Free Radical Research, April 2008; 42(4): 297–304

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healthcare

Differential roles for Nrf2 and AP-1 in upregulation of HO-1 expression by arsenite in murine embryonic fibroblasts

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Accepted by Professor G. Mann

(Received 25 October 2007; in revised form 15 January 2008)

Abstract
Heme oxygenase-1 (HO-1) is markedly upregulated by sodium arsenite and previous studies implicated the transcriptional enhancers Nrf2 and AP-1 in arsenite-induced *ho-1* gene expression in murine cells. To further evaluate the role of Nrf2 and its signalling pathway in the induction of HO-1 in response to low levels of arsenite, this paper studied wild-type and Nrf2-deficient murine embryonic fibroblasts. It was found that Nrf2 plays a crucial role in the early activation of *ho-1* transcription and that increased Nrf2 levels returned to basal levels within 24 h. In $Nrf2^{-/-}$ cells, *ho-1* gene activation increased gradually and HO-1 protein levels were approximately half of those attained in $Nrf2^{+/+}$ cells. The tyrosine kinase inhibitor genistein and JNK inhibitor SP600125 significantly attenuated arsenite induced increases in *ho-1* mRNA levels in Nrf2 deficient cells but had negligible effects on Nrf2 activation, suggesting tyrosine kinase/JNK/c-Jun plays a key role in the HO-1 upregulation via AP-1.

Keywords: *heme oxygenase-1, Nrf2, AP-1, JNK, Src, genistein, PD153035, arsenite*

Abbreviations: *AP-1, activator protein-1; ARE, antioxidant response element; EpRE, electrophile response element; HO-1, heme oxygenase-1; JNK, Jun N-terminal kinase; Nrf2, NF-E2-related factor 2; TRE, TPA-response element; EGFR, epidermal growth factor receptor.*

Introduction

Heme oxygenase (HO) catalyses the initial and rate-limiting reaction in heme catabolism [1,2]. HO-1 is a stress-inducible HO isoform that is recognized as a cellular stress marker, because a variety of agents strongly activate its gene expression [3–5]. As induction of HO-1 protects cells and tissues from oxidative stress, studies on the functions of HO-1 reaction products and on the regulation of *ho-1* gene expression by stress agents have attracted considerable interest.

Arsenic compounds are potent HO-1 inducers [6] and inorganic arsenic is an environmental contaminant and a known human carcinogen. Arsenite

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ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.1080/10715760801975735

(trivalent) is thiol-reactive and more toxic than arsenate (pentavalent). Low levels of arsenite cause oxidative stress in cells, most likely by reacting with cysteine residues in proteins and evoking cellular defense responses that lead to the upregulation of heat-shock family proteins [7] and other stress-response proteins [8,9]. Although HO-1 is known as Hsp32 [10], murine and human *ho-1* genes are not sensitive to heat-induction or to HSF-mediated gene activation (see review by Alam and Cook [11]).

Previous studies established that stress-response elements (StREs) are major *cis*-elements for *ho-1* gene activation in response to various stress agents including arsenite [12,13]. The consensus sequence of the StREs is similar to that of antioxidant response elements/electrophile response elements (ARE/EpRE) and all five StREs (AREs) contain an AP-1 (Fos/Jun)-binding consensus sequence, TRE [13]. Previous studies implicated AP-1 in arsenite-induced *ho-1* gene activation [14–16]. However, recent studies highlighted the importance of the Nrf2-ARE/EpRE pathway [17] in arsenite mediated HO-1 induction [18–20]. Thus, Nrf2/small Maf heterodimers and AP-1 complexes appear to be the two major transcription factors participating in the arsenite-induced murine *ho-1* gene activation, but the role of kinases in regulation of these two pathways by arsenite remains unclear.

Tyrosine kinase activity is clearly important in arsenite-induced HO-1 upregulation [21]. Kinase activity seems to be important for AP-1 activation, since arsenite activates JNK through tyrosine kinase [22] and JNK regulates AP-1 activation [23]. However, the tyrosine kinase responsible for gene activation has not yet been identified, although Chen et al. [24] first showed that arsenite activated EGF receptor tyrosine kinase played a crucial role for Ras/ERK signalling cascade. It is not known whether tyrosine kinase activity affects arsenite induced Nrf2 activation. In this context, recent studies report that JNK and ERK are involved in Nrf2-mediated gene activation [25,26].

Arsenite activates *ho-1* gene expression through multiple signalling pathways. Dissection of these pathways using wild-type cells alone is not straightforward and can lead to incorrect conclusions. Therefore, we compared wild-type and Nrf2-deficient murine embryonic fibroblasts to define the roles of Nrf2 and tyrosine kinase activity in *ho-1* gene activation by low levels of arsenite. We report that Nrf2 plays an essential role in the early and transient phase of *ho-1* gene induction by arsenite, but that Nrf2-independent pathway(s) is important for delayed and long-lasting *ho-1* gene expression. Our results also indicate that genistein-sensitive but PD153035-insensitive tyrosine kinase activity is crucial for arsenite-mediated JNK activation.

Experimental procedures

Chemical agents

Sodium arsenite ($NaAsO_2$) was purchased from Wako Chemical Co. (Osaka). SP600125 was from Tocris Co. (Ellisville, MO). Genistein and other kinase inhibitors were from Calbiochem (Tokyo). Western blotting reagents were from Amersham (Tokyo). Anti-actin was from Sigma (Tokyo). Anti-*p*-c-Jun was from Cell Signaling (Danvers, MA). The RNeasy Micro Kit was from QIAGEN (Tokyo). Other chemicals were from Nacalai Tesque (Kyoto). Rabbit anti-HO-1 and anti-Nrf2 sera were prepared using purified rat HO-1 [21] and recombinant murine Nrf2 [18], respectively.

Cell culture and treatment

Embryonic fibroblasts derived from Nrf2-deficient and wild-type C57BL mice were cultured in Iscove's Modified Dulbecco's Medium containing 10% (v/v) foetal bovine serum under 95% air and 5% CO_2. The cells were sub-cultured twice a week and for experiments cells were seeded at 1.0×10^5 cells/ml and used after 1–2 days when still pre-confluent. The growth rate of $Nrf2^{-/-}$ cells was similar to that of wild type cells (data not shown).

Immunoblotting

Whole-cell proteins were solubilized with SDS-sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol), boiled for 5 min and stored at $-20^{\circ}C$. Protein concentrations were estimated by the BCA protein assay (Pierce). 2-mercaptoethanol and a marker dye were added to protein solutions and the samples were boiled, separated by SDS-polyacrylamide gel electrophoresis and electrotransferred onto a PVDF membrane [18]. Immunoreactive proteins were detected with horseradish peroxidase-conjugated anti-rabbit IgG and the Chemi-Lumi One (Nacalai, Kyoto) or ECL-plus Western blotting system (Amersham Pharmacia Biotech).

Quantitative real-time PCR

Total RNA was isolated from cells and steady-state mRNA levels in cells were determined by quantitative real-time PCR using 25 ng RNA in 25 μl reaction mixture per assay. The PCR thermocycling parameters were 48°C for 30 min, 95°C for 10 min and 40 cycles (95°C for 15 s, 60°C for 1 min). Real-time PCR was carried out using an Applied Biosystems 7000 sequence detector. The primers and probe for

HO-1 were designed with Primer Express (Applied Biosystems, Tokyo) as follows:

- forward 5′-CCTCACTGGCAGGAAATCATC-3′,
- reverse 5′-CCTCGTGGAGACGCTTTACATA-3′, and
- probe FAM5′-TGCACGCCAGCCACACAGCA-3′TAM.

For GAPDH, the primers and probe in the predeveloped TaqMan assay reagents (Applied Biosystems) were used and the reverse-transcriptase was the qPCR Master Mix (Eurogentec SA, Seraing). Each sample was assayed in duplicate and normalized to GAPDH.

MTT assay

Cell sensitivity to arsenite was analysed by the spectrophotometric measurement of mitochondrial dehydrogenase activity with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) as the substrate. Cells were seeded at 1.0×10^4 cells/100 μl/well (96-well plates), cultured for 24 h, treated with arsenite and incubated for another 24 h in the presence of arsenite. MTT solution (5.0 mg/ml) was added (10 μl/well) and the cultures were incubated for 4 h. The solutions were removed, formazan salts produced by the reaction were dissolved in DMSO and the absorbance at 540 nm was determined. Cells treated without arsenite were used as the 100% control.

Results

We compared the toxicity of 1, 2.5 and 5 μM sodium arsenite in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ murine embryonic fibroblasts using the MTT assay. The OD_{540} measurements of $Nrf2^{+/+}$ cells were significantly higher than those of $Nrf2^{-/-}$ cells, when these cells were treated with 2.5 and 5 μM arsenite for 24 h, respectively (Figure 1A). These results suggest that Nrf2 activation contributes significantly to cytoprotective mechanisms (Figure 1A), most likely by upregulating expression of phase 2 detoxifying [16] and antioxidant proteins [18].

We next compared concentration-dependent effects of arsenite on HO-1 induction in $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cells after 8 h of exposure to arsenite (Figure 1B). HO-1 levels increased dose-dependently, with a maximal expression induced by 2.5 μM arsenite in both cell types. Arsenite also evoked concentration-dependent increases in Nrf2 (apparent mass, 110 kDa) in $Nrf2^{+/+}$ fibroblasts. Notably, HO-1 protein expression was ~2-fold higher in the $Nrf2^{+/+}$ cells compared to $Nrf2^{-/-}$ cells, implicating Nrf2 as an important transcription factor in HO-1

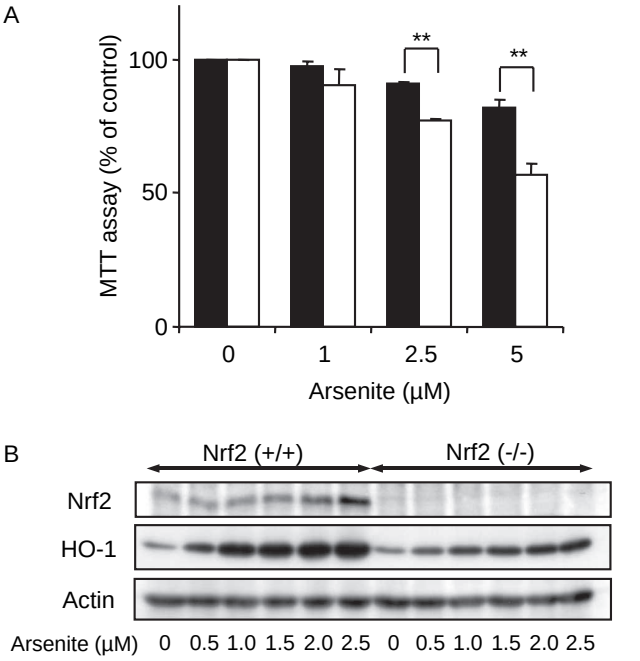

Figure 1. Concentration dependent effects of arsenite on cell viability and HO-1 expression in wild-type and Nrf2-deficient murine embryonic fibroblasts. (A) MTT assay to estimate the sensitivity of the cells to sodium arsenite. Embryonic fibroblasts from wild-type and Nrf2-deficient mice were seeded in 96-well plates (1×10^4 cells/well), incubated for 24 h and then exposed to different concentrations of arsenite for another 24 h. Untreated $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cells served as the 100% control. The growth rates of the two cell types were similar. Data represent the mean ± SE of four replicate measurements in three different cultures, ** $p < 0.01$, determined by Student's *t*-test, respectively. $Nrf2^{+/+}$, denoted by filled bars; $Nrf2^{-/-}$, denoted by unfilled bars. (B) HO-1 and Nrf2 expression levels detected by immunoblotting. Cells were treated with different concentrations of arsenite for 8 h, as described in Experimental Procedures. The blot shows representative immunostained bands. Immunostained HO-1 protein bands were quantified by densitometry using actin as an internal control, with data expressed as means ± SE of measurements in three different cell cultures. The increase in HO-1 protein expression was significant at 0.5 μM ($Nrf2^{+/+}$ cells, $p < 0.01$; $Nrf2^{-/-}$ cells, $p < 0.05$) and 1.0 μM arsenite ($Nrf2^{-/-}$ cells, $p < 0.01$).

upregulation. All subsequent experiments were performed in fibroblasts treated with 2.5 μM arsenite.

Time-dependent changes in HO-1 protein expression were subsequently examined in response to 2.5 μM arsenite (Figure 2A). In $Nrf2^{+/+}$ cells, HO-1 protein levels started to increase within 2 h, peaked at 12 h and then decreased marginally by 24 h. In the $Nrf2^{-/-}$ cells, HO-1 protein expression increased gradually after 4 h, peaked at 12 h and remained elevated at 24 h. In the $Nrf2^{+/+}$ cells, cellular Nrf2 levels reached their maximum 2 h after arsenite treatment, returning to baseline within 24 h (Figure 2A, upper panel). As an indicator of Nrf2 activation, we monitored protein levels in total cell lysates, since these values reflect relative Nrf2 levels in the nuclear fractions of arsenite treated cells [19].

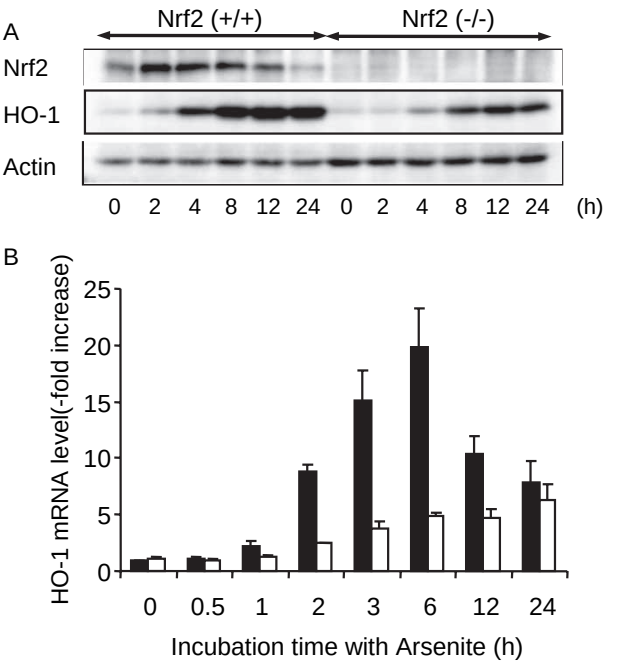

Figure 2. Time dependent effects of arsenite on HO-1 and Nrf2 protein and *ho-1* mRNA expression. (A) representative immunoblots for HO-1 and Nrf2 expression in murine embryonic fibroblasts stimulated with 2.5 μM arsenite. Densitometric analysis of HO-1 protein levels relative to actin were calculated from three different cultures (data not shown). The increase of HO-1 level was significant at 2 h in Nrf2$^{+/+}$ cells ($p<0.01$) and at 8 h ($p<0.05$) and at 12 h ($p<0.01$) in Nrf2$^{-/-}$ cells. (B) time-dependent increase in *ho-1* mRNA expression determined by quantitative real-time PCR. *ho-1* mRNA expression was normalized against *GAPDH* mRNA expression. The data show the means ± SE of duplicate determination in three different cell cultures. The increase of HO-1 mRNA level was significant at 2 h in both cell types ($p<0.01$). Nrf2$^{+/+}$, filled bars; Nrf2$^{-/-}$, unfilled bars.

HO-1 mRNA levels in the Nrf2$^{+/+}$ cells were clearly higher by 1 h of exposure to arsenite, reached a maximum at 6 h and then decreased gradually. In contrast, in Nrf2$^{-/-}$ cells, HO-1 mRNA levels increased gradually during 24 h incubation with arsenite (Figure 2B). These results indicate that Nrf2 is essential for the early, marked *ho-1* gene activation, whilst *ho-1* activation in the absence of Nrf2 is relatively slow and significantly less strong.

To establish a role for tyrosine kinases and JNK in *ho-1* gene activation, we compared the effects of relatively specific inhibitors, genistein and SP600125, in Nrf2$^{+/+}$ and Nrf2$^{-/-}$ cells. Both genistein (100 μM) and SP600125 (40 μM) caused similar inhibition of arsenite stimulated *ho*-1 mRNA upregulation in Nrf2$^{+/+}$ and Nrf2$^{-/-}$ fibroblasts (Figure 3A). The percentage inhibition of the increase in *ho-1* mRNA levels was ~2-fold greater in Nrf2$^{-/-}$ cells (40%) compared to wild-type cells (21%), suggesting these kinases are important for the Nrf2-independent pathways (Figure 3A).

Genistein and SP600125 caused partial and similar inhibition of arsenite stimulated HO-1 protein expression in both Nrf2$^{+/+}$ and Nrf2$^{-/-}$ cells (Figure 3B). Moreover, both genistein and SP600125 inhibited the phosphorylation of c-Jun (Figure 4A). Densitometric analyses showed that genistein (100 μM) and SP600125 (40 μM) treatments, respectively, decreased arsenite-induced p-c-Jun levels from 1 to 0.66 ± 0.17 (mean ± SD, $n=3$) and 0.27 ($n=2$) in Nrf2$^{+/+}$ cells (representative blot shown in Figure 4A) and to 0.53 ± 0.21 ($n=3$) and 0.36 ± 0.10 ($n=3$) in Nrf2$^{-/-}$ cells, respectively. These results are consistent with a previous study that JNK activation is a downstream effect of tyrosine kinase activation [22]. We observed that SP600125 (10–40 μM) dose-dependently blocked arsenite-induced c-Jun phosphorylation and HO-1 induction in Nrf2$^{-/-}$ cells (Figure 4B), suggesting that JNK-mediated phosphorylation of c-Jun significantly contributes to the *ho-1* gene activation in the absence of Nrf2. Notably, genistein and SP600125 did not reduce Nrf2 levels in the absence or presence of arsenite (Figure 4C and D).

To identify the tyrosine kinase that regulates JNK activation, we examined another tyrosine kinase inhibitor, PD153035, which is highly specific to EGFR [27]. Notably, PD153035 negligibly inhibited arsenite mediated HO-1 induction in either cell type (Figure 5A). These results suggest that EGFR tyrosine kinase activity is not involved in the *ho-1* gene activation by arsenite.

Inhibitors for ERK1/2 (PD98059), p38MAPK (SB202190 and SB203580) and pan-PKC (GF109203X) also had negligible effects on arsenite induced increases in HO-1 expression in both cell types (representative blots are shown in Figure 5B, C and D). Therefore, these kinase activities may not play important roles in HO-1 induction, at least under our experimental conditions.

Discussion

We have characterized arsenite-triggered signalling pathways leading to *ho-1* gene activation in wild-type versus Nrf2-deficient murine embryonic fibroblasts. Our results clearly show the following: (i) Nrf2$^{-/-}$ cells are more sensitive to arsenite than Nrf2$^{+/+}$ cells (Figure 1A), (ii) Nrf2 plays a major role in the early phase of *ho-1* gene expression and is responsible for ~ 50% of the HO-1 upregulation (Figures 1 and 2), (iii) genistein-sensitive and PD153035-insensitive tyrosine kinase(s) and JNK constitute TK/JNK/c-Jun/AP-1 signalling pathway (Figures 3–5A) and (iv) negligible roles for ERK1/2, p38MAPK and PKC in *ho-1* gene activation (Figure 5 B, C and D).

The importance of Nrf2 in the induction of HO-1 by arsenite has been reported previously. Alam et al. [20] used a transient trans-activation system to establish the inhibitory effect of mutant Nrf2 lacking a N-terminal activation domain (Nrf2M) in murine fibroblast

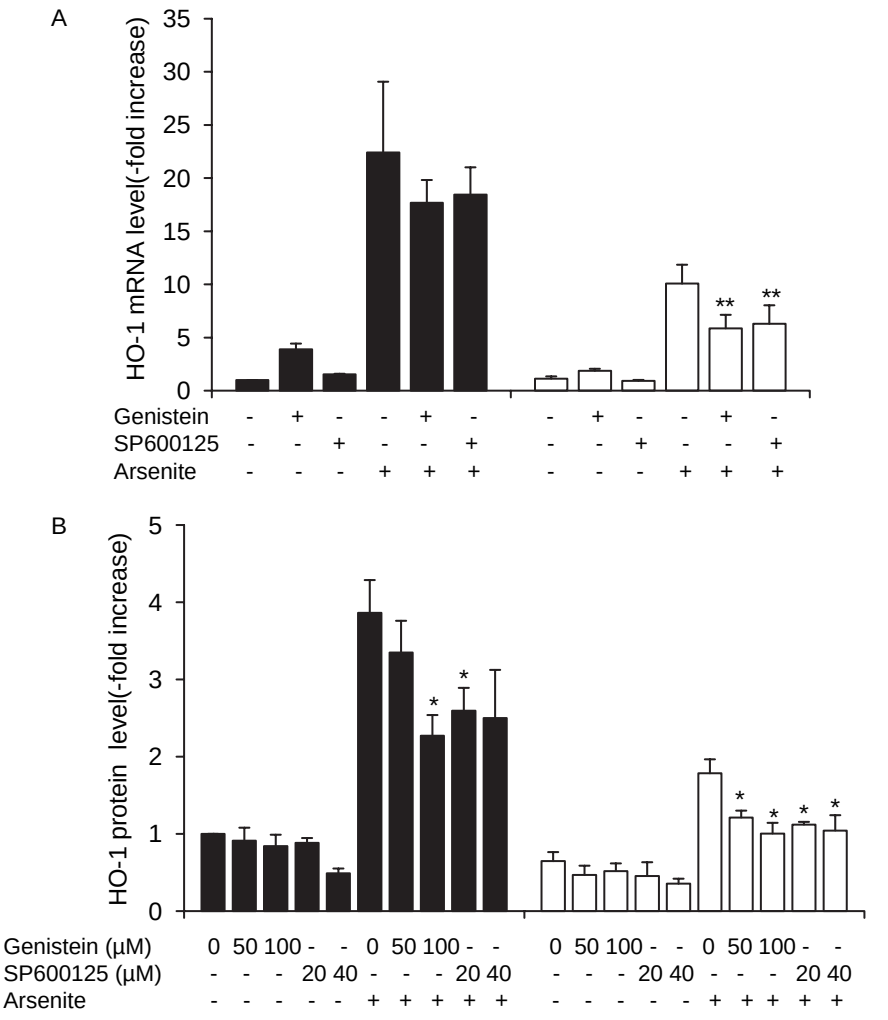

Figure 3. Effects of tyrosine kinase and JNK inhibitors on arsenite-induced mRNA and HO-1 protein expression. (A) $Nrf2^{+/+}$ and $Nrf2^{-/-}$ fibroblasts were treated with 2.5 μM sodium arsenite or left untreated for 6 h in the absence or presence of genistein (100 μM) or SP600125 (40 μM). Each inhibitor was added to the culture medium 1 h prior to the treatment with arsenite. *ho-1* mRNA expression was normalized against the *GAPDH* mRNA expression. The data denote means ± SE of duplicate determination in three different cell cultures, ** $p < 0.01$. (B) Cells were treated with 50 and 100 μM genistein or 20 and 40 μM SP600125 1 h before treatment with 2.5 μM arsenite. HO-1 protein levels in $Nrf2^{-/-}$ and $Nrf2^{+/+}$ cells were determined by immunostaining and quantified by densitometry. Data denote means ± SE of three different cell cultures, * $p < 0.05$. $Nrf2^{+/+}$, filled bars; $Nrf2^{-/-}$, unfilled bars.

L929 cells. Over-expression of Nrf2M inhibited 100 μM arsenite-induced HO-1 mRNA accumulation in 3 h in serum-free medium by 85–90% [20]. In murine peritoneal macrophages, upregulation of HO-1 expression by 2.5 μM arsenite (8 h) was > 5 times higher in $Nrf2^{+/-}$ cells compared to $Nrf2^{-/-}$ cells [18]. In the present study, we confirmed the importance of Nrf2 and have further established that Nrf2 contributes only to early and transient *ho-1* gene activation (Figure 2A and B). Concerning the mode of Nrf2 activation by arsenite, recent studies have shown that arsenite stabilizes Nrf2 in mouse hepa1c1c7 cells [28] and upregulates mRNA levels in human keratinocytes [29]. In this study, we could not detect contribution of tyrosine kinase and JNK on Keap1/Nrf2 pathways (Figure 4C and D), although previous studies suggested activation of Nrf2 by JNK [25,26].

Although arsenic activates EGFR kinase [22,24], our results do not support involvement of the tyrosine kinase activity of EGFR in the *ho-1* gene activation in murine embryonic fibroblasts since PD153035 did not inhibit HO-1 induction (Figure 5A). PD153035 is known as a very potent inhibitor of tyrosine kinase activity of the EGFR (IC50 = 0.025 nM) [27], but genistein is known to inhibit autophosphorylation of EGFR kinase and v-Src activities [30]. Notably, H_2O_2 activates JNK through Src/Cas pathway [31]. It was further shown that Src-mediated activation of JNK required interaction of Src with EGFR, but autophosphorylation of EGFR (EGF-mediated activation) was not required for Src-mediated JNK activation [32]. A recent study [33] has established roles for tyrosine kinase in the activation of AP-1 by H_2O_2 that involves the transcriptional activation of

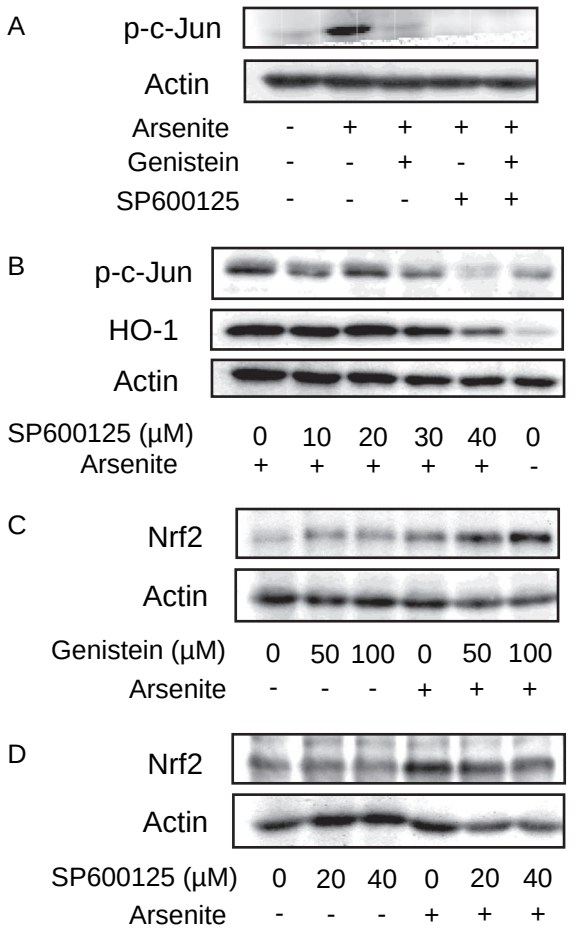

Figure 4. Effects of tyrosine kinase and JNK inhibitors on arsenite-induced c-Jun phosphorylation and Nrf2 levels. Cells were treated with 2.5 μM sodium arsenite or left untreated for 8 h in the absence or presence of the inhibitors as indicated and immunostaining was performed. Each inhibitor was added to the culture medium 1 h prior to the treatment with arsenite. Actin served as an internal loading control. Representative immunoblots of similar results from two-to-three different cell cultures are shown. (A) $Nrf2^{+/+}$ cells were treated with arsenite or left untreated in the absence or presence of genistein (100 μM) or SP600125 (40 μM). Immunoblotting was performed using antiserum specific to phosphorylated c-Jun (see text for more details). (B) $Nrf2^{-/-}$ cells were treated with arsenite in the presence of 0, 10, 20, 30 and 40 μM SP600125 and phospho-c-Jun and HO-1 levels monitored by immunoblotting. (C) $Nrf2^{+/+}$ cells were treated with arsenite or left untreated for 8 h in the absence or presence of genistein (50 and 100 μM). Nrf2 expression levels in whole cell extracts were monitored. (D) $Nrf2^{+/+}$ cells were treated with arsenite or left untreated for 8 h in the absence or presence of SP600125 (20 and 40 μM) and Nrf2 levels in whole cell extracts determined by immunoblotting.

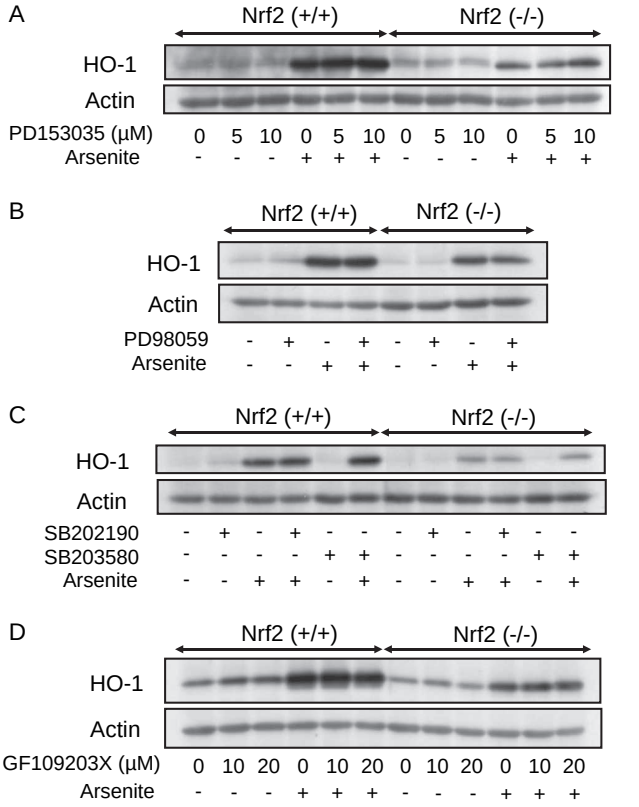

Figure 5. Effects of other kinase inhibitors on arsenite-induced HO-1 expression. All inhibitors were added 1 h prior to the start of an 8-h treatment with 2.5 μM sodium arsenite. Representative immunoblots of similar results from two different cell cultures are shown. Actin served as an internal loading control. (A) Cells were treated with PD153035 (inhibitor of EGFR) and/or arsenite or left untreated. (B) Cells were treated with PD98059 (50 μM, inhibitor of ERK1/2 activation) and/or arsenite or left untreated. (C) Cells were treated with p38MAPK inhibitor SB202190 or SB203580 at 10 μm and/or arsenite or left untreated. (D) Cells were treated with GF109203X (pan-PKC inhibitor) at 10 or 20 μM and/or with arsenite or left untreated.

c-*jun* and c-*fos* genes and phosphorylation of c-Jun. It is possible that arsenite could activate JNK through similar manner as H_2O_2, since H_2O_2 is produced in cells by arsenite treatment [34,35]. These studies and our results using kinase inhibitors support the hypothesis that the Src/JNK/c-Jun/AP-1 signalling pathway contributes *ho-1* gene activation induced by low levels of arsenite.

Interestingly, in $Nrf2^{-/-}$ cells, activation of *ho-1* gene expression levels increased gradually and remained elevated after 24 h of arsenite treatment (Figure 2B). This long-lasting *ho-1* gene activation by arsenite in the absence of Nrf2 is most likely mediated by AP-1, since previous studies showed that JNK activation is sustained under oxidative stress [36] and expression of multiple AP-1 family proteins is increased during the course of gene activation by arsenite [16]. Activation/phosphorylation of JNK by arsenite seems to be regulated through two cooperating mechanisms: inhibition of JNK phosphatase [14] and activation of an upstream tyrosine kinase.

In summary, we demonstrated that Nrf2 mediates the early induction of HO-1 and that Nrf2 contributes to approximately half of the HO-1 upregulation in embryonic fibroblasts exposed to low levels of arsenite. We also showed that genistein-sensitive but PD153035-insensitive tyrosine kinase, probably Src, plays a role in the JNK/AP-1 pathway, which seems to be important for the long-term induction of HO-1 by

arsenite. Our study provides insights for strategies to increase cellular protection against toxic agents such as arsenite. Furthermore, our findings suggest that the Nrf2 pathway may be a potential as a target for potential therapies [37], as we previously reported a beneficial effect of the Nrf2 activator sulphoraphane in reducing the toxicity of arsenite [38,39].

Acknowledgements

We thank Professor Giovanni E. Mann, Professor Yoshito Kumagai and Dr Baojie Li for their encouragement during this study. This study was supported by grants from the Japan Society for the Promotion of Science (TI) and the British Heart Foundation (RCMS).

References

[1] Abraham NG, Drummond GS, Lutton JD, Kappas A. The biological significance and physiological role of heme oxygenase. Cell Physiol Biochem 1996;6:129–168.

[2] Maines MD. The heme oxygenase system: a regulator of second messenger gases. Annu Rev Pharmacol Toxicol 1997;37:517–554.

[3] Keyse SM, Tyrrell RM. Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. Proc Natl Acad Sci USA 1989;86:99–103.

[4] Anwar AA, Li FYL, Leake DS, Ishii T, Mann GE, Siow RCM. Induction of heme oxygenase 1 by moderately oxidized low-density lipoproteins in human vascular smooth muscle cells: Role of mitogen-activated protein kinases and Nrf2. Free Radic Biol Med 2005;39:227–236.

[5] Choi AMK, Alam J. Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am J Respir Cell Mol Biol 1996;15: 9–19.

[6] Sardana MK, Drummond GS, Sassa S, Kappas A. The potent heme oxygenase inducing action of arsenic and parasiticidal arsenicals. Pharmacology 1981;23:247–253.

[7] Wang C, Gomer RH, Lazarides E. Heat shock proteins are methylated in avian and mammalian cells. Proc Natl Acad Sci USA 1981;78:3531–3535.

[8] Del-Razo LM, Quintanilla-Vega B, Brambila-Colombres E, Calderon-Aranda ES, Manno M, Albores A. Stress proteins induced by arsenic. Toxicol Appl Pharmacol 2001;177:132–148.

[9] Liu J, Kadiiska MB, Liu Y, Lu T, Qu W, Waalkes MP. Stress-related gene expression in mice treated with inorganic arsenicals. Toxicol Sci 2001;61:314–320.

[10] Shibahara S, Muller RM, Taguchi H. Transcriptional control of rat heme oxygenase by heat shock. J Biol Chem 1987;262:12889–12892.

[11] Alam J, Cook J. How many transcription factors does it take to turn on the heme oxygenase-1 gene? Am J Respir Cell Mol Biol 2007;36:166–174.

[12] Alam J, Camhi S, Choi AM. Identification of a second region upstream of the mouse heme oxygenase-1 gene that functions as a basal level and inducer-dependent transcription enhancer. J Biol Chem 1995;270:11977–11984.

[13] Inamdar NM, Ahn YI, Alam J. The heme-responsive element of the mouse heme oxygenase-1 gene is an extended AP-1 binding site that resembles the recognition sequences for MAF and NF-E2 transcription factors. Biochem Biophys Res Commun 1996;221:570–576.

[14] Cavigelli M, Li WW, Lin A, Su B, Yoshioka K, Karin M. The tumor promoter arsenite stimulates AP-1 activity by inhibiting a JNK phosphatase. EMBO J 1996;15:6269–6279.

[15] Simeonova PP, Wang S, Torium W, Kommineni V, Matheson J, Unimye N, Kayama F, Harki D, Ding M, Vallyathan V, Luster MI. Arsenic mediates cell proliferation and gene expression in the bladder epithelium: association with activating protein-1 transactivation. Cancer Res 2000;60:3445–3453.

[16] Gong P, Stewart D, Hu B, Vinson C, Alam J. Multiple basic-leucine zipper proteins regulate induction of the mouse heme oxygenase-1 gene by arsenite. Arch Biochem Biophys 2002;405:265–274.

[17] Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 1997;236:313–322.

[18] Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, Bannai S, Yamamoto M. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem 2000;275:16023–16029.

[19] Aono J, Yanagawa T, Itoh K, Li B, Yoshida H, Kumagai Y, Yamamoto M, Ishii T. Activation of Nrf2 and accumulation of ubiquitinated A170 by arsenic in osteoblasts. Biochem Biophys Res Commun 2003;305:271–277.

[20] Alam J, Stewart D, Touchard C, Boinapally S, Choi AM, Cook JL. Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J Biol Chem 1999;274:26071–26078.

[21] Masuya Y, Hioki K, Tokunaga R, Taketani S. Involvement of the tyrosine phosphorylation pathway in induction of human heme oxygenase-1 by hemin, sodium arsenite, and cadmium chloride. J Biochem 1998;124:628–633.

[22] Nuntharatanapong N, Chen K, Sinhaseni P, Keaney JF Jr. EGF receptor-dependent JNK activation is involved in arsenite-induced p21 Cip1/Waf1 upregulation and endothelial apoptosis. Am J Physiol Heart 2005;289:99–107.

[23] Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 1995;270:16483–16486.

[24] Chen W, Martindale JL, Holbrook NJ, Liu Y. Tumor promoter arsenite activates extracellular signal-regulated kinase through a signaling pathway mediated by epidermal growth factor receptor and Shc. Mol Cell Biol 1998;18:5178–5188.

[25] Xu C, Yuan X, Pan Z, Shen G, Kim JH, Yu S, Khor TO, Li W, Ma J, Kong AN. Mechanism of action of isothiocyanates: the induction of ARE-regulated genes is associated with activation of ERK and JNK and the phosphorylation and nuclear translocation of Nrf2. Mol Cancer Ther 2006;5: 1918–1926.

[26] Yuan X, Xu C, Pan Z, Keum YS, Kim JH, Shen G, Yu S, Oo KT, J. Ma J, Kong AN. Butylated hydroxyanisole regulates ARE-mediated gene expression via Nrf2 coupled with ERK and JNK signaling pathway in HepG2 cells. Mol Carcinog 2006;45:841–850.

[27] Fry DW, Kraker AJ, McMichael A, Ambroso LA, Nelson JM, Leopold WR, Connors RW, Bridges AJ. A specific inhibitor of the epidermal growth factor receptor tyrosine kinase. Science 1994;265:1093–1095.

[28] He X, Chen MG, Lin GX, Ma Q. Arsenic induces NAD(P)H-quinone oxidoreductase I by disrupting Nrf2/Keap1/Cul3 complex and recruiting Nrf2/Maf to the anti-

oxidant response element enhancer. J Biol Chem 2006;281: 23620–23631.
[29] Pi J, Qu W, Reece JM, Kumagai Y, Waalkes MP. Transcription factor Nrf2 activation by inorganic arsenic in cultured keratinocytes: involvement of hydrogen peroxide. Exp Cell Res 2003;290:234–245.
[30] Akiyama T, Ogawara H. Use and specificity of genistein as inhibitor of protein-tyrosine kinases. Methods Enzymol 1991; 201:362–370.
[31] Yoshizumi M, Abe J, Haendeler J, Hung Q, Berk BC. Src and Cas mediate JNK activation but not ERK1/2 and p38 kinases by reactive oxygen species. J Biol Chem 2000;275:11706–11712.
[32] Chen K, Vita JA, Berk BC, Keaney JF Jr. c-Jun N-terminal kinase activation by hydrogen peroxide in endothelial cells involves SRC-dependent epidermal growth factor receptor transactivation. J Biol Chem 2001;276:16045–16050.
[33] Li DW, Spector A. Hydrogen peroxide-induced expression of the proto-oncogenes, c-jun, c-fos and c-myc in rabbit lens epithelial cells. Mol Cell Biochem 1997;173:59–69.
[34] Wang TS, Huang H. Active oxygen species are involved in the induction of micronuclei by arsenite in XRS-5 cells. Mutagenesis 1994;9:253–257.
[35] Barchowsky A, Klei LR, Dudek EJ, Swartz HM, James PE. Stimulation of reactive oxygen, but not reactive nitrogen species, in vascular endothelial cells exposed to low levels of arsenite. Free Radic Biol Med 1999;27:1405–1412.
[36] Chen YR, Wang W, Kong AN, Tan TH. Molecular mechanisms of c-Jun N-terminal kinase-mediated apoptosis induced by anticarcinogenic isothiocyanates. J Biol Chem 1998; 273:1769–1775.
[37] Yu X, Kensler T. Nrf2 as a target for cancer chemoprevention. Mutat Res 2005;591:93–102.
[38] Aono J, Yanagawa T, Taketani S, Yoshida H, Kumagai Y, Ishii T. Sulforaphane protects cultured osteoblasts against arsenic damage. Proc Free Radic Meeting Europe 2005;29–32.
[39] Shinkai Y, Sumi D, Fukami I, Ishii T, Kumagai Y. Sulforaphane, an activator of Nrf2, suppresses cellular accumulation of arsenic and its cytotoxicity in primary mouse hepatocytes. FEBS Lett 2006;580:1771–1774.