Stabilization of Nrf2 by tBHQ Confers Protection against Oxidative Stress-Induced Cell Death in Human Neural Stem Cells

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Recent studies indicate that NF-E2 related factor 2 (Nrf2) is a substrate for the ubiquitin-proteasome pathway. The present study is aimed to determine whether increased protein stability is a mechanism by which quinone compounds, like tert-butylhydroquinone (tBHQ), may enhance Nrf2-mediated transcriptional activation and subsequent antioxidant protection. H2O2-induced necrotic cell death, evidenced by transmission electronic microscope (TEM) imaging with no caspase 3 activation and PARP cleavage, was significantly attenuated by pretreatment with tBHQ or overexpression of Nrf2 through adenovirus-mediated infection in human neural stem cells (hNSCs). Microarray analysis showed that those identified antioxidant genes, responsible for antiapoptotic action in IMR-32 cells (J. Li et al., 2002, J. Biol. Chem. 277, 388–394), were also coordinately upregulated through Nrf2-dependent antioxidant responsive element (ARE) activation in hNSC. The stabilization of Nrf2 by tBHQ in IMR-32 cells was evidenced by a pulse-chase assay showing no significant increase in Nrf2 protein synthesis after tBHQ treatment, and by ubiquitin immunoprecipitation showing that tBHQ stabilized ubiquitinated Nrf2. An in vitro proteasomal activity assay showed that tBHQ did not act as a 20S/26S proteasome inhibitor. Nrf2 stabilization by tBHQ also was observed in hNSCs. Taken together, this study suggests that identified antioxidant genes, which were upregulated through tBHQ induced Nrf2 stabilization, confer protection on target cells against H2O2-induced apoptotic cell death in neuroblasts cells as well as the necrotic cell death in the hNSC. Nrf2 stabilization by pharmacological modulation or adenovirus-mediated Nrf2 overexpression, therefore, might be viable strategies to prevent a wide-spectrum of oxidative stress-related neuronal cell injuries.

Key Words: Nrf2; tert-butylhydroquinone; apoptotic cell death; human neural stem cells.

The transcriptional activation of the phase II detoxification enzymes and/or antioxidant genes by quinone compounds has been traced to a cis-acting element (RTGACnnnGC motif) called the ARE or the electrophile response element (EpRE), which regulates either or both constitutive and inducible gene expression (Rushmore et al., 1991; Wasserman and Fall, 1997). Several ARE-binding proteins have been proposed and/or identified (Motohashi et al., 2002). Nrf2 has been demonstrated to play a central role in the gene expression of phase II detoxification enzymes and some antioxidant genes.

Human Nrf2 consists of 605 amino acids (aa) with a molecular weight of 67 KDa and exhibits strong interspecies homology at specific regions such as Nrf2-ECH homology (Neh2), CNC, DNA binding, and leucine zipper domains (Moi et al., 1994). Nrf2 is the actual transactivation factor responsible for upregulating ARE-driven gene expression (Nguyen et al., 2003; Venugopal and Jaiswal, 1996). Recent studies with Nrf2 knockout mice support this conclusion. Nrf2-deficient mice have lower basal levels of expression of phase II enzymes and lack the ability to induce them (Chan et al., 2001; Li et al., 2004).

Nrf2 is localized mainly in the cytoplasm bound to a repressor, KIAA0132 (also called iNrf2, the human homolog to Keap1). The Keap1-Nrf2 complex is proposed to be a candidate for the cytoplasmic sensor system that recognizes and reacts with inducers/stressors leading to the release of Nrf2 and subsequent ARE activation (Dinkova-Kostova et al., 2002; Itoh et al., 2003). Several protein kinase pathways, including mitogen-activated protein kinase (MAP kinase) and protein kinase C, have been implicated in transducing signals that control Nrf2 dependent gene expression (Huang et al., 2002; Kong et al., 2001; Lee et al., 2001). Nrf2 has a short half-life, T1/2 = 15 min (Nguyen et al., 2003) to 3 h (Stewart et al., 2003). The fast turnover of Nrf2 is a result of degradation through the ubiquitin-proteasome pathway and this degradation can be inhibited by KIAA0132 (Sekhar et al., 2002). A link between Nrf2 phosphorylation, Nrf2 stability, and the MAP kinase pathway is proposed (Nguyen et al., 2003). Since we have shown that phosphatidylinositol 3-kinase (PI3-kinase), not extracellular signal-regulated kinase (Erk1/2), is involved in the Nrf2-ARE activation pathway in IMR-32 cells (Lee et al., 2001), it would be of great interest to determine if PI3 kinase has a similar effect on Nrf2 stability in cells of neural origin. Alternatively, could tBHQ prevent Nrf2 ubiquitination or act as a 26S/20S proteasome inhibitor through directly targeting the proteasome?

Human neural stem cells (hNSCs) are generated from fetal human brain and can be expanded for extended periods of time in...
cell injury in H2O2-treated neuroblastoma cells or primary demonstrated that the apoptotic process and development of antioxidant defenses in the CNS as well. Recent reports hepatic detoxification; it most likely contributes significantly impaired therapeutic effectiveness. Conferring antioxidant properties to these cells may result in potential strategies to combat existing oxidative stimulus. Although Nrf2-dependent, ARE-driven gene expression has been studied extensively in hepatic detoxification; it most likely contributes significantly to antioxidant defenses in the CNS as well. Recent reports demonstrated that the apoptotic process and development of cell injury in H2O2-treated neuroblastoma cells or primary neural cultures were attenuated by tBHQ activation of the ARE (Lee et al., 2003; Li et al., 2002). In addition, primary neuronal or astrocytic cultures from Nrf2−/− mice showed increased sensitivity to H2O2, rotenone, or MPP⁺-induced cytotoxicity (Lee et al., 2003). High-throughput microarray technology has been applied to dissect a cluster of detoxification enzymes and antioxidant genes, or termed programmed cell life genes, which may be involved in the protective effect observed by treatments with tBHQ (Kraft et al., 2004; Lee et al., 2003; Li et al., 2002; Shih et al., 2003). In this study, we investigate the mechanism by which tBHQ activates Nrf2-dependent ARE-driven gene expression by evaluating whether tBHQ or overexpression of Nrf2 through adenovirus-mediated infection may attenuate oxidative stress induced cell injury in hNSCs and whether tBHQ modulates Nrf2 degradation through the ubiquitin proteasome pathway in human neuronal cells.

MATERIALS AND METHODS

Plasmids. pEF mammalian expression plasmids carrying cDNA encoding mouse Nrf2 (aa residues 1–597) and dominant-negative (DN) Nrf2 (aa residues 409–597) were a generous gift from Dr. Jawed Alam (Alton Ochsner Medical Foundation, New Orleans, LA). Recombinant adenoviral vectors were constructed using the Cre-lox system were purchased for the Canadian Stroke Foundation, New Orleans, LA). Recombinant adenoviral vectors were constructed using the Cre-lox system were purchased for the Canadian Stroke Foundation, New Orleans, LA). Recombinant adenoviral vectors were constructed using the Cre-lox system were purchased for the Canadian Stroke Foundation, New Orleans, LA). Recombinant adenoviral vectors were constructed using the Cre-lox system were purchased for the Canadian Stroke Foundation, New Orleans, LA). Recombinant adenoviral vectors were constructed using the Cre-lox system were purchased for the Canadian Stroke Foundation, New Orleans, LA). Recombinant adenoviral vectors were constructed using the Cre-lox system were purchased for the Canadian Stroke Foundation, New Orleans, LA).

Materials and Methods

Cell Culture, Adenovirus Infection, and in Vitro Differentiation Studies

IMR-32 human neuroblastoma cells were grown in Dulbecco’s modified eagle’s medium (DMEM) supplemented with fetal calf serum (10%), 100 U/ml penicillin, and 100 mg/ml streptomycin. Cultures were maintained at 37°C in a humidified 10% CO₂ atmosphere.

Human fetal cortical tissue (between 8 and 13 weeks post-conception) was collected following routine terminations of pregnancy. All fetal tissue was kindly provided to the laboratory of Dr. Clive Svendsen’s laboratory while at the MRC Center for Brain Repair, University of Cambridge by Dr. Eric Jauniaux, Department of Obstetrics and Gynaecology, University College, London. Full ethical approval had been granted by the Local Research Ethics Committee, University College Hospital, London. The methods of collection conform with the arrangements recommended by the Polkinghorne Committee and NIH for the collection of such tissues and to the guidelines set out by the United Kingdom Department of Health and as well as the University of Wisconsin. The human neural stem cell line CTX066 was established by Dr. Clive Svendsen’s laboratory at the University of Cambridge on 19 July 2000. The cell line was a gift to our laboratory in May 2001. CTX066 cells were passaged every 14 days by sectioning into 150 μm sections, which were then reseeded into fresh growth medium containing 70% DMEM, 30% Hams F12, 1% penicillin/streptomycin/amphotericin B supplemented with 20 ng/ml EGF, 10 ng/ml LIF, and 1% v/v N2 at a density equivalent to 200,000 cells per ml. Half the growth medium was replenished every fourth day (Wright et al., 2003).

Cell migration assay. The cell migration assay, which has been described in detail (Wright et al., 2003), was performed to determine the differentiation potential of stem cells. The neurospheres were plated directly onto poly-L-lysine/laminin-coated glass coverslips in serum-free medium (DMEM/HAMS F-12) containing B27 supplement (2%, v/v) plus NT-4 (10 ng/ml) without mitogens (EGF). Over a seven-day period following plating, cells migrated away from the sphere and formed a differentiating neuronal and glial monolayer.

Virus infection assay. IMR-32 cells or human neurospheres were passaged 48 h before infection with adenovirus in 96-well (at a density of 5 × 10⁵ cells/ml) or 24-well dishes (at a density of 5 × 10⁶ cells/ml). The cell density of human neural stem cells was determined by trypan blue exclusion assay (Invitrogen) after complete dissociation of spheres with Accutase (U.S. Bio-technologies Inc, Parker Ford, PA). After 1 h of incubation, the virus-containing medium was
replaced by fresh complete growth medium and placed in a 37°C humidified 5% CO2 incubator for 48 h. The infection rate with the virus was determined by the percentage of GFP-positive cells. The modified constructs contained Nrf2 (or DN-Nrf2) coupled to Green Fluorescent Protein (GFP) in separate expression cassettes. Therefore, we surmised that GFP-positive cells also overexpressed Nrf2 or DN form.

Transmission electronic microscope (TEM). The cultures that were treated with H2O2 (100, 200, 400, and 800 μM for 12 h) and Camptothecin (CA, 10 μM for 24 h) were processed at the University of Wisconsin Medical School Electron Microscope Facility for TEM imaging. The sections were post-stained in uranyl acetate and Reynolds lead citrate and imaged using a Philips CM120 STEM operating at 80 kV.

Immunohistochemistry. Cells were fixed in 4% paraformaldehyde in PBS. For neurospheres, cryosections were made and immunocytochemistry was carried out using standard protocols. After blocking with 3% normal goat serum, cells were incubated with primary antibodies at 4°C for 16 h. Cells were then reacted with appropriate secondary antibodies (Vector Laboratories) conjugated to biotin, fluorescein isothiocyanate (FITC) or Texas Red for 1 h at room temperature. Biotin-conjugated antibodies were then visualized using the avidin-biotin-peroxidase complex (ABC) method (Vector Laboratories). Hoechst 33258 or hematoxylin counterstaining was included after the final antibody applications.

Cell viability. Cytotoxicity was measured using an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) assay kit (Promega, Madison, WI) according to the manufacturer’s instructions. After 3 h incubation with MTS, the absorbance at 490 nm was measured. Cell death rate was calculated by [(A490 (control)−A490 (treatment))/A490 (control)] × 100%.

IMR-32 cells, at a density of 5 × 10⁵ cells/ml were passaged 48 h before infection with adenovirus for 48 h or treatment with tBHQ (10 μM) for 24 h in 96-well dishes. These cells were then treated with H2O2 (200 μM) for only 1 h as previously described (Li et al., 2002). Capped neurospheres were seeded at a density of 5 × 10⁵/well in 96-well dishes and let the cells recover for one week. Cells were treated with tBHQ for 48 h at the final concentration varied from 2.5 μM to 80 μM with the serial dilution. The result from the MTS assay indicated that there was no significant decrease in cell viability at doses up to 40 μM (data not shown). Therefore, the neural stem cells were pretreated with tBHQ (20 μM) for 48 h before H2O2 (200 μM) treatment for 12 h.

Microarray analysis. Details for sample preparation and microarray processing are available from Affymetrix and have been described previously (Li et al., 2002). This array contains approximately 12,000 probe sets corresponding to 9,670 full-length human DNAs. Affymetrix Microarray Suite 5.0 was used to scan and analyze the relative abundance of each gene. Data were analyzed by rank analysis as previously described (Li et al., 2002). Dchip software (www.dchip.org) was also used to analyze the data. Model-based (PM/MM or PM-only) data normalization was performed prior to the statistic analysis (Li and Wong, 2001). The final gene list was comprised of significantly increased or decreased genes (p < 0.05) by treatment with tBHQ. This microarray experiment conforms to standards by the Microarray Gene Expression (MGED) Society and the raw data are available from GEO (http://ncbi.nlm.nih.gov/geo) under the following accession numbers, GSE 759.

Western blot and immunoprecipitation. Protein concentrations were estimated by the BCA method (Pierce). Nuclear extraction was performed by using Celllytic nuclear extraction kit (Sigma). Whole cell (60 μg) or enriched nuclear extracts (30 μg) were resolved by 10% SDS-PAGE, transferred to PVDF membrane, and blocked with 5% non-fat milk. The PVDF membranes were incubated with primary antibodies overnight at 4°C. Membranes were washed and incubated with anti-rabbit (1:2000) or anti-mouse (1:2000) IgG labeled with horseradish peroxidase (Vector Laboratories) for 1 h and visualized using an enhanced chemiluminescence Western blotting detection system (Pierce). Representative Western blots are shown in the figures. For the immunoprecipitation (IP), the cell lysates were incubated with anti-ubiquitin antibodies (1 μg/100 μg cell lysate) at 4°C for 16 h and the immune complexes were precipitated with Protein G-Agarose (Roche Diagnostics GmbH) at 4°C for an additional 4 h. The precipitates then were washed extensively with IP buffer before fractionation by SDS-PAGE and subsequent immunoblotting with anti-Nrf2 antibodies. Densitometry was used for quantification of results. We used antibodies to cytochrome C (TH8.2C12, BD Pharmingen) and histone 1 (Santa Cruz) as cytosolic and nuclear markers. The data suggested that these are highly enriched nuclear extracts that have little cross contaminants.

NQO1 enzymatic activity assay. Endogenous NQO1 enzymatic activity was measured using a colorimetric method using menadione as a substrate (Lind et al., 1990). Data are presented as the change in absorbance per minute per ng protein (ΔA570/min/ng protein).

Total glutathione levels. Total glutathione levels (GSH and GSSG) were determined using a modified Tietze method by spectrophotometrically measuring the reduction of GSH by dithionitrobenzoic acid (DTNB) coupled to the recycling of GSSG to GSH with glutathione reductase (Tietze, 1969).

Pulse-chase labeling assay. Pulse-chase assay was performed as described with some adjustments (Stewart et al., 2003). IMR-32 cells were plated as 1 × 10⁶ cells/10 cm dish and cultured until ~80% confluent. Culture media were changed to DMEM (met/cys-free)/5% FCS for 60 min at 37°C. 50 μl of Tran®-S-label methionine (at roughly 10 mCi/ml; total of 500 μCi, Amersham Biosciences) were added to the cultures, followed by treatments with vehicle or tBHQ (10 μM). Cells were incubated in the Shoebox for 0, 15, 30, 60, and 120 min. After pulse-chase for up to 2 h, the media was removed and the labeling was terminated by addition of cold methionine from a 200 mM stock to the culture media. Cells were then washed with 5 ml ice-cold PBS, harvested, sonicated in 0.5 ml ice-cold PBS, and then preserved at ~80°C and later subjected to immunoprecipitation. For immunoprecipitation, cell lysates were incubated with 1 μg of rabbit IgG (5 mg/ml) and Protein G-Agarose (Roche Diagnostics GmbH) at 4°C for 4 h, and then 2 μg of anti-Nrf2 (H300, 5 μg/ml) were added for overnight incubation. The precipitates were washed extensively with IP buffer before fractionation by SDS-PAGE. The gels were subsequently vacuum dried. Signals were detected after two days of exposure and quantified using a phosphorimager (Packard Instruments).

RT-PCR. Total RNA, extracted from cell pellets with Trizol Reagent (Invitrogen), was subjected to reverse transcription-polymerase chain reaction (RT-PCR) using the Promega Transcription System. A set of PCR primers for Nrf2 (5'-CTGCTTTTATAGCTGAGCC-3', 5'-CCTGAGATGTGACAGGTT-3') was used to yield 356 bp product.

Statistical analysis. All the experimental data shown were repeated at least three times, unless otherwise indicated. Results are presented as mean ± SEM. Experimental groups were compared by a one-tailed, unequal SD, t-test. Statistical probability of p < 0.05 was considered significant.

RESULTS

Stabilization of Nrf2 Protein by tBHQ Treatment Confers Protection against H2O2-Induced Cytotoxicity in hNSCs

hNSCs were grown for more than 300 days by using a novel method ofpassaging that maintains cell-to-cell contact in media that contain LIF (10 ng/ml) and EGF (20 ng/ml) (Fig. 1A). This lengthy in vitro growth period provides sufficient time to perform toxicity studies on this model. After plating intact neurospheres on poly-L-lysine and laminin-coated slides, cells continuously migrated from the core (Figs. 1B and 1C) and differentiated into neurons (Figs. 1D and 1E) and astrocytes (Figs. 1D and 1F).

Previous reports from our laboratory indicated that Nrf2-ARE-driven genes coordinate function to protect cells
from H2O2-induced apoptosis in human neuroblastoma cells, rat primary neuronal cultures, mouse primary astrocytic and neuronal cultures (Kraft et al., 2004; Lee et al., 2003; Li et al., 2002; Shih et al., 2003). These findings lead us to investigate whether it is possible to manipulate the level of Nrf2 in normal hNSCs and reproduce the protection against H2O2-induced cytotoxicity in this model. Toxic doses of H2O2 vary with the cell density, components in culture media, and the cell type studied. The following oxidative stress may result in apoptotic and/or necrotic cell death depending on a variety of factors. This information led us to carefully evaluate the effects of cell density, dose, and time of exposure on H2O2-mediated cytotoxicity. Neurospheres were passaged into 24-well dishes at a density of 5 × 10^7 cells/ml. Doses ranging from 100 μM to 800 μM H2O2, which have been reported previously to induce apoptosis in human neuroblastoma cells, were applied to hNSCs for 12 h. TEM, a classical method to differentiate apoptotic from necrotic cell death in the central nervous system, was used to aid in our determination of H2O2-induced cell death. Camptothecin (CA), a well characterized DNA topoisomerase I inhibitor, has been widely used as an apoptotic inducer and is considered to establish positive control for apoptosis. Here, hNSCs were incubated with vehicle or CA (10 μM) for 24 h, and subjected to TEM. In CA treated samples, a large number of cells showed condensation of nuclear chromatin (Fig. 2A middle panel, arrowhead) or even nuclear fragmentation (data not shown), combining with intact cell membrane (arrow). The ultrastructural damages identified in CA treated samples were distributed evenly through the whole neurosphere and these ultrastructural characteristics were consistent with apoptosis. In contrast, H2O2 (200 μM) treated samples showed a dramatically different pattern of cell death. Severe damage, characterized as a loss of membrane integrity (arrow), was easily observable (Fig. 2A right panel). Unlike CA treated samples, nuclei varied from still intact (arrowhead) to completely dissolved (data not shown) with no evidence of nuclear condensation or fragmentation. All of these features identified by TEM, associated with H2O2 treatment, are consistent with definitions of necrotic cell death.

Biochemical pathway studies showed that a time or dose dependent caspase 3 activation (Fig. 2B) and an increase in PARP cleavage (Fig. 2C) were detected after treatment with CA but not H2O2, further suggesting that H2O2, unlike CA, induced a typical necrotic cell death in hNSCs.

Pretreatment with 20 μM tBHQ for 48 h significantly attenuated this H2O2-induced cytotoxicity in a time-dependent manner as shown in Figure 3. After 12 h of H2O2 (200 μM) treatment, cells pretreated with tBHQ demonstrated a 20% greater cell survival rate than the vehicle pretreatment group. Oligonucleotide microarray analysis identified 72 genes that are up (40) or down (32)-regulated by tBHQ treatment (24 h; Fig. 4). Interestingly,
a cluster of detoxification enzymes and antioxidant genes, which was regarded as programmed cell life genes, previously identified as being protective against H2O2-induced apoptosis in IMR-32 cells, were also upregulated by tBHQ in hNSCs (Supplemental Table 1). The most significant change within this cluster was heme oxygenase 1 (HO1, increased 43-fold) (Supplemental Table 1). Immunocytochemistry confirmed the significant increase of the HO1 expression product throughout the entire neurosphere (Fig. 5A). GCLR mRNA levels were also increased according to the microarray data (5.0-fold). This change should lead to increased levels of glutathione (GSH), a molecule that is one of the major intracellular contributors to a cell’s reducing potential. As shown in Figure 5B, total glutathione levels were increased by more than two-fold after treatment with tBHQ for 24 h and 48 h. In addition, Western blot demonstrated increased GCLR protein level (Fig. 5C) while the catalytic subunit, GCLC, was not significantly changed, suggesting that the regulatory subunit was transcriptionally regulated more tightly by tBHQ than the catalytic subunit. Another well-characterized Nrf2-ARE-driven gene, NQO1, also was upregulated (3.2-fold). Increased NQO1 enzymatic activity (Fig. 5D) as well as NQO1 protein levels (Fig. 5E) were detected.
confirming the gene expression data. Other genes involved in the antioxidant defenses such as thioredoxin/thioredoxin reductase, NAD(P)H producing system (malic enzyme and aldehyde dehydrogenase), ferritin, and multiple heat shock proteins (70 KDa and 90 KDa) also were increased suggesting that multiple antioxidant systems are hardwired and activated by a mechanism common to mouse, rat, and human.

Gene expression levels of Nrf2 binding partners showed no significant change except for MafF (4.45 ± 0.94), one of the small MAF family members. KIAA0132, the human homolog of mouse Keap1, showed no change in neurospheres whereas our previous report indicated that it was upregulated 3.0 fold after tBHQ treatment in IMR-32 cells (Li et al., 2002).

Overexpression of Nrf2 through Adenovirus-Mediated Infection Protects Human Neural Cells from Oxidative Stress Induced Cytotoxicity

Nrf2 is a major player in transcriptional regulation of those identified genes through activation of the ARE. Hence, overexpression of Nrf2 may serve to protect against H$_2$O$_2$–induced cytotoxicity similar to that of tBHQ-induced Nrf2 stabilization. Here, adenovirus constructs were used to overexpress Nrf2 in both neuroblastoma cells and the neural stem cells. As mentioned in the Materials and Methods section, three different vectors containing CMV-eGFP, CMV-Nrf2/CMV-eGFP, or CMV-DN-Nrf2/CMV-eGFP were constructed. The toxicity of this replication-deficient adenovirus was evaluated on IMR-32 cells. The virus applied at a titer of 50 MOI (multiplicity of infection) resulted in almost 100% infection rate with no significant increase in viral toxicity.

IMR-32 cells infected with adenovirus at 50 MOI for 48 h were then treated with H$_2$O$_2$ (200 μM) for 1 h. As shown in Table 1, the Ad-Nrf2 group (8.4% cell death) had significantly decreased cell death compared to Ad-eGFP (34%) or Ad-DN-Nrf2 (30%) infected cultures, as assessed by MTS assay. There was no significant difference in the cell death rate between the latter two groups. Similar to IMR-32 cells, Ad-Nrf2 infected spheres, treated with H$_2$O$_2$ for 12 h, showed significantly reduced cell death rate compared to Ad-DN-Nrf2 or Ad-eGFP infected spheres (Table 1).

Stabilization of Nrf2 Protein by tBHQ Is a Common Effect Found in IMR-32 Cells as Well as hNSCs

In contrast to the hNSCs, IMR-32 neuroblastoma cells are fast growing, relatively homogeneous, and easy to manipulate. To explore the mechanism(s) by which tBHQ stabilizes Nrf2, we first examined mRNA and protein levels in IMR-32 cells after tBHQ treatment. As shown in Figure 6A, IMR-32 cells treated with tBHQ (10 μM) for up to 24 h did not alter Nrf2 transcript levels. However, the abundance of Nrf2 protein in whole cell extracts increased by 4.7-fold at 2 h treatment and 3.4-fold at 24 h treatment according to densitometry analysis (Fig. 6A). c-Fos, a defined short half-life transcription factor that has been proven to be degraded through the ubiquitin-proteasome pathway (He et al., 1998), was not affected by tBHQ treatment (Fig. 6A).

To examine if this dramatic increase in protein levels of Nrf2 was a result of accelerated protein synthesis and/or decreased protein degradation by tBHQ treatment, a pulse-chase labeling assay was preformed in the absence or presence of tBHQ (10 μM) for up to 2 h. As shown in Figure 6B, there was no significant difference of [35S] labeled Nrf2 bands between vehicle or tBHQ treated samples in each time point, suggesting that Nrf2 protein synthesis may not be accelerated by tBHQ treatment.

The degradation of cellular proteins, particularly transcriptional factors, is a highly complex, temporally controlled, and tightly regulated process that plays major roles in efficiently controlling a variety of cellular pathways. The ubiquitin-proteasome system is actively involved in this process. Hence, we were curious about whether decreased protein degradation through interfering with ubiquitin-proteasome system may be responsible for the tBHQ induced Nrf2 stabilization. Ubiquitin immunoprecipitation was performed and subsequent immunoblotting with the Nrf2 N-terminal antibody (H300) revealed a 100 KDa band (Fig. 6C). Following treatment with tBHQ for up
FIG. 4. Hierarchical clustering of tBHQ induced gene expression profiles in hNSCs. Dchip software was applied for data analysis and hierarchical gene clustering. Model-based (PM-only or PM/MM) data normalization was performed prior to the statistic analysis. The genes listed at final showed significant increase or decrease ($p < 0.05$) by tBHQ treatment in both model systems. Those genes were recruited and trained for hierarchical clustering.
to 24 h, the ubiquitinated Nrf2 showed a significant increase by 6.6-fold at 2 h treatment and 3.6-fold at 24 h treatment (Fig. 6C), suggesting that tBHQ could stabilize ubiquitinated Nrf2 from degradation whereas it could not prevent Nrf2 from being ubiquitinated. We proposed that this 100 KDa band represents poly-ubiquitinated Nrf2. Two well-characterized 26S/20S proteasome inhibitors, MG132 (10 μM, 4 h treatment) and lactacystin (5 μM, 4 h treatment) were used to further confirm the
Overexpression of Nrf2 Through Adenovirus-Mediated Infection Attenuated H2O2-Induced Cytotoxicity in hNSCs

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Ad-eGFP</th>
<th>Ad-Nrf2/eGFP</th>
<th>Ad-DN-Nrf2/eGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMR-32</td>
<td>34.14% ± 5.81%</td>
<td>8.45% ± 3.59%*</td>
<td>29.63% ± 3.55%</td>
</tr>
<tr>
<td>hNSCs</td>
<td>46.62% ± 0.9%</td>
<td>27.47% ± 1.96%*</td>
<td>46.26% ± 4.33%</td>
</tr>
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Note. IMR-32 cells were challenged with adenovirus (Ad-eGFP, Ad-Nrf2/eGFP, and Ad-DN-Nrf2/eGFP) at 50 MOI for 48 h prior to H2O2 (200 μM) treatment for 1 h. Human neural progenitors were infected with adenovirus at 50 MOI for 48 h prior to H2O2 (200 μM) treatment for 12 h. MTS assay was developed for 2 h and the cell death rate was calculated with the algorithm, \(((A490_{\text{control}}) - A490_{\text{treatment}})/A490_{\text{control}}) \times 100\%. Each data bar represents the mean ± SE (n = 4).

*Represents the statistic significance based on one-tailed, unpaired t-test (p < 0.05).

Involvement of the 26S proteasome in Nrf2 degradation. Both inhibitors significantly enhanced Nrf2 as well as c-Fos protein levels (Fig. 6D) suggesting that tBHQ has a unique ability to stabilize Nrf2.

Adenoviral infection of IMR-32 cells and subsequent visualization with Nrf2-Zurich antibody was used to detect Nrf2 subcellular localization. This antibody can detect both Nrf2 full-length cDNA or DN-Nrf2 cDNA -encoded products (Nguyen et al., 2002). The increased mouse Nrf2 band, located at ~100 Kda beneath the human Nrf2 band, and DN-Nrf2, located at ~20KDa, were detected respectively after infection with Ad-Nrf2/eGFP or Ad-DN-Nrf2/eGFP for 48 h (Fig. 6E, upper panels). Immunoprecipitation showed the ~20 KDa DN-Nrf2 could not be detected by the Nrf2-Zurich antibody after ubiquitin pull-down (Fig. 6E, lower right panel), whereas the ~100 KDa Nrf2 band (mouse + human) could still be detected (Fig. 6E, lower left panel). This data suggests that the DN-Nrf2 is not ubiquitinated, thus narrowing the Nrf2 ubiquitination site to N-terminal aa residues 1–408.

Since our previous studies reported that the PI3 kinase pathway regulated tBHQ-induced ARE activation in IMR-32 cells, we tested whether the PI3 kinase pathway is involved in Nrf2 stabilization by tBHQ. Western blots of whole cell lysates with or without anti-ubiquitin immunoprecipitation showed there were no differences between the groups treated with and without LY294002, a selective PI3 Kinase inhibitor (Fig. 7A). However, Nrf2 immunoblots of nuclear extracts showed a decrease in tBHQ-induced nuclear accumulation of Nrf2 in the presence of LY294002 (Fig. 7B). Pretreatment with LY294002 significantly decreased Nrf2 levels in nuclear extract without changing the overall level of Nrf2 in the cell. Therefore, it is possible that PI3 kinase may only be involved in stimulating Nrf2 nuclear translocation, not Nrf2 stabilization.

In addition, tBHQ induced Nrf2 protein stabilization was also observed in hNSCs. Immunoblotting results using three antibodies (C20, H300, and Zurich) showed Nrf2 protein levels were dramatically increased by tBHQ (Fig. 8A) without changes in mRNA level (data not shown). A band at ~100 KDa was easily identified since overexpression of Nrf2 using adenovirus-mediated infection as a positive control showed a significantly increased band at the same location (Fig. 3A). This observation implies that in human neuronal cells, the steady-state Nrf2 has been physically modified at the post-translational level. Ubiquitin immunoprecipitation showed tBHQ increased ubiquitinated Nrf2 (~100 KDa) and that both MG132 (4 h) and Lactacystin (4 h) could also prevent Nrf2 degradation (Fig. 8B).

**DISCUSSION**

Nrf2 Is a Ubiquitin-Proteasome Substrate

Nrf2 was first reported as a substrate for the ubiquitin proteasome pathway (Sekhar et al., 2002). Others showed a physically high rate of intracellular Nrf2 turnover in hepatoma cells (Nguyen et al., 2003; Stewart et al., 2003). Here we report that there is an increase in protein levels of Nrf2 in response to tBHQ, without an increase in Nrf2 mRNA level. This finding is consistent with other studies demonstrating that Nrf2 mRNA levels were unaffected by various inducers of ARE activity. All of the above evidence led us to propose that quinone compounds, like tBHQ, may stabilize Nrf2 by inhibition of the ubiquitin-proteasome pathway. However, the mechanism by which tBHQ targets the ubiquitin-proteasome pathway is still unknown.

Through ubiquitin antibody immunoprecipitation or HA tagged polyubiquitin pull-down (Sekhar et al., 2002), immunoblots with Nrf2 antibody demonstrated that Nrf2 physically binds the polyubiquitin molecule in vitro. This observation was confirmed by overexpression of Nrf2 through adenovirus-mediated infection. The theoretical MW of Nrf2 is about 67 KDa for humans, mice, and rats. It has been noted, however, that the apparent molecular weight of human or murine Nrf2 in SDS-PAGE ranges from 57 KDa to 110 KDa. We used both the Nrf2 N- and C-terminal antibodies from Santa Cruz as well as a laboratory generated Nrf2 antibody (Zurich) for Western blots. With overexpressed Nrf2 or dominant negative Nrf2 as positive/negative controls, we found that all the antibodies recognized a cluster of significantly increased bands at ~100 KDa in multiple neural cultures including IMR-32 cells (Fig. 6D), mouse primary neuronal and astrocytic cultures (data not shown), and hNSCs (Fig. 8A) after tBHQ treatment. Other bands, typically with MW < 60 KDa, were not changed by tBHQ treatment or Nrf2 overexpression and were considered to be nonspecific binding. The laboratory generated antibody also identified DN-Nrf2 which migrates to ~20 KDa and is similar to the predicted MW (~22 KDa).

Polypeptide sequences enriched in proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST regions) target proteins for rapid destruction (Rogers and Rechsteiner, 1986). Scientific research has provided strong evidence that PEST regions do in
fact serve as proteolytic signals. Using the PEST find algorithm, Stewart et al. (2003) identified mouse Nrf2 as having several poor PEST candidates and one potential PEST sequence at position 350–380 aa. Serine and threonine residues within PEST domains are potential phosphorylation sites that may be necessary for degradation. This region is, however, deleted in the DN construct (which contains only the C-terminal 189 aa of Nrf2) suggesting that DN-Nrf2 ubiquitination is not possible (Fig. 6E). Recent reports suggested that the variable turnover of Nrf2 is accomplished through the use of at least two degrons.

**FIG. 6.** tBHQ uniquely stabilizes ubiquitinated Nrf2 in IMR-32 cells. (A) IMR-32 cells were treated with vehicle (0.01% EtOH) or tBHQ (10 μM) for 2 h or 24 h. Whole cell lysates were resolved by 10% SDS-PAGE and immunoblotted with Nrf2 (H300) antibody or c-Fos antibody. Total RNA was also harvested with Trizol reagent and RT-PCR for Nrf2 was performed. (B) Pulse-chase assay was performed in the absence or presence of tBHQ (10 μM) for the indicated times. The 100-KDa band representing Nrf2 was marked. (C) IMR-32 cells were treated with vehicle (0.01% EtOH), tBHQ (10 μM), lactacystin (5 μM), MG132 (10 μM) for the times indicated in the Figure. The whole cell lysates were immunoprecipitated with anti-ubiquitin antibody and subsequently immunoblotted with anti-Nrf2 (H300) antibody. (D) Whole cell lysates generated from Lactacystin (5 μM), MG132 (10 μM), or vehicle (0.01% DMSO) treated samples were resolved by SDS-PAGE and immunoblotted with Nrf2 antibody or c-Fos antibody. (E) IMR-32 cells were infected with adenoviral constructs containing Nrf2/eGFP, DN-Nrf2/eGFP, or eGFP-only for 48 h at the titer of 50 MOI. Whole cell extracts were immunoprecipitated with ubiquitin antibody. The whole cell extracts and immunoprecipitates were resolved by SDS-PAGE and immunoblotted with Nrf2-Zurich antibody. Upper panels, Nrf2 immunoblot; Lower panels, Ubiquitin IP + Nrf2 immunoblot. Some of the representative Western blots were semi-quantified by the densitometry.
C.

IP: anti-ubiquitin
IB: anti-Nrf2 (H300)

D.

IB: anti-Nrf2 (H300)
IB: anti-c-Fos

E.

E1. IB: anti-Nrf2 (Zurich)
Human Nrf2
Mouse Nrf2

E2. IP: anti-ubiquitin
IB: anti-Nrf2 (Zurich)
Human Nrf2
Mouse Nrf2

FIG. 6—Continued.
A. Whole cell Extracts

![Whole cell Extracts](image)

IB: anti-Nrf2 (H300)

IB: anti-UBiquitin

IB: anti-Nrf2 (H300)

B. Nuclear Extracts

![Nuclear Extracts](image)

IB: anti-Nrf2 (H300)

IB: Histone 1

![Nuclear Extracts](image)

IB: anti-Nrf2 (H300)

IP: anti-Ubiquitin

IB: anti-Nrf2 (H300)

![Nuclear Extracts](image)

IP: anti-Ubiquitin

IB: anti-Nrf2 (H300)

FIG. 7. tBHQ stabilizes Nrf2 independent of PI3 kinase phosphorylation in IMR-32 cells. (A) IMR-32 cells were pretreated with LY294002 (25 μM) or vehicle (0.01% DMSO) for 40 min prior to tBHQ (10 μM) or vehicle (0.01% EtOH) treatment for 2 h or 24 h. Whole cell lysates were resolved by SDS-PAGE and immunoblotted with anti-Nrf2 (H300) or immunoprecipitated with anti-ubiquitin antibody and subsequently immunoblotted with anti-Nrf2 (H300) antibody (B) Enriched nuclear extraction was performed and immunoblotted with anti-Nrf2 (H300) antibody (upper panel) and anti-histone 1 antibody (lower panel) as the equal loading control. The representative Western blots were semi-quantified by the densitometry.
Neh2 (17–32 aa) and Neh6 (329–379 aa), which were proposed to regulate two modes of Nrf2 degradation, either homeostatic Keap1-dependent degradation or Keap1-independent degradation under oxidative stress conditions (Itoh et al., 2003; McMahon et al., 2003). The previously uncharacterized, redox-insensitive Neh6 degron is essential to ensure Nrf2 turned-over in stressed cells (McMahon et al., 2004). tBHQ induced Nrf2 stabilization may possibly be controlled via both pathways. Clarification of these two pathways involved in quinone compound induced Nrf2 stabilization will help us understand how these chemicals interfere with the ubiquitin-proteasome pathway.

Ubiquitin is a small protein with 76 aa (~8.6 KDa). The manner in which ubiquitin is coupled to a substrate has a direct impact on how a cell interprets the ubiquitin signal. When a chain of four or more ubiquitin monomers are linked to a substrate through Lys48 of ubiquitin, the substrate is marked for proteolysis by the 26S proteasome (Thrower et al., 2000). This mechanism leads us to deduce that after tetraubiquitin covalently
binds Nrf2, the MW of this complex would be \(~100\) Kda. However, this remained to be determined in the subsequent experiment. The polyubiquitin-conjugated Nrf2 is presumably more sensitive to proteasome mediated degradation and would resolve on SDS-PAGE to the same apparent MW recognized by Nrf2 antibodies presented herein.

We have demonstrated that tBHQ does not prevent Nrf2 ubiquitination but in fact it appears to stabilize the ubiquitinated Nrf2. Questions then arise as to how tBHQ prevents Nrf2 degradation and/or how tBHQ acts as a 26S proteasome inhibitor. In vitro proteasome activity assays clearly demonstrated that tBHQ was not a general 20S/26S proteasome inhibitor. Trypsin-like, chymotrypsin-like, and peptidyl-glutamyl peptide hydrolase activities were measured with the corresponding substrates, Leu-Leu-Arg-AMC, Suc-Leu-Leu-Val-Tyr-AMC, and Z-Leu-Leu-Glu-βNA. Relative activity (% control) was calculated for the 20S and the 26S proteasomes. As shown in Supplemental Table 2, MG132, a potent 20S/26S proteasome inhibitor (positive control), dramatically suppressed 20S and 26S proteasome activities. However, tBHQ treatment for 2 h or 24 h did not inhibit their activity clearly indicating that tBHQ is not a proteasome inhibitor. In fact, the data would suggest that tBHQ actually increases 20S and 26S proteasome activity.

Additional evidence showed that unlike the reversible 26S proteasome inhibitor MG132, tBHQ could not stabilize other short half-life transcriptional factors like c-fos suggesting its specificity in stabilization of Nrf2. Recent reports indicated that Keap1 (KIAA0132) appears to be involved in Nrf2 stabilization. Keap1-dependent proteasomal degradation of Nrf2 contributes to the negative regulation of ARE-driven gene expression (McMahon et al., 2003). All of these findings suggest that tBHQ may regulate Nrf2 stabilization through manipulation of its binding partner(s), and that this action occurs upstream of the ubiquitin-proteasome pathway.

We also tracked mRNA level changes of genes related to ubiquitin-proteasome pathway using microarray analysis. Interestingly, one of the ubiquitin C-terminal hydrolases (UCHs), UCH-L1 (also called PGP 9.5), was upregulated both in IMR-32 cells and hNSCs after tBHQ treatment. UCH-L1 is an abundant neuronal enzyme (1–2% of brain protein) of unknown function (Wilkinson et al., 1989). UCHs are generally small (200–300 aa) and function to cleave ubiquitin from protein adducts regenerating monomeric ubiquitin molecules leading to ubiquitin recycling (Larsen et al., 1998). The high steady-state level of ubiquitinated Nrf2 maintained after tBHQ treatment for up to 24 h indicates a precise balance between the rates of ubiquitination and deubiquitination. UCH-L1 may play a role in keeping this balance.

**Nrf2-Dependent ARE Activation, a Potential Antioxidant Response**

Treating cells with tBHQ, a strong inducer of phase II detoxification enzymes via activation of ARE, can protect cells from oxidative stress (Duffy et al., 1998; Murphy et al., 1989). The protective effect conferred by tBHQ may not simply be due to an increase in one gene but the coordinate up-regulation of many genes. We performed H2O2-induced oxidative stress and subsequent apoptosis in cultured human neuroblastoma cells and mouse primary neural cultures (Lee et al., 2003; Li et al., 2002). The apoptotic process and development of cell injury in H2O2-treated cells was attenuated through tBHQ activation of ARE. Microarray analysis dissected a cluster of detoxification enzymes and antioxidant genes, termed programmed cell life genes, which were coordinately upregulated and found responsible for tBHQ’s protective effect against oxidative damage. Previous studies showed that the majority of identified genes were actually Nrf2 dependent, as evidenced by microarray profiling of Nrf2 overexpression in glial-enriched cultures and mixed neuronal/glial cultures (Shih et al., 2003) as well as sorted glia and neurons treated with tBHQ (Kraft et al., 2004). All of these findings suggest that Nrf2 dependent ARE activation by tBHQ is responsible for upregulation of many key genes, such as HO1, NQO1, GCLR, Ferritin, thioredoxin reductase (TR), glutathione reductase (GR), copper/zinc superoxide dismutase (SOD1), and multiple heat shock proteins, that together construct a potent antioxidant network.

In CNS-related acute or chronic disorders, it is quite common for one affector, such as oxidative stress, to have the capacity to trigger either or both apoptotic and necrotic cell death. A combination of apoptosis and necrosis may occur in the same tissue in response to the same acute insult resulting in apoptotic and non-apoptotic neuronal death. Therefore, finding a common therapeutic strategy that may potentially attenuate multiple modes of cell death simultaneously would be an ideal approach for modulating to deal neurodegeneration. Nrf2 stabilization by pharmacological modulation or adeno virus-mediated Nrf2 overexpression, therefore, might be viable strategies to prevent a wide-spectrum of oxidative stress-related neuronal cell injuries. Furthermore, hNSCs in culture provide a source of tissue for toxicity assessment, developmental studies, and cell therapy (Wright et al., 2003). However, in vitro migration studies demonstrate hNSCs and their differentiated cells are sensitive to all types of stress including media changes, temperature, and chemical treatment (Carpenter et al., 1999; Studer et al., 2000; Svendsen et al., 1996). It also is difficult to maintain viability in vivo, where there is acute or chronic oxidative stress. Therefore, conferring the precursor cells with high antioxidant capacity may allow them to overcome the endogenous and exogenous oxidative stressors. This study is a first step to achieve this goal. Cells with this enhanced antioxidant potential could be valuable for clinical transplantation of stem cells in a variety of diseases.

**SUPPLEMENTARY DATA**

Supplementary data are available online at www.toxsci. oupjournals.org.
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