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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.

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**GCA**CC**TTA**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**GGG**GAA**CC**TTC**AGA**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**GCA**CC**TTA**TG**G**GACGCCT-3′
Reverse: 5′-AGAAGGCGTCCCATAAGGTGCCAGAAAG-3′
Hsp70/HSE: HSE (−114 to −87) of the human Hsp70 gene promoter
Forward: 5′-GAAACCC**CTG**GAA**TA**TTC**CCG**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.

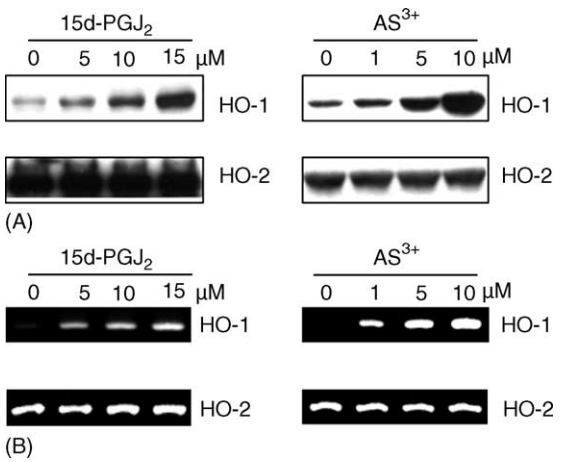


Fig. 1. 15d-PGJ_2 and arsenite induction of HO-1 expression. Cells were treated with 15d-PGJ_2 (5, 10, or 15 μM), or arsenite (1, 5, or 10 μM) for 14 (A) or 8 h (B), as indicated. Expressions of HO-1 and HO-2 were determined by Western blot (A) or by RT-PCR (B).

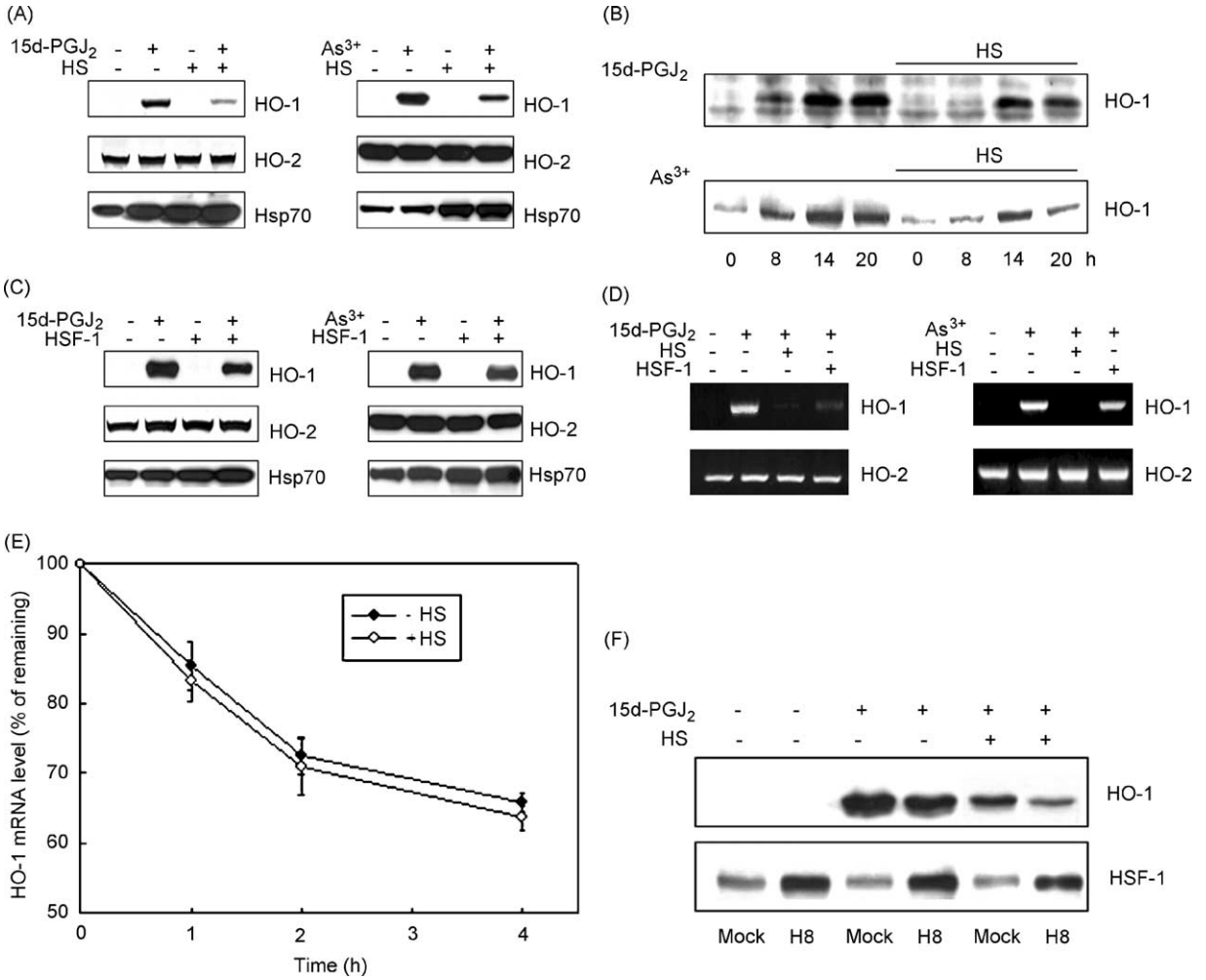
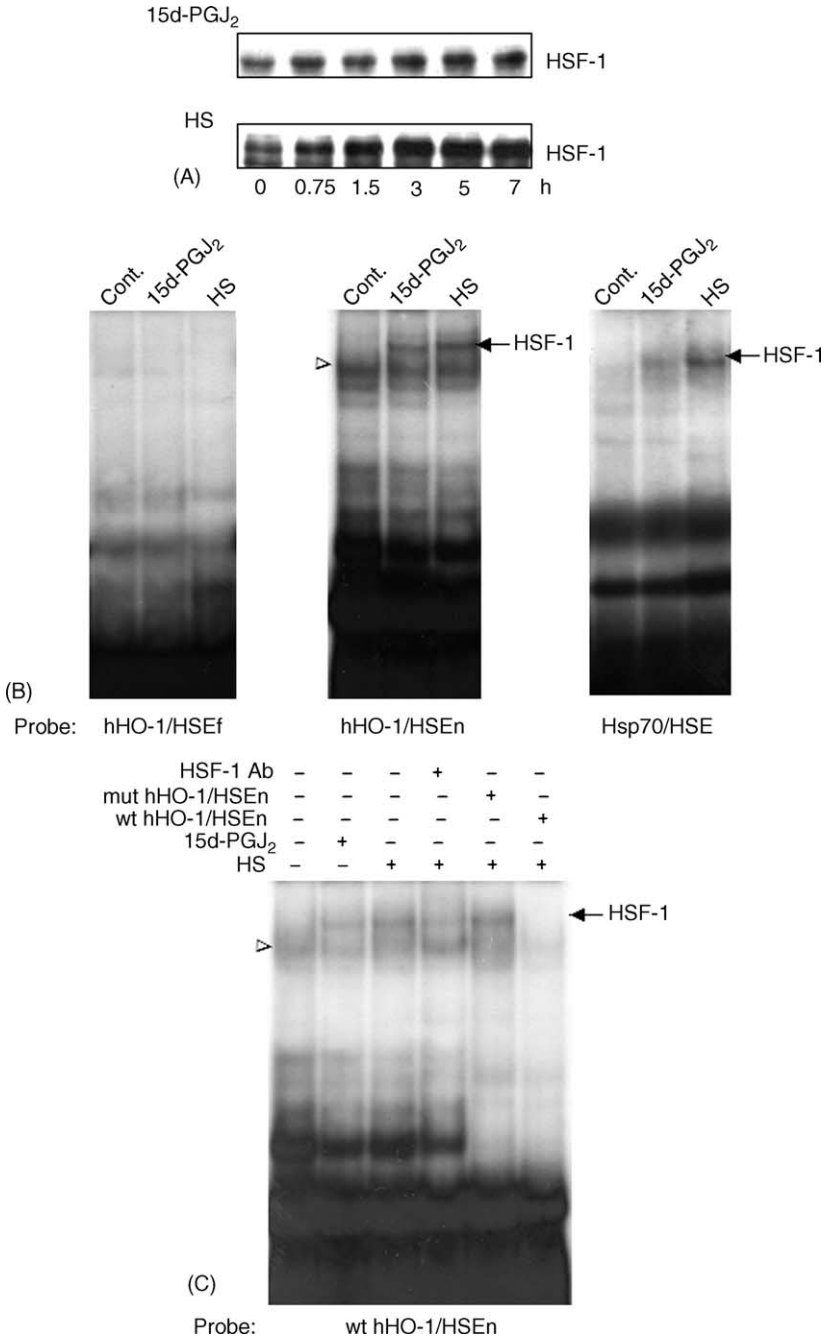


Fig. 2. Down-regulation of HO-1 expression by activation of HSF-1. (A & B) Cells were undisturbed (37 °C) or subjected to heat shock (HS) before addition of 15d-PGJ_2 (10 μM) or arsenite (5 μM). After 14 h (A) or indicated times (B), expressions of HO-1, HO-2, and/or Hsp70 were determined by Western 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.

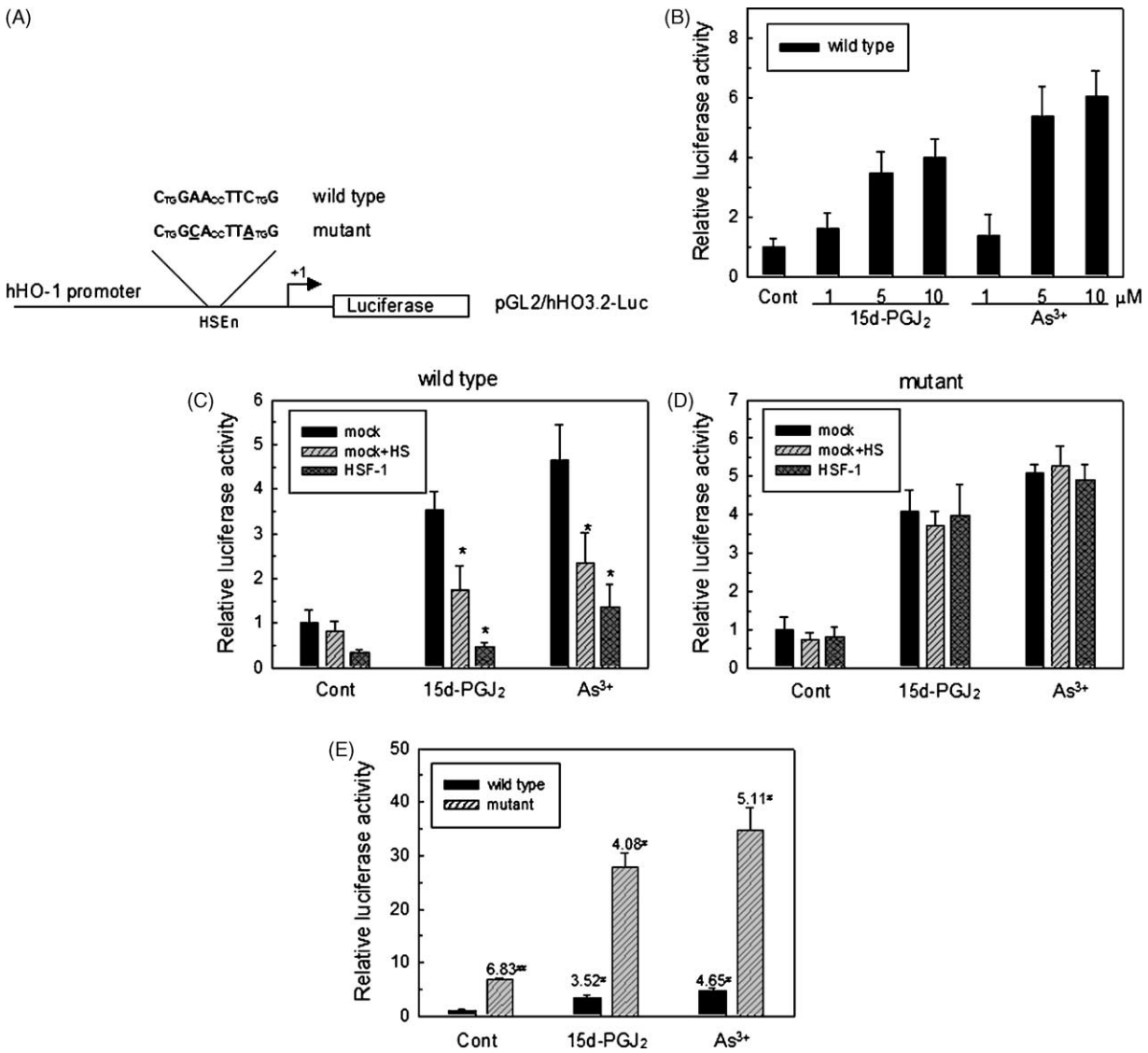


Fig. 4. Elimination of HSF-1-mediated repression by mutation of HSE consensus sequences in the human *HO-1* promoter. (A) The human *HO-1* promoter-luciferase reporter construct (pGL2/hHO3.2-Luc) contains an HSE site in the region from −369 to −382. The site-directed mutant contains a difference of two-base 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 expres-

sion 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.

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