Nrf2 protects against As(III)-induced damage in mouse liver and bladder

Tao Jiang a,b,1, Zheping Huang a,1, Jefferson Y. Chan c, Donna D. Zhang a,*

a Department of Pharmacology and Toxicology, University of Arizona, 1703 E. Mabel St, Tucson, AZ 85721, USA
b Department of Pathology, Shanghai Medical College, Fudan University, 138 Yixueyuan Rd., Shanghai, 200032, China
c Department of Pharmacology and Toxicology, University of Arizona, 1703 E. Mabel St., Tucson, AZ 85721, USA

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Arsenic compounds are classified as toxicants and human carcinogens. Environmental exposure to arsenic imposes a big health issue worldwide. Arsenic elicits its toxic efforts through many mechanisms, including generation of reactive oxygen species (ROS). Nrf2 is the primary transcription factor that controls expression of a main cellular antioxidant response, which is required for neutralizing ROS and thus defending cells from exogenous insults. Here, we demonstrated a protective role of Nrf2 against arsenic-induced toxicity using a cell culture model. In this report, we present evidence that Nrf2 protects against liver and bladder injury in response to six weeks of arsenic exposure in a mouse model. Nrf2−/− mice displayed more severe pathological changes in the liver and bladder, compared to Nrf2+/+ mice. Furthermore, Nrf2−/− mice were more sensitive to arsenic-induced DNA hypomethylation, oxidative DNA damage, and apoptotic cell death. These results indicate a protective role of Nrf2 against arsenic toxicity in vivo. Hence, this work demonstrates the feasibility of using dietary compounds that target activation of the Nrf2 signaling pathway to alleviate arsenic-induced damage.

Introduction

Groundwater contaminated with arsenic is the major source of human exposure to arsenic. Chronic arsenic poisoning poses a global health issue (Tchounwou et al., 2003; Walvekar et al., 2007). Therefore, the World Health Organization (WHO) has set the standard limit for arsenic to 10 parts per billion (ppb) (Kayajanian, 2003). However, approximately 57 million people are drinking groundwater with arsenic concentrations above 10 ppb. Arsenic has caused poisonings in Bangladesh, Bengal, Thailand, Finland, Hungary, Chile, Taiwan, Vietnam, Cambodia, Mexico, Argentina, and China, where geological environments are conducive to generate high amounts of arsenic compounds in groundwater (Smith et al., 2000; Kayajanian, 2003; Tchounwou et al., 2003). Furthermore, many states within the United States also have significant concentrations (up to 50 ppm) of arsenic in the groundwater (Tchounwou et al., 2003; Knobloch et al., 2006).

The most common species of arsenic in groundwater are arsenate (As(V)) and arsenite (As(III)). Arsenic is able to undergo redox conversion between As(V) and As(III). Following uptake from drinking water, inorganic arsenic species are converted in the liver into methylated arsenic species that are excreted from the bladder. Thus, the bladder is the major target organ that is exposed to methylated arsenic species in populations who consume arsenic-contaminated water (Tchounwou et al., 2003; Kenyon et al., 2005).

Chronic exposure to arsenic can cause skin, lung and bladder cancers (Hsu et al., 1995; Brown et al., 1997; Cohen et al., 2000; Smith et al., 2006). A small but measurable increase in the incidence of bladder cancer was associated with exposure to concentration as low as 10 ppm of inorganic arsenic (Chu and Crawford-Brown, 2006). In addition to cancers, epidemiological studies have also established a strong correlation between chronic arsenic exposure and various non-cancer human diseases, such as hyperkeratosis, atherosclerosis, diabetes, and chronic obstructive pulmonary diseases (Tseng, 2002; Tchounwou et al., 2003; Navas-Acien et al., 2006).

The transcription factor, Nrf2, controls a cellular antioxidant response through transcriptional upregulation of an array of downstream genes, such as glutamate cysteine ligase (GCL), heme oxygenase-1 (HO-1), glutathione s-transferase (GST), multidrug-resistance proteins (MRPs), and NAD(P)H quinone oxidoreductase-1 (NQO1) (Lau et al., 2008; Hayes and McMahon, 2009). Coordinated upregulation of the Nrf2-mediated endogenous antioxidants, phase II detoxifying enzymes, and drug transporters is essential in defending cells or animals from damage by environmental insults. This has been clearly demonstrated in many studies using Nrf2−/− mice. Nrf2−/− mice displayed a greater sensitivity to toxic effects induced by benzo[a]pyrene, diesel exhaust, pentachlorophenol, and bleomycin (Aoki et al., 2001; Ramos-Gomez et al., 2001; Cho et al., 2004; Umemura et al., 2006).

Arsenic exerts its toxicity in part by generation of ROS (Hei et al., 1998; Kitchin and Ahmad, 2003; Liu et al., 2003; Das et al., 2005). Consistent with the role of ROS in arsenic toxicity/carcinogenicity, endogenous sulfhydryl groups and the non-protein sulfhydryl glutathione (GSH) have been reported to play an important role in...
detoxification of arsenic (Duyndam et al., 2001). The exogenous antioxidant N-acetylcysteine is also able to prevent arsenic-induced toxicity (Liu et al., 2003). Arsenic itself was reported to induce the Nrf2-dependent antioxidant response, although the detailed mechanism of Nrf2 induction by arsenic remains to be explored (He et al., 2006; Wang et al., 2008). Numerous studies have been performed to elucidate events associated with arsenic-induced damage, both in animal and cell culture models. Results from these studies have revealed that arsenic induces multiple biological effects, such as DNA damage, apoptotic cell death, and global DNA hypomethylation, which represent both genotoxic and non-genotoxic mechanisms of action (Huang et al., 1999; Kirkpatrick et al., 2003; Chen et al., 2004).

Methylation is a covalent modification of genomic DNA and occurs on cytosines followed by guanines (CpG). DNA methylation appears to influence gene expression through alteration in chromatin structure and in DNA protein interaction. An overall decrease in the content of 5-methylcytosine (5-mc) in DNA has been viewed as an early event in tumor formation in humans and animal models (Lapeyre et al., 1981; Jones and Buckley, 1990; Umemura et al., 1990).

Recently, by using both genetic and biochemical approaches, we demonstrated a protective role of Nrf2 against arsenic-induced toxicity in a cell culture model (Wang et al., 2007). In vitro, even though Nrf2−/− mice have been demonstrated to display increased sensitivity to chemical carcinants and carcinogens (Chan and Kan, 1999; Aoki et al., 2001; Enomoto et al., 2001; Ramos-Gomez et al., 2001; Cho et al., 2002; Yu and Kessler, 2005), currently no study has been carried out to determine the effect of Nrf2 deficiency on arsenic-induced toxicity and carcinogenicity. In this report, we hypothesized that Nrf2−/− mice are more vulnerable to the arsenic-induced toxicity, compared to Nrf2+/+ mice. Our results confirmed that Nrf2−/− mice were more sensitive to arsenic-induced DNA hypomethylation, oxidative DNA damage, and apoptotic cell death. Furthermore, more severe pathological alterations were observed in the liver and bladder of Nrf2−/− mice, compared to Nrf2+/+ mice.

Materials and Methods

Animals and treatments. Nrf2−/− mice were originally generated in Dr. Kan’s laboratory (University of California, San Francisco). Mice were housed in polycarbonate cages (4/cage), provided AIN-76A diet and water ad libitum and maintained on a 12–12 h light-dark cycle at 22 ± 5°C and 50 ± 20% relative humidity. At 8 weeks of age, Nrf2+/+ and Nrf2−/− mice (4 mice per group) were treated with 1 ppm, 10 ppm or 100 ppm of sodium arsenite (As(III), Sigma, St Louis, MO, USA) through drinking water for six weeks. All 32 mice survived arsenic treatment.

Tissue collection and hematoxylin and eosin (HE) staining. Following six-week treatment, mice were euthanized and the liver, kidney, lung and bladder were isolated. Tissues were cut into two sections; one section was frozen in liquid nitrogen for total RNA extraction, and another section was fixed in 10% buffered formalin. The bladders were reversed; one section was fixed in formalin, and another was spread flat and scrubbed by a pestle to loosen up bladder epithelium. The bladder epithelium was then collected in Trizol solution (St. Louis, MO, USA) for total RNA extraction. The formalin-fixed tissues were embedded in paraffin and cut into 4 μm sections. Various sections were stained with HE.

DNA methylation in bladder tissue. DNA methylation in bladder epithelium tissues was detected by immunohistochemistry using a monoclonal antibody against 5-methylcytosine (Calbiochem, San Diego, CA, USA). Antigen retrieval of formalin-fixed paraffin-embedded tissue sections was carried out by microwave heating (7 min at the high setting to boil the retrieval solution), followed by 10 min at the low setting (the retrieval solution was maintained at the boiling temperature). The retrieval solution contains 1× TBS with 0.1% Tween 20 (TBS-T) in 1 mol/l sodium citrate. Following antigen retrieval, tissue sections were exposed to 3.5 M HCl for 15 min at room temperature (RT) and washed in TBS-T. Subsequently, tissue sections were treated with 0.3% peroxidase to quench endogenous peroxidase activity. Tissue sections were incubated with 5% normal goat serum for 30 min followed by 2 h incubation with an anti-5-methyl-C monoclonal antibody at 1:100 dilution at RT, followed by 1 h sequential incubation with a biotinylated goat anti-mouse secondary antibody and ABC kit (Vector Lab, Burlingame, CA, USA) at 1:100 dilution at RT. Finally, tissue sections were developed for 30 s using the 3, 3’-diaminobenzidine staining kit (DAKO, Carpinteria, CA, USA) and counterstained with hematoxylin.

Oxidative DNA damage in bladder tissue. A monoclonal antibody against 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-Oxo-dG) (Treivgen, Gaithersburg, MD) was used for detecting oxidative DNA damage in bladder epithelium. Formalin-fixed paraffin-embedded tissue sections were incubated with proteinase K (10 μg/ml) in PBS for 30 min at 37°C, and then exposed to 2 M HCl for 5 min at RT. Following quench of the endogenous peroxidase activity by 0.3% peroxidase, tissue sections were first incubated with anti-8-Oxo-dG at 1:250 dilution at RT for 2 h, followed by sequential incubation with the secondary antibody and ABC kit. Finally, tissue sections were developed and counterstained with hematoxylin.

Apoptotic cell death in bladder tissue. In situ cell death detection kit (Roche, IN, USA) was used for detecting apoptotic cell death in bladder epithelium according to the manufacturer’s instructions. Briefly, tissue sections were pretreated with proteinase K (15 μg/ml) in 10 mM Tris/HCl (pH 7.8) at 37°C for 30 min. After washing 3 times with PBS, tissue sections were incubated with TUNEL reaction mixture for 1 h at 37°C in the dark. Tissue sections were then rinsed with PBS 3 times, and analyzed under a fluorescence microscope (Zeiss LSM 510 NLO Meta confocal system). The excitation wavelength is in the range of 450–500 nm and the detection wavelength is in the range of 515–565 nm.

qRT-PCR analysis. Total RNA from liver tissues and bladder epithelial cells were extracted using Trizol. Equal amounts of RNA (2 μg) were reverse-transcribed into cDNA using the Transcriptor First Strand cDNA synthesis Kit (Roche). Taqman probes were from the universal probe library (Roche) and primers were from Integrated DNA Technologies (Corvalle, IA, USA). The Taqman probes used were NQO1(50), HO-1 (25), and β-actin (56). The primers used were NQO1: forward (aggtctggattgaacag) and reverse (agctaactggctctcttgct), HO-1: forward (ctgtcgcttggtgga) and reverse (cacaaggtcaggtga), β-actin: forward (agccaaactggcaagatg) and reverse (gggtcagccaggtcct).

The qPCR conditions were: 1 cycle of initial denaturation (95°C for 10 min), 40 cycles of amplification (95°C for 10 s and 60°C for 20 s), and a cooling period (50°C for 5 s). The data presented were relative mRNA levels normalized to β-actin, and the value from the Nrf2−/+ control group was set as 1.

Statistics. Two-way Student’s t test was used to determine the significant difference between two samples. Body weight gained and water consumption/week were average values from four different mice within the same group. Pooled mRNAs from four mice within the same group were analyzed by qPCR analysis. qPCR analysis was run in triplicate and data were expressed as mean ± SD.

Results

There was no difference in body weight or water consumption between Nrf2+/+ and Nrf2−/− mice. All 32 mice survived the 6-week As(III) treatment, with no obvious systemic toxicity, such as diarrhea, hair loss, or nasal hemorrhaging.

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Body weight gained during the 6-week period was recorded at the day of harvest. The weight gained was reduced as the doses of As(III) increased (Fig. 1A). In addition, no difference in weight gained between Nrf2+/+ and Nrf2−/− mice was observed (Fig. 1A). The amount of water intake in Nrf2+/+ and Nrf2−/− mice was similar, except that the Nrf2+/+ and Nrf2−/− mice treated with 100 ppm As(III) showed a decrease in water intake (Fig. 1B).

As(III) activated the Nrf2-dependent antioxidant response in a dose-dependent manner in Nrf2+/+ mice

As(III) has been reported to be an inducer of Nrf2 in vitro. Here, the ability of As(III) to activate the Nrf2 signaling pathway in vivo was tested. mRNA was extracted from both liver tissues and the bladder epithelial cells. qRT-PCR was performed to measure relative transcriptional levels of NQO1 and HO-1, two Nrf2 target genes. As expected, Nrf2+/+ mice had higher basal levels of NQO1 and HO-1 expression, compared to Nrf2−/− mice (Figs. 1C and D). As(III) enhanced the transcription of both NQO1 and HO-1, in a dose-dependent manner in Nrf2+/+ (Figs. 1C and D). Conversely, Nrf2−/− mice had very low basal and induced levels of NQO1 and HO-1 (Figs. 1C and D). In Nrf2−/− mice, As(III) induced NQO1 expression slightly at 10, and 100 ppm in the liver, presumably through an Nrf2-independent mechanism. Interestingly, induction of NQO1 by As(III) is more prominent in bladder epithelial cells than in liver tissues (compare Figs. 1C with D, NQO1 panels). In contrast, induction of HO-1 by As(III) was more significant in liver tissues, compared to that in bladder epithelial cells (Figs. 1C and D, HO-1 panel). Currently, the reason for this organ-specific induction of a particular gene is unclear.

Pathological changes induced by As(III) were more prominent in liver and bladder tissues from Nrf2−/− mice

The pathological changes in response to As(III) treatments were examined and compared among different groups in four different organs, lung, liver, kidney, and bladder. In the kidney, pathological changes, such as cell proliferation in glomeruli and tubular epithelial cell degeneration, were not observed in either Nrf2+/+ or Nrf2−/− mice. In
lung tissues, 1 ppm As(III) did not elicit distinct pathological changes (Fig. 2, lung panel, A, B, E and F). However, proliferation of fibroblasts and thickening of alveolar wall were observed in both Nrf2+/+ and Nrf2−/− mice at high doses of As(III), although there was no significant difference between Nrf2+/+ and Nrf2−/− groups. (Fig. 2, lung panel, compare C and D with A; G and H with E). The liver is a primary defense organ that detoxifies drugs and xenobiotics, which predispose it to injuries caused by excessive toxic exposure. Uptake of As(III) via drinking water caused severe lesions in the liver. Liver tissues in untreated mice have normal morphology (Fig. 2, liver panel, A and E). 1 ppm As(III) induced vacuolar degeneration in Nrf2+/+ mice (Fig. 2, liver panel, B), whereas it caused hepatic spotty and lytic necrosis that is accompanied by infiltrated macrophages and lymphocytes in Nrf2−/− mice (Fig. 2, liver panel, F). At 10 and 100 ppm As(III), necrosis was induced even in Nrf2+/+ mice (Fig. 2, liver panel, C and D). However, the same doses of As(III) elicited severe necrosis in Nrf2−/− mice with more infiltrated inflammatory cells including eosinophils (Fig. 2, liver panel, G and H). This indicates progression to liver disease, since eosinophilic infiltration was recently found to be associated with liver steatosis and liver fibrosis (Tarantino et al., 2008). As expected, there was no

Fig. 2. Effects of daily exposure of As(III) on histological changes in the kidney, liver, lung and bladder. The four mice in each group all underwent pathological analysis to avoid individual variation. Panels A, B, C and D show representative photomicrographs of HE staining from individual Nrf2−/− mice, and panels E, F, G, and H show representative tissues from individual Nrf2−/− mice. Spotty necrosis in the liver and interstitial edema in the bladder were observed in many fields.
significant malignant hyperplasia observed in bladder epithelium in either Nrf2+/+ or Nrf2−/− mice following six weeks of As(III) exposure. In Nrf2+/+ mice exposed to 100 ppm As(III) and Nrf2−/− mice exposed to 10 or 100 ppm, minimal epithelial proliferation in the bladder was observed (Fig. 2, bladder panel, D, G, and H). However, 10 or 100 ppm As(III) caused severe interstitial edema and congestion in Nrf2−/− mice (Fig. 2, bladder panel, G and H) while only 100 ppm As(III) caused a similar change in Nrf2+/+ mice (Fig. 2, bladder panel, D). Nrf2−/− mice were more susceptible to As(III)-induced DNA hypomethylation

The DNA methylation status of bladder epithelium was examined by immunohistochemical analysis with an anti-5-methylcytosine antibody. In untreated groups, DNA was heavily methylated, indicated by brown nuclear staining (Fig. 3, 5′-mC panel, A and E). Following As(III) treatment, the number of positive stained cells, and the intensity of staining decreased in a dose-dependent manner in both Nrf2+/+ or Nrf2−/− mice. These results indicate that As(III) induced global DNA hypomethylation. Furthermore, As(III)-induced hypomethylation is more substantial in Nrf2−/− than in Nrf2+/+ mice as shown by a significant decrease in brown staining in 10 or 100 ppm As-treated Nrf2−/− mice, compared to the same dose treatment in the Nrf2+/+ group (in Fig. 3, 5′-mC panel, compare G with C, and H with D). Collectively, these results indicate that Nrf2−/− mice are more susceptible to As-induced DNA hypomethylation than Nrf2+/+ mice.

Nrf2−/− mice were more susceptible to oxidative DNA damage in response to As(III) treatment

8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-Oxo-dG) is a mutagenic base modification that is generated by the reaction of reactive oxygen species with DNA and is a quantitative index of oxidative DNA damage (Wainfan and Poirier, 1992). Formation of 8-Oxo-dG in bladder tissues of mice from different groups was detected using immunohistochemical analysis with an antibody against 8-Oxo-dG. There was no detectable staining in the control and 1 ppm-treated Nrf2+/+ or Nrf2−/− groups (Fig. 3, 8-Oxo-dG panel, A, B, E, and F). However, 10 and 100 ppm As(III) induced extensive staining in bladder epithelium in both Nrf2+/+ and Nrf2−/− mice, and higher staining was observed in Nrf2−/− mice, compared to Nrf2+/+ mice (Fig. 3, compare C with G, and D with H).

Fig. 3. Daily exposure of As(III) caused damage in bladder epithelium. The four mice in each group all underwent immunohistochemical and apoptotic cell death analyses to avoid individual variation. As(III) induced damage was evaluated by levels of DNA methylation detected by an anti-5-methylcytosine antibody (α-5′mc), oxidative DNA damage by an anti-8-Oxo-dG antibody (α-8-Oxo-dG panel), and apoptotic cell death by TUNEL labeling (apoptosis panel).
Nrf2−/− mice were more susceptible to apoptotic cell death induced by As(III)

Apoptotic cell death in bladder epithelium was measured by TUNEL labeling. Similar to the formation of 8-Oxo-dG within bladder epithelium, the number of apoptotic epithelial cells increased in response to As(III) in a dose-dependent manner in both Nrf2+/+ and Nrf2−/− mice. There were no apoptotic cells observed in control or 1 ppm As(III)-treated groups (Fig. 3, apoptosis panel, A, B, E and F). Apoptotic epithelial cells appeared in response to 10 ppm As(III) only in the Nrf2−/−, but not in the Nrf2+/+ group (Fig. 3, apoptosis panel, compare C with G). At 100 ppm As(III), a considerable number of apoptotic epithelial cells were induced in both Nrf2+/+ and Nrf2−/− groups (Fig. 3, apoptosis panel, D and H).

Discussion

Many previous findings suggest that an adaptive response exists in mammals, i.e. previous exposure to low toxic reagents can elicit a beneficial effect that reduces damage by subsequent insults. We postulated that the adaptive response is derived from activation of the Nrf2 pathway based on the following observations: (i) resveratrol and epigallocatechin gallate, two known inducers of Nrf2, prevented arsenite-induced transformation of human osteogenic sarcoma cells. (Yang et al., 2005). (ii) Tert-butyldihydroquinone, an potent inducer of Nrf2, was found to be capable of reversing arsenite-induced gene expression in mouse embryo fibroblasts (Kann et al., 2005). (iii) Interestingly, a recent epidemiological study showed that populations exposed to low dose arsenic from their drinking water had reduced risk for cancer, compared to unexposed populations (Lamm et al., 2004). This “anticarcinogenic” effect of arsenic has also been demonstrated in animal and cell culture models. For example, arsenic inhibited formation of GST-P-positive hepatic foci in rats treated with chemical carcinogens (Pott et al., 1998); lymphocytes from individuals pre-exposed to arsenic-contaminated drinking water were more resistant to subsequent arsenic exposure (Mahata et al., 2004); 0.1 to 1 μM arsenite exposure provided a protective effect against oxidative stress and DNA damage in human keratinocyte and fibroblast cells (Snow et al., 2005); arsenic or arsenic-containing metal mixtures inhibited malignant transformation of RHEK-1, human keratinocyte cells (Bae et al., 2002); and chronic low dose arsenite exposure enhanced self tolerance in liver epithelial cells (Romach et al., 2000).

Arsenic can elicit both a beneficial Nrf2-dependant antioxidant response and a cell damaging effect. Given the complexity of the cell, it is highly likely that toxic effects in any given situation are dependent on many genes working in concert and not just a single gene. Activation of an array of Nrf2-dependent down-stream genes on many genes working in concert and not just a single gene. The initial attempt to counteract deteriorative effects induced by arsenic, ultimately resulting in toxicity.

In this study, we used Nrf2−/− mice to demonstrate that Nrf2 is able to alleviate damage in response to arsenic exposure. Arsenic appeared to be more toxic to Nrf2−/− mice compared to Nrf2+/+ mice, as measured by physiological changes, DNA methylation, oxidative DNA damage, and apoptotic cell death. Arsenic caused detectable physiologic alterations, especially in the liver and bladder in both Nrf2−/− and Nrf2+/+ mice. Although skin damage is frequently observed in the arsenic-exposed population, we did not observe any skin lesions during the course of our study. Two rational reasons can be envisioned: (1) in addition to exposure through drinking water, human exposure to arsenic also includes direct contact with the skin; however, in our study arsenic was solely administered through the digestive system, and (2) the duration of arsenic exposure was not long enough to induce skin damage. More importantly, these results indicate a protective role of Nrf2 against arsenic toxicity in vivo. Therefore, we believe that specific activation of the ARE−Nrf2−Keap1 pathway by a reagent with low toxicity should be a great strategy to combat arsenic-induced damage. Our findings will lay the groundwork for dietary and therapeutic interventions against arsenic adverse effects.

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References


