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*Research Paper*

# Induction of Heme Oxygenase-1 (HO-1) and NAD[P]H: Quinone Oxidoreductase 1 (NQO1) by a Phenolic Antioxidant, Butylated Hydroxyanisole (BHA) and Its Metabolite, *tert*-Butylhydroquinone (tBHQ) in Primary-Cultured Human and Rat Hepatocytes

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***Purpose.*** This study was aimed to investigate the effects of a phenolic antioxidant, butylated hydroxyanisole (BHA) and its metabolite, *tert*-butylhydroquinone (tBHQ) on the induction of HO-1, NQO1 and Nrf2 proteins and their regulatory mechanisms in primary-cultured hepatocytes.
***Methods.*** After exposure of BHA and tBHQ to primary-cultured rat and human hepatocytes and mouse neonatal fibroblasts (MFs), Western blot, semi-quantitative RT-PCR and microarray analysis were conducted.
***Results.*** Induction of HO-1, NQO1 and Nrf2 proteins and activation of ERK1/2 and JNK1/2 were observed after BHA and tBHQ treatments in primary-cultured rat and human hepatocytes. Semi-quantitative RT-PCR study and microarray analysis revealed that HO-1 and NQO1 were transcriptionally activated in primary-cultured rat hepatocytes and a substantial transcriptional activation, including HO-1 occurred in primary-cultured human hepatocytes after BHA treatment. Whereas BHA failed to induce HO-1 in wild-type and Nrf2 knock-out MFs, tBHQ strongly induced HO-1 in wild-type, but not in Nrf2 knock-out MFs.
***Conclusions.*** Our data demonstrate that both BHA and tBHQ are strong chemical inducers of HO-1, NQO1 and Nrf2 proteins in primary-cultured human and rat hepatocytes with the activation of MAPK ERK1/2 and JNK1/2. However, in MFs, BHA failed to induce HO-1, whereas tBHQ strongly induced HO-1 in Nrf2 wild-type but not in Nrf2 knock-out, suggesting that Nrf2 is indispensable for tBHQ-induced HO-1 in MF.

**KEY WORDS:** butylated hydroxyanisole (BHA); mitogen-activated protein kinases (MAPKs); Nrf2; *tert*-butylhydroquinone (tBHQ).

## INTRODUCTION

Induction of phase II detoxifying and anti-oxidant enzymes, such as heme oxygenase-1 (HO-1), NAD[P]H:quinone oxidoreductase 1 (NQO-1), γ-glutamylcysteine ligase (γ-GCL), and glutathione *S*-transferases (GSTs) represents an important cellular defense system in response to oxidative and electrophilic insults (1). Several lines of evidence clearly indicate that a coordinated regulation of these genes is mediated at the transcriptional level, in part, by a *cis*-acting element in the promoter, i.e., the antioxidant response element (ARE) (2). NF-E2-related factor 2 (Nrf2) is a basic leucine zipper transcriptional factor that plays a key role in ARE-mediated gene expression (3). Under basal conditions, Nrf2 is sequestered in the cytoplasm as an inactive complex with Kelch-like ECH-associated protein (Keap1). Upon exposure to oxidative stresses or chemical inducers, Nrf2 dissociates from Keap1, translocates into the nucleus and activates ARE-dependent phase II gene expression (4).

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme that catalyzes the degradation of heme, resulting in the production of equimolar quantities of biliverdin, carbon monoxide (CO) and free iron in the ferrous form ($Fe^{2+}$) (5). Because HO-1 expression is inducible in response to various forms of cellular insults and the end-products exhibit substantial anti-oxidative, anti-inflammatory and anti-apoptotic effects (6), HO-1 induction is thought to represent one of prime components of cellular defensive mechanisms. For example, biliverdin

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can scavenge intracellular peroxyradicals, inhibit lipid peroxidation (7) and protect against hydrogen peroxide (8). Intracellular CO also exhibits strong anti-inflammatory (9), vasodilatory (10) and immunomodulatory effects (11) and protects against TNF-α-mediated liver injury (12). In addition, $Fe^{2+}$ formed after heme catabolism is not regarded as harmful because ferritin is concomitantly upregulated and quickly sequesters intracellular iron (13), which otherwise participates in the Fenton reaction and generates intracellular reactive oxygen species (ROS).

NAD[P]H:quinone oxidoreductase 1 (NQO1), also known as DT-Diaphorase or quinone reductase, is another cellular enzyme that protects cells against deleterious reactive semiquinones by converting exogenous quinones into hydroquinones via a single-step two-electron reduction (14). Besides metabolizing foreign compounds, NQO1 also allows the cells to withstand a wide array of oxidative stress by maintaining certain endogenous antioxidants, such as ubiquinone (co-enzyme Q) and vitamin E (α-tocopherol) in their reduced and active states (15). Moreover, NQO1 was recently shown to stabilize p53 protein by blocking Mdm2-dependent proteosomal degradation (16,17), suggesting that stabilization of p53 by NQO1 could serve as another cellular defensive mechanism against carcinogens and their metabolites that cannot be metabolized by NQO1.

Butylated hydroxyanisole (BHA) is a synthetic phenolic antioxidant that is commonly used as a food preservative to extend the shelf life of many food commodities by preventing oxidative rancidity of fats. Studies in many carcinogen-induced animal models have demonstrated that BHA is an effective chemopreventive agent (18–21). BHA can undergo several metabolic pathways in the body, such as dimerization, conjugation and *O*-demethylation (22). One of the major metabolites of BHA, as shown in rats and human, is the demethylated product, *tert*-butylhydroquinone (tBHQ) (23). Because tBHQ exhibits its chemopreventive effects in a similar manner to those by BHA, including the modulation of the enzyme systems responsible for metabolic activation or deactivation of chemical carcinogens (24), it is believed that metabolic transformation of BHA into tBHQ might be an important process for chemoprevention by BHA. In the present study, we examined whether HO-1, NQO-1 and Nrf2 proteins are inducible by BHA and tBHQ treatments and, if so, what signaling pathways could potentially mediate the induction of these proteins in primary-cultured rat and human hepatocytes. In addition, we also report here for the first time that in MFs, Nrf2 might be indispensable for the induction of HO-1 protein by tBHQ.

## MATERIALS AND METHODS

### Chemicals and Antibodies

BHA and tBHQ were purchased from Sigma (St. Louis, MO) and dissolved in DMSO. Polyclonal antibodies against ERK1/2 and JNK1/2 and phospho-specific antibodies against ERK1/2, JNK1/2, and Akt were purchased from Cell Signaling Technologies (Beverly, MA). Polyclonal antibodies against Nrf2, HO-1, NQO1 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### Primary-Cultured Human Hepatocytes

Primary human hepatocytes for Western blots were purchased from *In Vitro* Technology (Baltimore, MD) and Tissue Transformation Technology (Edison, NJ). Primary human hepatocytes for microarray analysis were obtained from Cellzdirect (Plainsboro, NC). All subjects had no recorded liver disease history or damage.

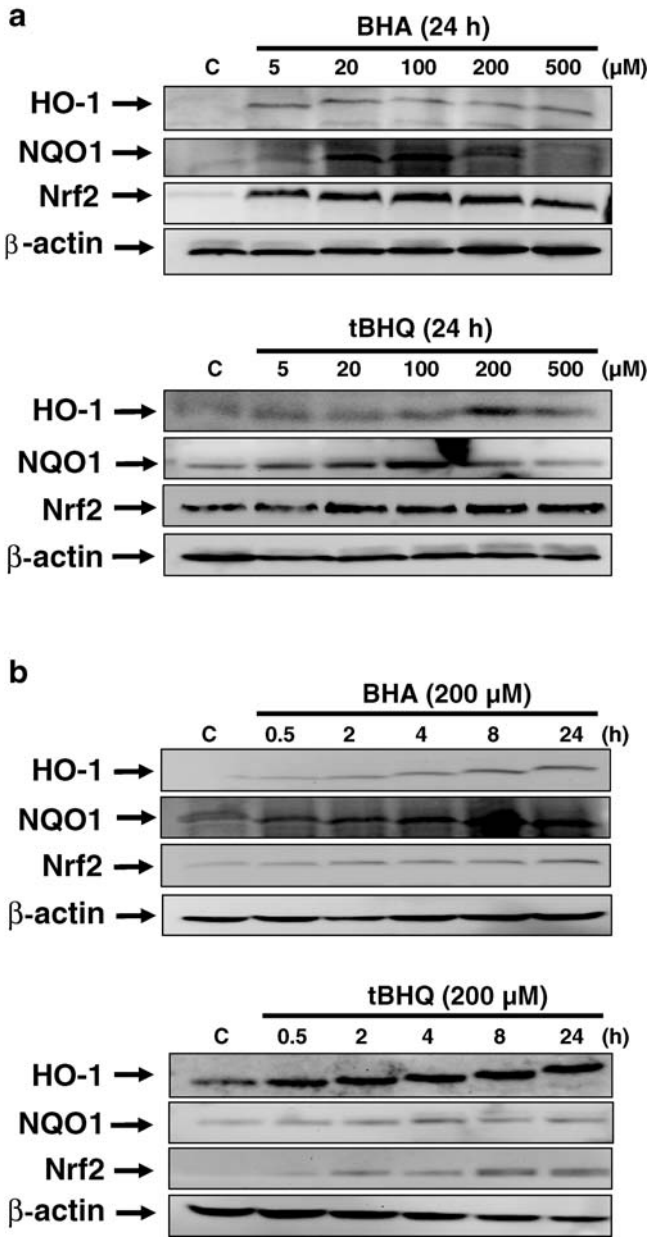


**Fig. 1.** BHA and tBHQ treatments cause dose-dependent (a) and time-dependent (b) induction of HO-1, NQO1 and Nrf2 proteins in primary-cultured rat hepatocytes. After preparation of primary-cultured rat hepatocytes (24 h), they were exposed to BHA and tBHQ at different concentrations (a) or for a period of times as indicated (b). After cell lysis, the protein expression of HO-1, NQO1 and Nrf2 proteins was measured by Western blotting. β-Actin was included as a loading control.

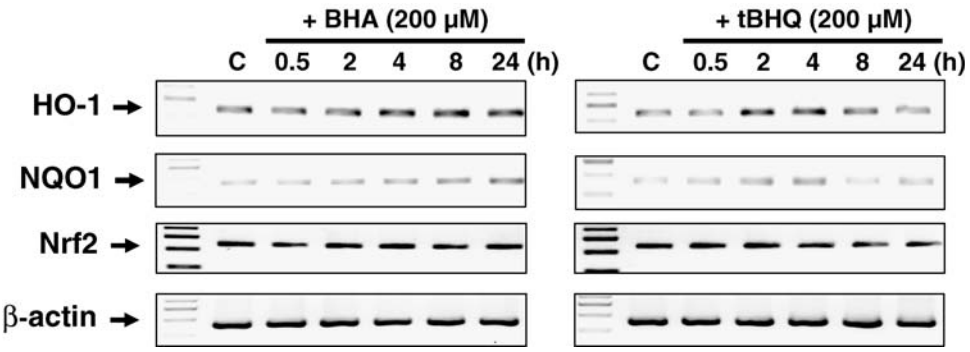


**Fig. 2.** BHA- and tBHQ-mediated induction of HO-1 and NQO1 proteins in primary-cultured rat hepatocytes is regulated at the transcriptional level. After preparation of primary-cultured rat hepatocytes (24 h), they were treated with BHA and tBHQ for a period of times as indicated and total RNA was extracted. Semi-quantitative RT-PCR analysis of HO-1 (25 cycles), NQO1 (25 cycles), Nrf2 (27 cycles) and β-actin (25 cycles) was performed.

### Preparation of Primary-Cultured Rat Hepatocytes and Mouse Neonatal Fibroblasts (MFs)

Rat hepatocytes were harvested from adult Sprague–Dawley rats weighing 250–300 g by way of a two-step *in situ* collagenase perfusion technique. In brief, rats were anesthetized with ketamine and xylazine (60 and 12 mg/kg i.p., respectively) and the liver was perfused *in situ* with oxygenated $Ca^{2+}$-free Krebs–Henseleit bicarbonate buffer containing 5.5 mM glucose for 10 min at 37°C followed by perfusion with the same buffer containing 5 mM $CaCl_2$ and 0.5 mg/ml collagenase type I for 10 min. After perfusion, the liver was removed and hepatocytes were released into 100 ml of DMEM by gently tearing the capsule of the liver. The cells were filtered through a nylon mesh and centrifuged at 50×*g* for 2 min. The pellet was resuspended in 25 ml of DMEM and 25 ml of 90% isotonic Percoll and centrifuged at 100×*g* for 5 min. The cells were resuspended in DMEM and seeded on collagen-precoated 6-well Biocoat® (Becton Dickinson Labware, Bedford, MA) at a density of $2 \times 10^6$ cells per well. Medium was changed on a daily basis thereafter. Mouse neonatal fibroblasts (MFs) from wild-type and Nrf2 knockout C57BL/6 mice were prepared as described previously (25) and maintained in modified F-12 medium supplemented with 10% FBS, 1.17 g/l sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% essential amino acids, and 0.1% insulin, in a humidified atmosphere of 5% $CO_2$ at 37°C.

### Western Blotting

After treatments, cells were washed with ice-cold PBS (pH 7.4) and harvested with 200 μl of a whole cell lysis buffer (pH 7.4), containing 10 mM Tris-HCl, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovanadate, 2 mM iodoacetic acid, 5 mM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5% Triton-X 100. Cell lysates were vigorously vortexed and incubated on ice for 30 min. The homogenates were centrifuged at 13,000 rpm for 10 min at 4°C and the supernatants were collected. After mixing equal amounts of the samples in loading buffer, followed by heating at 95°C for 5 min, the samples were resolved in a 10% SDS-polyacrylamide gel electrophoresis at 200 V and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA) for 2 h at 200 mA, using a semi-dry

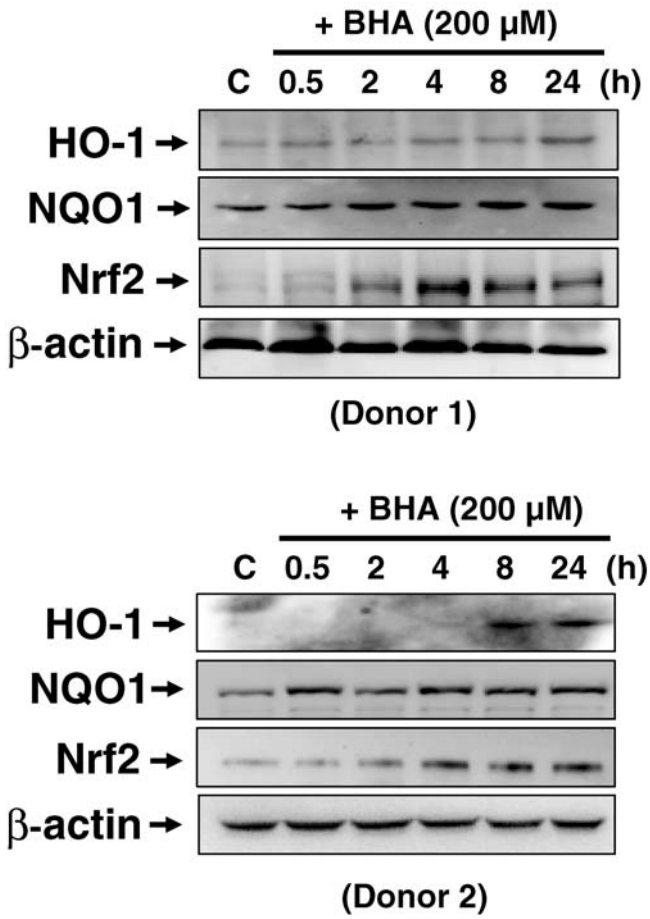


**Fig. 3.** BHA treatment results in a time-dependent induction of HO-1, NQO1 and Nrf2 proteins in primary-cultured human hepatocytes from two different donors. After arrival, primary-cultured human hepatocytes were stabilized in the incubator for 24 h and BHA was treated for a period of times as indicated. Western blotting was conducted to measure the protein expression of HO-1, NQO1 and Nrf2 proteins. β-Actin was included as a loading control.

**Table I.** BHA-Induced Gene Expression Profiles in Primary-Cultured Human Hepatocytes

| Gene description | Name | GenBank | Fold induction |
|---|---|---|---|
| *Detoxification* | | | |
| heme oxygenase (decycling) 1 | HMOX1 | NM_002133 | 8.862 |
| thioredoxin domain containing 4 (endoplasmic reticulum) | TXNDC4 | AI827677 | 5.47 |
| thioredoxin domain containing 5 | TXNDC5 | AI123471 | 10.37 |
| thioredoxin reductase 1 | TXNRD1 | BC042974 | 13.75 |
| NADPH oxidase 4 | NOX4 | NM_016931 | 2.036 |
| *Cell cycle and apoptosis* | | | |
| caspase 10, apoptosis-related cysteine protease | CASP10 | AF111345 | 2.755 |
| caspase 8, apoptosis-related cysteine protease | CASP8 | NM_033358 | 2.049 |
| CASP8 associated protein 2 | CASP8AP2 | BC027850 | 6.845 |
| cyclin B2 | CCNB2 | BF509102 | 2.377 |
| cyclin C | CCNC | BC026272 | 3.014 |
| cyclin D2 | CCND2 | AW026491 | 4.153 |
| cyclin E1 | CCNE1 | AW104515 | 4.556 |
| cyclin G2 | CCNG2 | BC032518 | 3.164 |
| cyclin 1 | CCNI | AA808018 | 4.684 |
| cell division cycle 25A | CDC25A | AY137580 | 24.28 |
| cell division cycle 25C | CDC25C | AF277724 | 3.212 |
| cell division cycle 2-like 5 (cholinesterase-related cell division controller) | CDC2L5 | BC001274 | 2.654 |
| cyclin-dependent kinase 5, regulatory subunit 1 (p35) | CDK5R1 | NM_003885 | 4.607 |
| cyclin-dependent kinase 6 | CDK6 | AI738463 | 2.502 |
| cell death-regulatory protein GRIM 19 | GRIM19 | AI871270 | 8.226 |
| p53-regulated apoptosis-inducing protein 1 | P53AIP1 | NM_022112 | 6.325 |
| *Cytochrome P450s* | | | |
| cytochrome P450, family 11, subfamily A, polypeptide 1 | CYP11A1 | NM_000781 | 2.945 |
| cytochrome P450, family 19, subfamily A, polypeptide 1 | CYP19A1 | NM_000103 | 22.91 |
| cytochrome P450, family 1, subfamily B, polypeptide 1 | CYP1B1 | N21019 | 8.039 |
| cytochrome P450, family 21, subfamily A, polypeptide 2 | CYP21A2 | M17252 | 3.331 |
| cytochrome P450, family 2, subfamily B, polypeptide 6 | CYP2B6 | NM_000767 | 2.237 |
| cytochrome P450, family 2, subfamily F, polypeptide 1 | CYP2F1 | NM_000774 | 9.597 |
| cytochrome P450, family 2, subfamily S, polypeptide 1 | CYP2S1 | AF335278 | 8.706 |
| cytochrome P450, family 2, subfamily U, polypeptide 1 | CYP2U1 | BC012027 | 4.144 |
| cytochrome P450, family 46, subfamily A, polypeptide 1 | CYP46A1 | NM_006668 | 2.21 |
| cytochrome P450, family 4, subfamily F, polypeptide 8 | CYP4F8 | AF133298 | 3.102 |
| cytochrome P450 4Z1 | CYP4Z1 | AV700083 | 7.878 |
| *Kinase and phosphatase* | | | |
| calcium/calmodulin-dependent protein kinase (CaM kinase)II alpha | CAMK2A | NM_015981 | 4.362 |
| calcium/calmodulin-dependent protein kinase (CaM kinase)II beta | CAMK2B | AF081924 | 5.138 |
| calcium/calmodulin-dependent protein kinase (CaM kinase)II delta | CAMK2D | AI829910 | 5.019 |
| p21 (CDKN1A-activated kinase 7) | PAK7 | AB040812 | 4.188 |
| phosphoinositide-3-kinase, class 3 | PIK3C3 | AU148957 | 3.572 |
| phosphoinositide-3-kinase, catalytic, gamma polypeptide | PIK3CG | AF327656 | 5.429 |
| phosphoinositide-3-kinase, regulatory subunit, polypeptide 2 (p85 beta) | PIK3R2 | AI684344 | 2.194 |
| protein phosphatase 2, regulatory subunit B (B56), gamma isoform | PPP2R5C | BC016183 | 11.86 |
| protein phosphatase 4, regulatory subunit 1 | PPP4R1 | AL833163 | 3.548 |
| protein kinase, cAMP-dependent, catalytic, alpha | PRKACA | M80335 | 2.204 |
| protein kinase, cAMP-dependent, catalytic, gamma | PRKACG | NM_002732 | 3.701 |
| protein kinase, AMP-activated, gamma 2 non-catalytic subunit | PRKAG2 | BE466158 | 2.75 |
| protein kinase, AMP-activated, gamma 3 non-catalytic subunit | PRKAG3 | AA002166 | 2.015 |
| protein kinase C, alpha | PRKCA | NM_002737 | 4.99 |
| protein kinase C, epsilon | PRKCE | AA626142 | 6.067 |
| protein kinase C, mu | PRKCM | AW085172 | 8.219 |
| *Receptors and transcription factors* | | | |
| E2F transcription factor 2 | E2F2 | NM_004091 | 2.585 |
| FOXJ2 forkhead factor | FHX | NM_018416 | 2.081 |
| Estrogen receptor 1 | ESR1 | AI073549 | 14.83 |
| hepatocyte nuclear factor 4, gamma | HNF4G | NM_004133 | 2.233 |
| Retinoic acid receptor, beta | RARB | NM_000965 | 2.356 |
| Sp1 transcription factor | SP1 | AU121035 | 2.071 |
| *Transporters* | | | |
| ATP-binding cassette, sub-family B (MDR/TAP), member 4 | ABCB4 | BC027586 | 2.798 |
| ATP-binding cassette, sub-family B (MDR/TAP), member 5 | ABCB5 | U66692 | 16.66 |
| ATP-binding cassette, sub-family C (CFTR/MRP), member 12 | ABCC12 | AF395909 | 7.186 |

**Table I.** Continued

| Gene description | Name | GenBank | Fold induction |
|---|---|---|---|
| ATP-binding cassette, sub-family C (CFTR/MRP), member 9 | ABCC9 | AK056519 | 4.005 |
| solute carrier family 13 (sodium/sulfate symporters), member 1 | SLC13A1 | NM_022444 | 2.056 |
| solute carrier family 15 (H+/peptide transporter), member 2 | SLC15A2 | BF223679 | 2.663 |
| solute carrier family 22 (organic anion transporter), member 6 | SLC22A6 | AJ271205 | 4.405 |
| solute carrier family 29 (nucleoside transporters), member 2 | SLC29A2 | AF034102 | 6.144 |
| solute carrier family 30 (zinc transporter), member 5 | SLC30A5 | NM_024055 | 2.063 |
| solute carrier family 30 (zinc transporter), member 6 | SLC30A6 | NM_017964 | 9.422 |
| solute carrier family 30 (zinc transporter), member 8 | SLC30A8 | AW300204 | 5.328 |
| solute carrier family 31 (copper transporters), member 2 | SLC31A2 | AI800670 | 13.67 |

transfer system (Fisher Scientific, Pittsburgh, PA). The membranes were blocked with 5% nonfat dry milk in 1× PBST buffer (0.1% Tween 20 in PBS) for 1 h at room temperature and incubated with primary antibodies in 3% nonfat dry milk of 1× PBS (1:1,000 dilution) for polyclonal antibodies or in 3% bovine serum albumin (BSA) of 1× PBS for phospho-specific antibodies overnight at 4°C. After hybridization with primary antibody, membranes were washed three times with 1× PBST and then incubated with secondary antibody conjugated with horseradish peroxidase for 1 h at room temperature and washed with 1× PBST three times. Final detection was performed with enhanced chemiluminescence Western blotting reagents (Amersham Pharmacia, Piscataway, NJ) and the bands were visualized with BioRad ChemiDoc XRS system (Hercules, CA).

### RNA Extraction and Semi-Quantitative RT-PCR

Total RNA from rat hepatocytes was isolated with a combined method using Trizol (Invitrogen, Carlsbad, CA) and RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA samples were converted to single-stranded cDNA by the Superscript First-Strand Synthesis System III (Invitrogen) and the resulting cDNA was amplified by the PCR supermix kit (Invitrogen). PCR conditions are as follows: 94°C for 10 min followed by cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 45 s, and a final extension at 72°C for 10 min. The 5′ and 3′ primers used for amplifying HO-1 were 5′-AGCATGTCCCAGGA TTTGTC-3′ and 5′-AAGGCGGTCTTAGCCTCTTC-3′. The 5′ and 3′ primers used for amplifying NQO1 were 5′-CG CAGAGAGGACATCATTCA-3′ and 5′-GTGGTGATG GAAAGCAAGGT-3′. The 5′ and 3′ primers used for amplifying Nrf2 were 5′-GAGACGGCCATGACTGATTT-3′ and 5′-TGGGTCTCCGTAAATGGAAG-3′. β-Actin was used as an internal control and was amplified with the 5′ and 3′ primers: 5′-TGTTACCAACTGGGACGACA-3′ and 5′-TC TCAGCTGTGGTGGTGAAG-3′. PCR products were resolved on 1.5% agarose gels and visualized under UV lamps.

### Microarray Analysis

After RNA isolation, the samples were sent to the Cancer Institute of New Jersey (CINJ) Core Expression Array Facility at the Robert Wood Johnson Medical School (New Brunswick, NJ) where all the microarray analysis procedures were performed, including RNA quality control and concentration measurement, cDNA synthesis and cRNA biotin-labeling, hybridization, and array scanning. GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA) with over 54,000 probe sets representing about 38,500 well-characterized human genes was used for analyzing the gene expression profiles of the human hepatocyte samples. After array scanning, the expression analysis file created from each sample (chip) scanning was imported into GeneSpring 6.1 software (Silicon Genetics, Redwood City, CA) for further data characterization. A new experiment was generated after importing data in which data were normalized to the 50th percentile of all measurements on that array. Data filtration based on flags present in at least one of the samples was generated and the lists of genes that were induced over twofold after treatment were created by filtration-on-fold function within the presented flag list, as we have described recently (26).

## RESULTS

### BHA and tBHQ Treatments Result in Dose- and Time-Dependent Induction of HO-1, NQO1 and Nrf2 Proteins in Primary-Cultured Rat Hepatocytes

The induction of phase II drug metabolizing and antioxidant enzymes represents a primary cellular defensive mechanism. However it is still unclear whether these enzymes are inducible in normal hepatocytes by BHA and/or tBHQ treatments. As seen in Fig. 1a, we found that BHA treatment for 24 h significantly induced the protein expression of HO-1 (5–500 μM), NQO1 (5–200 μM) and Nrf2 (5–200 μM) in primary-cultured rat hepatocytes (upper panel). Treatment with tBHQ, a demethylated metabolite of BHA could also up-regulate HO-1, NQO1 and Nrf2 proteins in primary-cultured rat hepatocytes. Whereas HO-1 was selectively inducible at 200 μM, NQO1 and Nrf2 proteins were increased at 5–200 and 20–500 μM, respectively (lower panel). Supporting this observation, time-kinetic studies have demonstrated that exposure of BHA and tBHQ (200 μM each) to primary-cultured rat hepatocytes increased the protein expressions of HO-1, NQO1 and Nrf2 in a time-dependent manner (Fig. 1b). These data suggest that both BHA and tBHQ treatments can increase the expression of HO-1, NQO1 and Nrf2 proteins in primary-cultured rat hepatocytes.

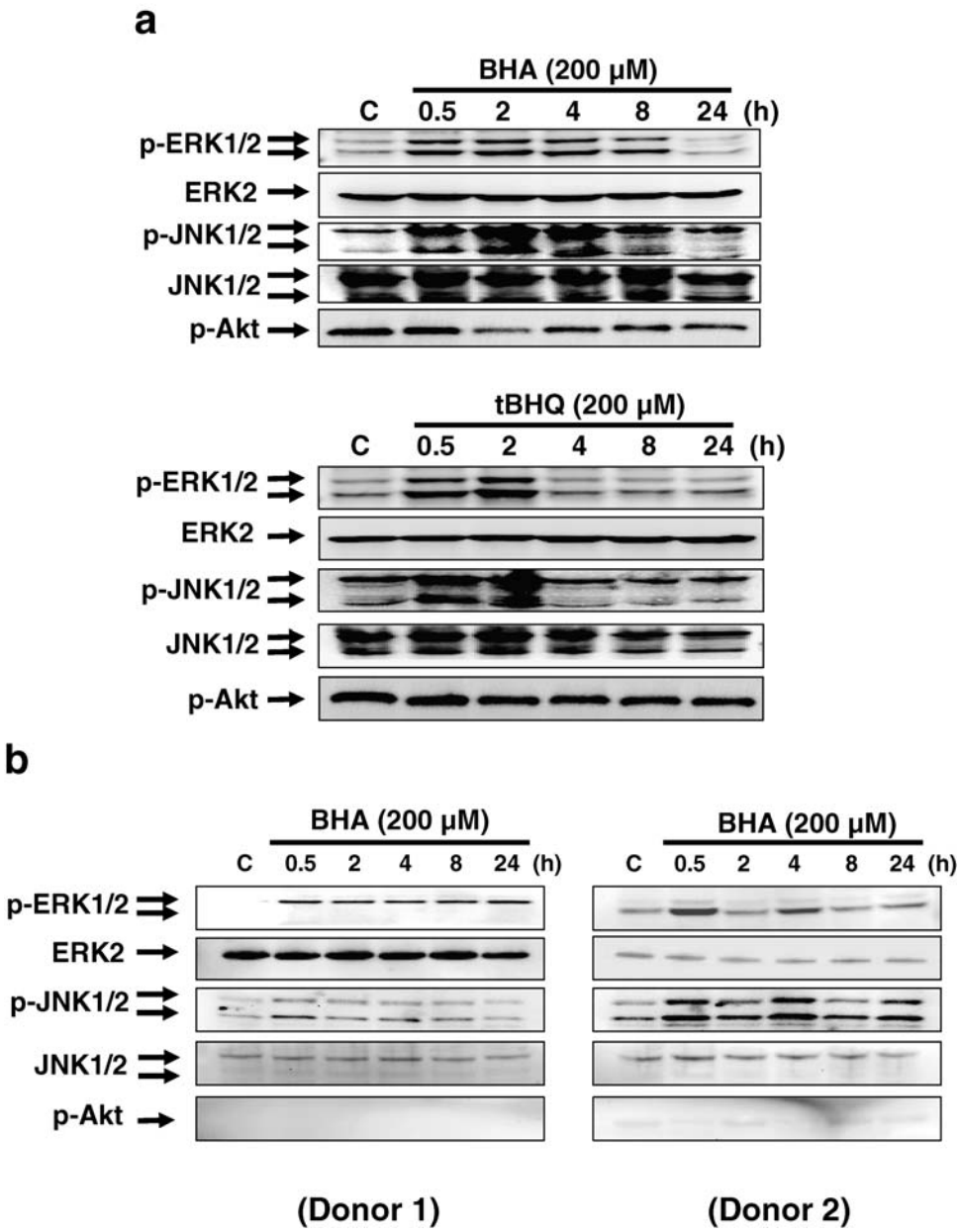


**Fig. 4.** Effects of BHA and tBHQ on the activation of ERK1/2, JNK1/2 and Akt in primary-cultured rat (a) and human hepatocytes (b). The same cell lysates as shown in Figs. 1b (a) and 3 (b) were used to analyze the phosphorylation changes of ERK1/2, JNK and Akt, using phospho-specific ERK1/2, JNK and Akt antibodies. Endogenous levels of ERK2 and JNK1/2 were also measured using polyclonal antibodies against ERK2, JNK1/2. β-Actin was included as a loading control.

### Induction of HO-1 and NQO1 Proteins by BHA and tBHQ Is Transcriptionally Regulated in Primary-Cultured Rat Hepatocytes

Because the induction of HO-1 and NQO1 proteins by BHA and tBHQ was accompanied with an increase of the transcription factor, Nrf2 (Fig. 1), we predicted that the induction of HO-1 and NQO1 proteins might occur through transcriptional activation. In order to address this question, we treated BHA and tBHQ in primary-cultured rat hepatocytes for different time points and extracted RNA to conduct semi-quantitative RT-PCR. As observed in Fig. 2, BHA and tBHQ treatments increased HO-1 and NQO1 mRNA levels in primary-cultured rat hepatocytes. While BHA caused a steady increase of HO-1 and NQO1 mRNA up to 24 h, the maximal transcriptional activation of HO-1 and NQO1 by tBHQ was observed after 4 h treatment and then decreased gradually thereafter. However, we found that Nrf2 mRNA was barely affected by BHA and tBHQ treatments. Since the amount of cellular Nrf2 protein is generally regulated at the post-translational level by modulating proteosomal degradation (27,28), we presume that the same mechanism might

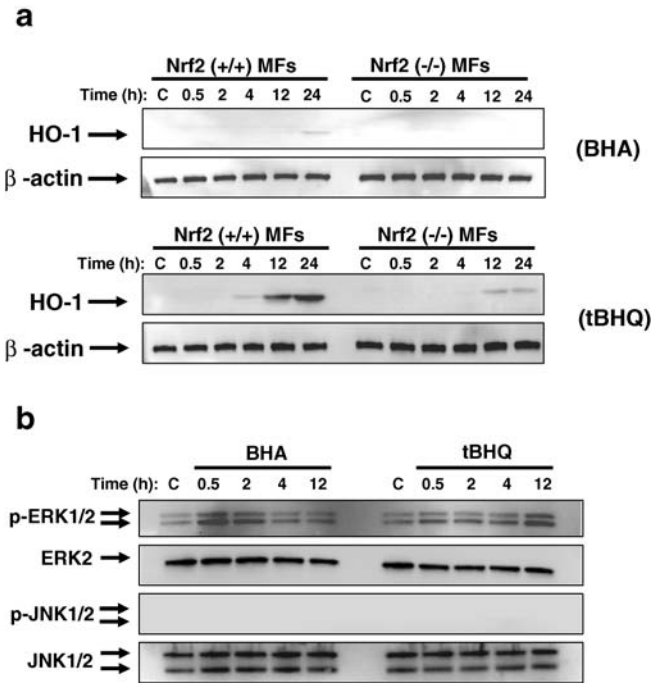


**Fig. 5.** Effects of BHA (200 μM) and tBHQ (200 μM) on (a) the induction of HO-1 protein in wild-type and Nrf2 knock-out MFs and (b) the phosphorylation of ERK1/2 and JNK1/2 in wild-type MFs. Wild-type and Nrf2 knock-out MFs were treated with BHA and tBHQ for various time periods as indicated and Western blotting was performed. β-Actin was included to serve as a loading control.

have contributed to BHA- and tBHQ-mediated induction of Nrf2 protein in primary-cultured rat hepatocytes. Taken together, our data suggest that the induction of HO-1 and NQO1 by BHA and tBHQ was transcriptionally regulated.

### BHA-Mediated Induction of HO-1, NQO1 and Nrf2 Proteins and Global Gene Expression in Primary-Cultured Human Hepatocytes

In order to investigate whether HO-1, NQO1 and Nrf2 proteins are inducible in human normal hepatocytes after BHA treatment, we have purchased primary-cultured human hepatocytes from two different donors and treated them with BHA (200 μM) at different time points. Similar to rat hepatocytes, BHA treatment caused a time-dependent induction of HO-1, NQO1 and Nrf2 proteins in both donors' human hepatocytes (Fig. 3), implying that these proteins are also inducible in primary cultured human hepatocytes by BHA treatment. In order to examine the global gene expression changes by BHA in primary-cultured human hepatocytes, we purchased additional primary cultured human hepatocytes and exposed them to BHA (185 μM) for 10 h. Then, RNA was isolated and subjected to Affymetrix microarray analysis. After data normalization, we found that 38.4% (20,997) of the probes passed through the filtration. Among them, transcriptional expression of 462 well-defined genes was elevated but that of 837 well-defined genes was suppressed more than two fold. Selected examples of genes, induced by BHA are listed in Table I. While BHA treatment significantly increased the mRNA level of HO-1, it failed to increase NQO1 mRNA in this sample. Although the exact reason for this is unclear at present, it is possible that BHA activated transcription of NQO1 at time point(s) other than 10 h or not at all, due to some possible genetic polymorphism in this donor.

### BHA Treatment Activates ERK and JNK, but not Akt in Primary Cultured Human and Primary-Cultured Rat Hepatocytes

It is known that multiple signaling kinase cascades are involved in the regulation of Nrf2/ARE-dependent gene expression, including MAPKs, PI3K, PKC and PERK (29). In order to explore the possible involvement of MAPKs and PI3K in the induction of HO-1 and NQO1 proteins by BHA and tBHQ treatments, we monitored the phosphorylation level changes of ERK1/2, JNK1/2, p85 and Akt, a direct downstream target of PI3K, using the same protein extracts in Figs. 1b and 3. As seen in Fig. 4, exposure of BHA and tBHQ strongly activated phosphorylation of ERK1/2 and JNK1/2 in both primary-cultured rat and human hepatocytes. However, we found that BHA and tBHQ treatment failed to activate p85, the catalytic subunit of PI3K, in both hepatocytes (data not shown). In addition, BHA and tBHQ treatments inhibited phosphorylation of Akt, a direct downstream target of PI3K, in primary-cultured rat hepatocytes (Fig. 4a). Moreover, we found that Akt phosphorylation was not observed in human hepatocytes from donor 1 and weak phosphorylation of Akt occurred in hepatocytes from donor 2, which was unaffected by BHA treatment (Fig. 4b). These results imply that the activation of ERK1/2 and JNK1/2, but not PI3K might be contributing to BHA- and tBHQ-mediated induction of HO-1 and NQO1 proteins in primary-cultured rat and human hepatocytes.

### Nrf2 Is Indispensable for tBHQ-Mediated Induction of HO-1 Protein in Mouse Neonatal Fibroblasts (MFs)

We next prepared mouse neonatal fibroblasts (MFs) from wild-type and Nrf2-null mice and measured the induction of HO-1 and NQO1 proteins by BHA and tBHQ. As shown seen in Fig. 5a, BHA treatment failed to induce HO-1 protein in both wild-type and Nrf2-deficient MFs. In addition, while tBHQ treatment strongly induced HO-1 protein in wild-type MFs, no significant induction of HO-1 protein was observed in Nrf2-deficient MFs after tBHQ treatment. Since MFs generally lack many metabolizing enzymes unlike the hepatocytes, it is speculated that BHA might not be able to induce HO-1 protein in MFs due to a lack of metabolic transformation of BHA into tBHQ or some other active metabolites. In addition, our data clearly demonstrates that Nrf2 is an essential transcription factor, required for the induction of HO-1 protein.

Next, we investigated whether the selective activation of ERK1/2 and/or JNK1/2 might contribute to the induction of HO-1 protein by tBHQ, but not by BHA in wild-type MFs and found that both BHA and tBHQ could activate ERK1/2 in MFs, while no activation of JNK1/2 was observed (Fig. 5b). However, interestingly, while the phosphorylation kinetics of ERK1/2 by BHA was transient (up to 2 h), that of ERK1/2 by tBHQ was sustained up to 12 h. Considering that the

induction of HO-1 protein by tBHQ occurred at 4 h and was maximal after 24 h (Fig. 5a), it is possible that the prolonged activation of ERK1/2 might play an important role in the induction of HO-1 protein in Nrf2 wild-type MFs. On the other hand, we observed that BHA and tBHQ treatments caused a very weak induction of NQO1 protein in both wild-type and Nrf2-deficient MFs (data not shown). The reason for this is unclear at present. Since aromatic hydrogen receptor (AhR) and estrogen receptor beta (ERβ) can bind to the promoter regions of NQO1 and regulate its transcription (30), it will be interesting to examine in the future whether these nuclear receptors cooperatively play important roles in the induction of NQO1 by BHA and tBHQ.

## DISCUSSION

Our present study demonstrates that BHA and tBHQ treatments increased HO-1, NQO1 and Nrf2 proteins in both primary-cultured rat and human hepatocytes (Figs. 1 and 3). RT-PCR results revealed that BHA- and tBHQ-mediated induction of HO-1 and NQO1 proteins occurred through transcriptional activation in primary-cultured rat hepatocytes (Fig. 2). In addition, we have demonstrated that induction of HO-1 and NQO1 proteins in both rat and human hepatocytes was closely correlated with activation of ERK1/2 and JNK1/2. Based on these data, we speculate that the activation of ERK1/2 and JNK1/2 by BHA and tBHQ might contribute to the transcriptional activation of ARE/Nrf2 module and subsequent induction of HO-1 and NQO1 proteins. However, we failed to detect any phosphorylation activation of the p85 protein of PI3K by BHA and tBHQ treatments in either rat or human hepatocytes. Rather, we observed that phosphorylation of Akt, a direct downstream regulator of PI3K was unaffected or substantially decreased (Fig. 4). Considering that PI3K could play a positive role in the transcriptional activation of Nrf2/ARE in some other cell lines (31), it appears that the PI3K might not be involved nor contributing to the activation of ARE/Nrf2 and induction of HO-1 and NQO1 proteins in our primary-cultured human and rat hepatocytes. In fact, this observation appears to be consistent with many recent studies that inhibition of Akt phosphorylation contributes to apoptotic induction in tumor cell lines by many chemopreventive agents (32–34).

Using microarray analysis, we have acquired the global gene expression profiles in primary-cultured human hepatocytes in response to BHA treatment. Based on the biological functions, genes could be classified into categories, including detoxification, cell cycle and apoptosis, cytochrome P450s, kinase and phosphatases, receptors and transcription factors and transporters (Table I). To the best of our knowledge, this is the first demonstration of gene expression profiles in normal human hepatocytes after BHA treatment. Nonetheless, due to the fact that the current expression profiles were obtained from hepatocytes of a single donor and human hepatic gene expression is highly variable between the individuals, additional microarray studies of different donors will be required to decipher the exact molecular mechanisms as to how BHA would exert its cancer protective effects in human hepatocytes.

BHA is typically metabolized by cytochrome P450s or monooxygenase to tBHQ and that in turn, is converted to *tert*-butylquinone either through autooxidation or by some of other enzymes in the hepatocytes. Formation of *tert*-butylquinone results in a redox-cycling and subsequent production of reactive oxygen species (35). Alternatively, BHA can be metabolized by peroxidases such as horseradish peroxidase and cyclooxygenase to phenoxyl free radicals that are capable of binding to macromolecules (36,37). Many *in vivo* studies have demonstrated that BHA is sometimes carcinogenic in certain animal models, especially at very high dose levels (38,39). As a possible mechanism, we have reported that the induction of apoptosis in primary-cultured rat hepatocytes at very high concentration of BHA (500 μM) did not occur through metabolic transformation into tBHQ, but rather involved a sequential apoptotic process, such as generation of intracellular oxygen species, loss of mitochondrial membrane, activation of caspases and the direct release of cytochrome *c* (40). Since tBHQ but not BHA strongly induced HO-1 protein in wild-type MFs (Fig. 5a), it is possible that the mechanisms that are responsible for the induction of HO-1 *versus* that of induction of apoptosis in primary-cultured hepatocytes could be different, in that BHA by itself induces apoptosis in the hepatocytes at very high concentration, however it might need to be metabolized to tBHQ for the induction of HO-1 protein in the hepatocytes. This would warrant further investigations in the future to better understand how BHA can be carcinogenic and/or anti-carcinogenic *in vivo.*

In summary, we have demonstrated that treatments of BHA and tBHQ can induce HO-1 and NQO1 in primary-cultured human and rat hepatocytes. In addition, activation of ERK1/2 and JNK1/2, but not of PI3K appears to be closely correlated with BHA and tBHQ-mediated induction of HO-1 and NQO1. Finally, we found that the BHA failed to induce HO-1 in MFs, whereas, tBHQ strongly induced HO-1 in Nrf2 wild-type but not in Nrf2 knockout MFs, suggesting that biotransformation of BHA to tBHQ might be required and that Nrf2 is indispensable for the induction of HO-1, at least in the mouse fibroblasts. Taken together, our present study shows that for the first time the induction of phase II drug metabolizing and antioxidant enzymes in human and rat hepatocytes by BHA and tBHQ, the activation of MAPK signaling pathways and the requirement of Nrf2 in the induction of these genes, which would culminate in the overall cellular protective effect and the subsequent cancer chemopreventive pharmacological response elicited by these phenolic antioxidant compounds.

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