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# The biphasic effects of cyclopentenone prostaglandins, prostaglandin $J_2$ and 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ on proliferation and apoptosis in rat basophilic leukemia (RBL-2H3) cells

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**Abstract**

Mast cells produce chemical mediators, including histamine and arachidonate metabolites such as prostaglandin $D_2$ ($PGD_2$) after antigen stimulation. Cyclopentenone prostaglandins of the J series, prostaglandin $J_2$ ($PGJ_2$) and 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$), are thought to be derivatives of $PGD_2$. In this study, the biphasic effects of the $PGJ_2$ and 15d-$PGJ_2$ on proliferation and apoptosis in rat basophilic leukemia cells (RBL-2H3), a tumor analog of mast cells, were examined. At low concentrations, 1 or 3 μM $PGJ_2$ and 15d-$PGJ_2$ induced cell proliferation, respectively. At high concentrations (10–30 μM) both the inhibition of viability and decrease in histamine content in RBL-2H3 cells were dose dependent. These effects were independent of the nuclear hormone receptor, peroxisome proliferator-activated receptor γ (PPARγ), since troglitazone, an agonist of PPARγ did not cause any effects in RBL-2H3 cells. Cell death induced by $PGJ_2$ and 15d-$PGJ_2$ was the result of apoptotic processes, since RBL-2H3 cells treated with 30 μM of the prostaglandins had condensed nuclei, DNA fragmentation and increase in activities of caspase-3 and -9. Moreover, $PGJ_2$ or 15d-$PGJ_2$-induced apoptotic effects were prevented by the caspase inhibitor, z-VAD-fmk. In conclusion, the $PGJ_2$ or 15d-$PGJ_2$-induced apoptosis in RBL-2H3 cells occurs mainly via mitochondrial pathways instead of by PPARγ-dependent mechanisms.


*Keywords:* Mast cells; Prostaglandin $J_2$; Proliferation; Apoptosis; PPAR; Histamine

## 1. Introduction

Mast cells are regarded as critical effector cells in the inflammatory reaction that underlies immediate hypersensitivity-mediated clinical conditions such as bronchial asthma, pollinosis and atopic dermatitis. After activation by cross-linking of FcεRI IgE receptors, mast cells initiate the production and secretion of chemical mediators, such as histamine, lipid-derived PGs and leukotrienes, as well as several types of cytokines. The soluble mediators and cytokines recruit cells to the site of inflammation, resulting in an inflammatory response [1].

$PGD_2$ is a major cyclooxygenase product in various kinds of cells, including mast cells [2,3]. $PGD_2$ has potent pharmacological actions such as bronchoconstriction, peripheral vasodilation, inhibition of platelet aggregation and neuromodulation, so on [4,5]. Two different types of $PGD_2$ synthesizing enzymes, PGD synthetase (PGDS) have been characterized, purified and cloned by Urade *et al*. [6]. One type of PGDS is a glutathione requiring enzyme, hematopoietic PGDS that is present in mast cells. $PGD_2$ is readily dehydrated to the cyclopentenone prostaglandins of the J series ($PGJ_2$ series), such as $PGJ_2$ and 15d-$PGJ_2$, via an albumin-independent mechanism [7,8]. The $PGJ_2$ series display several unique characteristics associated with cell proliferation and apoptosis. Specifically, a cytotoxic effect of $\Delta^{12}$-$PGJ_2$ is readily induced after translocation, accumulation in nuclei, and binding to nuclear proteins [9]. In addition the electrophilic carbons in cyclopentenone rings of the 15d-$PGJ_2$ can directly conjugate with a thiol residue and to modify the function of proteins [10]. Furthermore, 15d-$PGJ_2$ is a natural ligand of the nuclear receptor, PPARγ.

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*Abbreviations:* PG, prostaglandin; RBL, rat basophilic leukemia; PCR, polymerase chain reaction; 15d-$PGJ_2$, 15-deoxy-$\Delta^{12,14}$-$PGJ_2$; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; DP receptor, $PGD_2$ receptor; CRTH-2, chemoattractant receptor homologous molecule expressed on Th2 cells.

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The mechanisms involved in PPARγ-dependent [11,12] and PPARγ-independent cytotoxic effects [13] are unknown.

Because so little is known about the biological significance of the $PGJ_2$ series in mast cells [14–16], we have studied the cytotoxic effects of the $PGJ_2$ and 15d-$PGJ_2$ in mast cells.

Apoptosis is the physiological process of cell death that occurs in all multicellular organisms. In mast cells, apoptosis is also a key process in the regulation of inflammation and proliferation. It is also responsible for maintaining a constant cell number in tissues under normal conditions. Mast cell hyperplasia occurs in several clinical conditions; for example, host responses to parasites and the chronic inflammatory conditions that are present during either Crohn's disease or rheumatoid arthritis [17]. In contrast, mast cells also express Fas antigen and undergo apoptosis by direct activation of Fas antigen [18]. In many cases, apoptotic pathways are mediated via the p53 tumor suppressor protein [19]. The involvement of p53 in initiating apoptosis results in the up-regulation of proapoptotic members of the bcl-family, such as bax, in mitochondria [20]. The role of survival/apoptosis-regulated genes in mast cells activation is now being investigated [17].

In this study, the exogenous $PGJ_2$ and 15d-$PGJ_2$ both induced bifunctional effects on RBL-2H3 cells. Specifically, proliferation occurred at the lower concentrations and apoptosis was observed at the higher concentrations. These concentration-dependent bifunctional effects were independent of PPARγ and DP-receptor. And the apoptotic effects were mainly mediated by mitochondrial pathways, not involved in p53 accumulation.

## 2. Materials and methods

### 2.1. Assay of cell proliferation and viability

The rat mast cell line RBL-2H3 was kindly supplied from Dr. Michael A. Beaven (National Institutes of Health). The incorporation of 5-bromo-2′-deoxyuridine (BrdU) in place of thymidine is carried out to monitor cell proliferation. Briefly, RBL-2H3 cells ($2 \times 10^4$ cells/200 μL/well) were cultured with BrdU in 96-well culture plate for 24 hr at 37°, and BrdU assay was carried out according to manufacture's protocol. The optical density was then measured on a microplate reader with a wavelength of 450 nm.

Cell viability was assayed using the cell proliferation reagent WST-1. In contrast with MTT, different tetrazolium reagent which has been used for the measurement of cell proliferation and viability, WST-1 is more stable. The viable cells cleave the tetrazolium salts to formazan by cellular enzyme, mitochondrial dehydrogenases [22]. The augmentation in enzyme activity leads to an increase in the amount of formazan dye formed, which directly correlates to the number of metabolically active cells in the sample. RBL-2H3 cells were cultured in 96 well plates at a density of $1.25 \times 10^5$ cells/0.2 mL/well, then the WST dye solution was added directly to the 96-well plate. After 2 hr incubation at 37°, the formazon was quantified by absorption values detected at 450 nm by a microplate autoreader.

### 2.2. Histamine measurement

RBL-2H3 cells were cultured in 96-well plates ($1.25 \times 10^5$ cells/0.2 mL/well) in growth media in the presence of PGs or drugs, and incubated in a humidified atmosphere of 5% of $CO_2$ at 37° for 24 hr. After sonication 100 μL of the cell medium was collected, centrifuged at 1000 *g* for 5 min, and 50 μL of the supernatants was collected for testing. The histamine contents were determined by an HPLC-fluorometry technique described by Yamatodani *et al.* [21]. Samples were mixed with 250 μL of 3% perchloric acid in 5 mM $Na_2EDTA$, 30 μL of 2 M KOH/1 M $KH_2PO_4$ was added, then mixtures were centrifuged at 10,000 *g* for 15 min at 4°. Fifty μL of supernatant was injected directly into a column packed with cation exchanger, TSK gel SP-2SW (150 × 6 mm i.d.). The histamine was eluted with 0.25 M potassium phosphate at a flow rate of 0.6 mL/min, post-labeled with *o*-phtalaldehyde under alkaline conditions and detected fluorometrically in an F1080 fluorometer (Hitachi) using wavelength of 360 nm for excitation and 450 nm for emission.

### 2.3. Detection of cytotoxicity by staining with Hoechst 33258

For Hoechst staining, RBL-2H3 cells ($5 \times 10^5$ cells/0.4 mL/well) were seeded on a cover glass (14 mm in diameter) in a 4-well culture plate and treated with $PGJ_2$ and 15d-$PGJ_2$ for 8 hr. Cells were fixed in 4% paraformaldehyde and stained with 50 μM Hoechst 33258 for 30 min. The morphological changes in chromatin were analyzed with a phase contrast and fluorescent light microscope (Olympus) connected to a computer via a CCD camera.

### 2.4. Caspase activity assay

After treatment of $0.5 \times 10^6$ cells with the indicated concentrations of $PGJ_2$ and 15d-$PGJ_2$, cells were added with 50 μL of lysis buffer containing 50 mM HEPES pH 7.4, 0.1% CHAPS, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 10% glycerol on ice for 10 min. The lysates from both adherent and floating cells were pooled and centrifuged, and the supernatant was stored at −80°. Caspase-9 activity was measured fluorometically using 200 μM Ac-LEHD-MCA as a substrate. After 10 min incubation, 100 μL of lysate and a substrate were incubated for 1 hr at 37°. The fluorescence was measured in a spectrofluorometer (F2000, Hitachi) with wavelengths of 380 nm for excitation and 460 nm for emission. After 3 hr incubation with caspase-1, -3 and -8 substrates, enzyme activities were measured colorimetrically at 405 nm in a microplate

autoreader, using 200 μM of Ac-YVAD-pNa, Ac-DEVD-pNa and Ac-IETD-pNa as substrates, respectively.

### 2.5. Inhibition of caspase activity

To confirm which types of caspases are active, 20 μM of Ac-YVAD-CHO, Ac-DEVD-CHO, Ac-IETD-CHO and Ac-LEHD-CHO were used as inhibitors for caspase-1, -3, -8 and -9, respectively. Furthermore, to investigate whether a caspase inhibitor blocks the apoptotic process *in vivo*, RBL-2H3 cells were seeded in 96-well plate ($2.5 \times 10^5$ cells/0.2 mL/well) and preincubated for 1 hr with 50 μM of Ac-DEVD-CHO, Ac-LEHD-CHO and z-VAD-fmk, a irreversible caspase inhibitor, then incubated with various concentrations of $PGJ_2$ and 15d-$PGJ_2$ for 24 hr. The cell viability was determined by the WST-1 assay and the apoptotic changes were determined by TUNEL assay.

### 2.6. Analysis of the mRNA expression by RT-PCR

The total RNAs from RBL-2H3 cells were prepared according to the methods described previously [23] and used for RT-PCR analysis. cDNA was synthesized from 5 μg of total RNA with a random primer and reverse transcriptase (superscript II, GIBCO,). cDNA was amplified with the following specific primers; rat β-actin, ATG-GAT-GAC-GAT-ATC-GCT-GCG (upstream) and TCA-GCT-GTG-GTG-GTG-AAG-CTG-T (downstream); rat p53, ACG-TAC-TCA-ATT-TCC-CTC-AA (upstream) and TCC-AAG-GCC-TCA-TTC-AGC-TC (downstream) [24]. Taq polymerase was purchased from Takara Shuzo Co. Each PCR cycle was composed of denaturation at 94° for 30 s, annealing at 55° for 30 s, and extension at 72° for 2 min. The PCR products were visualized by agarose gel electrophoresis followed by ethidium bromide staining.

### 2.7. TUNEL assay

For *in situ* apoptotic cell detection, TUNEL assays were used. Nuclear DNA fragmentation, an important biochemical indicator of apoptosis in many cell types, were measured by labeling free 3′-OH DNA ends with the enzyme TdT using the Dead End™ Colorimetric Apoptosis Detection System (Promega) according to the manufacture's protocol. RBL-2H3 cells at a concentration of $5 \times 10^5$ cells/0.4 mL/well were seeded in individual wells of 24-well plates, preincubated for 1 hr with 50 μM z-VAD-fmk or vehicle, and finally incubated with 30 μM $PGJ_2$, 15d-$PGJ_2$ or vehicle for 24 hr. Subsequently, cells were fixed in 4% paraformaldehyde at room temperature for 15 min, and then cell membranes were permeabilized with 0.2% triton X-100 for 5 min at room temperature. DNA was labeled with biotinylated nucleotide in the presence of TdT for 1 hr at 37°. Cells were washed in PBS and incubated with 0.3% hydrogen peroxide for 5 min at room temperature to block the endogenous peroxidase. Cells are then washed and the horseradish-peroxidase-labeled-streptavidin that is bound to the biotinylated nucleotides, is detected colorimetrically using the hydrogen peroxide and the stable chromogen, diaminobenzidine as substrates for peroxidase. Apoptotic nuclei are stained dark brown against the background of cells that are counterstained with methyl green.

### 2.8. Drug used

PGs and arachidonic acid were purchased from Cayman Chemical Co except for $PGD_2$ and BW245C, which were generous gifts from Ono Pharmaceutical Co. These were stored in 20 mmol/L aliquots in ethanol at −80°, and diluted immediately before use. Troglitazone and ciglitazone were from Bio Mol Research laboratories, Inc. Drugs were dissolved in a final concentration of 0.5% ethanol, which had no effect on the experiments. The cell proliferation reagent WST-1 and cell proliferation ELISA, BrdU were from Roche. Caspase Colorimetric Substrate/Inhibitor Quanti PaK™ was from Bio Mol Research. Ac-LEHD-MCA, Ac-LEHD-CHO, as a substrate and inhibitor of caspase-9 and z-VAD-fmk were from PEPTIDE Institute. The bisbenzimide H 33258 fluorochrome, 3 HCl, was from Calbiochem, DeadEnd colorimetric Apoptosis Detection System was from Promega. All other chemicals were highest grade of available.

### 2.9. Statistical analysis

Results are expressed as means ± SEM of N experiments. Results were analyzed by repeated measures analysis of two-tailed Student's *t*-test.

## 3. Results

### 3.1. Biphasic effects of $PGJ_2$ and 15d-$PGJ_2$ on proliferation of RBL-2H3 cells

The incorporation of BrdU in place of thymidine is measured to monitor cell proliferation. Cell proliferation was enhanced by $PGJ_2$ at 1 μM (1.23-fold compared with the control) and by 15d-$PGJ_2$ at 3 μM (1.27-fold), showing that the potency of cell proliferative effect induced by 15d-$PGJ_2$ was slightly less than that of $PGJ_2$ (Fig. 1). Incubation of RBL-2H3 cells with high concentrations of either $PGJ_2$ or 15d-$PGJ_2$ caused significant cytotoxic effects. Cell proliferation was suppressed by 48.4 ± 9.6%, 72.1 ± 6.6% and 80.3 ± 8.4% with 3, 10 and 30 μM $PGJ_2$, respectively, and by 47.3 ± 10.1% and 79.9 ± 8.3% with 10 and 30 μM 15d-$PGJ_2$, respectively after treatment for 24 hr. WST assay showed almost same biphasic pattern as shown in BrdU incorporation after treatment with $PGJ_2$ and 15d-$PGJ_2$. No significant effects on cell viability were detected after treatment with arachidonic acid, $PGD_2$, $PGE_2$, BW245C for DP receptor agonist, and indomethacin for CRTH-2

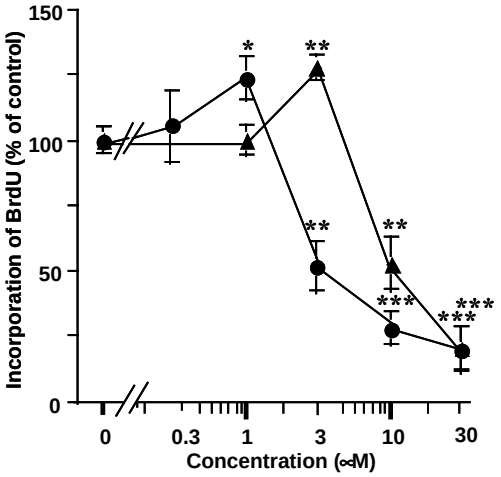


Fig. 1. Biphasic effects of $PGJ_2$ and 15d-$PGJ_2$ on RBL-2H3 cells. Cells were incubated for 24 hr with various concentrations of $PGJ_2$ (●), 15d-$PGJ_2$ (▲). Cell proliferations were examined by BrdU assay. Results are the mean± SEM of three experiments $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; significantly different from control group. The *x*-axis is logarithmic scale.

agonist, monitored by WST assay. The effect on cell viability and cytotoxicity of the PPARγ agonist, troglitazone and ciglitazone were also examined because 15d-$PGJ_2$ is a specific activator of the isoform of PPARs, PPARγ. Although 30 μM of troglitazone decreased cell viability by 30.5 ± 2.0%, ciglitazone induced no effect on cell viability (data not shown).

### 3.2. Effect of $PGJ_2$ and 15d-$PGJ_2$ on histamine contents in RBL-2H3 cells

Histamine contents were decreased at 24 hr by 91.1 ± 1.0% and 96.9 ± 0.3% with 10 and 30 μM $PGJ_2$, respectively, and 98.7 ± 0.3% with 30 μM 15d-$PGJ_2$ (Fig. 2). The decrease in histamine contents is paralleled to the decrease in viable cell number detected by trypan blue staining, 13.6 ± 1.5% and 3.6 ± 2.0% of the control after treatment with 30 μM of $PGJ_2$ or 15d-$PGJ_2$, respectively (data not shown). In this data, no increase in histamine contents was observed at the low concentration. Treatment with $PGD_2$, $PGE_2$, arachidonic acid, BW245C, troglitazone, ciglitazone and indomethacin had no detectable effect on histamine levels (data not shown).

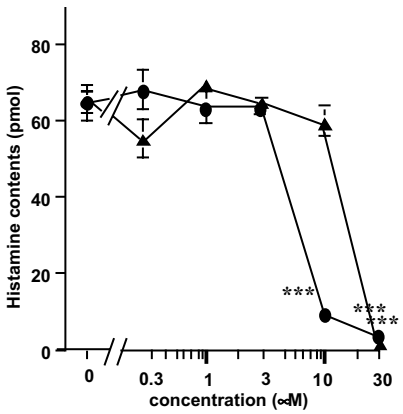


Fig. 2. Histamine contents after treatment with $PGJ_2$ and 15d-$PGJ_2$ on RBL-2H3 cells. Cells were incubated for 24 hr with various concentrations of $PGJ_2$ (●), 15d-$PGJ_2$ (▲). Results are the mean ± SEM of three experiments. $^{***}P < 0.001$; significantly different from control group. The *x*-axis is logarithmic scale.

### 3.3. Cellular and nuclear morphological changes

RBL-2H3 cells that were cultured for 8 hr in the presence of 30 μM of either $PGJ_2$ or 15d-$PGJ_2$ showed

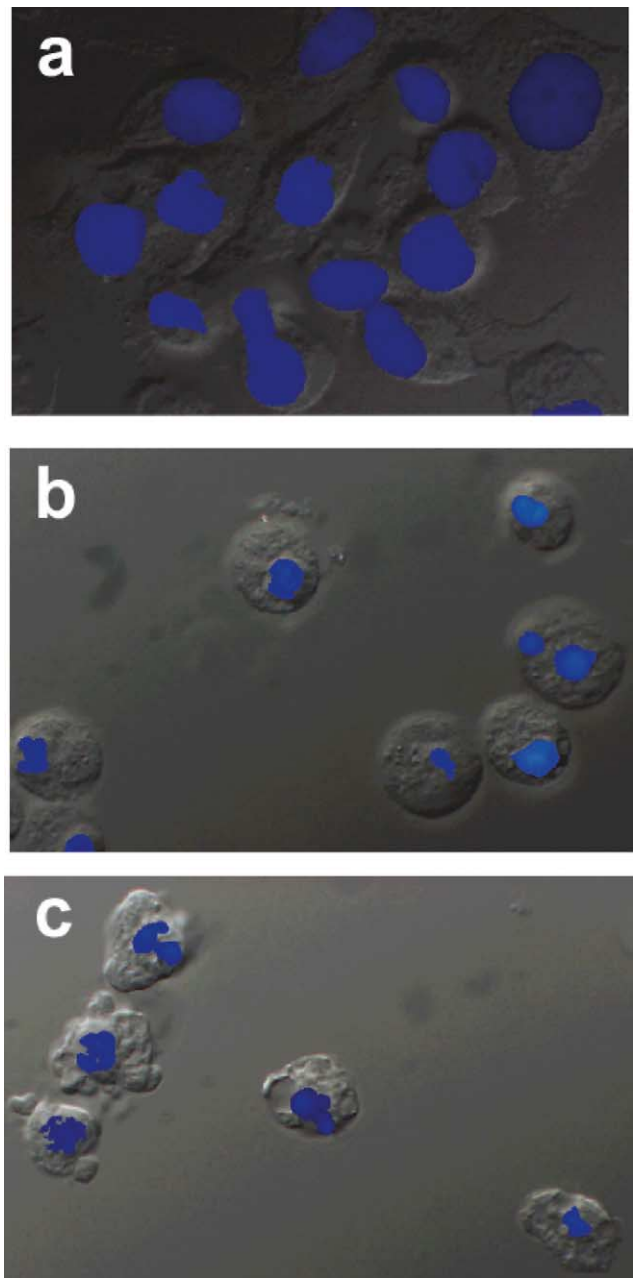


Fig. 3. Morphological changes in nucleus. Hoechst 33258 staining of the nuclei (800×). Cells were incubated for 8 hr in the presence of 30 μM $PGJ_2$ (a), 15d-$PGJ_2$ (b) or vehicle (c), fixed, then stained with Hoechst 33258. Phase contrast analysis (800×) combined images with nuclear fluorescence staining.

apoptotic changes. Hoechst 33258 fluorescent staining and phase contrast analysis revealed nuclear condensation and blebbing, cell shrinkage and rounding of RBL-2H3 cells (Fig. 3).

### 3.4. $PGJ_2$ and 15d-$PGJ_2$ induce apoptotic cell death in RBL-2H3 cells by activation of caspase-3 and -9

To further characterize the apoptotic effect of $PGJ_2$ and 15d-$PGJ_2$ on RBL-2H3 cells, caspase activities were monitored. Both $PGJ_2$ and 15d-$PGJ_2$ caused potent activation of caspase-3-like activity and caspase-9-like activity as assessed by DEVDase and LEHDase activity (Fig. 4A). At concentrations of 30 μM $PGJ_2$ and 15d-$PGJ_2$, the caspase-3-like enzyme activities were $687.1 \pm 93.4$ pmol/min/mg protein for $PGJ_2$ and $582.6 \pm 79.0$ pmol/min/mg for 15d-$PGJ_2$. The values were higher by 7.3-fold for $PGJ_2$ and 6.2-fold for 15d-$PGJ_2$ when compared with the control value of $93.6 \pm 18.3$ pmol/min/mg protein. $PGJ_2$ induced caspase-9-like activity to levels of $671.5 \pm 34.1$ pmol/min/mg protein and 15d-$PGJ_2$ induced activation to $513.1 \pm 73.2$ pmol/min/mg protein. These values were higher by 9.3-fold for $PGJ_2$ and 7.1-fold for 15d-$PGJ_2$ when compared with the control value of $71.9 \pm 37.0$ pmol/min/mg protein. The activation of both enzymes was inhibited by a caspase-3 inhibitor (Ac-DEVD-CHO) or a caspase-9 inhibitor (Ac-LEHD-CHO), respectively. However, both $PGJ_2$ and 15d-$PGJ_2$ failed to activate either caspase-1- or caspase-8-like activity (data not shown). The relationship between p53 expression and the apoptotic response following $PGJ_2$ or 15d-$PGJ_2$ treatments was further investigated (Fig. 4B). The decreases in expression of p53 mRNA were time-dependent following treatment of cells with $PGJ_2$ or 15d-$PGJ_2$. $PGJ_2$ treatment was followed by a decrease of p53 mRNA expression to 22.2% of control values at 4 hr and 15.3% after 8 hr. A decrease to 9.3% was detected after treatment for 8 hr with 15d-$PGJ_2$. These data indicated that in RBL-2H3 cells apoptosis that is induced by $PGJ_2$ and

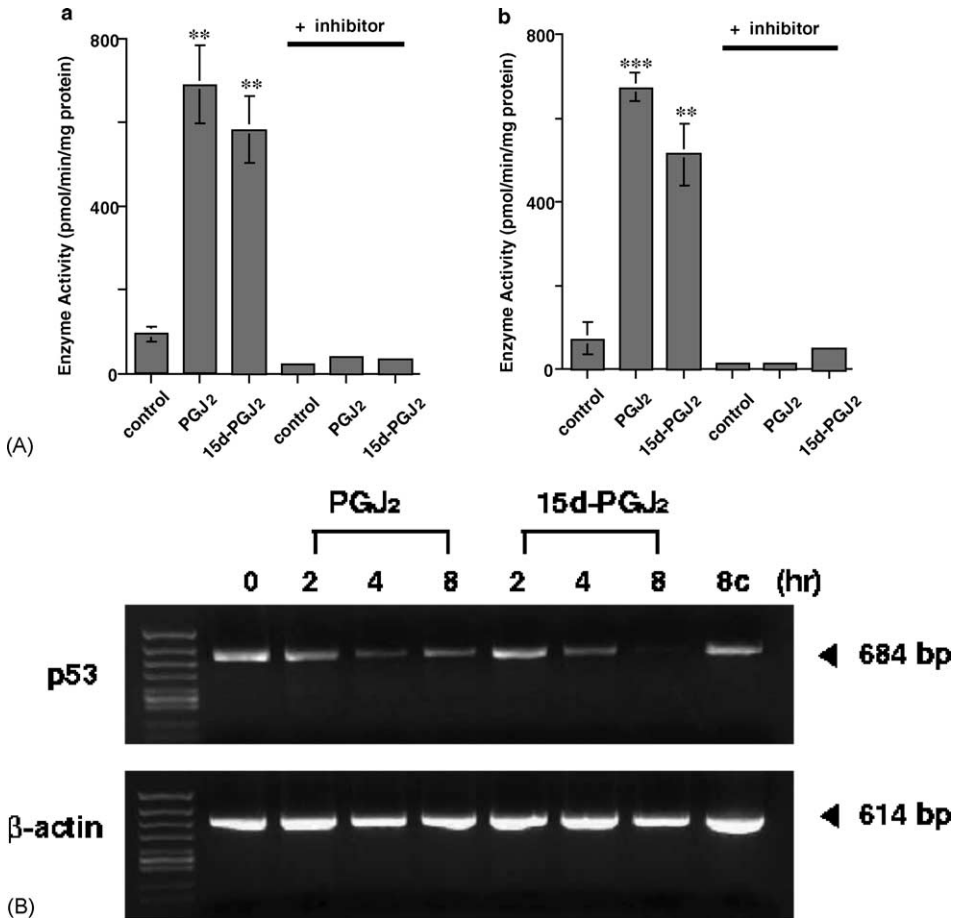


Fig. 4. Apoptotic pathway induced by $PGJ_2$ and 15d-$PGJ_2$. (A) Activation of caspase-3 and caspase-9 during apoptosis in RBL-2H3 cells after exposure to $PGJ_2$ and 15d-$PGJ_2$. (a) Caspase-3-like enzyme activity. (b) Caspase-9-like enzyme activity. Cells were treated with 30 μM of $PGJ_2$ and 15d-$PGJ_2$. Lysed cells were preincubated for 10 min at 37°, then incubated for an additional 30 min at 37° with Ac-DEVD-pNa for caspase-3-like activity, Ac-LEHD-MCA for caspase-9-like activity and Ac-LEHD-CHO as a caspase-9 inhibitor and Ac-DEVD-CHO as a caspase-3 inhibitor. Each caspase-like activity was measured as described in Section 2. Results are the mean ± SEM of four experiments. ($^{**}P < 0.01$ and $^{***}P < 0.001$; significantly different from control group). (B) Expression of p53 mRNA in RBL-2H3 cells after $PGJ_2$ or 15d-$PGJ_2$ treatment. Cells were treated with 30 μM of PGs for the incubation times indicated here. (8C; the control group in which cells were incubated with vehicle for 8 hr), and p53 mRNA expression was examined by RT-PCR analysis as described in Section 2.

15d-$PGJ_2$ follows the caspase-9-dependent, mitochondrial pathway rather than the pathway dependent upon p53.

### 3.5. A caspase inhibitor blocks apoptosis in RBL-2H3 cells induced with $PGJ_2$ and 15d-$PGJ_2$

The decrease in cell viability, as well as the fragmentations of DNA induced with $PGJ_2$ and 15d-$PGJ_2$, was prevented using a caspase inhibitor z-VAD-fmk (Fig. 5A). Without caspase inhibitors, treatment with 20 and 30 μM of $PGJ_2$ decreased cell viability by $84.4 \pm 4.0\%$ and $100 \pm 4.2\%$, respectively and 20 and 30 μM of 15d-$PGJ_2$ gave decreases of $67.2 \pm 4.0\%$ and $98.9 \pm 4.1\%$. With 50 μM Ac-DEVD-CHO as a caspase-3 inhibitor and Ac-LEHD-CHO as a caspase-9 inhibitor, cell viabilities were decreased by $29.9 \pm 6.9\%$ and $9.4 \pm 2.6\%$ after treatment with 20 μM of $PGJ_2$ and by $0.4 \pm 19.8\%$ and $26.0 \pm 3.3\%$ after treatment with 20 μM of 15d-$PGJ_2$. However, these inhibitions were reversible and the inhibition of cell viability was induced with 30 μM of $PGJ_2$ and 15d-$PGJ_2$. In the presence of 50 μM z-VAD-fmk which has a irreversible property for inhibition, cell viability was decreased by $17.6 \pm 18.6\%$ after treatment with 30 μM $PGJ_2$ and $21.2 \pm 7.1\%$ after treatment with 15d-$PGJ_2$.

Histamine contents were recovered after treatment with z-VAD-fmk. The histamine contents per well in the presence of a caspase inhibitor ($17.6 \pm 0.9$ pmol and $21.2 \pm 0.1$ pmol after treatment with $PGJ_2$ and 15d-$PGJ_2$, respectively) were significantly higher than those obtained without an inhibitor ($3.1 \pm 0.1$ pmol and $11.1 \pm 0.4$ pmol after treatment with $PGJ_2$ and 15d-$PGJ_2$, respectively). The percent of the attached TUNEL positive cells after treatment was $91.9 \pm 2.9\%$ for $PGJ_2$ and $91.4 \pm 2.7\%$ for 15d-$PGJ_2$ (Fig. 5B). However, z-VAD-fmk inhibited the

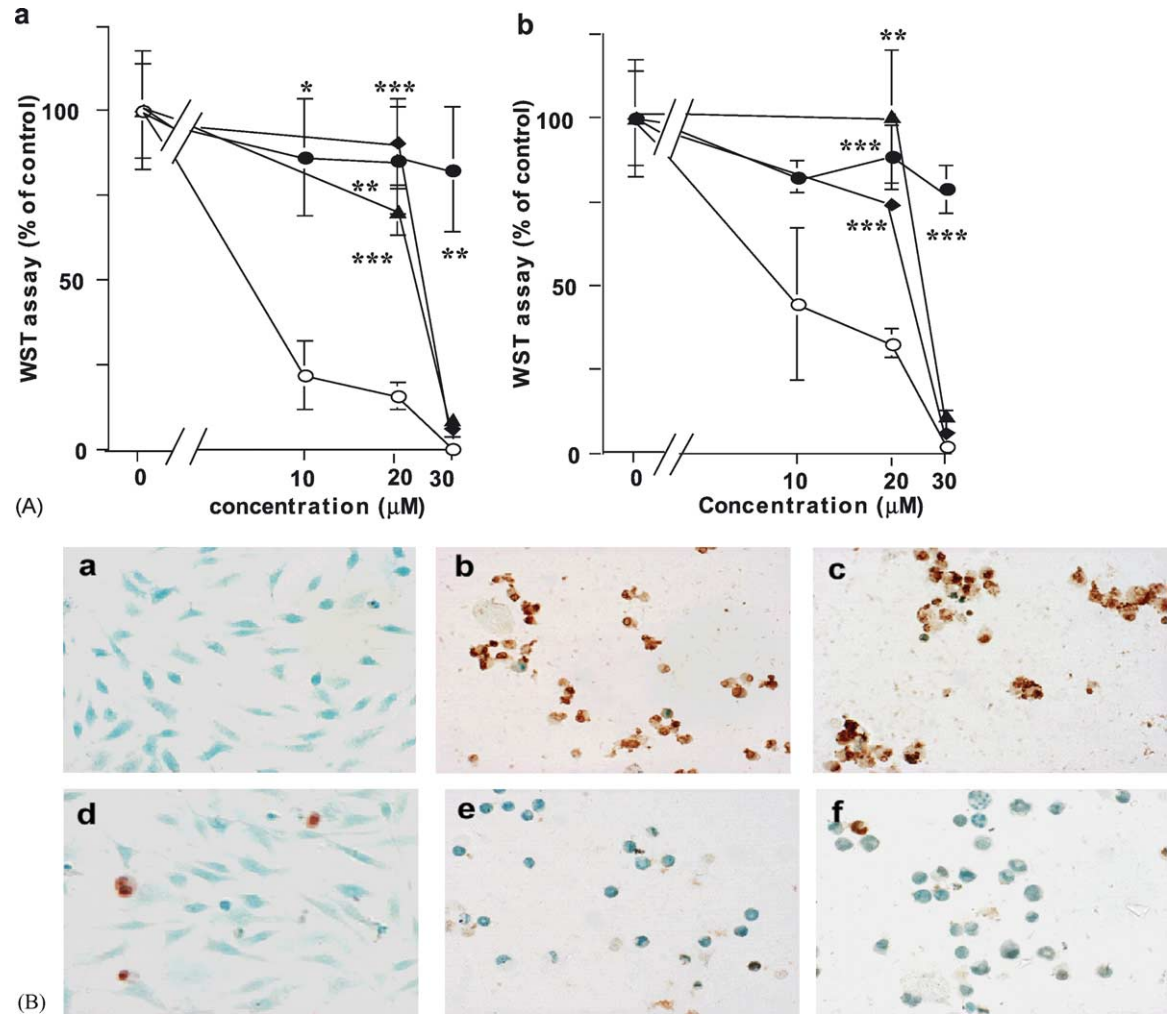


Fig. 5. $PGJ_2$ or 15d-$PGJ_2$-induced apoptosis is prevented by caspase inhibitor. (A) WST assay. RBL-2H3 cells were preincubated for 1 hr with 50 μM caspase inhibitor z-VAD-fmk, Ac-DEVD-CHO and Ac-LEHD-CHO or vehicle, and then incubated for an additional 24 hr with various concentrations of $PGJ_2$ (a) and 15d-$PG_2$ (b). The symbols show $PGJ_2$ or 15d-$PGJ_2$ alone (○), $PGJ_2$ or 15d-$PGJ_2$ with z-VAD-fmk treatment (●), $PGJ_2$ or 15d-$PGJ_2$ with Ac-LEHD-CHO treatment (▲) and $PGJ_2$ or 15d-$PGJ_2$ with Ac-DEVD-CHO treatment (◆). Each assay was performed as described in Section 3. Results are the mean ± SEM of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$; significantly different from the groups without inhibitors. The *x*-axis is logarithmic scale. (B) TUNEL staining (800×). (a) and (d) Control, (b) and (e) $PGJ_2$, (c) and (f) 15d-$PGJ_2$, (a)–(c) Without z-VAD-fmk treatment, (d)–(f) with z-VAD-fmk treatment.

TUNEL staining and only 8.1 ± 2.9% and 8.6 ± 2.7% of the attached cells were positive after treatment with $PGJ_2$ and 15d-$PGJ_2$, respectively. Thus, it appears that $PGJ_2$ and 15d-$PGJ_2$-induced apoptosis are likely to be mediated by the caspase pathway. In conclusion, considering the results of caspase-9 activation, the apoptotic effects of $PGJ_2$ or 15d-$PGJ_2$ were mediated via mitochondrial pathway.

## 4. Discussion

Mast cells play key roles in allergy and inflammatory responses via synthesis and release of inflammatory mediators. Some of the mediators are preformed and stored in secretory granules. Others such as the PGs, which are arachidonic acid metabolites, are generated *de novo*. $PGJ_2$ and 15d-$PGJ_2$, dehydration of $PGD_2$, are known as cytotoxic inducer [7]. They also have been identified as ligands for PPARγ and regulate adipocyte differentiation and inflammatory response in macrophages [12].

In this study, we show that biphasic effects of $PGJ_2$ and 15d-$PGJ_2$ on proliferation and apoptosis. In RBL-2H3 cells, 1 μM of $PGJ_2$ or 3 μM of 15d-$PGJ_2$ induced enhancement of cell proliferation for 24 hr (Fig. 1). Despite the acceleration of cell proliferation, elevation of histamine levels could not be detected in RBL-2H3 cells at the same concentrations of $PGJ_2$ and 15d-$PGJ_2$ (Fig. 2). The mechanisms used by the low concentrations of $PGJ_2$ or 15d-$PGJ_2$ to induce cell proliferation is still unknown. However, 15d-$PGJ_2$ can readily form thiol conjugates with glutathione and cysteine by means of the electrophilic carbons in their cyclopentenone rings [25]. Conjugation with thiol residues of proteins, especially the active site of enzymes, may lead to the modification of protein function. In fact, 15d-$PGJ_2$ binds to and activates H-Ras, through the formation of covalent adduct of 15d-$PGJ_2$ with Cys-184 of H-Ras, but not with N-Ras or K-Ras [10]. 15d-$PGJ_2$ induced mitogen-activated protein kinase (MAPK) activation, regulated Ark-dependent proliferation in human mesangial cells [26] and induced IL-8 induction through the MAPK and NF-κB pathways in activated human T cells [27]. All of these reports point to the mediation of the $PGJ_2$ or 15d-$PGJ_2$-induced proliferation in RBL-2H3 cells by phosphorylation cascades.

Many of the key apoptotic proteins have been identified and the mechanisms of apoptosis are now well understood. The role of apoptosis in the cytotoxic effects caused by the $PGJ_2$ or 15d-$PGJ_2$ in a number of cell types, including endothelial cells, blood cells such as macrophages and lymphocytes and many tumor cells, has been reevaluated. However, the apoptotic signaling pathways that are related to the $PGJ_2$ or 15d-$PGJ_2$ is still poorly understood. 15d-$PGJ_2$ is a potent inducer of intracellular oxidative stress, such as reactive oxygen species (ROS) production, lipid peroxidation and redox alteration [28]. In human hepatic myofibroblasts, the apoptotic effects of 15d-$PGJ_2$ were generated by ROS production and blocked by the antioxidants *N*-acetylcysteine, *N*-(2-mercapto-propionyl)-glycine and pyrrolidine dithiocarbamate [13]. In RBL-2H3 cells, $H_2O_2$ also induced cytotoxicity that was blunted by catalase. However, it is known that the effective concentrations for cytotoxicity of $H_2O_2$ are higher than those necessary for cytotoxicity of $PGJ_2$ or 15d-$PGJ_2$ that were used in this study [29].

$\Delta^{12}$-$PGJ_2$ is transported in the cytoplasma, accumulates in nuclei in a temperature-dependent manner and binds to some nuclear components, leading to growth inhibition [9,30]. Moreover, 15d-$PGJ_2$-induced apoptosis occurs in association with direct modulation of nuclear transcription factors that include IκB kinase (IKK) [28] and nuclear factor-κB (NF-κB) [31], resulting in inhibition of the inflammatory response. The type of apoptotic pathways induced with 15d-$PGJ_2$ depends upon the cell types. $PGD_2$ and 15d-$PGJ_2$ induce caspase-dependent apoptosis via inhibition of IκBα degradation in eosinophils [32]. In RBL-2H3 cells, $PGJ_2$-induced apoptosis was primarily by caspase activation because of the enhancement of caspase-3 and -9 like enzymes activities. Further evidence supporting the role of caspase activation in apoptosis was the complete inhibition of DNA fragmentation by z-VAD-fmk treatment. Caspase activation has also been reported in SH-SY5Y neuroblastoma cells and human hepatic myofibroblast cells [13]. In SH-SY5Y neuroblastoma cells, 15d-$PGJ_2$ caused the accumulation of p53, resulting in the caspase cascade that is mediated by Fas and Fas ligand [33]. However, the apoptotic pathway in RBL-2H3 cells is independent of p53, Fas and Fas ligand. In IL-3-dependent mouse bone marrow-derived mast cells, p53 is not required for either regulation of apoptosis or protection from radiation after removal of IL-3 [34]. Therefore, the activation of p53 tumor suppressor gene induced either growth arrest (in $G_1$ and $G_2$) or apoptosis, depending on the cell types and the surrounding environment [19,35].

The $PGJ_2$ series are attracting increasing attention because 15d-$PGJ_2$ is a potent intrinsic nuclear receptor for PPARγ. PPARγ is expressed on a variety of cell types, including adipocytes, macrophages, T cells, B cells, endothelial cells, vascular smooth muscle cells and colonic tumor cells. It also regulates diverse cell functions, such as survival and death by modulation of cytokine production [36]. In the PPARγ-dependent mechanism of cytotoxicity, 15d-$PGJ_2$ inhibited both the TNF-α production in monocytes [11] and the iNOS expression in macrophages by inhibiting expression of the genes for transcription factors AP-1, STAT and NF-κB [12].

The involvement of PPARγ in biological functions of proliferative and apoptotic process in mast cells is controversial. PPARγ is also expressed in cultures of murine bone marrow-derived mast cells after stimulation with either antigen or A23187 [15]. In human cultured mast cells, the expression of PPARγ2 increased after IgE receptor aggregation and the PPAR agonists including 15d-$PGJ_2$

and troglitazone attenuated the production of granulocyte-macrophage colony-stimulating factor [14]. On the other hand, Diaz *et al*. reported that cytokine-dependent PGHS-2 expression and IL-6 generation in mouse bone marrow-derived mast cells induced by 15d-$PGJ_2$ is independent of PPARγ [16]. Although the mRNA of PPARγ is expressed in RBL-2H3 cells (data not shown), we could not find any effects of thiazolidinediones, troglitazone and ciglitazone on viability and apoptosis in RBL-2H3 cells.

Therefore, we conclude that exogenous 15d-$PGJ_2$ does not play a role in apoptosis and proliferation as a functional PPARγ ligand in RBL-2H3 cells. Furthermore, the DP receptor and CRTH-2 receptor, which have high affinity for $PGD_2$ and its metabolite, is not likely to be involved in $PGJ_2$ or 15d-$PGJ_2$-induced apoptosis because the DP receptor agonist, BW245C and CRTH-2 agonist, indomethcin had no effect on cell viability. It indicated that the enhancements of both cell viability and apoptosis, induced with the $PGJ_2$ and 15d-$PGJ_2$, were mediated by mechanisms that were independent of those involved in the DP and CRTH-2 receptor pathways.

Under normal condition, the number of mast cells is controlled by the balance between the proliferation and apoptosis. The increase in numbers of mast cells occurs in certain forms of systemic mast cell disorders [1]. Stem cell factor, a kit ligand is important for mast cell proliferation and suppression of apoptosis. The mechanism of proliferation promoted by stem cell factor is mediated by PI-3 kinase and Src kinase signaling pathway [37], while apoptosis is mediated by bcl-2 expression [38]. bax, a pro-apoptotic relative that dimerizes with bcl-2, also plays a role in regulating mast cell apoptosis [39].

In the present study we demonstrated that $PGJ_2$ and 15d-$PGJ_2$ induced apoptosis in RBL-2H3 cells at the higher concentration of 3 and 10 μM, respectively. Mast cells are major sources of the $PGJ_2$ series, which are produced after antigen stimulation. The concentration of $PGD_2$ is estimated to be about 15 μM in mouse bone marrow-derived mast cells if all produced $PGD_2$ (40 ng/$10^6$ cells) is distributed in a whole cell volume and the radius of cell is 10 μm [40]. Kondo *et al*. demonstrated the quarter amount of $PGD_2$ is converted to 15d-$PGJ_2$ in 24 hr incubation [41]. The question remains of how mast cells protect themselves from the cytotoxic effects of the $PGJ_2$ series that they are producing. One mechanism of protecting cells from apoptosis is thought to be by the regulation of bcl-2 family proteins. After aggregation of IgE receptors, mast cells express the prosurvival gene A1, one of the bcl-2 homologues resulting in cell survival [42]. Recent work also indicates that monomeric, uncrosslinked IgE could lead to cytokine production and cell survival by suppression of the apoptosis that is induced by growth factor deprivation [43].

In conclusion, in response to exogenous $PGJ_2$ and 15d-$PGJ_2$, RBL-2H3 cells show biphasic responses. Cell proliferation occurs at the lower concentration and apoptosis at the higher concentration of $PGJ_2$ or 15d-$PGJ_2$. Levonen *et al*. showed biphasic effect of 15d-$PGJ_2$ in endothelial cells, glutathione-dependent cytoprotection through the upregulation of glutamate-cystein ligase at the lower concentration and apoptosis at the higher concentrations [44]. Bureau *et al*. suggested the proinflammatory role of 15d-$PGJ_2$ at micromolar concentrations lower than required for NF-κB inhibition to cause attenuation of inflammatory response [45].

Finally, we propose that $PGJ_2$ or 15d-$PGJ_2$ produced from mast cells might function as autocrines for regulaing self-proliferation and self-apoptosis, or as paracrines for other cells, such as endothelial cells and afferent neurons that neighbor mast cells, although the concentrations of $PGJ_2$ or 15d-$PGJ_2$ at the local sites of inflammation in tissue are still under debate. The biphasic effects may provide a basis for a better understanding of cellular modification process by $PGJ_2$ or 15d-$PGJ_2$ during inflammation.

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