

15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂-induced apoptosis does not require PPAR γ in breast cancer cells

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Abstract Naturally occurring derivatives of arachidonic acid are potent agonists for the nuclear hormone receptor peroxisome proliferator-activated receptor gamma (PPAR γ) and block cancer cell proliferation through the induction of apoptosis. We have previously reported that induction of apoptosis using cyclopentenone prostaglandins of the J series, including 15deoxy $\Delta^{12,14}$ PGJ₂ (15dPGJ₂), is associated with a high degree of PPAR-response element (PPRE) activity and requires early de novo gene expression in breast cancer cells. In the current study, we used pharmacologic and genetic approaches to test the hypothesis that PPAR γ is required for 15dPGJ₂-induced apoptosis. The PPAR γ agonists 15dPGJ₂, troglitazone (TGZ), and GW7845, a synthetic and highly selective tyrosine-based PPAR γ agonist, all increased transcriptional activity of PPAR γ , and expression of CD36, a PPAR γ -dependent gene. Transcriptional activity and CD36 expression was reduced by GW9662, a selective and irreversible PPAR γ antagonist, but GW9662 did not block apoptosis induced by 15dPGJ₂. Moreover, dominant negative expression of PPAR γ blocked PPRE transcriptional activity, but did not block 15dPGJ₂-induced apoptosis. These studies show that while 15dPGJ₂ activates PPRE-mediated transcription, PPAR γ is not required for 15dPGJ₂-induced apoptosis in breast cancer cells. Other likely mechanisms through which cyclopentenone prostaglandins induce apoptosis of cancer cells are discussed.—Clay, C. E., A. Monjazebe, J. Thorburn, F. H. Chilton, and K. P. High. 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂-induced apoptosis does not require PPAR γ in breast cancer cells. *J. Lipid Res.* 2002. 43: 1818–1828.

Supplementary key words cyclopentenone prostaglandins • arachidonic acid metabolism • peroxisome proliferator-activated receptor γ

Peroxisome proliferator-activated receptor gamma (PPAR γ) is a ligand activated transcription factor that induces expression of PPAR-response element (PPRE) con-

taining genes critical to diabetes, obesity, inflammation, and cancer (1). PPAR γ is activated by a diverse array of synthetic compounds including thiazolidinediones (TZDs), triterpenoids and tyrosine-based compounds, and naturally occurring lipid compounds including derivatives of fatty acid metabolism and oxidized fractions of LDL. The tyrosine based PPAR γ agonists (GW7845 and GW1929) induce neuroblastoma differentiation (2), inhibit mammary carcinogenesis (3), reverse the diabetic phenotype in mouse models (4), and block atherosclerosis (5) in part by inhibiting vascular smooth muscle cell proliferation and neointima formation (6). The synthetic triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) induces differentiation and apoptosis in human osteosarcoma and myeloid leukemia cells (7, 8). The TZDs, troglitazone (TGZ, Rezulin[®]), rosiglitazone (Rosi, BRL49653, Avandia[®]), and pioglitazone (Pio, Actos[®]) are effective anti-diabetes drugs and reduce the growth of several cancer cell types (9). However, their clinical application as chemotherapeutic drugs has been discouraging to date, due to unpredictable clinical performance and lack of efficacy in human trials (10–12). However, very recent data suggest that some properties of these drugs may not be related to their capacity to activate PPAR γ (13), suggesting there may be opportunities to enhance the anti-cancer activity of these compounds by better understanding their mechanism of action while maintaining their relative safety versus conventional chemotherapeutic agents.

Of the naturally occurring PPAR γ agonists, the cyclopentenone prostaglandin, 15deoxy $\Delta^{12,14}$ PGJ₂ (15dPGJ₂), is among the most potent for both transactivating PPAR γ

Abbreviations: CDDO, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid; DN, dominant negative PPAR γ ; 15dPGJ₂, 15deoxy $\Delta^{12,14}$ PGJ₂; PPAR γ , peroxisome proliferator-activated receptor gamma; PPRE, PPAR-response element; ROS, reactive oxygen species; TGZ, troglitazone; TZD, thiazolidinedione; WT, wild type PPAR γ .

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(14, 15) and inducing apoptosis (16). However, controversy exists as to the molecular mechanism(s) of 15dPGJ₂ activity. Clearly, 15dPGJ₂ is an effective PPAR γ agonist, but it also exerts effects that are independent of PPAR γ (17). Two electrophilic carbonyls within the ring structure of 15dPGJ₂ can form covalent Michael adducts with cysteine containing proteins. In this way, 15dPGJ₂ negatively regulates NF κ B activity by covalent inhibition of the IKK, I κ B α , and the DNA binding domain of NF κ B (18–21). Additionally, the immediate precursor to 15dPGJ₂ biosynthesis, Δ 12PGJ₂, inhibits ubiquitin isopeptidase activity of the proteasome pathway (22). 15dPGJ₂ may also induce the formation of reactive oxygen species that lead to cell death (23, 24).

In addition to its diverse mechanisms of action, the concentration of 15dPGJ₂ dictates opposing biologic outcomes in several types of cancer cells and cell lines (9, 25). Specifically, low concentrations of 15dPGJ₂, increase cellular proliferation, and moderate concentrations induce cell cycle arrest and cellular differentiation, while higher concentrations induce apoptosis. However, it is clear that 15dPGJ₂ induces apoptosis only when expression of critical gene products occurs, since inhibition by actinomycin D or cycloheximide blocks 15dPGJ₂-induced apoptosis (26). Thus, transcriptional activation is required for 15dPGJ₂-induced apoptosis and it is reasonable to suspect PPRE containing genes are the most likely mediators. It is clear from knockout studies that PPAR γ is required for differentiation of adipose tissue (27, 28) and perhaps differentiation of cancer cells. However, in the current study, we show that while PPAR γ does account for the PPRE-mediated transcriptional activation of 15dPGJ₂, it does not mediate 15dPGJ₂-induced apoptosis in breast cancer cells. Other plausible mechanisms of 15dPGJ₂-induced apoptosis are discussed.

MATERIALS AND METHODS

Cells and reagents

MDA-MB-231 breast cancer epithelial cells were maintained in DMEM supplemented with 10% fetal calf serum, 1% penicillin, 1% streptomycin, and 1% L-glutamine (Life Technologies, Rockville, MD). 15dPGJ₂ was purchased from Cayman Chemical (Ann Arbor, MI). 15dPGJ₂ is rapidly inter-converted to a mixture of at least five active isomers (29). Troglitazone was a generous gift from Parke Davis Warner Lambert (Plainsboro, NJ) and GW7647 (30), GW7845 (31), GW0742 (32), and GW9662 (33) were generous gifts from Dr. Timothy M. Willson and Dr. Peter J. Brown (Glaxo Smith Kline, Research Triangle Park, NC). Each compound was used at a concentration where it is selective for the indicated receptor subtype. All tissue culture experiments were done in humid 5% CO₂ atmosphere at 37°C.

Breast cancer cellular responses to 15deoxy Δ ^{12,14}PGJ₂

15dPGJ₂ was submitted to the Developmental Therapeutics Program (National Institutes of Health, National Cancer Institute, Bethesda, MD, <http://dtp.nci.nih.gov>) for in vitro screening against 60 human tumor cell lines (34–36). Briefly, the human tumor cell lines were grown at 37°C, 5% CO₂ and 100% relative humidity in 100 μ l of RPMI 1640 medium containing 5%

FBS and 2 mM L-glutamine in 96-well microtiter plates at densities ranging from 5,000 to 40,000 cells/well depending on the doubling time of individual cell lines. After 24 h, two plates of each cell line were fixed in situ with TCA, to represent a measurement of the cell population for each cell line at the time of drug addition. One hundred microliters aliquots of 15dPGJ₂ in growth media was added to the appropriate microtiter wells already containing 100 μ l of media, resulting in the indicated final drug concentrations. Plates were incubated for 48 h at 37°C, 5% CO₂ and 100% relative humidity. Adherent cells were fixed in situ by the gentle addition of 50 μ l of cold 50% (w/v) TCA (final concentration, 10% TCA) and suspension cells were fixed by gently adding 50 μ l of 80% TCA (final concentration, 16% TCA). Cells were then incubated for 60 min at 4°C, washed five times with tap water, and air-dried. Sulforhodamine B (SRB) solution (100 μ l) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air-dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. Using the seven absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth is calculated at each of the drug concentrations.

Relative levels of PPAR γ mRNA expression in breast cancer cells

Relative PPAR γ mRNA in breast cancer cell lines was determined by the Developmental Therapeutics Program (National Institutes of Health, National Cancer Institute, Bethesda, MD, <http://dtp.nci.nih.gov>). Briefly, mRNA was isolated from logarithmically growing cells and labeled cDNA was prepared by reverse transcription of test cell mRNA in the presence of Cy5-dUTP. Reference probes were made by pooling equal amounts of mRNA from HL-60, K562, NCI-H226, COLO205, SNB-19, LOX-IMVI, OVCAR-3, OVCAR-4, CAKI-1, PC-3, MCF7, and Hs578T cell lines. Labeled cDNA was prepared from the pooled reference cell mRNA by reverse transcription in the presence of Cy3-dUTP. Test and reference probes were combined, denatured, and hybridized overnight to Synteni microarrays (Incyte Genomics, Fremont, CA) containing cDNA from 9,703 human clones, including PPAR γ . Arrays were scanned using a laser-scanning microscope, the ScanAlyze program was used to analyze the microarray images and relative RNA level were determined by log (test cell line mRNA levels/reference pool RNA level).

Cell proliferation assays

1×10^4 MDA-MB-231 cells were seeded in 1 ml of medium in each well of a 24-well plate. After 24 h, the indicated concentration of drug was added. After 96 h, medium was removed, cells were washed with PBS, and stained with 0.16% w/v methylene blue in methanol. After 10 min, cells were washed with PBS and digital images were obtained.

Transcriptional activity assays

2×10^5 MDA-MB-231 cells were seeded in 2 ml of media in a 35 mm dish. After 24 h, cells were transfected with 1.0 μ g of a 3 \times PPRE-tk-luciferase vector, which has three copies of PPRE upstream of the TK promoter/luciferase fusion gene (37), a kind gift from Dr. Bruce Spiegelman, and 1 μ g of β -galactosidase as an internal control using Fugene 6 (Roche, Indianapolis, IN). After 24 h, cells were incubated for 1 h with or without the PPAR γ antagonist, GW9662 (10 μ M), and the indicated PPAR γ agonists were provided, 15dPGJ₂ (10 μ M), TGZ (100 μ M), or GW7845 (10 μ M). After 24 h, cells were scraped, transferred to microfuge

tubes, and luciferase activity was measured using a Luciferase Assay Kit (Promega, Madison, WI) according to manufacturer's protocol. Light intensity was measured using a Turner 20E luminometer (Turner Designs, Sunnyvale, CA). All experiments were done in triplicate. Luciferase activity was standardized to β -galactosidase activity and reported as mean fold increase over control with standard deviation.

Apoptosis assays

5×10^5 MDA-MB-231 cells were seeded in 3 ml of media in 60 mm dishes. After 24 h, cells were incubated for 1 h with or without the PPAR γ antagonist, GW9662 (10 μ M) and the indicated concentration of PPAR γ agonist was provided, 15dPGJ $_2$ (10 μ M), TGZ (100 μ M), or GW7845 (10 μ M). After 24 h, cells were collected by trypsinization, pelleted, and the percentage of cells undergoing apoptosis was determined by flow cytometry using a TACS Annexin V-FITC Kit (Trevig, Gaithersburg, MD) according to manufacturer's protocol. Fluorescent intensity was measured using a Coulter Epics XL-MCL flow cytometer (Hialeah, FL).

Microinjection

1×10^4 MDA-MB-231 cells were seeded in 35 mm dishes. After 24 h, cells were injected as described previously (38) with 0.25 μ g/ μ l of yellow fluorescent protein and 0.25 μ g/ μ l of either the wild type form of PPAR γ (WT) or the dominant negative form of PPAR γ (DN), a generous gift of Dr. VKK Chatterjee (39), using a Zeiss Aviovert microscope equipped with an Eppendorf FemtoJet and Injectman (Brinkman Instruments, Westbury, NY). After 24 h, the number of live cells was determined by counting fluorescent cells, and the indicated PPAR γ agonists were provided, 15dPGJ $_2$ (10 μ M), TGZ (100 μ M), or GW7845 (10 μ M). Twenty-four hours and 48 h after the addition of PPAR γ agonist, the number of surviving cells was determined by counting and digital images were obtained using a Hamamatsu C4742-95 digital camera (Bridgewater, NJ) and OpenLab software (Improvision, Warwick, UK).

Immunofluorescence

Cells were transfected with 1.0 μ g of FLAG-tagged WT or DN using Fugene 6 (Roche, Indianapolis, IN) and after 24 h, the expression and localization of PPAR γ was determined. Cells were washed with PBS, fixed in 3.7% formaldehyde in PBS for 10 min at room temperature (RT), washed with PBS, permeabilized with 0.3% Triton X-100 in PBS for 10 min at RT and washed in PBS-0.1% Tween. Cells were blocked with 10% goat serum in PBS-0.1% Tween for 10 min at RT and incubated with M2-FLAG primary antibody (25 μ g/ml) (Sigma, St. Louis, MO) for 1 h at 37°C in humid atmosphere. Cells were washed with PBS-0.1% Tween and incubated with rhodamine red-X-conjugated anti-mouse IgG secondary antibody (1:100, v/v) (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at 37°C in humid atmosphere. Cells were washed with PBS-0.1% Tween and digital images were obtained as described above.

RESULTS

PPAR γ expression does not correlate with 15dPGJ $_2$ -induced apoptosis

The proliferation of breast cancer cell lines exposed to various concentrations of 15dPGJ $_2$ for 48 h was determined and these same breast cancer cell lines were screened for relative PPAR γ expression (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD) (Fig. 1). All breast cancer cell lines tested

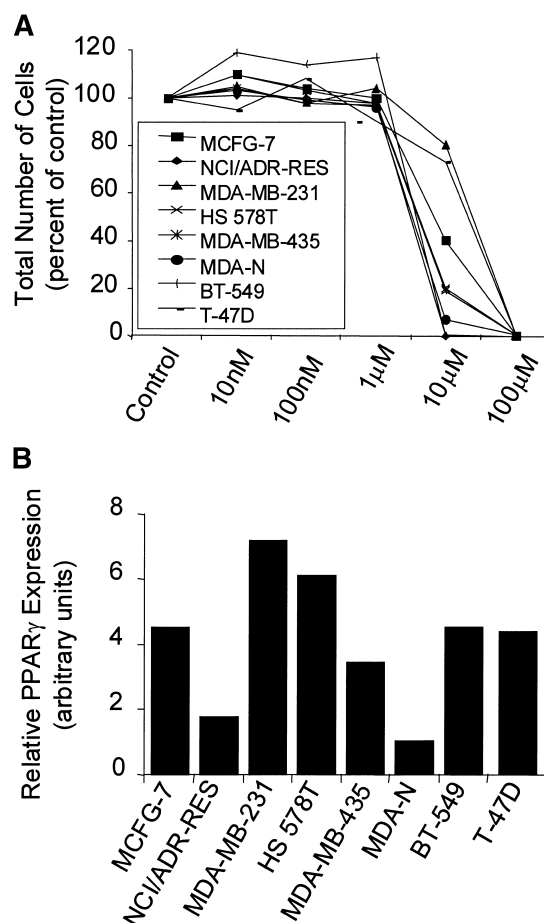


Fig. 1. Peroxisome proliferator-activated receptor gamma (PPAR γ) mRNA expression does not correlate with 15deoxy $\Delta^{12,14}$ PGJ $_2$ (15dPGJ $_2$)-induced inhibition of cellular proliferation. A: The indicated breast cancer cell lines were incubated with the indicated concentration of 15dPGJ $_2$ for 48 h and cell viability was determined using a sulforhodamine B (SRB) assay as described in Materials and Methods. B: Relative RNA level of PPAR γ in logarithmically growing breast cancer cells was determined using a microarray experiment as described in Materials and Methods. The cell line MDA-N was used as reference.

were sensitive to 15dPGJ $_2$ -induced apoptosis (Fig. 1A) independent of PPAR γ mRNA expression level (Fig. 1B). These early data suggested that PPAR γ might not play a pivotal role in 15dPGJ $_2$ -induced apoptosis.

Pharmacologic PPAR γ antagonism does not block 15dPGJ $_2$ -induced apoptosis

The structure of the PPAR γ agonists and antagonists tested, as well as the structure of WT and DN constructs used is shown in Fig. 2. We tested three different classes of PPAR γ agonists, 15dPGJ $_2$, TGZ, and GW7845, and an irreversible PPAR γ antagonist, GW9662, for their capacity to alter cellular proliferation and induce apoptosis of MDA-MB-231 breast cancer epithelial cells. We have previously reported that 15dPGJ $_2$ and TGZ induce rapid and irreversible apoptosis in this cell line (16). Here we show that 15dPGJ $_2$ and TGZ block cellular proliferation of MDA-MB-231 cells, but the selective PPAR α agonist, GW7647,

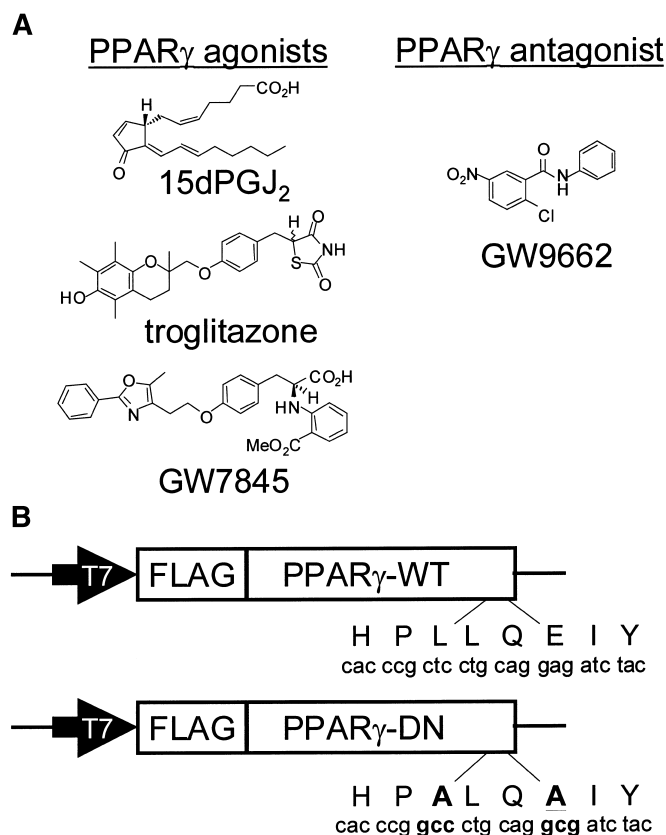


Fig. 2. Structure of PPAR γ agonists, an antagonist, and wild type and dominant negative PPAR γ constructs. A: The structures of the PPAR γ agonists 15dPGJ₂, troglitazone (TGZ), and GW7845 and the structure of the irreversible PPAR γ antagonist GW9662 are shown. B: The functional structures of the wild type PPAR γ (WT) and the dominant negative PPAR γ (DN) constructs are shown. The L486A and E471A mutations are underlined.

the selective PPAR γ agonist, GW7845, the selective PPAR β / δ agonist, GW0742, and the selective and irreversible PPAR γ antagonist, GW9662, did not alter cellular proliferation compared to control cells in a clonogenic assay (**Fig. 3**). Consistent with this finding, studies by Gupta and colleagues have shown that GW7845 and the PPAR γ selective thiazolidinedione rosiglitazone (Rosi, BRL49653, Avandia[®]) slowed, but did not completely stop proliferation and did not induce apoptosis of colon cancer cells (40). Pretreatment of MDA-MB-231 cells with the irreversible PPAR γ antagonist, GW9662, did not block 15dPGJ₂ or TGZ-induced inhibition of cellular proliferation (**Fig. 4A, B**). Moreover, 15dPGJ₂-induced apoptosis was not significantly reduced ($P = 0.07$) and GW7845 did not induce apoptosis in this cell line. In contrast, TGZ-induced apoptosis was reduced by nearly 50% by GW9662 ($P = 0.04$) (**Fig. 4C**). Together these data show that selective synthetic activators of different PPARs do not block cellular proliferation and that the anti-cancer effects of less selective PPAR ligands may be independent of PPARs. Furthermore, these data show that pharmacologic inhibition of PPAR γ does not rescue cells from apoptosis induced by 15dPGJ₂ and that TGZ and 15dPGJ₂ may have different mechanisms through which they induce apoptosis, some

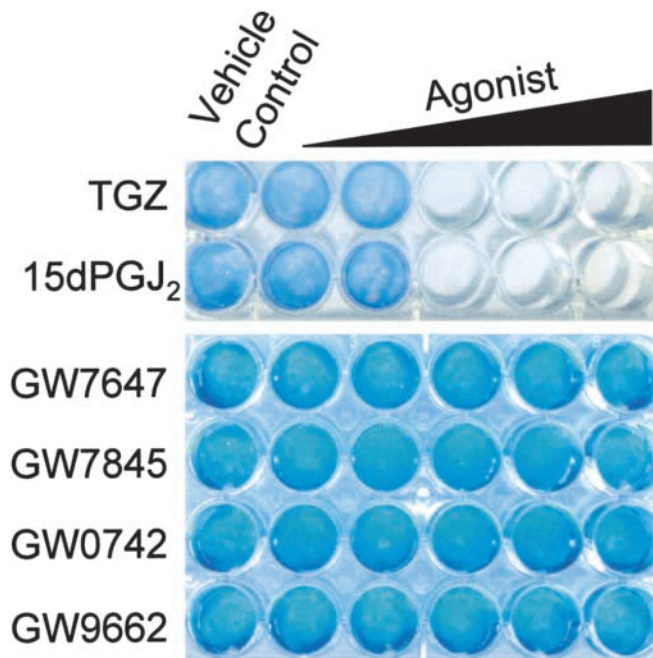


Fig. 3. Effect of PPAR ligands on cellular proliferation. MDA-MB-231 cells were grown in the presence of various concentrations of the indicated PPAR agonists and cell proliferation was determined by methylene blue staining. PPAR agonist concentration ranges are TGZ (PPAR γ agonist), 10, 20, 50, 100, 200 μ M; 15dPGJ₂ (PPAR γ agonist), 0.5, 1, 2.5, 5, 10 μ M; GW7647 (PPAR α agonist), GW7845 (PPAR γ agonist), GW0742 (PPAR β / δ agonist), and GW9662 (PPAR γ antagonist), 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} M.

independent of PPAR γ and some potentially involving PPAR γ .

15dPGJ₂-induced PPRE-mediated gene transcription is incompletely blocked by pharmacologic antagonism

PPAR γ antagonism using GW9662 had no effect on blocking cellular proliferation of MDA-MB-231 cells, but was able to markedly reduce PPRE-mediated gene transcription. MDA-MB-231 cells were transiently co-transfected with a PPRE driven luciferase construct and a β -galactosidase construct as an internal control. TGZ and GW7845-induced transcriptional activity was completely blocked by GW9662, but 15dPGJ₂-induced transcriptional activity was only blocked by half and was still higher than levels achieved by either TGZ or GW7845 in the absence of inhibitor (**Fig. 5A**). However, expression of DN by transient transfection reduced PPRE-mediated gene transcription induced by all three agonists to baseline values (**Fig. 5B**). While GW9662 is a selective and irreversible inhibitor of PPAR γ (33, 41), over-expression of DN more effectively blocked PPRE-mediated gene transcription in the results presented, perhaps by competing with other PPARs or related transcription factors, for binding to PPRES and/or enhanced recruitment of transcriptional co-repressors (39). These findings are consistent with our earlier data that show 15dPGJ₂ is the most potent activator of PPRES-mediated gene transcription (16, 25, 26). Surprisingly, the expression of CD36, a reported PPAR γ -dependent gene

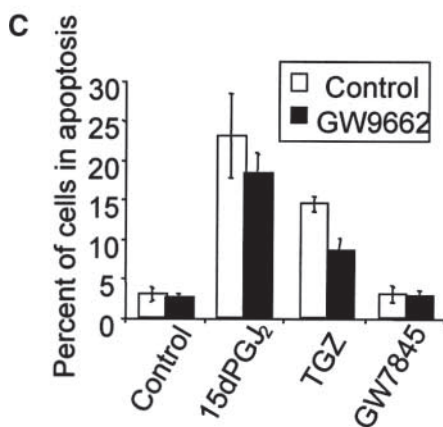
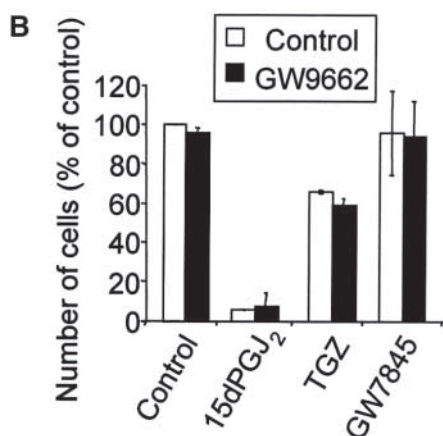
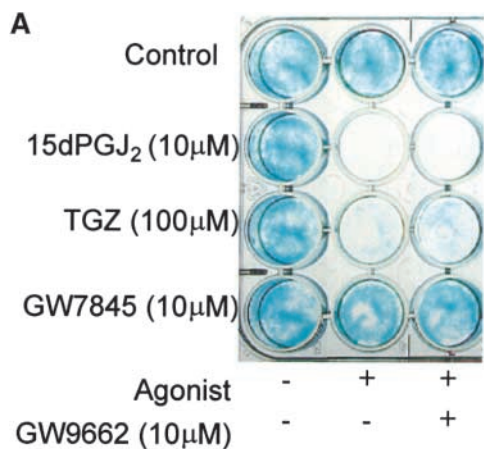


Fig. 4. Pharmacologic antagonism of PPAR γ ligands has different effects on 15dPGJ₂- versus TGZ-induced apoptosis. **A:** MDA-MB-231 cells were grown in the presence of vehicle or the indicated PPAR γ agonist with or without the PPAR γ antagonist GW9662. Cellular proliferation was determined by methylene blue staining. Data are representative of three separate experiments. **B:** 1×10^4 MDA-MB-231 cells were grown in the presence of vehicle or the indicated PPAR γ agonists at the same concentrations as in 4A with or without GW9662. Total cell number was determined after 96 h using a hemacytometer. Data are expressed as the mean \pm SD of three separate experiments. **C:** 5×10^5 MDA-MB-231 cells were grown in the presence of vehicle or the indicated PPAR γ agonists with or without GW9662 for 36 h and the number of cells undergoing apoptosis was determined by flow cytometry using an annexin V-FTIC kit. Data are expressed as the mean \pm SD of three separate experiments. *Apoptosis was significantly reduced by GW9662, $P < 0.05$.

product (17), was inversely correlated with PPAR γ activation (Fig. 5C). 15dPGJ₂ induced only 1.5-fold increase in CD36 expression whereas TGZ and GW7845 increased CD36 expression by nearly 3-fold and the expression of CD36 was blocked by GW9662, no matter which agonist was used. These data suggest that all three PPAR γ agonists tested enhance PPRE-mediated gene transcription, but 15dPGJ₂-mediated PPRE activity cannot be fully blocked by pharmacologic PPAR γ antagonism and the protein levels of PPRE-containing genes do not always correlate with luciferase reporter measures of gene expression.

Dominant negative PPAR γ localizes to the nucleus but does not rescue cells from 15dPGJ₂-induced apoptosis

We have shown here that the irreversible antagonist GW9662 reduces transcriptional activation of PPAR γ and expression of CD36, but does not rescue cells from 15dPGJ₂-induced apoptosis. However, since residual PPRE activity could be measured, we could not rule out PPAR γ -mediated transcription as a mediator of 15dPGJ₂-induced apoptosis. Furthermore, since PPAR γ must bind DNA for transcriptional activation of PPRE-containing genes, we identified the localization of PPAR γ expression in MDA-

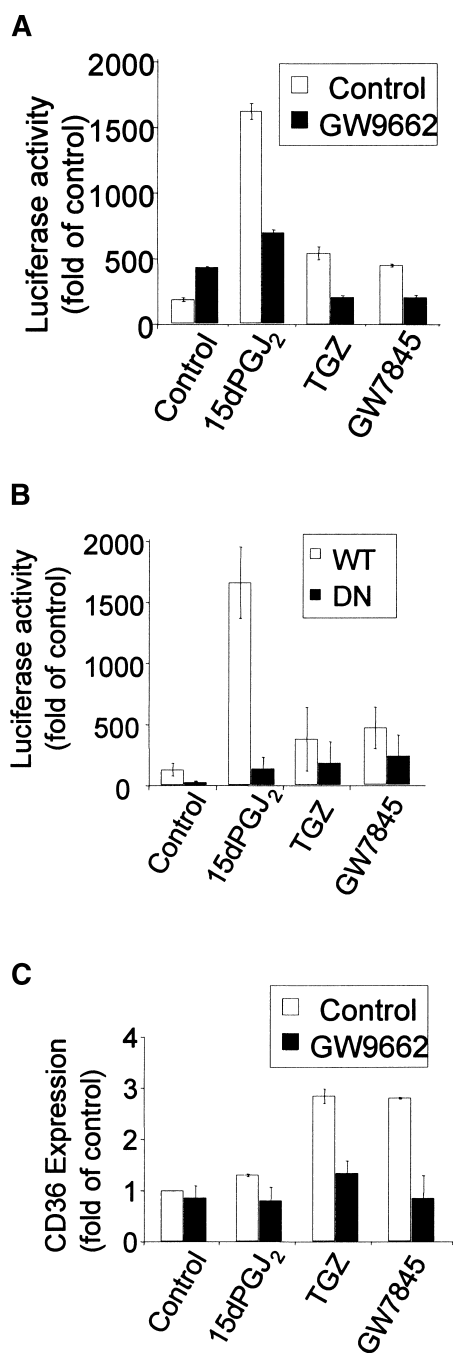


Fig. 5. PPAR γ antagonism blocks PPAR-response element (PPRE)-mediated gene transcription and expression of CD36. **A:** MDA-MB-231 cells were transiently transfected with a PPRE-driven luciferase reporter construct and the degree of PPRE-mediated gene transcription was determined after 24 h exposure to 15dPGJ₂ (10 μ M), TGZ (100 μ M), and GW7845 (10 μ M) with or without GW9662 (10 μ M). Data are the mean \pm SD of three separate experiments. *Luciferase activity was significantly higher versus TGZ or GW7845 without GW9662, $P < 0.05$. **B:** WT or DN was co-transfected with a PPRE-driven luciferase reporter construct and the degree of PPRE-mediated gene transcription was determined after 24-h exposure to 15dPGJ₂ (10 μ M), TGZ (100 μ M), or GW7845 (10 μ M). Data are the mean \pm SD of three separate experiments. **C:** MDA-MB-231 cells were treated for 1 h with or without GW9662, then grown in the presence of the indicated PPAR γ agonists for 12 h and the expression of CD36 was determined by flow cytometry. PPAR γ agonist and antagonist concentrations are 15dPGJ₂, 1 μ M; TGZ, 25 μ M; GW7845, .5 μ M; GW9662, 1 μ M. Data are the mean \pm SD of three separate experiments.

MB-231 cells. WT or DN was co-injected by single cell microinjection with YFP and visualized by immunofluorescence. Both WT and DN localized to the nucleus of MDA-MB-231 cells suggesting that WT and DN are functionally active in these cells (**Fig. 6A**). However, consistent with the data from clonogenic and apoptosis assays using the selective antagonist GW9662, dominant negative expression of PPAR γ by single-cell microinjection does not rescue cells from 15dPGJ₂ or TGZ-induced apoptosis. MDA-MB-231 cells expressing either WT or DN showed morphologic characteristic of apoptosis 24 and 48 h after cells were exposed to 15dPGJ₂ or TGZ (**Fig. 6B**) and the total number of surviving 15dPGJ₂ or TGZ-treated cells was decreased at both 24 h and 48 h (**Fig. 6C**). However, the selective PPAR γ agonist, GW7845, did not induce apoptosis in these cells. Taken together, these data suggest that 15dPGJ₂-induced apoptosis is not mediated by PPAR γ in breast cancer cells.

DISCUSSION

PPAR γ dependent gene expression and PPAR γ agonist-induced apoptosis using fatty acid derivatives, thiazolidinediones and tyrosine-based agonists has been reported in several cancer cell types (42–52). This is the first report, however, that specifically addresses the role of PPAR γ in apoptosis of breast cancer cells. Using three classes of PPAR γ agonists and an irreversible antagonist, we show that selective activation of PPAR γ (via GW7845) does activate PPRE-driven gene transcription but does not induce apoptosis. Furthermore, inhibition of PPAR γ (via GW9662 or a DN) in the presence of 15dPGJ₂ or TGZ reduces PPRE-mediated transcription but does not rescue cells from apoptosis. These results show that 15dPGJ₂ and TGZ have PPAR γ -independent effects in breast cancer cells and suggest that PPAR γ does not mediate 15dPGJ₂-induced apoptosis. Moreover, these data highlight the need for both a better understanding of cyclopentenone-induced apoptosis and the role of PPAR γ in cancer.

Most PPAR γ agonists, including 15dPGJ₂, 15(s)-HETE, TGZ, and BRL49653, have PPAR γ -dependent and PPAR γ -independent effects that result in variable biologic effects (9, 25). In mouse models of colon cancer, TGZ was reported to increase aberrant crypt foci and colon polyp number in one model (53, 54), but induce differentiation and a reversal of the malignant phenotype in another model (55). In humans, TGZ was reported to increase, rather than decrease, the size of liposarcomas (12). These disparate results may be due, in part, to PPAR γ -dependent and -independent pathways. We have previously shown that 15dPGJ₂-blocks the progression of breast tumors in a mouse model (16), and that 15dPGJ₂-induced apoptosis requires early de novo gene transcription (26). However, here we report that PPAR γ is not required for, and thus not the mediator of, 15dPGJ₂-induced apoptosis in breast cancer cells. Alternate proposed mechanisms are represented in **Fig. 7**. One possible mechanism is inhibition of NF κ B-mediated survival pathways. The exocyclic electoro-

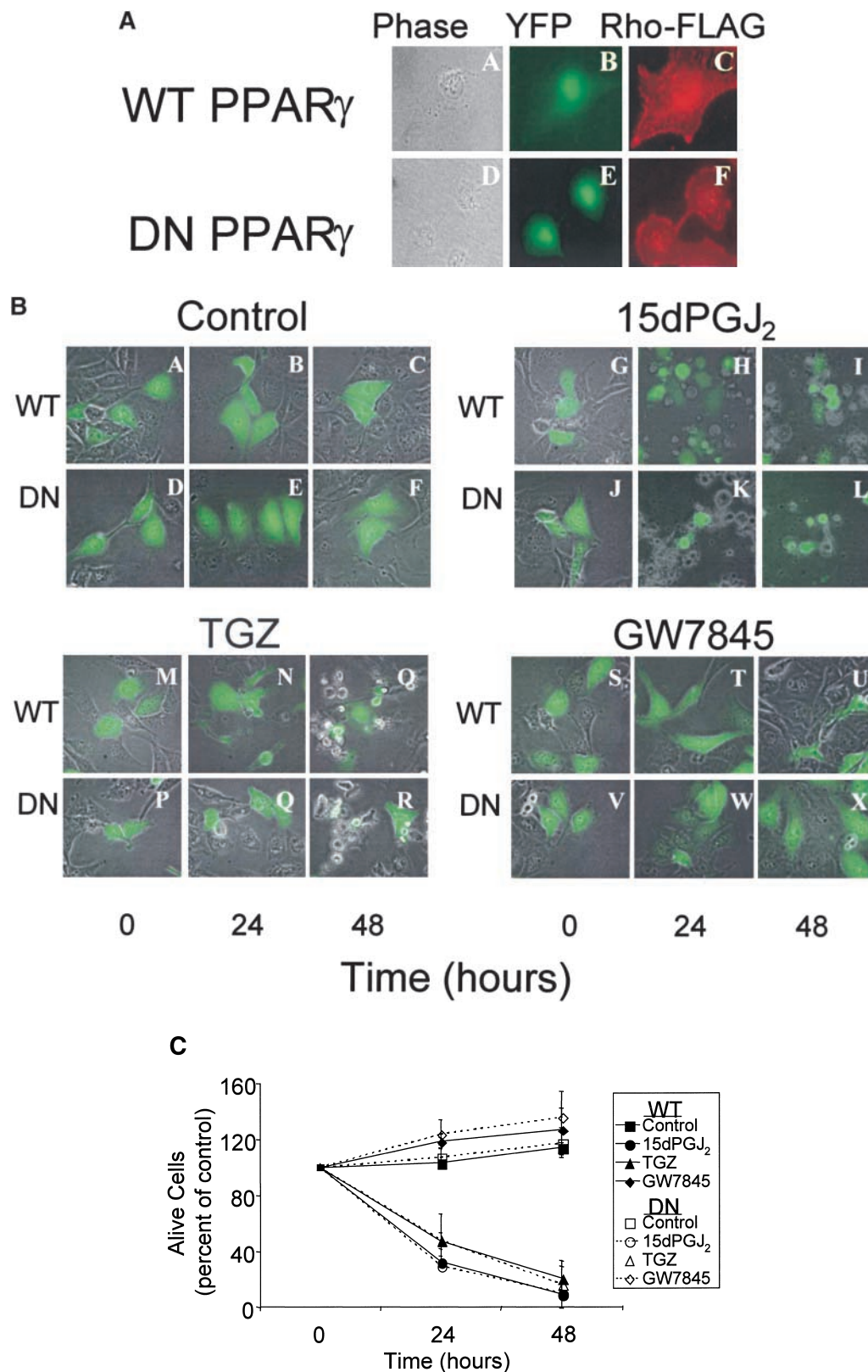


Fig. 6. DN localizes to the nucleus but fails to rescue cells from 15dPGJ₂ and TGZ-induced apoptosis. **A:** WT or DN and YFP were co-injected into MDA-MB-231 cells and the expression and localization of PPAR γ was determined by immunofluorescent staining. Images are representative of three separate experiments. **B:** WT or DN and YFP were co-injected into MDA-MB-231 cells. Digital images of the morphology of successfully injected cells were obtained 12 h after injection at which time cells were provided the indicated PPAR γ agonist (T = 0). Digital images were obtained 24 and 48 h after addition of the PPAR γ agonists. Images are representative of three separate experiments. **C:** The number of surviving cells was determined 12 h after injection (T = 0) and 24 and 48 h after addition of the indicated PPAR γ agonists. Data are the mean \pm SD of three separate experiments.

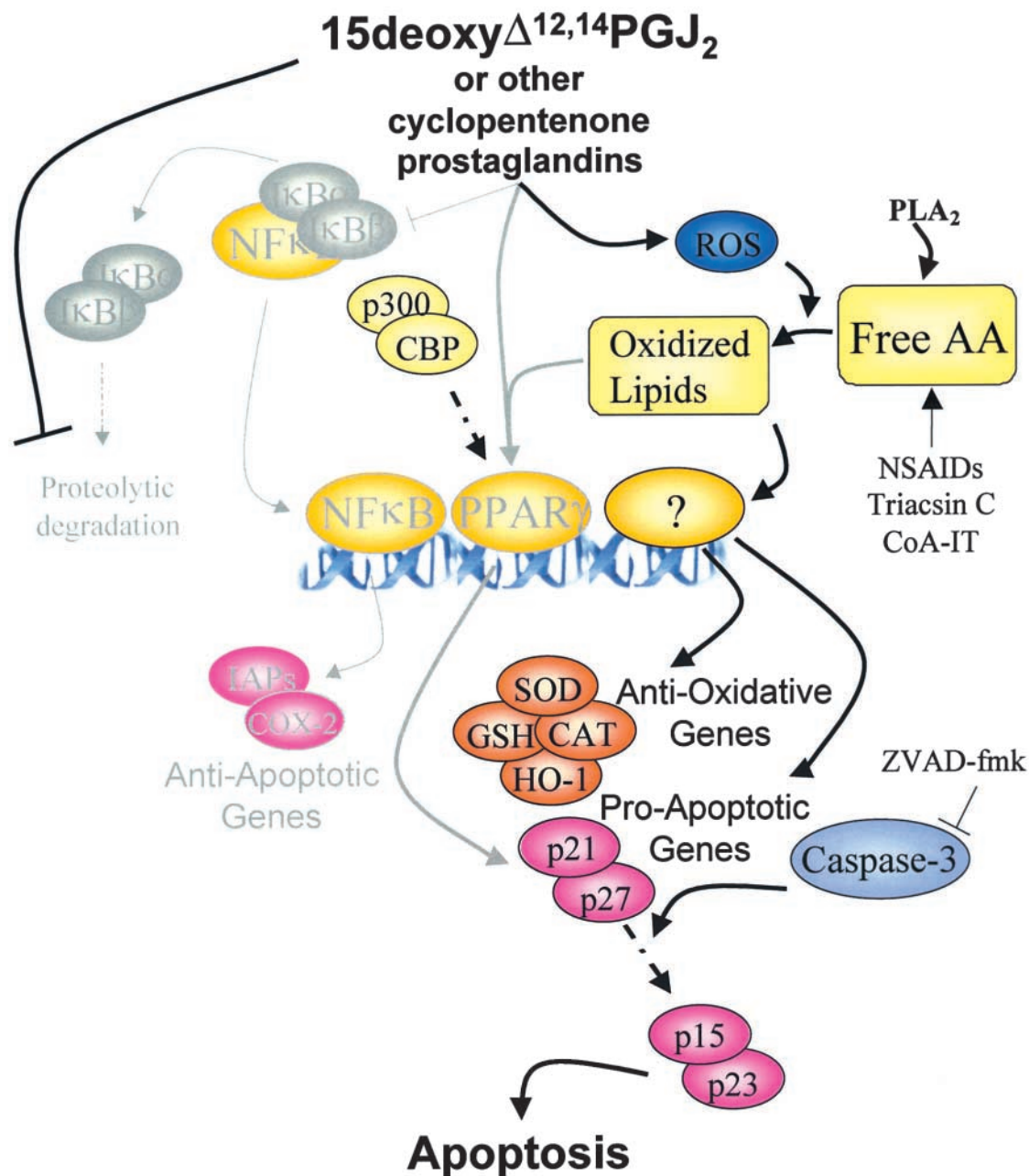



Fig. 7. Potential mechanisms for cyclopentenone prostaglandin-induced apoptosis. The exocyclic electrophilic carbonyl of J series cyclopentenone prostaglandins confers unique pro-apoptotic activity in part, perhaps, by inhibition of isopeptidase activity of the ubiquitin proteasome and covalent inactivation of NF κ B-mediated survival pathways. 15dPGJ₂ and other cyclopentenone prostaglandins mediate transcriptional inhibition of COX-2 and induce reactive oxygen species (ROS). COX-2 inhibition may lead to increased intracellular levels of free arachidonic acid and increased intracellular oxidative stress may lead to the production of oxidized lipids and lipid modified proteins could account for some of the 15dPGJ₂-induced PPRE-mediated gene transcription and/or result in cell death. 15dPGJ₂ and other cyclopentenone prostaglandins, increase expression of glutamate-cysteine ligase, GSH reductase, superoxide dismutase, heme oxygenase-1, and catalase, which may be cyto-protective at low levels, but higher expression levels are cytotoxic. Black lines represent likely pathways for 15dPGJ₂-induced apoptosis. Light gray lines represent less likely pathways based on current data.

philic carbonyl of 15dPGJ₂ covalently inactivates IKK, I κ B α , and I κ B β NF κ B (18–21). However, if this were a major initiator of apoptosis, inhibition of new RNA and protein would be expected to enhance apoptosis. We found the opposite to be true (26). A second mechanism could be inhibition of the ubiquitin proteasome, which would lead to accumulation of unmodified proteins and signal

cell death. The immediate precursor to 15dPGJ₂ synthesis, Δ^{12} -PGJ₂, blocks polyubiquitin disassembly by inhibition of isopeptidase activity (22); however these events occurred at very high concentrations of Δ^{12} -PGJ₂. Nonetheless, inhibition of the proteasome is a focus of novel drug design and cancer therapy (56, 57). Third, 15dPGJ₂ inhibits transcriptional activation of COX-2, and perhaps other

arachidonic acid metabolizing enzymes (58, 59) that may lead to increased intracellular levels of free arachidonic acid, an event known to induce apoptosis (60–62).

Finally, 15dPGJ₂ and other cyclopentenone prostaglandins induce reactive oxygen species (ROS), in part by the generation of superoxide anion (63), leading to the production of oxidized lipids and lipid modified proteins resulting in cell death (23, 24). ROS could oxidize LDL, a potent anti-inflammatory PPAR γ agonist (64, 65) or oxidize fatty acid derivatives that activate PPRE-mediated gene transcription and exert potent anti-neoplastic activity (Chilton, unpublished observations) (66). Lipid oxidation induced by cyclopentenone prostaglandins can lead to increased expression of cyto-protective enzymes in normal cells, but may represent a novel approach to the treatment of cancer cells. For example, Levonen and colleagues showed that low concentration of 15dPGJ₂, and other cyclopentenone prostaglandins, increase expression of glutamate-cysteine ligase as well as GSH reductase independent of PPAR γ (67). However, the cyto-protection was overwhelmed by higher concentrations of 15dPGJ₂. Robbins and colleagues showed that γ -linolenic acid and 15dPGJ₂ increase the expression of catalase and other antioxidant enzymes in normal astrocytes, but not in glioma cells (68) (unpublished observations). In addition, 15dPGJ₂ induces expression of heme oxygenase-1 (22), which is cyto-protective at low levels while higher expression levels are cyto-toxic (69–71).

PPAR γ is clearly involved in lipid metabolism and is essential for cellular differentiation (27, 28). However, the current study shows that PPAR γ is not required for 15dPGJ₂-induced apoptosis in breast cancer cells. Furthermore, these studies show that PPAR γ specific agonists, and likely the endogenous PPAR γ ligand(s), may not be pro-apoptotic, but may be anti-angiogenic (58) and protective against ischemia/reperfusion injury (72), inflammatory diseases (73–75), and the complications associated with diabetes (76–78). The synthesis and activity of endogenous PPAR γ ligands such as 15dPGJ₂ has been a matter of debate. However, the identification of increased in vivo production of 15dPGJ₂ in lipopolysaccharide-stimulated RAW264.7 macrophages and in macrophages of human atherosclerotic plaques (79) provides better clues to the site-specific production and biologic activity of 15dPGJ₂. Clearly, the pleiotropic nature of PPAR γ signaling and the mechanisms by which fatty acid derivatives, particularly the cyclopentenone prostaglandins, exert anti-inflammatory and anti-neoplastic activity warrants additional investigation. 

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