Sequential Upregulation of Superoxide Dismutase 2 and Heme Oxygenase 1 by *tert*-Butylhydroquinone Protects Mitochondria during Oxidative Stress^S

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ABSTRACT

Oxidative stress is linked to mitochondrial dysfunction in aging and neurodegenerative conditions. The transcription factor nuclear factor E2–related factor 2 (Nrf2)–antioxidant response element (ARE) regulates intracellular antioxidative capacity to combat oxidative stress. We examined the effect of *tert*-butylhydroquinone (tBHQ), an Nrf2–ARE signaling pathway inducer, on mitochondrial function during oxidative challenge in neurons. tBHQ prevented glutamate-induced cytotoxicity in an HT-22 neuronal cell line even with an 8-hour exposure delay. tBHQ blocked glutamate-induced intracellular reactive oxygen species (ROS) and mitochondrial superoxide accumulation. It also protected mitochondrial function under glutamate toxicity, including maintaining mitochondrial membrane potential, mitochondrial Ca²⁺ hemostasis, and mitochondrial respiration. Glutamate-activated, mitochondria-mediated

apoptosis was inhibited by tBHQ as well. In rat primary cortical neurons, tBHQ protected cells from both glutamate and buthionine sulfoximine toxicity. We found that tBHQ scavenged ROS and induced a rapid upregulation of superoxide dismutase 2 (SOD2) expression and a delayed upregulation of heme oxygenase 1 (HO-1) expression. In HT-22 cells with a knockdown of SOD2 expression, delayed treatment with tBHQ failed to prevent glutamate-induced cell death. Briefly, tBHQ rescues mitochondrial function by sequentially increasing SOD2 and HO-1 expression during glutamate-mediated oxidative stress. This study is the first to demonstrate the role of tBHQ in preserving mitochondrial function during oxidative challenge and provides a clinically relevant argument for using tBHQ against acute neuron-compromising conditions.

Introduction

Intracellular energy supply is highly dependent on oxidative phosphorylation in mitochondria. During ATP production, reactive oxygen species (ROS) are unavoidably generated as intermediates of oxygen reduction (Cadenas and Davies, 2000). Oxidative stress, caused by the failure of antioxidative defense against excessive ROS, leads to dysfunction of mitochondria and other subcellular organelles, and further triggers cell death. Thus, oxidative stress has been linked to mitochondrial dysfunction in aging and neurodegenerative conditions (Barnham et al., 2004).

Glutamate-induced oxidative stress is a known cause of pathologic cell death in neurons. This process is initiated by the depletion of antioxidant glutathione (GSH) synthesis by

blocking cysteine uptake, and is followed by an accumulation of ROS (Coyle and Puttfarcken, 1993; Choi, 1994). The ROS accumulation causes mitochondrial dysfunction and the release of apoptosis-inducing factor (AIF) from the mitochondria to the cytosol and nucleus and further leads to cell death (Landshamer et al., 2008; Fukui et al., 2009). In contrast, superoxide dismutase 2 (SOD2) acts as a primary mitochondrial antioxidative enzyme and protects against glutamate-induced oxidative damage (Fukui and Zhu, 2010).

The transcription factor nuclear factor E2–related factor 2 (Nrf2)–antioxidant response element (ARE) regulates intracellular antioxidative capacity to combat oxidative stress (Jaiswal, 2004). Under normal conditions, Nrf2 remains inactivated by binding to Kelch-like ECH-associated protein 1, which serves as a sensor of intracellular redox status (Itoh et al., 1999). Upon sensing of oxidative stress, phosphorylated Nrf2 dissociates with Kelch-like ECH-associated protein 1, translocates into the nucleus, and activates the transcription of ARE-driven genes (Huang et al., 2002; Apopa et al., 2008). ARE-driven genes are involved in production of a battery of antioxidant and phase 2 enzymes, which is a potent strategy

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ABBREVIATIONS: AIF, apoptosis-inducing factor; ARE, antioxidant response element; BSO, buthionine sulfoximine; AM, acetoxymethyl ester; DCF, dichlorofluorescein; ETC, electron transport chain; FACS, fluorescence-activated cell sorting; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; GSH, glutathione; H₂DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; NAO, nonylacridine orange; OCR, oxygen consumption rate; PBS, phosphate-buffered saline; ROS, reactive oxygen species; siRNA, small interfering RNA; tBHQ, *tert*-butylhydroquinone; TMRE, tetramethylrhodamine, ethyl ester.

to repress oxidative damage (Calkins et al., 2009). Among Nrf2-regulated phase 2 enzymes, heme oxygenase 1 (HO-1), the rate-limiting enzyme in catalysis of heme, has been reported to be critical for the protective effect of the Nrf2-ARE signaling pathway in neurodegenerative diseases (Sato et al., 2006; Li et al., 2012; Alfieri et al., 2013).

tert-Butylhydroquinone (tBHQ), an Nrf2 inducer, is a widely used food antioxidant (Yu et al., 1997). Previous studies have shown that tBHQ exerts protective effects in multiple neurodegenerative conditions, including stroke (Shih et al., 2005), traumatic brain injury (Jin et al., 2011; Lu et al., 2014), Parkinson's disease (Hara et al., 2003), and Alzheimer's disease (Eftekharzadeh et al., 2010; Akhter et al., 2011). However, the underlying mechanisms of tBHQ's protective role have not been elucidated. Using a glutamate-induced oxidative toxicity model in a mouse hippocampal neuronal cell line (HT-22 cells), we here demonstrate that tBHQ prevented glutamate-induced cell death even with an 8-hour treatment delay. tBHQ exerted protection against glutamate-induced mitochondrial dysfunction and inhibited mitochondria-mediated apoptosis. In addition, tBHQ protected primary cortical neurons from both glutamate and buthionine sulfoximine (BSO) toxicity. Interestingly, tBHQ not only scavenged free radicals but quickly activated the Nrf2-ARE signaling pathway. tBHQ rapidly upregulated SOD2 level followed by a delayed increase in HO-1 expression. By knocking down SOD2, we demonstrate that SOD2 is necessary for the early-phase protection of tBHQ and that SOD2 coordinates with HO-1 to defend against glutamate-induced oxidative damage. Our study clarifies the effect of tBHQ on mitochondrial function under conditions of oxidative challenge and provides a potential new therapeutic target for neurodegenerative disease.

Materials and Methods

Cell Culture. HT-22 cells were the generous gift of Dr. David Schubert (Salk Institute, San Diego, CA). Cells were maintained in

high-glucose Dulbecco's modified Eagle's medium (HyClone, South Logan, UT) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) in 75-mm tissue culture flasks (Corning, Tewksbury, MA) at standard cell culture conditions (5% CO₂, 95% air). HT-22 cells used were between passages 8 and 28.

Primary cortical neurons were prepared from embryonic day 17 Sprague-Dawley rats. The cortices were dissected and placed in Hanks' balanced salt solution (HyClone). Cells were mechanically dissociated by titration and filtered through 70- μ m cell strainers (BD Biosciences, San Jose, CA). Cells were maintained in minimal essential medium (American Type Culture Collection, Manassas, VA) supplemented with 4.4 g/l glucose and 10% heat-inactivated horse serum (Life Technologies, Carlsbad, CA). Cells (8×10^4 cells per well) were plated in poly-L-lysine (Sigma-Aldrich, St. Louis, MO)-coated 48-well plates (Corning). After 1 day in *in vitro* culture, cells were treated for 24 hours and morphologic changes were observed microscopically (EVOS FL Auto Imaging System; Life Technologies, Bothell, WA).

Cell Viability Assay. HT-22 cells were seeded in 96-well or 6-well plates (Corning) and were incubated overnight. After respective treatments, medium was removed and cells were incubated with 1 μ M calcein-acetoxymethyl ester (calcein-AM) (Molecular Probes, Grand Island, NY) in phosphate-buffered saline (PBS) for 15 minutes at 37°C. Calcein-AM, a nonfluorescent dye, is converted to a green fluorescent calcein by intracellular esterases. Fluorescence was measured using the BioTek Synergy H1 Hybrid plate reader (BioTek, Winooski, VT; excitation, 495 nm; emission, 516 nm). Calcein-AM led to the detachment of cells from the bottom of the wells after small interfering RNA (siRNA) transfection, an effect that compromises the assay. As such, to measure cell viability of siRNA-transfected cells, we used morphologic changes of cells after respective exposures as observed microscopically. For each well, pictures were randomly taken in three different fields. Based on the morphology, cells in each photomicrograph were counted and categorized into live cells and dead cells by UVP (Upland, CA) imaging software. The average cell number from three different pictures was calculated to represent cell viability. For the primary cortical neurons, viability of the cells was assessed using calcein-AM and imaged using fluorescent microscopy.

ROS Detection. Changes in intracellular ROS were measured by the ROS-reactive fluorescent indicator 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Molecular Probes). The nonfluorescent H₂DCFDA is converted to the highly fluorescent dichlorofluorescein

Fig. 1. tBHQ exerts a neuroprotective effect against glutamate toxicity in HT-22 cells. (A) HT-22 cells were exposed to glutamate (Glut) (5 mM) and tBHQ (1–25 μ M) for 24 hours. Cell viability was detected by calcein-AM assay ($n = 8$). (B) Cells were treated with glutamate at a concentration of 5 mM for 24 hours. tBHQ (10 μ M) treatment was applied at 0, 4, 8, 10, 12, or 14 hours after glutamate. Cell viability was detected by calcein-AM assay ($n = 8$), and (C) morphologic changes of cells were observed microscopically. Representative experiments were independently repeated three times. Results are reported as mean $\pm$ S.E.M. *** $P < 0.001$ compared with glutamate-treated cells (one-way analysis of variance, Tukey's test).

(DCF) by ROS (Fukui et al., 2010; Tobaben et al., 2011; Kang et al., 2014). Briefly, HT-22 cells were plated overnight at a density of 5000 cells/well in a 96-well plate. After respective exposures, the medium was removed and the cells were washed once with PBS and then incubated with 10 μ M H₂DCFDA for 30 minutes at 37°C. Mean fluorescence intensity of DCF was measured using the BioTek Synergy H1 Hybrid plate reader (excitation, 485 nm; emission, 530 nm). DCF fluorescence was standardized based on cell viability.

Mitochondrial Superoxide Measurement. MitoSOX Red (Molecular Probes) is a fluorogenic dye targeted to mitochondria and generates red fluorescence after oxidation by superoxide (Mukhopadhyay et al., 2007; Fukui and Zhu, 2010; Pfeiffer et al., 2014). The fluorescence signal was measured by fluorescence-activated cell sorting (FACS) analysis. Cells were harvested, washed once with ice-cold PBS, and stained with 5 μ M MitoSOX Red in Hanks' balanced salt solution for 10 minutes at 37°C. Cells were then washed twice with PBS before the red fluorescence intensity was analyzed using a flow cytometer (BD FACSCalibur; BD Biosciences, San Jose, CA). In each analysis, 10,000 events were recorded.

Mitochondrial Membrane Potential Analysis. HT-22 cells were plated at a density of 5000 cells/well and exposed to glutamate alone or in combination with tBHQ. The medium was then removed, and cells were incubated in PBS containing 1 μ M nonylacridine orange (NAO) (Molecular Probes) and 1 μ M tetramethylrhodamine, ethyl ester (TMRE) (Sigma-Aldrich) for 20 minutes at 37°C. Under normal mitochondrial intermembrane potential, TMRE enters into

mitochondria and quenches NAO. Collapse of mitochondrial membrane potential promotes NAO fluorescence. NAO fluorescence was measured using the BioTek Synergy H1 Hybrid plate reader (excitation, 485 nm; emission, 530 nm) and standardized based on cell viability.

Mitochondrial Ca²⁺ Detection. Mitochondrial Ca²⁺ was measured using Rhod-2 AM, a fluorogenic dye specifically targeted to mitochondrial Rhod-2 AM (Molecular Probes), which exhibits fluorescence upon binding Ca²⁺. Cells were incubated with 2 μ M Rhod-2 AM for 15 minutes at 37°C, washed with PBS twice, and analyzed immediately by flow cytometry (BD FACSCalibur). In each analysis, 10,000 events were recorded.

Mitochondrial Respiration Measurement. HT-22 cells were plated at a density of 15,000/well in an XF⁹⁶ plate (Seahorse Bioscience, North Billerica, MA). After respective exposures, the medium was exchanged 1 hour prior to the assay with XF assay medium (Seahorse Bioscience). Oligomycin (1 μ M), carbonilcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 0.5 μ M), and antimycin and rotenone mixture (1 μ M) (Sigma-Aldrich) were diluted into XF⁹⁶ medium and loaded into the accompanying cartridge. Injections of the components into the wells occurred at the time points specified. Oxygen consumption rate (OCR) was monitored using a Seahorse Bioscience XF⁹⁶ Extracellular Flux Analyzer.

Caspase-3/7 Activity. Caspase-3 and -7 activities were measured using a luminescence-based assay, Caspase-Glo 3/7 Assay (Promega, Madison, WI). According to the manufacturer's protocol, cells were

Fig. 2. tBHQ prevents glutamate-induced ROS and mitochondrial superoxide in HT-22 cells. (A) HT-22 cells were treated with glutamate (Glut) (5 mM) for 10 hours. tBHQ (10 μ M) was applied at 0 or 6 hours after glutamate exposure. ROS levels were detected by H₂DCF, and fluorescence was measured by plate reader ($n = 8$). MFI, mean fluorescence intensity. (B) tBHQ (10 μ M) was applied at 0 or 6 hours after glutamate (5 mM) exposure. Mitochondrial ROS was measured by MitoSOX after an 11-hour treatment and quantified by FACS analysis ($n = 3$). (C) Overlay of the FACS tracings for HT-22 cells stained with MitoSOX. All experiments were repeated three times, and the results indicate the mean $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with glutamate-treated cells (one-way analysis of variance, Tukey's test).

incubated with proluminescent caspase-3/7 substrate for 1 hour at room temperature. Following caspase cleavage, a substrate for luciferase is released, resulting in the luciferase reaction and the production of light. Luminescence was measured with the BioTek Synergy H1 Hybrid plate reader.

Immunocytochemistry. HT-22 cells were fixed with 4% para-formaldehyde (Sigma-Aldrich) in PBS for 15 minutes after respective exposures. The cells were permeabilized with 0.25% Triton X-100 for 10 minutes and were then incubated in 10% serum (Sigma-Aldrich) blocking solution containing 0.3 M glycine for 30 minutes. Cells were exposed to anti-AIF antibody or anti-Nrf2 antibody (1:100 in blocking solution; Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C, followed by incubation with appropriate fluorescence-conjugated secondary antibodies (Molecular Probes) for 1 hour. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Molecular Probes). Images were acquired using a fluorescence confocal microscope (Zeiss Violet Confocal; Zeiss, Oberkochen, Germany) with a 40× objective.

Nuclear Isolation. Cells were collected and resuspended in lysis buffer A (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.05% NP40; pH 7.9). Samples were left on ice for 10 minutes and centrifuged (Allegra 64R centrifuge; Beckman Coulter, Irving, TX) at 4°C at 3000 rpm for 10 minutes. Supernatant was removed; cell pellets were resuspended in lysis buffer B (5 mM HEPES, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 26% glycerol, 300 mM NaCl; pH 7.9) and homogenized with 20 full strokes in a glass homogenizer on ice. All the chemicals were ordered from Sigma-Aldrich. After the 30-minute incubation on ice, samples were

centrifuged at 24,000g for 20 minutes at 4°C. Supernatants were collected as the nuclear fraction.

Western Blot. For whole-cell lysis, cells were lysed in radio-immunoprecipitation assay buffer with cocktail protease inhibitors (EMD Millipore, Billerica, MA). Briefly, blots were probed with anti-AIF antibody (1:1000 dilution; Santa Cruz Biotechnology), anti-SOD2 antibody (1:1000 dilution; Santa Cruz Biotechnology), or anti-HO-1 antibody (1:1000 dilution; Abcam, Cambridge, MA) at 4°C overnight. Membranes were then exposed to the appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), followed by chemiluminescence detection (Fisher, Waltham, MA) of antibody binding. Equal protein loading was controlled by reprobing the membrane with anti-β-actin antibody or an anti-histone deacetylase 1 antibody (1:1000 dilution; Santa Cruz Biotechnology). Chemiluminescence was detected using the UVP ChemiDoc-It TS2 Imager, and UVP software was used for quantification of Western blot signals.

GSH Measurement. GSH level was measured using a luminescence-based assay, GSH-Glo Glutathione Assay (Promega). The assay was based on the conversion of a luciferin derivative into luciferin in the presence of GSH, catalyzed by glutathione S-transferase. According to the manufacturer's protocol, cells were incubated with GSH-Glo reagent for 30 minutes at room temperature, followed by a 15-minute incubation with luciferin detection reagent, and luminescence was measured with the BioTek Synergy H1 Hybrid plate reader.

Transfection. HT-22 cells were seeded at 4 × 10⁴ cells/well in 6-well plates at the time of transfection. siRNA selectively targeting

Fig. 3. tBHQ prevents glutamate-induced mitochondrial membrane potential disruption and mitochondrial Ca²⁺ overload in HT-22 cells. (A) Cells were treated with 5 mM glutamate (Glut). tBHQ (10 μM) was applied simultaneously or 6 hours after glutamate treatment. After an 11-hour treatment with glutamate, mitochondrial membrane potential was measured by NAO/TMRE assay. Fluorescence of NAO was measured by a plate reader (*n* = 8). (B) Cells were treated with glutamate (5 mM), and tBHQ (10 μM) was added at either 0 or 6 hours after glutamate. Mitochondrial Ca²⁺ was measured by Rhod-2 AM after an 11-hour treatment and quantified by FACS analysis (*n* = 3). (C) Histogram overlay representing the Rhod-2 levels in HT-22 cells. All experiments were repeated three times, and the results indicate the mean ± S.E.M. ***P* < 0.01; ****P* < 0.001 compared with glutamate-treated cells (one-way analysis of variance, Tukey's test).

mouse SOD2 (Santa Cruz Biotechnology) was used for transfection, and a scrambled nontargeting siRNA was used as the control. Transfections of the siRNA targeting SOD2 (50 pmol) or the scrambled control siRNA were performed using a siRNA transfection reagent (Santa Cruz Biotechnology) based on the protocol provided by the manufacturer. Transfection efficiency was observed microscopically, and SOD2 protein expression was determined by Western blot analysis after 24- or 36-hour transfection.

Statistical Analysis. The data are shown as means $\pm$ S.E.M. Statistical analyses were performed using one-way analysis of variance with Tukey's post hoc test or two-way analysis of variance with Bonferroni's post hoc test for multiple comparisons. GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA) was used for statistical analyses.

Results

tBHQ Protects HT-22 Cells against Glutamate-Induced Cytotoxicity. To evaluate tBHQ's protection against glutamate-induced cytotoxicity, we performed a calcein-AM cell viability assay in HT-22 cells. Glutamate (5 mM) reduced cell viability to 20% of control after a 24-hour exposure. With simultaneous exposure to tBHQ (1–25 μ M), glutamate-induced cell death was significantly ameliorated (Fig. 1A). At a concentration of 10 μ M, tBHQ reached the maximal protective effect without inducing cytotoxicity (Supplemental Fig. 1). Therefore, we selected an exposure of 10 μ M tBHQ for the following studies. To determine the duration of treatment delay with tBHQ, we exposed cells to 5 mM glutamate and applied tBHQ at 0–14 hours after the glutamate treatment. Cell viability was measured 24 hours after glutamate exposure. Surprisingly, with up to an 8-hour

delay in exposure, tBHQ rescued >75% of the cells from glutamate-induced cell death. This protection was attenuated when the exposure delay was prolonged to 10 hours. tBHQ also failed to protect cells against glutamate toxicity with 12-hour and 14-hour delayed exposure (Fig. 1B). Morphologic changes after glutamate and delayed exposure to tBHQ were observed microscopically (Fig. 1C).

tBHQ Prevents Glutamate-Induced ROS and Mitochondrial Superoxide Generation. Because ROS accumulation is a hallmark of glutamate-induced cell death in HT-22 cells (Tan et al., 1998), we determined whether tBHQ inhibited glutamate-induced intracellular ROS accumulation using an ROS-sensitive fluorescence indicator, H₂DCFDA. The accumulation of ROS was elevated by 2-fold after a 10-hour exposure to glutamate. Application of tBHQ at 0 and 6 hours after glutamate abrogated this ROS accumulation (Fig. 2A). A previous study revealed that glutamate also induced an increase in superoxide level in the mitochondria (Fukui et al., 2012). We then measured the mitochondrial superoxide level using the mitochondria-specific superoxide indicator MitoSOX. Using FACS analysis, we observed that glutamate induced a 2-fold increase of mitochondrial superoxide production. Even with a 6-hour exposure delay, tBHQ attenuated the accumulation of mitochondrial superoxide (Fig. 2, B and C).

tBHQ Prevents Mitochondrial Membrane Potential Disruption and Mitochondrial Calcium Overload Induced by Glutamate. It is well known that glutamate-induced oxidative damage causes the impairment of mitochondrial membrane potential and an increase of mitochondrial Ca²⁺ influx (Chen et al., 2003; Fukui et al., 2009). Thus, we

Fig. 4. tBHQ prevents the impairment of mitochondrial metabolism induced by glutamate. After a 12-hour treatment with 10 mM glutamate (Glut) and 10 μ M tBHQ, OCR was recorded by a Seahorse XF[®]96 flux analyzer ($n = 8$). (A) OCR recording at baseline and subsequent to treatment with 1 μ M oligomycin, 0.5 μ M FCCP, and a 1 μ M rotenone and antimycin mixture. ATP production (B), spare capacity (C), maximum respiration (D), and proton leak (E) were calculated. All experiments were repeated three times, and the results indicate the mean $\pm$ S.E.M. *** $P < 0.001$ compared with glutamate-treated cells (one-way analysis of variance, Tukey's test).

evaluated the role of tBHQ in glutamate-compromised mitochondrial membrane potential using the NAO/TMRE fluorescence resonance energy transfer assay, and we measured mitochondrial calcium levels using the fluorescent indicator Rhod-2 AM. Glutamate exposure induced a collapse of the mitochondrial intermembrane potential (Fig. 3A) and a mitochondrial Ca^{2+} overload (Fig. 3, B and C). Both simultaneous and delayed tBHQ exposures attenuated glutamate-induced mitochondrial membrane potential reduction and the increase in mitochondrial calcium levels. tBHQ alone did not have an appreciable effect on mitochondrial Ca^{2+} dynamic.

tBHQ Attenuates the Exacerbation of Mitochondrial Respiration under Glutamate Toxicity. Mitochondrial respiration deficiency is a key index of mitochondrial failure (Lin and Beal, 2006). To determine the effect of glutamate and tBHQ exposure on mitochondrial respiration, we measured OCR using a Seahorse XF[®]96 analyzer (Fig. 4A). Based on the OCR after application of stimuli, four parameters were calculated to evaluate mitochondrial respiration. Glutamate alone led to a 60% reduction in ATP production-linked respiration and fully abolished maximal respiration and spare capacity; cotreatment with tBHQ ameliorated these effects of glutamate (Fig. 4, B–D). Proton leak was not affected by glutamate or tBHQ (Fig. 4E).

tBHQ Blocks Mitochondria-Mediated Apoptosis under Glutamate Toxicity. Glutamate-induced oxidative stress triggers mitochondria-mediated apoptosis by activating caspase-3/7 and AIF translocation to the nucleus (Landshamer et al., 2008; Fukui et al., 2009). We explored the regulation by tBHQ of glutamate-induced mitochondria-mediated apoptosis. Caspase-3/7 was not activated upon exposure of HT-22 cells to glutamate (Fig. 5A), whereas the AIF level in the nuclear fraction was increased by almost 3-fold after 16 hours of exposure to glutamate. tBHQ coexposure inhibited glutamate-induced AIF translocation to the nucleus (Fig. 5B). These findings were confirmed by immunocytochemistry. There was a clear translocation of AIF (Fig. 5C, red) into the nucleus (Fig. 5C, blue) at 12 hours after glutamate exposure; cotreatment with tBHQ prevented AIF translocation and preserved cell morphology.

tBHQ Is Comparatively Ineffective against Electron Transport Chain Blockage-Induced Toxicity. In view of the ability of tBHQ to prevent mitochondrial dysfunction in response to glutamate, we determined if tBHQ protected cells from mitochondria-specific toxins. Exposure to oligomycin (20 μM), an ATP synthase inhibitor, increased cell death by ~40%, and this effect was partially rescued by tBHQ exposure (Fig. 6A). FCCP, a protonophore, disrupts the mitochondrial proton gradient by transporting protons across the membrane. Exposure of cells to FCCP (10 μM) alone reduced cell viability to 20% of control 24 hours after exposure; coexposure with tBHQ exerted moderate protection (Fig. 6B). Rotenone reduces oxidative phosphorylation by inhibition of mitochondrial complex I activity. Exposure of cells to rotenone (10 μM) induced 20% cell death, but tBHQ did not protect cells from rotenone-induced cytotoxicity in HT-22 cells (Fig. 6C). Compared with the efficacy of tBHQ's protection against glutamate-induced cytotoxicity, we conclude that tBHQ is comparatively ineffective against electron transport chain (ETC) blockage-induced toxicity in HT-22 cells.

Fig. 5. tBHQ inhibits glutamate-induced, mitochondrial AIF-mediated apoptosis. (A) After an 11-hour treatment with glutamate (Glut) (5 and 10 mM) and tBHQ (10 μM), caspase-3/7 activities were measured ($n = 3$). (B) After a 16-hour treatment with 15 mM glutamate and 10 μM tBHQ, AIF level in nuclear fraction was measured by Western blot. Quantitation of AIF was normalized to histone deacetylase 1 (HDAC₁). Bars represent normalized relative densities plotted as mean $\pm$ S.E.M. calculated from four independent experiments. (C) Immunocytochemistry for AIF (red) and 4',6-diamidino-2-phenylindole (DAPI; blue). Images were captured at a 12-hour exposure time to 5 mM glutamate and 10 μM tBHQ by confocal microscopy. All experiments were repeated at least three times, and the results indicate the mean $\pm$ S.E.M. *** $P < 0.001$ compared with glutamate-treated cells (one-way analysis of variance, Tukey's test).

tBHQ Causes a Rapid Upregulation of SOD2 Expression and a Delayed Upregulation of HO-1 Expression.

The neuroprotective treatment of tBHQ can be delayed up to 8 hours, the time before cells lose normal cell morphology, and we observed cell death after 11 hours of glutamate exposure (Supplemental Fig. 2). These data indicate that the protection of tBHQ is initiated within 3 hours after application. Next, we investigated the underlying mechanisms accounting for the

Fig. 6. tBHQ is comparatively ineffective against mitochondrial ETC blockage-induced toxicity. HT-22 cells were treated with 10 μ M oligomycin (Oligo) (A), 10 μ M FCCP (B), or 10 μ M rotenone (Rote) (C), plus 1–10 μ M tBHQ for 24 hours. Cell viability was measured by calcein-AM assay. Each experiment was repeated at least three times. Results are reported as mean $\pm$ S.E.M. * P < 0.05; *** P < 0.001 compared with ETC stimuli (oligomycin, FCCP, rotenone) treatment-only group (one-way analysis of variance, Tukey's test).

time course of this protection. Because GSH depletion is the primary cause of glutamate-induced toxicity, we first measured the GSH level after tBHQ and glutamate exposure. Glutamate induced a profound reduction in GSH synthesis, which was not ameliorated by tBHQ. However, tBHQ alone increased the intracellular GSH level by 20% (Fig. 7A). tBHQ, a phenolic compound, has been reported to be a free radical scavenger (Fig. 7B) (Alamed et al., 2009). We then determined if tBHQ served as an antioxidant by scavenging ROS. Within 30 minutes of exposure, tBHQ prevented H_2O_2 -induced

intracellular ROS accumulation (Fig. 7C). These data indicate that tBHQ does not prevent the reduction of GSH level induced by glutamate, but does function as a free radical scavenger to eliminate glutamate-induced oxidative stress. Further, we examined if tBHQ's protective effect worked through its free radical-scavenging activity. HT-22 cells were pretreated with tBHQ for 12 hours, and then cultures were incubated with glutamate in fresh medium without tBHQ. After 24-hour exposure to treatments, cell viability was measured. As shown in Fig. 7D, the protective effect was

Fig. 7. tBHQ fails to block glutamate-induced GSH depletion, but scavenges intracellular ROS. (A) GSH level was detected at 8 hours after treatment with 5 mM glutamate (Glut) and 10 μ M tBHQ (n = 5). (B) Chemical structure of tBHQ. (C) HT-22 cells were treated with H_2O_2 (12.5–100 μ M) and tBHQ (10 μ M) from 30 minutes. ROS levels were detected by H_2DCF , and fluorescence was measured by plate reader (n = 8). MFI, mean fluorescence intensity. (D) HT-22 cells were pretreated with tBHQ for 12 hours, and then cultures were incubated in glutamate in fresh medium without tBHQ. After 24 hours of exposure to treatments, cell viability was measured by calcein-AM assay (n = 8). Simultaneous treatment with tBHQ was used as a positive control. (E) Morphologic changes of cells were observed microscopically. Results are reported as mean $\pm$ S.E.M. ** P < 0.01, *** P < 0.001 compared with glutamate treatment-only group (one-way analysis of variance, Tukey's test).

observed 24 hours after removal of tBHQ. This indicates that the protection of tBHQ is not due to a radical-scavenging effect for this chemical. Morphologic changes after tBHQ and glutamate exposure were observed microscopically (Fig. 7E).

Next, we investigated if activation of Nrf2 and its regulated gene expression by tBHQ contributes to its protection. There was a clear translocation of Nrf2 (Fig. 8A, red) into the nucleus (Fig. 8A, blue) at 1.5 hours after tBHQ exposure; this colocalization was diminished with prolonged tBHQ exposure. HO-1 has been reported as an important target to prevent glutamate-induced oxidative damage in HT-22 cells (Satoh et al., 2003; Rössler et al., 2004). Therefore, we monitored the time-dependent change in HO-1 protein expression following tBHQ exposure. There was no significant change in HO-1 level within 3 hours of exposure of tBHQ; however, a 30-fold increase in HO-1 expression was observed at 12 hours after tBHQ treatment (Fig. 8B). Previous studies have demonstrated that the Nrf2-ARE signaling pathway regulates SOD2 expression (Dong et al., 2008; Piantadosi et al., 2008; Yan et al., 2010), and SOD2 plays a critical role in protecting HT-22 cells against glutamate-mediated cytotoxicity (Stocker et al., 1987). Our data showed a 2-fold increase in SOD2 expression after 3 hours of exposure to tBHQ (Fig. 8C), while SOD2 level returned to normal with prolonged tBHQ exposure (unpublished data). As such, we asked if this rapid upregulation of SOD2 expression is a key factor contributing to the protection offered by tBHQ.

Delayed Treatment with tBHQ Fails To Prevent Glutamate-Induced Cell Death in SOD2-Knockdown HT-22 Cells. To characterize the role of SOD2 in tBHQ-mediated protection, we transfected HT-22 cells with siRNA targeting SOD2. After 24- or 36-hour exposure to siRNA, a transfection efficiency of >90% was achieved. SOD2 expression was reduced by 45% after 24-hour transfection (Supplemental Fig. 3). As shown in Fig. 9A, SOD2 protein level was reduced by 65% at 36 hours after transfection, the time we selected for the following study. We compared the protection efficacy of tBHQ in scrambled siRNA-transfected and SOD2-knockdown HT-22 cells. After 18 hours of treatment with glutamate, cell morphologic changes were observed (Fig. 9B). In SOD2-knockdown HT-22 cells, simultaneous treatment with tBHQ was still able to protect cells from glutamate toxicity. However, silencing SOD2 attenuated the protective effect of delayed tBHQ exposure (Fig. 9C).

tBHQ Reduces Both Glutamate- and BSO-Mediated Cytotoxicity in Primary Cortical Neurons. Exposure of immature cortical neurons to glutamate or BSO has been previously shown to result in a time-dependent depletion of GSH (Li et al., 1997b). We exposed primary rat cortical neurons to either glutamate or BSO. A 24-hour exposure to 5 mM glutamate caused the disruption of neurites and the shrinkage of cell bodies. With simultaneous exposure to tBHQ (2.5–10 μ M), glutamate-induced cell damage was significantly ameliorated. Glutamate induced a marked decrease in calcein-AM fluorescence, which was protected with tBHQ exposure (Fig. 10A). Light microscopic analysis of cultures exposed to BSO (500 μ M) identified the disruption of neural networks and morphologic changes consistent with the calcein-AM data. Cotreatment with tBHQ (2.5–10 μ M) protected neurons from BSO-mediated cytotoxicity (Fig. 10B).

Fig. 8. tBHQ induces a rapid increase of SOD2 expression followed by a delayed upregulation of HO-1 expression. HT-22 cells were treated with 10 μ M tBHQ. (A) Immunocytochemistry for Nrf2 (red) and 4',6-diamidino-2-phenylindole (DAPI; blue). Images were captured at 1.5, 3, 6, 9, and 12 hours of exposure to tBHQ by confocal microscopy. Samples were also collected at 1.5, 3, 12, and 24 hours after tBHQ exposure. Cell extracts were subjected to immunoblot with antibodies specific for HO-1 (B) and SOD2 (C). Quantitation of HO-1 and SOD2 was normalized to β -actin. Bars represent normalized relative densities plotted as mean $\pm$ S.E.M. calculated from four independent blots. ** P < 0.01; *** P < 0.001 compared with control group (one-way analysis of variance, Tukey's test).

Fig. 9. Delayed treatment with tBHQ fails to overcome glutamate-induced cell death in a SOD2-knockdown HT-22 cell line. (A) HT-22 cells were transfected with scrambled and SOD2 siRNA for 36 hours. Transfection efficiency was measured by Western blot with antibody specific for SOD2. β -Actin was used to normalize loading. Bars represent normalized relative densities plotted as mean $\pm$ S.D. calculated from three independent blots (one-way analysis of variance [ANOVA], Tukey's test). (B) Both scrambled and SOD2 siRNA-transfected HT-22 cells were treated with 5 mM glutamate (Glut). tBHQ was applied either simultaneously or 8 hours after glutamate exposure. Morphologic changes of cells after respective treatments were observed microscopically. (C) Based on the morphology, cells in each photomicrograph were counted and calculated to represent cell viability. Experiments were repeated three times independently. Results are reported as mean $\pm$ S.E.M. *** P < 0.001 compared with glutamate treatment-only group (two-way ANOVA, Bonferroni's test).

Discussion

The present study demonstrates that tBHQ prevents cell death by GSH depletion-induced oxidative toxicity in both HT-22 cells and primary cortical neurons. In addition, this protection is observed with an 8-hour tBHQ treatment delay through blocking of glutamate-induced intracellular ROS accumulation and rescuing of mitochondrial function in HT-22 cells. Glutamate activates mitochondria-mediated apoptosis, which is also inhibited by tBHQ. Further, tBHQ activates the expression of the antioxidative enzymes SOD2 and HO-1, which contributes to its protective effect. This study is the first to demonstrate the role of tBHQ in preserving mitochondrial function during oxidative challenge.

Glutamate-induced excessive ROS accumulation leads to the loss of the proton gradient and disruption of the mitochondrial membrane potential. Our data demonstrate that tBHQ stabilizes mitochondrial membrane potential and maintains mitochondrial respiration under glutamate toxicity. The chemiosmotic hypothesis, identified by Peter Mitchell, describes the importance of mitochondrial membrane potential for mitochondrial ATP production (Mitchell, 1966). We speculate that tBHQ prevents mitochondrial membrane potential collapse by eliminating excessive ROS, which is positively correlated with improved mitochondrial metabolism. It is notable that tBHQ fully preserves ATP production-linked respiration with a mild recovery of mitochondrial spare capacity (Fig. 4). This indicates that tBHQ blocks glutamate-induced energy crisis, but the amount of extra ATP, which is produced in case of a sudden increase in energy demand, is not

fully recovered. It is well known that mitochondrial Ca^{2+} uptake regulates intracellular Ca^{2+} homeostasis (Rizzuto et al., 2012); mitochondrial Ca^{2+} overload induced by oxidative stress orchestrates execution of apoptosis (Mattson and Chan, 2003; Orrenius et al., 2003; Ott et al., 2007). Previous studies suggest that the truncation of AIF by calpain is necessary for its release from mitochondria and triggering apoptotic cell death (Susin et al., 1999; Cregan et al., 2002). Calpains are a family of Ca^{2+} -dependent cysteine proteases, which can be activated upon mitochondrial Ca^{2+} overload (Smith and Schnellmann, 2012). tBHQ blocks glutamate-induced mitochondrial Ca^{2+} overload (Fig. 3), which may contribute to its prevention of calpain activation. This provides an explanation of how tBHQ restrains AIF-mediated apoptosis under glutamate toxicity. Consistent with previous reports, no significant activation of caspase-3/7 was observed during glutamate-induced oxidative toxicity (Tan et al., 1998; van Leyen et al., 2005; Zhang and Bhavnani, 2006). Caspase activation during the initiation of apoptosis requires ATP (Li et al., 1997a; Hu et al., 1999; Fukui et al., 2009). Fukui et al. (2010) reported that the lack of caspase-3/7 activation may result from rapid onset of mitochondrial dysfunction and energy depletion induced by glutamate.

It has been shown that ROS accumulation adversely affects the mitochondrial ETC (Tan et al., 1998). Our observation that fully blocking glutamate-induced mitochondrial superoxide generation by tBHQ (Fig. 2) prompted us to investigate the regulation of tBHQ on ETC function. As previously reported, oligomycin impedes the conversion of ADP to ATP and induces a burst of cellular ROS levels (>10-fold) in HT-22

Fig. 10. tBHQ reduces both glutamate- and BSO-mediated cell death in immature primary rat cortical neurons. One-day-old primary cultures prepared from embryonic day 17 rats were treated with glutamate (5 mM), BSO (500 μ M), and tBHQ (2.5–10 μ M) for 24 hours. Phase-contrast images and calcein-AM staining fluorescence pictures were photographed. Representative experiments were repeated three times independently.

cells (Liu and Schubert, 2009). Similarly, inhibition of mitochondrial complex I activity by rotenone leads to a 3-fold increase in ROS, and cell death in HT-22 cells (Panee et al., 2007; Poteet et al., 2012). High concentration of FCCP causes a complete disruption of mitochondrial membrane potential and triggers the apoptotic signaling cascade (Dispersyn et al., 1999). Briefly, ETC is associated with ROS accumulation, and blockage of ETC leads to cell death. Our results reveal that tBHQ is comparatively ineffective against direct mitochondrial ETC inhibitors (Fig. 6). These data argue that tBHQ indirectly protects mitochondria against glutamate-induced toxicity. However, Holmström et al. (2013) reported that Nr2f2 directly regulates cellular energy metabolism through modulation of the availability of substrates for mitochondrial respiration.

Consistent with previous findings, we have observed that HT-22 cell death following exposure to 5 mM glutamate is delayed until 11 hours and maximal by 16 hours after exposure (Supplemental Fig. 2) (Tobaben et al., 2011). With up to an 8-hour exposure delay, tBHQ prevented glutamate-induced cell death. This indicates that, within 3 hours after application, tBHQ efficiently maintains mitochondrial function and further prevents cell damage. Upregulation of HO-1 expression has been shown to prevent glutamate-induced oxidative toxicity in HT-22 cells (Rössler et al., 2004; Son et al., 2013; Chao et al., 2014). However, the temporal profile of expression of SOD2 and HO-1 shows that peak SOD2 expression occurs at 3 hours but HO-1 expression does not peak until 12 hours following exposure to tBHQ (Fig. 8). This

indicates that elevation of HO-1 level is not the primary factor contributing to its acute protective effect. These data are similar to those from previous studies showing the time course of increased expression of SOD2 (Fukui et al., 2010) and HO-1 (Chao et al., 2014) in response to other polyphenols, suggesting that this temporal profile is a generalizable phenomenon. For simultaneous treatment of HT-22 cells with glutamate and tBHQ, both SOD2 and HO-1 expression were increased before cell death, which allows either or both to protect cells. However, only SOD2 expression was upregulated by delayed treatment with tBHQ and able to offer protection to cells. In SOD2-knockdown cells, simultaneous treatment with tBHQ was still protective to cells through an increase in HO-1 expression, which peaked at about the time that HT-22 cells began to die. However, with tBHQ exposure delay, absent a SOD2 response and given the long delay for the HO-1 response, cells are not protected (Fig. 9). In summary, tBHQ ameliorates glutamate-mediated cytotoxicity by sequentially increasing SOD2 and HO-1 expression. This coordinated activation of HO-1 with SOD2 is consistent with their roles as antioxidative enzymes. As shown in Fig. 11, SOD2 scavenges the highly cytotoxic mitochondrial superoxide ($O_2^{\cdot-}$) and converts it to hydrogen peroxide (H_2O_2). However, the detoxification of H_2O_2 requires biliverdin and bilirubin to serve as scavengers of the mitochondrial H_2O_2 . The formation of bilirubin relies on catalysis of HO-1 during heme metabolism (Stocker et al., 1987; Dore and Snyder, 1999). Therefore, we conclude that tBHQ activates the expression of antioxidative enzymes in a time-dependent

Fig. 11. Diagram of the protective effect of tBHQ against glutamate-induced oxidative stress in mitochondria. Through depletion of GSH synthesis, glutamate induces oxidative damage to mitochondria in HT-22 cells. Mitochondrial $O_2^{\cdot -}$ is eliminated by SOD2 and converted to H_2O_2 . The detoxification of H_2O_2 requires the participation of biliverdin and bilirubin, ROS scavengers, which rely highly on HO-1 activity. tBHQ induces a rapid increase of SOD2 expression and a delay in upregulation of HO-1 level. Sequential cooperation of SOD2 and HO-1 improves mitochondrial antioxidative ability and redox balance, which extricates cells from glutamate-induced oxidative stress.

sequence based on their physiologic function. In our study, we also found that tBHQ serves as an antioxidant by scavenging ROS (Fig. 7). However, our results showed that the protection of tBHQ was abolished in SOD2-knockdown cells when tBHQ exposure was delayed for a time that prevented HO-1 expression (Fig. 9). This indicates that free radical scavenging is not sufficient for tBHQ to rescue cells from glutamate toxicity. Overall, our study allows us to speculate that the protective effect of tBHQ is achieved by two mechanisms: a rapid upregulation of SOD2 and a delayed activation of HO-1 expression.

In our study, a major limitation was the method used to evaluate ROS generation. Even though DCF is a general indicator of the level of intracellular oxidative stress, as it is routinely used for this indication (Fukui et al., 2010; Tobaben et al., 2011; Kang et al., 2014), DCF does not identify the species of reactive oxygen that is elevated. Similarly, MitoSOX is routinely used to assay superoxide (Mukhopadhyay et al., 2007; Fukui and Zhu, 2010; Pfeiffer et al., 2014), and as it is taken up by mitochondria, it assays superoxide in this organelle (Robinson, et al., 2008). However, the MitoSOX indicator does not determine if the superoxide originates in the mitochondria. As the vast majority of superoxide is produced in mitochondria as a result of electron leak during oxidative phosphorylation (Brand et al., 2004; Rössler et al., 2004; Brand, 2010), we assumed that the identified superoxide came from mitochondria. The second limitation is that our study mainly focused on SOD2 and HO-1, which was based on our review of the literature and evidence that overexpression of SOD2 or HO-1 attenuated glutamate-induced cell death in HT-22 cells (Rössler et al., 2004; Fukui and Zhu, 2010). However, it is well known that Nrf2 induces the expression of

a wide range of enzymes involved in the maintenance of mitochondrial and cellular redox homeostasis (Panee et al., 2007; Bell and Hardingham, 2011; Ray et al., 2012). Notably, we found that a 6-hour delay of exposure to tBHQ inhibits glutamate-induced ROS generation at 7.5 hours after glutamate exposure (Supplemental Fig. 4). This indicates that glutamate-induced ROS accumulation was attenuated by tBHQ even before SOD2 expression was elevated. Our results suggest that other antioxidative enzymes may mediate protection by tBHQ. In addition, multiple pathways regulate the expression of SOD2 and HO-1 (Immenschuh and Ramadori, 2000; Miao and St. Clair, 2009). Therefore, the Nrf2-ARE signaling pathway may not be the only factor activating the transcription of these enzymes in the current model, which needs to be addressed in future studies.

It is very important to note that tBHQ also protects against oxidative stress-induced death in primary cortical neurons. This experiment was done in an effort to confirm that the profound protective effect of tBHQ on oxidative stress-mediated cell damage was not specific to a transformed cell line. These significant data may provide a clinically relevant argument for using tBHQ against acute neuron-compromising conditions.

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Authorship Contributions

Participated in research design: Sun, Ren, Simpkins.

Conducted experiments: Sun, Ren.

Performed data analysis: Sun, Ren, Simpkins.

Wrote or contributed to the writing of the manuscript: Sun, Ren, Simpkins.

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