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Prostaglandin D_2 and J_2 induce apoptosis in human leukemia cells via activation of the caspase 3 cascade and production of reactive oxygen species

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Abstract

The presence of prostaglandins (PGs) has been demonstrated in the processes of carcinogenesis and inflammation. In the present study, we found that 12-*o*-tetradecanoylphorbol 13-acetate (TPA) induced cyclooxygenase 2 (COX-2), but not COX-1, protein expression in HL-60 cells, and the addition of arachidonic acid (AA) in the presence or absence of TPA significantly reduced the viability of HL-60 cells, an effect that was blocked by adding the COX inhibitors, NS398 and aspirin. The AA metabolites, PGD_2 and PGJ_2, but not PGE_2 or $PGF_{2\alpha}$, reduced the viability of the human HL60 and Jurkat leukemia cells according to the MTT assay and LDH release assay. Apoptotic characteristics including DNA fragmentation, apoptotic bodies, and hypodiploid cells were observed in PGD_2- and PGJ_2-treated leukemia cells. A dose- and time-dependent induction of caspase 3 protein procession, and PARP and D4-GDI protein cleavage with activation of caspase 3, but not caspase 1, enzyme activity was detected in HL-60 cells treated with PGD_2 or PGJ_2. Additionally, DNA ladders induced by PGD_2 and PGJ_2 were significantly inhibited by the caspase 3 peptidyl inhibitor, Ac-DEVD-FMK, but not by the caspase 1 peptidyl inhibitor, Ac-YVAD-FMK, in accordance with the blocking of caspase 3, PARP, and D4-GDI protein procession. An increase in intracellular peroxide levels by PGD_2 and PGJ_2 was identified by the DCHF-DA assay, and anti-oxidant *N*-acetyl cysteine (NAC), mannitol (MAN), and tiron significantly inhibited cell death induced by PGD_2 and PGJ_2 by reducing reactive oxygen species (ROS) production. The PGJ_2 metabolites, 15-deoxy-$\Delta^{12,14}$-PGJ_2 and Δ^{12}-PGJ_2, exhibited effective apoptosis-inducing activity in HL-60 cells through ROS production via activation of the caspase 3 cascade. The proliferator-activated receptor-γ (PPAR-γ) agonists, rosiglitazone (RO), troglitazone (TR), and ciglitazone (CI), induced apoptosis in cells which was blocked by the addition of the PPAR-γ antagonists, GW9662 and BADGE, via blocking of caspase 3 and PARP cleavage. However, neither GW9662 nor BADGE showed any protective effect on PGD_2- and PGJ_2-induced apoptosis. A differential apoptotic effect of PGs through ROS production, followed by activation of the caspase 3 cascade, was demonstrated.

Keywords: Apoptosis; Caspase 3; ROS; Prostaglandin; Cyclooxygenase; PPAR-γ

1. Introduction

Prostaglandins (PGs) are a family of oxygenated metabolites of arachidonic acid (AA), and have a diverse range of actions depending on the PG type and cell target. PGs are divided into two groups, conventional PGs such as PGE_2, $PGF_{2\alpha}$, and PGD_2 and cyclopentenone PGs such as PGJ_2, PGA_1, and PGA_2 [1,2]. AA is the precursor of PGs, and is primarily converted to PGH_2 by

Abbreviations: MTT, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxyMethoxy-phenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; TPA, 12-*o*-tetradecanoylphorbol 13-acetate; Ac-DEVD-FMK, acetyl-Asp-Glu-Val-Asp-fluoromethylketone; Ac-YVAD-FMK, acetyl-Tyr-Val-Ala-Asp-fluoromethylketone; DCHF-DA, dichlorodihydrofluorescein diacetate; PARP, poly (ADP-ribose) polymerase; NAC, *N*-acetyl-cysteine; ALL, allopurinol; DPI, diphenylene iodonium; ROS, reactive oxygen species; PGs, prostaglandins; BADGE, biphenol A diglycidyl ether; GW9662, 2-chloro-5-nitrobenzanilide

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cyclooxygenases followed by conversion of PGH_2 to several related PGs including PGD_2, PGJ_2, $PGF_{2\alpha}$, and PGE_2 by tissue-specific isomerase. Several physiological effects of PGs have been identified. PGE_2 production is increased in colon, gastric, and lung carcinomas with an increase in COX-2 protein levels [3,4]. Our previous data demonstrated that PGE_2 is involved in 12-*o*-tetradecanoylphorbol 13-acetate (TPA)- and epidermoid growth factor (EGF)-induced proliferation [5,6]. PGD_2 is a major product in a variety of tissues or cells, and has significant effects including platelet aggregation and vasorelaxation [7]. In vivo and in vitro studies have shown that PGD_2 readily undergoes dehydration to yield active PGs of the J_2 series including PGJ_2, Δ^{12-14} PGJ_2, and 15-deoxy-Δ^{12-14} PGJ_2 [8,9]. Members of PGJ_2 contain a reactive α,β-unsaturated ketone in the cyclopentenone ring that is important for their biological activities including anti-tumor, anti-inflammation, and antiviral replication effects [10–12].

Many types of mammalian cells undergo apoptosis during normal development or in response to a variety of stimuli, including DNA damage, oxidative stress, growth factor deprivation, and chemical treatment. Apoptosis induced by these agents appears to be regulated by a set of downstream genes such as p53, p21, caspases, and Bcl-2 family genes [13,14]. Human caspase-1 to -10 have been described, and previous studies demonstrated that activation of the caspase cascade is involved in chemical- and agent-induced apoptosis [15,16]. Caspase 3 exists as an inactive pro-caspase 3 in the cytoplasm and is proteolytically converted to active caspase 3 by a single cleavage event in cells undergoing apoptosis. After caspase 3 activation, some specific substrates for caspase 3 such as PARP and D4-GDI proteins are cleaved, and these are important for the occurrence of apoptosis [17,18].

Several previous studies suggested that PGs might reduce cell viability via apoptosis induction, but their mechanisms of action are complex and not well defined. Our previous study demonstrated that apoptosis induced by chemicals was mediated by activation of the caspase 3 cascade through a distinct ROS-dependent or -independent pathway [19,20]. In the present study, we obtained evidence that PGD_2 and PGJ_2, but not PGE_2 or $PGF_{2\alpha}$, exhibited effective apoptosis-inducing activities through ROS production and caspase 3 activation in human leukemia cells. ROS-dependent caspase 3 activation was identified in PGD_2- and PGJ_2-induced apoptosis.

2. Materials and methods

2.1. Cell culture

HL-60 and Jurkat human promyeloleukemic cells were obtained from ATCC (American Type Culture Collection; Rockville, MD). HL-60 and Jurkat cells were grown in RPMI1640 containing 10% heat-inactivated fetal bovine serum (FBS) and maintained at 37 °C in a humidified incubator containing 5% CO_2. Exponentially growing cells were exposed to drugs for the indicated time periods. All culture reagents were purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Chemicals

The colorigenic synthetic peptide substrates, Ac-DEVD-pNA, Ac-YVAD-pNA, Ac-DEVD-FMK, and Ac-YVAD-FMK, were purchased from Calbiochem. Propidium, iodide, PGs, TPA, and AA were obtained from Sigma Chemical (St. Louis, MO). Rosiglitazone (RO), troglitazone (TR), ciglitazone (CI), GW9662, and BADGE were obtained from Cayman Chemical. Antibodies for PARP, caspase 3, and D4-GDI detection in Western blotting were obtained from IMGENEX. Antibodies for detecting Bcl-2 family proteins and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dichlorodihydrofluorescein diacetate (DCHF-DA) was obtained from Molecular Probes.

2.3. Cell viability

Cell viability was assessed by MTT staining as described previously [21]. Briefly, cells were plated at a density of 10^5 cells/well in 24-well plates. After overnight growth, cells were treated under various conditions for 12 h. At the end of treatment, 30 μl of the tetrazolium compound, MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), and 270 μl of fresh RPMI medium were added. The supernatant was removed, and formazan crystals were dissolved in DMSO. After incubation for 4 h at 37 °C, 200 μl of 0.1 N HCl in 2-propanol was placed in each well to dissolve the tetrazolium crystals. At the end, the absorbance at a wavelength of 600 nm was recorded using an ELISA plate reader.

2.4. Determination of ROS production

ROS production was monitored by flow cytometry using DCFH-DA [19]. This dye is a stable compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within cells. Hydrogen peroxide or low-molecular-weight peroxides produced by cells oxidize DCFH to highly fluorescent compound, 2′,7′-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. In the present study, HL-60 cells were treated with each of the indicated compounds for 2 h and washed twice with PBS to remove the extracellular compounds. DCHF-DA (100 μM) was added for an additional hour. Green fluorescence was excited using an argon laser and was detected using a 525-nm band-pass filter by flow cytometric analysis.

2.5. Western blots

Total cellular extracts (30 μg) were prepared and separated on 8% SDS-polyacrylamide mini gels for PARP detection and 12% SDS-polyacrylamide minigels for caspase 3, cleaved D4-GDI, Bcl-2 family, and α-tubulin detection, and then transferred to Immobilon polyvinylidene difluoride membranes (Millipore). The membrane was incubated at 4 °C with 1% bovine serum albumin at room temperature for 1 h and then incubated with the indicated antibodies for a further 3 h at room temperature followed by incubation with alkaline phosphatase-conjugated anti-mouse IgG antibody for 1 h. Protein was visualized by incubating with the colorimetric substrates, nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), as described in our previous paper [20,21].

2.6. DNA gel electrophoresis

Cells (10^6/ml) under different treatments were collected, washed with PBS twice, and then lysed in 100 ml of lysis buffer [50 mM Tris, pH 8.0; 10 mM ethylenediaminetetraacetic acid (EDTA); 0.5% sodium sarkosinate, and 1 mg/ml proteinase K] for 3 h at 56 °C and treated with 0.5 mg/ml RNase A for another hour at 56 °C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) before loading. Samples were mixed with loading buffer [50 mM Tris, 10 mM EDTA, 1% (w/w) low-melting-point agarose, and 0.025% (w/w) bromophenol blue] and loaded onto a presolidified 2% agarose gel containing 0.1 μg/ml ethidium bromide. The agarose gels were run at 50 V for 90 min in TBE buffer, then observed and photographed under UV light [22].

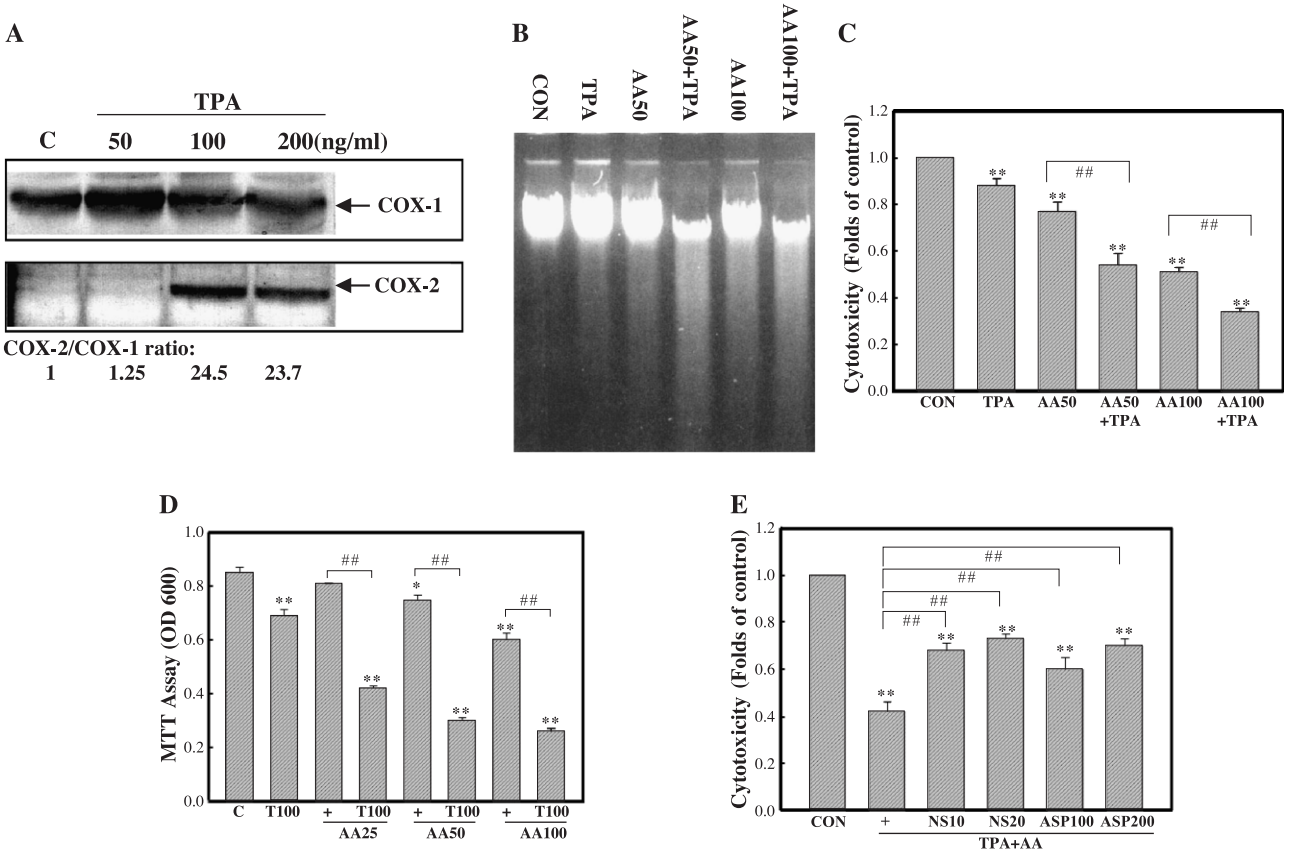

Fig. 1. TPA and arachidonic acid (AA) induce apoptosis with increasing COX-2 protein expression in HL-60 cells. (A) Cells were treated with TPA (50, 100, and 200 ng/ml) for 12 h, and expression of COX-1 and COX-2 protein was detected by Western blotting using specific antibodies. The ratio COX-2/COX-1 was measured by densitometric analysis, and described as folds of control. (B) Effects of TPA (100 ng/ml) and AA (AA50, 50 μM; A100, 100 μM) on DNA fragmentation in HL-60 cells. Cells were treated with different compounds for 12 h, and the integrity of DNA in cells was analyzed by electrophoresis in 1.8% agarose gels. (C) Cells were treated as described in (B), and the cytotoxicity under different treatments was detected by LDH release. (D) As described in (C), HL-60 cells were treated with TPA (100 ng/ml) in the presence or absence of different doses of AA (25, 50, and 100 μM); the viability of cells was examined by the MTT assay. (E) The COX inhibitors, NS398 (NS10, 10 μM; NS20, 20 μM) and aspirin (ASP100, 100 μM; ASP200, 200 μM), inhibited TPA (100 ng/ml) plus AA (50 μM)-induced cytotoxicity in HL-60 cells. HL-60 cells were treated with indicated doses of NS398 or aspirin for 1 h followed by TPA (100 ng/ml) plus AA (50 μM) treatment. The cytotoxic effect of TPA plus AA in the presence or absence of NS398 or aspirin was evaluated by LDH release. Data are expressed as the mean±S.E. of three independent experiments. *$p<0.05$, **$p<0.01$, significantly differs from the control (CON) group, as analyzed by Student's t-test. $^{\#\#}p<0.01$, significantly differs between indicated groups, as analyzed by Student's t-test. Western blotting and DNA ladder analyses were performed at least three times, and the results shown are representative of all of the data.

2.7. Analysis of respective caspase activities

Ac-DEVD-pNA for caspase 3 and Ac-YVAD-pNA for caspase 1 were used as colorimetric protease substrates. After different treatments, cells were collected and washed three times with PBS and resuspended in 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 10 mM ethyleneglycoltetra-acetic acid (EGTA). Cell lysates were clarified by centrifugation at 15,000 rpm for 3 min, and clear lysates containing 50 μg of protein were incubated with 100 μM of the indicated specific colorimetric substrates at 37 °C for 1 h. Activity of caspase 1 and 3 enzymes was described as the cleavage of colorimetric substrate by measuring the absorbance at 405 nm.

2.8. Flow cytometric analysis

Trypsinized cells were washed with ice-cold PBS and fixed in 70% ethanol at −20 °C for at least 1 h. After fixation, cells were washed twice, incubated in 0.5 ml of 0.5 ml of 0.5% Triton X-100/PBS at 37 °C for 30 min with 1 mg/ml of RNase A, and stained with 0.5 ml of 50 mg/ml propidium iodide for 10 min. Fluorescence emitted by the propidium-DNA complex was quantitated after excitation of the fluorescent dye by FACScan flow cytometry (Becton Dickenson, San Jose, CA).

2.9. LDH release assay

Cells were treated under different conditions, and medium was collected for the LDH release assay. The amount of LDH in the medium was measured by the protocol suggested by the manufacturer (Roche Applied Science). The addition of 2% Triton X-100 to the cells was used as a positive control of the total amount of LDH in cells. The resulting cytotoxicity values were calculated by the following equation:

$$\text{Cytotoxicity}\ (\%) = (\text{EXP. value} - \text{CON}/\text{Triton X} - 100\ \text{value} - \text{CON}) \times 100\%.$$

2.10. Statistics

Values are expressed as the mean±S.E. The significance of the difference from the respective controls for each experimental test condition was assayed using Student's *t*-

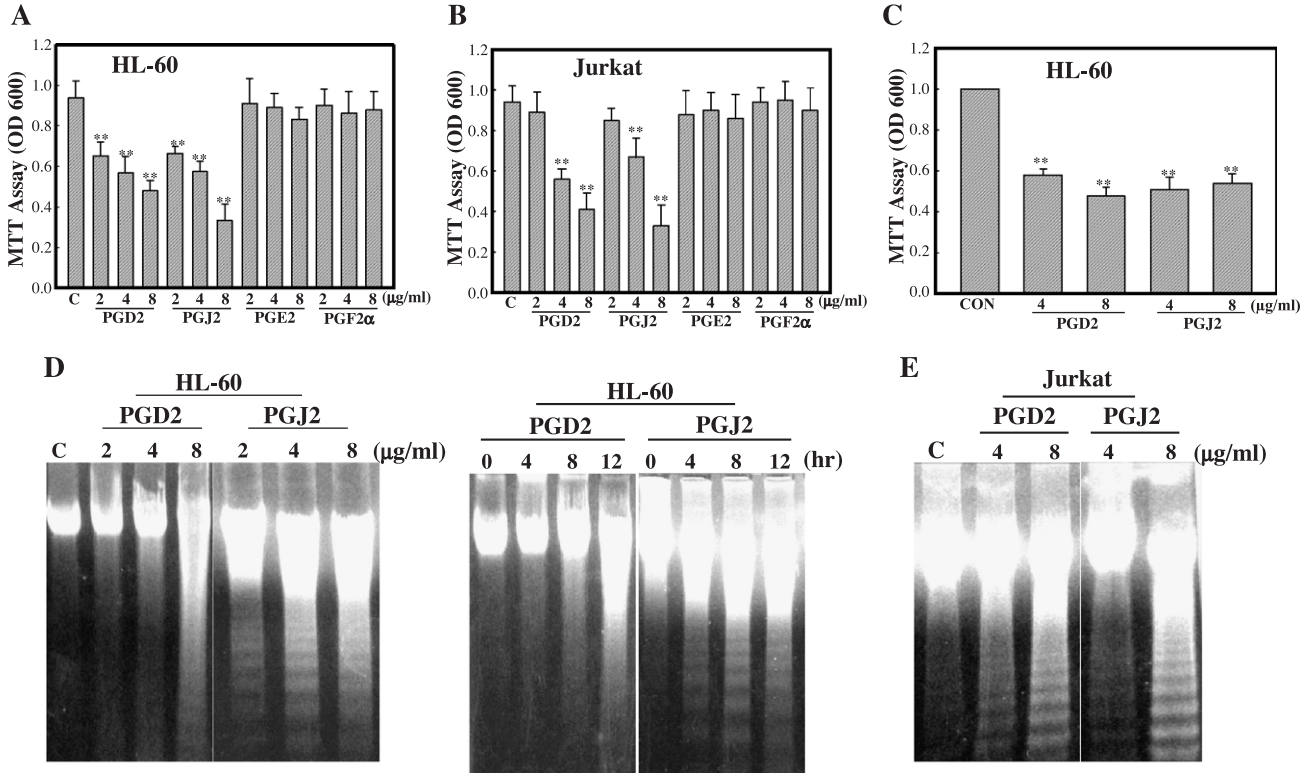

Fig. 2. Cytotoxic effect of PGD_2 and PGJ_2, but not of PGE_2 or $PGF_{2\alpha}$, in human HL-60 and Jurkat leukemia cells. (A, B) Cells were treated with different doses (2, 4, and 8 μg/ml) of PGs for 12 h, and the viability of cells was detected by the MTT assay. (C) HL-60 cells were treated with different doses (4 and 8 μg/ml) of PGD_2 and PGJ_2 for 12 h, and cytotoxicity was evaluated by LDH release. (D) HL-60 cells were treated with different doses (2, 4, and 8 μg/ml) of PGD_2 or PGJ_2 for 12 h (left panel), or PGD_2 or PGJ_2 (8 μg/ml; right panel) for different time points (4, 8, and 12 h). The integrity of DNA was analyzed by electrophoresis in 1.8% agarose gels. (E) Jurkat cells were treated with different doses (4 and 8 μg/ml) of PGD_2 or PGJ_2 for 12 h, and the integrity of DNA was analyzed. $^{**}p<0.01$, significantly differs from the control (CON) group, as analyzed by Student's *t*-test.

test for each paired experiment. A P value of <0.01 or <0.05 was regarded as indicating a significant difference.

3. Results

3.1. TPA induction of COX-2 protein expression stimulates the occurrence of apoptosis induced in human HL-60 leukemia cells

Conversion of arachidonic acid (AA) to PGH_2 is catalyzed by cyclooxygenases, and PGH_2 is isomerized into two groups of PGs including $PGE_2/PGF_{2\alpha}$ and PGD_2/PGJ_2. In the present study, we found that the addition of TPA at the doses of 100 and 200 ng/ml significantly induced COX-2, but not COX-1, protein expression in HL-60 cells by Western blot analysis (Fig. 1A). Addition of AA did not change the COX-2 protein expression induced by TPA (Data not shown). Results of the DNA fragmentation assay showed that TPA (100 ng/ml) exhibited a slight DNA laddering effect, and the addition of AA at the doses of 50 or 100 μM significantly enhanced the occurrence of the intensity of DNA fragmentation in cells (Fig. 1B). We further analyzed the viability of cells under different treatments by MTT and LDH release assays. As elucidated in Fig. 1C and D, cotreatment of HL-60 cells with AA/TPA showed a more significant decrease in the viability of cells, compared with the AA- or TPA-treated groups. Furthermore, the COX enzyme inhibitors, NS398 and aspirin, significantly attenuated the cytotoxic effect of AA/TPA in cells (Fig. 1D). These data suggest that activation of COX-2 enzyme activity might be involved in cell death induced by AA and TPA.

3.2. Differential cytotoxic effect of PGs in the human HL-60 and Jurkat leukemia cells

Previous data indicated that AA potentiated cell death in the presence of TPA in HL-60 cells. Therefore, we investigated the cytotoxic effects of four major AA metabolites

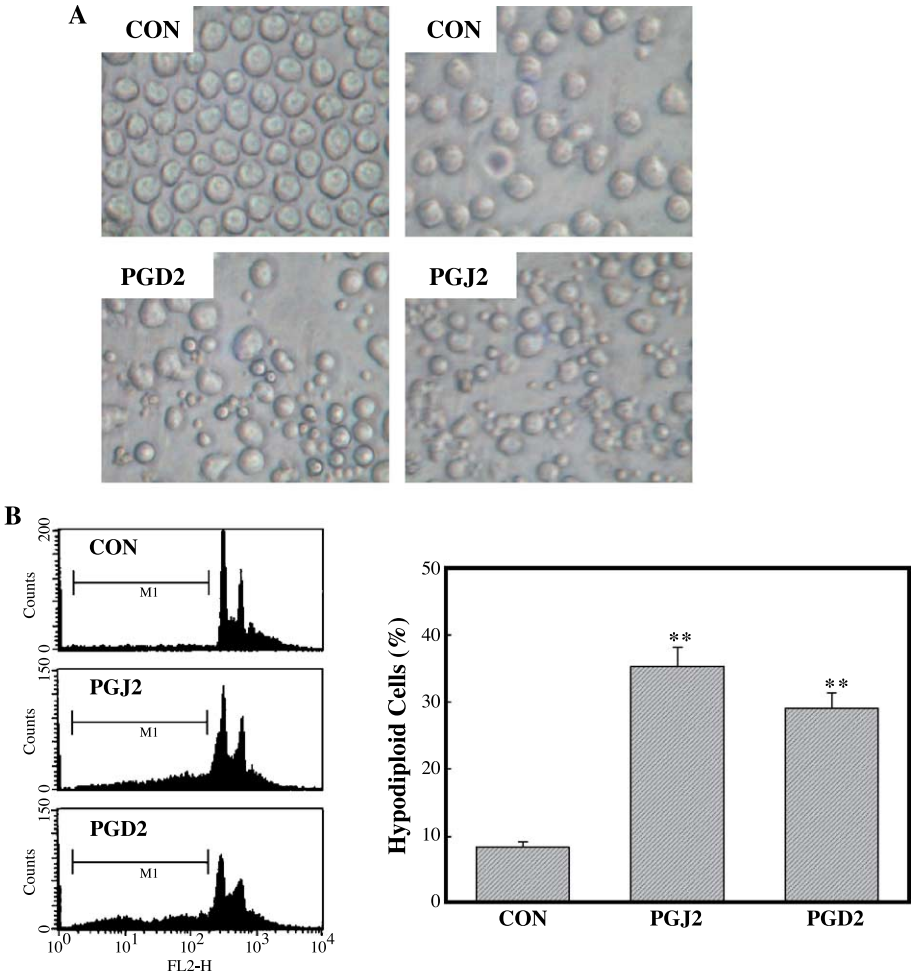

Fig. 3. Increased hypodiploid cells and apoptotic bodies in PGD_2- or PGJ_2-treated HL-60 cells by flow cytometric analysis and microscopic observations. HL-60 cells were treated with PGD_2 or PGJ_2 (8 μg/ml) for 12 h. (A) Apoptotic bodies in PGD_2- and PGJ_2-treated cells were detected by microscopic observations. (B) The sub-G1 peak (hypodiploid cells) was examined by flow cytometric analysis, and the percentage of hypodiploid cells (the ratio of the sub-G1 peak) was quantitated from three independent experiments. **p<0.01, significantly differs from the control (CON) group, as analyzed by Student's t-test.

PGE_2, $F_{2\alpha}$, D_2, and J_2 on the human leukemia cells, HL60 and Jurkat. As shown in Fig. 2, PGD_2 and PGJ_2, but not PGE_2 or $PGF_{2\alpha}$, dose-dependently reduced the viability of HL-60 and Jurkat cells by the MTT assay (Fig. 2A and B). Similarly, the LDH release assay showed that PGD_2 and PGJ_2 increased the release of LDH into the medium in HL-60 and Jurkat cells (Fig. 2C and data not shown). Furthermore, several apoptotic characteristics were investigated in the present study. Both PGD_2 and PGJ_2 induced DNA fragmentation in human HL-60 leukemia cells in a time- and dose-dependent manner (Fig. 2D). Induction of DNA fragmentation was also observed in Jurkat cells (Fig. 2E). Results of microscopic observations showed that apoptotic bodies were detected in HL-60 cells under PGD_2 and PGJ_2 treatment (Fig. 3A). Under flow cytometric analysis, we found an increase in hypodiploid cells (sub-G1 peak) in PGD_2- and PGJ_2-treated HL-60 cells (Fig. 3B). These data indicate that reduction of cellular viability by PGD_2 and PGJ_2 in human leukemia cells occurs via apoptosis.

3.3. Induction of caspase 3 protein procession and PARP and D4-GDI protein cleavage by PGD2 and PGJ2

We investigated if activation of the caspase 3 cascade is involved in apoptosis induced by PGD_2 and PGJ_2. Activation of caspase 3 (CPP32/Yama) causes it to recognize the sequence Asp-Glu-Val-Asp (DEVD) and to cleave a number of proteins, such as PARP and D4-GDI, another hallmark of apoptosis. With exposure to PGD_2 and PGJ_2, degradation of 116-kDa PARP into 85-kDa fragments and production of

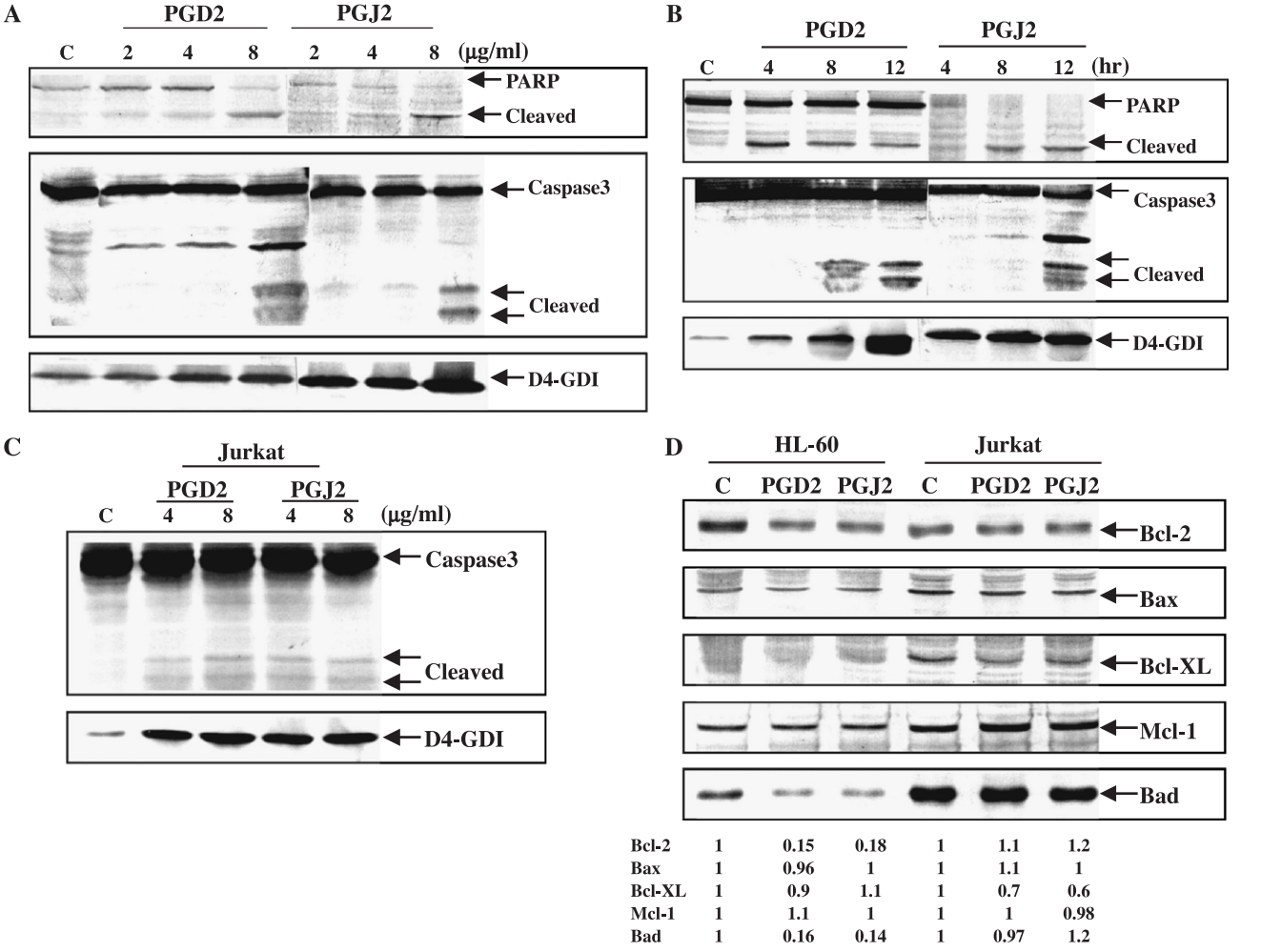

Fig. 4. Induction of the caspase 3 cascade was examined in PGD_2- or PGJ_2-induced apoptosis. (A) HL-60 cells were treated with different doses (2, 4, and 8 μg/ml) of PGD_2 or PGJ_2 for 12 h (left panel), and the expressions of caspase 3, PARP, and D4-GDI protein were detected by Western blotting using specific antibodies. (B) Cells were treated with PGD_2 or PGJ_2 (8 μg/ml; right panel) for different time points (4, 8, and 12 h), and the expressions of caspase 3, PARP, and D4-GDI protein were detected by Western blotting using specific antibodies. (C) Jurkat cells were treated with PGD_2 or PGJ_2 (4 and 8 μg/ml) for 12 h, and expressions of caspase 3 and D4-GDI protein were analyzed. (D) HL-60 and Jurkat cells were treated with PGD_2 and PGJ_2 (8 μg/ml) for 12 h, and the expression of Bcl-2 family proteins was detected by Western blotting using specific antibodies. The intensity of indicated Bcl-2 family proteins was detected by densitometric analysis, and expressed as folds of control. Western blotting was performed at least three times, and the results shown are representative of all of the data.

cleaved (23-kDa) D4-GDI proteins were found to be dose- and time-dependent in HL60 cells, and these were associated with activation of caspase 3 brought about by its cleavage, represented here as the production of cleaved fragments (Fig. 4A and B). Induction of caspase 3 protein procession, as well as PARP and D4-GDI protein cleavage, was also identified in Jurkat cells (Fig. 4C). Bcl-2 family proteins are important apoptotic regulators and are located upstream of caspase activation. In results of Western blotting, decreases in the antiapoptotic Bcl-2 protein level in HL-60 cells and the Bcl-XL protein level in Jurkat cells were detected with PGD_2 and PGJ_2 treatment. A decrease in Bad protein expression was found in HL-60, but not in Jurkat, cells; however, neither PGD_2 nor PGJ_2 induced the phosphorylation of Bad protein in both cells by Western blotting using a specific anti-body for phosphorylated Bad protein (Fig. 4D and data not shown). No significant change in Bax or Mcl-1 protein was observed. Furthermore, two colorimetric substrates, Ac-DEVD-pNA and Ac-YVAD-pNA, were used to detect caspase 1 and caspase 3 enzyme activities in HL-60 cells under PGD_2 and PGJ_2 treatment, respectively. As illustrated in Fig. 5A and B, PGD_2 and PGJ_2 dose-dependently induced DEVD-specific, but not YVAD-specific, caspase activity in HL-60 cells. These data suggest that activation of caspase 3, but not of caspase 1, was exhibited in PGD_2- and PGJ_2-induced apoptosis in human leukemia cells.

3.4. The caspase 3 peptidyl inhibitor, Ac-DEVE-FMK, inhibits apoptosis induced by PGD2 and PGJ2

In order to confirm if activation of caspase 3 is essential for the apoptosis induced by PGD_2 and PGJ_2, peptidyl

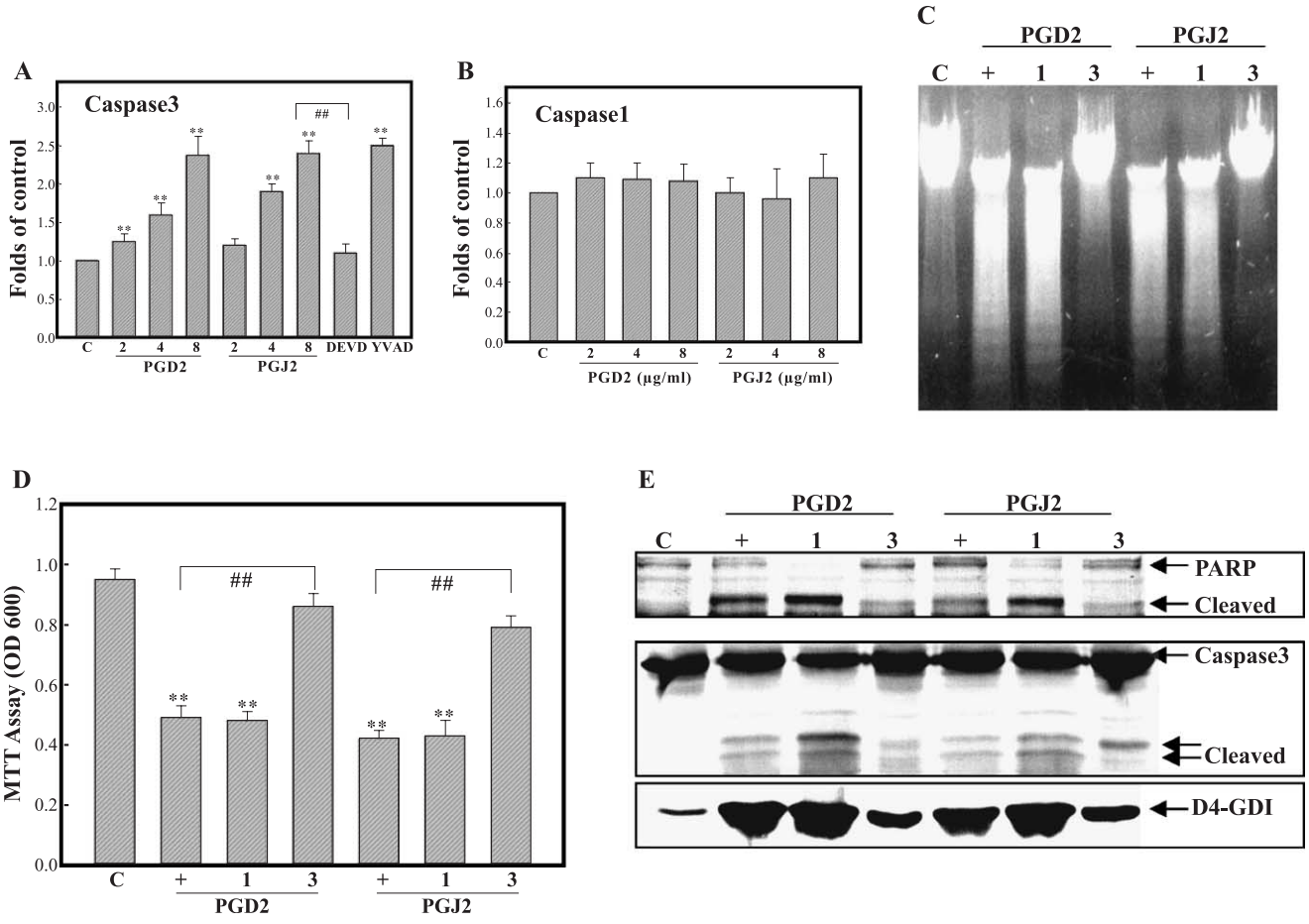

Fig. 5. Activation of caspase 3 enzyme plays an important role in PGD_2- and PGJ_2-induced apoptosis in HL-60 cells. (A, B) Activation of caspase 3, but not of caspase 1, enzyme was detected in PGD_2- and PGJ_2-treated HL-60 cells. Ac-DEVD-pNA for caspase 3 and Ac-YVAD-pNA for caspase 1 were used to detect respective enzyme activity. HL-60 cells were treated with different doses (2, 4, and 8 μg/ml) of PGD_2 or PGJ_2 for 12 h, and the enzyme activity was measured as described in Materials and methods. DEVD, addition of Ac-DEVD-FMK in the reaction; YVAD, addition of Ac-YVAD-FMK in the reaction. (C) The caspase 3 peptidyl inhibitor, Ac-DEVD-FMK, but not the caspase 1 peptidyl inhibitor, Ac-YVAD-FMK, inhibited PGD_2- or PGJ_2-induced apoptosis in HL-60 cells. HL-60 cells were treated with Ac-DEVD-FMK (3; 20 μM) or Ac-YVAD-FMK (1; 20 μM) for 1 h followed by PGD_2 or PGJ_2 (8 μg/ml) treatment. The integrity of DNA was analyzed by electrophoresis in 1.8% agarose gels. (D) Cells were treated as described in (C), and the viability of cells was detected by the MTT assay. (E) Cells were treated as described in (C), and expression of indicated proteins was evaluated by Western blotting using specific antibodies. $^{**}p<0.01$, significantly differs from the control (CON) group, as analyzed by Student's *t*-test. $^{\#\#}p<0.01$, significantly differs between indicated groups, as analyzed by Student's *t*-test. Western blotting and DNA fragmentation assay were performed at least three times, and the results shown are representative of all of the data.

inhibitors including Ac-DEVD-FMK for caspase 3 and Ac-YVAD-FMK for caspase 1 were used. In Fig. 5C, PGD_2 and PGJ_2 induced DNA fragmentation in HL-60 cells, which was blocked by adding Ac-DEVD-FMK, but not Ac-YVAD-FMK, to HL-60 cells. Results of the MTT assay showed that the addition of Ac-DEVD-FMK, but not of Ac-YVAD-FMK, protected HL-60 cells from PGD_2- and PGJ_2-induced cell death (Fig. 5D). Furthermore, results of Western blotting showed that caspase 3 protein procession, as well as PARP and D4-GDI protein cleavage induced by PGD_2 and PGJ_2, was significantly reduced by the addition of Ac-DEVD-FMK, but not Ac-YVAD-FMK (Fig. 5E). These data demonstrate that activation of caspase 3 is an essential event in apoptosis induced by PGD_2 and PGJ_2.

3.5. Production of reactive oxygen species (ROS) is involved in PGD_2- and PGJ_2-induced apoptosis and is located upstream of caspase 3 activation

ROS are important apoptosis mediators, therefore we investigated if ROS production involves PGD_2- and PGJ_2-induced apoptosis. The intracellular peroxide level in HL-60 cells under PGD_2 and PGJ_2 treatment was detected using DCHF-DA as a fluorescent ROS indicator. In results of flow cytometry analysis, increases in intracellular peroxide levels were found in PGD_2- and PGJ_2-treated HL-60 cells. The addition of *N*-acetyl cysteine (NAC), mannitol (MAN), and tiron significantly reduced the intracellular peroxide level induced by PGD_2 and PGJ_2 (Fig. 6A and B). The ratio of hypodiploid cells induced by PGD_2 and PGJ_2 was reduced by the addition of NAC, MAN, and tiron to HL-60 cells (Fig. 6C). In addition, NAC addition significantly inhibited PGD_2- and PGJ_2-induced apoptosis by DNA fragmentation and MTT assays (Fig. 7A and B). Activation of caspase 3 enzyme activity and protein procession and PARP and D4-GDI protein cleavage induced by PGD_2 and PGJ_2 were significantly blocked by the addition of NAC (Fig. 7C and D). These data indicated that ROS production is involved in PGD_2- and PGJ_2-induced apoptosis and is located upstream of caspase 3 activation.

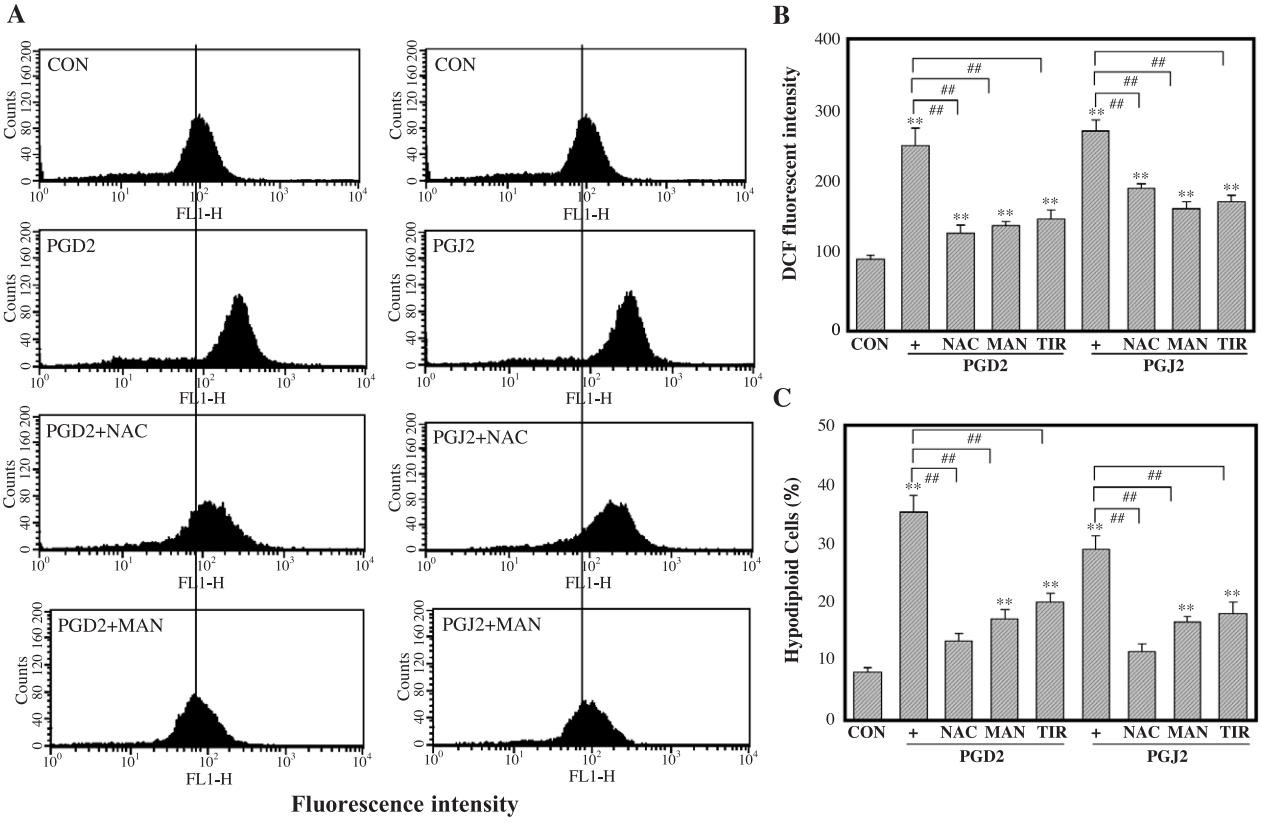

Fig. 6. Increased intracellular peroxide levels by PGD_2 and PGJ_2 in HL-60 cells. (A) HL-60 cells were pretreated with NAC (10 mM), mannitol (MAN; 20 μM), or tiron (TIR; 20 μM), followed by the addition of PGD_2 or PGJ_2 (8 μg/ml) for a further hour. DCHF-DA (100 μM) was added at the end of reaction for an additional hour. The level of intracellular peroxide was detected by flow cytometric analysis as described in Materials and methods. (B) Quantification of the fluorescent intensity from three independent experiments as described in (A) was performed, and results are expressed as the mean±S.E. (C) NAC, MAN, and TIR attenuated PGD_2- and PGJ_2-induced hypodiploid cells by flow cytometric analysis. Cells were treated with NAC (10 mM), NAM (20 μM), or TIR (20 μM) for 1 h followed by incubation with PGD_2 or PGJ_2 (8 μg/ml) for a further 12 h. The percentage of hypodiploid cells was quantitated as described in Fig. 4. $^{**}p<0.01$, significantly differs from the control (CON) group, as analyzed by Student's *t*-test. $^{\#\#}p<0.01$, significantly differs between indicated groups, as analyzed by Student's *t*-test.

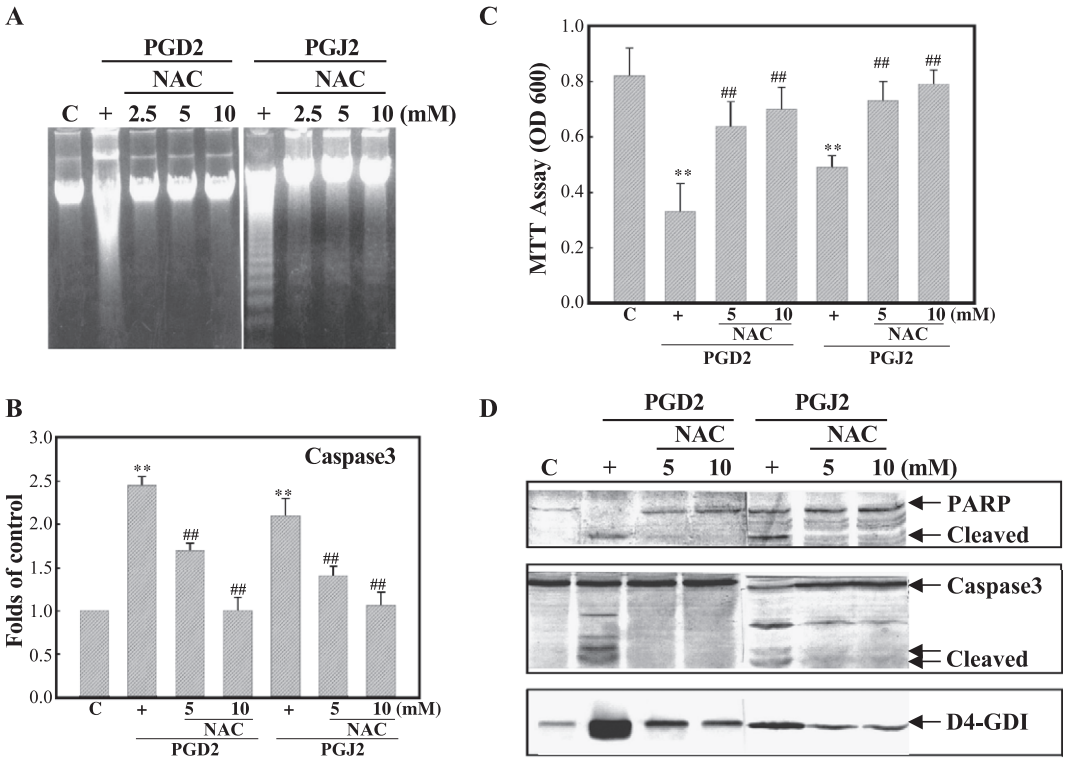

Fig. 7. NAC protects HL-60 cells from PGD_2- or PGJ_2-induced apoptosis via blocking caspase 3 activation. (A) NAC inhibits PGD_2- and PGJ_2-induced DNA fragmentation in HL-60 cells. Cells were treated with *N*-acetyl cysteine (NAC; 2.5, 5, and 10 mM) for 1 h followed by PGD_2 or PGJ_2 (8 μg/ml) treatment. The integrity of DNA was analyzed by electrophoresis. (B) Cells were treated with PGD_2 or PGJ_2 in the presence or absence of NAC pretreatment for 12 h, and the caspase 3 enzyme activity was measured as described in Fig. 6. (C) Cells were treated with PGD_2 or PGJ_2 in the presence or absence of NAC (5 and 10 mM) pretreatment for 12 h, and the viability of cells was detected by the MTT assay. (D) Cells were treated with PGD_2 or PGJ_2 in the presence or absence of NAC (5 and 10 mM) pretreatment for 12 h, and the expressions of caspase 3, PARP, and D4-GDI protein were detected. $^{**}p<0.01$, significantly differs from the control (CON) group, as analyzed by Student's *t*-test. $^{\#\#}p<0.01$, significantly differs from the PGD_2- or PGJ_2-treated group, as analyzed by Student's *t*-test. Western blotting and DNA fragmentation assay were performed at least three times, and the results shown are representative of all of the data.

3.6. Involvement of ROS production and caspase 3 protein procession in 15-deoxy-Δ^{1214} PGJ_2- and $\Delta^{12}PGJ_2$-induced apoptosis

$\Delta^{12}PGJ_2$ is a decomposition product of PGD_2 and may be converted to 15-deoxy-Δ^{12-14} PGJ_2 in an acidic condition. 15-Deoxy-Δ^{12-14} PGJ_2 is a metabolite of PGJ_2 and can be formed from PGD_2 by the elimination of two molecules of H_2O. Therefore, we investigated the apoptotic mechanism of 15-deoxy-Δ^{12-14} PGJ_2 and $\Delta^{12}PGJ_2$ in human HL-60 leukemia cells. As illustrated in Fig. 8, both 15-deoxy-Δ^{12-14} PGJ_2 and $\Delta^{12}PGJ_2$ showed effective cytotoxicity in HL-60 cells by the MTT assay in accordance with the induction of DNA fragmentation in cells (Fig. 8A and B). The IC_{50} values of 15-deoxy-Δ^{12-14} PGJ_2 and $\Delta^{12}PGJ_2$ were 2.8±0.2 and 2.3±0.4 μg/ml, much lower than those of PGD_2 (4.5±0.3 μg/ml) and PGJ_2 (4.1±0.5 μg/ml) in HL-60 cells. An increase in intracellular peroxide levels was detected in 15-deoxy-Δ^{12-14} PGJ_2- and $\Delta^{12}PGJ_2$-treated HL-60 cells. Furthermore, NAC significantly inhibited 15-deoxy-Δ^{12-14} PGJ_2- and $\Delta^{12}PGJ_2$-induced apoptosis with a reduction in intracellular peroxide levels (Fig. 8D and E). An increase in hypodiploid cells by 15-deoxy-Δ^{12-14} PGJ_2 and $\Delta^{12}PGJ_2$ was identified by flow cytometric analysis, which was blocked by the addition of NAC (Fig. 8C). Furthermore, induction of caspase 3 and PARP protein procession was detected in 15-deoxy-Δ^{12-14} PGJ_2- and $\Delta^{12}PGJ_2$-treated HL-60 cells, and those events were blocked by the addition of NAC (Fig. 8F).

3.7. The peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists, RO, TR, and CI, induce apoptosis in human HL-60 leukemia cells

J_2 series cyPGs have been identified as ligands of PPAR-γ, and cyPGs form from PGD_2 via dehydration. Therefore, we investigated if apoptosis induced by PGD_2 and PGJ_2 in human HL-60 leukemia cells occurs through activation of PPAR-γ. Three well-known PPAR-γ agonists, RO, TR, and CI, and two PPAR-γ antagonists, GW9662 and BADGE, were used in the study. Results in Fig. 9A show that RO, TR, and CI at doses of 10 and 20 μM induced DNA laddering in human HL-60 leukemia cells. DNA ladders induced by RO, TR, and CI were significantly reduced by the addition of the PPAR-γ antagonists, GW9662 (G) and

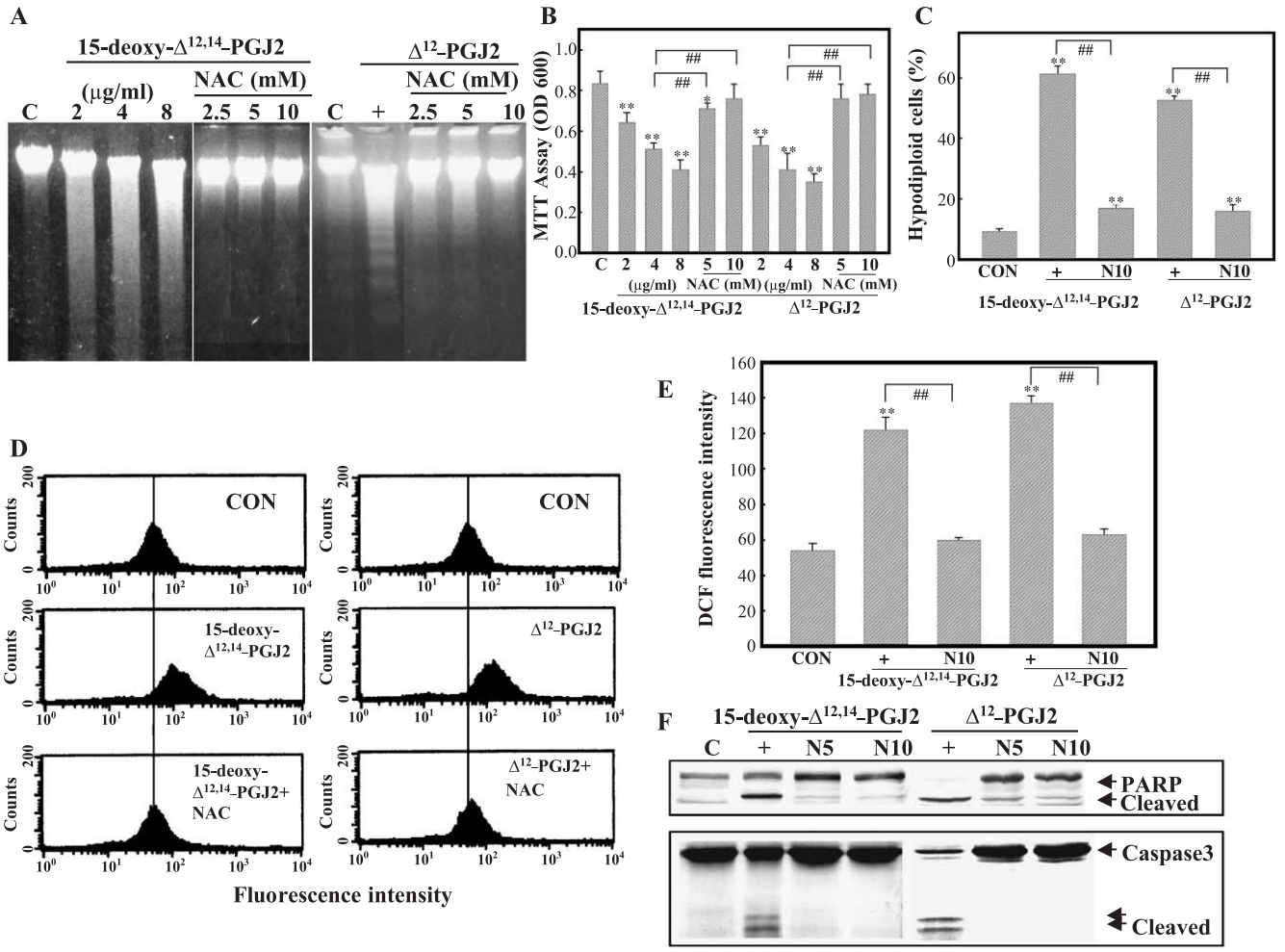

Fig. 8. PGJ_2 metabolites, 15-deoxy-Δ^{12-14} PGJ_2 and $\Delta^{12}PGJ_2$, induced apoptosis in HL-60 cells by ROS production and activation of the caspase 3 cascade. (A) HL-60 cells were treated with different doses of 15-deoxy-Δ^{12-14} PGJ_2 (2, 4, and 8 μg/ml) or $\Delta^{12}PGJ_2$ (4 μg/ml) for 12 h, and the integrity of DNA was analyzed by DNA electrophoresis. In the presence of NAC pretreatment for 1 h, cells were treated with 15-deoxy-Δ^{12-14} PGJ_2 or $\Delta^{12}PGJ_2$ (4 μg/ml) for 12 h, and the integrity of DNA was analyzed. (B) As described in A, the viability of cells under different treatments was measured by the MTT assay. (C) Similarly, hypodiploid cells induced by 15-deoxy-Δ^{12-14} PGJ_2 and $\Delta^{12}PGJ_2$ were detected by flow cytometric analysis, and that induction was prevented by the addition of NAC. (D) Increases in intracellular peroxide production were examined in 15-deoxy-Δ^{12-14} PGJ_2- and $\Delta^{12}PGJ_2$-treated HL-60 cells by the DCHF-DA assay. Addition of NAC (N10; 10 mM) significantly attenuated the peroxide production induced by 15-deoxy-Δ^{12-14} PGJ_2 and $\Delta^{12}PGJ_2$ (4 μg/ml). (E) Intracellular peroxide levels under different treatments were quantified, and results are presented as the mean±S.E. from three independent experiments. (F) Activation of the caspase 3 cascade was involved in 15-deoxy-Δ^{12-14} PGJ_2- and $\Delta^{12}PGJ_2$-induced apoptosis, which was blocked by the addition of NAC. Cells were treated with NAC (N5, 5 mM; N10, 10 mM) for 1 h followed by the addition of 15-deoxy-Δ^{12-14} PGJ_2 and $\Delta^{12}PGJ_2$ (4 μg/ml) for 12 h. Expression of PARP and caspase 3 protein was examined by Western blotting as described previously. $^{**}p<0.01$, significantly differs from the control (CON) group, as analyzed by Student's *t*-test. $^{\#\#}p<0.01$, significantly differs from indicated group, as analyzed by Student's *t*-test. Western blotting and DNA fragmentation assay were performed at least three times, and the results shown are representative of all of the data.

BADGE (B). However, neither GW9662 nor BADGE inhibited the occurrence of DNA ladders induced by PGD_2 and PGJ_2 (Fig. 9A, right panel). Results of the MTT assay indicated that GW9662 addition at doses of 10 and 20 μM protected HL-60 cells from RO-, TR-, or CI- but not PGD_2- or PGJ_2-induced cell death (Fig. 9B). Results of Western blotting showed that RO, TR, and CI induced caspase 3 and PARP protein procession, described herein as an increase in cleaved fragments (cleaved) and a decrease in intact protein (caspase 3 and PARP) (Fig. 9C). The addition of GW9662 or BADGE significantly reduced caspase 3 and PARP protein procession induced by RO, TR, and CI.

4. Discussion

TPA induction of COX-2 protein expression with reduction in the viability with or without AA was observed in human leukemia cells, and the cytotoxic effect induced by TPA and AA was attenuated by the COX inhibitors, NS398 and aspirin. PGD_2 and PGJ_2, but not PGE_2 or $PGF_{2\alpha}$, exhibited apoptosis-inducing activity through elevation of the intracellular ROS level, an event upstream of caspase 3 activation. Metabolites of PGD_2 and PGJ_2, including 15-deoxy-Δ^{12-14} PGJ_2 and $\Delta^{12}PGJ_2$, showed more effective apoptotic activity than PGJ_2 and PGJ_2, and induction of

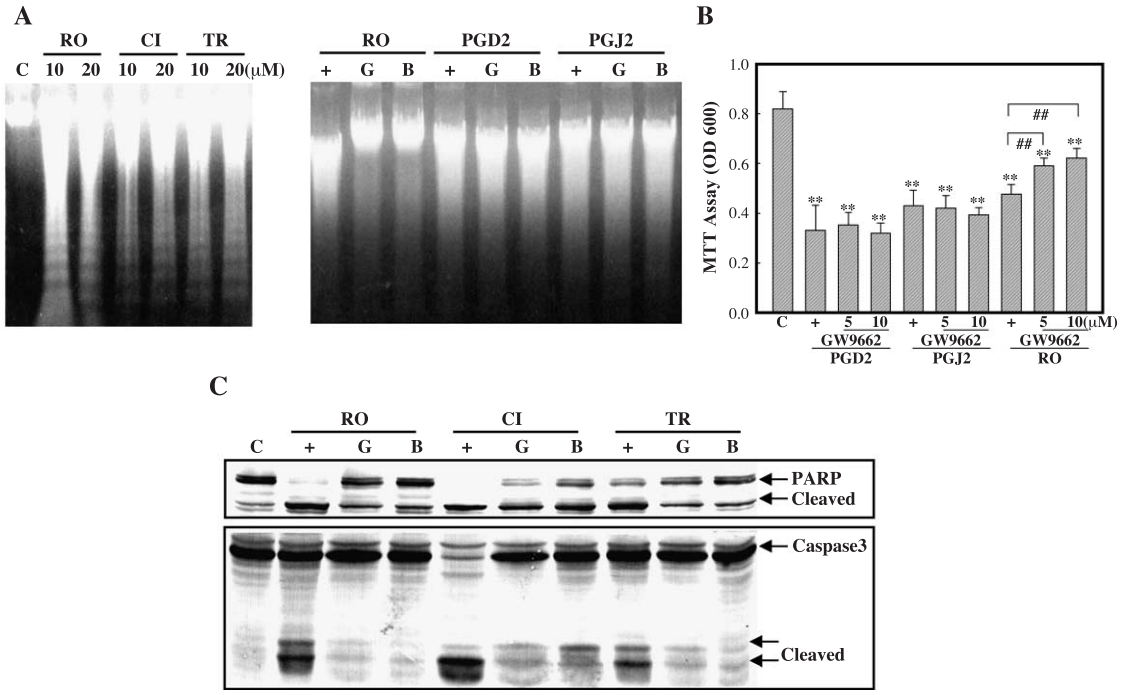

Fig. 9. The PPAR-γ agonists, RO, TR, and CI, induced DNA laddering with activation of caspase 3 and PARP protein procession. (A) Left panel: HL-60 cells were treated with different doses (10 or 20 μM) of RO, TR, or CI for 12 h, and the integrity of DNA was analyzed by agarose electrophoresis. Right panel: HL-60 cells were treated with GW9662 (G; 20 μM) or BADGE (B; 20 μM) for 1 h followed by incubation with RO (20 μM), PGD_2 (8 μg/ml), or PGJ_2 (8 μg/ml) for a further 12 h. The integrity of DNA was analyzed by agarose electrophoresis. (B) The viability of cells under different treatments was examined by the MTT assay. HL-60 cells were treated with the PPAR-γ, antagonist GW9662 (10 or 20 μM), for 1 h followed by the addition of PGD_2 (8 μg/ml), PGJ_2 (8 μg/ml), or RO (20 μM) for a further 12 h, and the viability of cells was detected by the MTT assay. (C) PARP and caspase 3 protein procession induced by the PPAR-γ agonists, RO, CI, and TR, was attenuated by the addition of GW9662 and BADGE (20 μM). Cells were treated with GW9662 or BADGE (20 μM) for 1 h followed by incubation with RO, TR, or CI (20 μM) for a further 12 h. The occurrence of caspase 3 and PARP protein procession were examined by Western blotting using specific antibodies as described previously. $^{**}p<0.01$, significantly differs from the control (CON) group, as analyzed by Student's *t*-test. $^{\#\#}p<0.01$, significantly differs from the indicated group, as analyzed by Student's *t*-test. Western blotting was performed at least three times, and the results shown are representative of all of the data.

ROS production and activation of the caspase 3 cascade were involved. PPAR-γ antagonists showed no protective effect on PGD_2- and PGJ_2-induced apoptosis. These data suggest the possibility that COX-2 up-regulation during inflammation is involved in the apoptotic process by enhancing the production of PGs such as PGD_2, PGJ_2, 15-deoxy-Δ^{12-14} PGJ_2, and $\Delta^{12}PGJ_2$, and that ROS production, upstream of the caspase 3 cascade, is involved in the apoptotic mechanisms.

Apoptosis induced by PGs, particularly J-series PGs, has been documented, and it has been shown that activation of PPAR-γ by J_2 series cyPGs such as 15d-PGJ_2 exhibits antiproliferative, anti-apoptotic, and anti-inflammatory properties in several types of cancer cells. Kondo et al. [10] demonstrated that 15d-PGJ_2 induces apoptosis via accumulation and phosphorylation of p53 and results in activation of the caspase cascade. Ward et al. [11] suggested that PGD_2 and 15d-PGJ_2 selectively induce apoptosis in eosinophils via inhibiting I kappa B degradation in a PPAR-γ-independent manner. In neuroblastoma cells, 15d-PGJ_2-induced apoptosis occurs through activation of the ERK pathway in a PPAR-γ-dependent manner [23]. Okano et al. [24] indicated that 15d-PGJ_2 induces apoptosis with suppression of NF-kappa B activation in a PPAR-γ-independent manner in SH-Hep1 cells, but in a PPAR-γ-dependent manner in Hep G_2 cells, through a caspase-3-independent pathway. Additionally, Castrillo et al. [25] provided evidence suggesting that 15d-PGJ_2 promotes apoptosis in activated macrophages via sustained activation of PKC zeta and JNK and inhibition of NF-kappa B activity. Nakamura et al. [26] indicated that inhibitors of the AA cascade modulate TPA-induced oxidative stress in mouse skin. These data indicate that apoptosis induced by PGs, especially 15d-PGJ_2, is very complicated, and dependent on the types of cells tested. However, the apoptotic mechanism induced by PGD_2 and PGJ_2 in HL-60 leukemia cells is still unclear. Constitutive expression of the PPAR-γ receptor has been identified in human HL-60 leukemia cells [27]. In the present study, we identified that PGD_2- and PGJ_2-induced apoptosis occurs through a caspase 3-dependent pathway in human leukemia cells. The PPAR-γ agonists, RO, TR, and CI, induced apoptosis in cells in accordance with caspase 3 and PARP, and this was blocked by the PPAR-γ antagonists, GW9662 and BADGE. However, neither GW9662 nor BADGE showed any preventive effect against PGD_2- or PGJ_2-induced apoptosis. This suggests that apoptosis

induced by PGD_2 and PGJ_2 might occur in a PPAR-γ-independent manner.

Oxidative stress is seen as an upstream event in the signaling cascade in many cellular functions such as proliferation, inflammatory responses, and apoptosis, and ROS produced during oxidative stress may play critical roles in these effects by damaging cellular components such as membrane lipids [28–30]. However, both ROS-dependent and -independent apoptosis has been found in a variety of chemical treatments [18,28]. Kondo et al. [31] reported that cyclopentenone PGs including PGA_2, PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-Δ^{12-14}-PGJ_2 showed a potent pro-oxidant effect in human neuroblastoma cells. A pro-oxidative characteristic of cyclopentenone PGs is that they contain α,β-unsaturated ketones for nucleophilic addition reactions with thiols. Although PGs have been reported to cause oxidative stress in neuroblastoma cells, the molecular mechanism underlying this has not been delineated. Based on the observations that (a) PGD_2 and PGJ_2 (but not PGE_2 or $PGF_{2\alpha}$) treatment resulted in apoptosis induction in human leukemia cells, (b) increases in intracellular peroxide levels in PGD_2- and PGJ_2-treated cells, and (c) NAC reduction of ROS production and apoptosis by PGD_2 or PGJ_2, these data suggest that PGD_2 and PGJ_2 possess the ability to induce apoptosis in human leukemia cells via their pro-oxidant activity. DPI and ALL are inhibitors of NADPH oxidase and xanthine oxidase, respectively. Our previous study demonstrated that DPI protected NIH3T3 cells from arsenic-induced apoptosis by decreasing ROS production [30]. In the present study, neither diphenyl iodine (DPI) nor allpurinol (ALL) showed an inhibitory effect on PGD_2- or PGJ_2-induced ROS production and apoptosis (data not shown). This suggests that increasing intracellular ROS levels by PGD_2 and PGJ_2 did not occur through activation of NADPH oxidase and xanthine oxidase in cells.

Activation of the caspase cascades has been shown in the process of apoptosis, and caspase 3 is an executioner caspase, and is extensively activated by proteolytical cleavage of its 32-kDa precursor to generate 17- or 15-kDa active forms in the apoptotic process. Cleavage of PARP (116 kDa) into 85- and 31-kDa fragments, and of D4-GDI (28 kDa) into 23- and 5-kDa fragments by activated caspase 3, has been identified in apoptosis in response to several stimuli such as chemicals, growth factor deprivation, and UV irradiation [32]. Its relation with the effect of PGs on apoptosis induction is still controversial. Ishaque et al. [33] reported that PGE_2 and PGI_2 protected renal glomerular mesangial cells against TNFα-mediated apoptosis, and down-regulation of PGs by the COX-2 inhibitors, SC-236 and NS398, may induce the occurrence of apoptosis [34]. However, Ragolia et al. [35]

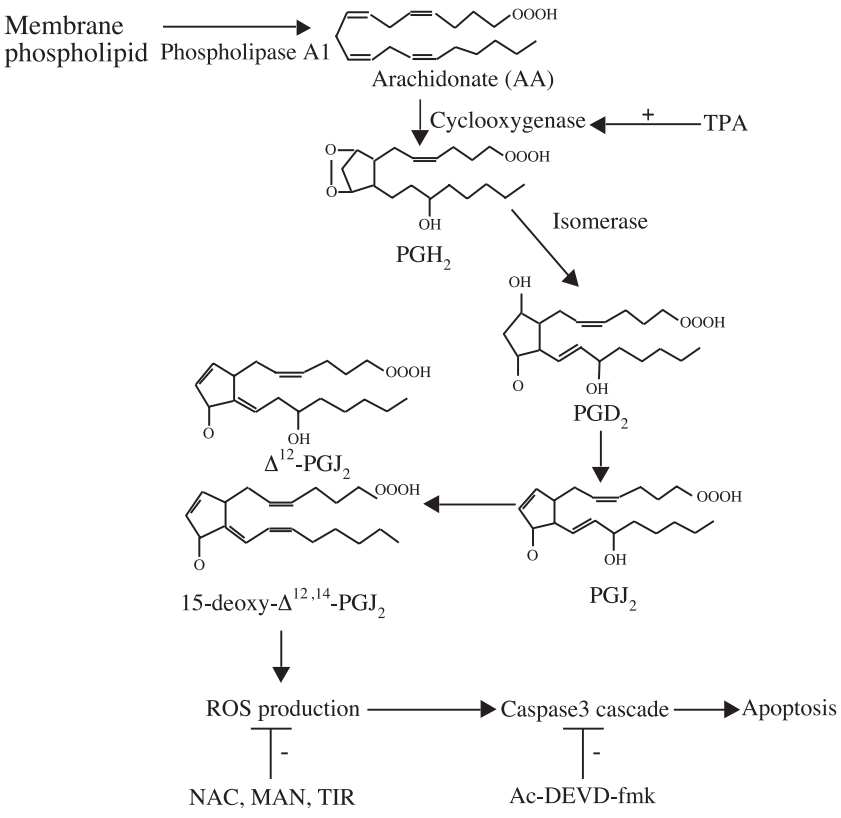

Fig. 10. A tentative model for PGs, proliferation, and apoptosis as proposed in the present study. TPA, 12-*o*-tetradecanoylphorbol 13-acetate; PGD_2, prostaglandin D_2; PGJ_2, prostaglandin J_2; ROS, reactive oxygen species; NAC, *N*-acetyl-cysteine; MAN, mannitol; TIR, tiron.

reported that PGD_2 synthase induced apoptosis in PC12 neuronal cells which was inhibited by the addition of PGE_1, E_2, and $F_{2\alpha}$. Based on the observations that (a) PGD_2 and PGJ_2 (but not PGE_2 or $PGF_{2\alpha}$) treatment resulted in caspase 3 activation in the presence of PARP and D4-GDI cleavage in leukemia cells, (b) the caspase 3 peptidyl inhibitor, Ac-DEVD-FMK, protected cells from PGD_2- and PGJ_2-induced apoptosis, and (c) NAC treatment significantly inhibited PGD_2- or PGJ_2-induced caspase 3 activation and the occurrence of apoptosis, these data indicate that apoptosis induced by PGD_2 and PGJ_2 is mediated by activation of the caspase 3 cascade located downstream of ROS production.

The structure–activity relationship (SAR) of PGs with apoptosis induction is still unclear. Vosseler et al. [36] indicated that 15-deoxy-$\Delta^{12,14}$-PGJ_2, but not others, markedly reduced cell viability in HUVECs, and that both 15-deoxy-$\Delta^{12,14}$-PGJ_2 and PGD_2 treatment induced apoptosis by the annexin V assay. However, apoptosis induced by PGD_2 seems to occur to a lesser degree than that induced by 15-deoxy-$\Delta^{12,14}$-PGJ_2. Results of the present study showed that both PGD_2 and PGJ_2 were effective apoptotic inducers in human leukemia cells, and that IC_{50} values for PGD_2 and PGJ_2 were 4.5 ± 0.3 and 4.1 ± 0.5 μg/ml in HL-60 cells, respectively. PGJ_2 has a reactive α,β-unsaturated carbonyl group in its cyclopentenone ring, and has been shown to exhibit several biological effects, including antiviral and antitumor activities. Interestingly, PGD_2 without a reactive α,β-unsaturated carbonyl group in its structure also showed apoptosis-inducing activity in cells in the present study. Shibata et al. [37] reported that PGD_2 might be non-enzymatically converted to PGJ_2 in the culture medium. Results of the present study demonstrated that the PGJ_2 metabolites, 15-deoxy-Δ^{12-14} PGJ_2 and $\Delta^{12}PGJ_2$, exhibited an effective apoptotic effect in cells, and the IC_{50} values of 15-deoxy-Δ^{12-14} PGJ_2 (2.8 ± 0.2 μg/ml) and $\Delta^{12}PGJ_2$ (2.3 ± 0.4 μg/ml) were much lower than those of PGD_2 and PGJ_2. This suggests that PGD_2 induction of apoptosis in human leukemia cells may be attributed to the conversion of PGD_2 to more reactive cyclopentenone PGs such as PGJ_2, 15-deoxy-Δ^{12-14} PGJ_2, and $\Delta^{12}PGJ_2$ which in turn induce DNA fragmentation in cells.

In summary, the findings described in the present study suggest that PGD_2 and PGJ products, including PGJ_2, 15-deoxy-Δ^{12-14} PGJ_2, and $\Delta^{12}PGJ_2$, possess apoptosis-inducing activity in human leukemia cells via activation of the caspase 3 cascade, and that production of ROS participates upstream of the caspase 3 cascade in the apoptotic mechanisms, which might be independent of activation of PPAR-γ. Therefore, involvement of PGs in apoptosis during inflammation through ROS production is proposed in the present study (Fig. 10). Understanding the roles of ROS in other biological functions of PGs such as regulation of cell growth, differentiation, and inflammation is an important topic and is reserved for future studies.

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