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# Essential role of Nrf2 in protection against hydroquinone- and benzoquinone-induced cytotoxicity

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ABSTRACT

Benzene is a well-established human carcinogen. Benzene metabolites hydroquinone (HQ) and benzoquinone (BQ) are highly reactive molecules capable of producing reactive oxygen species and causing oxidative stress. In this study, we investigated the role of the Nrf2, a key nuclear transcription factor that regulates antioxidant response element (ARE)-containing genes, in defense against HQ- and BQ-induced cytotoxicity in cultured human lung epithelial cells (Beas-2B). When the cells were exposed to HQ or BQ the activity of an ARE reporter was induced in a dose-dependent manner, meanwhile Nrf2 protein levels were elevated and accumulated in the nucleus. Increased expression of well-known Nrf2-dependent proteins including NQO1, GCLM, GSS and HMOX was also observed in the HQ/BQ-treated cells. Moreover, transient overexpression of Nrf2 conferred protection against HQ- and BQ-induced cell death, whereas knockdown of Nrf2 by small interfering RNA resulted in increased apoptosis. We also found that the increased susceptibility of Nrf2-knockdown cells to HQ and BQ was associated with reduced glutathione levels and loss of inducibility of ARE-driven genes, suggesting that deficiency of Nrf2 impairs cellular redox capacity to counteract oxidative damage. Altogether, these results suggest that Nrf2-ARE pathway is essential for protection against HQ- and BQ-induced toxicity.



## 1. Introduction

Air pollution is a worldwide problem and has become a major environmental health issue (Chen et al., 2007; WHO, 2005). Air pollution is defined as a mixture of particulate matter (PM) and gaseous chemicals consisting primarily of nitrogen oxides ($NO_X$), carbon monoxide (CO), sulfur dioxide ($SO_2$), ozone ($O_3$), and volatile organic compounds (VOCs) (Ferm et al., 2006; Han and Naeher, 2006). In recent years, due to increasing emissions from outdoor (Riediker et al., 2003; Rodolfo Sosa et al., 2009) and indoor sources (Carrer et al., 2000), and individual activities (e.g. smoking) (Serrano-Trespalacios et al., 2004), populations in large urban areas are exposed to high levels of VOCs (Tovalin et al., 2006), predominantly, monocyclic aromatic hydrocarbons, in particular benzene, toluene, ethylbenzene, and isomers of xylene (*m*-, *o*-, *p*-xylene) (Tovalin-Ahumada and Whitehead, 2007; Tovalin et al., 2006). Exposure to benzene has been associated with aplastic anemia, leukemia and lymphoma (Snyder, 2002; Yin et al., 1996). Besides its oncogenic effect on hematopoietic tissue, recent studies have correlated benzene exposure with tumor formation in human (Yin et al., 1996) and animal (Maltoni et al., 1989; Snyder et al., 1988) lungs, indicating that the lung is also a target of benzene-induced toxicity. Benzene toxicity is attributed to its metabolism, mainly in the liver (Koop et al., 1989; Nedelcheva et al., 1999; Ross, 2000; Snyder et al., 1989) and probably in the lungs (Powley and Carlson, 2000, 2001, 2002; Sheets et al., 2004), which leads to the formation of reactive metabolites hydroquinone (1,4-benzenediol or 1,4-hydroquinone; HQ) and its oxidized form benzoquinone

*Abbreviations:* ARE, antioxidant response element; BQ, benzoquinone; CO, carbon monoxide; ED50, effective dose 50; GCLM, glutamate cysteine ligase modifier subunit; GSH, glutathione; GSS, glutathione synthetase; GSTs, glutathione-S-transferases; HMOX1, heme oxygenase 1; $H_2O_2$, hydrogen peroxide; HQ, hydroquinone; $OH^{\cdot}$, hydroxyl radical; Keap1, Kelch-like ECH-associated protein 1; Maf, musculoaponeurotic fibrosarcoma oncogene; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; NQO1, NAD(P)H dehydrogenase quinone 1; $NO_X$, nitrogen oxides; Nrf2, nuclear factor (erythroid-derived 2)-like 2; $O_3$, ozone; ROS, reactive oxygen species; siRNA, short interference RNA; $SO_2$, sulfur dioxide; $O_2^-$, superoxide; UGTs, UDP-glucuronosyltransferases; VOCs, volatile organic compounds.

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(1,4-benzoquinone or *p*-benzoquinone; BQ). HQ and BQ are highly reactive molecules and, through redox cycling, they produce reactive oxygen species (ROS) (Bolton et al., 2000), including superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$), nitric oxide (NO) and ultimately hydroxyl radical ($OH^\cdot$), resulting in oxidative stress (Luo et al., 2008; Snyder and Hedli, 1996) and oxidative damage to DNA (Abernethy et al., 2004; Luo et al., 2008), proteins, and lipids (Gut et al., 1996; Winn, 2003). Moreover, addition of antioxidant enzymes (e.g. catalase) and *N*-acetyl cysteine, a glutathione precursor (GSH), has been shown to block oxidative damage induced by these metabolites (Barreto et al., 2009; Ruiz-Ramos et al., 2005) confirming the role of ROS production and oxidative stress in HQ and BQ cytotoxicity.

To counteract damage induced by oxidative stress, cells have developed an adaptive defense mechanism that leads to rapid and efficient induction of detoxifying enzymes (phase II enzymes) and antioxidants (Kang et al., 2005). Induction of these molecules is through a *cis*-acting element in the promoter region known as the antioxidant response element (ARE) (Lee et al., 2005). The nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a basic leucine zipper member of the cap 'n' collar family of transcription factors (Shen et al., 2004), is the principal regulator of the ARE-driven cellular defense system. Under homeostatic conditions, Nrf2 is present in the cytoplasm attaching to an actin-binding protein named Kelch-like ECH-associated protein 1 (Keap1) (Lee et al., 2007). Keap1 functions as a suppressor of Nrf2 by retaining it in the cytosol and enhancing its proteasomal degradation (Lo and Hannink, 2006). Exposure to electrophiles and ROS uncouples the Nrf2–Keap1 complex, leading to the release of Nrf2 and its nuclear translocation where it dimerizes with other transcription factors such as members of the small Maf (musculoaponeurotic fibrosarcoma oncogene) family (Motohashi et al., 2004). Binding of these heterodimers to ARE enables transcriptional activation of many target genes including those encoding antioxidants (e.g. GSH), drug-metabolizing enzymes (Phase I and Phase II), drug-efflux pumps (Phase III), 26S proteasome subunits, heat shock proteins, growth factors, and transcription factors (Hayes and McMahon, 2009; Itoh et al., 1997; Owuor and Kong 2002). The up-regulation of these genes promotes cell survival and protection against oxidative damage (Lee et al., 2004; Li et al., 2005).

It has been demonstrated that the Nrf2-dependent adaptive response provides a pivotal defense mechanism against environmental hazards, including various air pollutants (reviewed in (Osburn and Kensler, 2008) and (Rubio et al., 2010)). In this study, we have explored the role of Nrf2 in protection against benzene metabolites HQ and BQ in human lung cells. Our results demonstrate that these metabolites are able to induce ARE-driven gene expression through the activation of Nrf2. However, knockdown of Nrf2 greatly enhances HQ- and BQ-induced cytotoxicity and cell death, and the increased susceptibility of the Nrf2-knockdown cells is associated with reduced levels of GSH and loss of induction of ARE-driven genes, suggesting that Nrf2 is essential for the survival of lung cells against the toxic effects of these benzene metabolites.

## 2. Materials and methods

### 2.1. Chemicals and cell culture

All chemicals used in this study were purchased from Sigma–Aldrich (St. Louis, MO). Human bronchial epithelial cells (Beas-2B) were obtained from American Tissue Culture Collection (ATCC, Rockville MD). Beas-2B cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, South Logan UT) supplemented with 10% fetal bovine serum (FBS, Invitrogen Corporation, Carlsbad CA) and antibiotics (100 U penicillin/ml and 100 μg streptomycin/ml (Invitrogen Corporation, Carlsbad CA) at 37 °C in a 5% $CO_2$ incubator.

### 2.2. Plasmids and transient transfections

The plasmids pcDNA-Nrf2-V5 (referred as Nrf2-V5) (Jain et al., 2005) and pGL2B-NQO1-ARE-LUC (referred as ARE-LUC) (Dhakshinamoorthy and Jaiswal 2000) were a kind gift from Dr. Anil Jaiswal (University of Maryland). Transient transfection of Beas-2B cells were carried out using Lipofectamine 2000 following the manufacturer's instructions (Invitrogen Corporation, Carlsbad CA). Briefly, the cells were seeded in 6-well plates at a density of $3 \times 10^5$ cells/well (>90% confluence). Twenty-four hours after plating, cells were transfected with 4 μg of either the above plasmids or the pcDNA 3.1 empty vector diluted in Opti-MEM media (Invitrogen Corporation, Carlsbad CA).

### 2.3. siRNA transfection

Control siRNA (5′-UAACGACGCGACGACGUAATT-3′ and 5′-UUACGUCGUCGCGUCGUUATT-3′) and siRNA targeting human Nrf2 siRNA (Lee et al., 2008) (5′-GCUUUUGGCGCAGACAUUCTT-3′ and 5′-GAAUGUCUGCGCCAAAAGCTG-3′) were obtained from Ambion Inc. (Austin, TX). Beas-2B cells were transiently transfected with 100 nM of control siRNA or Nrf2 siRNA mixed with DharmaFect1 Transfection Reagent (Dharmacon, Lafayette, CO) according to the manufacturer's protocol.

### 2.4. Luciferase assay

Twenty-four hours after transfection with ARE-LUC, the cells were seeded in 96-well plates at a density of $1 \times 10^4$ cells/well and treated with conditions as elsewhere indicated. After the treatment, luciferase activity was determined using the Bright-Glo Luciferase Assay System (Promega Corporation, Madison WI) according to the manufacturer's instructions. Luminescence was recorded using a FLUOstar Optima plate reader (BMG Labtech Inc., Cary, NC).

### 2.5. Nuclear extraction

Nuclear extracts were prepared using the NE-PER system (Pierce Chemical Co., Rockford IL) following the manufacturer's recommendations. Briefly, $3 \times 10^5$ control and treated cells were harvested and suspended in 100 μl of the cytoplasmic extraction reagent I (CER I) and incubated on ice for 10 min. The cytoplasmic extraction reagent II (CER II, 5 μL) was then added, vortexed for 5 s, incubated on ice for 1 min and centrifuged (16,000×*g*) at 4 °C for 5 min. The pellet was suspended in 50 μl of nuclear extraction reagent (NER) and incubated on ice for 40 min, vortexing for 15 s every 10 min. After incubation, the sample was centrifuged at 4 °C (16,000×*g*) for 5 min and the supernatant was collected and frozen at −80 °C until further use.

### 2.6. Cell viability

Cell viability was assessed by the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)-based assay following the manufacturer's instructions (Promega Corporation, Madison, WI).

### 2.7. Flow cytometry

Analysis of different stages of apoptosis was performed using Annexin-V/PI and Annexin-V/7-AAD staining kits (BD Pharmigen, San Jose, CA) following manufacturer's protocol, and analyzed by