MOLECULAR CARCINOGENESIS 37:16–24 (2003)

# Involvement of c-jun N-Terminal Kinase Activation in 15-Deoxy-$\Delta^{12,14}$-Prostaglandin $J_2$– and Prostaglandin $A_1$–Induced Apoptosis in AGS Gastric Epithelial Cells

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Cyclopentenone prostaglandins (CyPGs), derivatives of arachidonic acid, have been suggested to exert growth-inhibitory activity through peroxisome proliferator-activated receptor (PPAR)-dependent and -independent mechanisms. Here we examined various eicosanoids for growth inhibition and found that the terminal derivative of prostaglandin (PG) $J_2$ metabolism, 15-deoxy-$\Delta^{12,14}$-$PGJ_2$ (15d-$PGJ_2$), and $PGA_1$ markedly inhibited the growth and induced apoptosis in AGS gastric carcinoma cells. There were no significant increases in cell death and DNA-fragmentation in the cells with overexpression of PPARα or PPARγ, indicating the possibility that 15d-$PGJ_2$ and $PGA_1$ induced apoptosis through PPAR-independent pathway. Moreover, 15d-$PGJ_2$ and $PGA_1$ activated the c-jun N-terminal kinase (JNK) and caspase-3 activity in dose- and time-dependent manners. To examine further the role of JNK signaling cascades in apoptosis induced by 15d-$PGJ_2$ and $PGA_1$, we transfected dominant-negative (DN) mutants of JNK plasmid into the cells to analyze the apoptotic characteristics of cells overexpressing DN-JNK following exposure to 15d-$PGJ_2$ and $PGA_1$. Overexpression of DN-JNK significantly repressed both endogenous JNK and caspase-3 activity, and subsequently decreased apoptosis induced by 15d-$PGJ_2$ and $PGA_1$. These results suggested that CyPGs, such as 15d-$PGJ_2$ and $PGA_1$, activated JNK signaling pathway, and that JNK activation may be involved in 15d-$PGJ_2$- and $PGA_1$-induced apoptosis. 

Key words: prostaglandin; apoptosis; JNK

## INTRODUCTION

The c-jun N-terminal kinases (JNKs), members of the mitogen-activated protein kinase family, are classic stress-activated protein kinases [1], which can be activated by proinflammatory cytokines [2,3] and environmental stress such as ultraviolet light [4], γ-irradiation [5], heat shock, osmotic stress [6], shear stress [7], growth factor withdrawal [8], ceramide [9], protein synthesis inhibitor [2], and treatment with chemotherapy drugs, including paclitaxel [10], adriamycin, vinblastine [11], and etoposide [12]. Recently, JNK has been found to be an important mediator of apoptosis signaling [13]. For example, JNK activation is associated with apoptosis in PC-12 cells deprived of nerve growth factor [14]. In addition, nonneuronal embryonic fibroblasts with targeted disruptions of the *JNK1* and *JNK2* genes are able to inhibit apoptosis [15]. These reports suggest that activation of JNK can be associated with induction of apoptosis; however, the mechanism(s) and functional role for JNK in the regulation of apoptosis are not clear [4].

Prostaglandins (PGs) are oxygenated metabolites of arachidonic acid (AA) and are divided into two groups, conventional PGs and cyclopentenone PGs (CyPGs), according to their mechanisms of action [16]. The conventional eicosanoids, such as $PGE_2$ and $PGD_2$, bind to cell surface receptors and transduce signal into cells. The CyPGs, such as the A and J series of PGs, lack cell surface receptors but directly enter the cells and exert their actions in the nuclei, including induction of cell growth arrest and cell differentiation [16]. It has been demonstrated that CyPGs induce several protein expressions, such as

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Received 18 September 2002; Revised 30 January 2003; Accepted 18 February 2003

Abbreviations: JNK, c-jun N-terminal kinase; PG, prostaglandin; AA, arachidonic acid; CyPGs, cyclopentenone PGs; 15d-$PGJ_2$, 15-deoxy-$\Delta^{12,14}$-$PGJ_2$; PPAR, peroxisome proliferator-activated receptor; DN, dominant-negative; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PARP, poly (ADP ribose) polymerase; Gst, glutathione S-transferase; PBS, phosphate buffered saline; $LTB_4$, leukotriene $B_4$; $TXB_2$, thromboxane $B_2$.

DOI 10.1002/mc.10119

heat shock proteins [17], γ-glutamylcysteine synthetase [18], collagen [19], growth arrest and DNA-damage inducible 45 [20], and heme oxygenase [21]. AA is first converted to $PGH_2$ by cyclooxygenase and subsequently converted to one of several related products, including $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $PGI_2$, and thromboxane $A_2$, through the action of specific PG synthases. 15-deoxy-$\Delta^{12,14}$-$PGJ_2$ (15d-$PGJ_2$) is derived from $PGD_2$ and involves sequential conversion of $PGD_2$, $PGI_2$, $\Delta^{12}$-$PGJ_2$, and 15d-$PGJ_2$ [16]. 15d-$PGJ_2$ has been showed to bind to and activate peroxisome proliferator-activated receptor γ (PPARγ) and is therefore an intracellular target of this CyPG [22].

The CyPGs have been shown to possess potent antiproliferative and antitumor activities, but their mechanisms of action are complex and not well understood. Recently it has been shown that $PGD_2$ metabolites, such as $PGJ_2$, $\Delta^{12}$-$PGJ_2$, and 15d-$PGJ_2$, are potential inducers of intracellular oxidative stress that mediate the cytotoxic effects in human neuroblastoma cells [23]. Kondo and colleagues have shown that the 15d-$PGJ_2$-induced accumulation of p53 results in the activation of a death-inducing caspase cascade mediated by Fas and the Fas ligand in neurons [24]. Other studies show that de novo gene transcription is necessary for 15d-$PGJ_2$–induced apoptosis in breast cancer cells [25]. The current investigation was undertaken to study the effects of AA and its metabolites on human AGS gastric cell death. These results demonstrated that 15d-$PGJ_2$ and $PGA_1$ induced apoptosis and activated JNK and caspase-3 activity in AGS cells. Moreover, the cells were decreased in apoptosis with overexpression of dominant-negative (DN)-JNK, indicating that induction of apoptosis by 15d-$PGJ_2$ and $PGA_1$ might mediate the activation of JNK.

## MATERIALS AND METHODS

### Materials

$PGA_1$, $PGJ_2$, and $LTB_4$ were purchased from Cayman Chemical (Ann Arbor, MI), and AA, $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $PGH_2$, $TXB_2$, and 15d-$PGJ_2$ were purchased from Biomol (Plymouth Meeting, PA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), poly (ADP ribose) polymerase (PARP), and JNK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-PPARα and PPARγ were purchased from Affinity Bioreagents (Golden, CO).

### Cell Culture

The human AGS gastric carcinoma cell lines (CCRC 60102) were obtained from Food Industry Research and Development Institute (Hsinchu, Taiwan) and cultured in Dulbecco's modified Eagle's medium/Nutrient mixture F-12 containing 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY). For all assays, cells were plated in 6-cm dishes at $5 \times 10^6$ cells per dish and allowed to grow for 24 h.

### Western Blotting

Equal amounts of total cellular protein (50 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto Immobilon-P membrane (Millipore, Bedford, MA) as described previously [26]. The membrane was then incubated with a primary antibody and subsequently probed with secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and visualized with enhanced chemiluminescence kits (Amersham, Arlington, IL). The densities of the bands were quantified with a computer densitometer (IS-1000 Digital Imaging System).

### Kinase Assay

Equal amounts of total cellular protein (200 μg) were immunoprecipitated with JNK1 antibody (Santa Cruz Biotechnology) and protein A/G-PLUS agarose for 12 h at 4°C. Kinase activity assay was performed in kinase buffer with glutathione S-transferase (Gst)-c-jun fusion protein as substrates as previously described [27]. The densities of the bands were quantified with a computer densitometer (IS-1000 Digital Imaging System).

### Plasmids and Transition Transfection

The DN-JNK plasmid was constructed from human JNK1 with double point mutant of Thr183 to Ala and Tyr185 to Phe [28]. The PPARγ and PPARα expression plasmid were generously provided by Professor Christopher K. Glass (University of California-San Diego) and Professor Bart Staels (Université de Lille) [29,30]. AGS cells were seeded in 6-cm dishes and transfected with the mock DN-JNK, PPARγ, or PPARα expression plasmid with LipofectAMINE$^{TM}$ 2000 (Gibco, BRL) [31]. After transfection, the cells were treated with 15d-$PGJ_2$ or $PGA_1$, and cells were collected for determination of cell viability, DNA fragmentation, caspase activity, or kinase assay. For the PPAR functional activity assay, PPARα or PPARγ expression or mock expression plasmid was cotransfected with AOx-TK reporter plasmid and pRL-TK plasmid as internal control. The AOx-TK reporter construct contained three copies of the acyl CoA oxidase PPAR responsive element (PPRE) driving luciferase gene expression [29]. Transfected cells were treated with 15d-$PGJ_2$ and $PGA_1$ for 9 h and PPAR transactivated activity was determined as described previously [31].

### DNA Fragmentation

After treatment with tested drugs, the cells were washed with phosphate buffered saline (PBS) and the DNA fragmentation detected by Cell Death Detection enzyme-linked immunosorbent assay kits

according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

### Caspase-3 Activity Assay

Briefly, cells were washed with cold PBS twice, and the cytosolic fraction extracted with extraction buffer (12.5 mM Tris, pH 7.4, 2 mM dithiothreitol, 0.125 mM EDTA, 5% glycerol, 1 μg/mL leupeptin, and 1 μg/mL aprotinin) by repeated cycles of freezing and thawing. Cell lysates (150 μg) were incubated with 10 μM fluorescence substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Calbiochem, La Jolla, CA) in assay buffer (50 mM Tris, pH 7.4, 1 mM EDTA and 10 mM EGTA) at 37°C for 30 min in the dark. The fluorescence intensity of the cleaved substrate was measured with a fluorescence spectrophotometer (Hitachi F-3000) by Ex 380 nm/Em 460 nm [32].

### Flow Cytometry

Sub-$G_1$ population of the cell cycle was analyzed by flow cytometry as described previously [26]. Briefly, cells were trypsinized, washed once with PBS, and fixed in 75% ethanol for 1 h at −20°C. Fixed cells were then washed with PBS, incubated with 0.5 mL PBS containing 0.05% RNase and 0.5% Triton X-100 for 30 min at 37°C, and stained with propidium iodide. The stained cells were analyzed with a FACscan laser flow cytometer and CellFIT DNA analysis software (Becton Dickinson, San Jose, CA).

## RESULTS

### Growth Inhibition of Human AGS Gastric Carcinoma Cells by 15d-$PGJ_2$ and $PGA_1$

To examine whether CyPGs inhibited the growth of AGS cells, we screened AA and a large number of its metabolites, and found that $PGA_1$, the $PGD_2$ itself, and its metabolites, such as $PGJ_2$ and 15d-$PGJ_2$, significantly inhibited the growth of AGS cells (Figure 1A). 15d-$PGJ_2$ and $PGA_1$ were more effective than the other PGs in the growth inhibition. Ten micromole 15d-$PGJ_2$ and 20 μM $PGA_1$ reduced the cell number by 80% and 50%, respectively (Figure 1A). The terminal metabolite of $PGJ_2$, 15d-$PGJ_2$, represented the most potent growth inhibitor. $PGD_2$ and $PGJ_2$, the precursors of 15d-$PGJ_2$, were less effective than 15d-$PGJ_2$. The inhibitory order was 15d-$PGJ_2$ > $PGA_1$ > $PGJ_2$ > $PGD_2$. AA, $PGE_2$, $PGF_{2\alpha}$, $PGH_2$, $LTB_4$, and $TXB_2$, were ineffective to inhibit the growth at the concentration of 50 μM. Thus, a reactive α, β-unsaturated carbonyl group in the cyclopentane ring, such as 15d-$PGJ_2$, $PGA_1$, and $PGJ_2$ (Figure 1B), which rendered this portion of the molecule able to form Michael's adducts with cellular nucleophilic and covalently modify specific protein [33,34], was essential for apoptosis.

We next examined whether 15d-$PGJ_2$ and $PGA_1$ inhibited cell growth through induction of apoptosis. AGS cells were treated with various concentrations of 15d-$PGJ_2$ and $PGA_1$ for 15 h and the DNA fragmentation was measured as an indicator of apoptosis. As shown in Figure 2A, 15d-$PGJ_2$ and $PGA_1$ induced DNA fragmentation in dose- and time- (data not shown) dependent manners. DNA fragmentation was approximately 75% and 68% after 12 h incubation with 10 μM 15d-$PGJ_2$ and 20 μM $PGA_1$, respectively. Moreover, we measured the numbers of cells with a sub-$G_1$ DNA content as a measure of

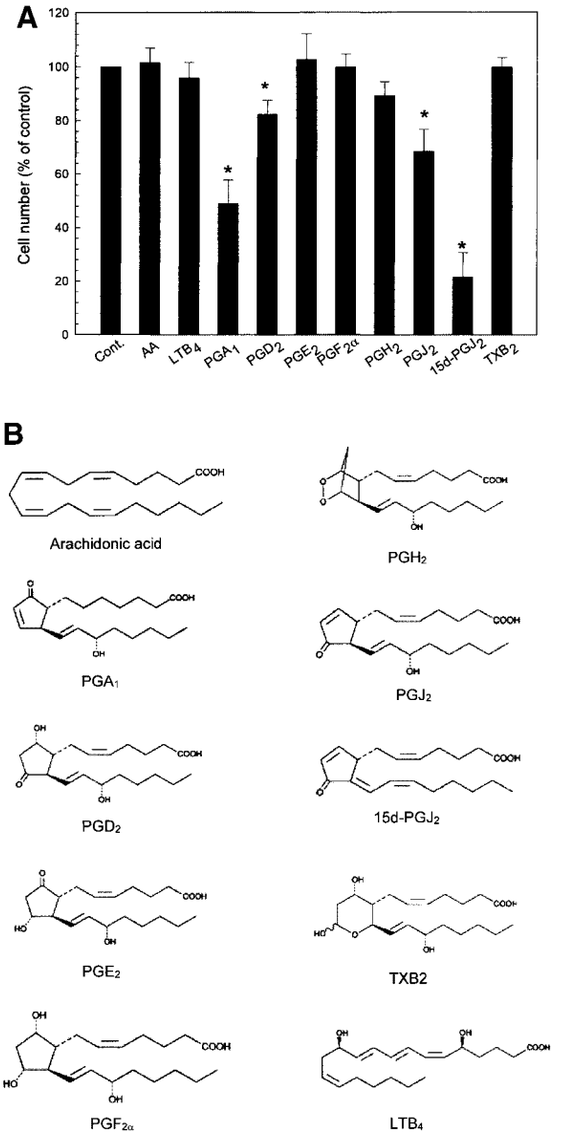


Figure 1. Effect of AA and its metabolites on the growth of AGS cells. (A) Cells were treated with 50 μM AA, $LTB_4$, $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $PGH_2$ or $TXB_2$, or 20 μM $PGA_1$, $PGJ_2$, or 10 μM 15d-$PGJ_2$ for 24 h. Cell numbers were measured by counting the number of trypan blue-excluding cells. The values were obtained in three independent experiments performed in triplicate and are represented as means ± SE. *$P < 0.05$ versus control. (B) Structures of AA and its metabolites [51].

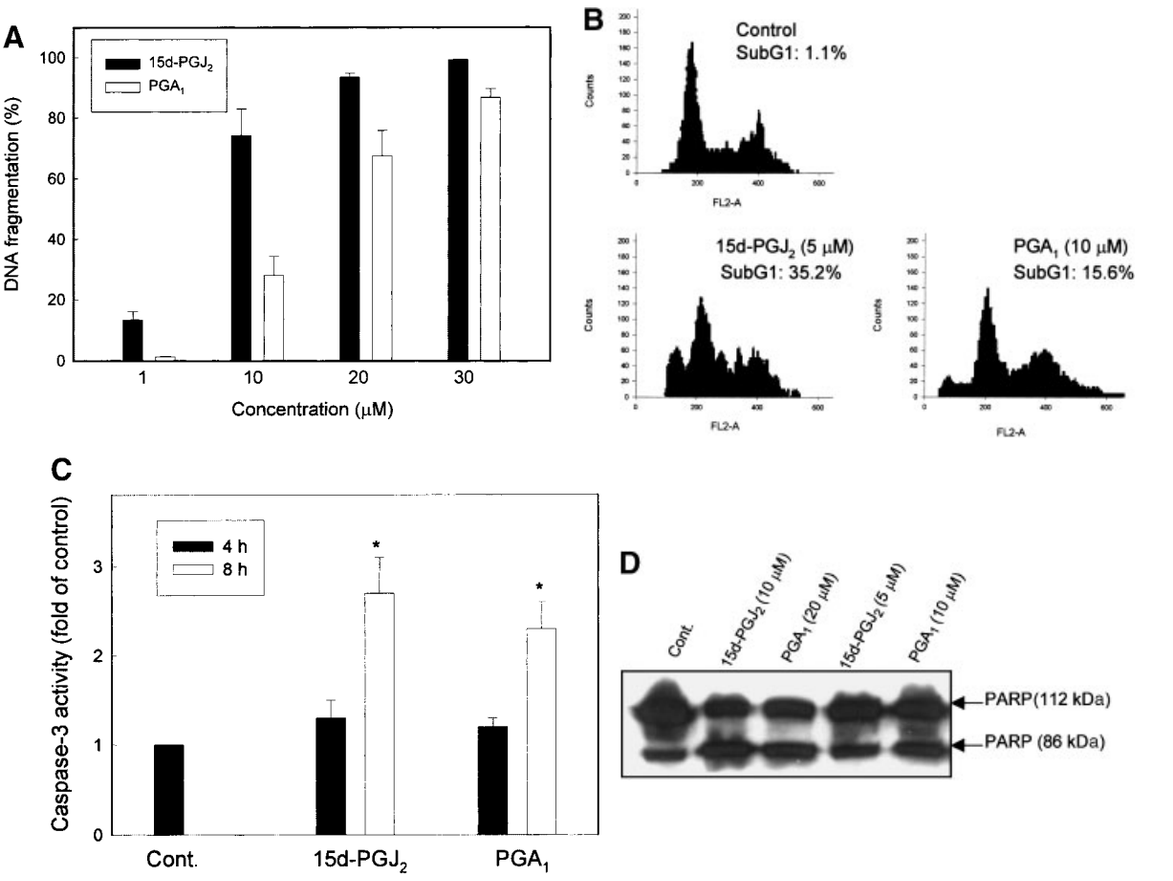


Figure 2. Effect of 15d-PGJ$_2$ and PGA$_1$ on the DNA fragmentation, caspase-3 activity, and PARP cleavage in AGS cells. (A) Cells were treated with various concentration of PGA$_1$ or 15d-PGJ$_2$ for 15 h. DNA fragmentation was determined by the histone-associated DNA-fragments as described in Materials and Methods. (B) Cells were treated with 5 μM 15d-PGJ$_2$ or 10 μM PGA$_1$, and subjected to DNA content analysis by flow cytometer. The percentages of total cells with sub-G$_1$ apoptotic DNA are indicated. (C) Cells were treated with 10 μM 15d-PGJ$_2$ or 20 μM PGA$_1$ for 4 h and 8 h as indicated, and cell extracts were subjected to caspase-3 activity assay as described in Materials and Methods. The values were obtained in two independent experiments performed in triplicate and represented as means ± SE. *$P < 0.05$ versus control. (D) Cells were treated with various concentrations of 15d-PGJ$_2$ or PGA$_1$ for 15 h. Total cellular proteins (50 μg) were subjected to Western blotting with anti-PARP antibody. The intact (112 kDa) and cleaved (86 kDa) species of PARP were indicated in the right of the panel.

apoptosis and observed an increase in the apoptosis cells in the cells with 15d-PGJ$_2$ and PGA$_1$ (Figure 2B). Previous studies have indicated that 15d-PGJ$_2$ activates caspase-3 in a variety of cancer cell types [35–37], so this parameter was monitored as a measure of the rate and extent of apoptosis. As shown in Figure 2C, caspase-3 activity was increased in cells with 10 μM 15d-PGJ$_2$ and 20 μM PGA$_1$ at 8 h. The substrate of caspase-3, PARP, also cleaved in 15d-PGJ$_2$- and PGA$_1$-treated cells (Figure 2D). These results indicated that 15d-PGJ$_2$ and PGA$_1$ inhibited the growth through induction of apoptotic cell death and the caspase-3 pathway.

### Induction of Apoptosis by 15d-PGJ$_2$ and PGA$_1$ Through PPAR-Independent Pathway

15d-PGJ$_2$ and PGA$_1$ were potent agonists of PPARα and PPARγ. Activation of PPARα or PPARγ can promote cell apoptosis in other cells [38]. To examine this possibility, AGS cells were transfected with mock, PPARα, or PPARγ expression plasmid, and treated with 15d-PGJ$_2$ or PGA$_1$. Western blot showed that the protein of PPARα or PPARγ was increased about threefold in the cells transfected with PPARα or PPARγ expression plasmid (Figure 3C and F). To be sure whether the transfection of PPARα or PPARγ expression plasmid was functional in AGS cells and increase the transactivated activity by treatment with 15d-PGJ$_2$ and PGA$_1$, the PPARα or PPARγ expression plasmid was cotransfected into AGS cells with a reporter construct containing three copies of the acyl CoA oxidase PPAR responsive element upstream of the thymidine kinase promoter driving luciferase gene expression. Cotransfection of PPAR mock expression plasmid, 15d-PGJ$_2$, and PGA$_1$ had approximately onefold to twofold activation of luciferase gene expression (Figure 3C and F, the number below the PPAR band). However, when

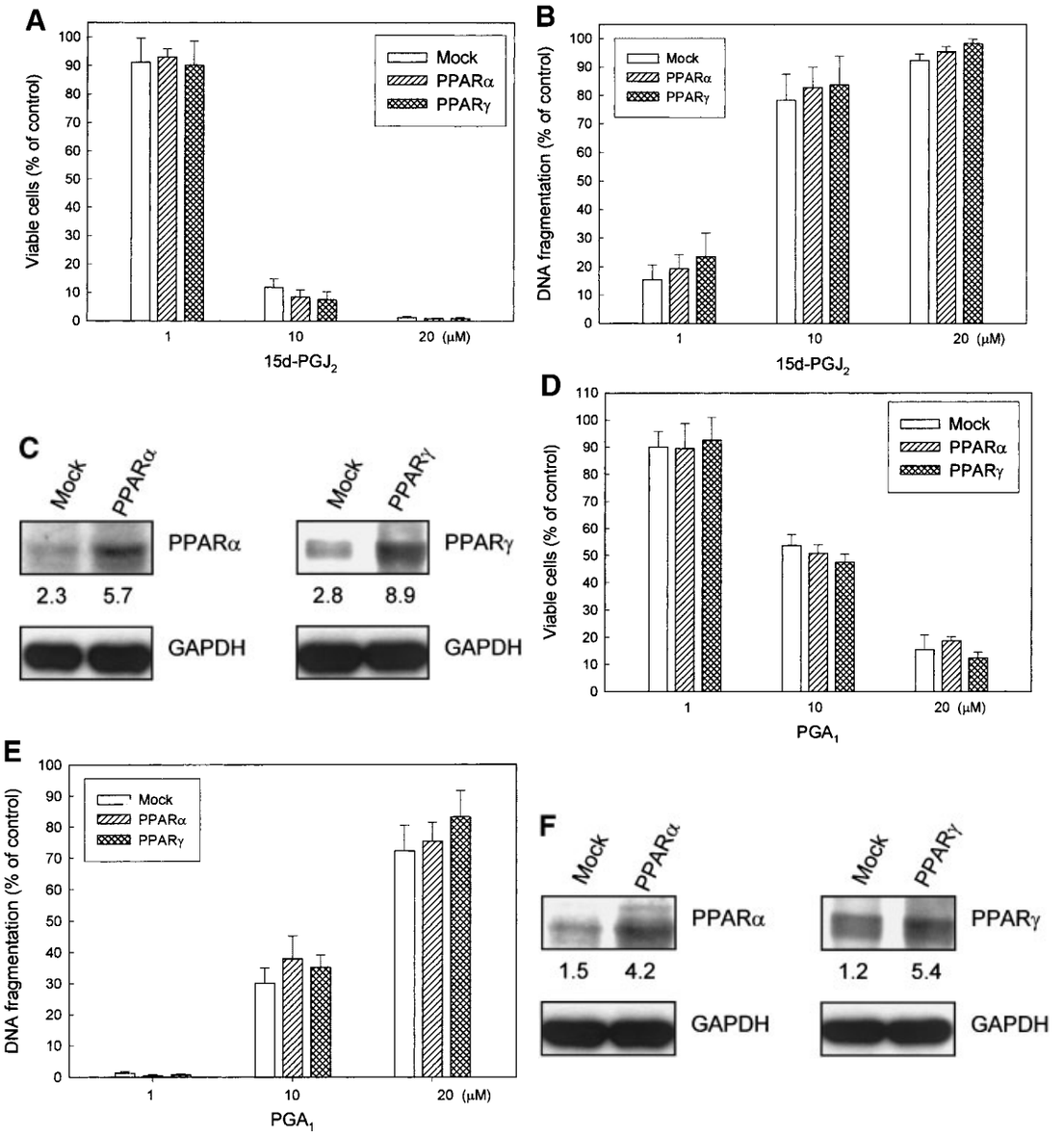


Figure 3. Effect of overexpression of PPARα or PPARγ on the cells' viability and DNA fragmentation by 15d-$PGJ_2$ or $PGA_1$. Cells were transfected with PPARα or PPARγ expression plasmid. After 48 h, the cells were treated with various concentrations of 15d-$PGJ_2$ or $PGA_2$ for 15 h. (A,D). Viable cells were measured by counting the number of trypan blue-excluding cells. (B,E). DNA fragmentation was determined by the histone-associated DNA-fragments as described in Materials and Methods. The values were obtained in two independent experiments performed in triplicate and are represented as means ± SE. $*P < 0.05$ versus mock control. (C,F). AGS cells were cotransfected with the PPARα or PPARγ expression plasmid or PPAR mock plasmid, AOx-TK reporter plasmid, and pRL-TK plasmid as internal control. After transfection, the cells were treated with 5 μM 15d-$PGJ_2$ (C) or 10 μM $PGA_1$ (F) for 9 h and luciferase activity determined as described in Materials and Methods. The values were obtained in two independent experiments performed in duplicate and represented as means. The relative luciferase activity of drug-treated transfected cells to nontransfected cells was indicated underneath PPAR bands. Total cellular proteins (50 μg) of transfected cells were subjected to Western blotting with anti-PPARα, PPARγ, or GAPDH antibody.

PPARα expression plasmid was cotransfected into the cells, 15d-$PGJ_2$ and $PGA_1$ significantly induced the luciferase gene expression about 5.7-fold and 4.2-fold, respectively (Figure 3C and F, left panel, the number below the PPAR band). Similar results were obtained in the cell cotransfection of PPARγ expression plasmid; 15d-$PGJ_2$ and $PGA_1$ significantly induced luciferase gene expression (about 8.9-fold and 5.4-fold, respectively) (Figure 3C and F, right panel, the number below the PPAR band). These results indicated that both PPARα and PPARγ significantly transactivated the luciferase reporter gene and actually were functional in these cells by treatment with 15d-$PGJ_2$ and $PGA_1$ in AGS cells.

15d-PGJ$_2$ and PGA$_1$ decreased the viability and induced DNA fragmentation in a dose-dependent manner; however, it was equally effective in PPARα or PPARγ overexpression of cells (Figure 3A, B, D, and E). Treatment of mock-expression cells with 10 μM 15d-PGJ$_2$ and 10 μM PGA$_1$ resulted in induction of DNA fragmentation by 75% and 28%, respectively. However, similar effects of 15d-PGJ$_2$ and PGA$_1$ were observed in PPARα or PPARγ overexpression cells. These results indicated that overexpression of PPARα or PPARγ did not enhance apoptosis, and induction of apoptosis may be mediated by PPAR-independent pathway in AGS cells with 15d-PGJ$_2$ or PGA$_1$.

### Activation of JNK and Caspase-3 Activity by 15d-PGJ$_2$ and PGA$_1$

JNK is a classic stress-activated protein kinase and plays an important role in drugs inducing apoptosis. To examine whether JNK activity participated in 15d-PGJ$_2$- and PGA$_1$-induced apoptosis, we determined the kinase activity by an immunocomplex kinase assay as described in Materials and Methods. As shown in Figure 4, the activation of JNK was observed at the earliest measured time point of 1.5 h at a concentration of 10 μM 15d-PGJ$_2$ and 20 μM PGA$_1$. The activation of JNK by 15d-PGJ$_2$ or PGA$_1$ was in a time- and dose-dependent manner (Figure 4A and B).

### Blockade of Apoptosis by Transfection of Dominant-Negative Mutants of JNK

To assess directly the involvement of JNK in apoptosis by 15d-PGJ$_2$ or PGA$_1$, the DN mutant of JNK plasmid was used. As shown in Figure 5, overexpression of DN-JNK significantly inhibited 15d-PGJ$_2$ and PGA$_1$-induced cell death, DNA fragmentation, and caspase-3 activity. To verify that overexpression of DN-JNK efficiently decreased endogenous JNK activity, we determined the total JNK activity in both mock and DN-JNK overexpression cells by immunocomplex kinase assay. As shown in Figure 5D, overexpression of DN-JNK significantly decreased the activation of JNK induced by 15d-PGJ$_2$ and PGA$_1$ by >50%. In addition, Western blotting showed that total JNK protein was increased approximately twofold to threefold in the cells transfected with DN-JNK expression plasmid, clearly indicating DN-JNK overexpression in AGS cells (Figure 5D). These results indicated that activation of JNK was important in the induction of apoptosis in AGS cells treated with 15d-PGJ$_2$ and PGA$_1$.

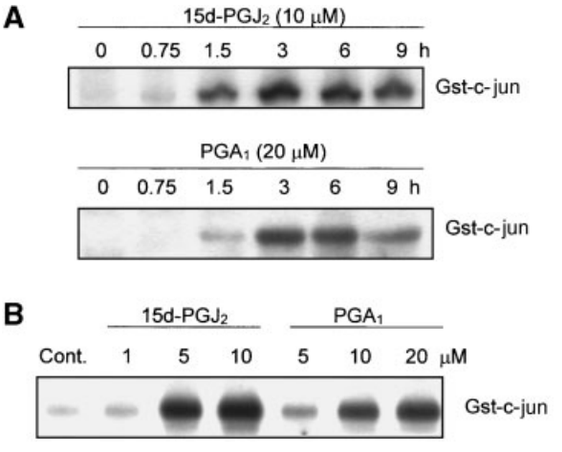


Figure 4. Effect of 15d-PGJ$_2$ and PGA$_1$ on the activation of JNK in AGS cells. (A) Cells were treated with 10 μM 15d-PGJ$_2$ or 20 μM PGA$_1$ for various times as indicated. (B) Cells were treated with various concentrations of 15d-PGJ$_2$ or PGA$_2$ for 3 h. JNK1 was immunoprecipitated from cell lysates and incubated with purified Gst-c-jun fusion protein as substrate in an in vitro kinase assay as described in Materials and Methods.

## DISCUSSION

We showed that 15d-PGJ$_2$- and PGA$_1$-induced apoptosis in human AGS gastric carcinoma cells required the activation of JNK. Activation of PPARγ has also been reported to induce apoptosis in various cell types, including choriocarcinoma cells, breast cancer cells, endothelial cells, and macrophages [39–41]. Recently, Harris and Phipps showed that PGD$_2$ and 15d-PGJ$_2$ have potent antiproliferative effects through activation of PPARγ [42]. However, here we described a different, PPAR-independent mechanism that explained the ability of 15d-PGJ$_2$ and PGA$_1$ to induce apoptosis in AGS cells.

The specified J$_2$-derivatives were potential metabolites of PGD$_2$ that were sequentially formed from this PG. A comparison of the PG biosynthetic pathway and the apoptosis induction profile revealed that induction of apoptosis was mediated mainly by the metabolites of PGD$_2$, the most active of which was the terminal metabolite of 15d-PGJ$_2$. The induction potency was 15d-PGJ$_2$ > PGJ$_2$ > PGD$_2$, indicating a gain in biological potency as the catabolism of PGD$_2$ proceeded. CyPGs are reactive compounds that possess an α, β-unsaturated carbonyl group in the cyclopentenone ring. These groups are very susceptible to nucleophilic addition reactions with sulfhydryl groups of cysteine residues of proteins or of the tripeptide glutathione [43]. It has been demonstrated that PGA$_1$ forms a bisconjugate with thiol-containing proteins [44], and 15d-PGJ$_2$ forms a covalent adduct with the p50 subunit of nuclear factor-κB [45], which results in modulation of protein functions. Based on functional evidence, a modification of IκB kinase [46] and of the p65 subunit of nuclear factor-κB [47] by 15d-PGJ$_2$ has also been proposed. In this study, we showed that 15d-PGJ$_2$ and PGA$_1$ activated the JNK pathway. Thus, whether 15d-PGJ$_2$ and PGA$_1$ activated JNK via interaction with JNK itself or with upstream proteins of JNK deserves further study.

Activation of the JNK signaling pathway has been mechanistically implicated in regulation of apoptosis. In contrast, several reports also indicate that JNK

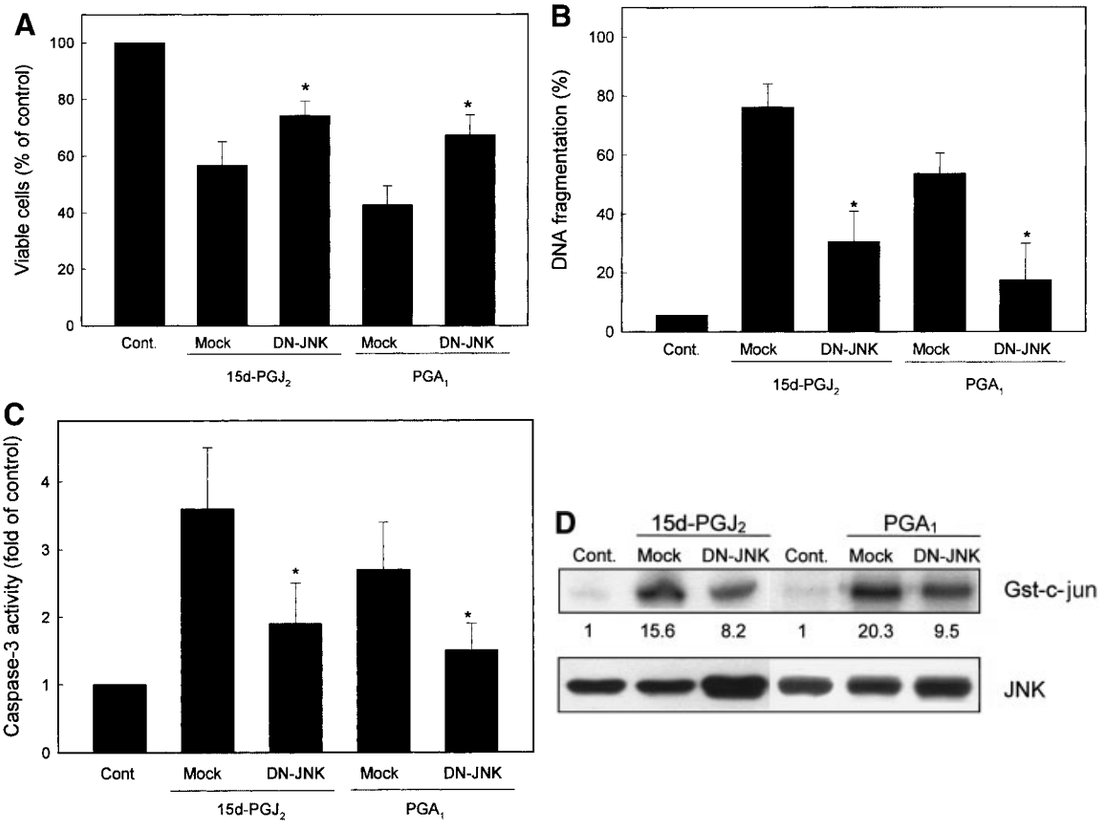


Figure 5. Effect of DN-JNK on the blocking of endogenous JNK activity, DNA fragmentation, and caspase-3 activity induced by 15d-$PGJ_2$ or $PGA_1$. Cells were transfected with mock or DN-JNK expression plasmid for 48 h. Transfected cells were treated with 5 μM 15d-$PGJ_2$ and 10 μM $PGA_2$ for 15 h. (A) Cells' viability was measured by counting the number of trypan blue–excluding cells. (B) DNA fragmentation was determined by the histone-associated DNA-fragments as described in Materials and Methods. The values were obtained in three independent experiments performed in triplicate and are represented as means ± SE. $^*P < 0.05$ versus mock control. (C) Transfected cells were treated with 5 μM 15d-$PGJ_2$ or 10 μM $PGA_1$ for 8 h and caspase-3 activity determined as described in Methods and Methods. The values were obtained in three independent experiments performed in triplicate and are represented as means ± SE. $^*P < 0.05$ versus mock control. (D) Transfected cells were treated with 5 μM 15d-$PGJ_2$ or 10 μM $PGA_1$ for 3 h, and determined the JNK activity as described in Materials and Methods. The number below the lanes indicate the relative intensities of JNK activity. Total cellular proteins (50 μg) of transfected cells were subjected to Western blotting with anti-JNK1 or GAPDH antibody.

plays a role in promoting or preventing apoptosis, depending on both cell type and apoptotic-triggering stimuli [48–50]. On the other hand, JNK activation may be a stress response secondary to apoptosis itself, rather than a primary mediator in the apoptotic pathway [50]. However, we showed that activation of JNK reached a peak as early as 3 h with 15d-$PGJ_2$ and $PGA_1$, at which time the apoptotic cells remained minimal (data not shown). These results suggested that JNK activation was not a secondary response to 15d-$PGJ_2$- and $PGA_1$-induced apoptosis. Several reports have demonstrated that the duration of JNK activation is a determining factor for cell proliferation or death [4]. Transient JNK activation, such as activation of JNK by phorbol 12-myristate 13-acetate plus ionomycin, resulted in cell proliferation not death. 15d-$PGJ_2$ and $PGA_1$ caused a more sustained activation pattern of JNK (Figure 4), suggesting that activation of JNK was directly linked to cell death in 15d-$PGJ_2$- and $PGA_1$-treated cells.

In summary, we found that 15d-$PGJ_2$ and $PGA_1$ represented the most potent inducers of apoptosis in AGS cells, and that activation of JNK was directly involved in 15d-$PGJ_2$- and $PGA_1$-induced apoptosis. The present study may therefore represent a first step in establishing a link between the activation of JNK and apoptosis induced by 15d-$PGJ_2$ and $PGA_1$.

## ACKNOWLEDGMENTS

This work was supported by a grant from the National Science Council (NSC 90-2320-B-038-050- and NSC 91-2320-B-038-030-), and a seed-grant from the Taipei Medical University (TMU 90-Y05-A101) and the Jin Lung Yen Foundation to J.-D. L.

## REFERENCES

1. Hibi M, Lin A, Smeal T, Minden A, Karin M. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Develop 1993;7:2135–2148.

2. Kyriakis JM, Banerjee P, Nikolakaki E, et al. The stress-activated protein kinase subfamily of c-Jun kinases. Nature 1994;369:156–160.
3. Raingeaud J, Gupta S, Rogers JS, et al. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 1995;270:7420–7426.
4. Chen YR, Meyer CF, Tan TH. Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in gamma radiation-induced apoptosis. J Biol Chem 1996;271:631–634.
5. Zanke BW, Boudreau K, Rubie E, et al. The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. Curr Biol 1996;6:606–613.
6. Galcheva-Gargova Z, Derijard B, Wu IH, Davis RJ. An osmosensing signal transduction pathway in mammalian cells. Science 1994;265:806–808.
7. Li YS, Shyy JY, Li S, et al. The Ras-JNK pathway is involved in shear-induced gene expression. Mol Cell Biol 1996;16: 5947–5954.
8. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 1995;270:1326–1331.
9. Verheij M, Bose R, Lin XH, et al. Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. Nature 1996;380:75–79.
10. Srivastava RK, Mi QS, Hardwick JM, Longo DL. Deletion of the loop region of Bcl-2 completely blocks paclitaxel-induced apoptosis. Pro Nat Acad Sci USA 1999;96:3775–3780.
11. Fan M, Goodwin ME, Birrer MJ, Chambers TC. The c-Jun NH(2)-terminal protein kinase/AP-1 pathway is required for efficient apoptosis induced by vinblastine. Cancer Res 2001; 61:4450–4458.
12. Osborn MT, Chambers TC. Role of the stress-activated/c-Jun NH2-terminal protein kinase pathway in the cellular response to adriamycin and other chemotherapeutic drugs. J Biol Chem 1996;271:30950–30955.
13. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 2000;103:239–252.
14. Le-Niculescu H, Bonfoco E, Kasuya Y, Claret FX, Green DR, Karin M. Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. Mol Cell Biol 1999;19:751–763.
15. Tournier C, Hess P, Yang DD, et al. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science 2000;288:870–874.
16. Fukushima M. Biological activities and mechanisms of action of PGJ2 and related compounds: An update. Prostagland Leukotri Essent Fatty Acids 1992;47:1–12.
17. Santoro MG, Garaci E, Amici C. Prostaglandins with antiproliferative activity induce the synthesis of a heat shock protein in human cells. Proc Nat Acad Sci USA 1989;86: 8407–8411.
18. Ohno K, Higaki J, Takechi S, Hirata M. Specific role of an alpha,beta-unsaturated carbonyl group in gamma-glutamylcysteine synthetase induction by prostaglandin A2. Chem Biol Interact 1990;76:77–87.
19. Tasaki Y, Takamori R, Koshihara Y. Prostaglandin D2 metabolite stimulates collagen synthesis by human osteoblasts during calcification. Prostaglandins 1991;41:303–313.
20. Ohtani-Fujita N, Minami S, Mimaki S, Dao S, Sakai T. p53-Independent activation of the gadd45 promoter by Delta12-prostaglandin J2. Biochem Biophy Res Commun 1998;251: 648–652.
21. Koizumi T, Odani N, Okuyama T, Ichikawa A, Negishi M. Identification of a cis-regulatory element for delta 12-prostaglandin J2-induced expression of the rat heme oxygenase gene. J Biol Chem 1995;270:21779–21784.
22. Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. Cell 1995;83:813–819.
23. Kondo M, Oya-Ito T, Kumagai T, Osawa T, Uchida K. Cyclopentenone prostaglandins as potential inducers of intracellular oxidative stress. J Biol Chem 2001;276:12076–12083.
24. Kondo M, Shibata T, Kumagai T, et al. 15-Deoxy-$\Delta^{12,14}$-prostaglandin $J_2$: The endogenous electrophile that induces neuronal apoptosis. Proc Nat Acad Sci USA 2002;99:7367–7672.
25. Clay CE, Atsumi GI, High KP, Chilton FH. Early de novo gene expression is required for 15-deoxy-Delta 12,14-prostaglandin J2-induced apoptosis in breast cancer cells. J Biol Chem 2001;276:47131–47135.
26. Liang YC, Lin-Shiau SY, Chen CF, Lin JK. Inhibition of cyclin-dependent kinases 2 and 4 activities as well as induction of Cdk inhibitors p21 and p27 during growth arrest of human breast carcinoma cells by (−)-epigallocatechin-3-gallate. J Cell Biochem 1999;75:1–12.
27. Liang YC, Huang YT, Tsai SH, Lin-Shiau SY, Chen CF, Lin JK. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis 1999;20:1945–1952.
28. Lin SY, Liang YC, Ho YS, Tsai SH, Pan S, Lee WS. Involvement of both extracellular signal-regulated kinase and c-Jun N-terminal kinase pathways in the 12-o-tetradecanoylphorbol-13-acetate-induced upregulation of $p21^{Cip1}$ in colon cancer cells. Mol Carcinogenesis 2002;35:21–28.
29. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature 1998;391: 79–82.
30. Chinetti G, Lestavel S, Bocher V, et al. PPAR-alpha and PPAR-gamma activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. Nature Med 2001;7:53–58.
31. Liang YC, Tsai DC, Lin-Shiau SY, Chen CF, Lin JK. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase through peroxisome proliferator-activated receptor-$\gamma$ by apigenin and related-flavonoids in macrophages. FEBS Lett 2001;496:12–18.
32. Shiah SG, Chuang SE, Kuo ML. Involvement of Asp-Glu-Val-Asp-directed, caspase-mediated mitogen-activated protein kinase kinase 1 cleavage, c-Jun N-terminal kinase activation, and subsequent bcl-2 phosphorylation for paclitaxel-induced apoptosis in HL-60 cells. Mol Pharmacol 2001;59:254–262.
33. Fukushima M. Prostaglandin J2—anti-tumour and anti-viral activities and the mechanisms involved. Eicosanoids 1990;3: 189–199.
34. Rossi A, Elia G, Santoro MG. 2-Cyclopenten-1-one, a new inducer of heat shock protein 70 with antiviral activity. J Biol Chem 1996;271:32192–32196.
35. Rohn TT, Wong SM, Cotman CW, Cribbs DH. 15-deoxy-delta12,14-prostaglandin J2, a specific ligand for peroxisome proliferator-activated receptor-gamma, induces neuronal apoptosis. Neuro Report 2001;12:839–843.
36. Chattopadhyay N, Singh DP, Heese O, et al. Expression of peroxisome proliferator-activated receptors (PPARS) in human astrocytic cells: PPARgamma agonists as inducers of apoptosis. J Biol Chem 2000;61:67–74.
37. Keelan JA, Sato TA, Marvin KW, Lander J, Gilmour RS, Mitchell MD. 15-Deoxy-Delta (12,14)-prostaglandin J(2), a ligand for peroxisome proliferator-activated receptor-gamma, induces apoptosis in JEG3 choriocarcinoma cells. Biochem Biophy Res Commun 1999;262:579–585.
38. Chinetti G, Griglio S, Antonucci M, et al. Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. J Biol Chem 1998;273:25573–25580.
39. Elstner E, Müller C, Koshizuka K, et al. Ligands for peroxisome proliferator-activated receptor$\gamma$ and retinoic acid

receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. Proc Nat Acad Sci USA 2001;95:8806–8811.
40. Bishop-Bailey D, Hla T. Endothelial cell apoptosis induced by the peroxisome proliferator-activated receptor (PPAR) ligand 15-deoxy-Delta12, 14-prostaglandin J2. J Biol Chem 1999; 274:17042–17048.
41. Chinetti G, Griglio S, Antonucci M, et al. Activation of proliferator-activated receptors alpha and gamma induces apoptosis of human monocyte-derived macrophages. J Biol Chem 1998;273:25573–25580.
42. Harris SG, Phipps RP. Prostaglandin D(2), its metabolite 15-d-PGJ(2), and peroxisome proliferator activated receptor-gamma agonists induce apoptosis in transformed, but not normal, human T lineage cells. Immunology 2002;105:23–34.
43. Atsmon J, Sweetman BJ, Baertschi SW, Harris TM, Roberts LJ 2nd. Formation of thiol conjugates of 9-deoxy-delta 9,delta 12(E)-prostaglandin D2 and delta 12(E)-prostaglandin D2. Biochemistry 1990;29:3760–3765.
44. Parker J, Ankel H. Formation of a prostaglandin A2-glutathione conjugate in L1210 mouse leukemia cells. Biochem Pharmacol 1992;43:1053–1060.
45. Cernuda-Morollon E, Pineda-Molina E, Canada FJ, Perez-Sala D. 15-Deoxy-Delta 12,14-prostaglandin J2 inhibition of NF-kappaB-DNA binding through covalent modification of the p50 subunit. J Biol Chem 2001;276:35530–35536.
46. Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M, Santoro MG. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. Nature 2000;403:103–108.
47. Straus DS, Pascual G, Li M, et al. 15-deoxy-delta 12,14-prostaglandin J2 inhibits multiple steps in the NF-kappa B signaling pathway. Proc Nat Acad Sci USA 2000;97:4844–4849.
48. Zanke BW, Lee C, Arab S, Tannock IF. Death of tumor cells after intracellular acidification is dependent on stress-activated protein kinases (SAPK/JNK) pathway activation and cannot be inhibited by Bcl-2 expression or interleukin 1beta-converting enzyme inhibition. Cancer Res 1998;58: 2801–2808.
49. Nishina H, Fischer KD, Radvanyi L, et al. Stress-signalling kinase Sek1 protects thymocytes from apoptosis mediated by CD95 and CD3. Nature 1997;385:350–353.
50. Ip YT, Davis RJ. Signal transduction by the c-Jun N-terminal kinase (JNK)—from inflammation to development. Curr Opin Cell Biol 1998;10:205–219.
51. www.CaymanChem.com