Nrf2 protects human bladder urothelial cells from arsenite and monomethylarsonous acid toxicity

Xiao-Jun Wang, Zheng Sun, Weimin Chen, Kylee E. Eblin, Jay A. Gandolfi, Donna D. Zhang

Department of Pharmacology and Toxicology, University of Arizona, 1703 E. Mabel St., Tucson, AZ 85721, USA

Received 6 July 2007; revised 25 July 2007; accepted 26 July 2007
Available online 7 August 2007

Abstract

Arsenic is widely spread in our living environment and imposes a big challenge on human health worldwide. Arsenic damages biological systems through multiple mechanisms including the generation of reactive oxygen species. The transcription factor Nrf2 regulates the cellular antioxidant response that protects cells from various insults. In this study, the protective role of Nrf2 in arsenic toxicity was investigated in a human bladder urothelial cell line, UROtsa. Using a UROtsa cell line stably infected with Nrf2-siRNA, we clearly demonstrate that compromised Nrf2 expression sensitized the cells to As(III)- and MMA(III)-induced toxicity. On the other hand, the activation of the Nrf2 pathway by tert-butylhydroquinone (tBHQ) and sulforaphane (SF), the known Nrf2-inducers, rendered UROtsa cells more resistant to As(III) and MMA(III). Furthermore, the wild-type mouse embryo fibroblast (WT-MEF) cells were protected from As(III)- and MMA(III)-induced toxicity following Nrf2 activation by tBHQ or SF, whereas neither tBHQ nor SF conferred protection in the Nrf2−/− MEF cells, demonstrating that tBHQ- or SF-mediated protection against As(III)- and MMA(III)-induced toxicity depends on Nrf2 activation. These results, obtained by both loss of function and gain of function analyses, clearly demonstrate the protective role of Nrf2 in arsenic-induced toxicity. The current work lays the groundwork for using Nrf2 activators for therapeutic and dietary interventions against adverse effects of arsenic.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Nrf2; Keap1; Arsenic; MMA(III); UROtsa

Arsenic is an environmental toxicant. Ground water contaminated with arsenic is the main source of human exposure worldwide and generates an important global health issue (Smith et al., 2000; Rossman, 2003; Tchounwou et al., 2003). Epidemiological studies have correlated arsenic exposure to various human diseases such as cancer, hyperkeratosis, atherosclerosis, diabetes and chronic obstructive pulmonary diseases (Cohen et al., 2000; Steinmaus et al., 2000; Tseng, 2002; Smith et al., 2006). Arsenic has been classified as a human carcinogen that induces tumors in the skin, lung, and bladder. However, it has been problematic to induce tumors in rodents using inorganic arsenic. Nevertheless, maternal exposure to inorganic arsenic induced several types of tumors in the offspring, demonstrating that arsenic is a complete carcinogen in animal models (Waalkes et al., 2003). Inorganic arsenic has also been demonstrated as a cocarcinogen to enhance ultraviolet-induced skin tumors in a hairless mouse model (Burns et al., 2004).

Many factors, such as chemical species and dose, determine the action of arsenic. High doses of arsenic mainly cause acute toxicity, whereas moderate doses of repetitive exposure are associated with various cancers. Different species of arsenic, such as As(III) vs. As(V) and inorganic vs. methylated organic arsenic, display varying potency of toxicities (Carter et al., 2003). Following uptake, inorganic arsenic is converted primarily in the liver to methylated metabolites, including monomethylarsonous acid [MMA(III)], monomethylarsonic acid [MMA(V)], dimethylarsinous acid [DMA(III)], and dimethylarsinic acid [DMA(V)] (Aposhian, 1997; Thomas et al., 2004; Kenyon et al., 2005). Trivalent arsenic species are more toxic than pentavalent species, and the methylated arsenic compounds are more toxic than the inorganic ones (Thomas et al., 2001; Aposhian et al., 2003).

Arsenic damages biological systems through multiple mechanisms, one of them being reactive oxygen species (ROS). ROS is generated by arsenic indirectly through many sources. For
example, arsenic was shown to increase NAD(P)H oxidase activity, resulting in superoxide accumulation (Smith et al., 2001). Through direct binding with the cysteinyl sulfhydryl group in glutathione, arsenic is able to conjugate with glutathione, which leads to subsequent removal of the arsenic–glutathione complex by multidrug associated transporters such as MRP1 and MRP2. As a consequence, glutathione is depleted, resulting in the formation of ROS (Scott et al., 1993; Delnomdedieu et al., 1994; Kala et al., 2000; Vernhet et al., 2001; Leslie et al., 2004).

In addition to the non-protein sulfhydryl glutathione, endogenous sulfhydryl groups in proteins have been reported to interact with arsenic and play an important role in the detoxification of arsenic (Scott et al., 1993; Thompson, 1993; Sakurai et al., 2005). The exogenous antioxidant N-acetylcysteine is able to prevent arsenic-induced toxicity, implicating a role of ROS in arsenic-induced toxicity/carcinogenicity (Liu et al., 2003).

Vertebrates have evolved several defense mechanisms to cope with environmental insults. The antioxidant response is the major one to neutralize ROS elicited by toxic exposure, and thus, to maintain cellular redox homeostasis (Venugopal and Jaiswal, 1996; Itoh et al., 1999). This antioxidant system is mediated by the transcription factor NF-E2-related factor 2 (Nrf2) through the antioxidant response element (ARE). The ARE is present in the promoters of genes encoding antioxidant enzymes and Phase II detoxification enzymes, such as glutathione S-transferase (GST), NAD(P)H quinone oxidoreductase (NQO1), γ-glutamylcysteine synthetase (γ-GCS), and heme oxygenase-1 (HO-1) (Wild et al., 1999; McMahon et al., 2001; Jaiswal, 2004; Kobayashi et al., 2004). These enzymes work in concert to elicit cytoprotective responses that defend cells from the toxic effects of environmental insults. Activation of the Nrf2 pathway has been clearly demonstrated to confer protection against toxic and carcinogenic effects of many environmental insults. This is illustrated by several findings indicating that Nrf2 knockout mice display increased sensitivity to chemical toxicants and carcinogens and are refractory to the protective actions of chemopreventive compounds (Chan and Kan, 1999; Aoki et al., 2001; Enomoto et al., 2001; Ramos-Gomez et al., 2001; Cho et al., 2002). Thus, it is believed that the activation of the Nrf2-mediated antioxidant response offers beneficial effects against many oxidative stress-related human diseases including cancer, neurodegenerative disease, aging, cardiovascular diseases, pulmonary fibrosis, and acute pulmonary injury (Motohashi and Yamamoto, 2004; Cho et al., 2006; Zhang, 2006). Fortunately, activity of Nrf2 can be modulated by dietary choice. Many health foods, for examples, curcumin, red grapes, green tea, cruciferous and leguminous vegetables, contain compounds that are able to activate the Nrf2-dependent cellular protective response (Surr, 2003; Lee and Surr, 2005).

Being the last target organ for arsenic exposure before excretion, the urinary bladder is exposed not only to inorganic arsenic but also to the organic metabolites. As reported in human studies, urinary arsenic species, including MMA(III), MMA(V), DMA(III), and DMA(V), were detected in populations exposed to inorganic arsenic in their drinking water (Hsieh et al., 1998; Aposhian et al., 2000; Mandal et al., 2001). Both MMA(III) and DMA(III) are significantly more toxic than inorganic arsenic. However, retention time for DMA(III) is very transient, which leads to the hypothesis that MMA(III) is the form of arsenic species responsible for the majority of toxicity and carcinogenicity in humans following the uptake of inorganic arsenic. MMA(III) comprises 7–11% of urinary arsenic and has been reported to be 20-fold more toxic than inorganic arsenic in various cell types (Aposhian et al., 2000; Petrick et al., 2000). In the current work, the functional role of Nrf2 in arsenic-induced toxicity was studied using both inorganic arsenic [As(III) in the form of sodium arsenite] and an organic arsenic metabolite [MMA(III)] in a human bladder urothelium cell line.

Materials and methods

Construction of retrovirus vectors containing Nrf2-shRNA. The vector pRS used for cloning Nrf2 siRNA was purchased from OriGene. Four different shRNA constructs were made. The 29-mer short hairpin sequence was inserted between the BamH1 and HindIII cloning sites. The 29-mer sequences used were:

- (i) TGGCATCACAGAACCTACTGTTGAACTC,
- (ii) CCTGGAAATGTCACAAGAAGTGTTCATAA,
- (iii) TGACCTTCAGTCACCGAGAAAGATATG, and
- (iv) CCAGTCTTCAAGTCACTAATCACGCTCA.

Chemicals, cell culture, transfection, and induction. Sodium arsenite, tBHQ, sulforaphane, polybrene and puromycin were purchased from Sigma. MMA(III) was synthesized in the Synthetic Chemistry Core at the University of Arizona. UROtsa cells were generously provided by Drs. Mary Ann and Donald Sens (University of North Dakota). The passage number of UROtsa cells used for this study was from 59 to 69. UROtsa cells were grown in DMEM medium enriched with 5% FBS according to the original condition described by Petzoldt et al. (1995)). All mammalian cells were incubated at 37 °C in a humidified incubator containing 5% CO2. For establishing stable cell lines harboring control siRNA and Nrf2-siRNA, five plasmids containing five different shRNA (four Nrf2 siRNAs and one control) were first transfected into SD-3443, an amphotrophic packaging cell line from ATCC. Viruses produced in the supernatant were collected and used to infect UROtsa cells in the presence of 2 μg/ml polybrene. Forty-eight hours after infection, cells were grown in the medium containing 3 μg/ml of puromycin for selection. Stable cell lines were established once all the cells in the negative control plate were killed. Stable cell lines were continuously grown in the medium containing 3 μg/ml of puromycin.

Cell viability. As(III)- and MMA(III)-induced toxicity was measured by functional change of mitochondria using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. For the MTT assay of UROtsa cells, approximately 8 × 103 cells per well were seeded in a 96-well plate and incubated overnight. Cells were treated with several concentrations of As(III) or MMA(III) for 24 h following the addition of 20 μl of 2 mg/ml MTT directly into the medium. After incubation (37 °C for 0.5–3 h), the plate was centrifuged and the medium removed. 100 μl of isopropanol/HCl was added into each well and crystals were dissolved by shaking the plate at room temperature. Absorbance was measured by a plate reader at 570 nm. Triplicate wells were used for each sample and the experiments were repeated at least three times to get means and standard deviations.

ROS detection. Cells were either left untreated or treated with 20 μM As(III) for 16 h. Following washing with PBS, fresh medium containing dichlorofluorescin (DCF) (Sigma, 10 μg/ml final concentration) was added and the plates were incubated for 20–60 min. Cells were then washed twice with PBS and trypsinized. After further washing with PBS, cells were resuspended in PBS to approximately 106 cells per ml. Fluorescence was measured using flow cytometry with excitation at 488 nm and emission at 515–545 nm. All steps were handled in the dark.

NQO1 enzymatic activity and glutathione concentration. NQO1 activity was measured as the dicoumarol-inhibitable fraction of DCIP reduction (De Fain et al., 2002). Cells were trypsinized, washed and resuspended in 2× TE buffer (20 mM Tris–HCl, 2 mM EDTA, pH 7.4). Cells were disrupted by three cycles of freeze/thaw and centrifuged for 5 min at 12,000×g. An aliquot of supernatant was mixed with the
reaction buffer (25 mM Tris–HCl [pH 7.4], 0.18 mM NADPH, 0.2 mg/ml BSA, and 0.01% Tween-20 in a total volume of 1 ml), in the absence or presence of 20 μM dicoumarol. The reaction was started by the addition of 40 μM DCPIP. Reduction of DCPIP was measured as the difference of two readings at 600 nm at room temperature between a 1-min interval. NQO1 activity was calculated as the dicoumarol-inhibitable part of DCPIP reduction (nmol DCPIP reduced/min/μg protein). Protein amounts were measured using Quant-iT™ Protein Assay Kit from Invitrogen. All the chemicals used for this assay were purchased from Sigma. Intracellular glutathione concentrations were measured using QuantiChrom glutathione assay kit from BioAssay Systems. The procedure was performed according to the manufacturer’s instructions. Three independent experiments were performed for the calculation of means and standard deviations.

Real-time RT-PCR. Total mRNAs were extracted using the TRIZOL reagent (Invitrogen) and equal amounts of RNA were reverse-transcribed to cDNA with Transcripter First Strand cDNA Synthesis Kit (Roche). Quantification of cDNA amount for Nrf2, NQO1, HO-1, and hGAPDH in each sample was performed with LightCycler 480 probes master kit (Roche). All primer sets were tested prior to use in this work to ensure that only a single product of the correct size was amplified. Taqman probes were from the universal probe library (Roche): hNrf2 (#70), hNQO1 (#25), hGAPDH (#25), and hkeap1 (#10). Oligos used for primers were synthesized by IDT.

Triplicate reactions were performed for each sample. Cycling was performed as follows: one 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 program (50 °C for 5 s). Mean crossing point (Cp) values and standard deviations were determined. Crossing point values were normalized to the respective crossing point values of the hGAPDH reference gene. Data are presented as a fold change in gene expression in Nrf2-siRNA cells compared to control-siRNA cells.

Antibodies and immunoblot analysis. The Nrf2 antibody was purchased from Santa Cruz. Cells were lysed in sample buffer (50 mM Tris–HCl [pH 6.8], 2% SDS, 10% glycerol, 100 mM DTT, 0.1% bromophenol blue). Following sonication, cell lysates were electrophoresed through SDS–polyacrylamide gels and subjected to immunoblot analysis.

Results

A UROtsa-derived cell line, UROtsa-725, has reduced endogenous Nrf2 and impaired Nrf2-mediated antioxidant response

The UROtsa cell line was used to examine the functional importance of Nrf2 activation in arsenic-induced toxicity. To overcome the problem of having low transfection efficiency, a retrovirus-mediated infection method was chosen for establishment of stable cell lines harboring Nrf2 shRNA. Five stable cell lines were established: four shRNAs targeting different regions of Nrf2 and one scrambled siRNA as a negative control. Once the selection was completed, endogenous levels of the Nrf2 protein were measured by immunoblot analysis with anti-Nrf2 antibodies. The UROtsa-725 (U725, UROtsa cells stably transfected with a vector containing Nrf2 siRNA: TGGCATCACCAGAACACTGGAATCT) had

Fig. 1. UROtsa-725 had reduced endogenous Nrf2. (A) UROtsa-725 and its negative control UROtsa-724 were derived from UROtsa cells stably infected with Nrf2-siRNA or scrambled-siRNA using retrovirus-based vectors. Equal amounts of total lysates were subjected to immunoblot analysis with anti-Nrf2 and anti-tubulin antibodies. (B) The mRNA levels of Nrf2, Keap1, NQO1, and HO-1 in UROtsa-724 and UROtsa-725 were compared by real-time RT-PCR. (C) NQO1 activities in the lysates of UROtsa-724 and UROtsa-725 were measured using DCPIP as a substrate. (D) Intracellular glutathione concentrations in UROtsa-724 and UROtsa-725 were measured using the QuantiChrom glutathione assay kit. (E) UROtsa-724 and UROtsa-725 cell lines were either left untreated or treated with 20 μM As(III) for 16 h. Following incubation of cells with DCF, ROS was detected using flow cytometry. The bar graph represents the means of fluorescence values.
maximum reduction of the Nrf2 protein with approximately 50% knockdown, compared to UROtsa-724 (U724, UROtsa cells stably infected with a vector containing a scrambled siRNA) (Fig. 1A). Thus, UROtsa-725 and UROtsa-724 were used for measuring mRNA of Nrf2 and the downstream effects of Nrf2 down-regulation: (i) mRNA expression of Nrf2, Keap1, NQO1, and HO-1 (Fig. 1B); (ii) enzymatic activity of NQO1 (Fig. 1C); (iii) intracellular glutathione concentration (Fig. 1D); and (iv) constitutive and induced levels of ROS following As(III) treatment (Fig. 1E). Stable transfection of Nrf2 siRNA reduced mRNA expression of Nrf2, as well as its downstream genes NQO1 and HO-1, while it had no effect on Keap1 mRNA (Fig. 1B). The 30–50% knockdown of the mRNAs of Nrf2 or its downstream target genes in URotsa-725 was not as pronounced as expected, which may be due to the factor that only a single copy of shRNA was incorporated into each cell, in addition to the weak U6 polymerase III promoter used. However, regardless of the moderate reduction of the Nrf2 mRNA expression, the functional difference of the Nrf2 downstream effects between UROtsa-724 and UROtsa-725 cell lines was significant by all the measurements tested. For example, when the cell lysates of UROtsa-724 and UROtsa-725 were tested for the enzymatic activity of NQO1 by DCPIP reduction, the NQO1 activity was decreased in UROtsa-725 and it was significant (Fig. 1C). Nrf2 is known to regulate cellular redox conditions by transcriptional activation of many enzymes involving glutathione biosynthesis such as gamma-glutamylcysteine synthetase, glutathione synthase, and xCT cysteine antiporter (Shih et al., 2003). Therefore, the intracellular glutathione concentrations of these two cell lines were compared. Impaired Nrf2 expression in UROtsa-725 resulted in a decrease of the reduced form of glutathione even in unchallenged cells, demonstrating the impact of Nrf2 on cellular redox homeostasis (Fig. 1D). To further verify that UROtsa-725 has reduced redox buffering capacity, intracellular ROS levels were measured by DCF. Fluorescence data from flow cytometry measurement were converted into bar graphic and presented in Fig. 1E. Both the basal and As(III)-induced levels of ROS in URotsa-725 were much higher, compared to UROtsa-724 (Fig. 1E). H2O2 was included as a positive control for the assay (data not shown). Taken together, these results demonstrate that a stable cell line with the impaired Nrf2-dependent antioxidant response was established.

**Down-regulation of Nrf2 sensitizes the human bladder urothelium cells to As(III)- and MMA(III)-induced toxicity**

To demonstrate the protective role of Nrf2 against arsenic-induced toxicity, cellular toxicity assays were performed in UROtsa-724 and UROtsa-725 following As(III) or MMA(III) treatment. Remarkably, the UROtsa-725 cell line had increased sensitivity to both As(III) and MMA(III) compared to the control cell line, UROtsa-724 (Figs. 2A and B). Consistence with the previous findings, MMA(III) is much more potent in inducing cell death than As(III) (Figs. 2A and B). These results indicate that a 30–50% reduction of the Nrf2-mediated response makes UROtsa cells more susceptible to As(III) and MMA(III), demonstrating the critical role of Nrf2 in cytoprotection against arsenic toxicity.

**Activation of Nrf2 by tBHQ and SF protects human bladder urothelium cells from As(III)- and MMA(III)-induced toxicity**

The results in Fig. 2 were obtained by reducing Nrf2 expression to make cells more sensitive to As(III)- and MMA(III)-induced toxicity. It is conceivable that activation of Nrf2 by its inducers should elicit the cytoprotective response, resulting in increased resistance of cells to the arsenic challenge. For this purpose, two well-studied Nrf2 activators tBHQ and SF were chosen. Because no data exists in terms of the duration of Nrf2 activation by tBHQ and SF in UROtsa cells, a set of pilot experiments was performed to define the optimal conditions for tBHQ and SF treatment in terms of concentration and duration of pretreatment and treatment. Optimal Nrf2 induction and duration were monitored by Nrf2 expression using immunoblot analysis (data not shown). 40 μM (24 h) pretreatment plus 5 μM (24 h) cotreatment with tBHQ significantly increased resistance of UROtsa cells to both As(III) and MMA(III) (Figs. 3A and B). In accordance with tBHQ, 8 μM (24 h) pretreatment plus 0.5 μM (24 h) cotreatment with SF also increased survival of UROtsa cells following As(III) or MMA(III) treatment (Figs. 3A and B).
To further confirm that the protective effect of tBHQ or SF on As(III)- and MMA(III)-induced toxicity is attributed to the specific activation of the Nrf2 pathway, the same experiments were carried out in the WT-MEF and Nrf2−/−-MEF cells with the same pretreatment and cotreatment conditions for tBHQ or SF as that used in UROtsa cells. tBHQ or SF rendered the WT-MEF cells more resistant to the toxic effects of both As(III) and MMA(III) (Figs. 3C and D). In sharp contrast, tBHQ or SF aggravated, rather than ameliorated, As(III)- and MMA(III)-induced toxicity in the Nrf2−/−-MEF cells (Figs. 3E and F), demonstrating that both tBHQ and SF are able to protect UROtsa cells from arsenic-induced toxicity through the Nrf2 pathway. Taken together, these data provide strong evidence that activation of the Nrf2 pathway protects cells from arsenic-induced toxicity.

**Discussion**

In the current study, the impact of Nrf2 on As(III)- and MMA (III)-induced toxicity has been elucidated by the finding that reduced expression of Nrf2 sensitized UROtsa cells to As(III)-
and MMA(III)-induced toxicity, whereas activation of Nrf2 by pretreatment and cotreatment with tBHQ or SF increased cellular resistance to As(III) and MMA(III). In accordance with our finding, SF was demonstrated to reduce intracellular arsenic concentration and increase resistance to arsenic toxicity in primary mouse hepatocytes (Shinkai et al., 2006). Thus, activation of Nrf2 represents a beneficial effect of cells to confer protection against arsenic toxicity. Paradoxically, As(III) was reported to activate the Nrf2 pathway (He et al., 2006; Kimura et al., 2006; Massrieh et al., 2006). The puzzling question is why does the highly toxic arsenic induce the Nrf2-mediated beneficial response? These seemingly conflicting results can be explained by the phenomena called adaptive response or hormesis, that is, preconditioning cells with sublethal doses of toxic compounds increases cellular resistance to similar types of toxic compounds (Mattson and Cheng, 2006). Hormesis has been described since 1956 although it is still not very clear how it works. It is speculated that the stress-inducible transcription factor Nrf2 contributes significantly to the hormesis phenomena. Activation of the ARE–Nrf2–Keap1 pathway by As(III) at either low or high concentration is beneficial and is likely to be an attempt of cells to counteract the damaging effects, although the protective mechanism of the Nrf2 pathway may be masked by cell death effects at high concentrations of arsenic. In the MTT assays performed with UROtsa cells, a peak at low concentrations of As(III) or MMA(III) was observed, which represents an increased rate of cell proliferation in response to low doses of As(III) or MMA(III) exposure (Figs. 2A and B). This beneficial low dose of As(III) exposure was also reported by others. For example, 0.1 to 1 μM As(III) exposure provides a protective effect against oxidative stress and DNA damage in human keratinocyte and fibroblast cells (Snow et al., 2005); Chronic low dose As(III) exposure enhances self-tolerance in liver epithelial cells (Romach et al., 2000). Obviously, given the complexity of gene expression profile in response to a toxicant, it is likely that the net outcome in a given exposure portfolio is dependent on batteries of genes working in concert. Nevertheless, activation of an array of Nrf2 downstream target genes preconditions cells to a defensive mode for toxic/carcinogenic insults. Following arsenic exposure, a number of pathways that lead to different responses are activated. The net outcome is dictated by species, dose of arsenic, and duration of exposure. With high concentrations or repetitive doses of arsenic exposure, the Nrf2-dependent defense response is outweighed by the deteriorative effects induced by arsenic, resulting in acute toxicity or cell transformation. Therefore, it is believed that specific and potent activation of Nrf2 to elicit cellular hormesis by a reagent with low toxicity such as sulforaphane should be a great strategy to combat arsenic-induced damage.

The current study clearly demonstrates that Nrf2 protects cells from acute toxicity of arsenic, as measured by the MTT assay. Previously, it has been reported that arsenic induced both forms of cell death in human osteogenic sarcoma cell line U-2OS cells: low doses of arsenic resulted in apoptosis, whereas high doses elicited necrosis. In addition, low-dose arsenic-induced apoptosis was a slow process and required at least 48 h for detection, whereas high-dose arsenic induced necrosis rapidly (Komissarova et al., 2005). Although whether Nrf2 protects cells from apoptotic cell death or necrotic cell death is not addressed in this study, it is envisioned that Nrf2 is able to protect both forms of cell death by acting on the early events of arsenic toxic effects, such as blockage of ROS formation through increased redox buffering capacity, detoxification/removal of arsenic and its reactive metabolites. Interestingly, a recent report indicates that arsenic was able to induce an apoptotic response through the NF-κB (P50)-dependent stabilization of GADD45α and subsequent induction of JNK (Song et al., 2006). JNK has been shown to be required for activation of Nrf2 by several Nrf2 inducers, which leads to the suggestion that JNK activates Nrf2 through direct phosphorylation of Nrf2 (Kong et al., 2001; Keum et al., 2003). However, the phosphorylation site in Nrf2 that is absolutely required for its activity in vivo has not been detected so far. It is not clear, at this point, whether JNK activation plays any role in the Nrf2-mediated protection against arsenic toxicity.

Many of the Nrf2 downstream-target genes seem to have apparent functions in inhibiting arsenic toxicity directly or indirectly. For example, (1) both subunits of γ-GCS, the rate-limiting enzyme in glutathione biosynthesis, were up-regulated by Nrf2, which is further supported by the finding that Nrf2 knockout cells had reduced intracellular glutathione levels (Wild et al., 1999; Chan and Kwong, 2000; Shih et al., 2003). Thus, Nrf2 likely increases the rate of glutathione synthesis in response to arsenic exposure to compensate for glutathione depletion during removal of the arsenic–glutathione complexes. By doing so, Nrf2 maintains cellular redox homeostasis and inhibits arsenic-induced ROS. Similarly, another intracellular redox-regulating protein thioredoxin is activated by tBHQ in an Nrf2-ARE-dependent manner. Working in concert, glutathione and thioredoxin redox buffering systems then counterbalance the production of ROS during arsenic exposure, resulting in enhanced resistance (Kim et al., 2003). (2) In addition to inducing γ-GCS, Nrf2 was shown to maintain intracellular glutathione levels through ARE-dependent induction of the gene encoding the xCT cysteine/glutamate exchange transported that is important in the uptake of cysteine (Sasaki et al., 2002; Shih et al., 2003). (3) Overexpression of glutathione S-transferase Pi, an Nrf2 downstream gene, increased cellular resistance to inorganic arsenic (Liu et al., 2001; Nishinaka et al., 2007). (4) Transcription of the multidrug resistance proteins (including Mrp1–6) and P-glycoprotein was reported to be upregulated by Nrf2 (Vernhet et al., 2001; Hayashi et al., 2003; Maher et al., 2005; Xu et al., 2005). Functioning as transporters, these proteins were shown to play crucial roles in arsenic transport (Kala et al., 2000; Leslie et al., 2004; Kimura et al., 2006). Evidently, detailed mechanistic investigations of Nrf2-dependent protection in response to arsenic is worth further investigation.

The finding that both tBHQ and SF are able to protect UROtsa cells from As(III)- and MMA(III)-induced toxicity provides a molecular basis for therapeutic or dietary intervention. Using Nrf2 inducers as a strategy for chemoprevention appears to be promising. Kensler and coworkers have pioneered clinical trials in residents of Qidong in China who are at high risk of aflatoxin-induced liver cancer. They have provided evidence that oral administration of Nrf2 inducers, such as
oltipraz (a synthetic anti-cancer drug) and SF-rich broccoli extract, offers beneficial effects (Wang et al., 1999; Kensler et al., 2000, 2005). Targeting the activation of Nrf2 may prove to be a superior approach for designing and developing more effective chemopreventive drugs. More significantly, many of the naturally occurring compounds isolated from plants have recently been identified as Nrf2 activators that include isothiocyanates (e.g. SF found in cruciferous vegetables) and polyphenols (epigallocatechin-3-gallate in green tea and caffeic acid phenethyl ester in honeybee propolis) (Zhang et al., 1994; Suganuma et al., 1999; Orsolic et al., 2003; Shen et al., 2005). Diet-based Nrf2 inducers offer greater advantages over therapeutic drugs due to their general acceptance, low toxicity, and cost-effectiveness. Thus, using natural products to boost the Nrf2-dependent antioxidant response in populations at high risk for arsenic exposure is an excellent choice to combat arsenic-induced damages.

Acknowledgments

This study was supported by the NIH grants ES015010-01 and ES04940. We thank Jefferson Y. Chan for his gift of the wild-type and Nrf2−/− MEF cells.

References


Keum, Y.S., Owuor, E.D., Hu, R., Kong, A.N., 2003. Involvement of Keap1 in the inducible expression of antioxidant and detoxifying enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper domain. J. Biol. Chem. 279, 33404–33408.


multidrug resistance transport proteins is associated with acquired tolerance to inorganic arsenic. Mol. Pharmacol. 60, 302–309.


