported to be more potent PPARγ activators than 15d-PGJ2, require significantly higher doses than 15d-PGJ2 to induce apoptosis of human B lymphocytes (12). Herein, we demonstrate that this difference is most likely due to the use of different apoptotic mechanisms that are independent of PPARγ. However, this does not rule out the possibility that PPARγ has other important functions in B lymphocytes. We have shown that B cells highly express PPARγ protein both in the cytoplasm and the nucleus (12) and PPARγ agonists activate PPARγ in human B cells (see Figs. 1 and 3). There is also evidence in PPARγ heterozygous mice that PPARγ is important in B cell responses, in particular for controlling proliferation and Ab production (40). Therefore, determining the role of PPARγ in B lymphocyte biology may have important implications for the use of PPARγ agonists in controlling B cell responses.

The cyclopentenone PGs, derivatives of PGD2 and PGJ2, have a unique structure that allows receptor-independent modification of cellular targets. It is this reactive nature of cyclopentenone PGs that is thought to be responsible for their potent antiviral and anti-inflammatory properties (32). The structure of 15d-PGJ2 is significantly different from the structures of the thiazolidinedione synthetic PPARγ agonists and it is most likely the nucleophilic ability...
of 15d-PGJ2 that gives it more potent apoptotic properties. The αβ-unsaturated ketones of the cyclopentenone PGs directly conjugate with cellular components involved in maintaining the cellular redox state. For example, thiols such as glutathione and cysteine, as well as thioredoxin are readily conjugated to cyclopentenone PGs (47, 48). It is most likely this reactivity of 15d-PGJ2 that caused depletion of intracellular GSH in the B lymphocytes (Fig. 7A). Not only can cyclopentenone PGs modify cellular components important for normal cellular defense mechanisms, but they can also modify proteins like NF-kB that are important for protecting against apoptosis (30, 31). Overall, 15d-PGJ2 is a potent inducer of intracellular oxidative stress as this PG not only depletes cellular anti-oxidant defenses, but also causes an increase in intracellular ROS (46) (see Figs. 5 and 6).

Induction of ROS has important implications for apoptosis. ROS levels may become so high as to overwhelm cellular oxidative defenses, such as GSH. The unstable nature of free radicals renders them highly reactive which results in cellular damage. ROS oxidize proteins resulting in protein degradation or fragmentation ultimately reducing protein function (49). Additionally, ROS cause lipid peroxidation and DNA damage which contribute to the cells destruction and ultimate apoptotic decision (49). Indeed, we found that ROS are rapidly induced in B lymphocytes within 2 h of 15d-PGJ2 exposure. Additionally, using a highly specific mitochondrial superoxide detection dye, MitoSOX Red, we found 15d-PGJ2 induced mitochondrial superoxide accumulation by 6 h of exposure. In fact, GSH-EE prevented total cellular ROS induction and mitochondrial superoxide accumulation. The ability of the antioxidant GSH-EE to rescue human B lymphocytes from 15d-PGJ2 exposure. Additionally, using a highly specific mitochondrial superoxide detection dye, MitoSOX Red, we found 15d-PGJ2 induced mitochondrial superoxide accumulation by 6 h of exposure. In fact, GSH-EE prevented total cellular ROS induction and mitochondrial superoxide accumulation. The ability of the antioxidant GSH-EE to rescue human B lymphocytes from 15d-PGJ2-induced apoptosis suggests that ROS are the major contributors to the apoptotic death mechanism.

In contrast to the ROS induction by 15d-PGJ2, we found that the synthetic PPARγ ligand ciglitazone did not induce intracellular ROS accumulation in B lymphocytes. Clearly, ciglitazone is a potent inducer of apoptosis in B cells. There are several possibilities for the mechanism of ciglitazone-induced apoptosis. First, we have shown that ciglitazone reduces mitochondrial membrane potential, a characteristic of apoptosis (50). Furthermore, we have previously shown that both 15d-PGJ2 and ciglitazone cause activation of caspases and cleavage of poly-ADP ribose polymerase in B lymphocytes (50). Therefore, the major difference between 15d-PGJ2 and ciglitazone is that 15d-PGJ2 also induces ROS and depletion of intracellular anti-oxidant defenses in B cells. This additional pathway may explain why 15d-PGJ2 is a more potent inducer of apoptosis in B lymphocytes.

The significance of these findings will be important in the design and application of PPARγ agonists for the treatment of B cell malignancies and inflammatory conditions. Our results clearly show that natural (15d-PGJ2) and synthetic (ciglitazone) PPARγ agonists are potent inducers of B cell apoptosis, even though they do so by a PPARγ-independent mechanism. The continued study of PPARγ in B lymphocyte development and function may reveal a yet undiscovered role for PPARγ in the immune system.

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Disclosures
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References


