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## Arsenic-Mediated Activation of the Nrf2-Keap1 Antioxidant Pathway

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### Abstract

Arsenic is present in the environment and has become a worldwide health concern due to its toxicity and carcinogenicity. However, the specific mechanism(s) by which arsenic elicits its toxic effects has yet to be fully elucidated. The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) has been recognized as the master regulator of a cellular defense mechanism against toxic insults. This review highlights studies demonstrating that arsenic activates the Nrf2-Keap1 antioxidant pathway by a distinct mechanism from that of natural compounds such as sulforaphane (SF) found in broccoli sprouts or tert-butylhydroquinone (tBHQ), a natural antioxidant commonly used as a food preservative. Evidence also suggests that arsenic prolongs Nrf2 activation and may mimic constitutive activation of Nrf2, which has been found in several human cancers due to disruption of the Nrf2-Keap1 axis. The current literature strongly suggests that activation of Nrf2 by arsenic potentially contributes to, rather than protects against, arsenic toxicity and carcinogenicity. The mechanism(s) by which known Nrf2 activators, such as the natural chemopreventive compounds SF and lipoic acid, protect against the deleterious effects caused by arsenic will also be discussed. These findings will provide insight to further understand how arsenic promotes a prolonged Nrf2 response, which will lead to the identification of novel molecular markers and development of rational therapies for the prevention or intervention of arsenic-induced diseases. The National Institute of Environmental Health Science (NIEHS) Outstanding New Environmental Scientist (ONES) award has provided the opportunity to review the progress both in the fields of arsenic toxicology and Nrf2 biology. Much of the funding has led to (1) the novel discovery that arsenic activates the Nrf2 pathway by a mechanism different to that of other Nrf2 activators, such as sulforaphane and tert-butylhydroquinone, (2) activation of Nrf2 by chemopreventive compounds protects against arsenic toxicity and carcinogenicity both in vitro and in vivo, (3) constitutive activation of Nrf2 by disrupting Keap1-mediated negative regulation contributes to cancer and chemoresistance, (4) p62-mediated sequestration of Keap1 activates the Nrf2 pathway, and (5) arsenic-mediated Nrf2 activation may be through a p62-dependent mechanism. All of these findings have been published and are discussed in this review. This award has laid the foundation for my laboratory to further investigate the molecular mechanism(s) that regulate the Nrf2 pathway and how it may play an integral role in arsenic toxicity. Moreover, understanding the biology behind arsenic toxicity and carcinogenicity will help in the discovery of potential strategies to prevent or control arsenic-mediated adverse effects.

### Keywords

Nrf2; Arsenic; Keap1; Oxidative stress; p62; Autophagy; Chemoprevention

## INTRODUCTION TO ARSENIC

Arsenic is a naturally occurring metalloid that exists in practically all environmental media, such as air, soil, and water. Mostly, it exists in two oxidative forms, trivalent arsenite (As(III)) and pentavalent arsenate (As(V)) [1]. Millions of people worldwide are exposed to arsenic by drinking contaminated water and inhalation of particulate matter [2, 3]. Arsenic is associated with a wide variety of adverse effects, such as skin lesions, peripheral vascular diseases, reproductive toxicity, and neurological effects [3]. In addition, several epidemiological studies have correlated arsenic exposure to various human malignancies in the skin, lung, urinary bladder, liver, and kidney [4]. Within the Past two decades, the World Health Organization (WHO), as well as the United States Environmental Protection Agency (EPA), reduced the allowable arsenic concentration in drinking water from 50 ppb to 10 ppb (WHO [5], 1993 and EPA [6], 2001). However, owing to the toxicity of arsenic, arsenic trioxide (ATO) is currently being used as a cancer chemotherapeutic for the treatment of a variety of human cancers, predominantly acute promyelocytic leukemia [7,8].

Arsenic can undergo a series of methylations and oxidative reductions to generate a number of metabolites, including monomethylarsonous acid (MMA(III)), monomethylarsonic acid (MMA(V)), dimethylarsinous acid (DMA(III)), and dimethylarsinic acid (DMA(V)), that are excreted from the bladder, making the bladder the major target organ that is susceptible to the toxic effects of arsenic [9]. Arsenic has also been shown to have multiple biological effects, including alterations in signal transduction pathways, damage to DNA, and inhibition of its repair, induction of apoptotic cell death, and effects on global DNA methylation [7]. Several studies have demonstrated that arsenic exposure results in the generation of reactive oxygen species (ROS) in various cellular systems. Moreover, addition of inhibitors of oxidative stress, such as catalase, superoxide dismutase or glutathione peroxidase, or antioxidants, such as glutathione or vitamin E, decreases the toxic effects caused by arsenic [3,10–13]. Therefore, the cytotoxic and genotoxic effects of arsenic are also attributed to its ability to be a potent inducer of ROS; however, the exact mechanism(s) by which arsenic causes its harmful effects are still under investigation.

## THE Nrf2-Keap1 PATHWAY

Nrf2 is a transcription factor that is activated in response to oxidative stress. Under unstressed conditions, Nrf2 is maintained at very low levels by its negative regulator, Keap1 [Kelch-like ECH associated protein 1], which forms an E3 ubiquitin ligase complex with Cullin 3 (Cul3) and Ring-box 1 (Rbx1) and facilitates the ubiquitination of Nrf2 [14, 15]. Subsequently, Nrf2 is targeted for degradation by the 26S proteasome. When cells are exposed to stress or electrophilic compounds, pivotal cysteine residues (C273, C288, and C151) in Keap1 act as “sensors” and are S-alkylated [16–18]. It is hypothesized that modification of the critical cysteine residues in Keap1 causes a conformational change in the Keap1-Cul3-Rbx1 E3 ubiquitin ligase complex, hindering ubiquitination of Nrf2 [19]. Subsequently, Nrf2 accumulates and translocates to the nucleus, dimerizes with a small Maf protein, and binds to the antioxidant response element in the promoter region of cytoprotective genes that are responsible for the detoxification and elimination of harmful substances, including arsenic. These genes include intracellular redox-balancing proteins (e.g., heme oxygenase-1 (HO-1) and thioredoxin reductase-1 (TrxR1)), phase I and II detoxication enzymes (e.g., NAD(P)H quinone oxidoreductase-1 (NQO1), glutathione S-transferase (GST), glutamate cysteine ligase catalytic subunit, and regulatory subunit (GCLM)), xenobiotic transporters (multidrug resistance-associated proteins (MRPs)), and other stress response proteins [20–22].

## ARSENIC ACTIVATES Nrf2 THROUGH A DISTINCT MECHANISM

Arsenicals have been shown to activate the Nrf2-Keap1 pathway in a variety of human cell lines including osteoblasts (MC3T3-E1) [23], keratinocytes [24], placental choriocarcinoma cells [25], HeLa [26], myeloma cells [27], bladder epithelial cells (UROtsa) [28], and breast cancer cells (MDA-MB-231) [29]. In 2008, studies conducted in our laboratory demonstrated that arsenic activates Nrf2 through a different mechanism than that of SF and tBHQ. SF- and tBHQ-mediated activation of Nrf2 is dependent upon modification of the cysteine 151 sensor in Keap1 (Keap1-C151), also known as the canonical mechanism of Nrf2 activation. However, As(III) and MMA(III) activate Nrf2 through a Keap1-C151 independent mechanism [29–31]. Recently, our group, along with three other laboratories, independently demonstrated that p62, a selective substrate adaptor protein that plays a critical role in autophagy (a bulk-lysosomal degradation pathway), directly binds to Keap1 [32–35]. Overexpression of p62 or an accumulation of p62 due to dysregulation of autophagy resulted in the sequestration of Keap1 in the autophagosomes and hindrance of the Keap1-Cul3 E3 ubiquitin ligase complex to properly ubiquitinate Nrf2 [32–35]. Furthermore, Aono et al. demonstrated that osteoblasts treated with arsenic activated Nrf2-dependent transcription of target genes, including HO-1, peroxiredoxin 1 (Prx1), and p62 [23]. An accumulation of p62 and ubiquitin-conjugated proteins was also observed [23]. p62 has also recently been confirmed to be a downstream target gene of Nrf2, creating a positive feedback loop [35]. Taken together, these studies highly suggest that autophagy and p62 may play a critical role in arsenic-mediated Nrf2 activation. Further studies are required to determine whether this p62-dependent, or noncanonical, mechanism of Nrf2 activation is the mechanism by which arsenic activates the Nrf2 pathway.

## THE “DARK SIDE” OF Nrf2

Nrf2 is also beneficial for cancer cells, providing an environment conducive for cell growth and protection against oxidative stress and chemotherapeutic agents [36]. Constitutive activation of Nrf2 due to somatic mutations in Keap1 or Nrf2 that disrupt Keap1-mediated Nrf2 regulation is prominent in several types of human cancer cell lines and tumors [37–40]. More specifically, mutations in Nrf2 have been found in lung, head/neck, esophagus, skin, and larynx cancers [41, 42]. Keap1 gene mutations were initially identified in lung cancer cell lines [43] and, thereafter, several reports have identified Keap1 mutations in breast cancer [44], gall-bladder cancer [45], prostate cancer [46], and many nonsmall cell lung cancer cell lines and tumors [40]. Moreover, several studies have demonstrated a correlation between high Nrf2 protein levels in cancer cells and chemoresistance [36,46–49]. For example, the lung cancer cell line, A549, contains a mutation in the Nrf2-binding domain in Keap1 (Kelch domain) that abolishes Keap1-mediated regulation of Nrf2. As a result, A549 cells have constitutively active Nrf2 and are resistant to a variety of chemotherapeutic agents, such as cisplatin, doxorubicin, and etoposide [36, 40]. A549 cells, as well as other cancer cells, can be sensitized to chemotherapeutic-induced apoptosis through knockdown or inhibition of Nrf2 [36,50].

In addition to somatic mutations of Keap1, epigenetic mechanisms and loss of heterozygosity of Keap1 have also been found to upregulate Nrf2 in different types of cancers due to reduced levels of Keap1 [37, 47, 51]. A comprehensive genetic and epigenetic analysis of the Keap1 gene in 47 nonsmall cell lung cancer tissues and specimens was performed. Interestingly, 22 of 47 of the tumor tissue were found to be methylated at the Keap1 promoter region, which was not observed in any of the normal samples and 10 of 47 had loss of heterozygosity [51].

## Nrf2: THE CULPRIT IN ARSENIC TOXICITY?

Uncovering the dual roles of Nrf2 in cancer has raised safety concerns with respect to the strategy of using natural compounds to activate Nrf2 for chemoprevention. Several studies, however, have shown that some Nrf2 chemopreventive compounds have short biological half-lives and that their ability to induce Nrf2 downstream genes ranges from hours to days [52]. In addition, although the activation of the Nrf2 pathway by these compounds is pronounced, it is transient and, therefore, intermittent dosing is suggested for chemopreventive use [53,54]. On the other hand, there is evidence suggesting that arsenic-mediated activation of Nrf2 is similar to genetic disruptions found in cancer cells, causing elevated and prolonged activation of the pathway. Human liver hepatocellular carcinoma (HepG2) cells exposed to 10  $\mu$ M inorganic arsenic not only caused persistent induction of HO-1 but also prolonged Nrf2 activation for up to 60 h [55]. When keratinocytes were exposed to 100 nM arsenic for 28 weeks, Nrf2 basal activity was higher than control cells [56]. However, there is some controversy as to whether Nrf2 protein levels elevate as a protective mechanism in response to arsenic-induced ROS, or persistent activation of Nrf2 is promoting the transformation of cells.

## CHEMOPREVENTIVE Nrf2 ACTIVATORS PROTECT AGAINST ARSENIC TOXICITY

Our laboratory has demonstrated the importance of Nrf2 against arsenic toxicity both *in vitro* and *in vivo*. Mouse embryonic fibroblasts (MEF) from Nrf2 wild-type mice were shown to be less susceptible to arsenic-induced toxicity compared to MEF cells from Nrf2 null mice [28]. *In vivo*, Nrf2 knockout mice exposed to drinking water containing 1, 10, or 100 ppm sodium arsenite for 6 weeks displayed more severe pathological changes in the bladder, liver, and lung compared to Nrf2 wild-type mice [57]. Furthermore, activation of Nrf2 by SF or tBHQ was shown to protect human bladder UROtsa cells from both arsenite and monomethylarsonous acid (MMA<sup>III</sup>) toxicity [28]. Recently, we demonstrated that SF-mediated activation of Nrf2 protects against arsenic-mediated inflammation using a whole body arsenic-inhalation model in Nrf2 wild-type mice [58]. The SF effects, however, were abrogated in Nrf2 knockout mice [58]. The concentrations of arsenic used in these studies are environmentally and biologically relevant. Our laboratory has also shown that Nrf2 is activated by tBHQ, as well as natural compounds, such as oridonin and cinnamaldehyde in several different cell lines. These compounds also protect against arsenic-induced toxicity [28, 59–62]. Lipoic acid, a thiol-compound that is a strong antioxidant, for example, induces Nrf2 in cells and protects against ATO-induced autophagic cell death in human glioma cells [63] and protects HepG2 cells from arsenic exposure [59]. Interestingly, a study done by Shinkai et al. showed that pretreatment of mouse hepatocytes with SF not only decreases arsenic toxicity but also inhibits accumulation of arsenic in the cells due to upregulation of  $\gamma$ -GCS, GST isoforms, and MRP1, all of which are important for the excretion of arsenic into the extracellular space [60]. These findings may suggest a possible mechanism by which the effects of SF could predominate over those of arsenic.

There are a few important differences to note among these studies. First, the concentrations of arsenic and/or chemopreventive compounds varied from the nanomolar to micromolar range and, second, the duration of the exposure to arsenic and/or chemopreventive compounds also varied, ranging from 2 to 48 h. Further studies are needed to determine whether dose and time of exposure by arsenic and/or chemopreventive compounds are determinants of Nrf2 activation being beneficial or detrimental to cell health and survival. Aside from the aforementioned differences, these studies seem to support the notion that activation of Nrf2 by the so-called beneficial compounds, such as tBHQ and SF, is through a Keap1-C151-dependent mechanism and is distinct from the p62-dependent mechanism that

has been identified for arsenic. These studies suggest that activation through the Keap1-C151-dependent mechanism may elicit a positive chemopreventive Nrf2 response, whereas the p62-dependent mechanism may mimic the constitutive activation observed in certain cancers, deemed the dark side of Nrf2.

## THE ROLE OF Nrf2 WHEN ARSENIC IS USED AS A CANCER THERAPEUTIC

Not only is arsenic present in the environment, but the metalloid, in the form of ATO, is also used in the treatment of several human malignancies (for a full review refer to [64]). ATO has also been shown to induce Nrf2 and its downstream cytoprotective genes, NQO1 and HO-1, in human oral squamous cell lines, multiple myeloma cell lines, rat cardiac myocytes, and liver epithelial cells [27, 65, 66]. cDNA microarray analysis revealed that Nrf2, along with HO-1, GCLM, NQO1, epoxide hydrolase 1, and thioredoxin reductase, were elevated in an ATO-resistant ovarian cancer cell line when compared to parental cells, which have low Nrf2 protein levels and are not resistant to ATO [67]. The cells resistant to ATO had continuous cancer cell growth, cell survival, tumor metastasis, and aggressiveness and were also resistant to cisplatin and paclitaxel [67]. In another study, microarray analysis of 59 cell lines from the NCI-60 tumor cell line panel that were resistant to ATO also revealed an enrichment of Nrf2 mRNA [68]. Supporting a role for Nrf2 in chemoresistance, knockdown of Nrf2 by shRNA was shown to sensitize A549 cells to ATO [68]. In addition, Nrf2 knockdown in glioma cells potentiated ATO-induced oxidative damage and cell death [69]. Morales et al. also demonstrated that ATO induces Nrf2 in multiple myeloma cell lines [70]. In the same study, inhibition of ATO-induced ROS with butylated hydroxyanisole did not affect Nrf2 activation or cell death, demonstrating that ATO-mediated induction of Nrf2 or cell death is not mediated through ROS [70]. More work is needed to determine the specific mechanisms of how ATO activates Nrf2 and how cancer cells become resistant to ATO. However, the results of these studies on ATO highly suggest that Nrf2 protects cells from the cytotoxic effects of chemotherapeutic arsenic. Therefore, inhibition of Nrf2 may sensitize cancer cells to chemotherapeutics, including ATO, and induce cell death. Taken together, the evidence supports the notion that arsenic-mediated activation of Nrf2 not only may cause toxicity and promote carcinogenicity but also contribute to chemoresistance.

## THE FUTURE OF ARSENIC AND Nrf2-Keap1 RESEARCH

Although much progress has been made in elucidating the role of Nrf2 in arsenic exposure within the past decade, a great deal still remains unknown. It is clear that arsenicals at environmentally relevant doses induce the Nrf2-Keap1 pathway as supported by much of the current literature. However, whether arsenic-mediated activation of Nrf2 protects or contributes to arsenic toxicity and carcinogenicity has not yet been clarified. Both long-term in vitro and in vivo arsenic studies are needed to determine whether arsenic-mediated autophagy and/or prolonged Nrf2 activation contributes to arsenic toxicity and carcinogenicity.

Our studies demonstrate that arsenic activates the Nrf2-Keap1 antioxidant pathway by a distinct mechanism from that of natural compounds such as SF or tBHQ. As shown in Figure 1, SF and tBHQ attenuate the deleterious effects of arsenic by activating the canonical Keap1-C151 Nrf2 pathway. This mechanism is dynamically regulated and provides intermittent increases in Nrf2. On the other hand, the mechanism by which arsenic activates Nrf2 is Keap1-C151 independent; instead, arsenic may activate Nrf2 through a p62-dependent mechanism. Unlike the Keap1-C151 dependent mechanism, this pathway results in prolonged activation of Nrf2.

Further investigation is required to determine whether p62 is indeed the dominant arsenic-mediated mechanism and whether this arsenic activation in fact mimics the dark side of Nrf2, as found in chemoresistant human cancer cell lines and tumors. Furthermore, there is a lack of sufficient evidence to suggest that the differentiation between the protective and the dark side effects of Nrf2 activators are determined by the mechanism of activation. Additional research may also reveal whether activation by different Nrf2 inducers may result in upregulation of differential downstream genes. If the two differentiated modes of Nrf2 activation determine whether or not Nrf2 is protective or harmful, then canonical Nrf2 activators (Keap1-C151 dependent) have the opportunity to be developed into therapeutics for the prevention or intervention of arsenic toxicity.

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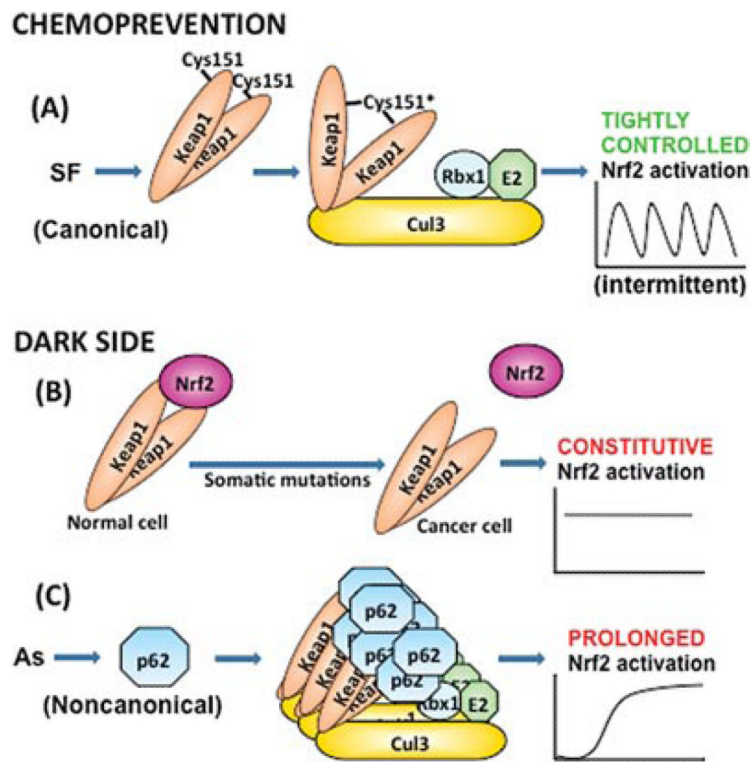
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**FIGURE 1.** The chemopreventive and dark side of Nrf2. (A) Nrf2 activation through the Keap1-C151 canonical pathway by chemopreventive compounds, such as sulforaphane (SF), is intermittent. (B) Somatic mutations in pro-carNrf2 or Keap1 found in human cancers and tumors have constitutive Nrf2 activation. (C) Arsenic-mediated Nrf2 activation is prolonged due to p2-Keap1 sequestration in autophagosomes.