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Study on the molecular mechanisms of anti-carcinogenic and anti-inflammatory effects of peroxisome proliferator-activated receptor (PPAR) ligands

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ABSTRACT

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 that various eicosanoids on the inhibition of cell proliferation, and found that the terminal derivative of PGJ_2 metabolism, 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2), and PGA_1 markedly inhibited growth and induced apoptosis in AGS gastric carcinoma cells. There were no significant increase in DNA-fragmentation in the cells with overexpression of PPARα or PPARγ plasmid, indicating 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 the caspase-3 activity in dose- and time-dependent manners. To further examine the role of JNK signaling cascades in apoptosis induced by 15d-PGJ_2 and PGA_1, we transfected dominant-negative (DN) mutants of JNK into the cells to analyze the apoptotic characteristics of cells expressing DN-JNK plasmid following exposure to 15d-PGJ_2 and PGA_1. Expression of DN-JNK proteins repressed both of endogenous JNK and caspase-3 activity, and subsequently inhibited apoptosis induced by 15d-PGJ_2 and PGA_1. These results suggest that CyPGs, such as 15d-PGJ_2 and PGA_1, activates the JNK signaling pathway, and that JNK activation may be involved in 15d-PGJ_2 and PGA_1 induced cell death.

Key words: prostaglandins; apoptosis; JNK

INTRODUCTION

The c-Jun N-terminal kinases (JNKs) members of mitogen-activated protein kinase (MAPKs), are classic stress-activated protein kinase (1), can be activated by proinflammatory cytokines (2, 3) and environmental stress such as UV 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 had play an important mediator of apoptosis signaling (13). For example, JNK activation was associated with apoptosis in PC-12 cells deprived of NGF (14). In addition, nonneuronal embryonic fibroblasts with targeted disruptions of the *jnk1* and *jnk2* genes, were able to inhibit apoptosis (15). These reports suggested 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 are oxygenated metabolites of arachidonic acid (AA), and divided into two groups, conventional prostaglandins and cyclopentenone prostaglandins (CyPGs) according to their mechanisms of action (16). The conventional eicosanoids, such as PGE_2 and PGD_2, bind to cell surface receptor and transduces 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). Several proteins have been identified to be induced by the CyPGs, such as heat shock proteins (17), γ-glutamylcysteine synthetase (18), collagen (19), gadd 45 (20), and heme oxygenase (21). Arachidonic acid 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. 15d-PGJ_2 is derived from PGD_2 and involves sequential conversion of PGD_2, PGI_2, $\triangle^{12}$-PGJ_2 and 15d-PGJ_2 (16). 15d-PGJ_2 has been shown to bind to and activate peroxisome proliferator-activated receptor γ (PPARγ) and is therefore an intracellular target of this cyclopentenone prostaglandin (22).

The CyPGs have been demonstrated that possess potent antiproliferative and antitumor activities, but their mechanisms of action are complex and not well understood. Recently it was shown that PGD_2 metabolites, such as PGJ_2, $\triangle^{12}$-PGJ_2, and 15d-PGJ_2, were potential inducers of intracellular oxidative stress that mediated the cytotoxic effects in human neuroblastoma cells (23). Uchida and colleagues showed that the 15d-PGJ_2-induced accumulation of p53 resulted in the activation of a death-inducing caspase cascade mediated by Fas and the Fas ligand in neurons (24). Another studies showed that de novo gene transcription was necessary for 15d-PGJ_2-induced apoptosis in breast cancer cells (25). The current investigation was undertaken to study the effects of arachidonic acid and its metabolites on human gastric cells death. The results demonstrated that 15d-PGJ_2 and PGA_1 induced apoptosis, activated JNK and caspase-3 activity in AGS cells. Moreover, the cells were decrease in apoptosis with overexpression of DN-JNK plasmid, indicating that induction of apoptosis by 15d-PGJ_2 and PGA_1may be mediated the activation of JNK.

MATERIALS AND METHODS

Materials

PGA_1, PGJ_2, and LTB_4 were purchased from Caymen Chemical (Ann Arbor, MI), and arachidonic acid, PGD_2, PGE_2, $PGF_{2\alpha}$, PGH_2, TBX_2 and 15d-PGJ_2 were purchased from Biomol (Plymouth Meeting, PA).

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 DMEM/F12 containing 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY). For all assays, cells were plated in 6-cm dishes at 5×10^6 cells per dish and allowed to grow for 24 h.

Western Blot

Equal amounts of total cellular protein (50 μg) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred onto Immobilon-P membrane (Millipore, Bedford, MA) as described previously (26). The membrane was then incubated with an anti-PARP or JNK antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) and subsequently probed with anti-mouse antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) and visualized using enhanced chemiluminescence kits (Amersham, Arlington, IL).

Kinase Assay

Equal amounts of total cellular protein (200 μg) were immunoprecipitated with JNK1 or ERK1 specific antibody (Santa Cruz Biotechnology) and protein A/G-PLUS agarose for 12 h at 4℃. Kinase assay was performed in kinase buffer with Gst-c-Jun fusion protein (for JNK, Santa Cruz Biotechnology) or myelin basic protein (for ERK, Sigma) as substrates as previously described (27).

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 expression plasmid and PPARα expression plasmid were generously provided by Professor Christopher K. Glass (California University) and Professor Bart Staels (Université de Lille), respectively (29, 30). AGS cells were seeded in 6-cm dishes, and transfected with the mock, DN-JNK, PPARγ, or PPARα expression plasmid using LipofectAMINE^{TM} 2000 (Gibco) for 48 h (31). After transfection, the cells were treated with 15d-PGJ_2 or PGA_1, and collected cells for DNA fragmentation, caspase activity, or kinase assay.

DNA Fragmentation

After treatment with tested drugs, the cells were washed with PBS and detected the DNA fragmentation by Cell Death Detection ELISA 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 extracted the cytosolic fraction with extraction buffer (12.5 mM Tris, pH7.4, 2 mM DTT, 0.125 mM EDTA, 5% glycerol, and 1 μg/ml leupeptin, and 1 μg/ml aprotinin) by repeated cycles of freezing and thawing. Cell lysate (150 μg) were incubated with 10 μM fluorescence substrate, Ac-DEVD-AMC (Calbiochem, La Jolla, CA) in assay buffer (50 mM Tris, pH7.4, 1 mM EDTA and 10 mM EGTA) at 37°C for 30 min in dark. The fluorescence intensity of the cleaved substrate was measured using a fluorescence spectrophotometer (Hitachi F-3000) by Ex 380 nm/Em 460 nm (32).

Flow Cytometry

SubG1 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 using a FACscan laser flow cytometer (Becton Dickinson, San Jose, CA).

RESULTS

15d-PGJ_2 and PGA_1 Inhibited the Growth of Human AGS Gastric Carcinoma Cells

To examine whether CyPGs inhibit growth of AGS cells, we screened a large number of arachidonic acid and 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 (Fig. 1A). Among the classes of PGs examined, 15d-PGJ_2 and PGA_1 were most effective. 10 μM 15d-PGJ_2 and 20 μM PGA_1 reduced the cell number (Fig. 1) by 80% and 50%, respectively. The terminal metabolites 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. Arachidonic acid, 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 (Fig. 1B), which renders this portion of the molecule able to form Michael assucts with cellular nucleophilics and covalently modify specific protein (33, 34), is essential for apoptosis.

We next examined the effects of different concentrations of 15d-PGJ_2 and PGA_1 on the induction of AGS cells apoptosis. As shown in Fig. 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 subG_1 DNA content as a measure of apoptosis and observed an increase in the apoptosis cells (Fig. 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 Fig. 2C, caspase-3 activity was detected in AGS cells with 10 μM 15d-PGJ_2 and 20 μM PGA_1 at 8 h. The substrate of caspase-3, poly(ADP ribose) polymerase (PARP) also cleaved in 15d-PGJ_2 (Fig. 2D) and PGA_1 (Data not shown). These results indicated that 15d-PGJ_2 and PGA_1 inhibited the growth through induction of apoptotic cell death and the caspase-3 pathway.

15d-PGJ_2 and PGA_1 Induced Apoptosis Through PPAR-Independent Pathway

Since 15d-PGJ_2 or PGA_1 was a potent agonist of $PPAR_\alpha$ and $PPAR_\gamma$. Activation of $PPAR_\alpha$ or $PPAR_\gamma$ can promotes cells apoptosis in other cells (38). To examine this possibility, AGS cells were transfected with mock, $PPAR_\alpha$, or $PPAR_\gamma$ expression plasmid, and treated with 15d-PGJ_2 or PGA_1. The DNA fragmentation were measured and shown the results in Fig. 3. 15d-PGJ_2 and PGA_1 induced DNA fragmentation in mock-expression AGS cells, however, it was equally effective in $PPAR_\alpha$ or $PPAR_\gamma$-overexpression AGS cells. Treatment of mock-expression AGS 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 $PPAR_\alpha$ or $PPAR_\gamma$-overexperssion AGS cells. These results indicated that overexpression of $PPAR_\alpha$ or $PPAR_\gamma$ did not enhance the apoptosis, and induction of apoptosis may be mediated PPAR-independent pathway in the cells with 15d-PGJ_2 or PGA_1.

15d-PGJ_2 and PGA_1 Activated the JNK and Caspase-3 Activity

Since JNK is a classic stress-activated protein kinase and play an important role in drugs inducing apoptosis. To examine whether activity of JNK participated in the 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 Fig. 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 a time- and dose-dependent manner (Fig. 4).

Dominant-Negative Mutants of JNK Blocked Apoptosis Induced by 15d-PGJ_2 or PGA_1

To assess directly the involvement of JNK in apoptosis by 15d-PGJ_2 or PGA_1, dominant-negative (DN) mutants of JNK plasmid were used. As shown in Fig. 5, overexpression of DN-JNK significantly inhibited 15d-PGJ_2 and PGA_1-induced 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 expression cells by immunocomplex kinase assay. As shown in Fig. 5C, transfection of DN mutant of JNK exactly resulted in the reduction of JNK activity about > 50% induced by 15d-PGJ_2 and PGA_1. These results indicated that the JNK-mediated induction of apoptosis is important for the treatment of 15d-PGJ_2 and PGA_1 in AGS cells.

DISCUSSION

We here show that 15d-PGJ_2 and PGA_1-induced apoptosis in human AGS gastric carcinoma cells requires the activation of JNK. Activation of $PPAR_\gamma$ 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 $PPAR_\gamma$ (42). However, here we have described a different, PPAR-independent mechanism that explains the ability of 15d-PGJ_2 and PGA_1 to induce apoptosis in AGS cells.

The specified J_2-derivatives are potential metabolites of PGD_2 that are sequentially formed from this prostaglandin. A comparison of the PG biosynthetic pathway and the induction of apoptosis profile revealed that induction of apoptosis was mediated mainly by the metabolites of PGD_2, the most active of which is 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 proceeds. CyPGs are reactive compounds that possess a 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). Since it has been demonstrated that PGA_1 formed a bisconjugate with thiol-containing proteins (44), and 15d-PGJ_2 formed a covalent adduct with the p50 subunit of NFκB (45), which resulted in modulation of protein functions. Based on functional evidence, a modification of IKK (46) and of the p65 subunit of NFκB (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 activate JNK via interaction with JNK itself or 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 indicated that JNK play 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 apoptotic pathway (50). However, we showed that activation of JNK reached a peak at early 2 h with 15d-PGJ_2 and PGA_1, in which the apoptotic cells are still minimal (Data not shown). These results suggested that JNK activation was not a secondary response to 15d-PGJ_2 and PGA_1-induced apoptosis. In addition, 15d-PGJ_2 and PGA_1 caused a more sustained pattern of JNK activation (Fig. 4), suggesting that activation of JNK was directly linked to cell death in 15d-PGJ_2 and PGA_1 treatment cells. Since several reports have demonstrated that the duration of JNK activation is a determining factor for cell proliferation or death (4). Transient JNK activation leaded cell proliferation not death, such as activation of JNK by PMA plus ionomycin.

In summary, we have found that 15d-PGJ_2 and PGA_1 represent the most potent inducers of apoptosis in AGS cells, and that JNK is directly involved in 15d-PGJ_2 and PGA_1-induced program cell death. 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.

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LEGENDS TO FIGURES

Fig. 1. Effect of arachidonic acid (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 expressed as mean ± S.E. of triplicate test. *, $p < 0.05$ versus control. (B). Structures of arachidonic acid and its metabolites.

Fig. 2. Effect of 15d-PGJ_2 and PGA_1 on the DNA fragmentation, caspase-3 activity, and PARP cleavage in AGS cells. Cells were treated with different concentration of PGA_1 or 15d-PGJ_2 for 15 h. (A). DNA fragmentation was determined by the histone-associated DNA-fragments as described in "Materials and Methods." The values were expressed as mean ± S.E. of triplicate test. *, $p < 0.05$ versus control. (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 subG1 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 assay as described in "Materials and Methods". The values were expressed as mean ± S.E. of triplicate test. *, $p < 0.05$ versus control. (D). Cells were treated with different concentrations of 15d-PGJ_2 for 15 h. Total cell lysates (50 μg) were subjected to Western blot by using anti-PARP antibody. The intact (112 kD) and cleaved (86 kD) species of PARP were indicated in right of the panel.

Fig. 3. Effect of overexpression of PPARα or PPARγ plasmid on the induction of DNA fragmentation by 15d-PGJ_2 or PGA_1. Cells were transfected with PPARα or PPARγ expression plasmid for 48 h, and treated with different concentrations of 15d-PGJ_2 or PGA_1 for 15 h. DNA fragmentation was determined by the histone-associated DNA-fragments as described in "Materials and Methods." The values were expressed as mean ± S.E. of triplicate test. *, $p < 0.05$ versus mock control.

Fig. 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 different concentrations of 15d-PGJ_2 or PGA_1 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”.

Fig. 5. Effect of dominant-negative 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 expression plasmid or DN-JNK expression plasmid for 48 h. (A). Transfected cells were treated with 10 μM 15d-PGJ_2 and 20 μM PGA_1 for 15 h. DNA fragmentation was determined by the histone-associated DNA-fragments as described in “Materials and Methods.” The values were expressed as mean ± S.E. of triplicate test. *, $p < 0.05$ versus control. (B). Transfected cells were treated with 10 μM 15d-PGJ_2 or 20 μM PGA_1 for 3 h or 8 h, and determined the caspase-3 activity (8 h), and JNK activity (3 h) as described in “Methods and Methods.” The values were expressed as mean ± S.E. of triplicate test. *, $p < 0.05$ versus control.

Fig. 1 Liu et al.

A.

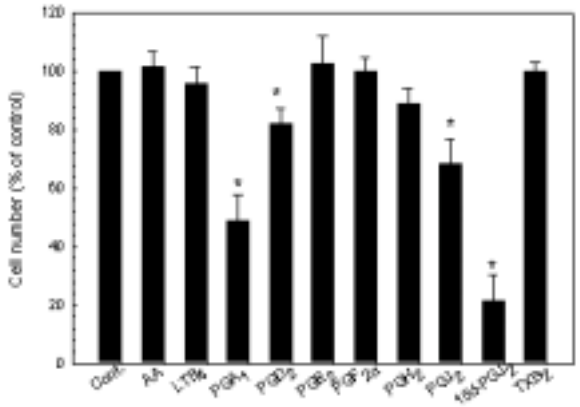
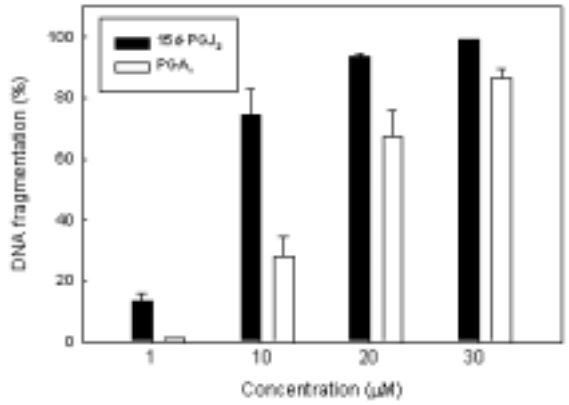


Fig. 1 Liu et al.

B.

Fig. 2 Liu et al.

A.



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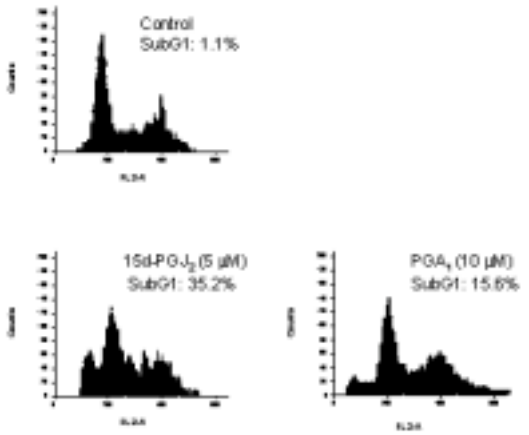
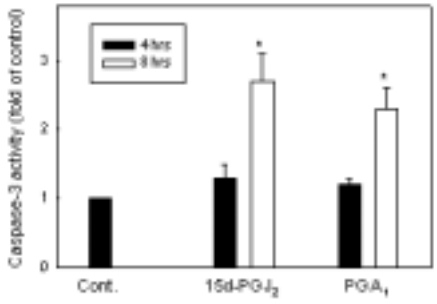


Fig. 2 Liu et al.

C.



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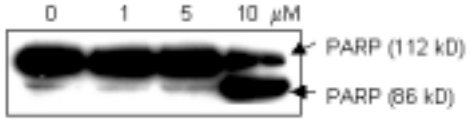
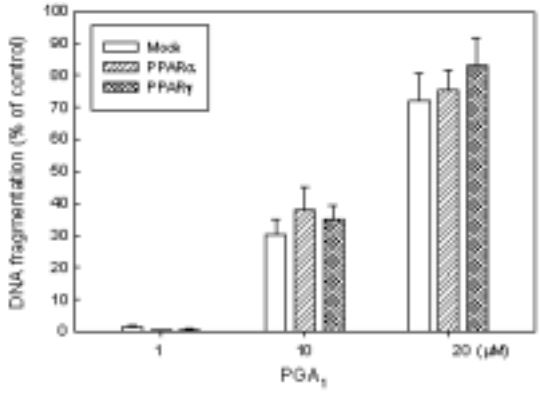


Fig. 3 Liu et al.

A.



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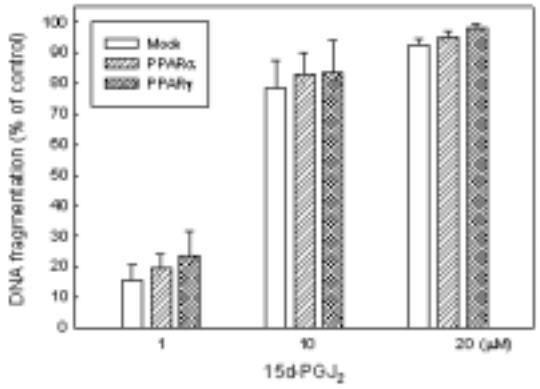

Fig. 4 Liu et al.

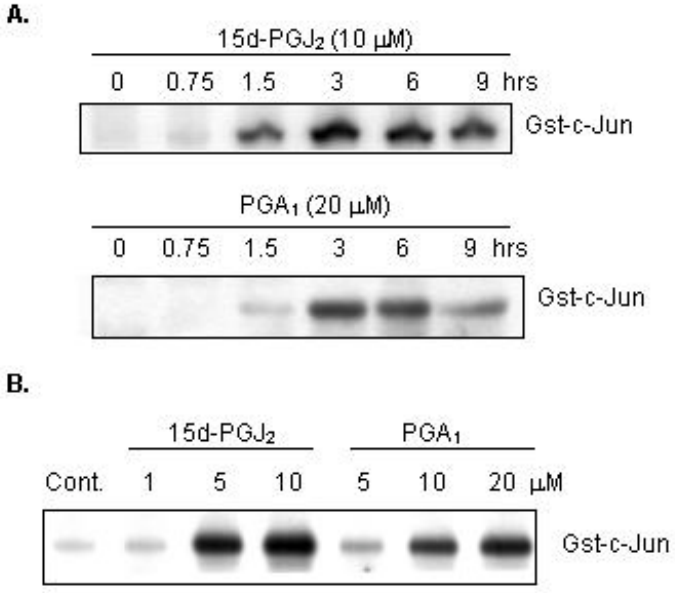
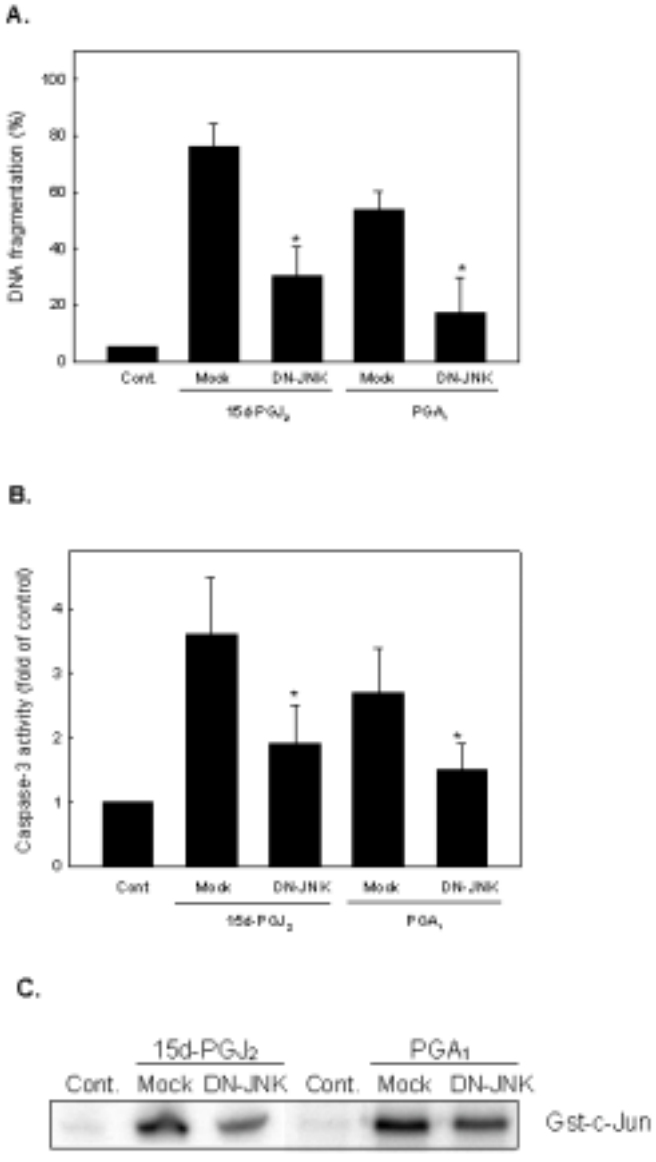
Fig. 5 Liu et al.