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行政院國家科學委員會補助專題研究計畫 ■ 成果報告
□期中進度報告

前列腺素及熱休克因子影響血基素氧化酶表現之機制探討(2/2)

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Thiol antioxidant and thiol-reducing agents attenuate 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2-induced heme oxygenase-1 expression

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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_2 (15d-PGJ_2)-induced HO-1 expression. PGH_2, PGD_2 and its metabolites of the PGJ_2 series, and PGA_1 markedly induced the protein expression of HO-1. Arachidonic acid (AA), docosahexaenoic acid (DHA), PGE_2, $PGF_{2\alpha}$, and thromboxane B_2 (TXB_2) were shown to have no effect on the induction of HO-1. 15d-PGJ_2 was the most potent activator achieving significance at 5 μM. Although 15d-PGJ_2 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_2 induced HO-1 expression through peroxisome proliferator-activated receptor (PPAR)-independent pathway. 15d-PGJ_2 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_2. Finally, NAC and DTT exhibited significant inhibition of HO-1 mRNA and HO-1 promoter reporter activity induced by 15d-PGJ_2. These results suggest that thiol antioxidant and reducing agents attenuate the expression

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of HO-1 induced by 15d-PGJ_2, and that the cellular thiol-disulfide redox status may be linked to HO-1 activation.

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Keywords: Heme oxygenase-1; 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2; N-Acetyl-L-cysteine; Dithiothreitol; Glutathione

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 guantities 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-1β, 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_2 by cyclooxygenase and subsequently converted to one of several related products, including PGD_2, PGE_2, PG$F_{2\alpha}$, PGI_2, and thromboxane A_2, through the action of specific PG synthases. 15d-PGJ_2 is derived from PGD_2 and involves the sequential conversion of PGD_2, PGI_2, Δ^{12}-PGJ_2, and 15d-PGJ_2 (Fukushima, 1992). Several proteins have been identified which are induced by cyclopentenone PGs, such as heat shock proteins (Santoro et al., 1989), γ-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_2 was shown to be a high-affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ) (Kliewer et al., 1995). Recently, PPARγ has been considered to have anti-inflammatory actions through activation by arachidonic acid metabolites, such as 15d-PGJ_2 (Jiang et al., 1998; Ricote et al., 1998, 1999). 15d-PGJ_2 represses several genes related to inflammation, including the inducible nitric oxide synthase (iNOS) and tumor necrosis factor α (TNF_α) genes in activated macrophages (Ricote et al., 1998). However, the repression is partly dependent on PPARγ expression (Chawla et al., 2001), and receptor-independent biological actions of 15d-PGJ_2 and other cyclopentenone PGs have been proposed (Rossi et al., 2000; Straus et al., 2000). Other studies have indicated that some cyclopentonene PGs induce the synthesis of HO-1 in cells of the mice and rat, but the mechanism underlying 15d-PGJ_2-induced HO-1 protein synthesis in human cells has been largely unexplored. We report herein that PGD_2, PGA_1, 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_2 may be mediated by modulation of the cellular thiol-disulfide redox status.

Materials and methods

Materials and cell culture

DHA, PGA_1, PGJ_2, 16,16-dimethyl PGD_2, and BRL49653 were purchased from Cayman Chemical (Ann Arbor, MI), while arachidonic acid, PGD_2, PGE_2, $PGF_{2\alpha}$, PGH_2, TXB_2, 15d-PGJ_2, 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-PPARγ (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′-CCGGTAGAGCTGCTTGAACT-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 transition 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 PPARγ 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 PPARγ plasmid using LipofectAMINE™ 2000 (Gibco) for 48 h (Liang et al., 2001). After transfection, cells were then treated with 15d-PGJ_2, 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 $CaCl_2$, and 1 mM $MgCl_2$). 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_1, PGH_2 metabolites including PGH_2 itself, PGD_2, PGJ_2,

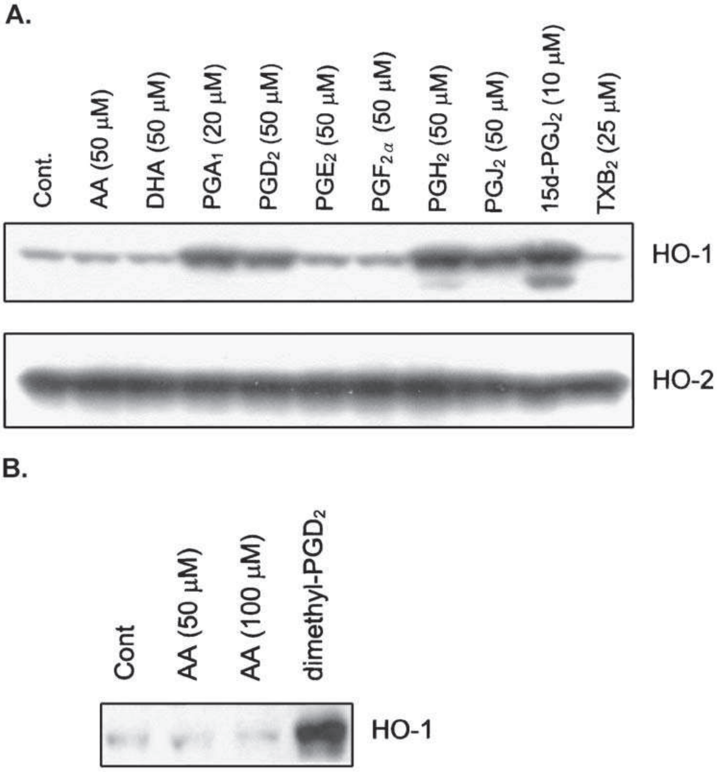
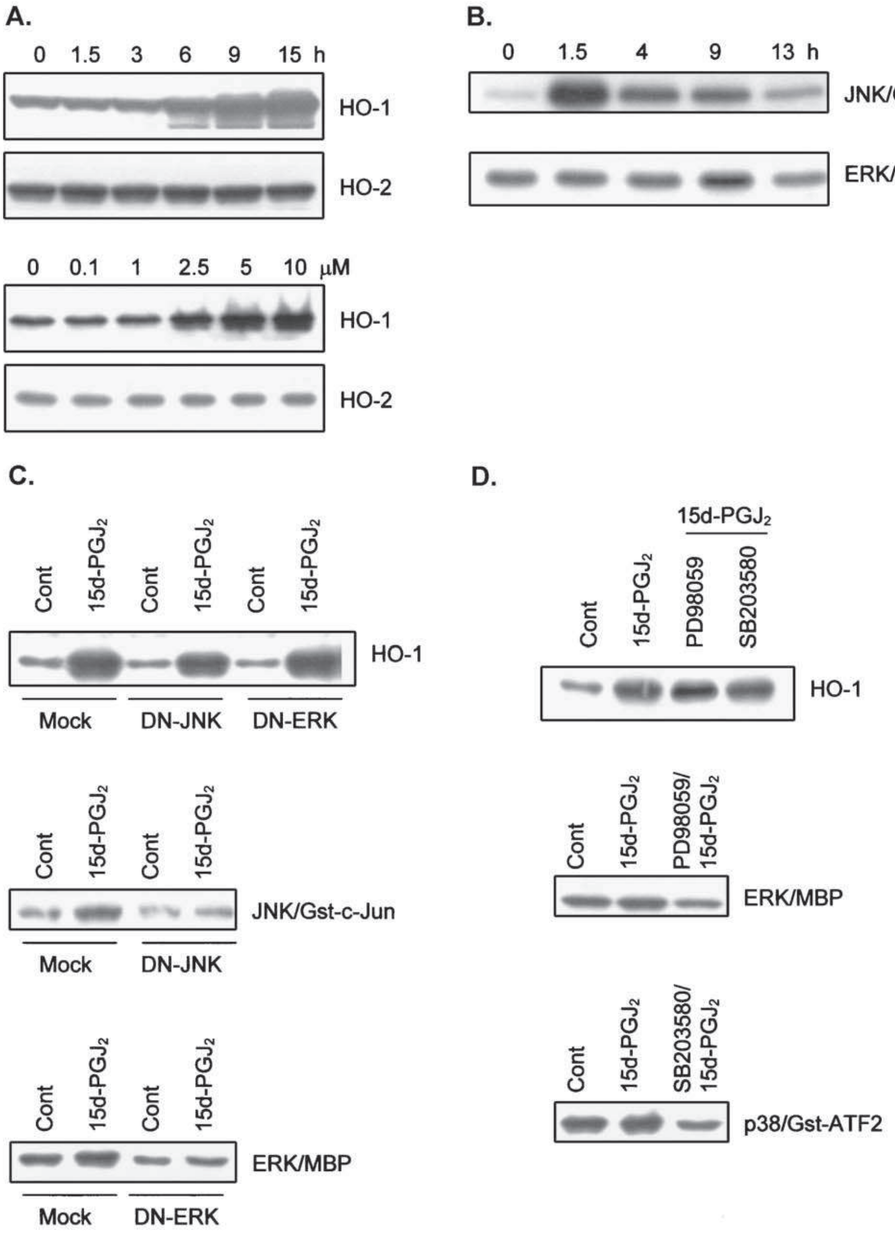


Fig. 1. Effect of arachidonic acid, docosahexaenoic acid, and various eicosanoids on the protein expression of HO-1 in HepG2 cells. (A) Cells were treated with various compounds or (B) with arachidonic acid and 50 μM 16,16-dimethyl-PGD$_2$ for 15 h; HO-1 and HO-2 proteins were detected by Western blotting. 15d-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-PGJ$_2$; AA, arachidonic acid; TXB$_2$, thromboxane B$_2$.

and 15d-PGJ$_2$. The induction potency was 15d-PGJ$_2$ > PGA$_1$ PGJ$_2$ ≒ PGD$_2$ ≒ PGH$_2$, with 15d-PGJ$_2$ being the most potent activator. Arachidonic acid, DHA, PGE$_2$, PGF$_{2\alpha}$, and TXB$_2$ had no significant effects on the induction of HO-1 expression. We examined the induction of HO-1 by PGD$_2$ through its metabolites, such as PGJ$_2$ and 15d-PGJ$_2$, in HepG2 cells. We used 16,16-dimethyl PGD$_2$, a metabolically stable synthetic analog of PGD$_2$, to examine the induction of HO-1. As shown in Fig. 1B, 16,16-dimethyl PGD$_2$ 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$_2$ 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$_2$ and PGD$_2$.

Induction of HO-1 by 15d-PGJ$_2$ 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$_2$ induces the expression of HO-1 through the MAPKs pathway, we performed the following experiments. 15d-PGJ_2 induced the expression of HO-1 in time- and dose-dependent manners (Fig. 2A). In the time-dependent experiment, we found that 15d-PGJ_2 was sufficient to activate JNK after 1.5 h. On the other hand, 15d-PGJ_2 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


A.
0 1.5 3 6 9 15 h
HO-1
HO-2
0 0.1 1 2.5 5 10 μM
HO-1
HO-2
B.
0 1.5 4 9 13 h
JNK/Gst-c-Jun
ERK/MBP
C.
Cont 15d-PGJ2 Cont 15d-PGJ2 Cont 15d-PGJ2
HO-1
Mock DN-JNK DN-ERK
Cont 15d-PGJ2 Cont 15d-PGJ2
JNK/Gst-c-Jun
Mock DN-JNK
Cont 15d-PGJ2 Cont 15d-PGJ2
ERK/MBP
Mock DN-ERK
D.
15d-PGJ2
Cont 15d-PGJ2 PD98059 SB203580
HO-1
Cont 15d-PGJ2 PD98059/15d-PGJ2
ERK/MBP
Cont 15d-PGJ2 SB203580/15d-PGJ2
p38/Gst-ATF2

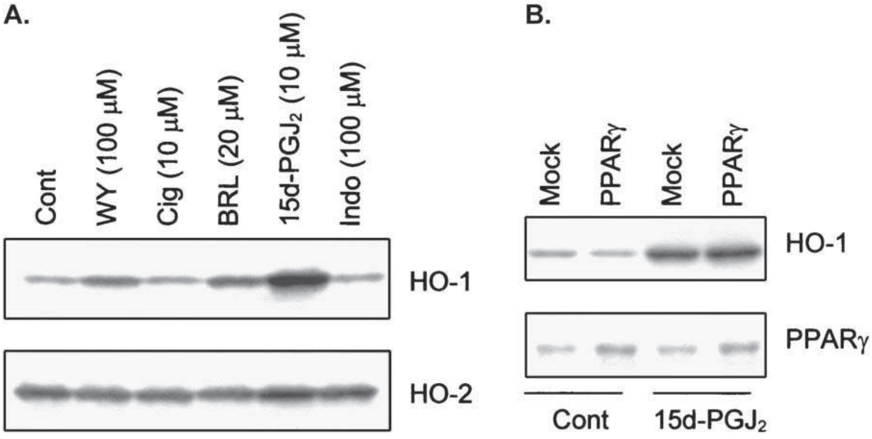


Fig. 3. Effect of PPAR agonists and overexpression of PPARγ on the induction of HO-1 expression. (A). Cells were treated with various PPAR agonists for 15 h, and the HO-1 and HO-2 protein expression were detected by Western blotting. WY, WY-14643; Cig, ciglitazone; BRL, BRL49653; Indo, indomethacin. (B). Transfected cells were treated with or without 10 μM 15d-PGJ_2 for 15 h, and the HO-1 and PPARγ protein expression were detected by Western blotting.

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_2. In addition, the MEK inhibitor, PD98059, and the p38 kinase inhibitor, SB203580, also exhibited an inefficient inhibition of HO-1 induced by 15d-PGJ_2. 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_2 might not be mediated by activation of the MAPKs pathway, and that there are clear differences between the activation pathways of 15d-PGJ_2 and the other treatments tested (cadmium and aresenite).

Fig. 2. Effect of MAPK pathways on the expression of HO-1 induced by 15d-PGJ_2 in HepG2 cells. (A) Cells were treated with 10 μM 15d-PGJ_2 for various times as indicated (top), or treated with various concentrations of 15d-PGJ_2 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 μM 15d-PGJ_2 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 μM 15d-PGJ_2 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 μM 15d-PGJ_2 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 μM PD98059 or 10 μM SB203580 and 10 μM 15d-PGJ_2 for 15 h, and the HO-1 protein was detected by Western blotting (top). Cells were treated with 50 μM PD98059 or 10 μM SB203580 and 10 μM 15d-PGJ_2 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".

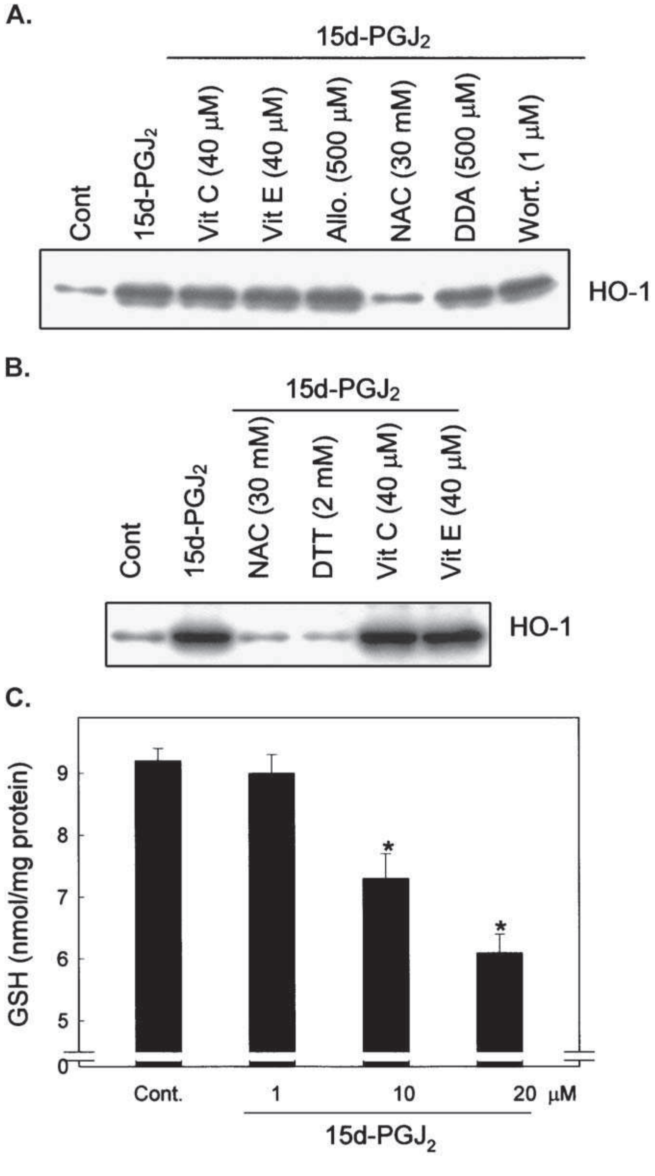


Fig. 4. Effect of various inhibitors on the protein expression of HO-1 induced by 15d-PGJ_2 in HepG2 cells. (A) Cells were pretreated with various drugs or (B) with NAC or DTT for 2 h, and 10 μM 15d-PGJ_2 was added for another 15 h. The HO-1 protein was detected by Western blotting NAC, N-acetyl-L-cysteine; 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_2 on glutathione level in HepG2 cells. Cells were treated with different concentrations of 15d-PGJ_2 for 3 h and assayed for GSH as described in ‘‘Materials and methods’’. The values were expressed as the mean ± S.E. of triplicate tests. *, $p < 0.05$ vs. the control.

15d-PGJ$_2$ as a potent agonist of PPAR$_\gamma$. To examine whether 15d-PGJ$_2$ 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$_2$ 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$_\gamma$ involved in the induction of HO-1, cells were transfected with PPAR$_\gamma$ expression plasmid and treated with 15d-PGJ$_2$. Western blot showed that PPAR$_\gamma$ was increase about two to three-fold in the cells with PPAR$_\gamma$ overexpression plasmid (Fig. 3B, lanes 2 and 4). However, no additional

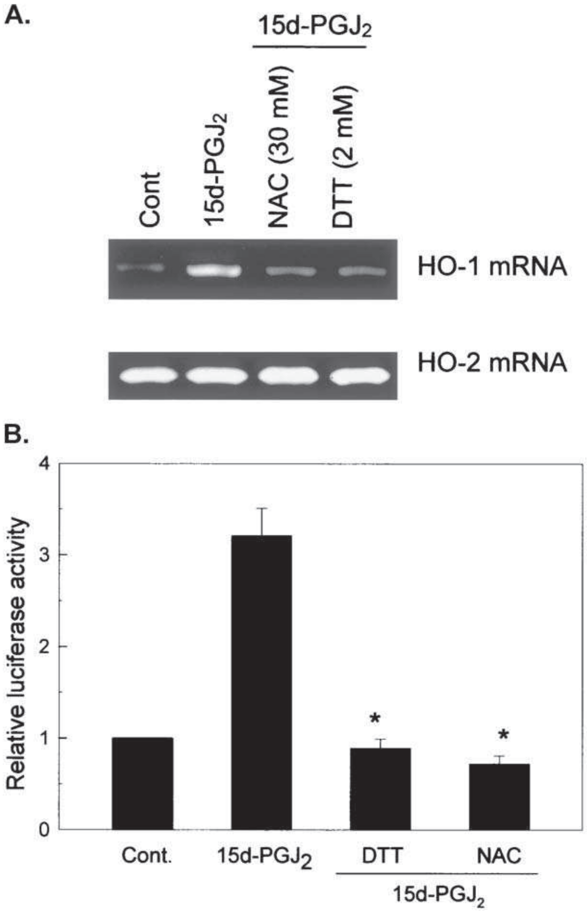


Fig. 5. Effect of NAC and DTT on mRNA levels and promoter activity of HO-1 induced by 15d-PGJ$_2$ in HepG2 cells. (A) Cells were pretreated with NAC or DTT for 2 h, 10 μM 15d-PGJ$_2$ was added for another 4 h, and HO-1 and HO-2 mRNA were detected by RT-PCR. (B) Cells were transfected with the pGL2/hHO3.2-Luc reporter plasmid and the phRL-TK internal control plasmid for 48 h, and 10 μM 15d-PGJ$_2$ 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$_2$ treatment.

induction of HO-1 was found in the $PPAR_\gamma$ overexpression cells with 15d-PGJ_2. These results suggested that induction of HO-1 expression might mediate PPAR-independent pathway in HepG2 cells with 15d-PGJ_2.

Thiol antioxidant and thiol-reducing agent attenuate 15d-PGJ_2-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_2 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_3K) inhibitor, wortmannin, with 15d-PGJ_2. As shown in Fig. 4A, 15d-PGJ_2-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_2 (Fig. 4B). Also to investigate whether 15d-PGJ_2 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_2. To examine whether DTT and NAC inhibited protein expression through transcriptional regulation, cells were pretreated with DTT or NAC for 2 h, 15d-PGJ_2 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-$PGJ_{2.}$ In addition, DTT and NAC also significantly inhibited the promoter activity of HO-1 induced by 15d-PGJ_2 (Fig. 5B).

Discussion

PGD_2 is known to be sequentially metabolized to PGJ_2, Δ^{12}-PGJ_2, and 15d-PGJ_2. 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_2, the most active of which is the terminal metabolite, 15d-PGJ_2 (Fig. 1A). However, 16,16-dimethyl prostaglandin D_2 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_2.

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^{12}-PGJ_2-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_2-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_2 (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$_2$ in human.

In this study, we found that 15d-PGJ$_2$ 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$_2$ (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$_2$ and the other treatments tested (cadmium and aresenite). However, we must mention that aresenite 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 aresenite occurs through increased intracellular oxidative stress, and results in changes in GSH levels as does 15d-PGJ$_2$. We have shown that 15d-PGJ$_2$-induced HO-1 expression is significautly 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$_2$. We also confirm the decrease in intracellular GSH levels produced by 15d-PGJ$_2$. A characteristic of cyclopentenone PGs is that they contain α, β-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$_2$ is likely to induce HO-1 expression through oxidizing cellular thiols, GSH, and proteins. Upon treatment of 15d-PGJ$_2$, a decrease in the GSH level may facilitate conversion of protein thiol groups to disulphide bonds, and the subsequent accumulation of malfolded polypeptides, which in turn induces HO-1 expression (Fig. 6). However, determining which proteins participate in this induction of HO-1 requires further investigation.

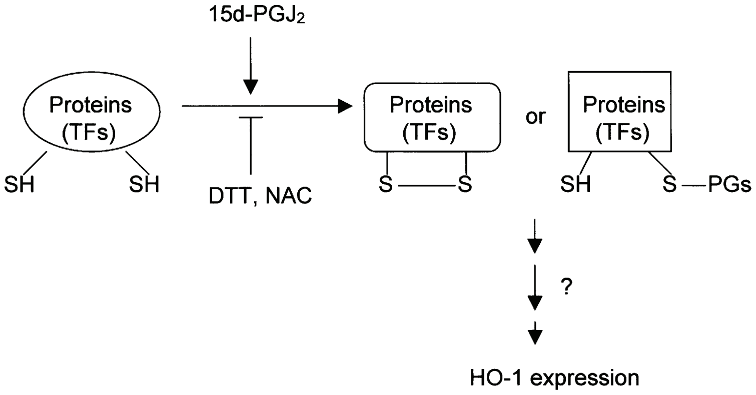


Fig. 6. Two possible mechanisms for the activation of HO-1 protein by 15d-PGJ$_2$. (i) 15d-PGJ$_2$ may form thiol conjugates with cysteine residue of proteins because of electrophilic carbons in their cyclopentenone ring (Atsmon et al., 1990a,b). Conjugation with proteins (including transcription factors) may lead to modification of protein functions, ultimately inducing HO-1 expression. (ii) 15d-PGJ$_2$ 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.

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References

Alam, J., Wicks, C., Stewart, D., Gong, P., Touchard, C., Otterbein, S., Choi, A.M., Burow, M.E., Tou, J., 2000. Mechanism of heme oxygenase-1 gene activation by cadmium in MCF-7 mammary epithelial cells. Role of p38 kinase and Nrf2 transcription factor. J Biol Chem 275, 27694–27702.

Atsmon, J., Sweetman, B.J., Baertschi, S.W., Harris, T.M., Roberts 2nd, L.J., 1990a. Formation of thiol conjugates of 9-deoxy-delta 9,delta 12(E)-prostaglandin D2 and delta 12(E)-prostaglandin D2. Biochemistry 29, 3760–3765.

Atsmon, J., Freeman, M.L., Meredith, M.J., Sweetman, B.J., Roberts 2nd, L.J., 1990b. Conjugation of 9-deoxy-delta 9,delta 12(E)-prostaglandin D2 with intracellular glutathione and enhancement of its antiproliferative activity by glutathione depletion. Cancer Res 50, 1879–1885.

Chawla, A., Barak, Y., Nagy, L., Liao, D., Tontonoz, P., Evans, R.M., 2001. PPAR-gamma-dependent and -independent effects on macrophage-gene expression in lipid metabolism and inflammation. Nat Med 7, 48–52.

Choi, A.M., Alam, J., 1996. Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am J Resp Cell Mol Biol 15, 9–19.

Elbirt, K.K., Whitmarsh, A.J., Davis, R.J., Bonkovsky, H.L., 1998. Mechanism of sodium arsenite-mediated induction of heme oxygenase-1 in hepatoma cells. Role of mitogen-activated protein kinases. J Biol Chem 273, 8922–8931.

Fukushima, M., 1992. Biological activities and mechanisms of action of PGJ2 and related compounds: an update. Prostagl and Leukotri Essent Fatty Acids 47, 1–12.

Gong, P., Stewart, D., Hu, B., Li, N., Cook, J., Nel, A., Alam, J., 2002. Activation of the mouse heme oxygenase-1 gene by 15-deoxy-Delta(12,14)-prostaglandin J(2) is mediated by the stress response elements and transcription factor Nrf2. Antioxid Redox Signal 4, 249–257.

Jiang, C., Ting, A.T., Seed, B., 1998. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature 391, 82–86.

Kim, S.H., Han, S.I., Oh, S.Y., Chung, H.Y., Kim, H.D., Kang, H.S., 2001. Activation of heat shock factor 1 by pyrrolidine dithiocarbamate is mediated by its activities as pro-oxidant and thiol modulator. Biochem Biophys Res Commun 281, 367–372.

Kim, U.J., Birren, B.W., Slepak, T., Mancino, V., Boysen, C., Kang, H.L., Simon, M.I., Shizuya, H., 1996. Construction and characterization of a human bacterial artificial chromosome library. Genomics 34, 213–218.

Kliewer, S.A., Lenhard, J.M., Willson, T.M., Patel, I., Morris, D.C., Lehmann, J.M., 1995. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. Cell 83, 813–819.

Koizumi, T., Odani, N., Okuyama, T., Ichikawa, A., Negishi, M., 1995. Indentification of a cis-regulatory element for delta 12-prostaglandin J2-induced expression of the rat heme oxygenase gene. J Biol Chem 270, 21779–21784.

Kondo, M., Oya-Ito, T., Kumagai, T., Osawa, T., Uchida, K., 2001. Cyclopentenone prostaglandins as potential inducers of intracellular oxidative stress. J Biol Chem 276, 12076–12083.

Lavrovsky, Y., Schwartzman, M.L., Levere, R.D., Kappas, A., Abraham, N.G., 1994. Identification of binding sites for transcription factors NF-κB and AP-2 in the promoter region of the human heme oxygenase 1 gene. Proc Natl Acad Sci USA 91, 5987–5991.

Lee, T.S., Chau, L.Y., 2002. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat Med 8, 240–246.

Liang, Y.C., Huang, Y.T., Tsai, S.H., Lin-Shiau, S.Y., Chen, C.F., Lin, J.K., 1999a. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis 20, 1945–1952.

Liang, Y.C., Lin-Shiau, S.Y., Chen, C.F., Lin, J.K., 1999b. 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 75, 1–12.

Liang, Y.C., Tsai, D.C., Lin-Shiau, S.Y., Chen, C.F., Lin, J.K., 2001. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase through peroxisome proliferator-activated receptor-γ by apigenin and related-flavonoids in macrophages. FEBS Lett 496, 12–18.

Lin, S.Y., Liang, Y.C., Ho, Y.S., Tsai, S.H., Pan, S., Lee, W.S., 2002. 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 Carcinogen 35, 21–28.

Lynn, S., Gurr, J.R., Lai, H.T., Jan, K.Y., 2000. NADH oxidase activation is involved in arsenite-induced oxidative DNA damage in human vascular smooth muscle cells. Circ Res 86, 514–519.

Maines, M.D., 1997. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol 37, 517–554.

McCoubrey Jr., W.K., Maines, M.D., 1994. The structure, organization and differential expression of the gene encoding rat heme oxygenase-2. Gene 139, 155–161.

McCoubrey Jr., W.K., Huang, T.J., Maines, M.D., 1997. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. Eur J Biochem 247, 725–732.

Minamino, T., Christou, H., Hsieh, C.M., Liu, Y., Dhawan, V., Abraham, N.G., Perrella, M.A., Mitsialis, S.A., Kourembanas, S., 2001. Targeted expression of heme oxygenase-1 prevents the pulmonary inflammatory and vascular responses to hypoxia. Proc Natl Acad Sci USA 98, 8798–8803.

Muller, R.M., Taguchi, H., Shibahara, S., 1987. Nucleotide sequence and organization of the rat heme oxygenase gene. J Biol Chem 262, 6795–6802.

Ohno, K., Higaki, J., Takechi, S., Hirata, M., 1990. Specific role of an alpha,beta-unsaturated carbonyl group in gamma-glutamylcysteine synthetase induction by prostaglandin A2. Chem Biol Interact 76, 77–87.

Ohtani-Fujita, N., Minami, S., Mimaki, S., Dao, S., Sakai, T., 1998. p53-Independent activation of the gadd45 promoter by Delta12-prostaglandin J2. Biochem Biophys Res Commun 251, 648–652.

Ponka, P., 1999. Cell biology of heme. Am J Med Sci 318, 241–256.

Poss, K.D., Tonegawa, S., 1997a. Heme oxygenase 1 is required for mammalian iron reutilization. Proc Natl Acad Sci USA 94, 10919–10924.

Poss, K.D., Tonegawa, S., 1997b. Reduced stress defense in heme oxygenase 1-deficient cells. Proc Natl Acad Sci USA 94, 10925–10930.

Ricote, M., Li, A.C., Willson, T.M., Kelly, C.J., Glass, C.K., 1998. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. Nature 391, 79–82.

Ricote, M., Huang, J.T., Welch, J.S., Glass, C.K., 1999. The peroxisome proliferator-activated receptor (PPARgamma) as a regulator of monocyte/macrophage function. J Leukocyte Biol 66, 733–739.

Robbins, D.J., Zhen, E., Owaki, H., Vanderbilt, C.A., Ebert, D., Geppert, T.D., Cobb, M.H., 1993. Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro. J Biol Chem 268, 5097–5106.

Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M., Santoro, M.G., 2000. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. Nature 403, 103–108.

Santoro, M.G., Garaci, E., Amici, C., 1989. Prostaglandins with antiproliferative activity induce the synthesis of a heat shock protein in human cells. Proc Natl Acad Sci USA 86, 8407–8411.

Stohs, S.J., Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metal ions. Free Rad Biol Med 18, 321–326.

Straus, D.S., Pascual, G., Li, M., Welch, J.S., Ricote, M., Hsiang, C.H., Sengchanthalangsy, L.L., Ghosh, G., Glass, C.K., 2000. 15-Deoxy-delta 12,14-prostaglandin J2 inhibits multiple steps in the NF-kappa B signaling pathway. Proc Natl Acad Sci USA 97, 4844–4849.

Takahashi, S., Takahashi, Y., Ito, K., Nagano, T., Shibahara, S., Miura, T., 1999. Positive and negative regulation of the human heme oxygenase-1 gene expression in cultured cells. Biochim Biophys Acta 1447, 231–235.

Tasaki, Y., Takamori, R., Koshihara, Y., 1991. Prostaglandin D2 metabolite stimulates collagen synthesis by human osteoblasts during calcification. Prostaglandins 41, 303–313.

Yet, S.F., Pellacani, A., Patterson, C., Tan, L., Folta, S.C., Foster, L., Lee, W.S., Hsieh, C.M., Perrella, M.A., 1997. Induction of heme oxygenase-1 expression in vascular smooth muscle cells. A link to endotoxic shock. J Biol Chem 272, 4295–4301.

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The possible role of heat shock factor-1 in the negative regulation of heme oxygenase-1

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Abstract

We examined a possible role for heat shock factor-1 (HSF-1) in the negative regulation of *HO-1* gene expression in human Hep3B hepatoma cells responding to stimulation with 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and arsenite. Overexpression of HSF-1 and heat-shock experiments indicated that HSF-1 repressed the 15d-PGJ_2-and arsenite-induced *HO-1* gene expression through directly binding to the consensus heat shock element (HSE) of the *HO-1* gene promoter. In addition, point mutations at specific HSE sequences of the HO-1 promoter-driven luciferase plasmid (pGL2/hHO3.2-Luc) abolished the heat shock- and HSF-1-mediated repression of reporter activity. Overall, it is possible that HSF-1 negatively regulates *HO-1* gene expression, and that the HSE present in the −389 to −362 region mediates HSF-1-induced repression of human *HO-1* gene expression.

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Keywords: Heme oxygenase-1; Heat shock factor-1; Hepatoma; Arsenite; 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2

1. Introduction

Heme oxygenase (HO) is a microsomal enzyme that degrades protoheme IX by cleaving its α-methene bridge into carbon monoxide (CO), free divalent iron, and biliverdin-IXα (Maines, 1997; Ponka, 1999). Three isoforms transcribed from separate genes have been characterized. HO-2 is a constitutively active form found mainly in the brain and testes (McCoubrey & Maines, 1994), and HO-3 has only recently been described, with properties similar to those of HO-2 (McCoubrey, Huang, & Maines, 1997). HO-1, known

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as heat shock protein 32, is induced by stressors, including its substrate heme, cytokines, heavy metals, and oxygen free radicals (Elbirt, Whitmarsh, Davis, & Bonkosky, 1998; Keyse & Tyrrell, 1989; Lee & Chan, 2002; Vile, Basu-Modak, Waltner, & Tyrrell, 1994). Much evidence has suggested that up-regulation of HO-1 plays an important role in the cytoprotective defense response against oxidative stress and inflammatory stimuli (Choi & Alam, 1996; Rizzardini, Terao, Falciani, & Cantoni, 1993). High expression of HO-1 in the liver suggests that the HO/CO system might serve as a modulator of hepatobiliary function. For example, induction of HO-1 by heme resulting in increased delivery of heme to the liver occurred as a result of several processes including a splenectomy, hemolysis, and rhabdomyolysis (Rizzardini, Carelli, Cabello Porras, & Cantoni, 1994; Suematsu & Ishimura, 2000). The products of CO may function as a gaseous regulator of cytochrome P450-dependent biotransformation, such as bile acid synthesis and xenobiotic catabolism (Sano et al., 1997; Shinoda et al., 1998). In contrast to the beneficial roles, induction of HO-1 expression may be associated with endotoxic shock in vascular smooth muscle cells (Yet et al., 1997), and repression of HO-1 expression may represent a defense strategy developed in humans (Shibahara, Nakayama, Kitamuro, Udono-Fujimori, & Takahashi, 2003).

Regulation of *HO-1* expression has been extensively studied, and several cis-acting promoter elements involved in its expression have also been elucidated (Lavrovsky, Schwartzman, Levere, Kappas, & Abraham, 1994; Lu et al., 1998; Takahashi et al., 1999). Specific sequences of a putative heat shock element (HSE) have been identified to be present in the HO-1 promoter. However, HSE seems to play a discrepant role in different species and cell types. A difference in the heat-mediated induction of HO-1 exists between rats and humans. In the rat, exposure of cells to elevated temperatures causes a rapid increase in HO-1 expression, indicating that the HSE is a functional element in response to heat shock (Raju & Maines, 1994; Shibahara, Muller, & Taguchi, 1987). In contrast to the rat gene, the heat-mediated induction of human HO-1 seems to be observed only in certain cell lines (Keyse & Tyrrell, 1989; Mitani, Fujita, Sassa, & Kappas, 1990). The different expression patterns in various human cell lines caused by heat shock suggest that induction of HO-1 occurs in a cell line-dependent manner, and the HSE seems to be a functional element of the human *HO-1* gene.

Heat shock factor-1 (HSF-1) is known to have the unique ability to bind to the HSE in a heat shock-dependent manner. Upon treatment with stress inducers, such as heat shock, activated HSF-1 translocates to the nucleus, binds as trimers to multiple arrays of the HSE, which are located in the promoter region of genes, and then regulates gene expression (Mosser, Theodorakis, & Morimoto, 1988). The HSE has been found to be present in the promoter of *Hsp* genes and several non-*Hsp* genes, such as TNFα and IL-1β (Cahill, Waterman, Xie, Auron, & Calderwood, 1996; Singh, He, Calderwood, & Hasday, 2002). Recent studies indicated that HSF-1 negatively regulates TNFα and IL-1β expression for which the HSE might serve as a negative regulatory element. In human monocytic cells, HSF-1 bound to the IL-1β promoter and repressed its activity in a manner dependent upon the presence of an intact HSE (Cahill et al., 1996). Several studies have reported a negative regulatory region in human HO-1 (Deramaudt, da Silva, Remy, Kappas, & Abraham, 1999; Lu, Pepe, Gildemeister, Tyrrell, & Bonkovsky, 1997), and the HSE possibly playing a role as a negative regulatory element in the human *HO-1* gene has not been well investigated as yet.

In this study, we investigated the possible role of HSF-1 in repression of HO-1 expression in human Hep3B hepatoma cells. Due to the discrepant roles of the HSE in the human *HO-1* gene and HSE's ability to act as a negative regulatory element, we examined the possibility that HSF-1 negatively regulates HO-1 expression through binding to the HSE. Our results demonstrated that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2)- and arsenite-induced upregulation of human HO-1 expression could be repressed by heat shock or overexpression of HSF-1. In another experiment, we used a mutated HO-1 promoter construct with specific point mutations in the HSE consensus sequences, which resisted repression by heat shock or the overexpression of HSF-1.

2. Materials and methods

2.1. Cell culture and treatments

Sodium arsenite and 15d-PGJ_2 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA) and Merck Taiwan Branch (Taipei, Taiwan), respectively. Human Hep3B hepatoma cells were cultured in MEM containing 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) and 1 mM sodium pyruvate. For heat shock (HS) treatments, cells were placed in a bath for 1 h at 42.5 °C and then allowed to recover for different times at 37 °C as described for individual examinations.

2.2. Plasmids and transfection assays

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 synthesized primers (Table 1). The PCR products were inserted into the pCR2.1-TOPO cloning vector (Invitrogen), and cut with KpnI/XbaI such that the resulting PCR products contained the KpnI/XbaI sites; these were ligated in frame into the unique KpnI/NheI sites present within the pGL2 plasmid (Promega, Madison, WI). Therefore, we obtained a pGL2/hHO3.2-Luc reporter construct containing an approximately 3.2 kb region of the human HO-1 promoter driving luciferase gene expression. Site-directed mutagenesis of the consensus HSE sequence (−389 to −362) of the human HO-1 promoter was made by sequential polymerase chain reaction steps from pGL2/hHO3.2-Luc. First, complementary oligonucleotides I and II were designed, and oligonucleotides III and IV (Table 1) were also synthesized for use as primers. Individual PCRs with primers I and IV as well as with primers II and III were performed using the pGL2/hHO3.2-Luc plasmid as a template. The amplification products were annealed with each other and extended by mutually primed synthesis. Fragments were then amplified by a second PCR step in the presence of primers III and IV. The products were then cut with *Apa*I and inserted into pGL2/hHO3.2-Luc, which had been cut with *Apa*I to eliminate the wild-type fragments.

The pcDNA3/HSF-1 expression plasmid, containing approximately 1.6 kb of full-length cDNA of human HSF-1, was amplified from the pHUHSF1 plasmid (Rabindran, Giorgi, Clos, & Wu, 1991) using the synthesized primers (Table 1). The PCR products were inserted into the pTARGET vector (Promega), cut with NheI/KpnI such that the resulting PCR products contained the NheI/KpnI sites, and ligated in frame into the unique NheI/KpnI sites present within the pcDNA3.1 vector (Invitrogen). All sequences of wild-type or mutant pGL2/hHO3.2-Luc and pcDNA3/HSF-1 were confirmed and verified by DNA sequencing. For establishment of stable expression of HSF-1 cell lines, Hep3B cells were transfected with pcDNA3/HSF-1 and selected with G418 for 4 weeks. One HSF-1-overexpressing clone (H8) was chosen to examine HO-1's expression.

For transfection, cells were seeded in 6 cm dishes (Western blot and RT-PCR) or 6-well plates (luciferase assay) at a density of 6×10^5 and 2.5×10^5, respectively. The next day, cells were transfected with either the wild-type or mutant pGL2/hHO3.2-Luc reporter plasmid, the pcDNA3/HSF-1 plasmid (when required), and phRL-TK (Promega) as the internal control plasmid using LipofetAMINE 2000™ (Invitrogen). After transfection (9 h), the medium was replaced with complete medium, and incubated was continued for another 39 h. Transfected cells were then directly treated with drugs, subjected to heat shock, or subjected to heat shock prior to drug treatment for Western blot, RT-PCR, or luciferase assay. Luciferase assays were performed using a FireLite™ dual luciferase assay kit (Packard Instrument Company, Meriden, CT) and a TopCount microplate scintillation and luminescence counter (Packard 9912v1) according to the manufacturer's instructions. Luciferase activities of the reported plasmid were normalized to luciferase activities of the internal control plasmid (Liang, Tsai, Lin-Shiau, Chen, & Lin, 2001).

2.3. Western blot analysis

Equal amounts of total cellular proteins or nuclear proteins (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, anti-Hsp70, or anti-HSF-1 (Santa Cruz Biotechnology, Santa Cruz, CA) antiserum as described previously (Liu et al., 2003).

2.4. RT-PCR and mRNA stability

Total RNA was isolated from both control and test cultured cells, and the mRNA level was de-

Table 1
Primer sequences[a] and their applications in this study

- Construction of plasmids
  - Human HO-1 promoter reporter plasmid
    - Forward: 5′-AGAGAACAGTTAGAAAAGAAAG-3′
    - Reverse: 5′-TACGGGCACAGGCAGGATCAGAA-3′
  - Human HO-1 promoter reporter plasmid with a mutant HSE (−389 to −362)
    - Oligonucleotide I: 5′-GCCTCCCAGCTTT**C**TG**G*C*A**CC**TT*A***TG**G**GACG-3′
- Oligonucleotide II: 5′-CAGGCGTCCCATAAGGTGCCAGAAAGCTGGGA-3′
    - Oligonucleotide III: 5′-AATCACAGTATTGGGAAAGGACTGTATGA-3′
    - Oligonucleotide IV: 5′-CTTTATGTTTTTGGCGTCTTCCA-3′
  - Human HSF-1 cDNA
    - Sense: 5′-TCCTTGCTCGAGATGGATCTG-3′
    - Antisense: 5′-CTAGGAGACAGTGGGGTCCTT-3′
- RT-PCR
  - HO-1 (nucleotides 676–1097 of GenBank accession nos. XM009946)
    - Sense: 5′-TGATAGAAGAGGCCAAGA-3′
    - Antisense: 5′-TTTCCAGAGAGAGGGACA-3′
  - HO-2 (nucleotides 338 to 693 of GenBank accession nos. NM002134)
    - Sense: 5′-TGGAGCGCAACAAGGACCAT-3′
    - Antisense: 5′-CCGGTAGAGCTGCTTGAACT-3′
- EMSA
  - hHO-1/HSEf: HSE (−1482 to −1455) of the human HO-1 gene promoter
    - Forward: 5′-TGGTTTTC**G**GG**GAA**CC**TTC**AG**A**GGAAGAA-3′
    - Reverse: 5′-AGATTCTTCCTCTGAAGGTTCCCCGAAAA-3′
  - Wt hHO-1/HSEn: wild HSE (−389 to −362) of the human HO-1 gene promoter
    - Forward: 5′-CAGCTTT**C**TG**GAA**CC**TTC**TG**G**GACGCCT-3′
    - Reverse: 5′-AGAAGGCGTCCCAGAAGGTTCCAGAAAG-3′,
  - Mut hHO-1/HSEn: mutant HSE (-389 to -362) of the human HO-1 gene promoter
    - Forward: 5′-CAGCTTT**C**TG**G*C*A**CC**TT*A***TG**G**GACGCCT-3′
    - Reverse: 5′-AGAAGGCGTCCCATAAGGTGCCAGAAAG-3′
  - Hsp70/HSE: HSE (−114 to −87) of the human Hsp70 gene promoter
    - Forward: 5′-GAAACCC**C**TG**GAA**TA**TTC**CC**G**ACCTGGC-3′
    - Reverse: 5′-AGAGCCAGGTCGGGAATATTCCAGGGGT-3′

[a] The underlining indicates HSE inverted repeats (**C**nn**GAA**nn**TTC**nn**G**). The italicized bases indicate a mutation to generate the mutated HSE probe or reporter.

tected by RT-PCR as described previously (Liang, Lin-Shiau, Chen, & Lin, 1999a). Two sets of primers were used to amplify the cDNA of HO-1 and HO-2 (Table 1). For measurement of mRNA stability, cells were treated with or without HS and then stimulated with 15d-PGJ$_2$ for 6 h, followed by treatment with actinomycin D (4 μg/ml). Total RNA was prepared, and mRNA was measured as described above.

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described in our previous report (Liang et al., 1999b). The partially complementary oligonucleotides were prepared (Table 1), and when required, filled in with [α-^{32}P] dCTP by the Klenow enzyme. For binding reactions, 10 μg of nuclear proteins was mixed with 3 μl of the labeled probe (approximately 5000 cpm) and 2 μg of poly(dI-dC) · poly(dI-dC) in a reaction buffer (10 mM Tris, pH 7.4, 50 mM NaCl, 5% glycerol, and 1 mM DTT) for 20 min at room temperature. To prove the specificity of binding, it was required that the reactions be carried out in the presence of a 50-fold excess of unlabeled wild-type or mutant HSE probe or in the presence of the anti-HSF-1 antibody. Complexes were separated by 4.5% native polyacrylamide gels and exposed to autoradiography.

3. Results

3.1. 15d-PGJ$_2$ and arsenite induce HO-1 expression

Our previous study found that 15d-PGJ$_2$ was a more effective activator than its precursor, Δ^{12}-PGJ$_2$ (Liu et al., 2004) in the induction of HO-1 expression. Therefore, 15d-PGJ$_2$ and another identified activator, arsenite (Keyse & Tyrrell, 1989), were used in this study. We examined the effects of 15d-PGJ$_2$ and arsenite on HO-1 expression in human Hep3B cells by Western blot analysis and RT-PCR. 15d-PGJ$_2$ and arsenite increased the protein and mRNA expressions of HO-1 in concentration-dependent manners (Fig. 1). On the other hand, the protein and mRNA levels of HO-2 did not change. These results suggest that 15d-PGJ$_2$ and arsenite induce HO-1 expression at the transcription levels.

3.2. Activation of HSF-1 down-regulates HO-1 expression

To investigate whether heat shock influences HO-1 expression induced by 15d-PGJ$_2$ and arsenite, cells were subjected to heat shock before being treated with 15d-PGJ$_2$ and arsenite. 15d-PGJ$_2$ and arsenite-induced HO-1 protein expression; however, elevated temperatures resulted in divergent changes in the expression of HO-1 and Hsp70 proteins (Fig. 2A). Heat shock did induce Hsp70 expression, but down-regulated the induction of HO-1. In addition, 15d-PGJ$_2$ alone increased Hsp70 protein expression. The time-course experiment indicated that both 15d-PGJ$_2$ and arsenite-induced HO-1 expression during 8–20 h, with a peak at 14 h. However, the pre-addition of HS down-regulated the induction of HO-1, and the maximal expression also was at 14 h after treatment with 15d-PGJ$_2$ and arsenite (Fig. 2B). Several lines of evidence have indicated that HSF-1 has the unique ability to bind to the HSE after heat shock. To provide direct evidence of whether HSF-1 is involved, we examined the effect of heat-independent HSF-1 overexpression on HO-1 expression. Cells were transfected with an HSF-1 expression plasmid and then treated with 15d-PGJ$_2$ or arsenite in the absence of heat shock. The induction of HO-1 by 15d-PGJ$_2$ and arsenite was down-regulated in cells transfected with the HSF-1 expression plasmid by 40 and 30%, respectively (Fig. 2C). Transfection of the HSF-1 expression plasmid alone resulted in slight activation of the Hsp70 protein, indicating that an observable amount of HSF-1 had been activated. Transfection with the HSF-1 expression plasmid or heat shock also down-regulated HO-1 mRNA expression induced by 15d-PGJ$_2$ or arsenite (Fig. 2D) similarly to the inhibition of HO-1 protein expression (Fig. 2A and C). We, next investigated whether HS affected HO-1 mRNA transcript stability. Cells were pre-treated with or without HS and with 15d-PGJ$_2$ to induce HO-1 mRNA expression. Transcription was then blocked with actinomycin D, and the half-lives of the HO-1 transcripts were assessed. The results showed that HS did not change the rate of HO-1 mRNA degradation (Fig. 2E). In additional experiments, we selected stable cell lines expressing HSF-1 to further examine the effect of HSF-1 on the down-regulation of HO-1 expression. Cells overexpressing HSF-1 (H8) had lower HO-1 expression than the parent cells in response to 15d-PGJ$_2$ (Fig. 2F, lanes 3 and 4). In addition, HO-1 expression was further decreased when HSF-1-overexpressing cells were subjected to heat shock (Fig. 2F, lanes 5 and 6). It is worthwhile noting that heat shock alone was insufficient to induce HO-1 expression under these experimental conditions.

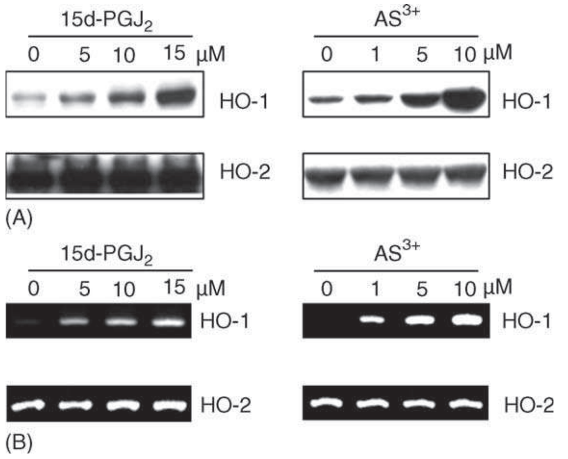


Fig. 1. 15d-PGJ$_2$ and arsenite induction of HO-1 expression. Cells were treated with 15d-PGJ$_2$ (5, 10, or 15 µM), or arsenite (1, 5, or 10 µM) for 14 (A) or 8 h (B), as indicated. Expressions of HO-1 and HO-2 were determined by Western blot (A) or by RT-PCR (B).

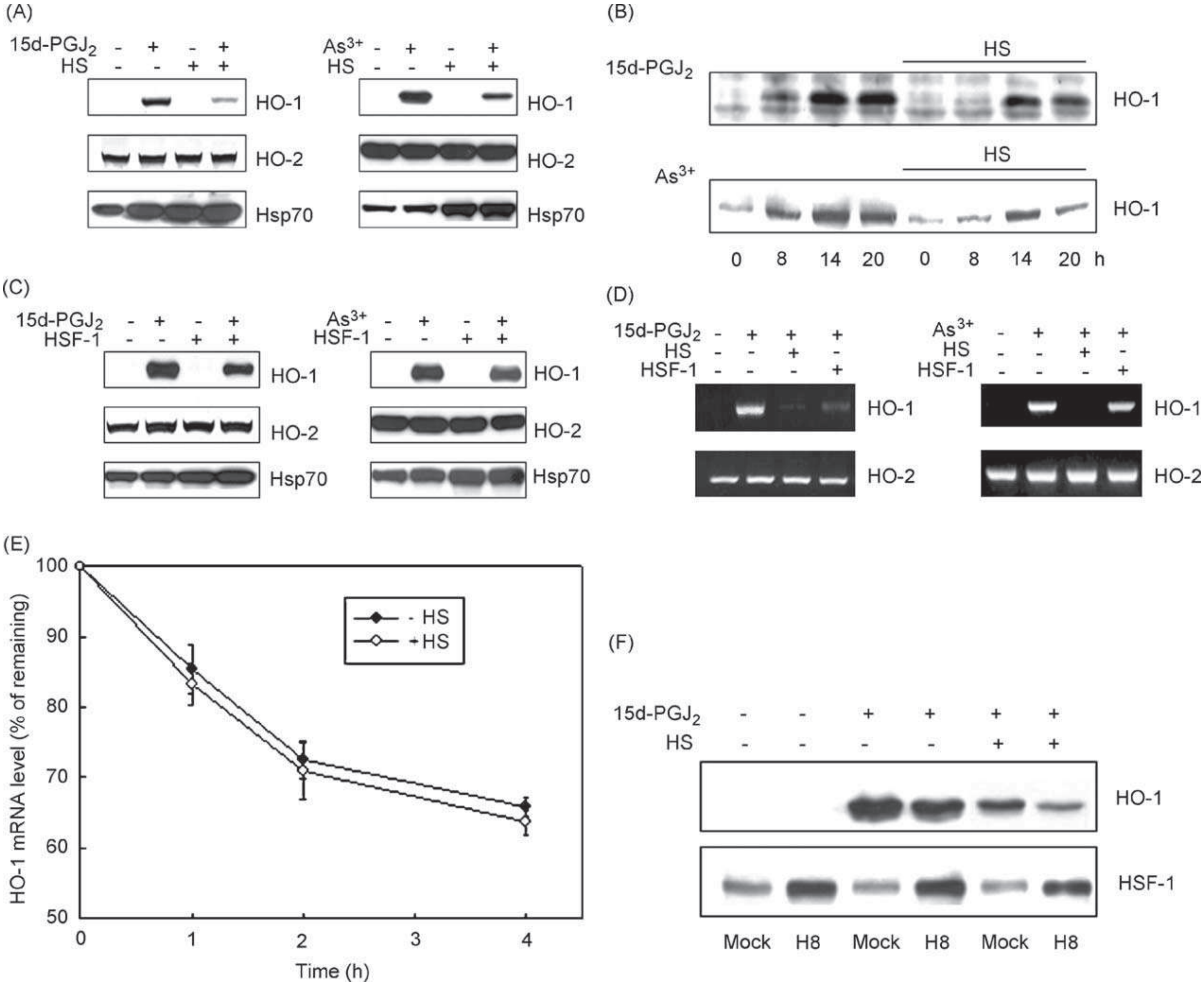
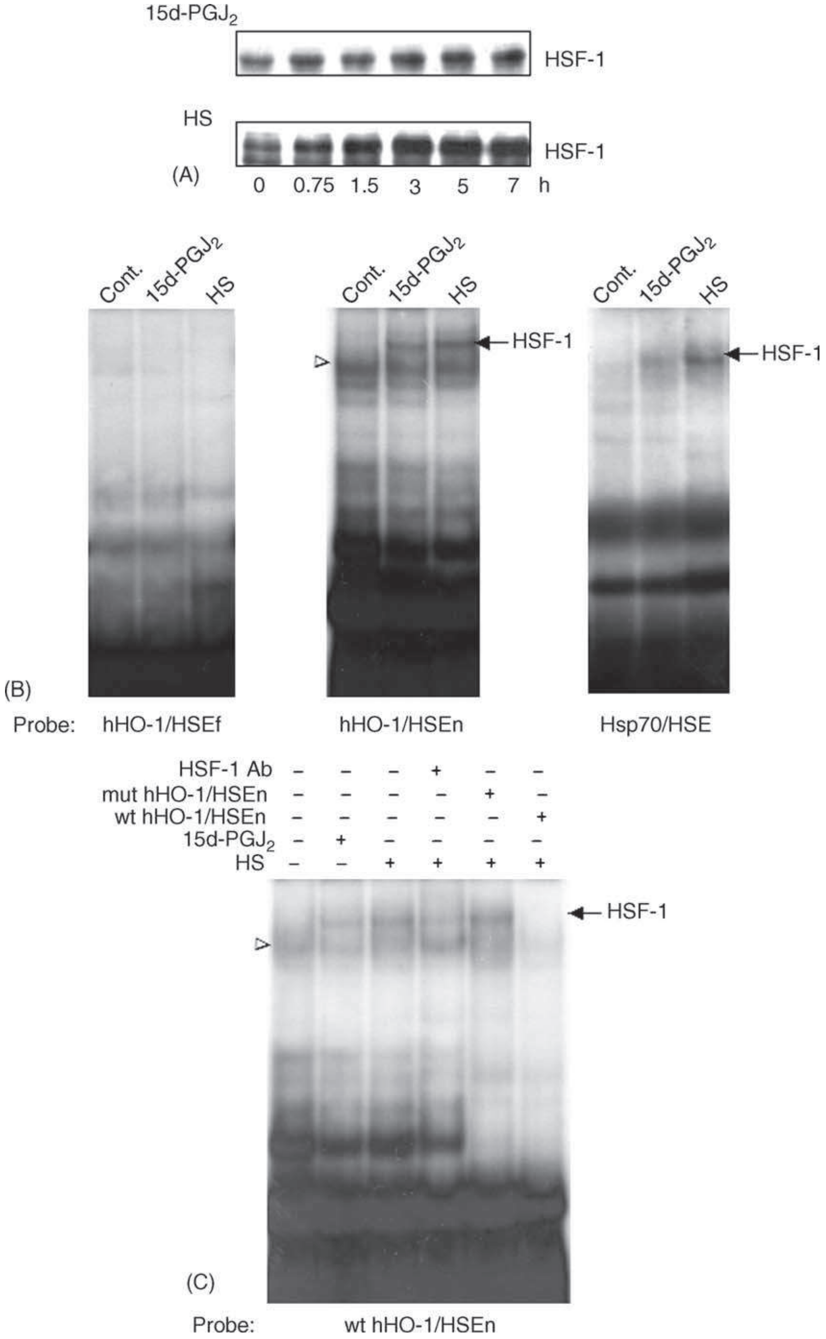


Fig. 2. Down-regulation of HO-1 expression by activation of HSF-1. (A & B) Cells were undisturbed (37 °C) or subjected to heat shock (HS) before addition of 15d-PGJ$_2$ (10 μM) or arsenite (5 μM). After 14 h (A) or indicated times (B), expressions of HO-1, HO-2, and/or Hsp70 were determined by Western blots. (C & D) Cells were transiently transfected with a mock or HSF-1 expression plasmid. Following transfection, cells were either directly treated with 15d-PGJ$_2$ (10 μM) or arsenite (5 μM), or subjected to HS before the addition of 15d-PGJ$_2$ (10 μM) or arsenite (5 μM), as indicated. After 14 (C) or 8 h (D), expressions of HO-1, HO-2, or Hsp70 were determined by Western blots (C) or by RT-PCR (D). (E) Cells were undisturbed (37 °C) or subjected to heat shock (HS) before addition of 15d-PGJ$_2$ (10 μ μM); after 6 h, cells were treated with actinomycin D (4 μg/ml) for 0–4 h. Cells were harvested, and total RNA was prepared. HO-1 mRNA levels were determined by RT-PCR normalized to GAPDH mRNA levels. Results are expressed relative to mRNA levels at 0 h, which were arbitrarily set to 100%. Values are expressed as the mean ± S.E. of triplicate tests. (F) Stable cell lines expressing HSF-1 (H8) and mock expression cells (mock) were undisturbed (37 °C) or subjected to HS before the addition of 15d-PGJ$_2$ (10 μM). After 14 h, expressions of HO-1 and HSF-1 were determined by Western blots.


15d-PGJ2
HSF-1
HS
HSF-1
(A)
0 0.75 1.5 3 5 7 h
Cont.
15d-PGJ2
HS
HSF-1
HSF-1
(B)
Probe: hHO-1/HSEf
hHO-1/HSEn
Hsp70/HSE
HSF-1 Ab – – – + – –
mut hHO-1/HSEn – – – – + –
wt hHO-1/HSEn – – – – – +
15d-PGJ2 – + – – – –
HS – – + + + +
HSF-1
(C)
Probe: wt hHO-1/HSEn

3.3. HSF-1 binds to HSE consensus sequences of the human HO-1 promoter

We attempted to confirm whether activated HSF-1 is translocated to the nucleus in 15d-PGJ$_2$- or heat shock-treated cells, and whether activated HSF-1 can bind to HSE located in the human HO-1 promoter. The first investigation was to detect if HSF-1 is present in the nucleus. 15d-PGJ$_2$ and heat shock increased HSF-1 translocation to the nucleus in a time-dependent manner, with the beginning increase seen at 45 min (Fig. 3A). Second, computer-assisted identification of the putative HSE (set at a cutoff score of > 85%) was performed using MOTIF (available at http://motif.genome.ad.jp), and we found the putative HSE in the region of −1482 to −1455 and −389 to −362 of the 5′-flanking region of human *HO-1*. Two putative HSE sequences (hHO-1/HSEf and hHO-1/HSEn) from the HO-1promoter and HSE sequences (Hsp70/HSE, as the positive control) from the *Hsp*70 promoter were selected as the EMSA probes (Table 1). No observable binding band was observed for nuclear extracts from 15d-PGJ$_2$- or heat shock-treated cells using the hHO-1/HSEf probe, indicating that the HSE sequence present in the −1482 to −1455 region of the human HO-1promoter seemed to be a pseudo-HSE for HSF-1 binding (Fig. 3B). On the other hand, nuclear extracts from 15d-PGJ$_2$- or heat shock-treated cells bound the hHO-1/HSEn probe with a similar pattern as in the case of the Hsp70/HSE probe, indicating that the HSE sequence present in the −389 to −362 region of the human HO-1promoter is a definite HSE for HSF-1 binding (Fig. 3B). In a further examination to determine if binding to the hHO-1/HSEn probe was specific, it was not detected when the incubation was carried out in the presence of the anti-HSF-1 antibody or an excess of the wild-type HSE probe (Fig. 3C). On the contrary, an excess of the mutant hHO-1/HSEn probe did not affect it.

3.4. Mutation of the HSE consensus sequences in the human HO-1promoter eliminated HSF-1-mediated repression

To investigate the functional role of the HSE site as a transcriptional element which may repress *HO-1* gene expression in response to 15d-PGJ$_2$ or arsenite, we chose the reporter construct, pGL2/hHO3.2-Luc, which contains a definite consensus HSE element located in the region from −389 to −362, upstream of the transcription start site. The mutant HSE of the pGL2/hHO3.2-Luc construct was also used to study HSE's role in regulating the *HO-1* gene, by making site-directed mutations in two of eight base pairs, as illustrated in Fig. 4A. Figure 4B shows the concentration dependence of the induction effect of 15d-PGJ$_2$ or arsenite on the reporter activity of pGL2/hHO3.2-Luc, with the maximum being reached for both at 10 μM. We next examined down-regulation of promoter activity by overexpression of HSF-1 or by heat shock. Both heat shock and overexpression of HSF-1 significantly down-regulated the induction of wild-type reporter activity induced by 15d-PGJ$_2$ or arsenite (Fig. 4C). On the other hand, the reporter construct containing the mutated HSE, although strongly induced by 15d-PGJ$_2$ or arsenite, was significantly resistant to repression by additional treatments of heat shock or overexpression of HSF-1 (Fig. 4D). These results demonstrate that repression of the HO-1promoter by heat shock or overexpression of HSF-1 is mediated through the HSE. Moreover, both 15d-PGJ$_2$ and arsenite treatment resulted in similar levels of induction of wild-type and mutant pGL2/hHO3.2-Luc reporter activities (Fig. 4E). Treatment with 15d-PGJ$_2$ resulted in 3.52- and 4.08-fold

Fig. 3. HSF-1 binding to HSE consensus sequences in the human *HO-1* promoter. (A) Cells were subjected to heat shock (HS) or treated with 15d-PGJ$_2$ (10 μM) for the indicated times. Nuclear proteins were used to detect the level of HSF-1 by Western blots. (B) Gel shift assays used different HSE probes, as well as nuclear extracts from control cells, HS-, or 15d-PGJ$_2$ (10 μM)-treated cells. (C) To examine the specificity of binding to the HSE sequence of the human *HO-1* promoter, the reaction was carried out in the absence of a competitor (lane 3), in the presence of anti-HSF-1 antibody (lane 4), or in the presence of a 50-fold excess of an unlabeled mutant HSEn (mut HSEn, lane 5) or a wild-type HSEn (wt HSEn, lane 6) probe. Nuclear extracts from cells treated with 15d-PGJ$_2$(10 μM, lane 2) or exposed to HS (lanes 3–6) were used as a source of HSF-1. Positions of the complexes of HSF-1 and ^{32}P-labeled HSE probes are marked by arrows. The open arrowhead shows additional factors, but not HSF-1, bound to HSE. The sequences of HSE probes are described in Table 1. hHO-1/HSEf, HSE sequence (−1482 to −1455) far away from the transcription start site of human *HO-1* gene; hHO-1/HSEn, HSE sequence (−389 to −362) near the transcription start site of the human *HO-1* gene; Hsp70/HSE, HSE sequence (−114 to −87) from the human *Hsp70* promoter.

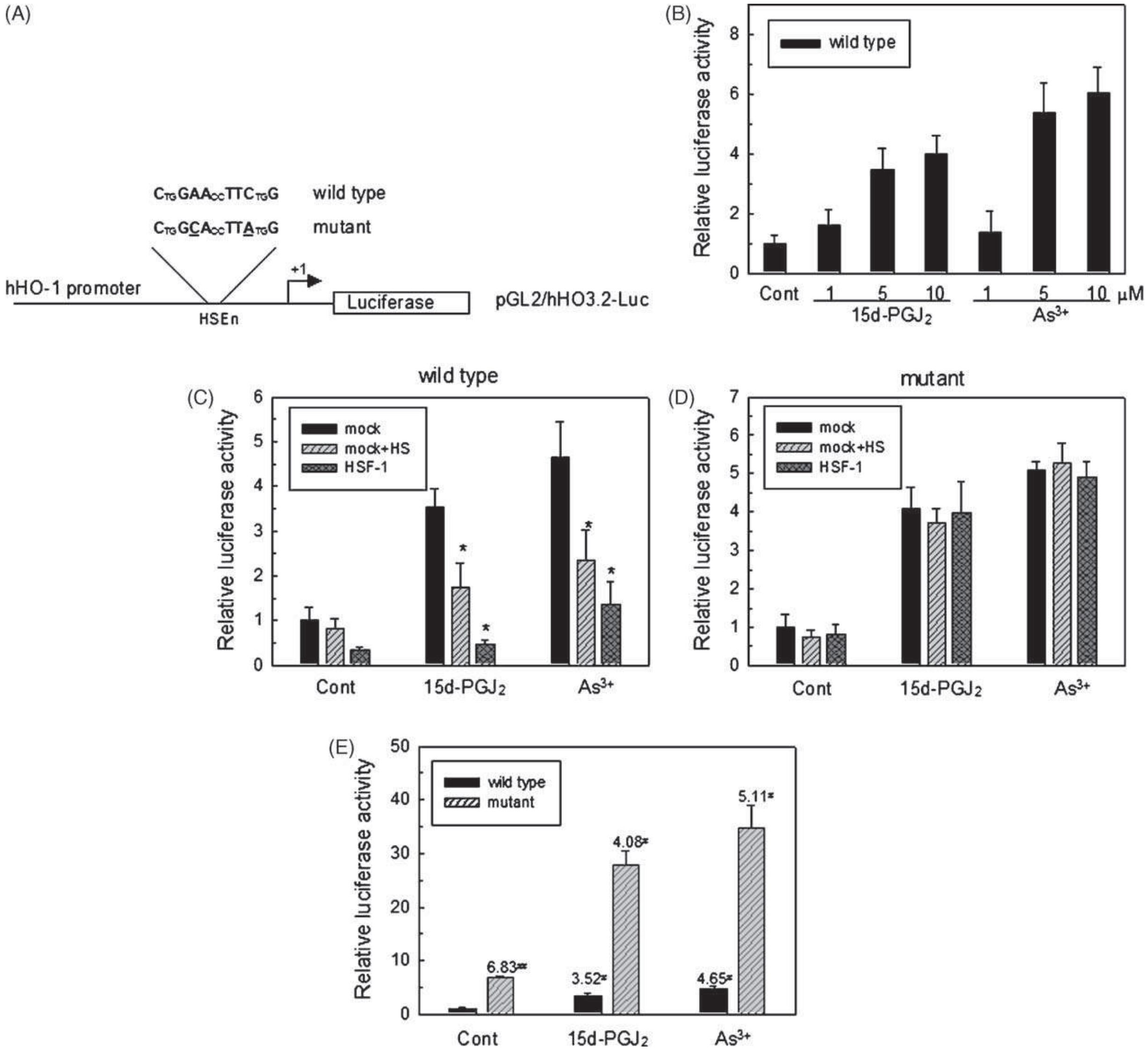


Fig. 4. Elimination of HSF-1-mediated repression by mutation of HSE consensus sequences in the human *HO-1* promoter. (A) The human *HO-1* promoter-luciferase reporter construct (pGL2/hHO3.2-Luc) contains an HSE site in the region from −369 to −382. The site-directed mutant contains a difference of two-base changes indicated by underlining. Cells were transiently co-transfected with wild-type (B, C, E) or mutant (D, E) pGL2/hHO3.2-Luc reporter and mock expression (C, D) or the HSF-1 expression (C, D) plasmid. Following transfection, cells were undisturbed (37 °C) or subjected to heat shock (HS) before the addition of 15d-PGJ$_2$ (10 μM) or arsenite (5 μM), as indicated. Total cell lysates were collected, and luciferase activities were determined. Values are expressed as the mean ± S.E. of triplicate tests. $^*p < 0.05$, compared with the individual test of mock expression alone (Student's *t*-test). The indicated numbers were obtained from $^{\#}$the induction fold of 15d-PGJ$_2$ or arsenite treatment to their control, and $^{\#\#}$the induction fold of mutant to wild-type in control cells.

activations of the wild-type and mutant reporter activities, respectively. Arsenite also produced 4.65- and 5.11-fold activations in the wild-type and mutant reporter, respectively. However, the mutant reporter had a high basal activity in the controls, and showed a 6.83-fold increase in comparison with the wild-type reporter (Fig. 4E, columns 1 and 2). These results suggest that basal expression of the *HO-1* gene may be limited by an existing HSE complex, and therefore it is expressed at a low level.

4. Discussion

In this study, we demonstrate that the negative transcriptional regulation of the *HO-1* gene involves an HSE conserved cis-acting sequence in human Hep3B hepatoma cells. Under normal conditions, low expression of HO-1 was found in adult human liver cells. It is therefore possible that (1) no significant transcriptional activator induces its expression; or (2) certain constitutive HSE-binding factors (Liu, Kim, Yang, & Li, 1993) bind with HSE, and limit *HO-1* expression at low levels. We found that mutations in the specific sequences of HSE resulted in high basal activity of the *HO-1* reporter (Fig. 4E). In addition, gel shift assays indicated that control cells contain a HSE sequence-specific binding band (Fig. 3B, C, open arrowhead), which was competed for by excess unlabeled HSE oligonucleotides (Fig. 3C, lane 6). These results suggest that certain constitutive HSE-binding factors really exist and bind with the HSE site. As mention earlier, some human cell lines can increase HO-1 expression after heat shock, while other human cell lines cannot. It is possible that components of HSE complexes, including constitutive HSE-binding factors, HSF-1, and other unknown factors, determine whether HO-1 is expressed after heat shock. It is known that there are a number of genes, apart from the classical heat shock genes, whose expression can be down-regulated by HSF-1 through interacting with another transcription factor, such as NF-IL6 or STAT-1 (Stephanou, Isenberg, Nakajima, & Latchman, 1999; Xie, Chen, Stevenson, Auron, & Calderwood, 2002). The activated transcription factors in response to 15d-PGJ_2 or arsenite may interact with HSF-1 in combination with heat shock, and ultimately repress the HO-1 expression. Therefore, our results suggest the possibility that HSF-1's partner determines the transactivation potential, but determining which of the transcription factor joins with HSF-1 in regulating the HO-1 promoter requires further study.

Various extracellular stresses, such as oxidants and heavy metals, induce HO-1 expression, and also simultaneously activate HSF-1 (Ozaki, Deshpande, Angkeow, Suzuki, & Irani, 2000; Vilaboa et al., 1995). In this study, we found that 15d-PGJ_2 induced both HO-1 and Hsp70 expressions (Fig. 2), and also activated HSF-1 (Fig. 3). The activation of HSF-1 may play an inhibitory feedback role on excess HO-1 expression. In liver cells, repression of excess HO-1 expression may reduce energy expenditure consumed for heme metabolism and prevent the local accumulation of carbon oxide, iron, and bilirubin. Shibahara et al. hypothesized that repression of HO-1 expression may represent a potential defense strategy in humans (Shibahara et al., 2003). The repression of HO-1 by HSF-1 may play an important role in the physiology of the liver, but this requires further study.

The ability of the precursor of 15d-PGJ_2, Δ^{12}-PGJ_2, to induce HO-1 expression in the rat has been examined (Koizumi, Odani, Okuyama, Ichikawa, & Negishi, 1995). In human hepatoma cells, we screened a large number of lipophilic chemicals, including arachidonic acid and its metabolites, and found that 15d-PGJ_2 increased HO-1 expression by about 5-fold higher than that of cells treated with Δ^{12}-PGJ_2 at the same concentration (Liu et al., 2004). Recently, 15d-PGJ_2 has been identified as a high-affinity ligand for peroxisome proliferator-activated receptor-γ (PPARγ). Activation of PPARγ by 15d-PGJ_2 has been considered to have anti-inflammatory actions, and the possible mechanism is linked to inhibition of multiple NFκB activation steps (Straus et al., 2000). However, the repression is partly dependent on PPARγ expression (Chawla et al., 2001), and some of the receptor-independent biological action of 15d-PGJ_2 and other cyclopentenone PGs have been proposed (Rossi et al., 2000). We hypothesize that the anti-inflammatory effects of 15d-PGJ_2 in response to inflammatory stimuli may in part be mediated by the induction of an anti-inflammatory enzyme, HO-1. Lee and Chau (2002) demonstrated that the anti-inflammatory action of interleukin-10 was mediated by the induction of HO-1. In this study, we found that 15d-PGJ_2 strongly increased HO-1 expression in human hepatoma cells. Although, the molecular mechanisms underlying the 15d-PGJ_2-induced HO-1 expression were not investigated in this study, the unpublished data suggest that induction of HO-1 expression by 15d-PGJ_2 did not mediate the activation of PPARα or PPARγ.

In conclusion, we provide evidence of a unique HSE sequence in the human *HO-1* promoter, which binds HSF-1 and is required for HSF-1-mediated transcriptional repression. This is further proof of the concept that HSF-1 cannot repress gene transcription by using the mutant HSE sequence in the HO-1 reporter plasmid.

Acknowledgments

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References

Cahill, C. M., Waterman, W. R., Xie, Y., Auron, P. E., & Calderwood, S. K. (1996). Transcriptional repression of the prointerleukin 1beta gene by heat shock factor 1. *Journal of Biological Chemistry*, *271*, 24874–24879.

Chawla, A., Barak, Y., Nagy, L., Liao, D., Tontonoz, P., & Evans, R. M. (2001). PPAR-gamma dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. *Nature Medicine*, *7*, 48–52.

Choi, A. M., & Alam, J. (1996). Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. *American Journal of Respiratory cell and Molecular Biology*, *15*, 9–19.

Csonka, C., Varga, E., Kovacs, P., Ferdinandy, P., Blasig, I. E., Szilvassy, Z., et al. (1999). Heme oxygenase and cardiac function in ischemic/reperfused rat hearts. *Free Radical Biology & Medicine*, *27*, 119–126.

Deramaudt, T. B., da Silva, J. L., Remy, P., Kappas, A., & Abraham, N. G. (1999). Negative regulation of human heme oxygenase in microvessel endothelial cells by dexamethasone. *Proceeding of Society for Experimental Biology and Medicine*, *222*, 185–193.

Elbirt, K. K., Whitmarsh, A. J., Davis, R. J., & Bonkosky, H. L. (1998). Mechanism of sodium arsenite-mediated induction of heme oxygenase-1 in hepatoma cells. Role of mitogen-activated protein kinases. *Journal of Biological Chemistry*, *273*, 8922–8931.

Keyse, S. M., & Tyrrell, R. M. (1989). Heme oxygenase is the major 32 kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proceedings of the National Academy of Sciences of the United States of America*, *86*, 99–103.

Kim, U. J., Birren, B. W., Slepak, T., Mancino, V., Boysen, C., Kang, H. L., et al. (1996). Construction and characterization of a human bacterial artificial chromosome library. *Genomics*, *34*, 213–218.

Koizumi, T., Odani, N., Okuyama, T., Ichikawa, A., & Negishi, M. (1995). Identification of a cis-regulatory element for delta 12-prostaglandin J2-induced expression of the rat heme oxygenase gene. *Journal of Biological Chemistry*, *270*, 21779–21784.

Lavrovsky, Y., Schwartzman, M. L., Levere, R. D., Kappas, A., & Abraham, N. G. (1994). Identification of binding sites for transcription factors NF-kappa B and AP-2 in the promoter region of the human heme oxygenase 1 gene. *Proceedings of the National Academy of Sciences of the United States of America*, *91*, 5987–5991.

Lee, T. S., & Chau, L. Y. (2002). Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. *Nature Medicine*, *8*, 240–246.

Liang, Y. C., Lin-Shiau, S. Y., Chen, C. F., & Lin, J. K. (1999a). 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. *Journal of Cell Biochemistry*, *75*, 1–12.

Liang, Y. C., Huang, Y. T., Tsai, S. H., Lin-Shiau, S. Y., Chen, C. F., Lin, J. K., et al. (1999b). Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. *Carcinogenesis*, *20*, 1945–1952.

Liang, Y. C., Tsai, D. C., Lin-Shiau, S. Y., Chen, C. F., & Lin, J. K. (2001). Suppression of inducible cyclooxygenase and inducible nitric oxide synthase through peroxisome proliferator-activated receptor-γ by apigenin and related-flavonoids in macrophages. *FEBS Letters*, *496*, 12–18.

Liu, J. D., Lin, S. Y., Ho, Y. S., Pan, S., Hung, L. F., Tsai, S. H., et al. (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. *Molecular Carcinogenesis*, *37*, 16–24.

Liu, R. Y., Kim, D., Yang, S. H., & Li, G. C. (1993). Dual control of heat shock response: involvement of a constitutive heat shock element-binding factor. *Proceedings of the National Academy of Sciences of the United States of America*, *90*, 3078–3082.

Liu, J. D., Tsai, S. H., Lin, S. Y., Ho, Y. S., Hung, L. F., Pan, S., et al. (2004). Thiol antioxidant and thiol-reducing agents attenuate 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2-induced heme oxygenase-1 expression. *Life Science*, *74*, 2451–2463.

Lu, T. H., Lambrecht, R. W., Pepe, J., Shan, Y., Kim, T., & Bonkovsky, H. L. (1998). Molecular cloning, characterization, and expression of the chicken heme oxygenase-1 gene in transfected primary cultures of chick embryo liver cells. *Gene*, *207*, 177–1786.

Lu, T. H., Pepe, J. A., Gildemeister, O. S., Tyrrell, R. M., & Bonkovsky, H. L. (1997). Regulation of expression of the human heme oxygenase-1 gene in transfected chick embryo liver cell cultures. *Biochimica et Biophysics Acta*, *1352*, 293–302.

Maines, M. D. (1997). The heme oxygenase system: a regulator of second messenger gases. *Annual Review of Pharmacology and Toxicology*, *37*, 517–554.

McCoubrey, W. K., Jr., & Maines, M. D. (1994). The structure, organization and differential expression of the gene encoding rat heme oxygenase-2. *Gene*, *139*, 155–156.

McCoubrey, W. K., Jr., Huang, T. J., & Maines, M. D. (1997). Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *European Journal of Biochemistry*, *247*, 725–732.

Mitani, K., Fujita, H., Sassa, S., & Kappas, A. (1990). Activation of heme oxygenase and heat shock protein 70 genes by stress in human hepatoma cells. *Biochemical and Biophysical Research Communications*, *166*, 1429–1434.

Mosser, D. D., Theodorakis, N. G., & Morimoto, R. I. (1988). Coordinate changes in heat shock element-binding activity and HSP70 gene transcription rates in human cells. *Molecular and Cellular Biology*, *8*, 4736–4744.

Nath, K. A., Balla, G., Vercellotti, G. M., Balla, J., Jacob, H. S., Levitt, M. D., et al. (1992). Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat. *Journal of Clinical Investigation*, *90*, 267–270.

Nishizawa, J., Nakai, A., Matsuda, K., Komeda, M., Ban, T., & Nagata, K. (1999). Reactive oxygen species play an important role in the activation of heat shock factor 1 in ischemic-reperfused heart. *Circulation*, *99*, 934–941.

Ozaki, M., Deshpande, S. S., Angkeow, P., Suzuki, S., & Irani, K. (2000). Rac1 regulates stress-induced, redox-dependent heat shock factor activation. *Journal of Biological Chemistry*, *275*, 35377–35383.

Ponka, P. (1999). Cell biology of heme. *American Journal of Medical Science*, *318*, 241–256.

Rabindran, S. K., Giorgi, G., Clos, J., & Wu, C. (1991). Molecular cloning and expression of a human heat shock factor HSF1. *Proceedings of the National Academy of Sciences of the United States of America*, *88*, 6906–6910.

Raju, V. S., & Maines, M. D. (1994). Coordinated expression and mechanism of induction of HSP32 (heme oxygenase-1) mRNA by hyperthermia in rat organs. *Biochimica et Biophysics Acta*, *1217*, 273–280.

Rizzardini, M., Carelli, M., Cabello Porras, M. R., & Cantoni, L. (1994). Mechanisms of endotoxin-induced haem oxygenase mRNA accumulation in mouse liver: synergism by glutathione depletion and protection by N-acetylcysteine. *The Biochemical Journal*, *304*, 477–483.

Rizzardini, M., Terao, M., Falciani, F., & Cantoni, L. (1993). Cytokine induction of haem oxygenase mRNA in mouse liver. Interleukin 1 transcriptionally activates the haem oxygenase gene. *The Biochemical Journal*, *290*, 343–347.

Rossi, A., Kapahi, P., Natoli, G., Takahashi, T., Chen, Y., Karin, M., et al. (2000). Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IkappaB kinase. *Nature*, *403*, 103–108.

Sano, T., Shiomi, M., Wakabayashi, Y., Shinoda, Y., Goda, N., Yamaguchi, T., et al. (1997). Endogenous carbon monoxide suppression stimulates bile acid-dependent biliary transport in perfused rat liver. *American Journal of Physiology*, *272*, G1268–G1275.

Shibahara, S., Muller, R. M., & Taguchi, H. (1987). Transcriptional control of rat heme oxygenase by heat shock. *Journal of Biological Chemistry*, *262*, 12889–12892.

Shibahara, S., Nakayama, M., Kitamuro, T., Udono-Fujimori, R., & Takahashi, K. (2003). Repression of heme oxygenase-1 expression as a defense strategy in humans. *Experimental Biology and Medicine (Maywood)*, *228*, 472–473.

Shinoda, Y., Suematsu, M., Wakabayashi, Y., Suzuki, T., Goda, N., Saito, S., et al. (1998). Carbon monoxide as a regulator of bile canalicular contractility in cultured rat hepatocytes. *Hepatology*, *28*, 286–295.

Singh, I. S., He, J. R., Calderwood, S., & Hasday, J. D. (2002). A high affinity HSF-1 binding site in the 5'-untranslated region of the murine tumor necrosis factor-alpha gene is a transcriptional repressor. *Journal of Biological Chemistry*, *277*, 4981–4988.

Stephanou, A., Isenberg, D. A., Nakajima, K., & Latchman, D. S. (1999). Signal transducer and activator of transcription-1 and heat shock factor-1 interact and activate the transcription of the Hsp-70 and Hsp-90beta gene promoters. *Journal of Biological Chemistry*, *274*, 1723–1728.

Straus, D. S., Pascual, G., Li, M., Welch, J. S., Ricote, M., Hsiang, C. H., et al. (2000). 15-deoxy-delta 12, 14-prostaglandin J2 inhibits multiple steps in the NF-kappa B signaling pathway. *Proceedings of the National Academy of Sciences of the United States of America*, *97*, 4844–4849.

Suematsu, M., & Ishimura, Y. (2000). The heme oxygenase-carbon monoxide system: a regulator of hepatobiliary function. *Hepatology*, *31*, 3–6.

Takahashi, S., Takahashi, Y., Ito, K., Nagano, T., Shibahara, S., & Miura, T. (1999). Positive and negative regulation of the human heme oxygenase-1 gene expression in cultured cells. *Biochimica et Biophysica Acta*, *1447*, 231–235.

Vilaboa, N. E., Calle, C., Perez, C., de Blas, E., Garcia-Bermejo, L., & Aller, P. (1995). cAMP increasing agents prevent the stimulation of heat-shock protein 70 (HSP70) gene expression by cadmium chloride in human myeloid cell lines. *Journal of Cell Science*, *108*, 2877–2883.

Vile, G. F., Basu-Modak, S., Waltner, C., & Tyrrell, R. M. (1994). Heme oxygenase 1 mediates an adaptive response to oxidative stress in human skin fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, *91*, 2607–2610.

Xie, Y., Chen, C., Stevenson, M. A., Auron, P. E., & Calderwood, S. K. (2002). Heat shock factor 1 represses transcription of the IL-1beta gene through physical interaction with the nuclear factor of interleukin 6. *Journal of Biological Chemistry*, *277*, 11802–11810.

Yet, S. F., Pellacani, A., Patterson, C., Tan, L., Folta, S. C., Foster, L., et al. (1997). Induction of heme oxygenase-1 expression in vascular smooth muscle cells. A link to endotoxic shock. *Journal of Biological Chemistry*, *272*, 4295–4301.