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

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The Peroxisome Proliferator-Activated Receptor γ (PPAR γ) Ligands 15-Deoxy- $\Delta^{12,14}$ -Prostaglandin J₂ and Ciglitazone Induce Human B Lymphocyte and B Cell Lymphoma Apoptosis by PPAR γ -Independent Mechanisms¹

Denise M. Ray,* Filiz Akbiyik,*[†] and Richard P. Phipps^{2,*}

Peroxisome proliferator-activated receptor γ (PPAR γ) is a transcription factor important for adipogenesis and more recently has been shown to be an anticancer target. PPAR γ ligands, including the endogenous ligand 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (15d-PGJ₂) and synthetic ligands like ciglitazone and troglitazone, all induce apoptosis in normal and malignant human B lymphocytes, but the dependency of PPAR γ for apoptosis induction is unknown. In this study, we used a PPAR γ dominant-negative approach and a small molecule irreversible PPAR γ antagonist and found that these inhibitors prevented PPAR γ activation but did not prevent B cell apoptosis induced by 15d-PGJ₂ or ciglitazone. In addition, a PPAR γ agonist that is a structural analog of 15d-PGJ₂, and lacks the electrophilic carbon of the 15d-PGJ₂ cyclopentenone ring, activated PPAR γ but did not kill B lymphocytes, further supporting a non-PPAR γ -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 in B lymphocytes, 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 PPAR γ may not be predictive of whether a normal or malignant B lineage cell is sensitive to PPAR γ agonists. Furthermore, these new findings support continued investigation into the use of PPAR γ agonists as agents to attenuate normal B cell responses and as anti-B cell lymphoma agents. *The Journal of Immunology*, 2006, 177: 5068–5076.

Peroxisome proliferator-activated receptors (PPARs)³ are members of the nuclear receptor superfamily of ligand-activated transcription factors and are regulators of fat metabolism. The three PPAR subtypes, PPAR α , PPAR β/δ , and PPAR γ , are differentially expressed and form heterodimers with the retinoid X receptor (1, 2). The PPAR/retinoid X receptor heterodimer binds to *cis*-acting DNA elements to turn on transcription of genes. The two isoforms of PPAR γ , PPAR γ 1 and PPAR γ 2, result from differential promoter use and alternative RNA splicing (3) and are highly expressed in adipocytes (4). In contrast to adipose tissue, most other cell types only express PPAR γ 1 (5).

PPAR γ is expressed in diverse cell types including endothelial cells, fibroblasts, macrophages, dendritic cells, and T and B lymphocytes (6–12). PPAR γ has been implicated in several disease conditions including diabetes, atherosclerosis, and inflammation (13–15). Agonists of PPAR γ include the natural ligands, 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (15d-PGJ₂) (16, 17), lysophosphatidic acid (18), nitrooleic acid (19), as well as the synthetic thiazolidinedione class of antidiabetic drugs such as ciglitazone (14). PPAR γ agonists are potent inducers of adipogenesis in fibroblasts through PPAR γ -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 PG GH₂ 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- κ B (30, 31). It is this reactivity that is attributed to the potent antiproliferative and antiviral effects of cyclopentenone PGs (32).

PPAR γ agonists have both PPAR γ -dependent and -independent effects. PPAR γ agonists are reported to induce apoptosis of several types of cancer cells and normal cells independently of PPAR γ activation including breast cancer cells, dendritic cells, and hepatic

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³ Abbreviations used in this paper: PPAR, peroxisome proliferator-activated receptor; 15d-PGJ₂, 15-deoxy- $\Delta^{12,14}$ -PG J₂; GSH, glutathione; GSH-EE, glutathione-reduced ethyl ester; DN, dominant negative; PPRE, PPAR response element; ROS, reactive oxygen species; DiOC₆(3), 3,3'-dihexyloxycarbocyanide iodide; carboxy-H₂DCFDA, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate, β -gal, β -galactosidase.

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

We previously reported that both mouse and human normal and malignant B lymphocytes abundantly express PPAR γ and undergo apoptosis after exposure to both natural and synthetic PPAR γ agonists (11, 12, 41). However, it is not known if PPAR γ is required for apoptosis induction of human B lymphocytes. In this study, we investigate whether the PPAR γ agonists, 15d-PGJ₂ and ciglitazone, induce apoptosis independently of PPAR γ in normal and malignant human B lymphocytes. These findings are important for the potential use of PPAR γ 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-EE), β -NADPH, 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), sulfosalicylic acid, GSH reductase, dipotassium hydrogen orthophosphate (K₂HPO₄), anti-Flag M2 mAb peroxidase conjugate, PGF₂ α , MTT, 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), 3,3'-dihexyloxycarbonyl 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 μ g/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 $933 \times g$ 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 (Dyna). The CD19-positive cells were selected with a magnet, washed, and the CD19 magnetic beads were detached using CD19 Detachabead (Dyna). 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 PPAR γ dominant-negative (DN) and PPAR response element (PPRE)-LUC reporter constructs

The pcDNAFlag- γ 1 L466A/E469A DN human PPAR γ 1 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 PPAR γ 1 (42). A total of 8×10^5 Ramos B lymphoma cells per well of a 24-well plate were transfected with 1 μ g 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 PPAR γ agonists in an MTT assay for 48 h. To test for expression of the Flag-tagged DN PPAR γ , 8×10^5 cells were lysed in Nonidet P-40 lysis buffer containing a protease inhibitor mixture (4-(2-aminoethyl)-benzenesulfonyl fluoride, pepstatin A, transepsoxysuccinyl-L-leucylamide (4-guanidino) butane, bestatin, leupeptin, and aprotinin) (Sigma-Aldrich), and total protein was quantified using the bicinchoninic acid protein assay (BCA assay kit; Pierce). Briefly, 20 μ g of total protein was electrophoresed on a 10% polyacrylamide-stacking gel and transferred to nitrocellulose membrane. The membrane was blocked for 2 h with 10% Blotto (PBS/0.1% Tween 20, and 10% 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 PPAR γ 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 acyl CoA oxidase (a gift from Dr. B. Seed, Massachusetts General Hospital, Boston, MA) (35) and a β -galactosidase (β -gal) expression plasmid (Promega) to normalize the transfections, exposed to PPAR γ 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 pretreated with the PPAR γ antagonist GW9662 for 3 h. The cells were then exposed to the PPAR γ ligands for 8 h and luciferase and β -gal assays were performed. PPAR γ activation studies with the PPRE-LUC construct were also performed to determine the concentration of PPAR γ agonists required for maximal PPAR γ activation in human B lymphocytes. 15d-PGJ₂, ciglitazone, and CAY10410 all induced maximal PPAR γ activation at 1 μ M (data not shown), therefore this concentration was used for the transfection experiments.

Viability assays

A total of 6×10^4 Ramos cells or 8×10^4 human peripheral blood B cells per well were incubated with the PPAR γ agonists, CAY10410, or DMSO as a control for 48 h in a 96-well flat-bottom microtiter plate. A solution of 5 mg/ml MTT in PBS was added for 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 PPAR γ antagonist studies, cells were first pretreated with an optimal dose of 100 nM GW9662 for 3 h and then exposed to PPAR γ 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 PPAR γ agonists and some cell toxicity was observed at doses higher than 1 μ M (data not shown). The T0070907 PPAR γ antagonist was used at 1 μ M with a 3 h pretreatment. For the antioxidant treatments, cells were preincubated with 2 mM GSH-EE for 1 h and then exposed to the PPAR γ agonists.

Mitochondrial membrane potential

A total of 5×10^5 cells were treated with PPAR γ 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 carboxy-H₂DCFDA (43). When ROS are present in the cell, the carboxy-H₂DCFDA is modified and becomes fluorescent. A total of 5×10^5 cells were treated with PPAR γ agonists or DMSO for 12 h. Ten micromoles of carboxy-H₂DCFDA was

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 PPAR γ agonists, cells were washed in HBSS containing Mg²⁺ and Ca²⁺, resuspended in a 5 μ M 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 3×10^6 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 flow 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 PPAR γ does not prevent PPAR γ agonist-induced cell death of Ramos B lymphoma cells

Human B lymphocytes abundantly express PPAR γ and are killed by a variety of structurally dissimilar small molecule PPAR γ ago-

nists (12, 41). According to our previous studies and the data reported herein, the EC₅₀ for cell death induction by PPAR γ agonists in human B lymphocytes is 2 μ M for 15d-PGJ₂ and 7 μ M for ciglitazone (12). In the following set of experiments, we wanted to determine whether selected PPAR γ agonists killed human B cells by a PPAR γ -dependent or -independent mechanism. We first used a FLAG tagged DN human PPAR γ construct (DN PPAR γ). This DN contains two amino acid substitutions, which reduces coactivator recruitment and enhances corepressor recruitment and is a potent inhibitor of wild-type PPAR γ (42). Ramos B lymphoma cells were transfected with EV or the DN PPAR γ construct using Lipofectamine reagent. Twenty-four hours after transfection, expression of the Flag-tagged DN PPAR γ was confirmed by Western blot using an anti-Flag Ab (Fig. 1A). Next, the functional ability of the DN PPAR γ 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 μ M 15d-PGJ₂ or 1 μ M 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 PPAR γ 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 PPAR γ is indeed functional in the Ramos cells. Transfected Ramos cells were next exposed to PPAR γ agonists for up to 48 h and an MTT assay was performed to evaluate whether the DN PPAR γ was able to inhibit cell death induced by the PPAR γ agonists. Interestingly, at most doses of 15d-PGJ₂, the DN PPAR γ

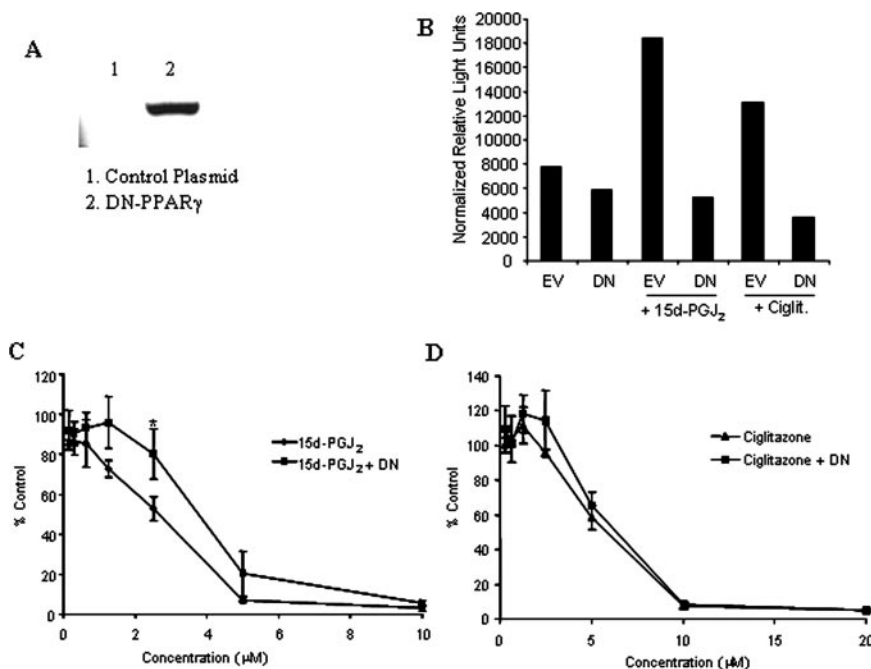


FIGURE 1. A DN PPAR γ does not prevent PPAR γ agonist-induced cell death in B cells. *A*, Ramos B lymphoma cells were transfected with a control plasmid (EV) or a DN PPAR γ plasmid. Twenty-four hours after transfection, the cells were lysed and equal amounts of total protein were loaded per lane of a Western blot for the Flag-PPAR γ fusion protein using an anti-FLAG Ab. The Flag-DN PPAR γ fusion protein was detected in the cells (lane 2), but not in the control transfection (lane 1). *B*, Ramos B cells were transfected as in *A*, except a PPRE-LUC reporter and a β -gal plasmid were cotransfected with the DN-PPAR γ and 24 h later the cells were treated with or without 1 μ M 15d-PGJ₂ or 1 μ M ciglitazone (ciglit.) for 8 h at which time a luciferase assay was performed. The values shown were normalized to β -gal activity. The DN PPAR γ completely inhibited the PPRE-LUC expression induced by both PPAR γ agonists. *C* and *D*, Ramos cells were transfected with the DN PPAR γ and 24 h later were exposed to 15d-PGJ₂ (*C*) or ciglitazone (*D*) for 48 h. An MTT assay was performed and the results presented as percent of the DMSO control. The DN PPAR γ significantly inhibited cell death at only one dose of 15d-PGJ₂ (2.5 μ M, *p* = 0.03), but there was no statistically significant inhibition with ciglitazone.

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 PPAR γ agonist ciglitazone, shown in Fig. 1D, there were no significant differences seen with the DN PPAR γ compared with ciglitazone alone. These findings support that PPAR γ is not a major contributor to B cell death induced by PPAR γ agonists.

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

To confirm the DN PPAR γ results, we next used the PPAR γ irreversible antagonist GW9662. GW9662 covalently modifies the PPAR γ 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 PPAR γ 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 PPAR γ findings in Fig. 1. Overall, these observations suggest that for the Ramos B lymphoma cells, there may be a small PPAR γ contribution to apoptosis. Normal human B cells do not appear to require PPAR γ for apoptosis induction by these PPAR γ agonists. An additional PPAR γ 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 PPAR γ ligands, we confirmed that GW9662 inhibited PPAR γ 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 PPAR γ antagonist in an adipogenesis assay and 100 nM GW9662 prevented 15d-PGJ₂ induced-adipogenesis in human orbital fibroblasts (data not shown).

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

To further dissect the involvement of PPAR γ in 15d-PGJ₂ induced-apoptosis of B lymphocytes, we used a 15d-PGJ₂ analog, CAY10410. CAY10410 is a potent PPAR γ agonist, but lacks the reactive electrophilic carbon in the cyclopentenone ring (Fig. 4A). To be certain of the PPAR γ 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-PPAR γ 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 PPAR γ DN demonstrating that CAY10410 activates PPAR γ in B cells. Additionally, CAY10410 induced adipogenesis of human orbital fibroblasts (data not shown); further evidence supporting CAY10410 is a PPAR γ agonist. Exposure to CAY10410, as shown in Fig. 4, B and C, did not kill either Ramos cells or normal human B cells, even

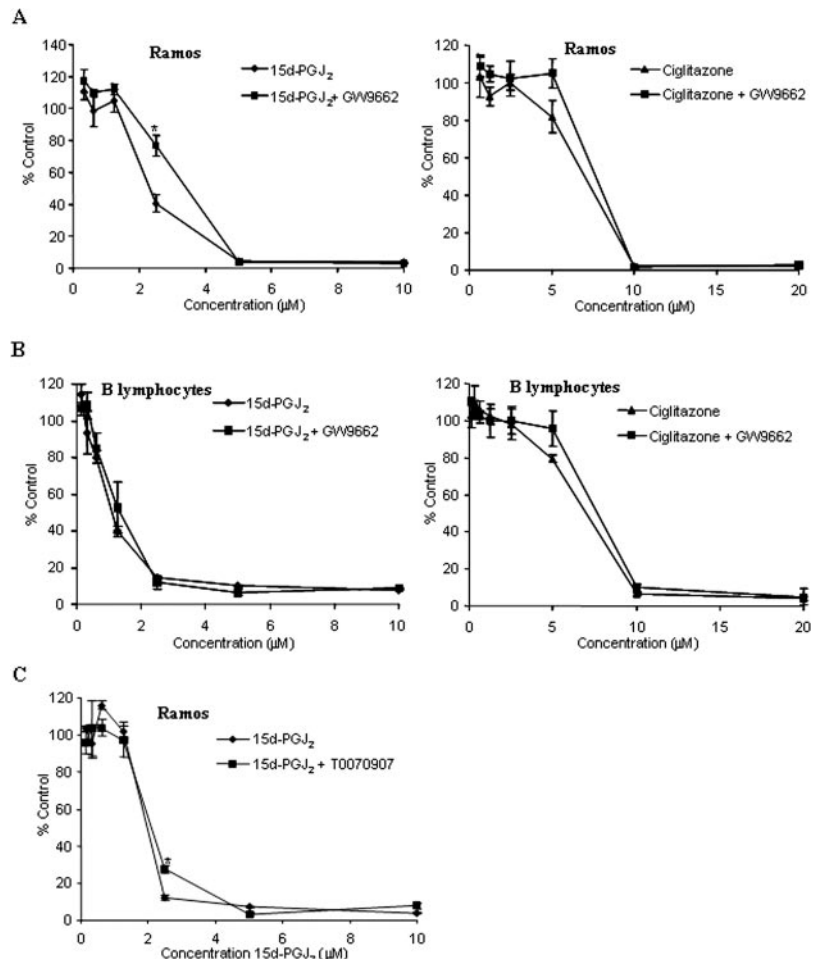
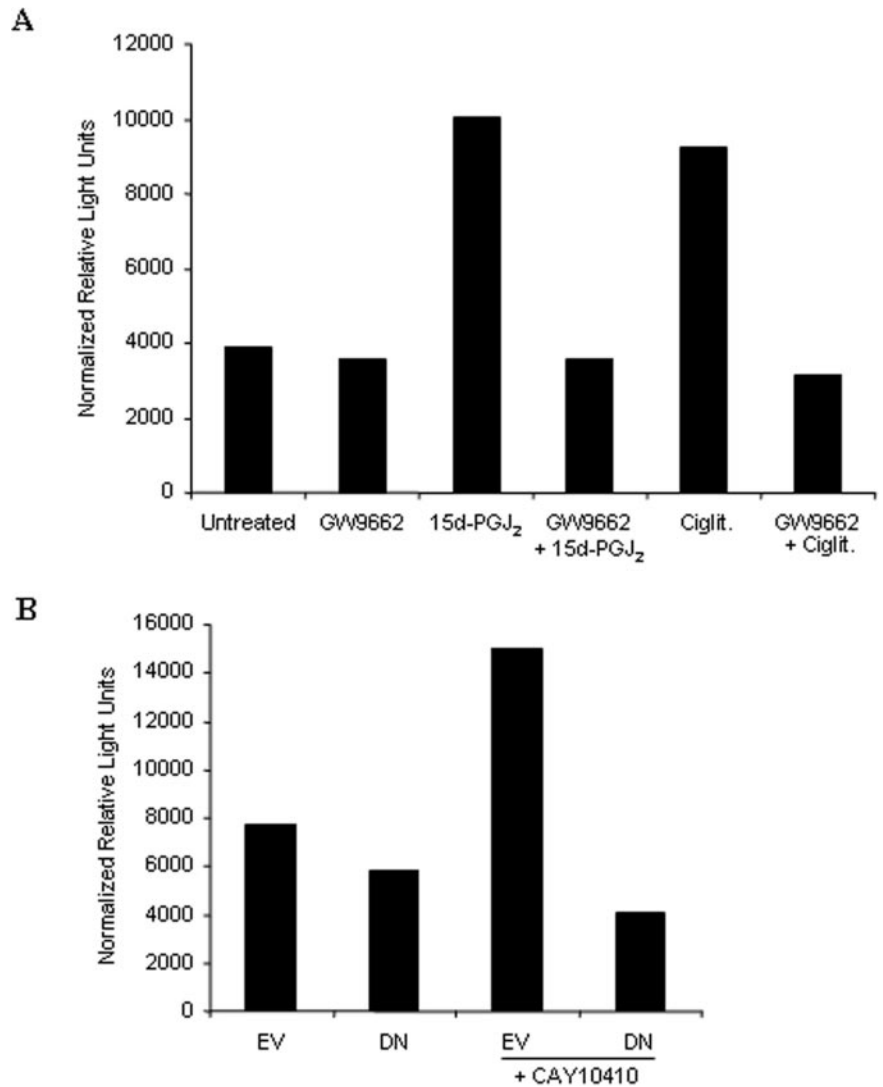


FIGURE 2. The irreversible small molecule PPAR γ antagonist GW9662 does not prevent cell death of B lymphocytes. Ramos B lymphoma cells (A) and purified normal human B cells (B) were pretreated for 3 h with an optimal dose (100 nM) of GW9662, and then exposed to PPAR γ agonist for 48 h at which time an MTT assay was performed. In Ramos cells, there was a significant prevention of cell death at only one dose of 15d-PGJ₂ (2.5 μM , $p = 0.003$). There were no statistically significant differences for the normal human B cells. C, Ramos B lymphoma cells were pretreated with the PPAR γ antagonist T0070907 (1 μM) for 3 h and then exposed to 15d-PGJ₂ for 48 h. The results of an MTT assay are shown. T0070907 significantly prevented cell death at 2.5 μM of 15d-PGJ₂ ($p = 0.003$).

FIGURE 3. The PPAR γ antagonist, GW9662, inhibits PPAR γ and the 15d-PGJ₂ analog, CAY10410, activates PPAR γ in human B cells. **A**, Ramos B lymphoma cells were transfected with a PPRE-LUC reporter construct and a β -gal construct. Twenty-four hours after transfection, the cells were exposed to 100 nM GW9662 (3 h pretreatment), or a combination of GW9662 and 1 μ M 15d-PGJ₂ or ciglitazone for 8 h at which time a luciferase assay was performed. Relative light units were normalized to β -gal activity. **B**, Ramos B lymphoma cells were cotransfected with PPRE-LUC and an empty vector control plasmid (EV) or a DN-PPAR γ (DN). CAY10410 (1 μ M) was added 24 h after transfection and a luciferase assay was performed after 8 h of exposure. The luciferase values were normalized to β -gal activity.



at doses up to 25 μ M, whereas 15d-PGJ₂ kills almost 100% of cells at doses <5 μ M. 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 PPAR γ , perhaps as a result of its reactive electrophilic properties.

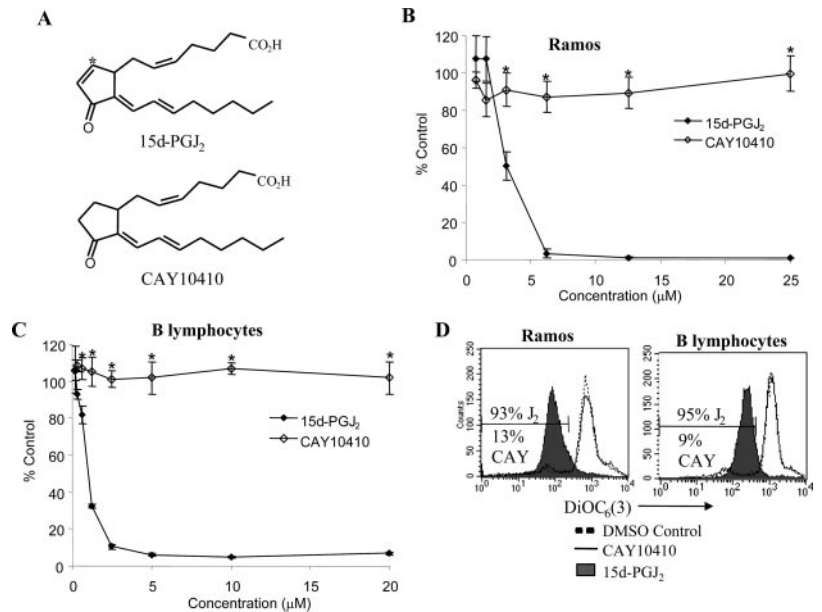
15d-PGJ₂, but not ciglitazone, induces ROS production in B lymphocytes

Because PPAR γ 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 in-

creasing doses of 15d-PGJ₂ and ciglitazone for 12 h at which time the cells were incubated with 10 μ M 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 μ M 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).

FIGURE 4. The PPAR γ agonist CAY10410, a 15d-PGJ $_2$ analog, does not kill B lymphocytes. *A*, The structures of 15d-PGJ $_2$ and its analog CAY10410 are identical except CAY10410, while still a potent PPAR γ agonist, lacks the electrophilic carbon in the cyclopentenone ring (depicted with an asterisk (*)). *B* and *C*, Ramos B lymphoma cells (*B*) and purified normal human B cells (*C*) were exposed to CAY10410 or 15d-PGJ $_2$ for 48 h. The results of an MTT assay are shown. *, $p < 0.05$ for CAY10410 vs 15d-PGJ $_2$ exposure. *D*, Ramos B lymphoma cells (*left panel*) and purified normal human B cells (*right panel*) were treated with 5 μ M 15d-PGJ $_2$ or 20 μ M CAY10410 for 12 h. The dye, DiOC $_6$ (3), was added for the last 15 min of culture and dye incorporation was analyzed on a flow cytometer. The percent of cells with decreased mitochondrial membrane potential was determined. The dotted line histogram represents DMSO-treated cells as a control (11% for Ramos and 9% for normal human B cells), the solid line histogram represents CAY10410 treatment (13% for Ramos and 9% for normal human B cells), and the shaded histogram for 15d-PGJ $_2$ treatment (93% for Ramos and 95% for normal human B cells). Results from one representative experiment are shown.



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 μ M 15d-PGJ $_2$ or 20 μ 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.

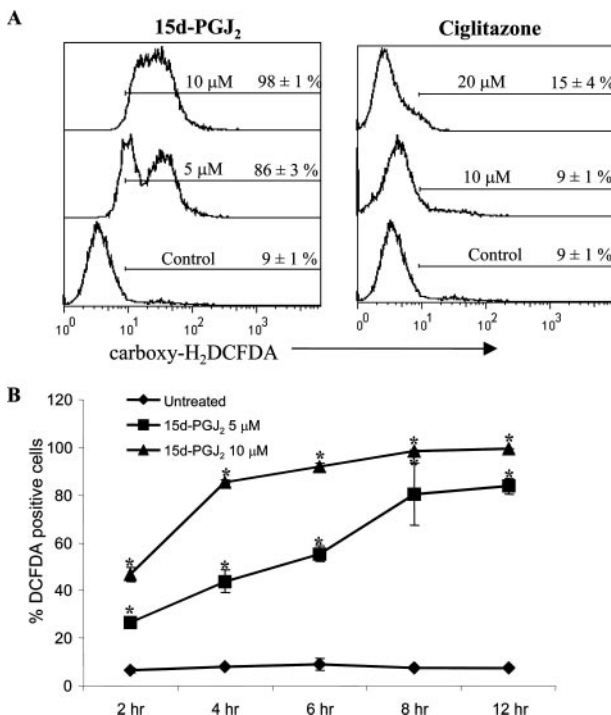


FIGURE 5. ROS production is induced by 15d-PGJ $_2$, but not by ciglitazone. *A*, Ramos B lymphoma cells were exposed to 15d-PGJ $_2$ (*right panel*) or ciglitazone (*left panel*) for 12 h. The dye carboxy-H $_2$ DCFDA was added for the last 30 min of culture, after which the cells were analyzed by flow cytometry for dye fluorescence. The percent of ROS-positive cells is shown. DMSO-treated cells had a background level of 9% ROS-positive cells. *B*, Ramos B lymphoma cells were exposed to 5 or 10 μ M 15d-PGJ $_2$ for 2, 4, 6, 8, or 12 h and analyzed for carboxy-H $_2$ DCFDA fluorescence by flow cytometry. The results were plotted as the average percent of cells positive for DCFDA and are representative of three independent experiments. *, $p < 0.05$ compared with untreated control.

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 anti-oxidant GSH-EE to block B cell death induced by PPAR γ agonists. Ramos and normal human B cells were first pretreated with 2 mM GSH-EE and then exposed to doses up to 20 μ M 15d-PGJ $_2$ or 25 μ 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 $_2$ exposure (data not shown). Moreover, the GSH-EE prevented ROS induction by 15d-PGJ $_2$, as determined by carboxy-H $_2$ DCFDA and MitoSOX Red staining, as shown in Fig. 7C.

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

PPAR γ Agonist	(μ M)	% ROS ^a
15d-PGJ $_2$	0	9 \pm 2
	1	66 \pm 4 ^b
	5	83 \pm 5 ^b
	10	94 \pm 3 ^b
Ciglitazone	1	5 \pm 2
	5	4 \pm 1
	10	9 \pm 2

^a The percent of ROS-positive B cells after a 12-h exposure to PPAR γ agonists.
^b A value of $p < 0.01$ for induction of ROS with 15d-PGJ $_2$ treatment.

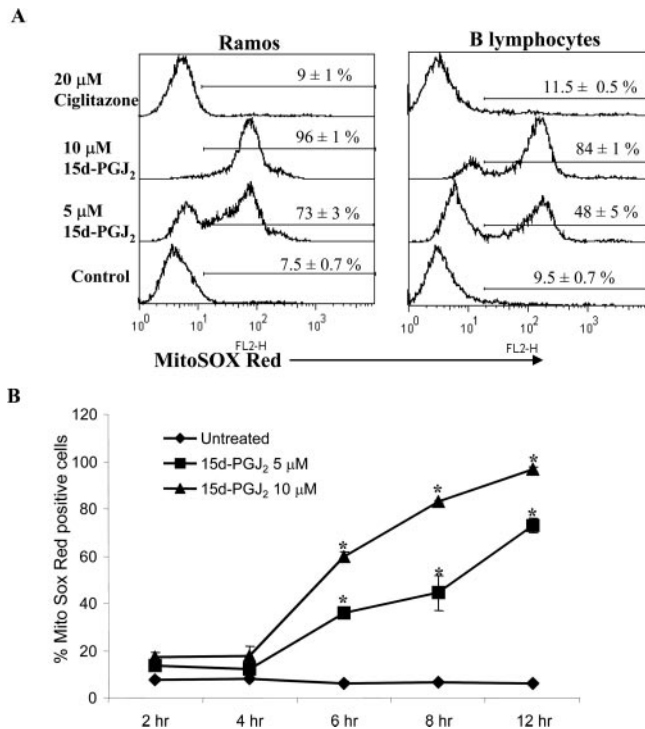


FIGURE 6. 15d-PGJ₂, but not ciglitazone, induces mitochondrial superoxide accumulation in B lymphocytes. *A*, Ramos B lymphoma cells (*left panel*) and purified normal human B cells (*right panel*) were exposed to 15d-PGJ₂ or ciglitazone for 12 h. The fluorescence of the MitoSOX Red dye was detected by flow cytometry. Results from one representative experiment are shown. *B*, Ramos B lymphoma cells were exposed to DMSO vehicle (untreated), 5 μ M or 10 μ M 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 PPAR γ 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 PPAR γ DN and the irreversible small molecule PPAR γ antagonist GW9662, both of which inhibit PPAR γ activation in human B cells, that both 15d-PGJ₂ and ciglitazone induced apoptosis of both normal and malignant human B lymphocytes independently of PPAR γ 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 PPAR γ 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 PPAR γ in B cells, have distinct apoptotic mechanisms in human B lymphocytes that are independent of PPAR γ .

This is the first report to examine the PPAR γ dependency of PPAR γ 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 PPAR γ agonists. In fact, the thiazolidinediones, which are reported to be more potent PPAR γ 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 PPAR γ . However, this does not rule out the possibility that PPAR γ has other important functions in B lymphocytes. We have shown that B cells highly express PPAR γ protein both in the cytoplasm and the nucleus (12) and PPAR γ agonists activate PPAR γ in human B cells (see Figs. 1 and 3). There is also evidence in PPAR γ heterozygous mice that PPAR γ is important in B cell responses, in particular for controlling proliferation and Ab production (40). Therefore, determining the role of PPAR γ in B lymphocyte biology may have important implications for the use of PPAR γ agonists in controlling B cell responses.

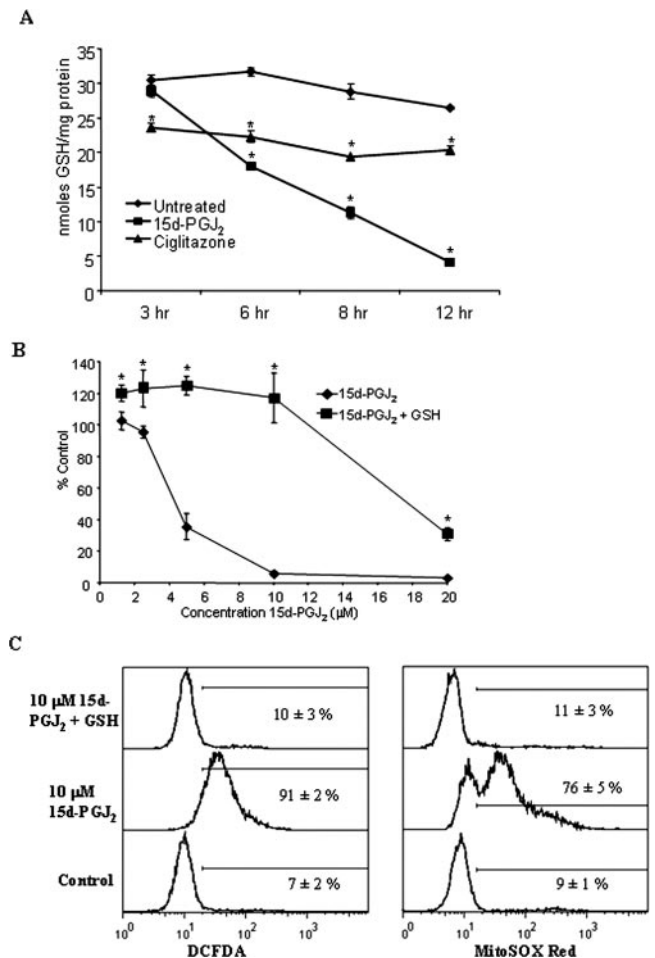


FIGURE 7. 15d-PGJ₂-, but not ciglitazone-, induced B cell death is prevented by the antioxidant GSH-EE. *A*, Ramos B lymphoma cells were exposed to DMSO vehicle (untreated), 10 μ M 15d-PGJ₂, or 20 μ M ciglitazone for 3, 6, 8, or 12 h after which total intracellular GSH was measured in the cell lysates. *, $p < 0.007$ compared with untreated control. *B*, Ramos B lymphoma cells were pretreated with GSH-EE for 1 h and subsequently exposed to 15d-PGJ₂ for 24 h at which time an MTT assay was performed. *, $p < 0.05$ for 15d-PGJ₂ + GSH compared with 15d-PGJ₂ alone. *C*, Ramos B lymphoma cells were treated as described in *B*. After 6 h, the cells were incubated with either carboxy-H₂DCFDA or MitoSOX Red and analyzed by flow cytometry. Results are representative of three independent experiments.

phocytes (12). Herein, we demonstrate that this difference is most likely due to the use of different apoptotic mechanisms that are independent of PPAR γ . However, this does not rule out the possibility that PPAR γ has other important functions in B lymphocytes. We have shown that B cells highly express PPAR γ protein both in the cytoplasm and the nucleus (12) and PPAR γ agonists activate PPAR γ in human B cells (see Figs. 1 and 3). There is also evidence in PPAR γ heterozygous mice that PPAR γ is important in B cell responses, in particular for controlling proliferation and Ab production (40). Therefore, determining the role of PPAR γ in B lymphocyte biology may have important implications for the use of PPAR γ 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 PPAR γ agonists and it is most likely the nucleophilic ability

of 15d-PGJ₂ that gives it more potent apoptotic properties. The α,β -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- κ B 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-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 PPAR γ 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 PPAR γ agonists for the treatment of B cell malignancies and inflammatory conditions. Our results clearly show that natural (15d-PGJ₂) and synthetic (ciglitazone) PPAR γ agonists are potent inducers of B cell apoptosis, even though they do so by a PPAR γ -independent mechanism. The continued study of PPAR γ in B lymphocyte development and function may reveal a yet undiscovered role for PPAR γ in the immune system.

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Disclosures

The authors have no financial conflict of interest.

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