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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 3×10^5 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 1×10^4 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, 3×10^5 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

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flow cytometry (BD LSR II cytometer). Briefly, 2×10^5 HQ or BQ-treated cells were harvested, washed with PBS, stained with either Annexin-V/PI or Annexin-V/7-AAD for 15 min at room temperature and analyzed by flow cytometry.

2.8. GSH measurement

GSH was assessed using the intracellular thiol probe ThiolTracker Violet (Invitrogen Corporation, Carlsbad CA) following the manufacturer's instructions. Briefly, Beas-2B cells were treated with various concentrations of either HQ or BQ in serum-free media for 2 h, washed twice with PBS, and stained with ThiolTracker Violet for 30 min at 37 °C. Then, the dye was replaced with pre-warmed PBS and fluorescence intensity (410 nm excitation/520 nm emission) was measured using a FLUOstar Optima plate reader (BMG Labtech Inc., Cary, NC).

2.9. Western blot

Cell lysates were prepared in lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with protease inhibitor cocktail (Thermo Fisher Scientific Inc., Rockford, IL) followed by centrifugation at 16,000×*g* for 10 min at 4 °C. Total protein was quantified using a bicinchoninic acid kit (Thermo Fisher Scientific Inc., Rockford, IL). Equal amounts of protein were loaded in a 4–20% gradient polyacrylamide gel (Invitrogen Corporation, Carlsbad, CA) and transferred to a PVDF membrane. The membrane was blocked with 5% non-fat milk solution and sequentially incubated with primary antibody and enzyme-conjugated secondary antibody. The bands were visualized using the chemiluminescence method according to the manufacturer's guidelines (GE Healthcare, Pittsburgh, PA). Antibodies used were purchased from the following suppliers: Nrf2, HMOX1, GSS, NQO1, GCLM and lamin A from Santa Cruz Biotechnology (Santa Cruz, Biotechnology Inc., Santa Cruz, CA); β-actin and GAPDH from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO); and anti-rabbit, anti-goat and anti-mouse IgG peroxidase linked whole antibody from GE Healthcare (GE Healthcare, Waukesha, WI).

3. Results

3.1. Induction of ARE-driven gene expression and Nrf2 activation by HQ and BQ

To examine the capability of HQ and BQ in the induction of ARE-driven gene expression, human bronchial epithelial cells, Beas-2B, were transiently transfected with a luciferase reporter plasmid driven by the NQO1 gene ARE (referred as ARE-LUC) (Dhakshinamoorthy and Jaiswal, 2000). The cells were exposed to various concentrations (5–20 μM) of HQ or BQ for 16 h and assayed for luciferase activity. These doses were found to produce mild to moderate stress to the cells without causing appreciable cytotoxicity that may interfere with the reporter gene assay (Crisman et al., 2007; Fan and Wood, 2007). Both HQ and BQ treatments resulted in a dose-dependent induction of ARE-driven luciferase activity (Fig. 1A). When used at a lower concentration (5 μM), HQ seemed to be a stronger ARE inducer than BQ. However, when a higher concentration (20 μM) was used, they induced luciferase activities at a comparable rate (~2.5-fold). We then evaluated if the observed ARE induction could correlate with the activation of the Nrf2

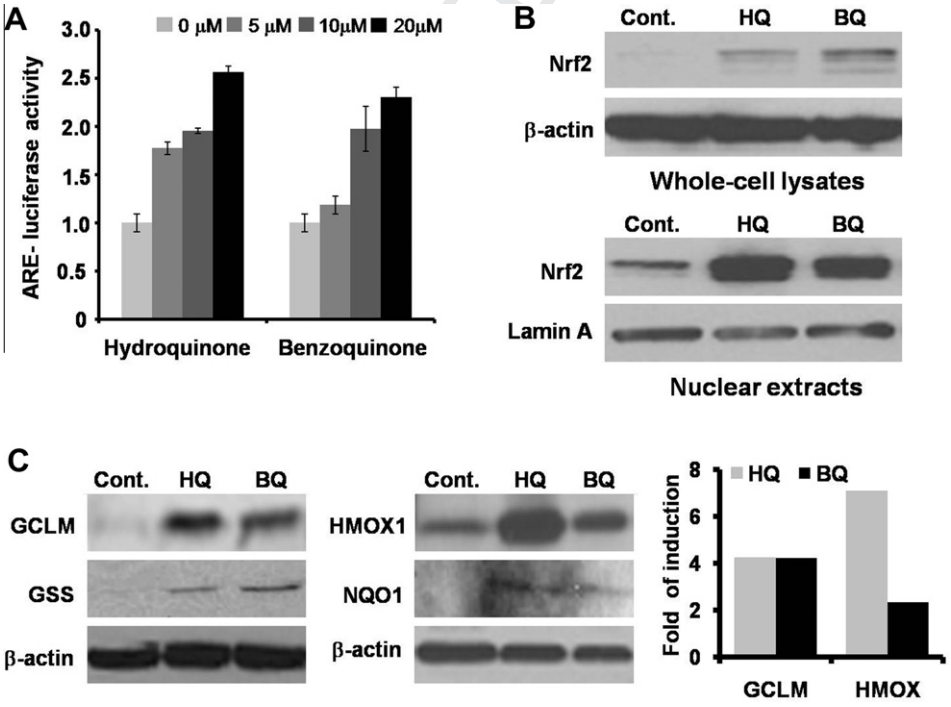

Fig. 1. Induction of ARE-driven gene expression and activation of Nrf2 by HQ and BQ. (A) Beas-2B cells were transfected with the ARE-LUC reporter vector. Forty-eight hours after transfection, the cells were treated with various concentrations of HQ or BQ for ~16 h and luciferase activity was measured. Data are expressed as fold of induction of luciferase activity compared to vehicle-control (dimethyl sulfoxide) (mean ± SD; $n = 3$). (B) Exposure to either HQ or BQ increases Nrf2 protein expression and causes Nrf2 nuclear accumulation. Beas-2B cells were treated with 20 μM of HQ or 10 μM of BQ for 4 h. Nuclear extracts and whole-cell lysates were prepared as described in Section 2. β-actin and lamin A antibodies were used as loading controls for whole-cell lysates and nuclear extracts respectively. (C) Up-regulation of Nrf2-dependent enzymes. Western blot analysis of Beas-2B cells treated with up to 20 μM of HQ or 10 μM of BQ for 16 h. β-actin antibody was used as loading control. Quantification of band intensity was performed by ImageJ version 1.42q software (developed by the National Institute of Health) (right panel) and expressed as percentage of induction compare to vehicle-treated cells (control). Data are representative of three independent experiments with similar results. GCLM, glutamate cysteine ligase modifier subunit; GSS, glutathione synthetase; HMOX1, heme oxygenase 1; NQO1, NAD(P)H dehydrogenase quinone 1.

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pathway. A rapid elevation of Nrf2 protein was detected in whole-cell lysates prepared after a short term treatment (4 h) with either HQ or BQ (Fig. 1B). Since Nrf2 nuclear translocation is a key event in the activation of this pathway, we examined Nrf2 nuclear accumulation in either HQ or BQ treated Beas-2B cells. Western blot analysis showed that HQ and BQ led to Nrf2 accumulation in nuclear extracts after 4 h treatment (Fig. 1B). These data indicate that Nrf2 is rapidly stabilized and mobilized to the nucleus in response to HQ and BQ treatments. Next, to determine the effect of Nrf2 activation on gene expression, levels of several well-known Nrf2 target proteins were analyzed. These included GSH synthesis enzymes glutamate cysteine ligase modifier subunit (GCLM) and glutathione synthetase (GSS), the drug-metabolizing enzyme NAD(P)H dehydrogenase quinone 1 (NQO1), and the antioxidant enzyme heme oxygenase 1 (HMOX1). As shown in Fig. 1C, induction of these proteins was confirmed in cells exposed to either HQ or BQ for 16 h. Levels of HMOX and GCLM were markedly increased by both compounds, whereas expression of GSS and NQO1 only became detectable after treatment with either HQ or BQ. Noteworthy, HQ treatment caused a greater accumulation of HMOX1 (7-fold induction) as compared to the BQ treatment (2-fold). These results demonstrate that HQ and BQ activate Nrf2 by triggering its nuclear translocation and protein accumulation leading to the transcriptional up-regulation of ARE-driven genes in Beas-2B cells.

3.2. Protection against HQ- and BQ-induced cytotoxicity and cell death by Nrf2

To evaluate the role of Nrf2 in HQ- and BQ-induced cytotoxicity, we explored whether Nrf2 overexpression could confer cytoprotection against these xenobiotics in lung epithelial cells. Beas-2B cells were transfected with Nrf2-V5 plasmid to establish the transient overexpression of wild-type Nrf2 (Fig. 2A). Transfected cells were treated with different concentrations of either HQ or BQ for 5 h and cell viability was assessed by MTS reduction assay. As shown in Fig. 2B, cells overexpressing Nrf2 exhibited significantly attenuated loss of viability caused by HQ or BQ treatments compared to control cells, as reflected by either raised viability curves (Fig. 2B) or increased effective dose 50 (ED50, yielding 50% cell viability) (Fig. 2C). To further stress the importance of Nrf2 in protection against HQ and BQ cytotoxicity, Nrf2 in Beas-2B cells was silenced by siRNA (short silencing RNA) and tested for their susceptibility to these metabolites. Nrf2 siRNA transfection led to a knockdown of Nrf2 protein to an undetectable level as determined

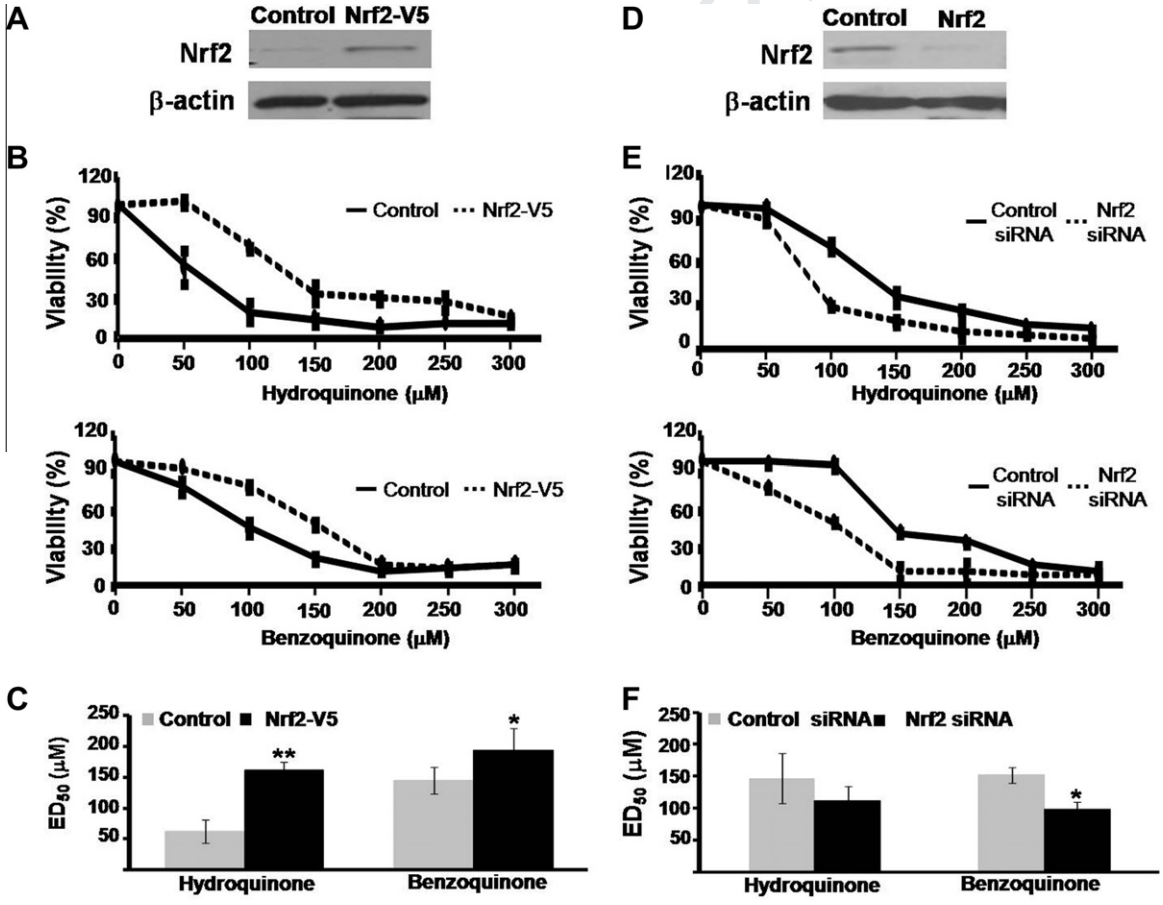

Fig. 2. The role of Nrf2 in protection against HQ- and BQ-induced cytotoxicity. Overexpression experiments (A–C). (A) Western blot analysis of Nrf2 expression in Beas-2B cells transiently transfected with pcDNA 3.1 or Nrf2-V5 plasmid. β-actin was used as loading control. (B) Beas-2B cells overexpressing Nrf2 were exposed to various concentrations of either HQ or BQ for 5 h and cytotoxicity was determined by MTS reduction assay. The curves represent the percentage of viability of treated cells relative to untreated-control cells (mean ± SD; $n > 3$). Note: data are representative of at least three independent experiments with similar results. (C) Based on the cytotoxicity assays, effective dose 50 (ED_{50}) was calculated for HQ and BQ in control and Nrf2-overexpressing cells (mean ± SD; $n > 4$). Knockdown experiment (D–F). (D) Western blot analysis of Nrf2 in Beas-2B cells transfected with control siRNA or siRNA targeting Nrf2. β-actin was used as loading control. (E) Forty-eight hours after siRNA transfection, Beas-2B cells were exposed to various concentrations of either HQ or BQ for 5 h and cytotoxicity was evaluated by MTS reduction assay. The curves represent the percentage of viability of treated cells relative to untreated-control cells (mean ± SD; $n > 3$). Note: data are representative of at least three independent experiments with similar results. (F) Based on the cytotoxicity assays, effective dose 50 (ED_{50}) was calculated for HQ and BQ in control and Nrf2-knockdown Beas-2B cells (mean ± SD; $n > 4$). Student's t-test $^{*}p < 0.05$, $^{**}p < 0.01$.

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by Western blot analysis (Fig. 2D). The Nrf2-knockdown cells exhibited increased susceptibility to either HQ- or BQ-induced cytotoxicity as reflected by lowered viability curves (Fig. 2E) and decreased ED50s for HQ and BQ as compared to control-siRNA transfected cells (Fig. 2F).

Since the above cell viability assay (MTS) was based on measuring mitochondrial function and unable to differentiate the processes of cell death in detail, we further assessed cytotoxicity of HQ and BQ in Nrf2 overexpressing and knockdown cells using cell death-based assays. Apoptosis was determined by annexin V/PI (overexpression experiments) and annexin V/7-AAD (knockdown experiments) staining followed by flow cytometry analysis. Although pronounced induction of cell death was observed in both controls and Nrf2 overexpressing Beas-2B cells after the treatment of either HQ or BQ (4 h, 50 μM) (Fig. 3), Nrf2 overexpressing cells showed a relatively lower rate of cell death compared to control cells (empty vector transfected cells) (Fig. 3). Noteworthy, early apoptotic cells (annexinV^+/PI^-) were, in fact, slightly increased in the Nrf2-overexpressing cells, but the overall apoptotic process was remarkably reduced in these cells. In addition, we also tested if lack of Nrf2 could enhance apoptotic cell death induced by either HQ or BQ (Fig. 4). After HQ treatment (4 h, 50 μM) ~40% of Nrf2-knockdown cells were dead or in later stages of apoptosis whereas only ~20% of control cells were dead or apoptotic (Fig. 4A and C). Moreover, the percentage of viable cells was significantly lower in Nrf2-knockdown cells than that of control cells. Similar results were obtained from the BQ treatment, in which ~60% of Nrf2-knockdown cells were dead or in later stages of apoptosis but only ~40% of control cells were the case (Fig. 4B and D). Taken together, these results demonstrate that Nrf2 overexpression attenuates cytotoxicity and cell death induced by both HQ and BQ whereas Nf2 deficiency increases susceptibility of Beas-2B cells to these compounds.

3.3. Reduced GSH levels and loss of inducibility of ARE-driven genes in Nrf2-knockdown cells

GSH is the most abundant cellular non-protein thiol, it plays a central role in maintaining cellular redox status and protecting against oxidative damage (Zhang et al., 2009). GSH depletion appears to be one of the key mechanisms underlying HQ and BQ toxicity in other human cells (Luo et al., 2008; Smith, 1999; Trush et al., 1996). We measured GSH levels in Beas-2B cells treated with various concentrations of either HQ or BQ for a short period (2 h). As shown in Fig. 5A, cellular GSH decreased in a dose–response manner in both treatments, with relatively greater depletion of GSH in BQ treatment. Since Nrf2 is a master regulator of antioxidant transcriptional response, we hypothesized that the initial depletion of GSH by HQ and BQ could trigger an Nrf2-dependent up-regulation of detoxifying enzymes and antioxidants, which could help detoxify these compounds (Bolton et al., 2000; Moran et al., 1999; Smith, 1999). In order to delineate the role of Nrf2 in this detoxification mechanism, we studied the effect of Nrf2-knockdown in the same cellular model. As expected, Nrf2-knockdown cells had lower basal levels of GSH compared to the control-transfected cells (80%) (Fig. 5B). When the siRNA transfected cells were treated with either 20 μM of HQ or 10 μM of BQ for 16 h, protein levels of HMOX and GSS were markedly increased in control-siRNA transfected cells, but this induction was totally absent in Nrf2-knockdown cells (Fig. 5C). These results suggest that the previously observed enhanced HQ/BQ cytotoxicity in Nrf2-knockdown cells is due to decreased production of GSH and

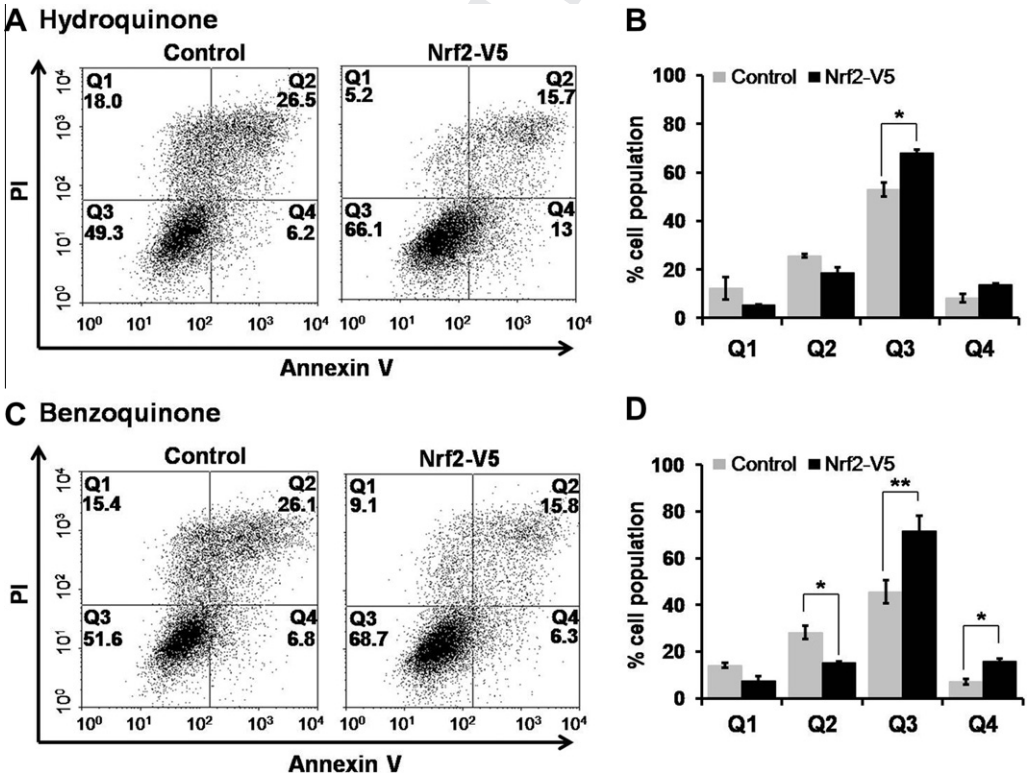

Fig. 3. Nrf2 overexpression renders protection against HQ- and BQ-induced apoptotic cell death induction. Forty-eight hours after transfection with control or Nrf2-V5 plasmid Beas 2-B cells were exposed to 50 μM of either HQ or BQ for ~4 h. Apoptosis and cell death induction was evaluated by Annexin-V/PI staining followed by flow cytometry analysis. Data from 1×10^4 cells were acquired. Representative flow cytometry profiles are presented for HQ (A) and BQ (C) treated cells. The percentage of cell population in each quadrant is indicated. (B) and (D) Data shown as mean ± SD of three independent experiments. Q1, dead cells (annexinV^-/PI^+); Q2, late apoptotic cells (annexinV^+/PI^+); Q3, viable cells (annexinV^-/PI^-); Q4, early apoptotic cells (annexinV^+/PI^-). Student's *t*-test $^{*}p < 0.05$, $^{**}p < 0.01$.

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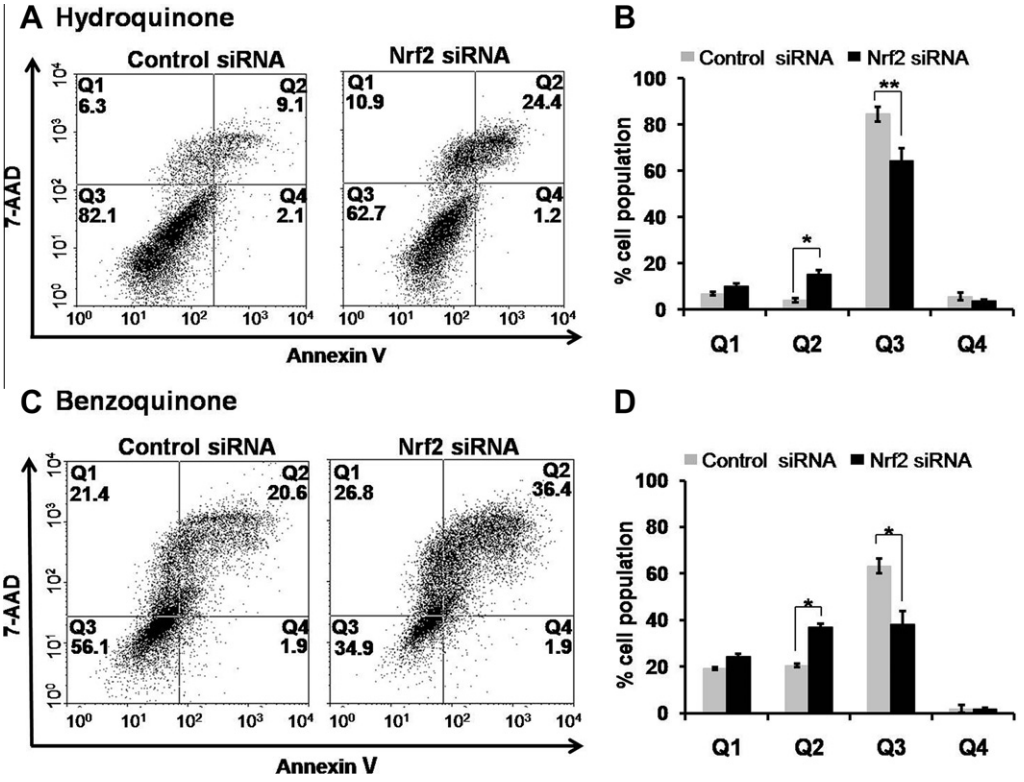

Fig. 4. Nrf2-knockdown enhances HQ- and BQ-induced apoptotic cell death. Forty-eight hours after transfection with control or Nrf2 siRNA, Beas 2-B cells were exposed to 50 μM of either HQ or BQ for ~4 h. Apoptosis and cell death induction was evaluated by Annexin-V/7-AAD staining followed by flow cytometry analysis. Data from 1×10^4 cells were acquired. Representative flow cytometry profiles are presented for HQ (A) and BQ (C) treated cells. The percentage of cell population in each quadrant is indicated. (B) and (D) Data shown as mean ± SD of three independent experiments. Q1, dead cells (annexinV$^-$/7AAD$^+$); Q2, late apoptotic cells (annexinV$^+$/7AAD$^+$); Q3, viable cells (annexinV$^-$/7AAD$^-$); Q4, early apoptotic cells (annexinV$^+$/7AAD$^-$). Student's *t*-test $^*p < 0.05$, $^{**}p < 0.01$.

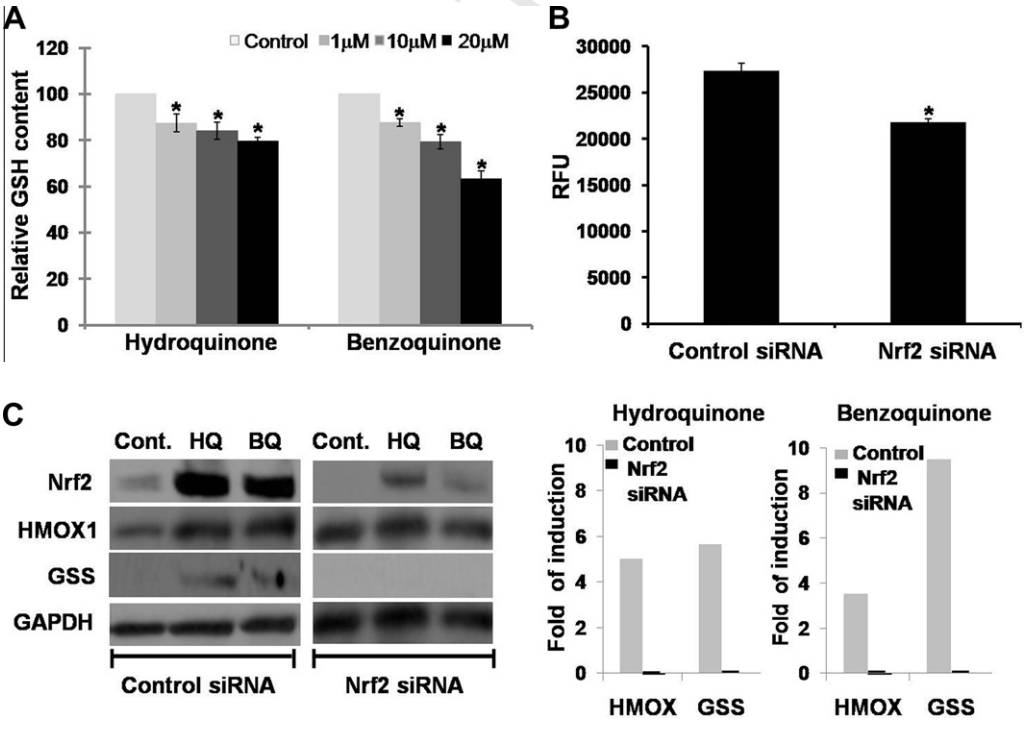

Fig. 5. Reduced GSH and loss of inducibility of HOMX1 and GSS in Nrf2-knockdown cells. (A) Beas-2B were treated at the indicated concentrations for 2 h followed by measurement of GSH intracellular content (mean ± SD; $n = 4$). (B) GSH content was measured 48 h after transfection with either control siRNA or Nrf2 siRNA (mean ± SD; $n = 3$) as described in Section 2. (C) Forty-eight hours after transfection, control and Nrf2-knockdown cells were treated with the indicated concentrations of HQ or BQ for 16 h. After treatment, protein levels of Nrf2, HMOX1 and GSS were determined by Western blot analysis. GAPDH was used as loading control. Quantification of bands intensity was performed by ImageJ version 1.42q software (developed by the National Institute of Health) (right panel) and expressed as percentage of induction compared to vehicle-treated cells (control). Data are representative of three independent experiments with similar results. Student's *t*-test $^*p < 0.05$.

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loss of inducibility of Nrf2-dependent antioxidant enzymes including HMOX and enzymes responsible for GSH metabolism (e.g. GSS). Therefore, the deficiency of Nrf2 reduces cellular redox capacity to counteract oxidative damage induced by HQ and BQ.

4. Discussion

In this study, we used human bronchial epithelial cells (Beas-2B) as a cellular model to explore the role of the Nrf2 pathway in the defense against HQ- and BQ-induced cytotoxicity in lung. We have shown that both HQ and BQ are able to induce ARE-driven gene expression in the lung cells, as reflected by an increase in ARE-dependant luciferase reporter activity (Fig. 1A). HQ and BQ exposures also lead to increased Nrf2 total protein levels, as well as Nrf2 nuclear accumulation (Fig. 1B). It is well established that Nrf2 nuclear accumulation and protein stabilization are indicators of Nrf2 activation (Li et al., 2005; Niture et al., 2009). Hence, these results reveal that HQ and BQ are Nrf2 activators, capable of inducing ARE-driven gene expression. Furthermore, we confirmed that HQ and BQ can induce endogenous ARE-driven genes, including those encoding GSH synthesis enzymes GCLM and GSS, the drug-metabolizing enzyme NQO1, and the antioxidant enzyme HMOX1. The induction of detoxifying enzymes (phase II enzymes) and antioxidants is critical for detoxification of benzene metabolites. NQO1 and GSH have been found to be key intrinsic molecules that defend against benzene-induced toxicity in hematopoietic cells (reviewed in (Bolton et al., 2000) and (Ross and Zhou, 2009)). NQO1 is a flavoprotein that catalyzes the beneficial two-electron reduction of benzoquinone to hydroquinone, preventing unwanted one-electron reduction which results in formation of ROS, thus avoiding redox cycling of semiquinone radicals and the subsequent covalent modification of cellular components (Vasiliou et al., 2006). It has been clearly demonstrated that NQO1 induction protects against cell death induced by HQ (Smith 1999) and BQ (Flescher and Snyder, 1995). Moreover, conjugation of HQ and BQ to GSH and other endogenous substrates (e.g. glucuronic acid) enables the formation of less reactive and toxic metabolites, with increased solubility and excretion (Snyder et al., 1993). Induction of the GSH synthesis enzymes and enzymes that catalyze the conjugation reactions (such as glutathione-S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs)) is mostly regulated by Nrf2 (Biswas and Rahman, 2009; Cho et al., 2002). Nrf2-dependent gene expression not only counteracts oxidative stress produced by HQ and BQ exposures, but also participates in the biotransformation and excretion of these metabolites. Our study demonstrated that exposure to either HQ or BQ causes a rapid GSH depletion (Fig. 5A) likely due to production of ROS or direct conjugation with the compounds (Luo et al., 2008; Smith, 1999; Trush et al., 1996), leading to Nrf2 activation and subsequent induction of GCLM and GSS (Fig. 1C), two major enzymes involved GSH synthesis (Biswas and Rahman, 2009). On the other hand, HQ and BQ are Michael reaction acceptors (Bolton et al., 2000), compounds that have been shown to react with SH groups of Keap1 leading to Nrf2 nuclear translocation and the subsequent activation of the pathway (Dinkova-Kostova et al., 2001; Wang et al., 2010). It is more likely that both of these two mechanisms of Nrf2 induction contribute to the observed Nrf2 activation. These results allow us to assemble a series of consequential events: exposure to HQ and BQ → ROS production → depletion of GSH and generation of oxidative stress → Nrf2 activation → induction of ARE-driven gene expression → detoxification of HQ and BQ and repair of oxidative damage (Fig. 6). This suggests that there exists an auto-regulation mechanism (Rubio et al., 2010) for the detoxification of HQ/BQ compounds and it is largely dependent on the activation of the Nrf2/ARE pathway.

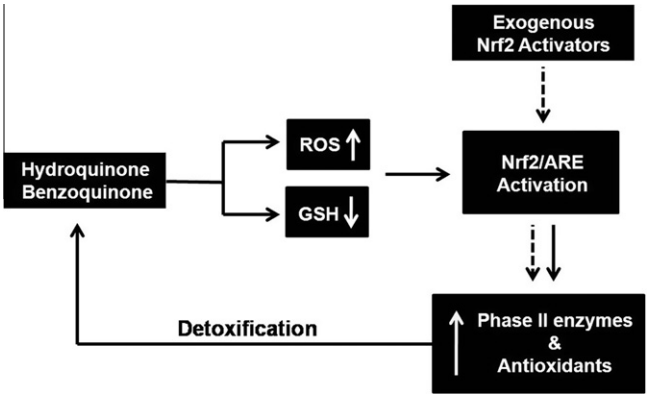

Fig. 6. Model detoxification pathway involved in the activation of Nrf2. Exposure to benzene metabolites HQ and BQ causes GSH depletion and ROS generation, resulting in changes in the redox status of the cell, which triggers nuclear translocation of Nrf2 and the activation of the Nrf2/ARE pathway, in turn leading to up-regulation of Phase II detoxification enzymes and enzymes responsible for the synthesis of endogenous antioxidants such as GSH. This auto-regulation mechanism is suggested as a therapeutic target to prevent or reduce benzene toxicity using pharmaceutical or natural dietary agents.

To further elucidate the protective role of Nrf2 against HQ- and BQ-induced cytotoxicity and cell death, two strategies were used to manipulate Nrf2 levels: gene overexpression and knockdown. While Nrf2 overexpression significantly reduced the cytotoxicity and apoptotic cell death induced by either HQ or BQ (Figs. 2 and 3), knockdown of Nrf2 by siRNA led to the opposite effects (Figs. 4 and 5). These results are in agreement with several previous reports in which neurons and kidney cells with Nrf2 overexpression showed enhanced resistance against oxidant-induced cell death (Chen and Shaikh, 2009; Shih et al., 2003), however, Nrf2-knockdown bone marrow stromal cells exhibited increased sensitivity to HQ and BQ (Zhu et al., 2006). Finally, we demonstrated that (1) reduced cellular GSH (Fig. 5B) and (2) impaired induction of ARE-driven genes including GSS and HMOX (Fig. 5C), both due to the loss of Nrf2, are the mechanistic explanations for the observed enhanced susceptibility of Nrf2-knowckdown cells to HQ and BQ. In other words, once the "auto-regulation" mechanism (Fig. 6) is disrupted, e.g., by inactivation of Nrf2, cells are more susceptible to these compounds. It is noteworthy that BQ appears to be relatively more potent than HQ in terms of depleting GSH and causing cytotoxicity (Figs. 1, 4 and 5). Compared to HQ, BQ is considered a less stable, but more reactive electrophile (Gaskell et al., 2005) capable of reacting directly with cellular components (e.g. DNA and proteins) and GSH (Snyder and Hedli, 1996). Nevertheless, manipulation of Nrf2 significantly affects cytotoxicity induced by either HQ or BQ (Figs. 2–4).

Our study demonstrated that the Nrf2/ARE pathway is essential in the intrinsic defense against inhaled benzene and its metabolites HQ and BQ. This knowledge is critical for the development of strategies to help susceptible populations exposed to these air pollutants. One of the most prominent developing strategies to prevent or reduce the benzene-induced damage is to enhance the Nrf2-dependent antioxidant response. It has been suggested that failure to induce functional NQO1 contributes to increased risk of benzene hematotoxicity (Moran et al., 1999) and induction of this enzyme by a well-known Nrf2 inducer, (Kwak et al., 2001) was proven to increase protein levels preventing HQ-induced apoptosis in hematopoietic cells. Furthermore, NQO1 can facilitate the formation of less toxic and soluble metabolites that are easier to be eliminated (Schrenk et al., 1996). Recently, several dietary chemopreventive compounds (e.g. curcuminoids, resveratrol, and isothiocyanates) have been reported to increase Nrf2 levels, disassociate

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Nrf2 of its cytosolic inhibitor Keap1, or/and enhance Nrf2 protein stabilization, resulting in the activation of Nrf2-dependent gene expression (reviewed in (Kwak et al., 2004) and (Jeong et al., 2006)). Use of these common and abundant compounds could prove to be a simple and safe strategy to provide protection against benzene- and benzene metabolite-induced toxicity. With tens of millions of people living and working in densely populated cities and industrial areas, such a preventive strategy – if successful – could have a significant impact on lessening the public health burden from exposure to oxidant pollutants.

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