Activation of Nrf2 by arsenite and monomethylarsonous acid is independent of Keap1-C151: enhanced Keap1–Cul3 interaction

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Drinking water contaminated with arsenic, a human carcinogen, is a worldwide health issue. An understanding of cellular signaling events in response to arsenic exposure and rational designing of strategies to reduce arsenic damages by modulating signaling events are important to fight against arsenic-induced diseases. Previously, we reported that activation of the Nrf2-mediated cellular defense pathway confers protection against toxic effects induced by sodium arsenite [As(III)] or monomethylarsonous acid [MMA(III)]. Paradoxically, arsenic has been reported to induce the Nrf2-dependent signaling pathway. Here, we report the unique mechanism of Nrf2 induction by arsenic. Similar to tert-butylhydroquinone (tBHQ) or sulforaphane (SF), arsenic induced the Nrf2-dependent response through enhancing Nrf2 protein levels by inhibiting Nrf2 ubiquitination and degradation. However, the detailed action of arsenic in Nrf2 induction is different from that of tBHQ or SF. Arsenic markedly enhanced the interaction between Keap1 and Cul3, subunits of the E3 ubiquitin ligase for Nrf2, which led to impaired dynamic assembly/disassembly of the E3 ubiquitin ligase and thus decreased its ligase activity. Furthermore, induction of Nrf2 by arsenic is independent of the previously identified C151 residue in Keap1 that is required for Nrf2 activation by tBHQ or SF. Distinct mechanisms of Nrf2 activation by seemingly harmful and beneficial reagents provide a molecular basis to design Nrf2-activating agents for therapeutic intervention.

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Introduction

Human exposure to arsenic is primarily through drinking contaminated water (Tchounwou et al., 1999; Smith et al., 2000; Tchounwou et al., 2003). High doses of arsenic cause acute toxicity while chronic arsenic exposure results in a high incidence of tumors in the skin, lung, kidney, liver, and bladder in populations living in the geographic areas where arsenic concentration is high in drinking water. (Chen et al., 1992; Byrd et al., 1996; Tchounwou et al., 2003; Chen et al., 2004). Numerous studies have been performed in elucidating molecular events associated with arsenic-induced tumor formation or malignant transformation, both in animal and cell culture models. Results from these studies have revealed that arsenic induces global DNA hypomethylation and modulates gene expression profiles, which implicates a non-genotoxic mechanism of carcinogenesis and thus emphasizes the importance of investigating molecular signaling events elicited by arsenic exposure. Aberrant expressed genes can be classified into many categories such as stress response genes, hormone-related genes, cytokines, apoptotic genes, cell cycle regulatory genes, proteolytic genes, and proto-oncogenes (Kitchin, 2001; Yih et al., 2002; Zheng et al., 2003; Chen et al., 2004). In particular, genes important in controlling cell proliferation and transformation are aberrantly expressed during the arsenic–induce carcinogenesis (Shimizu et al., 1998; Chen et al., 2001; Hamadeh et al., 2002; Li et al., 2003; Liu et al., 2004; Benbrahim-Tallaa et al., 2005).

The transcription factor Nrf2 regulates an antioxidant response that defends cells from toxic and carcinogenic effects of environmental pollutants (Kobayashi and Yamamoto, 2006; Zhang, 2006; Kessler et al., 2007). Activity of Nrf2 is tightly regulated by Keap1 at multiple levels: (i) Keap1 is able to sense a disturbance in the cellular redox condition to modulate the Nrf2 signaling pathway accordingly. Several cysteine residues have been proposed to play a role in the sensing mechanism. We and others have demonstrated that mutation of C151 in Keap1 adequately blocks tBHQ or SF-induced activation of Nrf2 (Zhang and Hannink, 2003; Levonen et al., 2004; Wakabayashi et al., 2004). (ii) Functioning as an E3 ubiquitin ligase, Keap1 constantly targets Nrf2 for ubiquitination and degradation to maintain low constitutive levels of Nrf2 under basal conditions (Cullinan et al., 2004; Kobayashi et al., 2004; Zhang et al., 2004; Furukawa and Xiong, 2005). (iii) Upon induction, E3 ubiquitin ligase activity is inhibited, leading to decreased degradation of Nrf2 and enhanced nuclear translocation of Nrf2 (Zhang et al., 2004; Jain et al., 2005; Zhang, 2006). (iv) At the post induction stage, Keap1 facilitates Nrf2 nuclear export and its association with the cytoplasmic ubiquitination and degradation machinery to turn off the Nrf2 signal (Karapetian et al., 2005; Nguyen et al., 2005; Velichkova and Hasson, 2005; Sun et al., 2007). These multiple control mechanisms rendered by Keap1 ensure prompt removal of hazardous reactive oxygen species to maintain cellular redox homeostasis.

Ubiquitination of Nrf2 is regulated by the Keap1–Cul3 E3 ubiquitin ligase complex in which Keap1 functions as a substrate adaptor (Zhang, 2006). Thus, the activity of the Keap1-containing E3 ubiquitin ligase...
complex is critical in maintaining the protein level of Nrf2. The E3 ubiquitin ligase complex consists of Keap1, Cul3, Rbx1, and a ubiquitin-charged E2 (Fig. 5 of the review article) [Zhang, 2006]. The assembly and disassembly of the complex is a tightly regulated event that is controlled by many other proteins or protein complexes. It is thought that the reason for the assembly/disassembly of the Cul-containing E3 ubiquitin complex is for many substrate adaptor proteins to share the common Cul–Rbx1 core complex. Proper assembly/disassembly of the Keap1–Cul3 E3 complex is important in controlling ubiquitination of Nrf2. Both increased and decreased association of Keap1 with Cul3, as demonstrated by modulating CAND1 levels with CAND1 si-RNA or CAND1 overexpression, impairs the ubiquitination of Nrf2, leading to stabilization of Nrf2 [Lo and Hannink, 2006]. In addition, Keap1 mutants that have increased association with Cul3 were shown to have a lower ability to target Nrf2 for ubiquitination [Zhang et al., 2004], further demonstrating the importance of having proper affinity of Keap1 for Cul3 to maintain dynamic assembly/disassembly of the E3 ligase complex.

Inorganic arsenic is the primary form of arsenic in drinking water. It is metabolized in the liver into organic metabolites including MMA (III) that is 20 times more potent than inorganic arsenic in eliciting toxic effects [Bredfeldt et al., 2006]. Recently, we have reported that activation of the Nrf2 pathway confers protection against toxic effects induced by both sodium arsenite [As(III)] and monomethylarsonous acid [MMA(III)], demonstrating the feasibility of using Nrf2 activators for intervention of arsenic-induced damage in populations at high risk [Wang et al., 2007]. Paradoxically, the Nrf2 pathway is also induced by for intervention of arsenic-induced damage in populations at high risk [Wang et al., 2007]. In the current work, the molecular mechanism of Nrf2 activation by arsenic was investigated. Our results clearly demonstrate that both As(III) and MMA(III) were able to activate Nrf2 by increasing association between Keap1 and Cul3, therefore disrupting the dynamic assembly/disassembly process of the Keap1–Cul3 E3 ubiquitin ligase complex. Reduced E3 ubiquitin ligase activity led to decreased degradation of Nrf2 and activation of the Nrf2 downstream effects. Furthermore, upregulation of Nrf2 by As(III) and MMA(III) was independent of the previously identified cysteine residue C151 in Keap1, which indicates a distinct mechanism by which As(III) and MMA(III) activate Nrf2 compared to other Nrf2 inducers, such as BHQ and SF.

**Materials and methods**

**Construction of recombinant DNA molecules.** Expression plasmids for Keap1-WT, Keap1-C151S, CBD-tagged version of Keap1-WT and Keap1-C151S, HA-Nrf2, HA-Cul3, HA-Keap1-C151S, CBH-tagged version of Keap1-WT and Keap1-C151S, and HA-Nrf2 were kindly provided by Drs. Mary Ann and Donald Sensi (University of North Dakota). NQO1, Keap1, and Gal4-Neh2 were purchased from the universal probe library (Roche). hNrf2: forward (acacgtgaagagggccag) and reverse (tcttcgagagagcctg). hNQO1: forward (atgatgatgagggccagcct) and reverse (tccttcgagagagcctg). hHO-1: forward (aacctcgaagggcaggccag) and reverse (tccttcgagagagcctg).

**Ubiquitination of Nrf2.** To detect ubiquitinated Nrf2 in vivo, cells were transfected with expression vectors for HA-ubiquitin, Keap1, and Gal4-Neh2. The transfected cells were exposed to 10 μM MMA(III) for 4 h. Cells were lyzed by boiling in a buffer containing 2% SDS, 150 mM NaCl, 10 mM Tris–HCl (pH 8.0), 100 mM sodium deoxycholate, 0.1% SDS containing 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (Sigma). Cell lysates were pre-cleared with protein A beads and incubated with 2 μg of affinity-purified antibodies for 2 h at 4 °C, followed by incubation at 4 °C for 1 h with protein A–agarose beads for 2 h. After washing with RIPA buffer, immunoprecipitated complexes were eluted in sample buffer by boiling for 4 min, electrophoresed through SDS-polyacrylamide gels, and subjected to immunoblot analysis. Antibodies specific for Nrf2, NQO1, and HO-1 were purchased from Santa Cruz, and the Myc and HA epitopes (Covance) were purchased from Covance.

**Results**

**Both As(III) and MMA(III) enhance the transcriptional activity and the protein level of ectopically expressed Nrf2.** Uppregulation of the Nrf2-mediated cellular antioxidant response by As(III) and MMA(III) was determined using an ARE-dependent firefly luciferase reporter gene assay in transient transfected MDA-MB-231 cells.
MB-231 cells following As(III) or MMA(III) treatment. It has been demonstrated that Nrf2 is maximally induced by tBHQ or SF in MDA-MB-231 cells and thus this cell line was used for testing activation of Nrf2 by arsenic. As(III) induced the activity of Nrf2 in a concentration-dependent manner (Fig. 1A, left panel) without obvious cell death. Induction was detected at a concentration as low as 20 μM and the fold of induction was significantly higher with 30–50 μM As(III) compared to that with 50 μM tBHQ. Likewise, MMA(III) activated the activity of Nrf2 in a concentration-dependent manner with approximately 10–20 times greater potency compared to As(III) (Fig. 1A, right panel). Previous work has demonstrated that tBHQ and SF activate Nrf2 through stabilization of the Nrf2 protein. Therefore, aliquots of lysates from the transfection vectors for HA-Cul3 and Keap1-CBD. The cell lysates were subjected to immunoblot analysis. Consistent with our previous findings with tBHQ or SF (Zhang et al., 2004). As expected, the Nrf2 protein level was low in the untreated sample, whereas chemical treatment induced Nrf2 protein levels without significant change in Keap1 levels (Fig. 3A, bottom three panels). Assembly/disassembly of Keap1 with Cul3 is important for the ligase function of the Keap1–Cul3 E3 complex and therefore ubiquitination of Nrf2. Next, the ability of these chemicals in modulating interaction of Keap1 with Cul3 was assessed by immunoprecipitation analysis. To assess the Nrf2–Keap1 interaction, the endogenous Nrf2 and Keap1 proteins were measured using lysates from MDA-MB-231 cells treated with the indicated chemicals. There were still significant amounts of Keap1–associated Nrf2 following treatment with As(III), MMA(III), tBHQ, or SF, indicating that none of the chemicals disrupt the Nrf2–Keap1 complex (Fig. 3A, top two panels), consistent with our previous findings with tBHQ or SF.

As(III) and MMA(III) have no effect on the Nrf2–Keap1 complex, but enhance the Cul3–Keap1 interaction

To further understand the action of As(III) and MMA(III) in Nrf2 stabilization, the interactions of Nrf2 with Keap1 and Keap1 with Cul3, were assessed by immunoprecipitation analysis. To assess the Nrf2–Keap1 interaction, the endogenous Nrf2 and Keap1 proteins were measured using lysates from MDA-MB-231 cells treated with the indicated chemicals. There were still significant amounts of Keap1–associated Nrf2 following treatment with As(III), MMA(III), tBHQ, or SF, indicating that none of the chemicals disrupt the Nrf2–Keap1 complex (Zhang et al., 2004). As expected, the Nrf2 protein level was low in the untreated sample, whereas chemical treatment induced Nrf2 protein levels without significant change in Keap1 levels (Fig. 3A, bottom three panels). Assembly/disassembly of Keap1 with Cul3 is important for the ligase function of the Keap1–Cul3 E3 complex and therefore ubiquitination of Nrf2. Next, the ability of these chemicals in modulating interaction of Keap1 with Cul3 was assessed by immunoprecipitation analysis. To assess the Nrf2–Keap1 interaction, the endogenous Nrf2 and Keap1 proteins were measured using lysates from MDA-MB-231 cells treated with the indicated chemicals. There were still significant amounts of Keap1–associated Nrf2 following treatment with As(III), MMA(III), tBHQ, or SF, indicating that none of the chemicals disrupt the Nrf2–Keap1 complex (Fig. 3A, top two panels), consistent with our previous findings with tBHQ or SF (Zhang et al., 2004). As expected, the Nrf2 protein level was low in the untreated sample, whereas chemical treatment induced Nrf2 protein levels without significant change in Keap1 levels (Fig. 3A, bottom three panels). Assembly/disassembly of Keap1 with Cul3 is important for the ligase function of the Keap1–Cul3 E3 complex and therefore ubiquitination of Nrf2. Next, the ability of these chemicals in modulating interaction of Keap1 with Cul3 was assessed by immunoprecipitation analysis. To assess the Nrf2–Keap1 interaction, the endogenous Nrf2 and Keap1 proteins were measured using lysates from MDA-MB-231 cells treated with the indicated chemicals. There were still significant amounts of Keap1–associated Nrf2 following treatment with As(III), MMA(III), tBHQ, or SF, indicating that none of the chemicals disrupt the Nrf2–Keap1 complex (Fig. 3A, top two panels), consistent with our previous findings with tBHQ or SF (Zhang et al., 2004). As expected, the Nrf2 protein level was low in the untreated sample, whereas chemical treatment induced Nrf2 protein levels without significant change in Keap1 levels (Fig. 3A, bottom three panels). Assembly/disassembly of Keap1 with Cul3 is important for the ligase function of the Keap1–Cul3 E3 complex and therefore ubiquitination of Nrf2. Next, the ability of these chemicals in modulating interaction of Keap1 with Cul3 was assessed by immunoprecipitation analysis. To assess the Nrf2–Keap1 interaction, the endogenous Nrf2 and Keap1 proteins were measured using lysates from MDA-MB-231 cells treated with the indicated chemicals. There were still significant amounts of Keap1–associated Nrf2 following treatment with As(III), MMA(III), tBHQ, or SF, indicating that none of the chemicals disrupt the Nrf2–Keap1 complex (Fig. 3A, top two panels), consistent with our previous findings with tBHQ or SF (Zhang et al., 2004). As expected, the Nrf2 protein level was low in the untreated sample, whereas chemical treatment induced Nrf2 protein levels without significant change in Keap1 levels (Fig. 3A, bottom three panels). Assembly/disassembly of Keap1 with Cul3 is important for the ligase function of the Keap1–Cul3 E3 complex and therefore ubiquitination of Nrf2.
in samples treated with As(III) or MMA(III) (Fig. 3B, top α-HA panel, compare lane 2 with lanes 3 and 4). Equal expression of HA-Cul3 or Keap1-CBD was observed when small aliquots of total lysates were analyzed with anti-HA, anti-CBD, and anti-α-tubulin antibodies (Fig. 3B, bottom three panels). These results suggest that As(III) and MMA(III) inhibit the activity of the Keap1–Cul3 E3 ubiquitin ligase by interfering with the dynamic assembly/disassembly of Keap1 with the Cul3-containing core complex.

As(III)- or MMA(III)-induced activation of Nrf2 is independent of C151 in Keap1

We have reported previously that the cysteine residue (C151) in Keap1 is required for activation of Nrf2 by tBHQ or SF (Zhang and Hannink, 2003). Thus, the necessity of this residue in Nrf2 activation by As(III) and MMA(III) was tested using the ARE-luciferase reporter gene assay system. In the presence of Keap1-WT, Nrf2 activity was substantially enhanced by As(III), MMA(III), tBHQ, and SF (Fig. 4A, black bars). Consistent with our previous report, mutation of C151 in Keap1 blocked activation of Nrf2 by tBHQ or SF (Fig. 4A, compare 1st white bar with last two white bars). Interestingly, this mutation had no effect on As(III)- or MMA(III)-induced activation of Nrf2 (Fig. 4A, 2nd and 3rd white bars). Aliquots of cell lysates were subjected to immunoblot analysis with anti-HA and anti-α-tubulin antibodies for detection of Nrf2 and Keap1. In Keap1-WT cotransfected cells, all treatments increased the steady-state levels of Nrf2 and the protein level of Nrf2 correlated well with the luciferase activity (Fig. 4B, α-HA panel, lanes 1–5). As expected, induction of the Nrf2 protein by tBHQ or SF was blocked in Keap1-C151S cotransfected cells while this mutation had no effect on Nrf2 induction by As(III) or MMA(III) (Fig. 4B, α-HA panel, compare lanes 4 and 5 with lanes 9 and 10; lanes 2 and 3 with lanes 7 and 8).

As demonstrated previously, tBHQ and SF stabilize Nrf2 through inhibition of the Keap1-mediated ubiquitination of Nrf2. To further understand activation of Nrf2 by As(III) and MMA(III), in vivo ubiquitination of Nrf2 was carried out in cells cotransfected with expression vectors for Gal4–Neh2 fusion protein, HA-ubiquitin, and either Keap1-WT or Keap1-C151S. Since Neh2 contains seven lysine residues that have been identified as the ubiquitin-conjugating sites of Nrf2, ubiquitination of the Gal4–Neh2 fusion protein was used as an index of Nrf2 ubiquitination (Zhang et al., 2004). In the presence of Keap1-WT, ubiquitination of Gal4–Neh2 was completely blocked by all the treatments (Fig. 4C, top panel, compare lane 2 with lanes 3–5). Amazingly, As(III) and MMA(III) were still able to block ubiquitination of Nrf2 while tBHQ lost its ability to inhibit ubiquitination of Gal4–Neh2 when Keap1-C151S was cotransfected (Fig. 4C, top panel, lanes 7–10). Equal expression levels of Gal4–Neh2 and Keap1 were shown in the bottom panels.
three panels of Fig. 4C. Collectively, these data consistently demonstrate that activation of Nrf2, by both As(III) and MMA(III), is independent of C151 in Keap1 and implicate that structurally diverse Nrf2 inducers activate Nrf2 through distinct mechanisms.

Discussion

Both As(III) and MMA(III) strongly induced the Nrf2-mediated antioxidant response in the human bladder urothelium, which is consistent with the reported activation of Nrf2 by As(III) in other cell types (Pi et al., 2003; He et al., 2006; Kimura et al., 2006; Massrieh et al., 2006). Activation of the Nrf2 pathway by arsenic is primarily through the prolonged half life of Nrf2, resulting in increased steady-state levels of Nrf2. Further investigation of the detailed actions of As(III) and MMA(III) in upregulating the Nrf2 pathway revealed a distinct mechanism of Nrf2 activation by As(III) and MMA(III). As illustrated, As(III) and MMA(III) had no effect on the interaction of Nrf2 with Keap1, which supports our previous conclusion that tBHQ or SF does not dissociate the Keap1-Nrf2 complex (Zhang et al., 2004). Surprisingly, both As(III) and MMA(III) inhibited the Keap1-dependent E3 ubiquitin ligase by augmenting the association of Keap1 with Cul3, which is in sharp contrast with the enhanced dissociation of Keap1 with Cul3 in response to tBHQ and SF. Keap1, functioning as a substrate adaptor for Nrf2, is constantly undergoing assembly/
Under the redox balanced conditions, Keap1 brings Nrf2 into the Nrf2-activators. Distinct mechanisms of Nrf2 activation by seemingly target genes to elicit speci
turally diverse chemicals activate different subsets of Nrf2 downstream
Keap1 rectly, rather, they affect the cycles of assembly/disassembly of the active E3 ubiquitin ligase, and therefore activation of the Nrf2 pathway. We have shown that mutations in the BTB domain of Keap1 (125A3 or 162A3) increased its affinity for Cul3 and decreased its ability in targeting Nrf2 for ubiquitination and degradation (Zhang et al., 2004). This scenario resembles the currently observed As(III) or MMA(III)-treated condition in which the dynamic cycles of assembly/disassembly are disrupted by the enhanced affinity of Keap1 for Cul3. The stronger association of Keap1 with Cul3 prevents Keap1 from bringing in another Nrf2 mole-
cule into the complex for degradation. In addition, increased association of Keap1 with the core enzyme results in Keap1 self-ubiquitination (Zhang et al., 2004). Together, these findings reveal a model by which both increased and decreased affinity of a substrate adaptor for the Cul-
containing core complex reduce the activity of Cul-containing E3 ubiquitin ligases. How arsenic enhances the binding of Keap1 to the Cul3–
Rbx1 complex requires further investigation.

In this report, we provide strong evidence that As(III) and MMA(III) are able to activate the Nrf2-mediated antioxidative response in human bladder urothelial cells, indicating that activation of Nrf2 by arsenic is not limited to a particular cell type. More significantly, we provide evidence that As(III) and MMA(III) activate the Nrf2 pathway through a novel mechanism, as demonstrated by the striking finding that induction of Nrf2 by As(III) and MMA(III) is independent of C151 in Keap1 while this cysteine residue is required for activation of Nrf2 by tBHQ and SF. It is likely that tBHQ and SF may directly act on the thiol groups of Keap1 with Cul3 prevents Keap1 from bringing in another Nrf2 mole-
cule into the complex for degradation. In addition, increased association of Keap1 with the core enzyme results in Keap1 self-ubiquitination (Zhang et al., 2004). Together, these findings reveal a model by which both increased and decreased affinity of a substrate adaptor for the Cul-
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Acknowledgments

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