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Nrf2-Dependent Activation of the Antioxidant Responsive Element by *tert*-Butylhydroquinone Is Independent of Oxidative Stress in IMR-32 Human Neuroblastoma Cells

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The antioxidant responsive element (ARE) is a *cis*-acting regulatory element located in the 5′-flanking region of several genes encoding phase II detoxification enzymes, including NAD(P)H:quinone oxidoreductase (NQO1). We report here that activation of the NQO1 ARE by *tert*-butylhydroquinone (tBHQ) is dependent on Nrf2 and not oxidative stress in IMR-32 human neuroblastoma cells. Overexpression of wild-type Nrf2 activated ARE in a dose-dependent manner, and ARE activation by tBHQ or diethyl maleate (DEM) was inhibited by dominant/negative Nrf2 not by dominant/negative c-Jun. According to our observation, the palindromic sequence (5′ to the core) and the GC box in the ARE core sequence are essential for maximal inducibility by tBHQ or DEM. Overexpression of Nrf2 selectively activated wild-type ARE up to 24 h. In addition, a dramatic nuclear translocation of Nrf2 by tBHQ supports a role for Nrf2 in ARE activation. Although oxidative stress is hypothesized to be a major driving force for ARE activation, pretreatment of antioxidant or antioxidant enzyme did not block tBHQ-mediated ARE activation. In contrast, ARE activation by DEM was inhibited by antioxidants or catalase. These results suggest that ARE activation signals from tBHQ and DEM converge at Nrf2 transcription factor through independent mechanisms.

The antioxidant responsive element (ARE), located in the 5′-flanking region of rat NQO1 (rNQO1) and human NQO1 (hNQO1) is a unique *cis*-acting regulatory element and activated by redox-cycling phenols and electrophiles (1–5). Based on mutational analysis of the hNQO1-ARE, we have demonstrated that the palindromic sequence (5′ to the ARE core sequence) and the GC box are required for maximal activation of the ARE in IMR-32 human neuroblastoma cells (6). The ARE sequence of hNQO1 contains one perfect (5′-TGACTCA-3′) and one imperfect AP-1 binding sequence (5′-GCAGTCA-3′) arranged as inverse orientation as well as shows strong similarity to the Nf-E2 binding sequence (5′-TGAGTCA-3′) (7, 8, 10). Although members of AP-1 family such as c-Jun, Jun-B, Jun-D, and c-fos have been reported to bind to the ARE of hNQO1 (8, 9), the functional consequence of this interaction is controversial. On the other hand, several lines of evidence suggest that Nrf2 is the actual transactivation factor responsible for upregulating ARE-driven gene expression (10–15). Human Nrf2 was first cloned in 1994 by Moi and coworkers (16) and was classified as a b-zip family member. Nrf2 has been reported to bind to the ARE sequence of human NQO1 and to regulate positively ARE-mediated NQO1 expression in hepatoma cells (8, 9, 22). In addition, Nrf2 knock out mice showed significantly decreased NQO1 expression in liver (15), and Keap1 protein was reported to bind to Nrf2, sequestering Nrf2 in the cytoplasm (11). These observations suggest that Nrf2 is the principle transcription factor necessary for ARE activation even though many other transcription factors can bind to the ARE sequence.

Oxidative stress has been hypothesized to be a main driving force for ARE activation (14, 17). Itoh and colleagues proposed that oxidative stress or electrophiles induce the release of Nrf2 from the Nrf2-Keap1 complex resulting in nuclear translocation of Nrf2 and subsequent activation of ARE (11). The exact mechanism by which oxidative stress induces release of Nrf2 from the Nrf2-Keap1 complex is not fully understood. The present investigation was designed to test the

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hypothesis that tBHQ- and DEM-mediated activation of the ARE in IMR-32 human neuroblastoma cells is a result of increased oxidative stress induced by these chemicals. Specifically, we evaluated (1) the effect of Nrf2 and c-Jun on ARE activation, (2) the subcellular localization of Nrf2 by tBHQ treatment, and (3) the effect of antioxidants and antioxidant enzymes on the inducibility of ARE-reporter construct and NQO1 protein level.

MATERIALS AND METHODS

Plasmids and expression vectors. The reporter gene fusion constructs for wild-type ARE (wt-ARE), GC mutant ARE (GC-ARE), and palindromic mutant ARE (Pal-ARE) were made as described previously (6). The following oligonucleotides were used (core and mutated sequences are underlined). wt-ARE, 5′-CTCAGCCTTCCAAATCGCAGTCACAGTGACTCAGCAGAATC-3′; GC-ARE, 5′-CTCAGCCTTCCAAATCGCAGTCACAGTGACTCA**AT**AGAATC-3′; Pal-ARE, 5′-CTCAGCCTTCCAAATCG**GGACTG**CAGTGACTCAGCAGAATC-3′. Mammalian expression plasmids (pEF, wild-type Nrf2, dominant/negative Nrf2, pRJB, wild-type c-Jun, and dominant/negative c-Jun) were kindly provided by Dr. Jawed Alam (Alton Ochsner Medical Foundation, New Orleans, LA) (12).

Transient transfection. IMR-32 human neuroblastoma cells (ATCC, #CCL-127) were plated at a density of 2.5×10^4 cells/well in 96-well plates and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transient transfections were performed using the calcium phosphate crystal method as described previously (6). For overexpression of wild-type (wt) or dominant/negative (DN) Nrf2 and c-Jun, cells were cotransfected with mammalian expression plasmids (wt-Nrf2, DN-Nrf2, wt-c-Jun or DN-c-Jun), hNQO1-ARE-luciferase (80 ng/well) and CMV-β-galactosidase (20 ng/well). Appropriate control plasmids (It-luciferase for ARE-luciferase; pEF for wt-Nrf2, DN-Nrf2, and DN-c-Jun; pRJB for wt-c-Jun) were used to ensure specificity. After 24 h of transfection, cells were treated with vehicle (ethanol, 0.1%), *tert*-butylhydroquinone (tBHQ, Fisher) or diethyl maleate (DEM, Aldrich). To determine the effect of antioxidants, cells were pretreated with vehicle (PBS), 5 mM glutathione (GSH, Sigma), 2.5 mM glutathione monoethyl ester (GSHEE, Sigma), 2.5 mM *N*-acetyl cysteine (NAC, Sigma), catalase (Sigma) or superoxide dismutase (SOD, Sigma) for 2 h prior to tBHQ (10 μM) or DEM (20 μM) treatment. After 24 h of treatment, medium was removed and cells were lysed in 70 μl of lysis buffer (6). Luciferase activity was determined using a luminometer as described previously (6). β-Galactosidase activity was measured using *O*-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) as substrate. Briefly, cell lysates were incubated with ONPG (0.4 mg/ml) in reaction buffer (0.1 M sodium phosphate pH 7.5, 10 mM KCl, 1 mM $MgCl_2$) for 1 h then absorbance at 405 nm was read. The data are expressed as a ratio of luciferase to β-galactosidase activity.

Preparation of whole cell, cytosolic, and nuclear extracts. IMR-32 human neuroblastoma cells were treated with vehicle (ethanol, 0.1%) or tBHQ (10 μM) and harvested for time-course study of Nrf2 nuclear translocation. Nuclear and cytosolic extracts were isolated as described previously (18) with modifications. Briefly, cells were washed with cold PBS and resuspended in cold buffer A (lysis buffer: 20 mM HEPES, pH 8.0, 1 mM EDTA, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM PMSF, 0.5 mg/ml benzamidine, 0.1 mg/ml leupeptin, and 1.2 mg/ml aprotinin). The cells were allowed to swell on ice for 15 min, then 7.5 μl of 10% NP-40 was added and vortex mixed vigorously for 10 s. The homogenate was centrifuged for 50 s at 16,000*g* and the supernatant was used as cytosolic extract. The nuclear pellet was resuspended in cold buffer B (extraction buffer: 20 mM HEPES, pH 8.0, 1 mM EDTA, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM PMSF, 0.5 mg/ml benzamidine, 0.1 mg/ml leupeptin, 1.2 mg/ml aprotinin, and 20% glycerol). To determine the effect of antioxidants, cells were pretreated with vehicle (PBS), GSH (5 mM), GSHEE (2.5 mM), NAC (2.5 mm), catalase (50–100 unit/ml) and SOD (50 unit/ml) for 2 h prior to treatment (vehicle, 10 μM tBHQ, or 20 μM DEM). After washing 3 times with cold PBS, whole cell extracts were prepared in lysis buffer (6). Protein concentrations were determined using BCA protein assay kit (Pierce, Rockford, IL).

Western immunoblot analysis. Whole cell extract, cytosolic fraction and nuclear fraction were resolved by SDS-PAGE, transferred to PVDF membrane and blocked with 5% non-fat milk. The membranes were probed with hNQO1 (provided by Dr. David Ross, University of Colorado) or Nrf2 antibody (Santa Cruz). Chemiluminescence was detected using SuperSignal West Pico chemiluminescent substrate (Pierce).

RESULTS

ARE Activation by tBHQ or DEM Is Nrf2-Dependent

To evaluate the involvement of Nrf2 and c-Jun in ARE activation in human neuroblastoma cells, we transfected IMR-32 cells with wt-Nrf2 or wt-c-Jun expression vector. As shown in Fig. 1A, overexpression of Nrf2 induced ARE activation in a dose-dependent manner and 10 ng/well of wild-type Nrf2 transfection caused a massive activation of ARE without any treatment (926-fold, vehicle at 10 ng/well divided by vehicle at 0 ng/well). Although c-Jun overexpression activated hNQO1-ARE reporter construct, the general transactivating potential of c-Jun was much less than that of Nrf2 (58-fold, same as above). tBHQ treatment caused an additional increase of ARE-luciferase expression in both wt-Nrf2 and wt-c-Jun transfected cells (Figs. 1A and 1B). The activation patterns, however, were quite different from each other. In the case of wt-Nrf2 + tBHQ, wt-Nrf2 appeared to saturate the system and compete with tBHQ (Fig. 1A), but in the c-Jun experiments, we could not detect a similar effect (Fig. 1B). To further characterize the roles of Nrf2 and c-Jun in ARE activation, IMR-32 cells were cotransfected with DN-Nrf2 or DN-c-Jun. The DN-Nrf2 lacks its transactivation domain thereby inhibiting endogenous Nrf2 function by sequestering Nrf2 binding partners (12). DN-Nrf2 decreased ARE activation induced by tBHQ (10 μM) and DEM (20 μM) in a dose-dependent manner (Fig. 2B). In contrast, DN-c-Jun did not inhibit either tBHQ- or DEM-mediated ARE activation up to 20 ng/well (Fig. 2C). Cotransfection of control vector (pEF) had no effect on the tBHQ- or DEM-induced ARE activation (data not shown). Together, these data imply that although both Nrf2 and c-Jun can increase hNQO1-ARE-luciferase in IMR-32 cells, the relative transactivating potential of Nrf2 is more pronounced than c-Jun. In addition, ARE activation by tBHQ and DEM is mediated via Nrf2 not c-Jun.

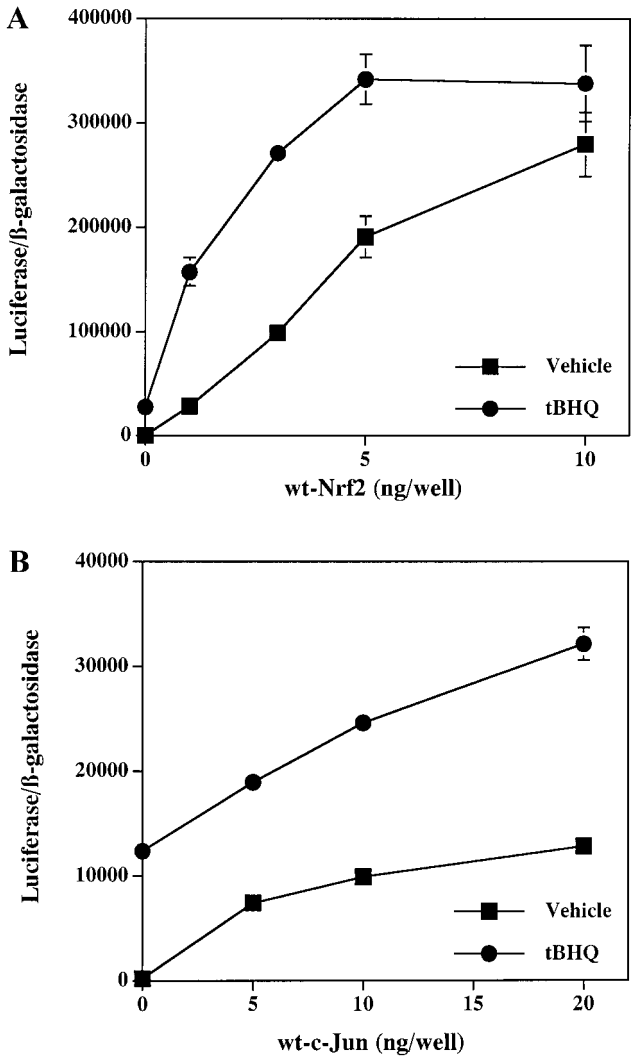

FIG. 1. Effect of Nrf2 and c-Jun on hNQO1-ARE activation in IMR-32 cells. IMR-32 human neuroblastoma cells were transfected with hNQO1-ARE-luciferase (80 ng/well), CMV-β-galactosidase (20 ng/well), and indicated amounts of wt-Nrf2 (A) or wt-c-Jun (B). After 24 h, cells were treated with vehicle (square, ethanol 0.1%) or tBHQ (circle, 10 μM) for an additional 24 h. Cells were lysed, and luciferase and galactosidase activities were determined as described under Materials and Methods. Each data point represents the mean ± SE (n = 6).

Activation of ARE by Nrf2 Is Specific to wt-ARE

The specificity of Nrf2 for the ARE sequence was determined by transfecting IMR-32 cells with luciferase reporter constructs containing wt-ARE or mutated-ARE (Pal-ARE and GC-ARE). The Pal-ARE has 6 mutations that eliminate the 5′ palindromic sequence upstream from the ARE core and the GC-ARE has 2 mutations in the GC box of the ARE core sequence. As shown in Fig. 3A, tBHQ alone selectively increased expression of the wt-ARE-luciferase (36.8-fold) and wt-Nrf2 overexpression activated the wt-ARE (230 fold). Surprisingly, Pal-ARE-luciferase (223-fold) and GC-ARE-luciferase (295-fold) were also significantly increased by wt-Nrf2 overexpression (Fig. 3A). These data suggested that simple overexpression of Nrf2 was not specific to the ARE sequence. The data in Fig. 3A were compiled from cells 48 h after transfection. A time-dependent analysis for wt-Nrf2 overexpression revealed that there was indeed specificity for the wt-ARE sequence at earlier times (18

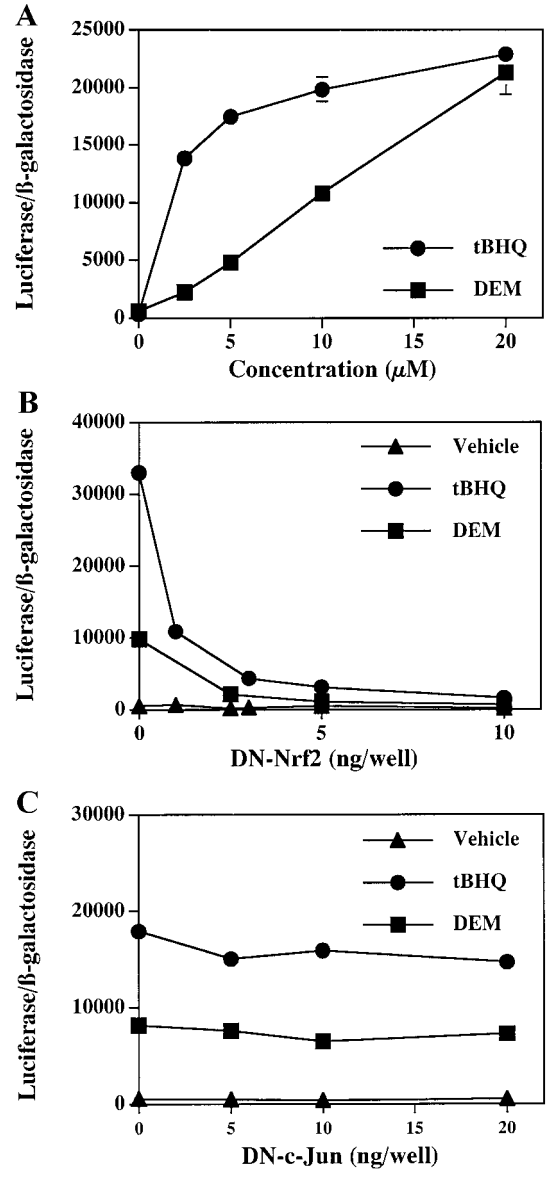

FIG. 2. Effect of dominant/negative Nrf2 and c-Jun on transactivation of hNQO1-ARE. (A) IMR-32 cells were transfected with hNQO1-ARE-luciferase (80 ng/well) and CMV-β-galactosidase (20 ng/well). After 24 h, cells were treated with increasing doses of tBHQ or DEM. For inhibition of normal Nrf2 or c-Jun function, cells were cotransfected with indicated concentrations of DN-Nrf2 (B) or DN-c-Jun (C) and then treated with vehicle (triangle), 10 μM tBHQ (circle) or 20 μM DEM (square). Cells were harvested and luciferase and galactosidase activities were determined as described under Materials and Methods. Each data point represents the mean ± SE (n = 6).

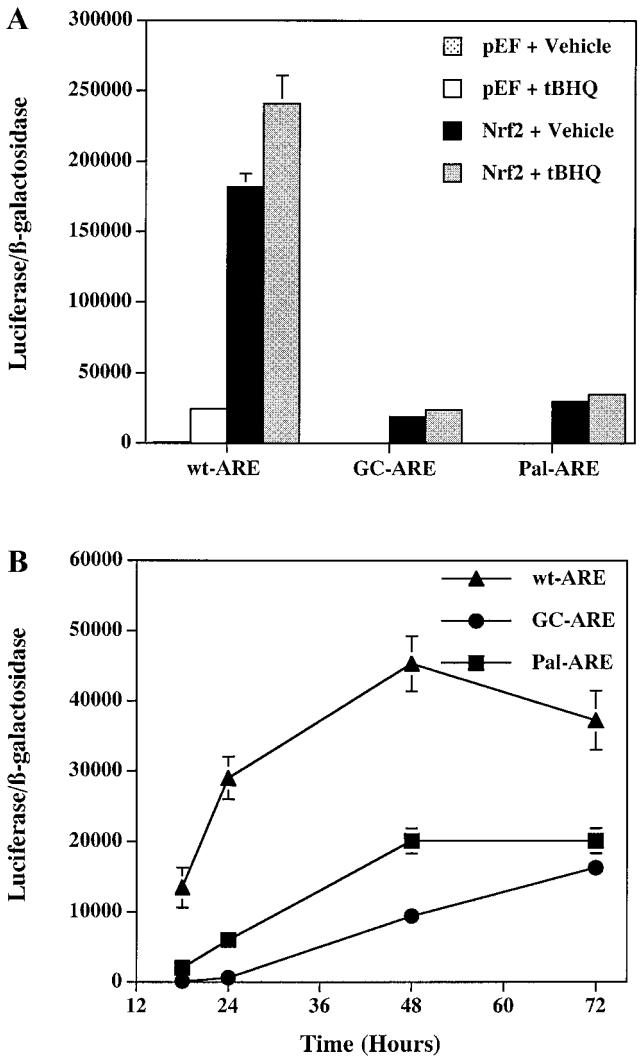

FIG. 3. Selective hNQO1-ARE activation by Nrf2. (A) IMR-32 cells were cotransfected with wt-, GC-, or Pal-ARE-luciferase construct (80 ng/well), CMV-β-galactosidase (20 ng/well), and wt-Nrf2 (Nrf2, 5 ng/well) or control vector (pEF, 5 ng/well). After 24 h, cells were treated with vehicle (ethanol, 0.1%) or tBHQ (10 μM) for an additional 24 h. (B) IMR-32 cells were cotransfected with wt-, GC-, or Pal-ARE-luciferase construct (80 ng/well), CMV-β-galactosidase (20 ng/well) and wt-Nrf2 (5 ng/well) or control vector (pEF, 5 ng/well). Cells were harvested at indicated time for time course study. Cells lysates were used to determine luciferase and galactosidase activity. Each data point represents the mean $\pm$ SE (n = 6). Control vector (pEF) had no effect on time course activation of hNQO1-ARE (data not shown).

and 24 h) whereas at longer times of transfection (48 and 72 h), this selective effect was lost (Fig. 3B). The pattern of expression for wt-ARE, pal-ARE, and GC-ARE at 18 h mimics the tBHQ-mediated induction of these same constructs.

TBHQ Induces Nuclear Translocation of Nrf2 Transcription Factor

There are several reports showing Nrf2 binds to ARE by gel mobility shift assay (8, 9, 13, 14). To show direct evidence for a critical role of Nrf2 in ARE activation, we used a different experimental approach other than mobility shift assay. We prepared cytosolic and nuclear extracts from vehicle- or tBHQ-treated IMR-32 cells and performed Western immunoblot analysis to determine whether tBHQ treatment leads to nuclear translocation of Nrf2. As shown in Figs. 4A and 4B, tBHQ treatment induced dramatic nuclear translocation of Nrf2. The molecular weight of human Nrf2 protein is 65 kDa (16) but the increased protein in the nuclear fraction from tBHQ-treated cells was approximately 100 kDa. In addition, the Nrf2 antibody recognizes many nonspecific proteins (Fig. 4C). Thus, we performed Western immunoblot analysis on the whole cell extract from wt-Nrf2 transfected IMR-32 cells and the nuclear fraction of tBHQ-treated cells for verification of the electrophoretic mobility of Nrf2 (Fig. 4C). In the transfected whole cell extract, Nrf2 protein was increased and migrated to approximately 100 kDa

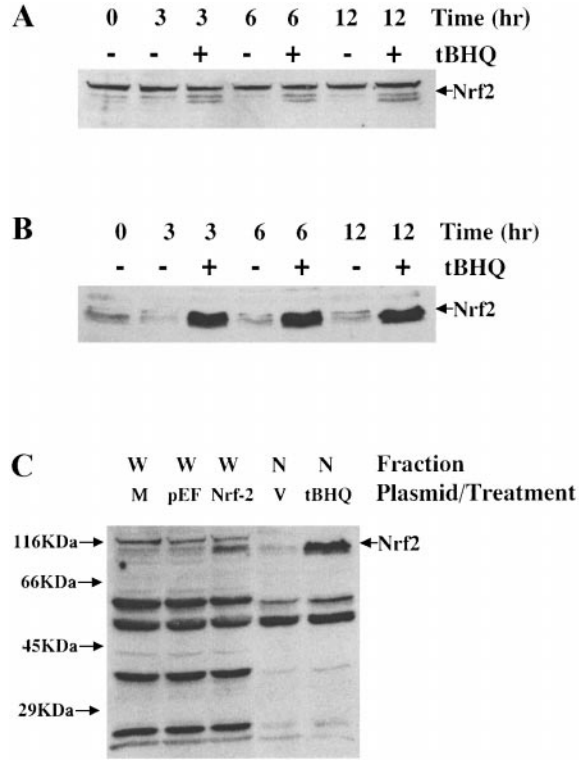

FIG. 4. TBHQ treatment induces nuclear translocation of Nrf2. IMR-32 cells were treated with vehicle (−) or 10 μM tBHQ (+) for the indicated time. Then cytosolic (A) and nuclear fraction (B) were isolated as described under Materials and Methods. Fifty micrograms of protein from each fraction was resolved by SDS-PAGE (10%), transferred to PVDF membrane, and probed with Nrf2 antibody (Santa Cruz). (C) For overexpression of Nrf2, cells were transfected with mock (M, calcium phosphate crystal), pEF (control vector), or wt-Nrf2 (8 μg/plate) for 3 days in 10-cm dishes. Cells were washed, harvested, and whole cell extracts (W) were prepared as described under Materials and Methods. For comparison, nuclear fraction (N) from vehicle- (V) or tBHQ (10 μM)-treated cells (for 6 h) were analyzed at a same time.

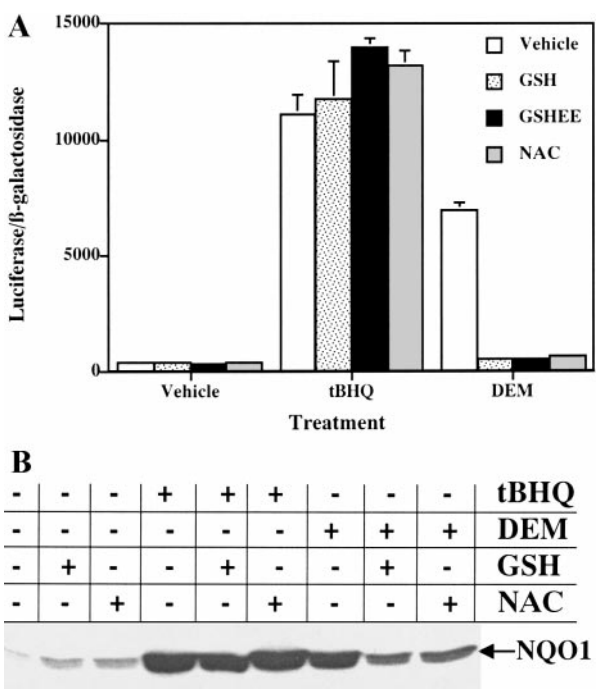

FIG. 5. ARE activation by tBHQ is not inhibited by antioxidants. (A) IMR-32 cells were transfected with hNQO1-ARE-luciferase construct (80 ng/well) and CMV-β-galactosidase (20 ng/well). After 24 h, cells were pretreated with vehicle (PBS), glutathione (GSH, 5 mM), glutathione monoethyl ester (GSHEE, 2.5 mM), or N-acetyl cysteine (NAC, 2.5 mM) 2 h prior to tBHQ (10 μM) or DEM (20 μM) treatment. Lysates were used to determine luciferase and β-galactosidase activity (n = 6). (B) IMR-32 cells were pretreated with glutathione (GSH, 5 mM) or N-acetylcysteine (NAC, 2.5 mM) for 2 h then treated with tBHQ (10 μM) or DEM (20 μM). After 24 h of treatment, whole cell extracts were prepared and 50 μg of protein was resolved by 12% SDS-PAGE. Transferred membrane was probed with hNQO1 antibody.

(Fig. 4C, lane 3). No other proteins recognized by Santa Cruz Nrf2 antibody were altered by Nrf2 transfection and the Nrf2 band did not increase in the mock- (Fig. 4C, lane 1) or pEF-transfected cells (Fig. 4C, lane 2). Furthermore, the nuclear translocated proteins recognized by the Nrf2 antibody comigrated with the overexpressed Nrf2 protein (Fig. 4C) verifying that the translocated protein is Nrf2. This abnormal mobility of Nrf2 protein in SDS-PAGE has been described by others (9, 16).

Activation of the hNQO1-ARE Is Not Dependent on the Production of Oxidative Stress

Oxidative stress has been thought to be a major driving force for ARE activation (11, 14, 17). To further characterize the involvement of oxidative stress in ARE activation, we pretreated IMR-32 cells with antioxidants or antioxidant enzymes. In Fig. 5A, we observed that pretreatment of antioxidants such as glutathione (GSH), glutathione monoethyl ester (GSHEE) or N-acetyl cysteine (NAC) did not inhibit ARE activation by tBHQ. In contrast, DEM-mediated ARE activation was completely inhibited by pretreatment of GSH, GSHEE, or NAC (Fig. 5A). In addition, the increase of NQO1 protein by tBHQ was not inhibited whereas the increase by DEM was significantly inhibited by these antioxidants. To evaluate the effects of antioxidant enzymes, we pretreated IMR-32 cells with either catalase or superoxide dismutase (SOD). ARE activation by tBHQ was not inhibited by any antioxidant enzyme pretreatment (Fig. 6A). However, ARE activation by DEM was decreased significantly by catalase. The inhibitory effect of catalase was dose-dependent and

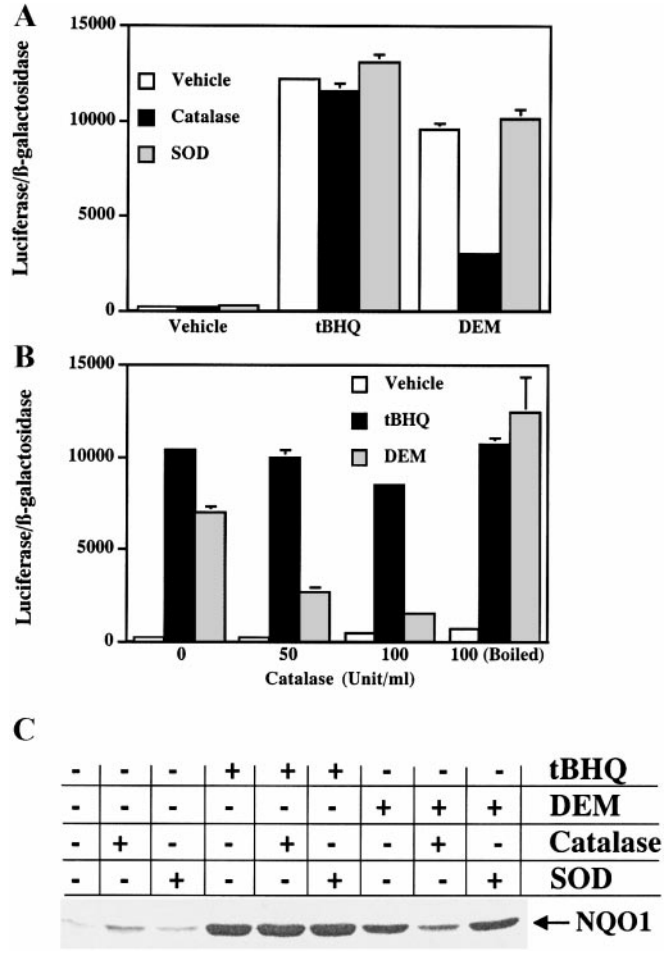

FIG. 6. ARE activation by tBHQ is not inhibited by antioxidant enzymes. (A) IMR-32 cells were transfected with hNQO1-ARE-luciferase reporter construct (80 ng/well) and CMV-β-galactosidase (20 ng/well). After 24 h, cells were pretreated with vehicle (PBS), catalase (50 unit/ml), or SOD (50 unit/ml) 2 h prior to tBHQ (10 μM) or DEM (20 μM) treatment. (B) For dose–response relationship, transfected cells were treated with 50 or 100 unit/ml of intact catalase or 100 unit/ml of boiled catalase then treated with tBHQ (10 μM) or DEM (20 μM) for 24 h. (C) For Western immunoblot analysis, IMR-32 cells pretreated with vehicle (PBS), catalase (100 unit/ml) or SOD (100 unit/ml) for 2 h then treated with tBHQ (10 μM) or DEM (20 μM). After 24 h of treatment, whole cell extracts were prepared, and 50 μg of protein was resolved by 12% SDS-PAGE. Transferred membrane was probed with hNQO1 antibody.

boiled catalase had no inhibitory effect on ARE-activation (Fig. 6B). In Western immunoblots, catalase decreased NQO1 protein induced by DEM but had no effect on NQO1 induction by tBHQ (Fig. 6C). SOD had no effect on ARE activation by either tBHQ or DEM (Figs. 6A and 6C).

DISCUSSION

The ARE sequence of human NQO1 gene contains one perfect and one imperfect AP-1 binding sequence (7, 8) and several AP-1 components have been shown to bind to the NQO1 ARE sequence (8, 9). We found that although c-Jun could induce ARE activation, c-Jun overexpression alone was not as effective as Nrf2 in ARE activation. Furthermore, only DN-Nrf2 blocked the activation of ARE by tBHQ or DEM (Figs. 2B and 2C). These observations support that the ARE activation by tBHQ or DEM is mediated by Nrf2. In addition, the observation that the rat NQO1-ARE (lacking AP-1 binding site) showed strong induction by tBHQ (6) supports that an AP-1 binding sequence in ARE core sequence is not essential for ARE activation by tBHQ.

According to our previous findings, the palindromic sequence and GC box in the ARE sequence are essential for maximal inducibility (6). As expected, tBHQ (Fig. 3A) and DEM (data not shown) selectively activated wt-ARE. Wt-Nrf2 transfection also activated wt-ARE selectively at earlier times (18–24 h), but GC-ARE and Pal-ARE were strongly activated by longer exposure of Nrf2 (Fig. 3B). These data suggest that overexpression of Nrf2 may be increasing expression of other transcription factors with the potential to interact with the wt- and mutated-ARE sequences. Due to the lack of specificity for the ARE, we speculate that these transcription factors would be AP-1 proteins interacting with the remaining intact AP-1 binding site found within the wt- and mutated-ARE constructs. This hypothesis is supported by preliminary data overexpressing wt-c-Jun in IMR-32 cells. The wt-c-Jun activates the wt-ARE, GC-ARE and Pal-ARE suggesting that increased AP-1 binding proteins can account for the loss of specificity (Lee and Johnson, unpublished data).

Since we observed that overexpression of wt-Nrf2 activated ARE and DN-Nrf2 inhibited ARE activation by tBHQ or DEM, we hypothesized that the signaling pathways leading to ARE activation by tBHQ or DEM converge on Nrf2 in human neuroblastoma cells. The large increase in nuclear Nrf2 associated with tBHQ treatment (Fig. 4) suggests that, similar to that seen for DEM in 293T cells (11), tBHQ treatment is probably stimulating the release of Nrf2 from Nrf2-Keap1 protein complex. Itoh and coworkers reported that DEM treatment (100 μM, 24 h) released Nrf2 protein from Nrf2-Keap1 complex resulting in nuclear localization of Nrf2 using immunocytochemistry (11). In our system, DEM (20 μM) also induced Nrf2 nuclear translocation in IMR-32 cells from 3 h (data not shown). Our finding complements that published by Itoh and colleagues (11) in that we concur with their conclusion that oxidative stress induced by treatment with DEM results in Nrf2-mediated activation of ARE-driven genes. In addition, our data clearly demonstrate that tBHQ treatment leads to Nrf2 accumulation in the nucleus via a signaling mechanism unique from that of DEM and presumably without an increase in oxidative stress.

Alternative mechanism for ARE activation by tBHQ can be found in specific kinase pathways. Yu and co-workers reported that mitogen-activated protein kinases MAPK) are involved in induction of phase II detoxifying enzymes by tBHQ (19, 20) and Huang and co-workers reported protein kinase C (PKC)-mediated Nrf2 phosphorylation by tBHQ (21). Since neither down-regulation nor chemical inhibition of PKC blocks tBHQ-mediated ARE activation or increase in NQO1 protein in IMR-32 cells (6), the latter possibility has been ruled out. TBHQ treatment resulted in an increase in the activity of two mitogen-activated protein kinases, extracellular signal-regulated protein kinase 2 (Erk 2) and c-Jun N-terminal kinase 1 (JNK 1) in HepG2 human hepatoma cells (19, 25). In a human macrophage cell line, tBHQ treatment also has been shown to result in an increase in JNK and p38 MAPK activity (26). Preliminary attempts to link ARE activation to MAPK or JNK by using the MEK inhibitor PD98059, phospho-ERK 1/2 antibodies, or overexpression of dominant/negative c-Jun, however, have been negative in human neuroblastoma cells (Lee *et al.*, unpublished data). Recently, however, our laboratory using tBHQ (23) and another laboratory using sulfur amino acid deprivation (24) have linked ARE activation and increased expression of ARE-driven genes to phosphatidylinositol 3-kinase (PI3-kinase). The PI3-kinase inhibitor LY 294002 blocked tBHQ-mediated ARE activation and increase in NQO1 protein in IMR-32 cells (23), as well as blocked sulfur amino acid deprivation-induced protein binding to the rat GSTA2 ARE in electrophoretic mobility shift assay and increase in rat GSTA2 mRNA (24).

The extent of overlap between signaling pathways activated by DEM (oxidative stress dependent) and tBHQ (oxidative stress independent) as well as the upstream branch point separating these pathways is under intensive investigation in our laboratory. The potential identification of a novel signal transduction cascade, not involving oxidative stress, which causes Nrf2 translocation and subsequent ARE-driven gene activation, is very intriguing. Such a pathway could lead to the development of drugs targeting this cascade to coordinately control expression of the ARE-driven gene family.

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