

# 行政院國家科學委員會專題研究計畫 成果報告

## 探討 PPAR 配位体抑癌作用及引起細胞分化之分子機制：体 外與体内試驗

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計畫參與人員：林仁混，劉正典，林時宜，何元順，潘憲，洪苓方，蔡淑慧

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## 中、英文摘要及關鍵詞 (keywords)

### Abstract

Heme oxygenase-1 (HO-1) is induced as a beneficial and adaptive response in cells and tissues exposed to oxidative stress. Herein we examined how various eicosanoids affect the induction of HO-1, and the possible mechanism underlying 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>)-induced HO-1 expression. PGH<sub>2</sub>, PGD<sub>2</sub> and its metabolites of the PGJ<sub>2</sub> series, and PGA<sub>1</sub> markedly induced the protein expression of HO-1. Arachidonic acid (AA), docosahexaenoic acid (DHA), PGE<sub>2</sub>, PGF<sub>2 $\alpha$</sub> , and thromboxane B<sub>2</sub> (TXB<sub>2</sub>) were shown to have no effect on the induction of HO-1. 15d-PGJ<sub>2</sub> was the most potent activator achieving significance at 5  $\mu$ M. Although 15d-PGJ<sub>2</sub> significantly activated the MAPKs of JNK and ERK, the activation of JNK and ERK did not contribute to the induction of HO-1 as determined using transfection of dominant-negative plasmids and MAPKs inhibitors. Additional experiment indicated that 15d-PGJ<sub>2</sub> induced HO-1 expression through peroxisome proliferator-activated receptor (PPAR)-independent pathway. 15d-PGJ<sub>2</sub> significantly decreased the intracellular level of reduced glutathione; and the thiol antioxidant, N-acetyl-L-cysteine (NAC), and the thiol-reducing agent, dithiothreitol (DTT), inhibited the induction of HO-1 by 15d-PGJ<sub>2</sub>. Finally, NAC and DTT exhibited significant inhibition of HO-1 mRNA and HO-1 promoter reporter activity induced by 15d-PGJ<sub>2</sub>. These results suggest that thiol antioxidant and reducing agents attenuate the expression of HO-1 induced by 15d-PGJ<sub>2</sub>, and that the cellular thiol-disulfide redox status may be linked to HO-1 activation.

*Keywords:* Heme oxygenase-1; 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub>; N-Acetyl-L-cysteine; Dithiothreitol; Glutathione.

### 摘要

細胞在受到氧化壓力時，會引起 heme oxygenase-1 (HO-1) 蛋白的表現，使細胞傷害降至最低。利用一系列前列腺素衍生物，我們發現 peroxisome proliferator-activated receptor (PPAR) 內生性配位體 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>)，可引起 HO-1 的大量表現。其可能機制與活化 MAPKs 路徑無關；並且不經由其受體 PPAR。進一步的研究發現，15d-PGJ<sub>2</sub> 可顯著降低細胞內還原態穀胱甘肽，若細胞先處理硫基還原劑，如乙醯胱胺酸及二硫鮮糖醇，則可有效恢復細胞內還原態穀胱甘肽，並且抑制 HO-1 的表現。結果顯示 PPAR 配位體 15d-PGJ<sub>2</sub> 是先影響細胞內硫基氧化還原狀態，進而引起 HO-1 的表現。

關鍵字：血基質氧化酶甲，前列腺素 J<sub>2</sub>，乙醯胱胺酸，二硫鮮糖醇，穀胱甘肽

### 報告內容

#### INTRODUCTION

Heme oxygenase (HO, EC 1.14.99.3) is a microsomal enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism, which converts heme to biliverdin and releases

equimolar amounts of carbon monoxide (CO) and free iron (Maines, 1997; Ponka, 1999). Three isoforms transcribed from separate genes have been characterized. HO-1 is an inducible form found in large quantities in the liver and spleen, and HO-2 is a constitutively active form found mainly in the brain and testes (McCoubrey and Maines, 1994). Another HO isoform, HO-3, has only recently been described with properties similar to those of HO-2 (McCoubrey et al., 1997). Poss and Tonegawa (1997a, b) showed that HO-1-deficient mice are hypersensitive to cytotoxicity when given additional hydrogen peroxide, indicating that HO-1 plays an important role in the cytoprotective defense response against oxidative stress (Choi and Alam, 1996). HO-1 gene expression is upregulated by both inflammatory mediators and anti-inflammatory cytokines, such as LPS, IL-1 $\beta$ , and IL-10 (Yet et al., 1997; Lee and Chau, 2002). However, much evidence has suggested that upregulation of HO-1 contributes to the anti-inflammatory action of cells and tissues. Minamino et al. (2001) demonstrated that HO-1 transgenic mice are protected from pulmonary inflammation and vessel wall hypertrophy induced by hypoxia. CO, a product of HO, inhibits the expression of LPS-induced proinflammatory cytokines (Muller et al., 1987), and the anti-inflammatory interleukin-10 mediates the induction of HO-1 (Lee and Chau, 2002).

Arachidonic acid is first converted to PGH<sub>2</sub> by cyclooxygenase and subsequently converted to one of several related products, including PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2</sub>, PGI<sub>2</sub>, and thromboxane A<sub>2</sub>, through the action of specific PG synthases. 15d-PGJ<sub>2</sub> is derived from PGD<sub>2</sub> and involves the sequential conversion of PGD<sub>2</sub>, PGI<sub>2</sub>,  $\Delta^{12}$ -PGJ<sub>2</sub>, and 15d-PGJ<sub>2</sub> (Fukushima, 1992). Several proteins have been identified which are induced by cyclopentenone PGs, such as heat shock proteins (Santoro et al., 1989),  $\gamma$ -glutamylcysteine synthetase (Ohno et al., 1990), collagen (Tasaki et al., 1991), gadd 45 (Ohtani-Fujita et al., 1998), and heme oxygenase (Koizumi et al., 1995). Although their intracellular receptor has not been described, 15d-PGJ<sub>2</sub> was shown to be a high-affinity ligand for peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) (Kliwer et al., 1995). Recently, PPAR $\gamma$  has been considered to have anti-inflammatory actions through activation by arachidonic acid metabolites, such as 15d-PGJ<sub>2</sub> (Jiang et al., 1998; Ricote et al., 1998; Ricote et al., 1999). 15d-PGJ<sub>2</sub> represses several genes related to inflammation, including the inducible nitric oxide synthase (iNOS) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) genes in activated macrophages (Ricote et al., 1998). However, the repression is partly dependent on PPAR $\gamma$  expression (Chawla et al., 2001), and receptor-independent biological actions of 15d-PGJ<sub>2</sub> and other cyclopentenone PGs have been proposed (Rossi et al., 2000; Straus et al., 2000). Other studies have indicated that some cyclopentenone PGs induce the synthesis of HO-1 in cells of the mice and rat, but the mechanism underlying 15d-PGJ<sub>2</sub>-induced HO-1 protein synthesis in human cells has been largely unexplored. We report herein that PGD<sub>2</sub>, PGA<sub>1</sub>, and the J series of PGs markedly induced HO-1 protein synthesis in human HepG2 hepatoma cells, and that the induction of HO-1 by 15d-PGJ<sub>2</sub> may be mediated by modulation of the cellular thiol-disulfide redox status.

## **MATERIALS AND METHODS**

### ***Materials and Cell Culture***

DHA, PGA<sub>1</sub>, PGJ<sub>2</sub>, 16,16-dimethyl PGD<sub>2</sub>, and BRL49653 were purchased from Cayman Chemical (Ann Arbor, MI), while arachonic acid, PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2</sub>, PGH<sub>2</sub>, TXB<sub>2</sub>, 15d-PGJ<sub>2</sub>,

PD98059, SB203580, 2',5'-dideoxyadenosine, WY-14643, ciglitazone, and indomethacin were purchased from Biomol (Plymouth Meeting, PA). Vitamin C, vitamin E, allopurinol, NAC, wortmannin, and DTT were purchased from Sigma Chemical (St. Louis, MO). The human HepG2 hepatoma cell lines were cultured in MEM containing 10% heat-inactivated fetal bovine serum and 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA).

### ***Western Blot Analysis***

Equal amounts of total cellular protein (50 µg) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto an Immobilon-P membrane (Millipore, Bedford, MA), and blotted with anti-HO-1, anti-HO-2 (BD Biosciences, Franklin Lakes, NJ), anti-JNK1, anti-ERK1 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-PPAR $\gamma$  (Affinity Bioreagents, Inc., Golden, CO) antiserum as described previously (Liang et al., 1999a).

### ***Reverse-Transcription Polymerase Chain Reaction (RT-PCR)***

Total RNA was isolated from both the control and tested cultured cells, and RT-PCR was performed as previously described (Liang et al., 1999b). Two sets of primers were used to amplify the mRNA of HO-1 and HO-2: for HO-1, they were 5'-TGATAGAAGAGGCCAAGA-3' and 5'-TTTCCAGAGAGAGGGACA-3'; and for HO-2, they were 5'-TGGAGCGCAACAAGGACCAT-3' and 5'-CCGGTAGAGCTGCTTGAAC-3'.

### ***Kinase Assay***

Equal amounts of total cellular protein (200 µg) were immunoprecipitated with JNK1-, p38-, or ERK1-specific antibodies (Santa Cruz Biotechnology) and protein A/G-PLUS agarose for 12 h at 4 °C. The kinase assay was performed in kinase buffer with Gst-c-Jun fusion protein (for JNK), Gst-ATF2 (for p38), or myelin basic protein (for ERK) as substrates as previously described (Liang et al., 1999b).

### ***Plasmids and Transient Transfection***

The dominant-negative (DN)-ERK1 expression plasmid was generously provided by Prof. Peter E. Shaw, The University of Nottingham (Robbins et al., 1993), and the DN-JNK expression plasmid was constructed from human JNK1 with a double-point mutation of Thr183 to Ala and Tyr185 to Phe (Lin et al., 2002). The PPAR $\gamma$  expression plasmid was generously provided by Professor Christopher K. Glass (University of California-San Diego).

The pGL2/hHO3.2-Luc reporter plasmid, containing a 3292-bp fragment, -3106 to +186 relative to the transcription start site of the human HO-1 gene, was amplified from the human BAC clone CTA-286B10 (Kim et al., 1996) using the primers 5'-AGAGAACAGTTAGAAAAGAAAG-3' and 5'-TACGGGCACAGGCAGGATCAGAA-3'. The PCR products were inserted into the pCR2.1-TOPO cloning vector (Invitrogen), and cut with Kpn I/Xba I such that the resulting PCR products contained the Kpn I/Xba I sites, and was ligated in-frame into the unique Kpn I/Nhe I sites present within the pGL2 plasmid (Promega, Madison, WI). Therefore, we obtained a pGL2/hHO3.2-Luc reporter construct containing about a 3.2-kb region of the human HO-1 promoter driving luciferase gene expression. Sequence identities were confirmed using an ABI PRISM 377 DNA analysis system (Perkin-Elmer Corp., Taipei, Taiwan).

HepG2 cells were seeded in 60-mm dishes and either mock-treated or transfected with DN-JNK, DN-ERK, or PPAR $\gamma$  plasmid using LipofectAMINE<sup>TM</sup> 2000 (Gibco) for 48 h (Liang et

al., 2001). After transfection, cells were then treated with 15d-PGJ<sub>2</sub>, and the cell lysate was collected for the kinase assay or Western blot analysis. For the reporter plasmid assay, HepG2 cells were seeded in 6-well plates for 24 h. Then cells were transfected with the pGL2e-hHO3.2 reporter plasmid, and phRL-TK (Promega) as an internal control using LipofectAMINE 2000™ (Invitrogen). After 12 h of transfection, the medium was replaced with complete medium and incubated for another 24 h. Transfected cells were then treated with drugs for the luciferase activity assay. Each well was washed twice with cold PBS and harvested in 100 µl of lysis buffer (0.5 M HEPES, pH 7.8, 0.5% Triton N-101, 1 mM CaCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>). Then 75 µl of cell lysate was placed into a 96-well white plate, and the luciferase activity was determined using a FireLite™ luciferase reporter gene assay kit (Packard Instrument Co., Meriden, CT). Luciferase activity was measured on a TopCount microplate scintillation and luminescence counter (Packard 9912V1) in the single-photon counting mode for 3 s/well. Luciferase activities of reporter plasmids were normalized to luciferase activities of the internal control plasmid.

### ***Glutathione Assay***

Cells were washed twice with PBS, extracted with a 25% (w/v) metaphosphoric acid solution containing 5 mM EDTA, and centrifuged at 12,000 rpm for 10 min to precipitate the proteins. The supernatant was incubated with 5 mM EDTA and 100 µg *O*-phthalaldehyde, and then the fluorescence intensity was determined by excitation at 350 nm and emission at 420 nm (Kim et al., 2001).

### ***Statistical Analysis***

Data are presented as the mean ± S.E. for the indicated number of independently performed experiments. Statistical analysis was done using one-way Student's *t* test.

## **RESULTS**

### ***Prostaglandins as Potential HO-1 Activators***

To identify the endogenous activator of HO-1 expression, we screened a large number of lipophilic chemicals, including DHA, arachidonic acid, and its metabolites, and found that HO-1 expression was potently induced by some of the PG derivatives. As shown in Fig. 1A, the protein expression of HO-1 in HepG2 cells was significantly induced by PGA<sub>1</sub>, PGH<sub>2</sub> metabolites including PGH<sub>2</sub> itself, PGD<sub>2</sub>, PGJ<sub>2</sub>, and 15d-PGJ<sub>2</sub>. The induction potency was 15d-PGJ<sub>2</sub> > PGA<sub>1</sub> >> PGJ<sub>2</sub> > PGD<sub>2</sub> > PGH<sub>2</sub>, with 15d-PGJ<sub>2</sub> being the most potent activator. Arachidonic acid, DHA, PGE<sub>2</sub>, PGF<sub>2</sub>, and TXB<sub>2</sub> had no significant effects on the induction of HO-1 expression. We examined the induction of HO-1 by PGD<sub>2</sub> through its metabolites, such as PGJ<sub>2</sub> and 15d-PGJ<sub>2</sub>, in HepG2 cells. We used 16,16-dimethyl PGD<sub>2</sub>, a metabolically stable synthetic analog of PGD<sub>2</sub>, to examine the induction of HO-1. As shown in Fig. 1B, 16,16-dimethyl PGD<sub>2</sub> exhibited significant induction of HO-1 in HepG2 cells. The PG precursor, arachidonic acid, failed to induce HO-1 expression up to a concentration of 100 µM. These results suggest that PGD<sub>2</sub> is not dependent on its metabolites, such as the J series of PGs, to induce the expression of HO-1, and that HepG2 cells may lack the conversion enzymes which catalyze the metabolites of arachidonic acid to PGH<sub>2</sub> and PGD<sub>2</sub>.

### ***Induction of HO-1 by 15d-PGJ<sub>2</sub> Does Not Mediate the MAPK and PPAR Pathways***

It has been demonstrated that activation of the MAPKs pathway contributes to the induction

of HO-1 by cadmium and arsenite (Elbirt et al., 1998; Alam et al., 2000). To examine whether 15d-PGJ<sub>2</sub> induces the expression of HO-1 through the MAPKs pathway, we performed the following experiments. 15d-PGJ<sub>2</sub> induced the expression of HO-1 in time- and dose-dependent manners (Fig. 2A). In the time-dependent experiment, we found that 15d-PGJ<sub>2</sub> was sufficient to activate JNK after 1.5 h. On the other hand, 15d-PGJ<sub>2</sub> slightly stimulated ERK activity at 6 h after drug treatment (Fig. 2B). To examine whether MAPKs, including ERK, JNK, and p38, could influence HO-1 expression, we transfected cells with dominant-negative (DN) mutants of the JNK or ERK expression plasmid. As shown in Fig. 2C (top), HO-1 levels were not significantly diminished by overexpression of the DN-JNK or DN-ERK. To verify that overexpression of dominant-negative JNK or ERK efficiently decreased endogenous JNK or ERK activities, we determined the total JNK and ERK activities by immunocomplex kinase assay. As shown in Fig. 2C (middle and bottom), overexpression of the DN-JNK or DN-ERK significantly decreased the JNK or ERK activity in the cells with or without 15d-PGJ<sub>2</sub>. In addition, the MEK inhibitor, PD98059, and the p38 kinase inhibitor, SB203580, also exhibited an inefficient inhibition of HO-1 induced by 15d-PGJ<sub>2</sub>. The immunocomplex kinase assay showed that ERK and p38 kinases activities were significantly inhibited by their inhibitors PD98059 and SB203580, respectively (Fig. 2D, middle and bottom). These data suggest that the induction of HO-1 by 15d-PGJ<sub>2</sub> might not be mediated by activation of the MAPKs pathway, and that there are clear differences between the activation pathways of 15d-PGJ<sub>2</sub> and the other treatments tested (cadmium and arsenite).

15d-PGJ<sub>2</sub> was a potent agonist of PPAR<sub>γ</sub>. To examine whether 15d-PGJ<sub>2</sub> induced HO-1 expression through activation of PPAR, cells were also treated with the other PPAR agonists, including WY-14643, ciglitazone, BRL49653, and indomethacin. As shown in the Fig. 3A, 15d-PGJ<sub>2</sub> strongly increased the HO-1 expression, and WY-14643 and BRL49653 slightly induced the HO-1 expression. On the contrary, ciglitazone and indomethacin had no effect on the HO-1 expression. To further examine the possibility of PPAR<sub>γ</sub> involved in the induction of HO-1, cells were transfected with PPAR<sub>γ</sub> expression plasmid and treated with 15d-PGJ<sub>2</sub>. Western blot showed that PPAR<sub>γ</sub> was increase about two to three-fold in the cells with PPAR<sub>γ</sub> overexpression plasmid (Fig. 3B, lanes 2 & 4). However, no additional induction of HO-1 was found in the PPAR<sub>γ</sub> overexpression cells with 15d-PGJ<sub>2</sub>. These results suggested that induction of HO-1 expression might mediate PPAR-independent pathway in HepG2 cells with 15d-PGJ<sub>2</sub>.

#### ***Thiol Antioxidant and Thiol-reducing Agent Attenuate 15d-PGJ<sub>2</sub>-induced HO-1 Expression***

There is evidence which indicates that cyclopentenone PGs, such as the J series of PGs, are potential inducers of intracellular oxidative stress in human neuroblastoma cells (Kondo et al., 2001). To examine whether 15d-PGJ<sub>2</sub> induced HO-1 expression through acting as a pro-oxidant or as a signal transducer, we treated cells with the antioxidants, vitamins C and E, with the xanthine oxidase inhibitor, allopurinol, glutathione precursor-N-acetyl-L-cysteine (NAC), and adenylate cyclase inhibitor-2',5'-dideoxyadenosine, and with the phosphatidylinositol 3-kinase (PI<sub>3</sub>K) inhibitor, wortmannin, with 15d-PGJ<sub>2</sub>. As shown in Fig. 4A, 15d-PGJ<sub>2</sub>-induced HO-1 expression was significantly inhibited by NAC, whereas it was not significantly inhibited by the other antioxidants or by the inhibitors of signal transduction, such as vitamins C and E,

allopurinol, 2',5'-dideoxyadenosine, and wortmannin. In addition, dithiothreitol (DTT), a reducing thiol agent, also exhibited inhibitory effects on HO-1 expression induced by 15d-PGJ<sub>2</sub> (Fig. 4B). Also to investigate whether 15d-PGJ<sub>2</sub> could influence the cellular thiol-disulfide redox status, we measured intracellular glutathione (GSH) levels. As shown in Fig. 4C, GSH levels were significantly diminished by treatment with 10 and 20 μM 15d-PGJ<sub>2</sub>. To examine whether DTT and NAC inhibited protein expression through transcriptional regulation, cells were pretreated with DTT or NAC for 2 h, 15d-PGJ<sub>2</sub> was added for 4 h, and the mRNA levels of HO-1 were detected by RT-PCR. As shown in Fig. 5A, DTT and NAC significantly inhibited the mRNA levels of HO-1 induced by 15d-PGJ<sub>2</sub>. In addition, DTT and NAC also significantly inhibited the promoter activity of HO-1 induced by 15d-PGJ<sub>2</sub> (Fig. 5B).

## DISCUSSION

PGD<sub>2</sub> is known to be sequentially metabolized to PGJ<sub>2</sub>, Δ<sup>12</sup>-PGJ<sub>2</sub>, and 15d-PGJ<sub>2</sub>. A comparison of the PG biosynthetic pathway with HO-1 inducer profiles reveals that induction of HO-1 might be mediated mainly by the metabolites of PGD<sub>2</sub>, the most active of which is the terminal metabolite, 15d-PGJ<sub>2</sub> (Fig. 1A). However, 16,16-dimethyl prostaglandin D<sub>2</sub> also markedly induced the expression of HO-1 (Fig. 1B). These results indicate that induction of HO-1 pathway potency is not dependent on the catabolism of PGD<sub>2</sub>.

The 5'-flanking region of the human HO-1 gene contains a number of DNA sequences of potential regulatory elements, such as AP-1 site, AP-2 like site, NF-κB site, STATx site, c-Rel site, HNF-1 site, HNF-4 site, HSE site, and GATA-X sites (Takahashi et al., 1999; Lavrovsky et al., 1994). In rat HO-1 promoter, the consensus E-box motif, CANNTG, seem to be essential for Δ<sup>12</sup>-PGJ<sub>2</sub>-induced the expression of rat HO-1 (Koizumi et al., 1995). However, we found several E-box like sequences in the 5'-flanking (-3106 to +186) of the human HO-1 gene. Further experiments are needed to determine which E-box like sequences is essential for 15d-PGJ<sub>2</sub>-induced the expression of human HO-1 gene. Recently, another report indicated that the mouse HO-1 promoter contains a stress-response element (StRE), which is required for induction of HO-1 gene by 15d-PGJ<sub>2</sub> (Gong et al., 2002). We performed the computer-assisted identification of putative StRE (set at a cutoff score of > 90) by MOTIF (<http://motif.genome.ad.jp>) and TRANSFAC (<http://transfac.gbf.de/TRANSFAC>), and not found putative StRE in the 5'-flanking region (-3106 to +186) of human HO-1 gene. These results suggest that StRE sequences might not be important to mediate the induction of HO-1 gene by 15d-PGJ<sub>2</sub> in human.

In this study, we found that 15d-PGJ<sub>2</sub> activated the MAP kinases, ERK and JNK, in Hep3B cells, while dominant-negative components could not block the induction of HO-1 by 15d-PGJ<sub>2</sub> (Fig. 2B, C). Other studies have demonstrated that activation of MAP kinase mediates the induction of HO-1 by arsenite or cadmium. Elbirt et al. (1998) found that the MAP kinases, ERK and p38, are involved in the induction of HO-1 by arsenite. Another study indicated that the MAP kinase, p38, is involved in the induction of HO-1 by cadmium (Alam et al., 2000). The results from this study showed that there were clear differences between the activation pathways of 15d-PGJ<sub>2</sub> and the other treatments tested (cadmium and arsenite). However, we must mention that arsenite and cadmium are strong inducers of intracellular oxidative stress (Stohs and Bagchi,



1995; Lynn et al., 2000). It is possible that induction of HO-1 by cadmium and arsenite occurs through increased intracellular oxidative stress, and results in changes in GSH levels as does 15d-PGJ<sub>2</sub>. We have shown that 15d-PGJ<sub>2</sub>-induced HO-1 expression is significantly inhibited by the thiol antioxidant, NAC, and by the thiol reducing agent, DTT. In contrast to NAC, non-thiol antioxidants such as vitamins C and E, and allopurinol were unable to inhibit the induction of HO-1 by 15d-PGJ<sub>2</sub>. We also confirm the decrease in intracellular GSH levels produced by 15d-PGJ<sub>2</sub>. A characteristic of cyclopentenone PGs is that they contain  $\alpha$ ,  $\beta$ -unsaturated ketones, which are very susceptible to nucleophilic addition reactions with thiol, and are essential for the actions of the PGs (Atsmon et al., 1990a, b). Therefore, 15d-PGJ<sub>2</sub> is likely to induce HO-1 expression through oxidizing cellular thiols, GSH, and proteins. Upon treatment of 15d-PGJ<sub>2</sub>, a decrease in the GSH level may facilitate conversion of protein thiol groups to disulphide bonds, and the subsequent accumulation of malformed polypeptides, which in turn induces HO-1 expression (Fig. 6). However, determining which proteins participate in this induction of HO-1 requires further investigation.

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## FIGURE LEGENDS

**Fig. 1.** Effect of arachidonic acid, docosahexaenoic acid, and various eicosanoids on the protein expression of HO-1 in HepG2 cells. (A) Cells were treated with various compounds or (B) with arachidonic acid and 50  $\mu$ M 16,16-dimethyl-PGD<sub>2</sub> for 15 h; HO-1 and HO-2 proteins were detected by Western blotting. 15d-PGJ<sub>2</sub>, 15-deoxy- $\Delta^{12,14}$ -PGJ<sub>2</sub>; AA, arachidonic acid; TXB<sub>2</sub>, thromboxane B<sub>2</sub>.

**Fig. 2.** Effect of MAPK pathways on the expression of HO-1 induced by 15d-PGJ<sub>2</sub> in HepG2 cells. (A) Cells were treated with 10  $\mu$ M 15d-PGJ<sub>2</sub> for various times as indicated (top), or treated with various concentrations of 15d-PGJ<sub>2</sub> for 15 h (bottom). The total cell lysates was used to detect HO-1 and HO-2 proteins by Western blot. (B) Cells were treated with 10  $\mu$ M 15d-PGJ<sub>2</sub> for various times as indicated, the total cell lysates was preformed the JNK (Gst-c-Jun as the substrate) and ERK (MBP as the substrate) kinases activity assays as described in “Materials and Methods”. (C) Cells were transfected with mock, dominant-negative (DN) JNK, or DN-ERK expression plasmid, and treated with 10  $\mu$ M 15d-PGJ<sub>2</sub> for 15 h. Total cells lysates was used to detect the HO-1 protein was detected by Western blotting (top). Transfected cells were treated with 10  $\mu$ M 15d-PGJ<sub>2</sub> for 1.5 h (middle) or 9 h (bottom), and determined the JNK (middle) and ERK (bottom) kinases activity assays as described in “Materials and Methods”. (D) Cells were treated with 50  $\mu$ M PD98059 or 10  $\mu$ M SB203580 and 10  $\mu$ M 15d-PGJ<sub>2</sub> for 15 h, and the HO-1 protein was detected by Western blotting (top). Cells were treated with 50  $\mu$ M PD98059 or 10  $\mu$ M SB203580 and 10  $\mu$ M 15d-PGJ<sub>2</sub> for 9 h, and determined the ERK (middle) and p38 (Gst-ATF2 as the substrate, bottom) kinase activity assays as described in “Materials and Methods”.

**Fig. 3.** Effect of PPAR agonists and overexpression of PPAR $\gamma$  on the induction of HO-1 expression. (A). Cells were treated with various PPAR agonists for 15 h, and the HO-1 and HO-2 protein expression were detected by Western blotting. WY, WY-14643; Cig, ciglitazone; BRL, BRL49653; Indo, indomethacin. (B). Transfected cells were treated with or without 10  $\mu$ M 15d-PGJ<sub>2</sub> for 15 h, and the HO-1 and PPAR $\gamma$  protein expression were detected by Western blotting.

**Fig. 4.** Effect of various inhibitors on the protein expression of HO-1 induced by 15d-PGJ<sub>2</sub> in HepG2 cells. (A) Cells were pretreated with various drugs or (B) with NAC or DTT for 2 h, and 10  $\mu$ M 15d-PGJ<sub>2</sub> was added for another 15 h. The HO-1 protein was detected by Western blotting NAC, N-acetyl-L-cysteine; DTT, dithiothreitol; Vit C, vitamin C; Vit E, vitamin E; Allo, allopurinol; NAC, N-acetyl-L-cysteine; DDA, 2',5'-dideoxyadenosine; Wort, wortmannin. (C) Effect of 15d-PGJ<sub>2</sub> on glutathione level in HepG2 cells. Cells were treated with different concentrations of 15d-PGJ<sub>2</sub> for 3 h and assayed for GSH as described in “Materials and Methods”. The values were expressed as the mean  $\pm$  S.E. of triplicate tests. \*,  $p < 0.05$  vs. the control.

**Fig. 5.** Effect of NAC and DTT on mRNA levels and promoter activity of HO-1 induced by 15d-PGJ<sub>2</sub> in HepG2 cells. (A) Cells were pretreated with NAC or DTT for 2 h, 10 μM 15d-PGJ<sub>2</sub> was added for another 4 h, and HO-1 and HO-2 mRNA were detected by RT-PCR. (B) Cells were transfected with the pGL2/hHO3.2-Luc reporter plasmid and the phRL-TK internal control plasmid for 48 h, and 10 μM 15d-PGJ<sub>2</sub> was added for another 9 h. Luciferase activity was measured as described in “Materials and Methods”. The values are expressed as the mean ± S.E. of triplicate tests. \*,  $p < 0.05$  vs. 15d-PGJ<sub>2</sub> treatment.

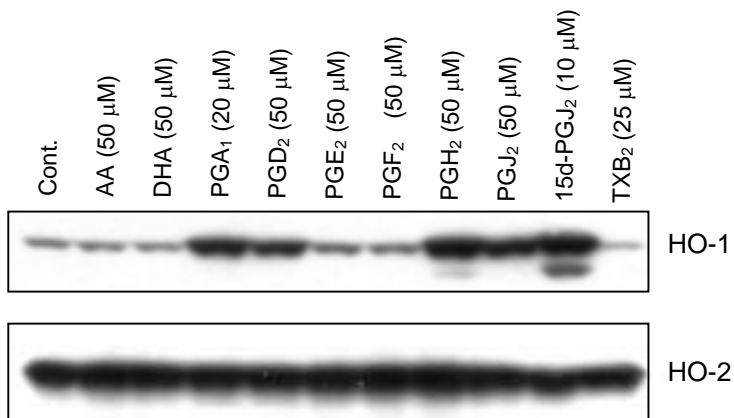
**Fig. 6.** Two possible mechanisms for the activation of HO-1 protein by 15d-PGJ<sub>2</sub>. (i) 15d-PGJ<sub>2</sub> may form thiol conjugates with cysteine residue of proteins because of electrophilic carbons in their cyclopentenone ring (Atsmon et al., 1990). Conjugation with proteins (including transcription factors) may lead to modification of protein functions, ultimately inducing HO-1 expression. (ii) 15d-PGJ<sub>2</sub> exerts a pro-oxidant effect, resulting in the conversion of a sulfhydryl group (-SH) into an oxidized disulfide (-S-S-) in cellular proteins (including transcription factors). This may lead to accumulation of denatured proteins, which in turn may activate HO-1.

### 計畫成果自評

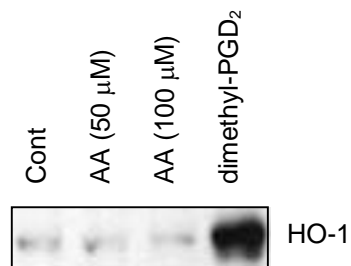
先前的研究我們發現 PPAR 配位體 15d-PGJ<sub>2</sub> 可活化 JNK，且活化 JNK 與造成細胞凋亡有相關性，已發表在 *Mol. Carcinogenesis*, 37: 16-24, 2003. 在我們一系列的研究 PPAR 配位體對腫瘤細胞毒殺作用時，發現 15d-PGJ<sub>2</sub> 的毒殺作用可能亦經由引起細胞氧化壓力增加；另一個蛋白 heme oxygenase-1 (HO-1) 已知在氧化壓力增加時會大量表現，而 15d-PGJ<sub>2</sub> 亦可大量增加 HO-1 的表現，所以本計畫的先前研究，為探討 15d-PGJ<sub>2</sub> 引起 HO-1 的可能路徑，本結果已在今年被 *Life Sciences* 雜誌所接受，目前在編排出版中。

**Fig. 1.**

**A.**

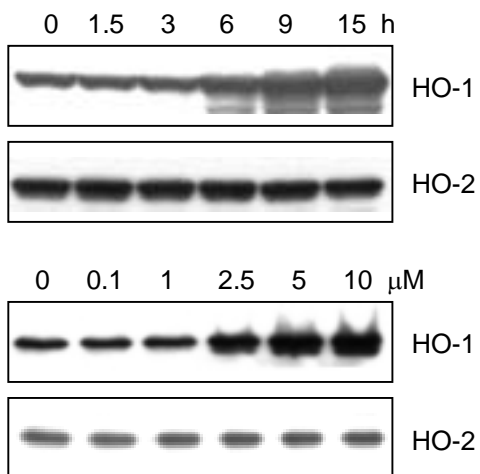


**B.**

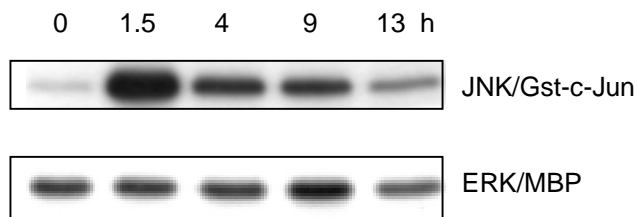


**Fig. 2.**

**A.**

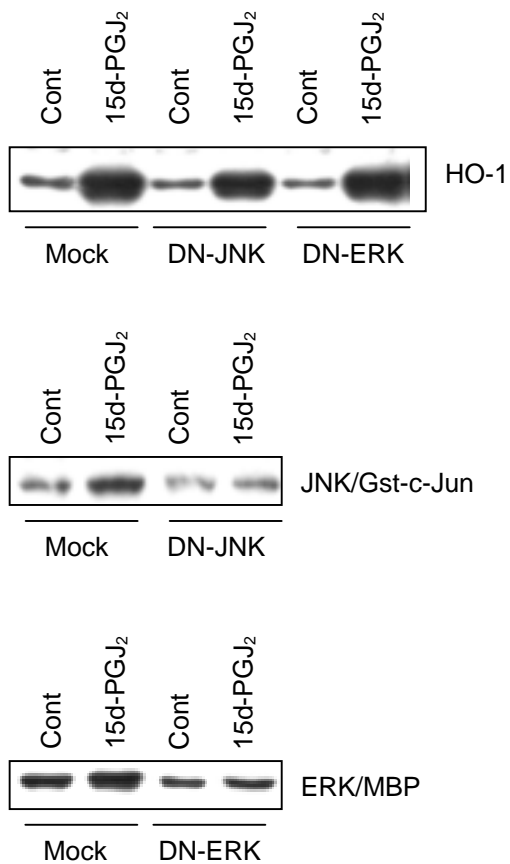


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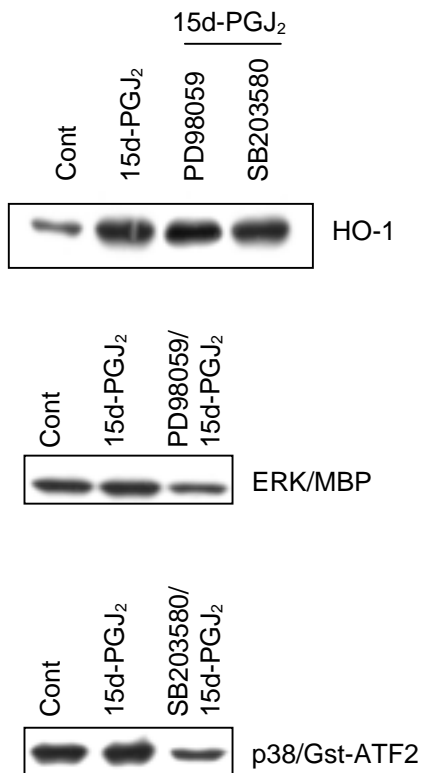


**Fig. 2.**

**C.**

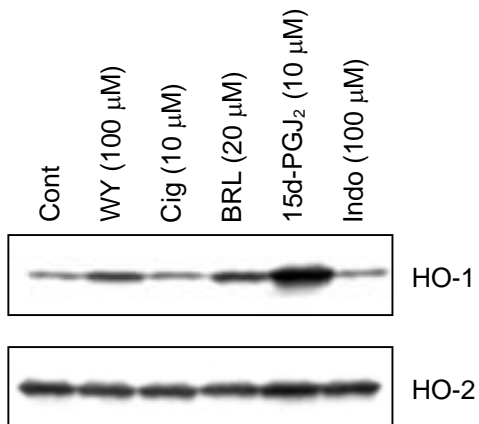


**D.**

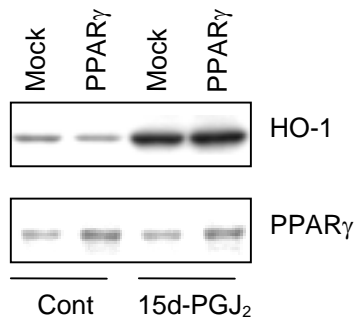


**Fig. 3.**

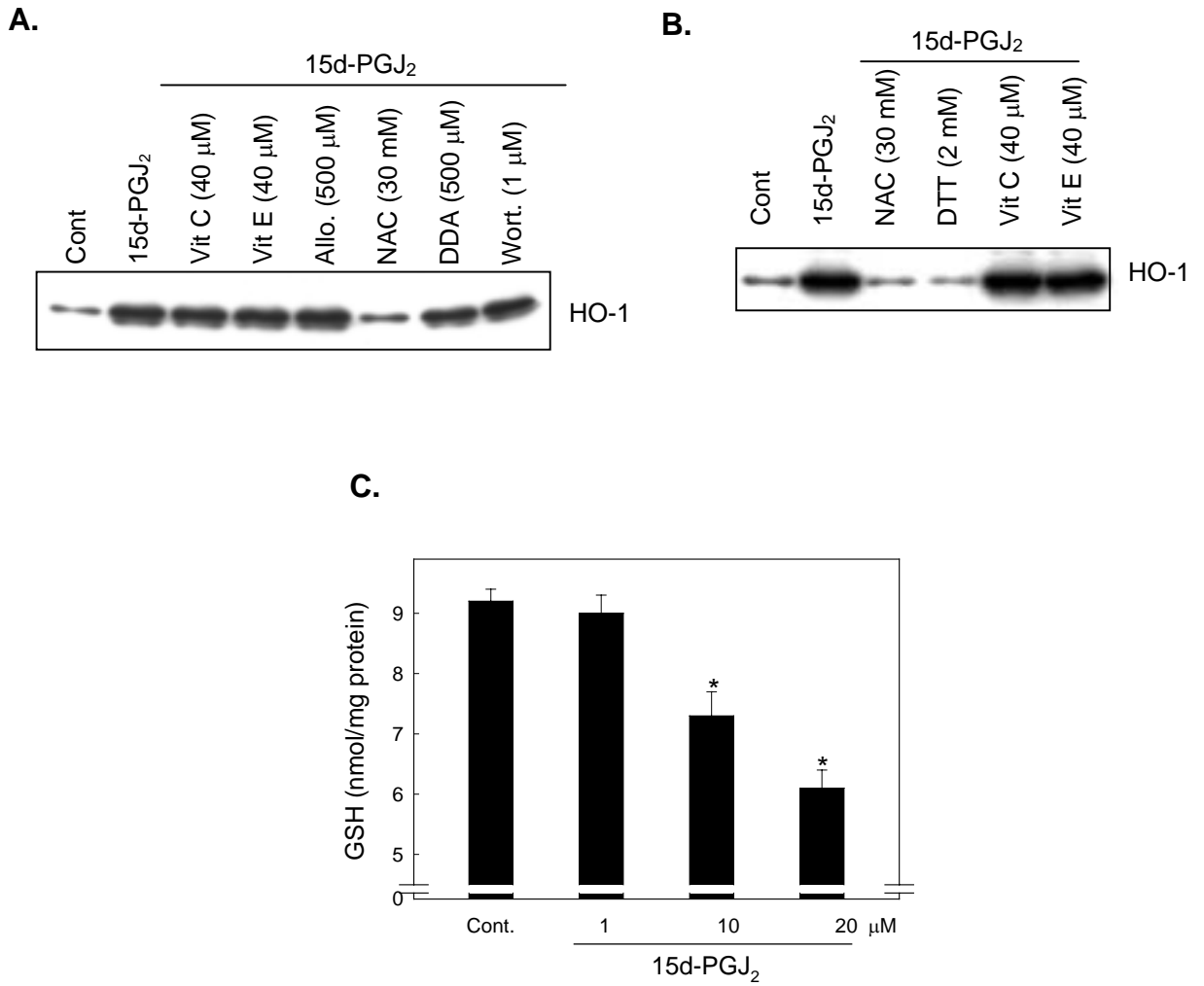
**A.**



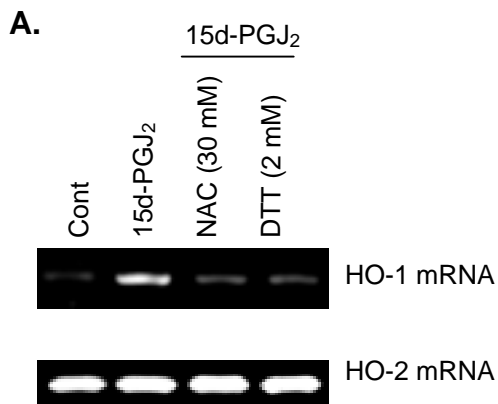
**B.**



**Fig. 4.**



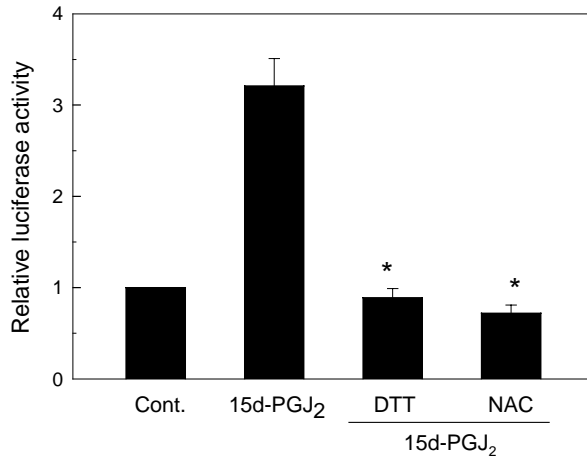
**Fig. 5.**





**Fig. 5.**

**B.**



**Fig. 6.**

