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The Cytoprotective Effect of N-acetyl-L-cysteine against ROS-Induced Cytotoxicity Is Independent of Its Ability to Enhance Glutathione Synthesis

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2,3,5-Tris(glutathion-*S*-yl)-hydroquinone (TGHQ), a metabolite of hydroquinone, is toxic to renal proximal tubule epithelial cells. TGHQ retains the ability to redox cycle and create an oxidative stress. To assist in elucidating the contribution of reactive oxygen species (ROS) to TGHQ-induced toxicity, we determined whether the antioxidant, N-acetyl-L-cysteine (NAC), could protect human kidney proximal tubule epithelial cells (HK-2 cell line) against TGHQ-induced toxicity. NAC provided remarkable protection against TGHQ-induced toxicity to HK-2 cells. NAC almost completely inhibited TGHQ-induced cell death, mitochondrial membrane potential collapse, as well as ROS production. NAC also attenuated TGHQ-induced DNA damage and the subsequent activation of poly (ADP-ribose) polymerase and ATP depletion. Moreover, NAC significantly attenuated c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase phosphorylation induced by TGHQ. In contrast, NAC itself markedly increased extracellular regulated kinase1/2 (ERK1/2) activation, and the upstream mitogen-activated protein/extracellular signal-regulated kinase kinase inhibitor, PD-98059, only partially inhibited this activation, suggesting that NAC can directly activate ERK1/2 activity. However, although NAC is frequently utilized as a glutathione (GSH) precursor, the cytoprotection afforded by NAC in HK-2 cells was not a consequence of increased GSH levels. We speculate that NAC exerts its protective effect in part by directly scavenging ROS and in part via ERK1/2 activation.

Key Words: **HK-2 cells; N-acetyl-L-cysteine; reactive oxygen species; 2,3,5-tris(glutathion-*S*-yl)-hydroquinone; glutathione; mitogen-activated protein kinase.**

Hydroquinone (1,4-benzenediol, HQ) is ubiquitously present in the environment as a natural ingredient in plants, including vegetables, fruits, grains, and in wine. It is also produced in large amounts and widely used in the food industry, in cosmetics, as well as the pharmaceutical industry (DeCaprio, 1999; Devillers *et al.*, 1990). Humans are also exposed to HQ indirectly via the biotransformation of benzene, which exists in our environment mainly from the inhalation of gasoline vapors. Accordingly, HQ is rapidly absorbed via either ingestion (dietary intake) or inhalation. After absorption, HQ is metabolized by conjugation with either sulfate or glucuronic acid. The fraction of HQ that escapes these conjugation reactions can be readily and sequentially oxidized to 1,4-benzoquinone (1,4-BQ) followed by the reductive addition of glutathione (GSH), leading to the eventual formation of 2,3,5-tris(glutathion-*S*-yl)-hydroquinone (TGHQ), a far more toxic compound than HQ (Lau *et al.*, 1988a,b; Monks, 1995). In particular, TGHQ targets the redox and electrophilic properties of HQ/1,4-BQ to specific cell types, including renal proximal tubular epithelial cells, resulting in nephrotoxicity (Dong *et al.* 2004a; Monks and Lau, 1994). Thus, TGHQ retains the ability to redox cycle and to create an oxidative stress (Towndrow *et al.*, 2000).

N-acetyl-L-cysteine (NAC) is frequently employed as a source of sulfhydryl groups to cells as an acetylated precursor of reduced GSH. NAC can also interact directly with reactive oxygen species (ROS) and nitrogen species because it is a scavenger of oxygen free radicals (Zafarullah *et al.*, 2003). The protective effect of NAC on renal injury has been demonstrated in various models, including ischemia-reperfusion injury (Nitescu *et al.*, 2006), cisplatin- (Luo *et al.*, 2008) and glycerol-induced acute kidney injury (Kim *et al.*, 2010), and chronic kidney disease (Shimizu *et al.*, 2005). Moreover, animal and human studies of NAC suggest that it is a very safe and effective tool for the treatment of many diseases considered to be mediated by oxidant free radical damage, and it has been used therapeutically in several disorders related to oxidative stress (Cotgreave, 1997). The present studies were therefore designed to determine the mechanisms by which NAC might provide protection against chemical-induced renal injury, using TGHQ as a model renal toxicant known to produce ROS.

MATERIALS AND METHODS

Chemicals and reagents. TGHQ was synthesized and purified in our laboratory as previously described (Lau *et al.*, 1988a). TGHQ is nephrotoxic and

nephrocarcinogenic in rats and therefore must be handled with protective clothing in a ventilated hood. PD-98059 and PJ34 were obtained from CalBiochem (La Jolla, CA). 5,5′6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Invitrogen (Carlsbad, CA). Antibodies for phospho-histone H2AX (Ser139), H2AX, phospho-extracellular regulated kinase1/2 (ERK1/2), ERK1/2, phosphor-p38 mitogen-activated protein kinase (p38 MAPK), p38 MAPK, phosphor-c-Jun NH2-terminal kinase (JNK)1/2, and JNK1/2 were all purchased from Cell Signaling Technology, Inc. (Danvers, MA). The antibody for poly(ADP-ribose) was from Biomol International (Plymouth Meeting, PA). Goat anti-rabbit immunoglobulin G (IgG) and goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Colorimetric Glutathione Detection kit was obtained from BioVision Corporate Headquarters. CellTiter 96 AQueous One Solution Cell Proliferation Assay 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) kit and CellTiter-Glo Luminescent Assay kit were products of Promega BioSciences (Promega, Madison, WI). All other chemicals were purchased from Sigma (St. Louis, MO).

Cell culture and treatment regimen. HK-2 cells, an immortalized proximal tubular epithelial cell line from normal adult human kidney, and were obtained from the American Type Culture Collection (Manassas, VA). HK-2 cells were cultured in Keratinocyte Serum-Free Medium supplemented with 0.05 mg/ml bovine pituitary extract and 5 ng/ml epidermal growth factor and were maintained at 37°C in a humidified atmosphere of 5% CO_2, and subcultured every 3 days at 90% confluence. Cells were plated in 96-well, 24-well, or 6-well plates and grown to ~80% confluence before treatment. Cells were then washed once and treated with various agents in Dulbecco's modified eagle medium (DMEM) containing 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).

Synthesis and purification of TGHQ. TGHQ was synthesized and purified according to established protocols (Lau *et al.*, 1988a). In brief, an equal volume of 167mM GSH solution was added drop wise to a 167mM 1,4-BQ solution with stirring. The reaction mixture was incubated at room temperature with constant stirring for an additional 30 min and then extracted twice with three volumes of ethyl acetate to remove the residual 1,4-BQ and HQ formed by reduction during the reaction. The aqueous phase was collected, rotary-evaporated, and lyophilized. The resulting light green powders containing multi-GSH-HQ conjugates were purified by high-performance liquid chromatography (HPLC).

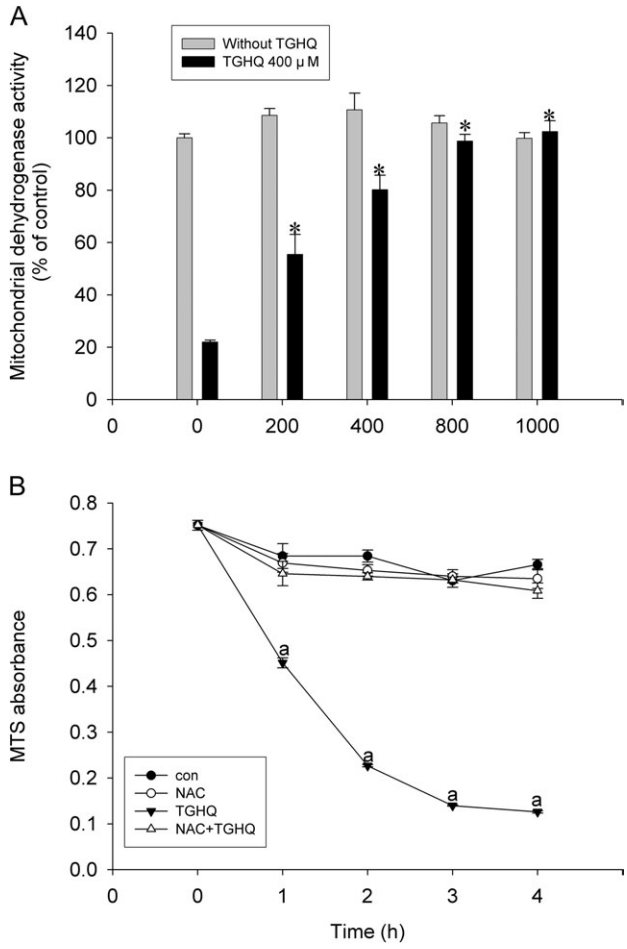

FIG. 1. NAC protects against TGHQ-induced HK-2 cell death. (A) Cells were treated with 400μM TGHQ with or without different concentrations of NAC for 4 h. Cytotoxicity was determined with MTS-based assay. *, $p < 0.001$, (TGHQ + NAC) treatment group compared with TGHQ treatment group. (B) Cells were treated with 1mM NAC, 400μM TGHQ, or NAC and TGHQ cotreatment for different time periods. Cytotoxicity was assayed with the MTS assay. Data represent the mean ± SE ($n \geq 3$). (a) $p < 0.001$, one-way ANOVA followed by a Dunnett's *post hoc* test compared with control group.

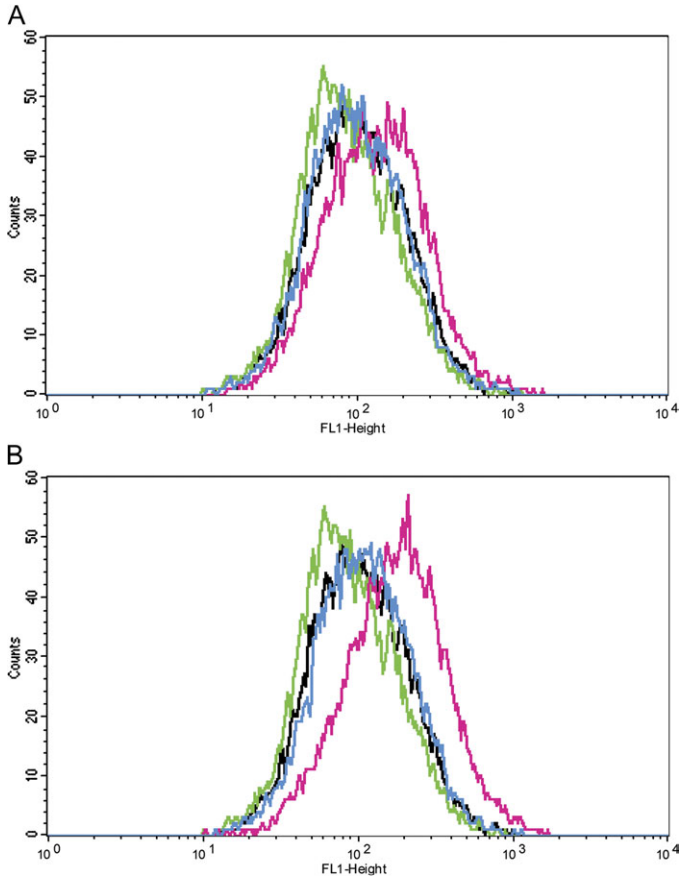

FIG. 2. NAC abolishes TGHQ-dependent ROS accumulation in HK-2 cells. ROS production was measured with DCFH-DA using flow cytometry. Cells were treated with 400μM TGHQ (A) or 600μM TGHQ (B) with or without 1mM NAC for 0.5 h or treated with 1mM NAC alone. In the graph, the middle graph represents control, the left graph represents NAC treatment alone, the right graph represents TGHQ treatment group, and the middle graph represents TGHQ and NAC cotreatment group. Data shown are typical representatives of three independent experiments.

The crude compound was dissolved in 1% acetic acid in water and injected onto a Beckman octadecylsilane reverse phase semipreparative column (10 mm × 25 cm × 5 μm). The compound was eluted with 1% acetic acid in water and 1% acetic acid in methanol mobile phases (2.7:0.3) at a flow rate of 3 ml/min. The elution is isocratic till 25 min when the elution becomes gradient from (water phase/methonal phase, 2.7:0.3) to (0:3.0) in 5 min and keeps this ratio for 5 min. Then it turns to gradient from (0:3.0) to (2.7:0.3) in about 3 min and keeps this ratio for about 3 min. Fractions containing TGHQ were collected according to the retention time of the peak, which has been verified by mass spectrometry, rotary-evaporated, frozen at −80°C, and lyophilized. The retention time for TGHQ is about 9.6 min in our system. The purity of TGHQ (> 98%) was confirmed by HPLC analysis.

Mitochondrial dehydrogenase cell viability assay. To determine the viability of HK-2 cells after treatment with various agents, cells were seeded in 96-well plates at a density of 1×10^4 cells per well and grown to 80% confluence. Cells were then washed once with DMEM, without phenol red, containing 25mM HEPES and treated with TGHQ dissolved in DMEM/HEPES (pH = 7.2–7.4) in the presence or absence of various chemical modulators. Cytotoxicity was assessed with the mitochondrial dehydrogenase activity assay according to the manufacturer's instructions (Promega Madison, WI), in which a tetrazolium compound, MTS, is reduced into a formazan product. After treatment for indicated time, cells were washed twice with DMEM/HEPES, and then 20 μl MTS solution was added to 100 μl DMEM/HEPES and incubated for 2 h at 37°C. The absorbance of the formazan at 490 nm was measured directly from 96-well plates using a microplate reader.

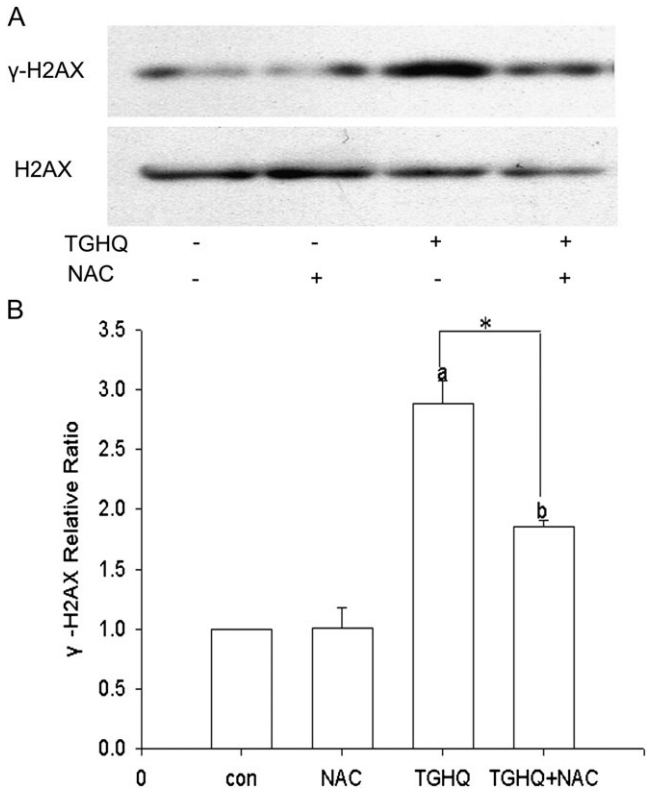

FIG. 3. NAC attenuates TGHQ-induced DNA damage (H2AX phosphorylation) in HK-2 cells. (A) Cells were treated with 400μM TGHQ for 1 h in the presence or absence of NAC. Histone was acid-extracted and examined by Western blot analysis. DNA strand breaks were determined using phospho-H2AX ser139 antibody. (B) Quantitative analysis of Western blots demonstrated in (A) by densitometry. Values are expressed as fold changes relative to the appropriate controls. Values are given as mean ± SE ($n = 3$). (a) $p < 0.001$ and (b) $p < 0.01$, one-way ANOVA followed by a Dunnett's *post hoc* test compared with control group; *, $p < 0.001$, (TGHQ + NAC) treatment group compared with TGHQ treatment group.

Detection of intracellular oxidative stress by flow cytometry. Analysis of TGHQ-induced generation of intracellular oxidative stress was determined by flow cytometry using DCFH-DA as a sensitive nonfluorescent precursor dye according to a published standard procedure (Yang *et al*., 2005). DCFH is generally considered a probe not only for H_2O_2 in presence of cellular peroxidases but also for the determination of $ONOO^{\bullet}$, and $HO^{\bullet}$. However, when used in cellular systems, DCFH is a general marker of oxidative stress rather than a specific indicator H_2O_2 formation or other ROS and reactive nitrogen species (Gomes *et al*., 2005). Particularly, HK-2 cells were seeded on 6-well plates at 2×10^5 cells per well and cultured for 48 h. Cells were washed with DMEM/HEPES and incubated with 10μM DCFH-DA for 30 min in the incubator before exposure to TGHQ in the presence or in the absence of various agents. Following treatment, cells were washed with Hank's Buffered Salt Solution (HBSS) and incubated with 0.25% trypsin-EDTA for 1–2 min, and trypsinization was quenched with DMEM with 10% fetal bovine serum (FBS). Detached cells were then collected by centrifugation at 120 × g for 5 min and resuspended in PBS. The fluorescence was determined by flow cytometery, with excitation at 495 nm and emission at 525 nm.

Histone extraction. HK-2 cells were washed with 3 ml PBS and lysed in 1 ml ice-cold hypotonic lysis buffer (10mM Tris-HCl, pH 8.0, 1mM KCl, 1.5mM $MgCl_2$, and 1mM dithiothreitol (DTT)). Histones were extracted with 0.4 N H_2SO_4 rotating overnight at 4°C and then precipitated in trichloroacetic acid on ice for 0.5 h. The resulting pellet was washed with ice-cold acetone. Pellets were dried in air and subjected to Western blot analysis.

Western blot analysis. Approximately 1×10^6 cells were lysed in buffer containing 20mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM Na_2EDTA, 1mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid, 1% Triton X-100, 5mM sodium pyrophosphate, 10mM sodium glycerophosphate, 50mM NaF, 1mM Na_3VO_4, 1mM PMSF, and complete protease inhibitor tablet (Roche, Indianapolis, IN). After incubation for 15 min on rotator in cold room, cell lysates were centrifuged to remove cell debris. Protein concentrations were determined with detergent-compatible reagent (Bio-Rad Laboratories, Hercules, CA). Samples were incubated with sample buffer and boiled for 5 min at 100°C and then resolved in SDS polyacrylamide gels. After electrophoresis, proteins were transferred to polyvinylidene fluoride Immobilon-P membranes (Millipore, Billerica, MA). Membranes were stained with 0.2% Ponceau S to assure equal loading of samples. After blocking with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20, membranes were incubated with primary antibody overnight at 4°C and then incubated with secondary antibody coupled with the horseradish peroxidase for 1 h at room temperature. Immunoblots were developed with enhanced chemoluminescence reaction (Amersham, Piscataway, NJ) and exposed to x-ray film.

Cellular ATP assay. HK-2 cells were seeded in opaque-walled 96-well plates at a density of 5000 cells/well and allowed to grow for 48 h. Cells were treated with test compound. At various time points, ATP content per well was determined using the CellTiter-Glo luminescent assay (Promega) according to the manufacturer's instructions. The luminescence was measured directly from 96-well plates using a microplate reader and differences expressed as means ± SEs ($n = 3$).

Determination of mitochondrial membrane potential. HK-2 cells were seeded at 2×10^5 cells per 6-well plate, and allowed to grow for 48 h. Log-phase cells were washed with DMEM/HEPES and treated with TGHQ in the presence or absence of different agents. Following treatment, cells were washed with HBSS and incubated with 0.25% trypsin-EDTA for 1–2 min, and trypsinization was quenched with DMEM with 10% FBS. Detached cells were collected by centrifugation at 120 × g for 5 min, resuspended in 5 μg/ml JC-1, and incubated in a 37°C humidified incubator with 5% CO_2 for 15 min. After the staining, cells were washed with PBS and then analyzed via

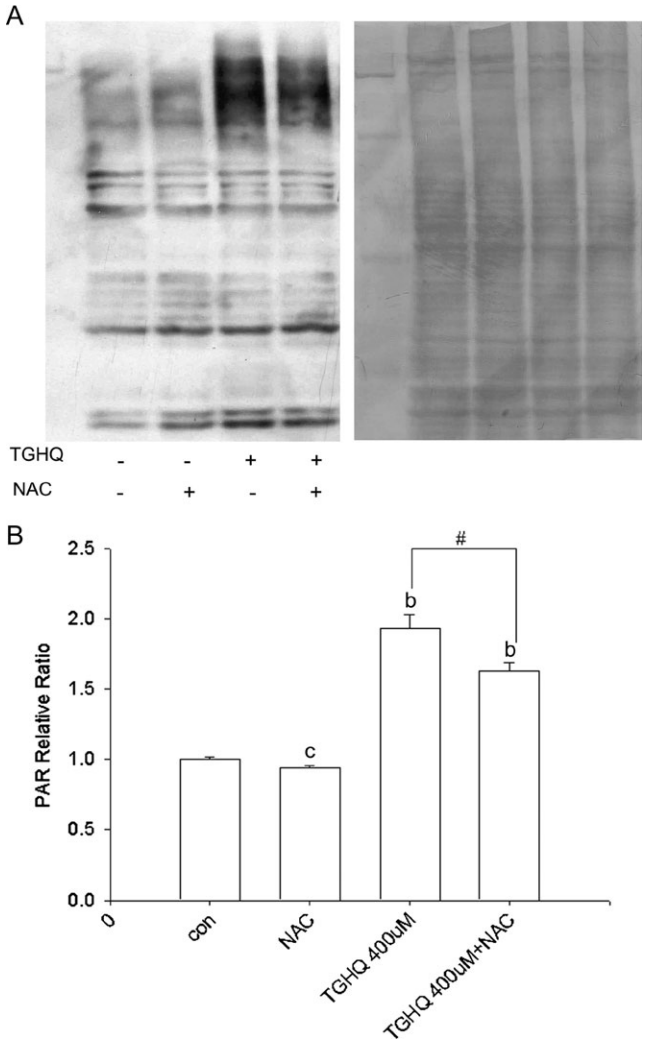

FIG. 4. NAC attenuates TGHQ-induced PAR accumulation. (A) Cells were treated with 400μM TGHQ for 20 min together with or without 1mM of NAC. PAR accumulation was determined with a PAR antibody, SA-216. Equal loading was confirmed by Ponceau S staining. (B) Quantitative analysis of Western blots demonstrated in (A) by densitometry. Values are expressed as fold changes relative to the appropriate controls. Values are given as mean ± SE ($n = 3$). (b) $p < 0.01$ and (c) $p < 0.05$, one-way ANOVA followed by a Dunnett's *post hoc* test compared with control; #, $p < 0.01$, (TGHQ+NAC) treatment group compared with TGHQ treatment group.

flow cytometry with excitation at 488 nm and emission at 530 nm at the Arizona Cancer Center.

Determination of total cellular GSH content. Pharmacological modulation of intracellular GSH content was analyzed using the colorimetric GSH detection kit from BioVision based on the enzymatic recycling method involving oxidized glutathione (GSSG) reductase and 5,5′-dithiobis-2-nitrobenzoic acid (Ellman's reagent). Briefly, 0.5×10^6 cells were lysed in ice-cold GSH buffer and centrifuged at 700 × g for 5 min at 4°C. Supernatants were used to assay GSH by mixing with a reduced form of nicotinamide adenine dinucleotide phosphate generating mix, (GSSG) reductase, and GSH reaction buffer according to the manufacturer's instructions. The absorbance was read at 405 nm using a microplate reader.

Statistical analysis. Results are expressed as mean ± SE. Statistical differences between the treated and control groups were determined by

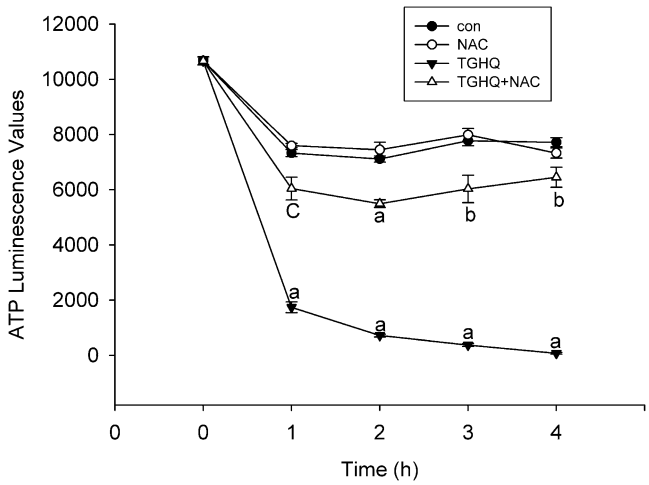

FIG. 5. NAC attenuates TGHQ-induced ATP depletion in HK-2 cells. Cells were treated with 400μM TGHQ for different time periods with or without NAC treatment. ATP content was determined using ATP Luminescent Assay. Data represent the mean ± SE ($n \geq 3$). (a) $p < 0.001$, (b) $p < 0.01$, and (c) $p < 0.05$, one-way ANOVA followed by a Dunnett's *post hoc* test compared with control.

Student's paired *t*-test. Differences between groups were assessed by one-way ANOVA using the SPSS software package for Windows. Differences between means were considered significant if $p < 0.05$.

RESULTS

NAC Protects against TGHQ-Induced HK-2 Cell Death

We first examined the effect of NAC on TGHQ-induced HK-2 cell death. As shown in Figure 1A, TGHQ (400μM) induced cytotoxicity in HK-2 cells, and cotreatment with NAC offered a concentration-dependent protection against TGHQ-induced toxicity. In particular, 1mM NAC provided complete protection against TGHQ. Figure 1B illustrates the time-dependent toxicity of TGHQ in HK-2 cells, as determined via the MTS assay, and similarly, NAC offered almost complete protection against TGHQ-induced toxicity.

NAC Inhibits TGHQ-Induced ROS Production in HK-2 Cells

TGHQ catalyzes the production of ROS, and TGHQ-induced cytotoxicity in LLC-PK1 cells is dependent upon the formation of ROS (Dong *et al.*, 2004b). We therefore examined the ability of TGHQ to induce ROS production in HK-2 cells using DCFH-DA as an indicator. TGHQ induced the dose-dependent formation of intracellular ROS (Fig. 2). Almost complete inhibition of TGHQ-induced ROS production was observed when exposure occurred in the presence of NAC (Fig. 2), providing pharmacological evidence in support of the protective mechanism of NAC against TGHQ toxicity. We also assayed for nitrite (NO_2^-), one of two primary stable and nonvolatile breakdown products of nitric oxide using the Griess Reagent System; however, there was no

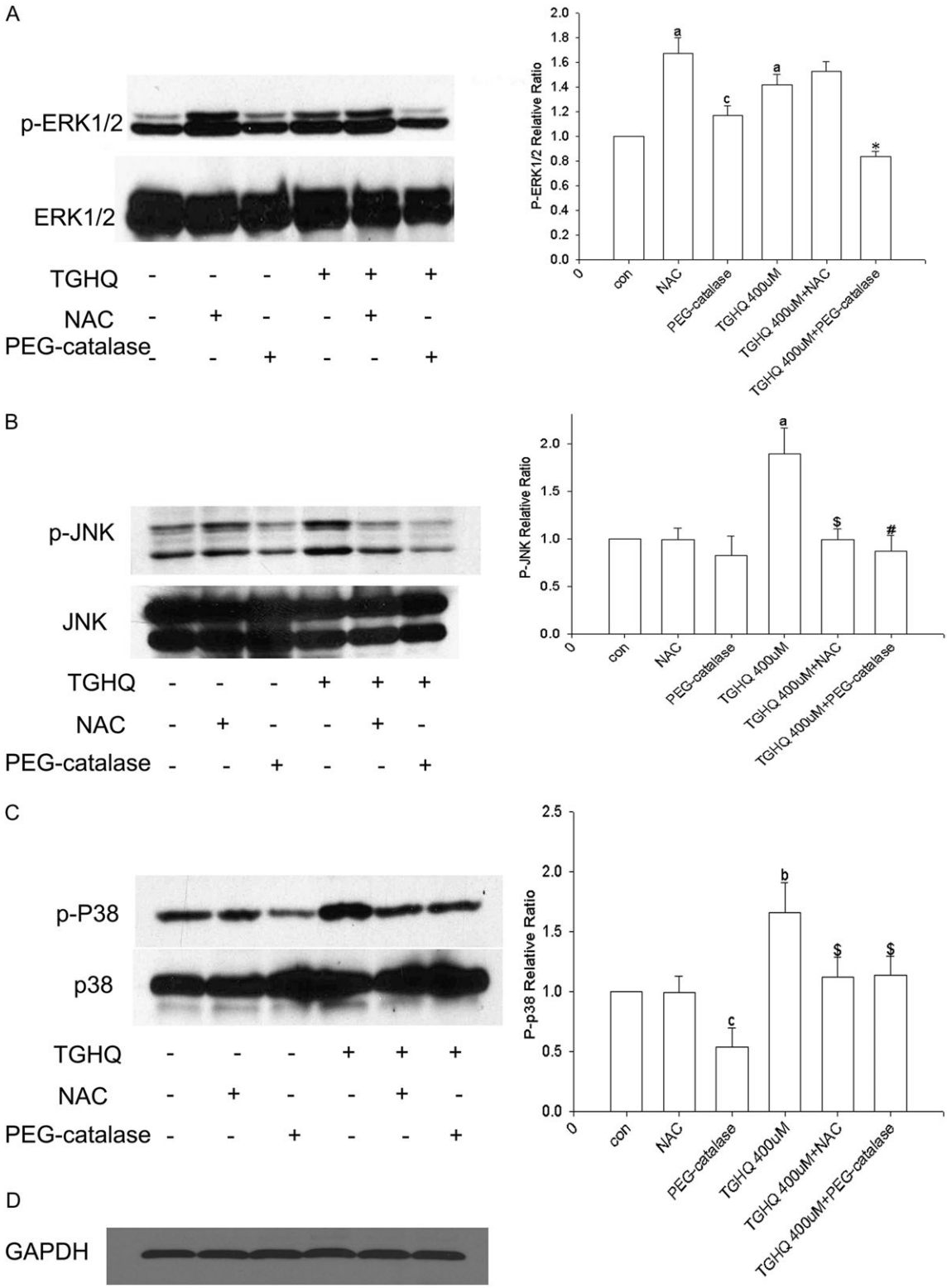

FIG. 6. NAC and Catalase-polyethylene glycol modulate TGHQ-induced MAPK activation in HK-2 cells. Cells were treated with 400μM TGHQ in the presence or absence of 1mM NAC or 17.5 IU Catalase-polyethylene glycol for 1 h. Total cell lysates were prepared, and Western blot analyses were performed. Phosphorylated and unphosphorylated forms of ERK1/2 (A), JNK-p54/p46 (B), and p38 (C) were detected accordingly. GAPDH was detected as loading control (D). Data shown are typical representatives of three independent experiments. The quantitative analyses of Western blots were analyzed by densitometry. Values are expressed as fold changes relative to the appropriate controls. Values are given as mean ± SE ($n = 3$). (a) $p < 0.001$, (b) $p < 0.01$, and (c) $p < 0.05$ compared with control. *, $p < 0.001$; #, $p < 0.01$; and $, $p < 0.05$ compared with TGHQ treatment group.

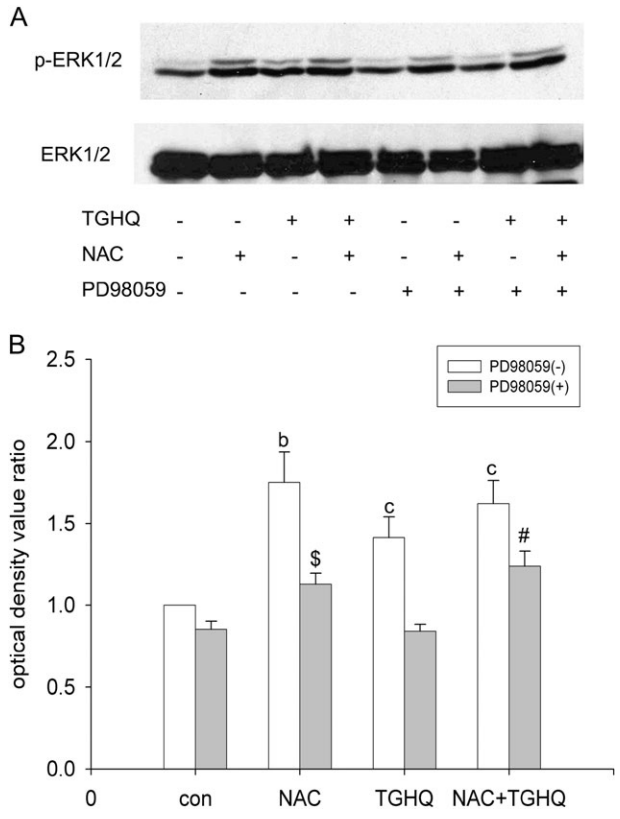

FIG. 7. PD-98059 modulates NAC- and TGHQ-induced ERK1/2 activation in HK-2 cells. (A) Cells were treated with 400μM TGHQ in the presence or absence of 1mM NAC. Alternatively, cells were pretreated with 50μM PD-98059 followed by 400μM TGHQ in the presence or absence of 1mM NAC. Total cell lysates were prepared, and Western blot analyses were performed. Phosphorylated and unphosphorylated forms of ERK1/2 were detected accordingly. (B) The intensity of the bands was represented by a percentage of control. Results shown are the means ± SEs of three independent experiments. (b) $p < 0.01$ and (c) $p < 0.05$ compared with control. #, $p < 0.01$ and $, $p < 0.05$ compared with control group pretreatment with PD-98059.

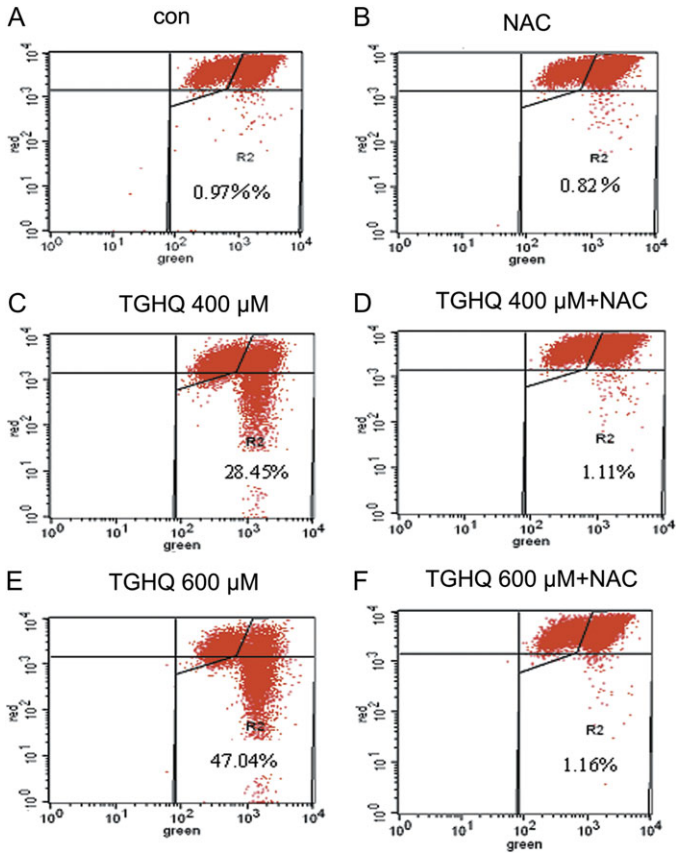

FIG. 8. NAC almost completely inhibits TGHQ-induced decreases in MMP. MMP was measured with JC-1 using flow cytometry. Cells were treated with different concentrations of TGHQ with or without 1mM NAC for 4 h or treated with 1mM NAC alone. Changes in MMP were determined. Cells in the lower right hand quadrant exhibit loss of MMP. Data shown are typical representatives of three independent experiments.

detectable nitrite found following exposure of cells to TGHQ (data not shown).

NAC Attenuates TGHQ-Induced H2AX Phosphorylation in HK-2 Cells

ROS are capable of damaging to macromolecules, such as proteins and DNA (Droge, 2002; Goodarzi *et al.*, 2010; Kulbacka *et al.*, 2009). We therefore determined whether TGHQ could induce DNA damage in HK-2 cells and, if so, whether NAC could protect against this damage. DNA damage was assessed by determining the phosphorylation of histone H2AX (gamma H2AX), a sensitive marker of DNA damage (Mah *et al.*, 2010). Massive induction of H2AX phosphorylation was observed in response to TGHQ exposure (Fig. 3) as early as 1 h after exposure to TGHQ, indicating a TGHQ-induced early impairment of genomic integrity in HK-2 cells. Importantly, NAC significantly inhibited gamma H2AX formation in HK-2 cells exposed to TGHQ.

NAC Attenuates TGHQ-Induced PAR Accumulation in HK-2 Cells

Upon DNA damage occurred, poly (ADP-ribose) polymerase-1 (PARP-1), a DNA nick sensor enzyme, is rapidly activated to catalyze the synthesis of PAR by cleaving nicotinamide adenine dinucleotide into nicotinamide and ADP-ribose. PAR is subsequently covalently attached to PARP-1 and to other suitable acceptor proteins (Erdelyi *et al.*, 2005; Strosznajder *et al.* 2005). We therefore examined the ability of TGHQ to induce PARP activation by immune detection of PAR-modified cellular proteins. Substantial PAR formation was observed in HK-2 cells within 20 min of exposure of HK-2 cells to TGHQ, indicating rapid activation of PARP by TGHQ (Fig. 4). Although the appropriate moderate activation of PARP facilitates DNA repair, excessive activation of PARP-1 can contribute to cell death by depleting cells of nicotinamide adenine dinucleotide+/ATP. NAC attenuated TGHQ-induced PAR accumulation in HK-2 cells, presumably a result of decreasing ROS production and the subsequent DNA damage.

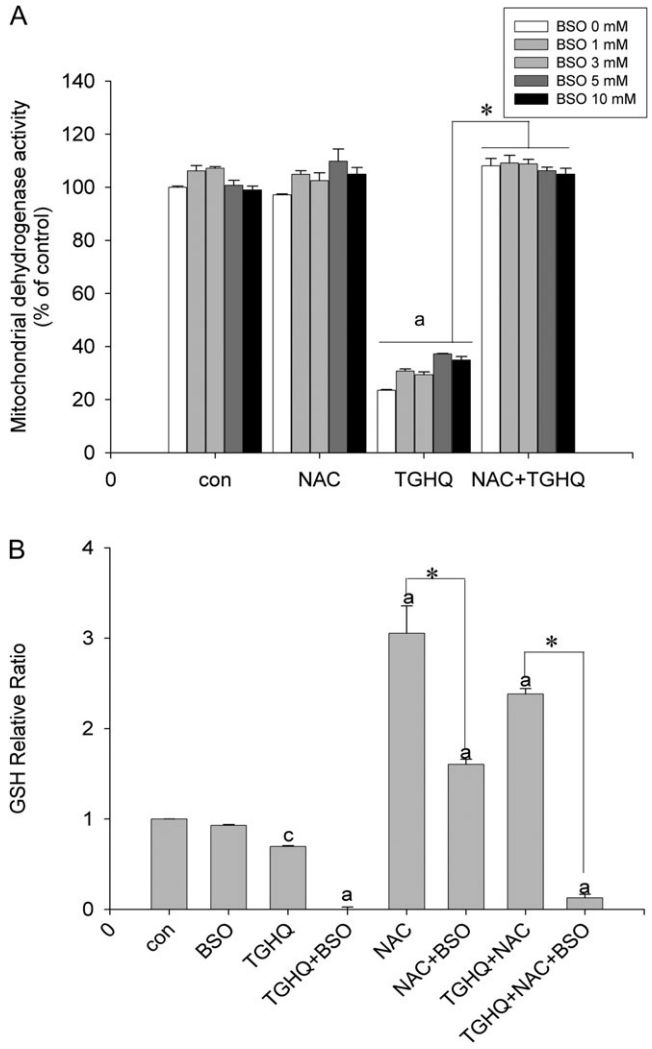

FIG. 9. BSO inhibits NAC-induced increases in GSH levels without affecting the protective effect of NAC against TGHQ-induced cell death. (A) Effect of BSO on protective effect of NAC against TGHQ-induced HK-2 cell death. Cells were incubated with different concentrations of BSO in the presence or absence of 1mM NAC, 400μM TGHQ, or 1mM NAC plus 400μM TGHQ for 4 h. Cytotoxicity was determined with the MTS-based assay. Data represent the mean ± SE ($n \geq 3$). (a) $p < 0.001$ compared with control at each concentration of BSO treatment; *, $p < 0.001$, (TGHQ+NAC) treatment group compared with TGHQ treatment group at each concentration of BSO treatment. (B) BSO inhibited the NAC-induced increase in GSH levels. Cells were incubated with 10mM BSO in the presence or absence of 1mM NAC, 400μM TGHQ, or 1mM NAC plus 400μM TGHQ for 4 h. Then, cell lysates were prepared, and the levels of GSH were estimated. Results were expressed as a percentage relative to control, and error bars represent SE ($n \geq 3$). (a) $p < 0.001$ and (c) $p < 0.05$ compared with control. *, $p < 0.001$, (NAC + BSO) group versus NAC treatment group or (TGHQ + NAC) group versus (TGHQ + NAC + BSO) treatment group.

NAC Attenuates TGHQ-Induced ATP Depletion in HK-2 Cells

The pronounced induction of DNA damage combined with rapid PARP activation led us to subsequently examine the effects of TGHQ on ATP concentrations in HK-2 cells (Kehe *et al.*, 2008). TGHQ caused the rapid depletion of cellular ATP in HK-2 cells within 4 h of exposure; ATP levels almost completely exhausted by 4 h (Fig. 5). In contrast, no depletion of ATP was observed with NAC treatment alone, and TGHQ-induced ATP depletion was significantly attenuated by co-administration of NAC (Fig. 5).

NAC and Catalase-polyethylene glycol on TGHQ-Induced MAPK Activation in HK-2 Cells

Because MAPKs play a key role in determining the fate of renal tubular cells after kidney injury and ROS modulate MAPK activation (di Mari *et al.*, 1999; Safirstein, 1997), we next examined the effect of TGHQ treatment on the phosphorylation status of various MAPKs in HK-2 cells. Phosphorylation of ERK1/2, c-Jun NH2-terminal kinase (JNK), and p38 were found to be significantly elevated after TGHQ (400μM) treatment (Fig. 6). Catalase-polyethylene glycol cotreatment, which inhibits ROS production by TGHQ (data not shown), also almost completely attenuated TGHQ-induced MAPK activation, indicating that TGHQ-induced MAPK activation is ROS dependent. Similarly, NAC cotreatment significantly attenuated TGHQ-induced JNK and p38 phosphorylation. In contrast, NAC could not attenuate TGHQ-induced ERK1/2 activation. Moreover, NAC alone markedly increased phosphorylation of ERK, which at least partially explains why NAC does not attenuate TGHQ-induced ERK1/2 activation.

NAC Induces Receptor-Independent ERK1/2 Activation in HK-2 Cells

To elucidate the mechanism of NAC-induced ERK1/2 activation, PD-98059, a specific inhibitor of the activation of the ERK kinases, via inhibition of its immediate upstream activators mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)-1 and MEK-2 (Alessi *et al.*, 1995; Dudley *et al.*, 1995), was employed to determine its ability to inhibit NAC-induced ERK activation. Pretreatment of cells with 50μM PD-98059 completely inhibited ERK activation in response to TGHQ injury in HK-2 cells (Fig. 7). However, PD-98059 only partially inhibited NAC-induced ERK activation.

NAC Attenuates TGHQ-Induced Decreases in the Mitochondrial Membrane Potential

Because high levels of ROS may damage mitochondria, leading to a loss of membrane potential, we next examined the effects of TGHQ on the mitochondrial membrane potential (MMP) as an indicator of mitochondrial health. The cationic dye JC-1 localizes to normal mitochondrial membranes as red aggregates and turns into a green monomer upon membrane depolarization (Moiseeva *et al.*, 2009). A substantial collapse of the MMP occurred in TGHQ-treated cells (Fig. 8), indicating that in TGHQ-treated cells mitochondria are dysfunctional. Moreover, NAC almost completely inhibited TGHQ-induced MMP decrease in HK-2 cells.

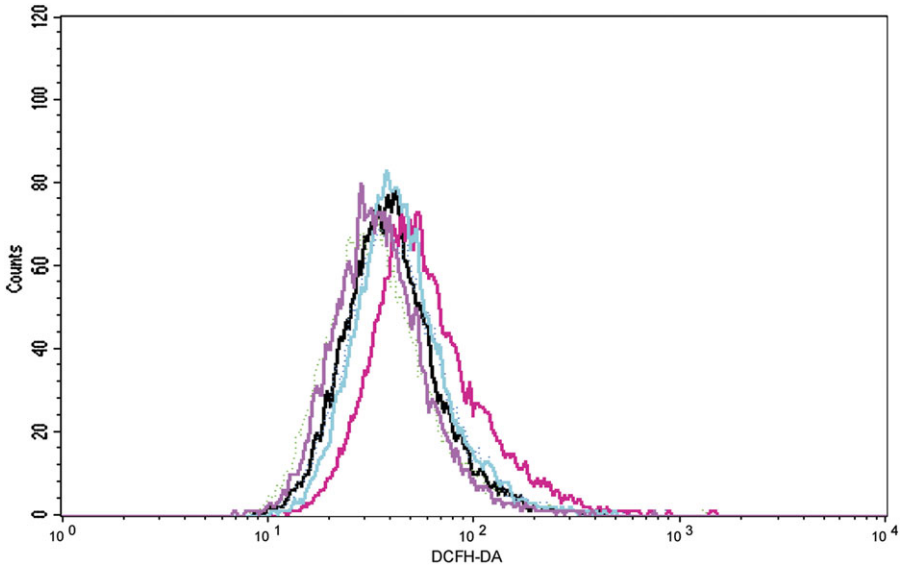

FIG. 10. The ROS-scavenging effect of NAC on TGHQ-induced ROS production is independent of GSH synthesis. ROS production was measured with DCFH-DA using flow cytometry. Cells were treated with 600μM TGHQ with or without 1mM NAC and/or 10mM BSO for 1 h or treated with 1mM NAC or 10mM BSO alone. In the graph, the second solid graph from the left represents control, the first dot graph from the left represents NAC treatment alone, the fourth solid graph from the left represents TGHQ treatment group, the second dot graph from the left represents BSO treatment group, the third solid graph from the left represents TGHQ and NAC cotreatment group, and the first graph from the left represents TGHQ and NAC and BSO cotreatment group.

The Protective Effects of NAC Are Independent of Intracellular GSH

NAC is a cell-permeable precursor of GSH (Saito *et al.*, 2010), which implies that the protective effects of NAC are coupled to either increases in or the ability to sustain intracellular GSH. To test this possibility, we determined whether buthionine sulfoximine (BSO), an inhibitor of GSH synthesis (Hamilton *et al.*, 1985), might abolish the cytoprotection afforded by NAC. Varying concentrations (1, 2, 5, and 10mM) of BSO were added to cell cultures containing TGHQ (400μM) or NAC (1mM), or TGHQ (400μM) plus NAC (1mM). Exposure of cells to NAC led to a large increase in intracellular levels of GSH (Fig. 9B), and BSO treatment predictably prevented the NAC-induced increase in intracellular GSH. However, NAC was still capable of protecting against TGHQ-induced HK-2 cell death, even when GSH synthesis was inhibited (Fig. 9A).

The ROS-Scavenging Effect of NAC Is Not Dependent on GSH Synthesis

We next determined the effects of BSO on TGHQ-induced ROS levels. NAC abolished TGHQ-induced ROS production in HK-2 cells even in the presence of BSO (Fig. 10), suggesting again that NAC-mediated reductions in oxidative stress are independent of intracellular GSH levels. Thus, cytoprotection provided by NAC is unlikely to be a result of increasing GSH levels but perhaps to the direct antioxidant/radical-scavenging properties of NAC.

The Antioxidant Effect NAC Might Play Important Roles in Protecting against TGHQ-Induced HK-2 Cell Death

To elucidate the precise mechanism by which NAC protects against TGHQ-induced cytotoxicity, we considered the chemical properties of the cysteinyl thiol of NAC, which permits the direct action of NAC as an antioxidant or free radical scavenger. We therefore determined the effects of some common thiols on TGHQ-induced cytotoxicity. As shown in Figure 11A, almost complete suppression of TGHQ-induced cytotoxicity was observed when exposure to TGHQ occurred in the presence of DTT or GSH. Similarly, DTT and GSH both suppressed TGHQ-induced ROS production in HK-2 cells (Fig. 11B). Taken together, the data suggest that NAC is cytoprotective via its direct scavenging of ROS.

DISCUSSION

ROS participate in the initiation and progression of a variety of human diseases (Kehrer, 1993). In particular, ROS are critical players in various renal diseases, including renal ischemia/reperfusion injury, acute nephrotoxic nephritis, complement-activated glomerular injury, and nephrotoxicities associated with chemical exposure (Andreoli and McAteer, 1990; Diamond, 1992; Khan, 2005). TGHQ-induced cytotoxicity in HK-2 cells is dependent upon the generation of ROS, and NAC almost completely abolishes TGHQ-induced cytotoxicity (Fig. 1). It is well known that NAC is frequently used as a precursor to GSH, supports the synthesis of GSH, and assists in replenishing GSH

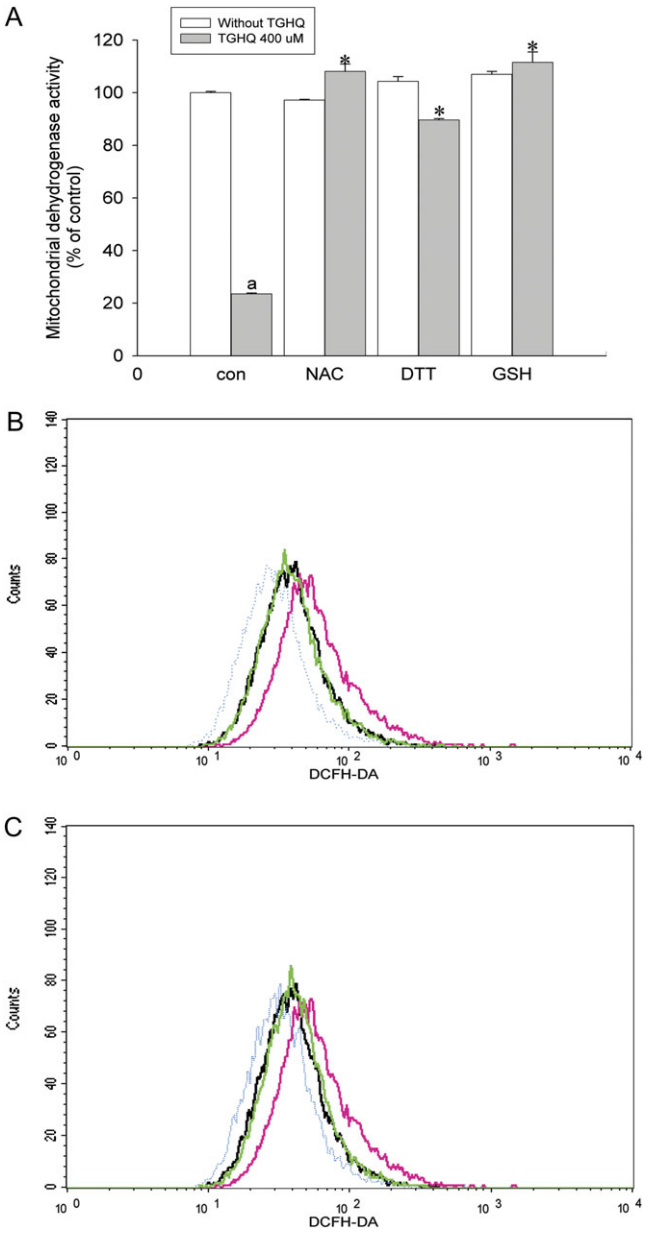

FIG. 11. The antioxidant effect of the sulfhydryl group in NAC plays an important role in protecting against TGHQ-induced HK-2 cell death. (A) Protective effect of sulfhydryl group–containing compounds (NAC, DTT, and GSH) against TGHQ-induced HK-2 cell death. Cells were treated with 1mM NAC, 0.5mM DTT, or 1mM GSH for 4 h. Alternatively, cells were treated with 400μM TGHQ in the presence or absence of 1mM NAC, 0.5mM DTT, or 1mM GSH for 4 h. Cytotoxicity was determined with the MTS-based assay. Data represent the mean ± SE ($n \geq 3$). (a) $p < 0.001$, significantly different from control; *, $p < 0.001$, (TGHQ + sulfhydryl group–containing compound) treatment groups compared with TGHQ treatment group. (B) Cells treated with DTT almost completely inhibited TGHQ-induced ROS production in HK-2 cells. ROS production was measured with DCFH-DA using flow cytometry. Cells were treated with 600μM TGHQ with or without 0.5mM DTT for 0.5 h or treated with 0.5mM DTT alone. In the graph, the middle graph represents control, the left graph represents DTT treatment alone, the right graph represents TGHQ treatment group, and the middle graph represents TGHQ and DTT cotreatment group. (C) GSH almost completely inhibited TGHQ-induced ROS production in HK-2 cells. ROS production was measured with DCFH-DA using flow cytometry. Cells were treated with 600μM TGHQ with or without 1mM GSH for 0.5 h or treated with 1mM GSH alone. In the graph, the middle graph represents control, the left graph represent GSH treatment alone, the right graph represents TGHQ treatment group, and the middle graph represents TGHQ and GSH cotreatment group.

when stores are compromised during oxidative or electrophilic stress. However, the ability of NAC to protect against TGHQ-induced cytotoxicity was independent of its ability to promote GSH synthesis in HK-2 cells because inhibition of GSH synthesis had no effect on the cytoprotective effects of NAC (Fig. 9). Rather, the ability of NAC to afford protection against TGHQ-induced cytotoxicity appears to be a consequence of its ability to directly scavenge ROS (Fig. 2).

Although NAC completely protected against TGHQ-induced cytotoxicity, as determined by measuring mitochondrial dehydrogenase activity (MTS assay), its ability to prevent TGHQ-induced DNA damage (Fig. 3), PARP overactivation (Fig. 4), and energy depletion (Fig. 5) was incomplete. Thus, factors in addition to ROS likely contribute to TGHQ-induced DNA damage, which is consistent with the notion that TGHQ retains the ability to form covalent adducts with a variety of cellular macromolecules (Kleiner *et al.*, 1998). Therefore, it is likely that the combination of nucleophilic adduction to cellular macromolecules and the generation of ROS both contribute to TGHQ-mediated DNA damage. However, the DNA damage alone seems insufficient to result in cytotoxicity because the cells remain viable in the context of increases in γ-H2AX (Fig. 3) and PAR-positive proteins (Fig. 4).

Because MAPKs play a key role in regulating the fate of renal tubular cells after injury and ROS can activate MAPKs (di Mari *et al.*, 1999; Safirstein, 1997), the effect of NAC on TGHQ-induced MAPK activation was subsequently investigated. NAC inhibited TGHQ-induced p38 MAPK and JNK activation (Fig. 6). Interestingly, NAC alone significantly activated the ERK signaling pathway (Fig. 6A), an MAPK cascade broadly associated with cell survival (Wang *et al.*, 1998). Indeed, NAC inhibits the activation of JNK completely in the post-ischemic kidney, and inhibition of JNK was accompanied by improved renal function and accelerated renal repair compared with saline-treated animals (di Mari *et al.*, 1999). Moreover, in cardiac myocytes, reoxygenation-induced ROS production and phosphorylation of JNK were inhibited by NAC (Laderoute and Webster, 1997). Similarly, NAC attenuated TNFα-induced p38 activity in human pulmonary vascular endothelial cells (Hashimoto *et al.*, 2001), and addition of NAC to mitogen-free human and bovine articular chondrocyte cultures rapidly activated the ERK signaling pathway (Li *et al.*, 2000). NAC was also found to activate the activator protein 1 via sequential activation of the ERK-MAPK pathway and Ets-like protein 1 transcription factor, which may lead to cell

growth and differentiation (Meyer *et al*., 1993; Muller *et al*., 1997).

The protective action of NAC is generally attributed to its ability to serve as a precursor to GSH. However, inhibition of GSH synthesis had no effect on the capacity of NAC to protect HK-2 cells against TGHQ-induced toxicity. Thus, in this system, enhanced GSH levels cannot account for NAC's protective actions. Consistent with this finding, BSO treatment of T cells decreased NAC-supported GSH levels by ~50% generated but had no effect on the capacity of NAC to protect these cells from apoptosis induced by exposure to anti-CD3 antibodies (Jones *et al*., 1995). Moreover, it has been generally assumed that the survival-promoting actions of NAC are due to its direct or indirect (via intracellular GSH) action as an antioxidant or free radical–scavenging agent (Atkuri *et al*., 2007). However, we found in the present study that it was the cysteinyl thiol of NAC that provided antioxidant properties.

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