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Letter

Reduction of arsenic-induced cytotoxicity through Nrf2/HO-1 signaling in HepG2 cells

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ABSTRACT — Our previous study indicated that Nrf2 is a key transcription factor in cellular defenses against inorganic arsenite (iAsIII). However, the role of heme oxygenase-1 (HO-1), which is regulated by Nrf2, in iAsIII-induced cytotoxicity is poorly understood. To address this issue, we examined the contribution of HO-1 to iAsIII-mediated Nrf2 activation and in protection against iAsIII cytotoxicity in HepG2 cells. Exposure of HepG2 cells to iAsIII (10 μM) caused persistent induction of HO-1 accompanied by prolonged Nrf2 activation, whereas siRNA-mediated knockdown of HO-1 decreased prolonged Nrf2 activation. Pretreatment with either HO-1 siRNA or HO inhibitor (tin protoporphyrin IX) significantly enhanced iAsIII-induced cytotoxicity. These results suggest that iAsIII-induced HO-1 appears, at least in part, to act as a positive feedback regulator of Nrf2 activation, thereby diminishing its cytotoxicity in HepG2 cells.

Key words: Arsenite, Nrf2, Heme oxygenase-1, Cytotoxicity

INTRODUCTION

Arsenic is a naturally occurring metalloid that is regarded as a ubiquitous contaminant (Nordstrom, 2002). Worldwide, millions of people are exposed to arsenic in drinking water. One of symptoms of chronic arsenic poisoning is liver damage (Guha Mazumder, 2001; Liu and Waalkes, 2008), and although inorganic arsenite (iAsIII) exposure causes liver injury in rodents (Bashir *et al*., 2006; Santra *et al*, 2007), the mechanisms underlying protection against iAsIII-induced cytotoxicity in hepatocytes are not fully understood.

Heme oxygenase-1 (HO-1) is a 32-kDa protein that is highly inducible in mammalian tissues by a wide variety of stimuli including arsenic (Otterbein and Choi, 2000). HO-1 catalyzes the rate-limiting step in heme catabolism and generates carbon monoxide (CO), iron, and biliverdin, leading to cellular protection against oxidant injury (Bauer and Bauer, 2002). Alam *et al.* (1999) reported that HO-1 is regulated by the transcription factor Nrf2, which recognizes the antioxidant response element in promoter regions and regulates basal and inducible expression of numerous antioxidant and detoxifying genes (Itoh *et al.*, 1997; Motohashi and Yamamoto, 2004).

We found that iAsIII activates Nrf2, resulting in up-regulation of phase II xenobiotic-metabolizing enzymes and phase III transporters (Aono *et al.*, 2003; Pi *et al.*, 2003; Kumagai and Sumi, 2007) and thus proposed the notion that there is an Nrf2-dependent cellular response to iAsIII through facilitation of formation of iAsIII-glutathione adduct, which rapidly excreted into the extracellular space. Although a marked induction of HO-1 was detected during iAsIII exposure, the protective role of HO-1 against iAsIII-induced cytotoxicity remains unclear. Interestingly, CO and ferric iron can activate Nrf2 (Lee *et al*., 2006; Kim *et al*., 2007; Tanaka *et al.*, 2008), suggesting that HO-1 acts as a cytoprotective factor through not only suppression of oxidative stress but also activation of Nrf2. This study examined the role of HO-1 in iAsIII-mediated activation of Nrf2 and iAsIII-induced cytotoxicity in HepG2 cells.

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MATERIALS AND METHODS

Materials

Sodium arsenite was purchased from Wako (Osaka, Japan). Tin protoporphyrin IX (SnPP) was obtained from Alexis (Lausen, Switzerland). Anti-Nrf2, anti-HO-1, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Stressgen (Victoria, BC, Canada), and American Research Products (Belmont, MA, USA), respectively. Horseradish peroxidase (HRP)-linked anti-rabbit IgG was obtained from Cell Signaling Technology (Beverly, MA, USA). All other reagents were of the highest grade available.

Cell culture and treatment

The human hepatocellular carcinoma cell line HepG2 was obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako) containing 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and 2 mM l-alanyl-l-glutamine (Invitrogen, Carlsbad, CA, USA) in an incubator supplemented with 5% CO_2 at 37°C. Cells were pre-incubated in serum-free medium for 12 hr before treatment with iAsIII. For inhibitor treatments, cells were incubated in serum-free medium with or without 50 μM SnPP for 12 hr before treatment with iAsIII.

Western blotting

After iAsIII treatment, cells were washed with ice-cold phosphate-buffered saline, collected by scraping into cell lysis buffer (20 mM Tris, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 2.5 mM sodium pyrophosphate; 1 mM β-glycerophosphate; and 1 mM Na_3VO_4), and sonicated on ice. Cell lysates were centrifuged at 13,000 × *g* for 5 min, and the resulting supernatants were transferred to new tubes. Protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL, USA). Each sample was mixed with a half volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (62.5 mM Tris-HCl, pH 6.8; 6% SDS; 24% glycerol; 15% 2-mercaptoethanol; and 0.015% bromophenol blue) and incubated at 95°C for 5 min. The cellular proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% skim milk in TTBS (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; and 0.1% Tween 20); this was followed by incubation with primary antibody in TTBS. To detect immunoreactive proteins, we used HRP-conjugated anti-rabbit IgG and an enhanced chemiluminescence system (Chemi-Lumi One; Nacalai, Kyoto, Japan).

Cytotoxicity assay

The MTT assay was performed as previously described (Shinkai *et al.*, 2009).

siRNA transfection

Predesigned, short interfering RNA (siRNA) against HO-1 (catalog no. SI02780533) and control siRNA (catalog no. 1022076) were purchased from Qiagen (Valencia, CA, USA). Transient transfection of siRNAs was performed in serum-free medium using the HiPerFect transfection reagent (Qiagen) with slight modification, according to the manufacturer's protocol. Briefly, cells were grown to approximately 40% confluence. For experiments in 35 mm dish, 3 μl of siRNA duplex (20 μM) and 12 μl of HiPerFect reagent were both mixed with OPTI-MEM (Invitrogen) in separate tubes. For experiments in 96-well plates, 0.25 μl of siRNA duplex (20 μM) and 0.75 μl of HiPerFect reagent were both mixed with OPTI-MEM in separate tubes. Before addition to the cells, the siRNA and transfection reagent solutions were mixed together and incubated for 5 min at room temperature to allow the formation of complexes.

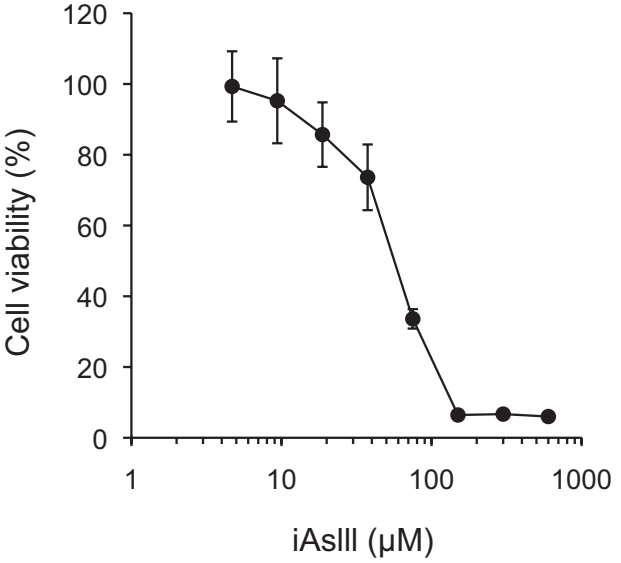

Fig. 1. Inorganic arsenite-induced cytotoxicity in HepG2 cells. Cells were exposed to iAsIII (5, 10, 20, 40, 80, 150, 300, and 600 μM) for 48 hr. Data are mean ± S.E. of six determinations.

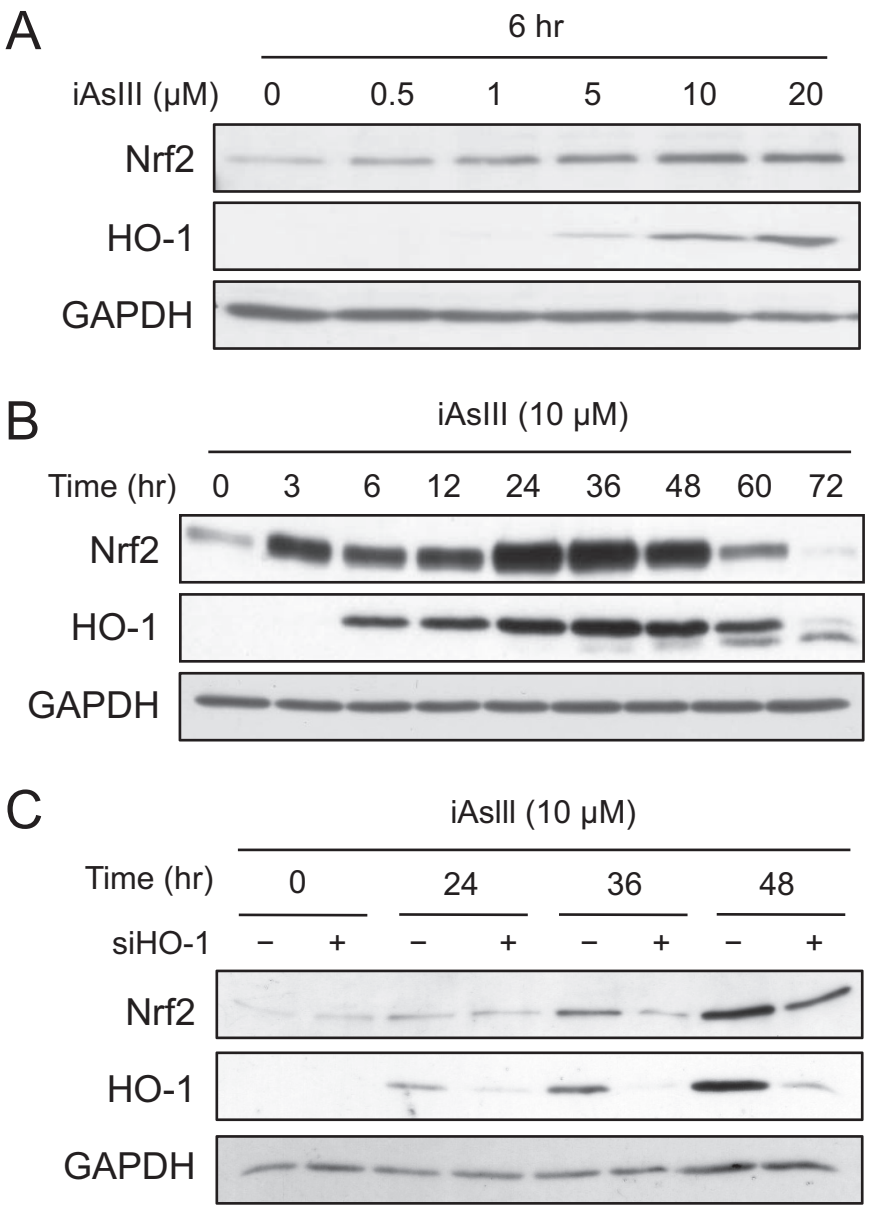

Fig. 2. Activation of the Nrf2/HO-1 pathway by iAsIII in HepG2 cells. (A) Cells were exposed to iAsIII (0.5, 1, 5, 10, or 20 μM) for 6 hr, and total cell lysates were subjected to Western blot analysis using the indicated antibodies. (B) Cells were exposed to iAsIII (10 μM) for 3, 6, 12, 24, 36, 48, 60, or 72 hr, and total cell lysates were subjected to Western blot analysis using the indicated antibodies. (C) Effect of knockdown of HO-1 on Nrf2 activation by iAsIII. Control siRNA- or HO-1 siRNA-transfected cells (24 hr) were exposed to iAsIII (10 μM) for 24, 36, or 48 hr, and total cell lysates were subjected to Western blot analysis using the indicated antibodies. GAPDH was used as an internal control.

Statistical analysis

Significance was assessed with a *t* test, and $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Fig. 1 shows iAsIII-induced cytotoxicity in HepG2 cells. Exposure of cells to iAsIII resulted in concentration-dependent cytotoxicity with an LD_{50} value of 56 ± 2.6 μM at 48 hr. Next, we examined the activation of

the Nrf2/HO-1 pathway by iAsIII under nontoxic conditions in HepG2 cells. As shown in Fig. 2A, iAsIII activated Nrf2 and up-regulated HO-1 protein expression in a concentration-dependent manner at 6 hr following iAsIII exposure. In the time-course experiments, iAsIII (10 μM) induced prolonged increases in Nrf2 and HO-1 protein levels (Fig. 2B). Since iAsIII causes not only activation of Nrf2 but also inactivation of Bach1, a transcriptional repressor of HO-1 (Reichard *et al*., 2008), the prolonged induction of HO-1 observed after iAsIII exposure in HepG2 cells may be due to Bach1 inactivation. In contrast, exposure to Nrf2 activators such as sulforaphane and diethyl maleate caused transient increases in the Nrf2 and HO-1 protein levels, which peaked at 3-6 hr and then declined in a time-dependent manner (data not shown). To examine whether HO-1 is involved in Nrf2 activation by iAsIII, HepG2 cells were transfected with HO-1 siRNA. Transfection of HepG2 cells with HO-1 siRNA resulted in decreased HO-1 induction by iAsIII (Fig. 2C). Under the same condition, Nrf2 activation was also suppressed by knockdown of HO-1. This suggests that HO-1 plays a role in the positive feedback regulation of Nrf2 activation in HepG2 cells. We speculate that CO or ferrous iron may be a mediator of this positive feedback system because these HO-1 metabolites can activate Nrf2 (Lee *et al*., 2006; Kim *et al*., 2007; Tanaka *et al*., 2008).

We reported that Nrf2 is important for reducing cellular accumulation of arsenic and its cytotoxicity (Shinkai *et al*., 2006; Kumagai and Sumi, 2007). To assess whether HO-1 contributes to cytoprotection from iAsIII toxicity, HO-1 siRNA-transfected cells were exposed to iAsIII, and then cell viability was monitored with the MTT assay. Knockdown of HO-1 resulted in a significant increase in iAsIII-induced cytotoxicity, suggesting that HO-1 has a protective effect against iAsIII in HepG2 cells (Fig. 3A). Furthermore, pretreatment with SnPP, an inhibitor of HO-1 activity, significantly enhanced iAsIII-induced cytotoxicity (Fig. 3B). These results suggest that HO-1 suppresses iAsIII-induced cytotoxicity through the heme degradation pathway that leads to Nrf2 activation in HepG2 cells.

In contrast to these results, Miralem *et al.* (2005) reported that knockdown of HO-1 did not result in a significant increase in iAsIII-induced apoptosis in HEK293 cells. A possible explanation for this discrepancy is that the efficacy of HO-1 may be dependent on the cell type (e.g., cellular heme content). Although the precise mechanism underlying HO-1–mediated protection against arsenic remains to be elucidated, our results suggests that, at least in HepG2 cells, activation of Nrf2/HO-1 signaling functions as a defensive mechanism against iAsIII-induced cytotoxicity.

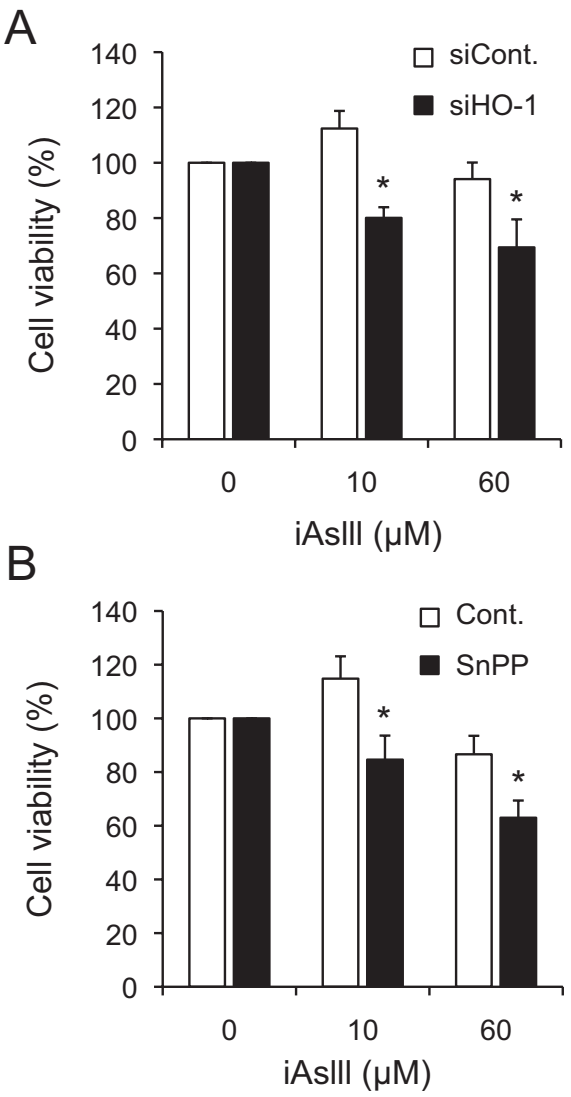

Fig. 3. Effects of HO-1 knockdown or SnPP on the cytotoxicity of iAsIII in HepG2 cells. (A) Control siRNA- or HO-1 siRNA-transfected cells (24 hr) were exposed to iAsIII (10 or 60 μM) for 48 hr. *, $P < 0.05$ compared with control siRNA-transfected cells. (B) Cells were pre-incubated with SnPP (50 μM) prior to exposure to iAsIII for 48 hr. Data are mean ± S.E. of three determinations. *, $P < 0.05$ compared with control.

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