Prostaglandin D$_2$ and Its Metabolites Induce Caspase-Dependent Granulocyte Apoptosis That Is Mediated Via Inhibition of IκBα Degradation Using a Peroxisome Proliferator-Activated Receptor-γ-Independent Mechanism

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Prostaglandin D$_2$ and Its Metabolites Induce Caspase-Dependent Granulocyte Apoptosis That Is Mediated Via Inhibition of I$_{kB}$α Degradation Using a Peroxisome Proliferator-Activated Receptor-γ-Independent Mechanism$^1$

Carol Ward,* Ian Dransfield,* Joanna Murray,* Stuart N. Farrow,† Christopher Haslett,* and Adriano G. Rossi$^2$*

Many inflammatory mediators retard granulocyte apoptosis. Most natural PGs studied herein (e.g., PGE$_2$, PGA$_2$, PA$_1$, PGF$_2$α) either delayed apoptosis or had no effect, whereas PGD$_2$ and its metabolite PGJ$_2$ selectively induced eosinophil, but not neutrophil apoptosis. This novel proapoptotic effect does not appear to be mediated via classical PG receptor ligation or by elevation of intracellular Ca$^{2+}$. Intriguingly, the sequential metabolites Δ$^{12}$PGJ$_2$ and 15-deoxy-Δ$^{12}$, Δ$^{14}$PGJ$_2$ (15dPGJ$_2$) induced caspase-dependent apoptosis in both granulocytes, an effect that did not involve de novo protein synthesis. Despite the fact that Δ$^{12}$PGJ$_2$ and 15dPGJ$_2$ are peroxisome proliferator-activated receptor-γ (PPAR-γ) activators, apoptosis was not mimicked by synthetic PPAR-γ and PPAR-α ligands or blocked by an irreversible PPAR-γ antagonist. Furthermore, Δ$^{12}$PGJ$_2$ and 15dPGJ$_2$ inhibited LPS-induced I$_{kB}$α degradation and subsequent inhibition of neutrophil apoptosis, suggesting that apoptosis is mediated via PPAR-γ-independent inhibition of NF-κB activation. In addition, we show that TNF-α-mediated loss of cytoplasmic I$_{kB}$α in eosinophils is inhibited by 15dPGJ$_2$ in a concentration-dependent manner. The selective induction of eosinophil apoptosis by PGD$_2$ and PGJ$_2$ may help define novel therapeutic pathways in diseases in which it would be desirable to specifically remove eosinophils but retain neutrophils for antibacterial host defense. The powerful proapoptotic effects of Δ$^{12}$PGJ$_2$ and 15dPGJ$_2$ in both granulocyte types suggest that these natural products control the longevity of key inflammatory cells and may be relevant to understanding the control and resolution of inflammation. The Journal of Immunology, 2002, 168: 6232–6243.

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can also bind to other PG receptors, thereby triggering several different signaling pathways. For example, binding to the DP receptor increases intracellular cAMP and/or cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$), whereas PGE receptor (EP receptor) 1, PGF$_2$α receptor (FP receptor), or thromboxane A$_2$ receptor (TP receptor) ligation increases [Ca$^{2+}$]$_i$, but has no direct effect on cAMP levels. Prostacyclin receptor (IP receptor) activation also increases intracellular cAMP (9, 21–23). The metabolites of PGD$_2$, Δ$^1$PGJ$_2$ and 15dPGJ$_2$, have been shown to activate intracellular peroxisome proliferator-activated receptors (PPARs) which are transducer proteins belonging to the steroid/thyroid/rexinoid receptor superfamily that regulate target genes by binding to PPAR response elements (24, 25). Three isoforms of PPAR (PPAR-γ, PPAR-δ, and PPAR-α) are present in human cells (26–28). PPAR-α is primarily expressed in tissues with high fatty acid metabolism; PPAR-γ is expressed in adipose tissue, adrenal gland, spleen, and several myeloid cell lines; and PPAR-δ is highly expressed in heart, kidney, and intestine (29–31).

In this study, we demonstrate differential effects of PGD$_2$ on granulocyte apoptosis; selectively inducing eosinophil but not neutrophil apoptosis. This intriguing result prompted us to examine the actions of this PG and its metabolites more closely. We show that the PGD$_2$ metabolites, Δ$^1$PGJ$_2$ and 15dPGJ$_2$, are powerful inducers of caspase-dependent granulocyte apoptosis. These data could not be mimicked using synthetic PPAR-γ agonists such as the thiazolidinediones BRL 49653 and ciglitazone (32), nor could the PPAR-γ antagonist GW9662 (33) prevent induction of cell death by 15dPGJ$_2$. PGS of the J series also activate PPAR-α; however, a synthetic ligand to this isoform, pinirinic acid (WY-14643), had no effect on granulocyte apoptosis. We also rule out a significant proapoptotic role for the other classical cell surface PG receptors in both neutrophils and eosinophils. We have recently shown that NF-κB plays a critical role in the regulation of granulocyte apoptosis where specific inhibition of NF-κB can directly induce granulocyte apoptosis and can enhance apoptosis induced by TNF-α and block the delayed apoptosis induced by LPS (8). In view of the suggestion that PPAR-γ ligands may inhibit NF-κB activation, we examine whether the effects of Δ$^1$PGJ$_2$ and 15dPGJ$_2$, are mediated via inhibition of this transcription factor. We demonstrate in the neutrophil that PGD$_2$ metabolites inhibit LPS-induced degradation of IκBα (the inhibitory subunit of NF-κB) and block LPS-mediated inhibition of apoptosis. Furthermore, TNF-α-induced IκBα breakdown in the eosinophil is also inhibited by PGD$_2$ metabolites. We conclude that the PPAR-γ ligands Δ$^1$PGJ$_2$, and 15dPGJ$_2$ influence granulocyte apoptosis by interfering with the prosurvival NF-κB pathway, an effect that is independent of PPAR-γ ligation.

Materials and Methods

**Neutrophil and eosinophil isolation and culture**

Neutrophils and eosinophils were isolated from the peripheral blood of normal donors by dextran sedimentation followed by centrifugation through discontinuous Percoll gradients (Amersham Pharmacia Biotech, Little Chalfont, U.K.) (34, 35). Only neutrophil preparations with a cell purity of ≥98% were used. Eosinophils were separated from contaminating neutrophils using immunomagnetic separation with sheep anti-mouse IgG-Dynabeads (Dynabeads M-450; Dynal, Merseyside, U.K.) coated with the murine anti-neutrophil Ab 3G8 (anti-CD16; a gift from Dr. J. Unkeless, Mount Sinai Medical School, New York, NY). Cells were mixed with washed Ab-coated magnetic beads at a bead-neutrophil ratio of 3:1 on a rotary mixer at 4°C for 20 min, and the beads were removed magnetically by two 3-min stationary magnetic contacts (Dyna Magnetic Particle Concentrator, MPC-1) to yield an eosinophil population of ≥98% purity. After purification, cells were washed twice in PBS without Ca$^{2+}$ and Mg$^{2+}$ before resuspending in IMDM (Life Technologies, Paisley, U.K.) supplemented with 10% autologous serum (unless otherwise stated in figures).

Both cell types were cultured in flat-bottom Falcon flexible well plates (BD Biosciences, Oxford, U.K.) at 37°C in a 5% CO$_2$ atmosphere; or in 2 ml round-bottom Eppendorf tubes in a shaking water bath at 37°C, with neutrophils 5 × 10$^6$/ml and eosinophils 2 × 10$^6$/ml. Cells were cultured in the absence or presence of test agents as described in the figures. All experiments were performed at least three times in triplicate.

**Assessment of granulocyte apoptosis**

**Morphology.** Cells were cytocentrifuged, fixed in methanol, stained with DiffQuik (Gamidor, Abingdon, U.K.) and counted using oil immersion microscopy (≥100 objective) to determine the proportion of cells with distinctive apoptotic morphology (8). At least 500 cells were counted per slide with the observer blinded to the experimental conditions. The results were expressed as the mean percent apoptosis ± SEM

**Annexin V binding and propidium iodide staining.** A separate and independent assessment of apoptosis was performed by flow cytometry using FITC-labeled recombinant human annexin V that binds to phosphatidylserine exposed on the surface of apoptotic cells and propidium iodide as an index of loss of cell membrane integrity (8). Stock annexin V (Bender MedSystems, Vienna, Austria) was diluted 1:200 with binding buffer and then added (25 μl) to 75 μl of the recovered cell samples. After a 10-min incubation at 4°C, these samples were treated with propidium iodide (final concentration, 10 μg/ml) for 2 min before flow cytometric analysis using an EPICS XL2.

**DNA fragmentation assay.** Cells were lysed, DNA was extracted and run on an agarose gel containing ethidium bromide, and DNA fragmentation (laddering) was visualized as described (36).

**Assessment of membrane integrity and cell recovery.**

To ensure that the cell death observed was due solely to apoptosis, the membrane integrity of treated and untreated cells was assessed using the vital dye trypan blue. In addition, cells were counted at the start of culture and at the end of the indicated period. Under all conditions and treatments used, there was no loss of cell membrane integrity, and cell loss was minimal.

**Measurement of [Ca$^{2+}$]$_i$.**

Freshly isolated granulocytes were washed (three times) in HBSS (Ca$^{2+}$- and Mg$^{2+}$-free) before being resuspended at 10$^6$/ml in HBSS (Ca$^{2+}$- and Mg$^{2+}$-free), for incubation with fura 2-acetoxymethyl ester (final concentration, 2 μM) for 30 min at 37°C (37, 38). The cells were then washed (twice) to remove fura 2-acetoxymethyl ester and left in HBSS (Ca$^{2+}$ and Mg$^{2+}$-free) for a further 10 min for optimal deesterification, before finally resuspending the granulocytes at 2 × 10$^6$/ml in HBSS (containing Ca$^{2+}$ and Mg$^{2+}$). Changes in fluorescence upon agonist addition were determined using a PerkinElmer (Wellesley, MA) LS520 fluorometer, with dual wavelength excitation (340 and 380 nm) and emission at 510 nm, fitted with a thermostated cuvette compartment and stirring attachment, to ensure complete mixing of reagents. [Ca$^{2+}$]$_i$ was calibrated as previously described (37, 38).

**Western blotting for IκBα.**

Cell samples (5 × 10$^6$/ml) were incubated in a shaking water bath at 37°C with the agents of interest as described in the figure legends. After treatment, cells were immediately placed on ice, and all lysates were prepared at 4°C. To minimize problems with proteolysis, lysates were prepared by methods normally used for EMSA preparations (8). Lysates were run on a 9% SDS gel and, after transfer, blocked by 5% milk protein before an overnight incubation with primary IκBα Ab (New England Biolabs, Beverly, MA) diluted 1/500. After washing, blots were incubated with HRP-conjugated anti-IκBα Ab diluted at 1/2500 and developed using standard ECL reagents (Amersham, Arlington Heights, IL).

**Other materials.**

Further specific materials were obtained as follows: LPS (Escherichia coli 0127:B8) (Sigma, Poole, U.K.); benzylcyclohexanecarboxylic acid fluoromethylketone (z-VAD-fmk; Bachem U.K., Saffron Walden, U.K.); Δ$^1$PGJ$_2$, 15dPGJ$_2$, PGD$_2$, PGA$_2$, PGA$_1$, U46619, ciglitazone, and BRL49653 was a gift from K. Chaterjee (University of Cambridge, Cambridge, U.K.). All other reagents were obtained from Sigma U.K. and were of the highest purity.
Statistical analysis

The results are expressed as mean ± SEM of the number (n) of independent experiments each using cells from separate donors with each treatment performed in triplicate. Statistical analysis was performed by ANOVA with comparisons between groups made using the Newman-Kuels procedure. Differences were considered significant when p < 0.05.

Results

Most PG either inhibit or have no direct effect on granulocyte apoptosis

The effects of PGs on granulocyte apoptosis are shown in Table I. PGE₂ and 11-deoxyPGE₂ delayed neutrophil apoptosis as did the dehydration product of PGE₁, PGA₁. PGA₂ had no significant effect on apoptosis in either cell type, whereas PGF₂α decreased the rate of constitutive apoptosis in both neutrophils and eosinophils. The thromboxane A₂ mimetic U-46619 had no direct effect on cell death. Only PGD₂ increased the constitutive rate of eosinophil apoptosis.

PGD₂ has differential effects on neutrophil and eosinophil apoptosis

PGD₂ did not enhance the rate of constitutive neutrophil apoptosis at 20 h (Fig. 1A) or at earlier time points (e.g., 2, 3, 4, or 6 h) when basal levels of apoptosis are much lower (data not shown). However, PGD₂ significantly increased the rate of eosinophil apoptosis, after both 20 and 40 h in culture, with levels of apoptosis in treated cells being ∼4 times higher than those in control untreated cells (Fig. 1B). As a further control in these experiments, when neutrophils were cultured at the same density as eosinophils (2 × 10⁶/ml), PGD₂ still did not induce neutrophil apoptosis (control, 85.9 ± 4.6; PGE₂ (10 μM), 87.9 ± 1.3 (n = 3, each experiment performed in triplicate). To directly demonstrate the efficacy of PGD₂ on eosinophils, apoptosis was induced by PGD₂ and compared with apoptosis induced by maximal concentrations of dexamethasone (1 μM), an established accelerator of eosinophil apoptosis (39), during concurrent experiments on cells from the same donor. PGD₂ (10 μM) induced apoptosis to a greater extent than dexamethasone at both time points (20 and 40 h) tested (Fig. 1B).

The increase in eosinophil apoptosis is not explained by binding to other surface PG receptors

PGD₂ at the concentrations used in this study, has the ability to bind other PG receptors, namely, the EP, FP, IP, and TP receptors (9). We therefore examined the effects of agonist binding to these receptors to determine whether the increase in apoptosis observed with PGD₂ could be reproduced. The FP receptor binds PGE₂α, which inhibits several functional activities in human neutrophils (41, 42); however, little is known of its effects on eosinophils. As shown in Table I, PGF₂α, significantly increased neutrophil survival, and by 40 h of culture, eosinophil apoptosis was almost 50% of control values. Had PGD₂ been acting via the FP receptor, apoptosis should have been inhibited in both eosinophils and neutrophils.

The use of U-46619, a thromboxane A₂ receptor agonist, demonstrated that activation of putative TP receptors on granulocytes had no significant effect on apoptosis in either neutrophils or eosinophils (Table I), again suggesting that PGD₂ is not acting via this receptor. Activation of the DP, IP, EP₂, EP₃, and EP₄ receptors has been demonstrated to increase intracellular cAMP levels in many cell types including granulocytes (9). To mimic elevation of cAMP, we used the stable, cell-permeable analog of cAMP, db-cAMP (dbcAMP), and show that this compound powerfully delays apoptosis in both neutrophilic (Fig. 1E) and eosinophilic (Fig. 1F) granulocytes. Therefore, elevation of cAMP by ligation of certain PG receptors cannot explain the dramatic augmentation of apoptosis induced by PGD₂.

Ligation of the EP₄ receptor has no reported effects on intracellular cAMP levels, but it does increase [Ca²⁺]i, in some cell systems. To investigate directly whether PGD₂ can influence [Ca²⁺]i,

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Table I. Effect of PG on neutrophil and eosinophil apoptosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neutrophils (20 h)</th>
<th>Eosinophils (20 h)</th>
<th>Eosinophils (40 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td>PGE₂</td>
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<td>7.7 ± 1.4</td>
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<td>11-DeoxyPGE₂</td>
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<td>50.3 ± 7.0b</td>
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<td>56.1 ± 0.9b</td>
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<td>71.4 ± 5.6</td>
<td>64.6 ± 9.4</td>
<td>7.7 ± 1.4</td>
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<td>PGE₂α</td>
<td>66.0 ± 6.7</td>
<td>57.7 ± 8.0b</td>
<td>7.7 ± 1.4</td>
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<tr>
<td>U-46619</td>
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<td>70.3 ± 3.2</td>
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</tr>
<tr>
<td>PGD₂</td>
<td>61.0 ± 6.2</td>
<td>65.1 ± 2.3</td>
<td>10.9 ± 2.4</td>
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</tbody>
</table>

a Neutrophils (5 × 10⁶/ml) or eosinophils (2 × 10⁶/ml) were incubated in IMDM supplemented with serum alone (control) or with PGE (10 μM) as detailed above and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils). Apoptosis was assessed morphologically. Data represent the mean ± SEM of at least three separate experiments. All experiments were performed in triplicate.

b Significant differences (p < 0.05) from the appropriate control.
in granulocytes, we performed studies using fura 2-loaded cells. Neutrophils (Fig. 2) and eosinophils (Fig. 3) respond to stimuli such as platelet-activating factor and leukotriene B<sub>4</sub>, which act on specific G protein-coupled receptors by a rapid and reversible elevation of \([\text{Ca}^{2+}]_i\). We found that PGD<sub>2</sub> at 10 \(\mu\text{M}\) induced a rapid and reversible elevation of \([\text{Ca}^{2+}]_i\) in eosinophils but not in neutrophils. These differential effects were mirrored by the PGD<sub>2</sub> sequential metabolites PGJ<sub>2</sub>, 12\(^{15}\)PGJ<sub>2</sub>, and 15dPGJ<sub>2</sub>. Interestingly, in eosinophils there was both homologous and heterologous desensitization between the different PGD<sub>2</sub> metabolites and indeed PGD<sub>2</sub> itself (Fig. 3 and data not shown). It has been recently reported that PGD<sub>2</sub> metabolites are potent selective activators of human eosinophils inducing calcium mobilization, actin polymerization, and CD11b expression by interacting with DP<sub>2</sub> (43). We have previously reported that increasing levels of \([\text{Ca}^{2+}]_i\) inhibits neutrophil apoptosis while accelerating this process in eosinophils (44, 45). However, use of calcium ionophores and other pharmacological agents (e.g., thapsigargin) that elevate \([\text{Ca}^{2+}]_i\), also cause degranulation in the eosinophil and cause necrosis rather than apoptosis by 40 h. Neither degranulation nor necrosis was observed in either cell type when treated with PGD<sub>2</sub> at any time point examined. This suggests that increases in \([\text{Ca}^{2+}]_i\) were not responsible for the proapoptotic effect of this PG. Therefore, the proapoptotic effect of PGD<sub>2</sub> on eosinophils does not appear to be mediated via any known classical cell surface PG receptor or by increasing intracellular cAMP or \([\text{Ca}^{2+}]_i\).

**The PGD<sub>2</sub> metabolites PGJ<sub>2</sub>, \(\Delta^{12}\text{PGJ}_2\), and 15dPGJ<sub>2</sub> induce granulocyte apoptosis**

PGJ<sub>2</sub>, an active PGD<sub>2</sub> metabolite, is also capable of selectively triggering the DP receptor (13, 22). As demonstrated in Fig. 4A, PGJ<sub>2</sub> produced results similar to those of PGD<sub>2</sub> in neutrophils,
causing no significant change in the rate of constitutive apoptosis at 20 h. However, PGJ2 significantly increased eosinophil apoptosis at 20 and 40 h, but this increase was markedly less than that induced by the parent compound, PGD2, when used at equimolar concentrations (Fig. 4B).

These results suggested that the differential effect of PGD2 on eosinophil and neutrophil apoptosis might be the result of differences in the metabolic products produced by these two cells. We therefore investigated whether the PGJ2 metabolites 15dPGJ2 and 15dPGJ2 could increase levels of constitutive granulocyte apoptosis. Fig. 4, C and D, illustrates that these metabolites are efficacious inducers of programmed cell death in both cell types causing increases in eosinophil apoptosis comparable with those observed with PGD2. However, 15dPGJ2 and 15dPGJ2 also had powerful proapoptotic effects on neutrophils, increasing control rates from 66 to ~90% at 20 h. These data support the possibility that neutrophils and eosinophils may metabolize PGD2 differently. The proportion of cells exhibiting classical pyknotic nuclei of apoptotic

neutrophils and eosinophils increases markedly in cells treated with 15dPGJ2 and 15dPGJ2 even at the early time points of 3–4 h (Fig. 6 and data not shown). When DNA was extracted from granulocytes treated with PGD2 metabolites for short period of culture (e.g. 4 h) and run on an agarose gel, a typical DNA ladder pattern was evident whereas there was no DNA “laddering” observed in control cells (data not shown). Moreover, the proportion of granulocytes binding FITC-labeled annexin V (indicative of cell surface changes associated with apoptosis) was increased when cells were treated with PGD2 and its metabolites (Fig. 5). A smaller increase in propidium iodide staining was also observed with the PGs. We believe that this increase is likely due to secondary necrosis, in that failure of apoptotic eosinophil clearance results in these cells quickly undergoing necrosis and it is possible that actual processing of eosinophils during the double staining technique can contribute to an abnormally high level of propidium iodide staining. This latter point is further supported by the observation that we failed to observe any significant uptake of the vital dye trypan blue when the cells are treated with these PGs.

The induction of granulocyte apoptosis by 15dPGJ2 and 15dPGJ2 is independent of synthesis of a death protein but dependent on activation of caspases

To investigate whether the proapoptotic effect of 15dPGJ2 and 15dPGJ2 requires synthesis of protein(s) (e.g., a death-inducing protein), cells were cultured with a protein synthesis inhibitor, cycloheximide. Although cycloheximide itself is a potent accelerator of granulocyte apoptosis at 20 h (46), experiments were performed at a 3-h time point at which cycloheximide alone has little influence on this process. Interestingly, the proapoptotic effect of 15dPGJ2 was apparent even at this short incubation period (Fig. 6A). Cycloheximide did not inhibit the induction of cell death but rather produced an additive increase in the levels of apoptosis produced by this metabolite. The increase in apoptosis induced by 15dPGJ2 was attenuated when cells were cotreated with the pan-caspase inhibitor z-VAD-fmk but not by the vehicle control (0.02% DMSO) (Fig. 6B). z-VAD-fmk

![FIGURE 2. Effect of PGD2, PGJ2, 15dPGJ2, and 15dPGJ2 on neutrophil [Ca2+]2: Fura 2-loaded neutrophils (2 x 10⁶/ml) were stimulated with platelet-activating factor (PAF; 100 nM), PGD2 (10 μM), PGJ2 (10 μM), 15dPGJ2 (10 μM), and 15dPGJ2 (10 μM) as indicated above. Changes in [Ca2+]2 were determined as described in Materials and Methods, and data are representative of three separate experiments.](http://www.jimmunol.org/)

<table>
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<th>Jurkat T Lymphocytes</th>
<th>Human Peripheral Lymphocytes</th>
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<td></td>
<td>Mean</td>
<td>SEM</td>
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<tr>
<td>Control</td>
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<tr>
<td>Dexamethasone (1 μM)</td>
<td>ND</td>
<td>6.8a</td>
</tr>
<tr>
<td>PGD2 (10 μM)</td>
<td>2.2</td>
<td>1.0</td>
</tr>
<tr>
<td>PGJ2 (10 μM)</td>
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<td>0.8</td>
</tr>
<tr>
<td>15dPGJ2 (10 μM)</td>
<td>17.0b</td>
<td>5.2</td>
</tr>
<tr>
<td>15dPGJ2 (10 μM)</td>
<td>2.5</td>
<td>0.9</td>
</tr>
</tbody>
</table>

* Jurkat T lymphocytes (5 x 10⁶/ml) in RPMI with 10% FBS and human peripheral lymphocytes (5 x 10⁶/ml) in IMDM with 10% autologous serum were treated with the reagents as indicated and cultured at 37°C for 20 h. The cells were then fixed and stained, and apoptosis was determined by morphological assessment. All values are from n = 3–5 separate experiments, each performed in triplicate.

b Represents significance differences (p < 0.05) from the appropriate control.

![Table II. Effects of PGD2 and PGD2 metabolites on lymphocyte apoptosis](http://www.jimmunol.org/)
also blocked the induction of eosinophil apoptosis by both metabolites (data not shown). Taken together, these results clearly demonstrate that the proapoptotic nature of the PGD₂ metabolites likely does not result from the synthesis of a death-inducing protein as has been suggested for the proapoptotic effect observed in other cells (47) but is, however, critically dependent on the activation of caspases.

Synthetic PPAR-γ activators do not induce granulocyte apoptosis

Because Δ¹²PGJ₂ and 15dPGJ₂ are known ligands for PPAR-γ, we investigated the effects of other known PPAR-γ activators on granulocyte apoptosis. The synthetic PPAR-γ ligands, BRL49653 and ciglitazone, used at concentrations ranging from 1 nM to 100 μM, did not affect the rate of constitutive apoptosis in either cell type (Fig. 6C). Moreover, GW9662, an irreversible PPAR-γ antagonist (33), did not prevent the induction of apoptosis induced by 15dPGJ₂ (Fig. 6D). Because some reports indicate that PGD₂ metabolites can also activate PPAR-γ at higher concentrations (48), we also incubated granulocytes with pirinixic acid (WY-14643), a PPAR-γ agonist. This compound did not influence apoptosis in either cell type over the wide range of concentrations examined (1–300 μM) (Fig. 6C). Our data strongly suggest the powerful

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Effect of PGI₂, Δ¹²PGJ₂, and 15dPGJ₂ on constitutive granulocyte apoptosis. Neutrophils (A and C; 5 × 10⁶/ml) and eosinophils (B and D; 2 × 10⁶/ml) were incubated in IMDM supplemented with serum alone (control) or with the indicated reagent and harvested at 20 h (neutrophils) and 20 and 40 h (eosinophils) and apoptosis was assessed morphologically. In all panels □ is vehicle control-treated cells. A, PGI₂ (10 μM)-treated cells (■); B, PGI₂ (10 μM) (■), PGD₂ (10 μM) (■), Δ¹²PGJ₂ (10 μM) (■), and 15dPGJ₂ (10 μM) (■). Data represent the mean ± SEM of three separate experiments (A and B) and four separate experiments (C and D). All experiments were performed in triplicate. * p < 0.05 compared with control values.

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Effect of PGD₂, PGJ₂, Δ¹²PGJ₂, and 15dPGJ₂ on eosinophil [Ca²⁺]ᵢ. Fura 2-loaded eosinophils (2 × 10⁶/ml) were stimulated with leukotriene B₄ (LTB₄; 100 nM), PGD₂ (10 μM), PGJ₂ (10 μM), Δ¹²PGJ₂ (10 μM), and 15dPGJ₂ (10 μM) as indicated above. Changes in [Ca²⁺]ᵢ were determined as described in Materials and Methods, and data are representative of three separate experiments.
induction of apoptosis by Δ12PGJ2 and 15dPGJ2 is not mediated via PPAR-α or PPAR-γ activation.

Δ12PGJ2 and 15dPGJ2 may induce apoptosis in granulocytes by inhibition of IkBα proteolysis

Recent work has demonstrated that 15dPGJ2 can inhibit activation of NF-κB (49), a transcription factor that we have recently shown to be critically involved in regulating granulocyte survival (8). Activation of NF-κB by LPS may explain the powerful inhibition of granulocyte apoptosis by this proinflammatory bacterial product. As shown in Fig. 7A, 15dPGJ2 completely prevented LPS-induced delay of apoptosis, indicating that the cyclopentenone PGs may be inhibiting NF-κB activation. We therefore examined this possibility directly by Western blot analysis of IkB degradation. As shown in Fig. 7B, 15dPGJ2 and Δ12PGJ2 did not cause IkBα degradation, but both metabolites inhibited the proteolytic breakdown of IkBα in response to LPS stimulation. Further studies determined that neither PGD2 nor PGJ2, which do not induce apoptosis in neutrophils, could prevent IkBα degradation in these cells (data not shown). It is therefore apparent that the proapoptotic effect of Δ12PGJ2 and 15dPGJ2 may be mediated by inhibition of NF-κB activation. This is further supported by the demonstration that the inhibition of LPS by Δ12PGJ2 in neutrophils (Fig. 8A) and TNF-α-mediated IkBα breakdown by 15dPGJ2 in eosinophils (Fig. 8B) is concentration dependent.

**FIGURE 5.** Effect of PGD2, PGJ2, Δ12PGJ2, and 15dPGJ2 on eosinophil apoptosis. Eosinophils (2 × 10⁶/ml) were incubated in IMDM supplemented with serum alone (control) or with PGD2, PGJ2, Δ12PGJ2 and 15dPGJ2 (all at 10 μM) for 20 h and incubated with FITC-labeled recombinant human annexin V (annV) to determine phosphatidylserine expression and propidium iodide (pi) to determine loss of cell membrane integrity. Fluorescence was assessed using a EPICS XL2 flow cytometer. Data from a minimum of 5000 cells were analyzed for each condition and the proportion of cells in each quadrant is indicated.
Although these metabolites induce apoptosis in neutrophils, they do not exhibit a Ca\(^{2+}\) mediated eosinophil proapoptotic effect because: 1) fura 2-loaded H\(_9251\) GW9662 on 15dPGJ\(_2\) H\(_11006\) Data represent the mean \(-300\) H\(_9262\) alone (control) with WY-14643 (10\(\mu\)M) were incubated in PBS with calcium and magnesium alone (control) or with 15dPGJ\(_2\) (10\(\mu\)M) \pm 100\(\mu\)M). Cells were harvested at 3 h, and apoptosis was assessed morphologically. Data represent the mean \pm SEM of three separate experiments. All experiments were performed in triplicate. A, Effect of the irreversible PPAR-\(\gamma\) antagonist GW9662 on 15dPGJ\(_2\)-induced neutrophil apoptosis. Neutrophils (5 \times 10\(^7\)/ml) were incubated in PBS with calcium and magnesium alone (control) or with 15dPGJ\(_2\) (10\(\mu\)M) \pm 100\(\mu\)M, ciglitazone, and BRL49653 (0.001–100\(\mu\)M), and harvested at 20 h. Apoptosis was assessed morphologically. Data represent the mean \pm SEM of three separate experiments. B, The effect of cycloheximide (CHX) and z-VAD-fmk on 15dPGJ\(_2\)-induced neutrophil apoptosis. Neutrophils (5 \times 10\(^7\)/ml) were incubated in IMDM supplemented with serum alone (control) with WY-14643 (1–300 \(\mu\)M), ciglitazone, and BRL49653 (0.001–100 \(\mu\)M), and harvested at 20 h. Apoptosis was assessed morphologically. Data represent the mean \pm SEM of three separate experiments. C, All experiments were performed in triplicate.

Discussion

This study demonstrates for the first time that PGD\(_2\) is a powerful selective inducer of eosinophil apoptosis while exerting no effect on survival in neutrophils, Jurkat T lymphocytes, monocytes, or macrophages. PGD\(_2\) has been shown to influence granulocyte responsiveness by binding to specific 7-transmembrane G protein-coupled DP receptors (42, 43, 50). Despite convincing evidence that ligand binding increased cAMP production in COS-M6 and HEK 293 cells transfected with the cloned human DP receptor (51), it is highly unlikely that PGD\(_2\) induces cell death in eosinophils via elevation of cAMP because this would delay granulocyte apoptosis (see Fig. 1, B and C) rather than induce apoptosis. PGD\(_2\) causes a rapid transient increase in [Ca\(^{2+}\)] in human eosinophils (Ref. 23; see Fig. 3) and primes eosinophils for enhanced release of LTC\(_4\) in response to the calcium ionophore A23187 (23). Although fura 2-loaded eosinophils exposed to PGD\(_2\) and its metabolites exhibit a rapid transient elevation of [Ca\(^{2+}\)] (Fig. 3), we believe that an increase in [Ca\(^{2+}\)] is not responsible for the PGD\(_2\)-mediated eosinophil proapoptotic effect because: 1) fura 2-loaded neutrophils do not exhibit a Ca\(^{2+}\) transient when exposed to the PGD\(_2\) metabolites although these metabolites induce apoptosis in neutrophils; 2) elevation of eosinophil [Ca\(^{2+}\)], by pharmacological agents such as A23187 and thapsigargin causes degranulation and necrosis at time points when PGD\(_2\) induces apoptosis; 3) receptor-directed stimuli such as IL-5 that increase eosinophil [Ca\(^{2+}\)] inhibit rather than induce apoptosis (52, 53); 4) PGD\(_2\) and PGJ\(_2\) exhibit similar Ca\(^{2+}\) responses whereas PGD\(_2\) is a more powerful inducer of eosinophil apoptosis; and 5) chemotactic agents such as eotaxin that cause an increase in [Ca\(^{2+}\)] (54) do not induce eosinophil apoptosis (C. Ward and A. G. Rossi, unpublished observations). A newly described receptor CTRH2/DP\(_2\) has been found to mediate [Ca\(^{2+}\)] flux and responsiveness to PGD\(_2\) (55, 56) and indeed Ca\(^{2+}\) mobilization in response to PGD\(_2\) metabolites (43) in eosinophils; however, it is unlikely that [Ca\(^{2+}\)] flux, and hence this receptor, are involved in the proapoptotic effect of the PGD\(_2\) metabolites. Furthermore, this receptor binds PGE\(_2\) and PGF\(_{2\alpha}\) with similar affinities to 15dPGJ\(_2\), and as illustrated in Table I both these PGs inhibit rather than induce eosinophil apoptosis. This receptor is not present on neutrophils; therefore, it cannot be responsible for the proapoptotic effects of \(\Delta^{12}\)PGJ\(_2\) and 15dPGJ\(_2\) observed in these cells.

PGD\(_2\) shows a promiscuous PG receptor binding profile (57, 58); and in the absence of direct evidence for the expression of IP, FP, or TP receptors on granulocytes, it is possible that other
receptors (e.g., EP) are mediating the proapoptotic activities of PGD2. However, the results obtained using a wide variety of PGs or PG analogs demonstrate that the proapoptotic effect of PGD2 could not be reproduced through triggering of any of the classical cell surface PG receptors for which it has a known affinity (Table I; Figs. 1 and 2). It is unlikely that the DP receptor is involved in mediating the proapoptotic effect in eosinophils because the stable PGD2 mimetic ZK118.182 induced minimal increases in eosinophil apoptosis and inhibited this process in the neutrophil. A plausible explanation for the observed differential proapoptotic effects of PGD2 could be that eosinophil and neutrophil granulocytes metabolize PGD2 differently, resulting in distinct functional outcomes. It is possible that neutrophils fail to metabolize PGJ2 to the Δ12-PGJ2 and 15dPGJ2 sequential metabolites and are thus protected from the proapoptotic effects of these products. It is also possible that eosinophils readily metabolize PGD2 into the active PGD2 proapoptotic metabolites or alternatively that eosinophil uptake of PGD2 and PGJ2 may differ from the process in neutrophils. These intriguing possibilities await further investigation. Interestingly, a very recent study has specifically and conclusively shown, using a newly described mAb raised against 15dPGJ2, that this metabolite is present in the cytoplasm of macrophages in human atherosclerotic plaques (59). Thus, these authors have set a precedent for the detection of PGD2 metabolites in vivo and specifically demonstrated that this metabolite can be generated during an inflammatory response in an important human disease. Although we and many others have used low micromolar concentrations of PGD2 and its metabolites, we believe that such levels could be achieved in vivo at their site of action. There is convincing evidence demonstrating that certain PGs can reach concentrations in the micromolar range at sites of acute inflammation (60) and in certain biological fluids, e.g., seminal fluid (61). Actual concentrations of PGD2 metabolites at relevant sites in vivo await confirmation. Because the metabolites can be produced intracellularly and extracellularly it would be difficult to estimate or assess actual concentrations at their target sites.

The mechanisms by which Δ12-PGJ2 and 15dPGJ2 induce granulocyte apoptosis involve caspase activation because death was inhibited by z-VAD-fmk. Although Δ12-PGJ2 and 15dPGJ2 may have their primary targets in the nucleus, where they regulate the expression of specific genes, e.g., via binding to PPAR-γ, we found that other synthetic agonists to PPAR-γ and PPAR-α could not mimic the proapoptotic effects of Δ12-PGJ2 and 15dPGJ2. Although the compound WY-14643 is an effective activator of PPAR-α, PPAR-γ is also activated by this agent at concentrations of 100 μM (32). Despite the use of WY-14643 concentrations up to 300 μM (Fig. 3C), apoptosis was not affected in either cell type. In addition, the thiazolinediones BRL49653 and ciglitazone, potent activators of PPAR-γ, did not induce apoptosis in either neutrophils or eosinophils despite the use of concentrations as high as 100 μM. Moreover, when PPAR-γ was blocked using the irreversible antagonist, GW9662 (32), the induction of apoptosis mediated by either of the PPAR-γ ligands was unaffected despite the use of concentrations in excess of those known to block PPAR-γ activation. Taken together, these data strongly indicate that the

![FIGURE 7](http://www.jimmunol.org/)

**FIGURE 7.** A. Effect of 15dPGJ2 on LPS-induced inhibition of neutrophil apoptosis. Neutrophils (5 × 10⁶/ml) were preincubated in PBS with calcium and magnesium alone (control) or with 15dPGJ2 (10 μM) for 1 h before the addition of LPS (1 μg/ml) and 10% autologous serum. Cells were harvested at 20 h. Apoptosis was assessed morphologically. Data represent the mean ± SD of a representative experiment (of six) performed in triplicate. All experiments were performed in triplicate. *, p < 0.05 compared with control values. B, Western blot analysis of 15dPGJ2 and Δ12-PGJ2 inhibition of LPS-induced IkBα degradation. Neutrophils (5 × 10⁶/ml) were preincubated in PBS with calcium and magnesium alone (control) or with 15dPGJ2 (10 μM) for 1 h before the addition of LPS (1 μg/ml) and 10% autologous serum in a shaking water bath at 37°C. After 15 min, cells were lysed using procedures detailed in Materials and Methods, and the resulting lysates were run on a 9% acrylamide gel. Data are representative of one experiment of at least five.

![FIGURE 8](http://www.jimmunol.org/)

**FIGURE 8.** A. Western blot analysis of the concentration dependency of Δ12-PGJ2-mediated inhibition of LPS-induced IkBα degradation in human neutrophils. Neutrophils (5 × 10⁶/ml) were preincubated in PBS with calcium and magnesium alone (control) or with Δ12-PGJ2 (3–30 μM) for 1 h before the addition of LPS (1 μg/ml) and 10% autologous serum in a shaking water bath at 37°C. After 15 min, cells were lysed using procedures detailed in Materials and Methods, and the resulting lysates were run on a 9% acrylamide gel. Data are representative of at least three. B, Western blot analysis of the concentration dependency of 15dPGJ2-mediated inhibition of TNF-α-induced IkBα degradation in human eosinophils. Eosinophils (5 × 10⁶/ml) were preincubated in PBS with calcium and magnesium alone (control) or with 15dPGJ2 (10 μM) for 1.5 h before the addition of TNF-α (10 ng/ml) and 10% autologous serum in a shaking water bath at 37°C. After 15 min, cells were lysed using procedures detailed in Materials and Methods, and the resulting lysates were run on a 9% acrylamide gel. These are results of a representative experiment of at least three.
proapoptotic effects of Δ12PGJ2 and 15dPGJ2 on granulocytes signal independently of PPAR-γ (or PPAR-α) and point to the existence of another mechanism. Evidence supporting this possibility has been reported by Thieringer et al. (62), who showed that 15dPGJ2, but not other PPAR-γ agonists, could inhibit cytokine production in primary human monocyte-derived macrophages and RAW 264.7 cells. However, the signaling mechanisms of cyclopentenone PGs appear to be cell type specific (49). For example, in activated macrophages, 15dPGJ2, as well as BRL49653 antagonize AP-1, STAT, and NF-κB in a PPAR-γ-dependent manner (63), whereas in human monocytes there is no inhibitory effect of PPAR-γ agonists on the induced expression of TNF-α and IL-6, products that are controlled by these transcription factors (64). In human monocyte-derived macrophages, both BRL49653 and 15dPGJ2 induced apoptosis (PPAR-γ-dependent) (65); and in endothelial cells, both 15dPGJ2 and ciglitazone caused cell death via a caspase and PPAR-γ-dependent mechanism (66). These putative PPAR-γ ligands can therefore act in some cells in a PPAR-γ-independent manner. Studies in human neutrophils have shown that 15dPGJ2 inhibits the β2 integrin-dependent respiratory burst via a PPAR-γ-independent pathway and also suggest the presence of an as yet unidentified receptor (67). Data obtained suggested that such a receptor may act via increases in cytosolic cAMP. Our data, however, indicate that such a mechanism could not be responsible for the proapoptotic effect because an elevation of cAMP strongly inhibits granulocyte apoptosis (Fig. 1, E and F and Refs. 68 and 69).

Because we have recently shown that activation of an inducible form of NF-κB is crucial to granulocyte survival (8), the reported inhibition of this transcription factor by these metabolites (49, 63, 70) made an attractive hypothesis to explain the increases in cell death observed in our studies. Thus, we examined the effects of Δ12PGJ2 and 15dPGJ2 on the activation of NF-κB in granulocytes and found that both metabolites could inhibit LPS-induced degradation of IkBα in neutrophils with concentrations reported in other studies (49, 63, 70). Interestingly, we show for the first time that TNF-α-mediated loss of cytoplasmic IkBα in eosinophils is also inhibited, in a concentration-dependent manner, by 15dPGJ2. Thus, we have shown in granulocytes that this inhibition was concentration dependent and correlated well with the proapoptotic effects observed. Recently, it has been demonstrated that the cyclopentenone PG, PGA1, inhibits IkB kinase (IKK) activity in Jurkat, HeLa, and COS cells transfected with IKKα. Moreover, in HeLa cells, which do not express PPAR-γ, 15dPGJ2 inhibits IKK and NF-κB activation by TNF-α (70). This group shows that A- and J-type PGs inhibited IKK activity and thus that a reactive αβ-unsaturated carbonyl group in the cyclopentane ring was critical for IKK inhibition (70). However, as shown in Table I, neither PGA1 nor PGA2, both of which contain this reactive carbonyl group (48, 70), induced apoptosis in neutrophils; indeed by 20 h, PGA1 had produced a small but significant decrease in neutrophil programmed cell death. Because we have previously shown that NF-κB activation is crucial for survival in these cells (8), these data also suggest a degree of specificity in the interaction of these cells with these PGs and that the effects of PGs on NF-κB may be dependent on cell type.

Knowledge of the mechanisms by which these metabolites exert their proapoptotic effects may be central to understanding why such products influence inflammation. For example, 15dPGJ2 suppresses adjuvant-induced arthritis in the rat (71), whereas in a rat model of pleurisy, increased levels of 15dPGJ2 and PGD2 have been shown to correlate with the resolution of inflammation (72). PPAR-γ ligands also affect the acquired immune response; e.g., ciglitazone and 15dPGJ2 inhibit helper T cell responses by inhibiting IL-2 secretion (73). However, whether this phenomenon is concurrent with apoptosis and clearance of inflammatory cells from the respiratory system, is currently unknown. Our results depicting PGD2, as a powerful selective inducer of eosinophil apoptosis may have implications where induction of apoptosis of a specific inflammatory cell type may be of importance in the control of eosinophilic type inflammation. The mechanism involved is likely to depend on the metabolism of this parent prostanoid to products such as PGJ2 and the PPAR-γ ligands Δ12PGJ2 and 15dPGJ2, which also induce eosinophil and neutrophil apoptosis. However, this proapoptotic effect is not mediated by activation of PPAR-γ or PPAR-α. Interestingly, cyclopentenone PGs have been shown to bind irreversibly to nascent proteins in the endoplasmic reticulum (74). In granulocytes, this mechanism could interfere with the function of survival proteins needed to prevent activation of the apoptotic pathway, a possibility that is currently under investigation.

In conclusion, we have shown for the first time that PGD2 is a powerful, selective accelerator of eosinophil apoptosis, the effects being more significant that those described previously for corticosteroids (50). A preliminary investigation of the underlying mechanisms has shown that its sequential metabolite PGJ2 also induces eosinophil cell death but that the sequential natural metabolites Δ12PGJ2 and 15dPGJ2, which inhibit the degradation of 1xβα in granulocytes, also accelerate apoptosis in both neutrophils and eosinophils. The proapoptotic effects of these cyclopentenone PGs are caspase dependent but do not involve ligation of PPAR-γ or PPAR-α receptors. Our experiments suggest that the mechanism involves inhibition of NF-κB activation, a central event in the control of granulocyte apoptosis (8), and thus these metabolites may fundamentally influence the resolution phase of inflammation.

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