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In vivo Pharmacokinetics, Activation of MAPK Signaling and Induction of Phase II/III Drug Metabolizing Enzymes/Transporters by Cancer Chemopreventive Compound BHA in the Mice

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Phenolic antioxidant butylated hydroxyanisole (BHA) is a commonly used food preservative with broad biological activities, including protection against chemical-induced carcinogenesis, acute toxicity of chemicals, modulation of macromolecule synthesis and immune response, induction of phase II detoxifying enzymes, as well as its undesirable potential tumor-promoting activities. Understanding the molecular basis underlying these diverse biological actions of BHA is thus of great importance. Here we studied the pharmacokinetics, activation of signaling kinases and induction of phase II/III drug metabolizing enzymes/transporter gene expression by BHA in the mice. The peak plasma concentration of BHA achieved in our current study after oral administration of 200 mg/kg BHA was around 10 μM. This *in vivo* concentration might offer some insights for the many *in vitro* cell culture studies on signal transduction and induction of phase II genes using similar concentrations. The oral bioavailability (F) of BHA was about 43% in the mice. In the mouse liver, BHA induced the expression of phase II genes including NQO-1, HO-1, γ-GCS, GST-pi and UGT 1A6, as well as some of the phase III transporter genes, such as MRP1 and Slco1b2. In addition, BHA activated distinct mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK), extracellular signal-regulated protein kinase (ERK), as well as p38, suggesting that the MAPK pathways may play an important role in early signaling events leading to the regulation of gene expression including phase II drug metabolizing and some phase III drug transporter genes. This is the first study to demonstrate the *in vivo* pharmacokinetics of BHA, the *in vivo* activation of MAPK signaling proteins, as well as the *in vivo* induction of Phase II/III drug metabolizing enzymes/transporters in the mouse livers.

Key words: Butylated hydroxyanisole, Pharmacokinetics, MAPKs, Phase II gene, Transporter

INTRODUCTION

Butylated hydroxyanisole (BHA), a synthetic phenolic antioxidant due to its chain-breaking action during the autooxidation of lipid, is widely used as a food preservative (Rehwoldt, 1986). In addition to the inhibition of lipid peroxidation, numerous studies in animals reveal that this compound exhibits a wide range of biological activities. BHA protects animals against radiation and the acute toxicity of various xenobiotics and mutagens (Kahl, 1984; Brekke *et al.*, 1992; Vercammen *et al.*, 1998). Wattenberg and coworkers were among the first to report that BHA and its metabolite *tert*-butylhydroquinone (tBHQ) to protect rodents against formation of tumors (Wattenberg, 1973, 1983, 1985). Subsequently, a large number of studies have established that phenolic compounds/antioxidants including BHA are effective cancer preventive (chemopreventive) agents for carcinogenesis/tumorigenesis induced by a variety of carcinogens at numerous organ sites (Talalay *et al.*, 1978; King and McCay, 1983). Dietary administration of BHA also leads to the protection against various carcinogens, presumably through the induction of many

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phase II detoxifying enzymes such as epoxide hydrolases (Cha *et al.*, 1978; Monroe and Eaton, 1987), glutathione *S*-transferases (GSTs) (Benson *et al.*, 1978), and UDP-glucuronosyltransferases (UGT) (Moldeus *et al.*, 1982), as well as through the inhibition of cytochrome P-450 monooxygenase (Cummings and Prough, 1983). In contrast to its beneficial effects, BHA is also found to be toxic and even carcinogenic in some animal models, especially after higher doses. For example, BHA induced papilloma and carcinoma formation in the forestomachs of rats, mice, and hamsters when fed continuously at high doses (Ito *et al.*, 1983a; Clayson *et al.*, 1990). Chronic dietary administration of BHA also enhances the development of preneoplastic and neoplastic lesions in the rat kidney and urinary bladder (Nera *et al.*, 1988; Peters *et al.*, 1996). Furthermore, BHA appears to have initiating activity in two-stage mouse skin carcinogenesis assay and in two-stage transformation of BALB/3T3 cells (Sakai *et al.*, 1990). Most notably, BHA induced proliferative effects not only in rodent forestomachs but also in the esophagus of pigs and primates (Iverson *et al.*, 1985; Wurtzen and Olsen, 1986). Consistent with these results, we have previously shown that BHA and its metabolite, tBHQ, exerted a dose-dependent toxic effect (especially at high doses) in human hepatoma HepG2 cells, human cervical squamous carcinoma HeLa cells, as well as in primary cultured of rat hepatocutes (Yu *et al.*, 1997, 2000). Thus, this well known antioxidant can exert opposing biological effects, beneficial as well as toxic effects. Although both anti-carcinogenic and carcinogenic effects of BHA are well described, the precise mechanisms as to how these effects are derived remain obscure but probably are dose- and/or tissue-dependent.

Phase II detoxifying enzymes include NAD(P)H:quinone oxidoreductase (NQO), GSTs, γ-glutamylcysteine synthetase (γ-GCS), heme oxygenase-1 (HO-1), UGTs, and epoxide hydrolases. These enzymes are capable of converting the reactive electrophiles to less toxic and more readily excretable products, thus protecting cells against various chemical stresses and mutagenesis and carcinogenesis induced by carcinogens (Benson *et al.*, 1978; Prochaska *et al.*, 1985; Wattenberg, 1985). Biochemical and genetic studies revealed that the induction of phase II detoxifying enzymes by various chemicals occurs at the transcriptional level and is regulated by a *cis*-acting regulatory element, defined as the antioxidant responsive element (ARE) or electrophile-responsive element (EpRE). This regulatory element was first detected in the 5'-flanking region of the rat and mouse GST-Ya subunit gene (Friling *et al.*, 1990; Rushmore and Pickett, 1990; Rushmore *et al.*, 1991) and human NQO-1 genes (Favreau and Pickett, 1991; Li and Jaiswal, 1992) and is also expected to be present in the promoters of epoxide hydrolase and UGT genes. Recent studies from several laboratories showed that the basic leucine zipper (bZIP) transcription factors, including nuclear factor-E2-related factor 1 (Nrf1) (Venugopal and Jaiswal, 1996), Nrf2 (Venugopal and Jaiswal, 1996; Itoh *et al.*, 1997) and small Mafs (Itoh *et al.*, 1997) were implicated in the binding to and transcriptional activation of the ARE sequences. In knock-out studies, the induction of QR and GST by BHA was largely eliminated in the liver and intestine of Nrf2-/- mice (Itoh *et al.*, 1997), suggesting the critical role of Nrf2 in the regulation of ARE-mediated phase II genes induction by BHA. Furthermore, the transcriptional activation of the Nrf2/ARE signaling pathway by phase II enzyme inducers can also be modulated by several cellular signaling molecules such as protein kinase C (PKC), phophainositol-3-kinase (PI3K), RNA-dependant protein kinase-like endoplasmic reticulum kinase (PERK) and mitogen-activated protein kinase (MAPK) (Bloom and Jaiswal, 2003; Chen *et al.*, 2004; Cullinan *et al.*, 2004; Kraft *et al.*, 2004).

MAPKs, which belong to the superfamily of serine/threonine kinases, are evolutionarily conserved in all eukaryotes and play a central role in transducing various extracellular signals into the nuclei (Cobb and Goldsmith, 1995). A typical MAPK cascade consists of three kinases: an MAPK kinase kinase, which phosphorylates and activates an MAPK kinase, which, in turn, phosphorylates and activates MAPK (Marshall, 1994). Extracellular signal-regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) are often responsive to different extracellular signals (Wu *et al.*, 1991; Kyriakis *et al.*, 1994). In contrast, the p38 pathways are activated primarily by a diverse array of cellular stresses (Ichijo, 1999). However, they can also be activated by the same stimuli such as mitogenic signals, growth factors, oncogenic Ras (Logan *et al.*, 1997; Vojtek and Der, 1998), stress signals, UV radiation, and oxidative stress (Guyton *et al.*, 1996; Liu *et al.*, 1996). We have previously shown that BHA and its metabolite t-BHQ activated MAPK in human hepatoma HepG2 and cervical squamous carcinoma HeLa cells, as well as in rat hepatocytes (Yu *et al.*, 1997, 2000), and demonstrated a positive role of MAPK in the regulation of phase II genes by tBHQ. However, the *in vivo* activation of these kinases has not been examined. In this study, we investigated the *in vivo* pharmacokinetics of BHA in mice, the *in vivo* MAPK activation as well as the induction of phase II drug enzymes and phase III drug transporters in mouse liver by BHA.

MATERIALS AND METHODS

Animals and drug treatments

Swiss Webster mice were obtained from Hilltop Laboratories (Wilmington, DE) and kept *ad libitum* with

food (AIN 76A) and water and housed in the Rutgers University Animal Facilities. BHA and the internal standard 4-methoxyphenol were purchased from Sigma (St. Louis, MO). Methanol was HPLC grade from Fisher (Fair Lawn, NJ). After acclimatization for 1 week, they were dosed by oral gavage with 200 mg/kg of BHA suspended in corn oil, or by intravenous (i.v.) bolus injection with 10 mg/kg BHA in 50% ethanol (Pharmaco Products, Brookfield, CT) and 50% sterile saline solution (Braun Medical, Irvine, CA). Four mice were randomly assigned to each group. At 0.5, 1, 2, 3, 4, 6, and 12 h after oral administration and at 5, 15, 30, 45 min, and 1, 1.5, 2, 3, 4, 6, and 12 h following i.v. dose, mice were sacrificed; plasma (both p.o. and i.v.) and liver (p.o. dose only) samples were obtained. The blood samples were placed in 0.5 ml heparinized micro-centrifuge tubes and the plasma were immediately separated from blood via centrifugation at 1,000 *g* (Eppendorf Centrifuge 5417R, Brinkmann Instruments Inc., Westbury, NY) for 5 min at 4°C and stored at -80°C until HPLC analyses. Liver samples were frozen in liquid nitrogen immediately and then stored in -80°C until further analyses.

HPLC assay

BHA concentrations in plasma samples were determined using a validated reversed-phase HPLC method with UV detection. The Shimadzu HPLC system (SCL-10A vp) consisted of a model FCV-10AL vp binary pump, a model SIL-10AD vp autosampler (a 250 μL injector and a 100 μL loop) configured with a heater/cooler, and a model SPD-10AV vp UV-vis detector. The column and autosampler temperatures were kept at room temperature (21 ± 1°C) and 4°C, respectively. The reversed phase chromatography was performed with a Shimadzu C-18 5 m column (4.6 mm i.d.) (Shimadzu, Columbia, MA), and the mobile phase was a gradient of methanol (solvent A) and 5% acetate in H_2O (solvent B). The initial mobile phase was 40% solvent A. Between 0 and 10 min, the percentage of solvent B was increased linearly from 60% to 100%. Between 10 to 20 min, the composition was maintained at 100% solvent B. Between 20 to 22 min, the percentage of solvent B was decreased from 100% to 60%. Bwtween 22 to 30 min, the composition was maintained at 60% solvent B. The flow rate was 1.0 mL/min and the injection volume was 50 μL. The UV detector was set at a single wavelength of 280 nm. The Class-VP software version 7.1.1 (Shimadzu) was used for instrument control and data analysis. Stock solutions of BHA and the internal standard (i.s.) 4-methoxyphenol were freshly prepared by dissolving a weighted amount of each compound in acetonitrile. The 0.5 mg/mL working solution of i.s. was prepared by diluting the stock solution with acetonitrile. Each blank plasma sample (60 μL) was spiked with 3 μL of the i.s. solution, 3 μL of varying concentrations of BHA, extracted with 200 μL ethyl acetate three times, the organic phase was then pooled and dried under nitrogen gas and dissolved in 60 μL methanol, to prepare a series of standards (final concentrations of 50, 100, 250, 500, 1000, 2000, and 5000 ng/mL) for the calibration curve. Each plasma sample (60 μL) from the pharmacokinetic studies was spiked with 3 μL i.s. solution, followed by the same extraction method used for preparing the standards. The detection limit for BHA is 50 ng/mL. The active metabolite, tBHQ, was not detected in any of the plasma samples collected in this study.

Pharmacokinetic analysis

BHA plasma concentration-versus-time data were analyzed by noncompartmental method, performed by WinNonlin (v 2.1) (Pharsight, Mountain View, CA). The peak plasma concentration (C_{max}) and the time to reach peak concentration (tmax) were determined directly from experimental observations. The area under the plasma concentration-time curve (AUC) was calculated by the log-linear trapezoidal method. The slope (*k*) of the terminal phase of concentration-time profile was determined by the log-linear regression of at least three data points. The value of *k*, determined from the terminal slope, was used to estimate the terminal half-life ($t_{1/2}$) by $t_{1/2} = 0.693/k$. Following intravenous dosing, total body clearance (CL) was estimated by dividing the administered dose by the calculated AUC. The area under the first moment curve (AUMC) was used to calculate the apparent volume of distribution as follows: Vss = CL* (AUMC/AUC). The absolute oral bioavailability (F) of BHA was estimated from the ratios of dose-normalized AUC values (AUC/dose) following oral administration over that obtained following intravenous administration.

MAPK Assay

The mouse liver samples were crushed into powder with a mortar and pestle. Following evaporation of most of the nitrogen, powdered tissues were lysed with lysis buffer (10 mM Tris-HCl, pH 7.4, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 mM sodium orthovanadate, 2 mM iodoacetic acid, 5 mM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton-X 100). The lysate was homogenized by passing through a 23 G needle three time, sonicating 10 sec, and kept in ice for 30 min. The homogenate was centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was transferred into a clean tube and stored in -80°C. The protein concentration of the whole lysates was determined by Bio-Rad protein assay kit. Equal amount of protein was mixed with 4 × loading buffer, and pre-heated at 95°C for 3 min. The samples were then loaded on a 10% mini

SDS-polyacrylamide gel, and ran at 200 V. The proteins were then transferred onto a PVDF membrane for 1.5 h using semi-dry transfer system (Fisher). The membrane was blocked in 5% bovine serum albumin (BSA) solution for 1 h at room temperature, then incubated overnight at 4°C with each of the three anti-phospho-MAPK primary antibodies (1:1,000 dilution, New England Biolabs, Inc., Beverly, MA), which specifically recognized phosphorylated ERK1/2 (Thr202/Tyr204), JNK 1/2 (Thr183/Tyr185), or p38 (Thr180/Tyr182). After hybridization with primary antibody, membrane was washed with TBST (Tris buffered-saline Tween-20) for three times, then incubated with HRP (Horseradish Peroxidase)-conjugated secondary antibody (1:10,000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 45 min at room temperature and washed with TBST three times. Final detection was performed with ECL (Enhanced Chemiluminescence) Western blotting reagents (Amersham Pharmacia Biotech., Piscataway, NJ).

Quantitative real time PCR (Q-RT-PCR)

A total number of 9 genes were examined, including 5 phase II genes and 4 phase III genes (Table I). Total RNA was purified from mouse liver with the RNeasy Mini kit (Qiagen, Valencia, CA) after isolation with TRIzol reagent (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's instructions. Total RNA was quantitated and checked for integrity on a 1% formaldehyde-agarose gel. First-strand cDNA was synthesized from total RNA using the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) with oligo-dT primers. The PCR reactions were carried out using 10 ng of cDNA, 50 nM of each primer, and SYBR Green master mix (Applied Biosystems, Foster City CA) in 10 μL reactions. Levels of Q-RT-PCR product were measured using SYBR Green fluorescence collected during real-time PCR on an Applied Biosystems PRISM 7900HT Sequence Detection System. A control cDNA dilution series was created for each gene to establish a standard curve. Each reaction was subjected to melting point analysis to confirm single amplified product. The data generated from each PCR were analyzed using SDS 2.0 software (Applied Biosystems).

Table I. Oligonucleotide primers used for real-time PCR. The nucleotide sequences of the respective up- and downstream primers are indicated

Genes	5' Primers	3' Primers
NQO1	AGCCCAGATATTGTGGCCG	CCTTTCAGAATGGCTGGCAC
GSTpi	TGGGCATCTGAAGCCTTTTG	CCAGCAAGTTGTAATCGGCAA
HO-1	TGCCCCACTCTACTTCCCTG	TGCAAGGGATGATTTCCTGC
UGT1A6	GGCGCTACACCGGAACTAGA	TGCCCGAGTCTTTGGATGAC
γ-GCS	TCCAGGTGACATTCCAAGCC	AGATGCAGCACTCAAAGCCA
MRP1	AGACAGCATTGAGCGGAGGT	TCAGTGTGGGAGGTTCACCC
MRP2	CGTGGCTGTTGAGCGAATAA	TCTCACCTTTTTTGGGCCAAT
Slco2b1	GCCCACCATAGCAACTCACC	AGGCGTAGCATGAGGCTACC
Slco1b2	ACCATCTTCCATTTCCGGTTT	TTGGTCGGTGTAGCTTGGATC
GAPDH	CACCAACTGCTTAGCCCCC	TCTTCTGGGTGGCAGTGATG

RESULTS

Pharmacokinetic analysis

In the mice the averaged plasma concentration versus time profiles for BHA following the i.v. (10 mg/kg) and oral (200 mg/kg) administrations are shown in Fig. 1. Following the oral administration, BHA concentrations could be determined over the 12 h period. However, after i.v. administration, BHA concentrations were below the limit of detection after 4 h. Noncompartmental analysis estimated a $t_{1/2}$ of 0.8 h, an AUC of 580 ng/mL*h, CL of 17.2 L/h/kg, and Vss of 43.3 L/kg (Table II). After oral administration, the plasma concentration peaked at 2,095 ng/mL at 1 h. Noncompartmental analysis estimated a $t_{1/2}$ of 2 h, and calculated an AUC of 4,968 ng/mL*h (Table II). To estimate the oral availability (F) after oral dosing, the normalized

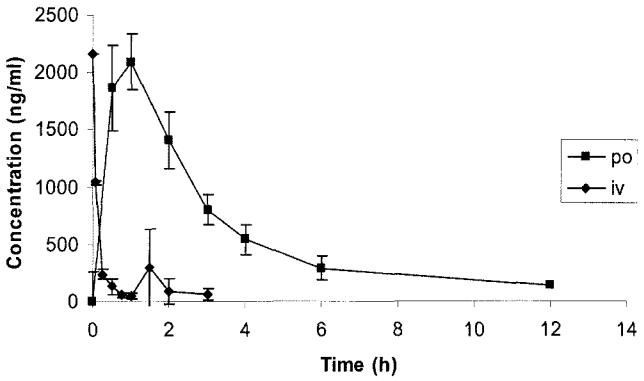

Fig. 1. Plasma concentration versus time profile of BHA following intravenous administration of 10 mg/kg and oral administration of 200 mg/kg to mice. Each point represents the mean ± S.D. of 4 mice.

Table II. Pharmacokinetic variables. Results of noncompartmental modeling of plasma BHA concentration versus time data in the mice given i.v. or p.o. doses of BHA

Species	Mouse	
Route	i.v.	p.o.
Dose (mg/kg)	10	200
Cmax (ng/mL)	1040	2095
Tmax (h)		1
t1/2 (h)	0.8	2
AUC (h*ng/mL)	580	4968
CL (L/h/kg)	17.2	
Vss (L/kg)	43.3	
F (%)		42.8

AUC (AUC/dose) value after the oral dose was divided by the normalized AUC (AUC/dose) value after the i.v. doses. The F value for BHA was 42% in the mice.

Gene expression induced by BHA

To examine the gene expression induced by BHA in the mouse liver, real-time PCR experiments utilizing specific primer pairs for the genes of interest were carried out. As shown in Fig. 2A, phase II drug metabolizing genes such as NQO-1, HO-1, γ-GCS, GST-pi and UGT-1A6 were induced time dependently after treatments with 200 mg/kg BHA in the mouse liver. The gene expression patterns were somehow different with different kinetics of induction (Fig. 2A). With respect to the three genes shown on the left panel of Fig. 2A (NQO-1, HO-1 and γ-GCS), they were up-regulated at relatively late time points (6-12 h), whereas the two genes on the right panel of Fig. 2A (GST-pi and UGT-1A6), they were up-regulated at earlier time points (1-3 h). This suggests that their potential different modes of regulation.

We next examined the effects of BHA on some of the phase III drug transporter genes. As shown in Fig. 2B, multidrug resistance protein 1 (MRP1) or ABCC1 (ATP-binding cassette, sub-family C, member 1) was induced

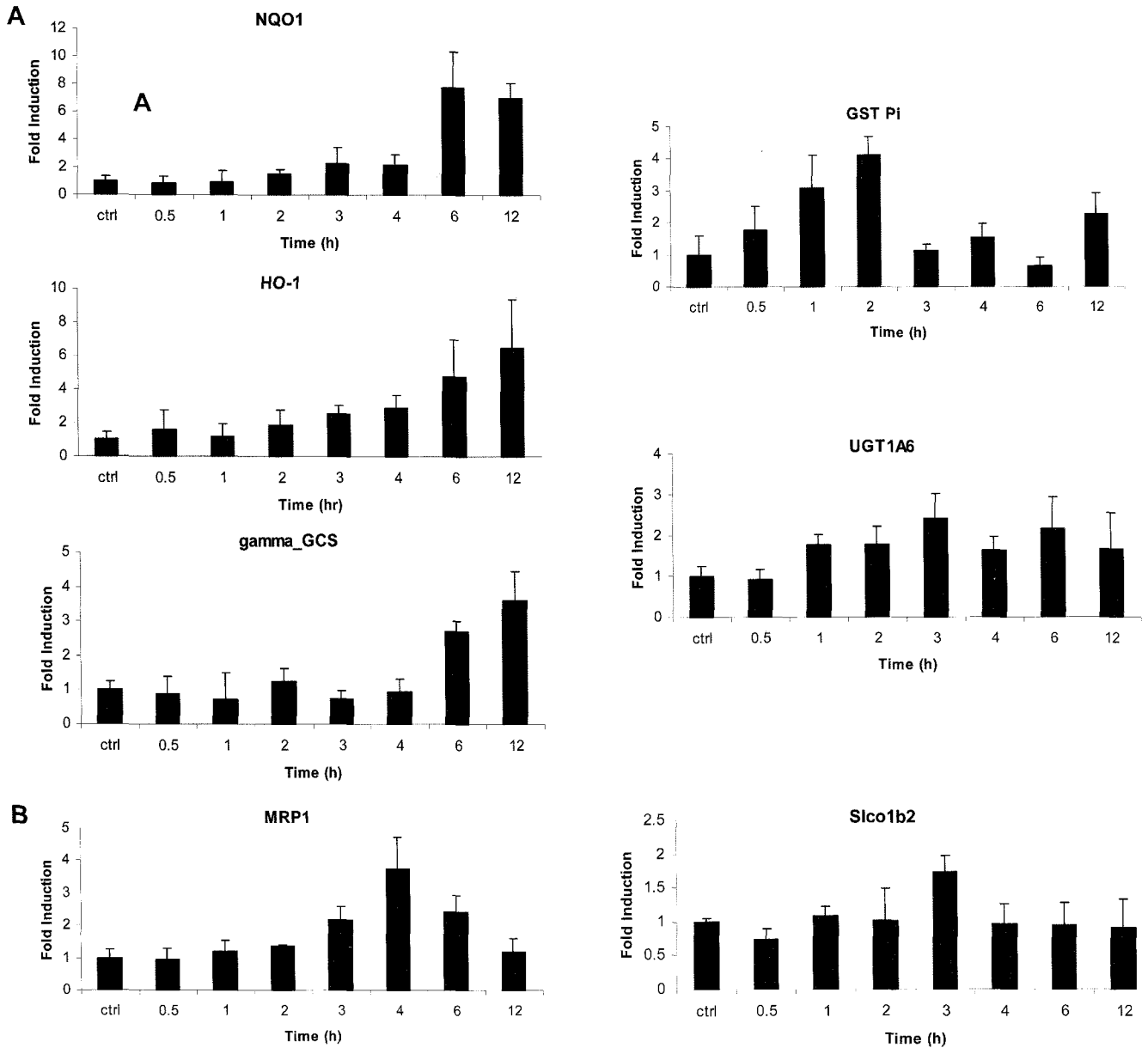

Fig. 2. Activation of some phase II and transporter genes by BHA in mouse liver. Gene expression levels were measured by real-time PCR and normalized against control as fold induction. (A) time-dependent activation of phase II genes; (B) time-dependent activation of transporter genes. Bars, SD of triplicate data points.

by BHA in the liver starting at 3 h and peaked at 4 h. For the solute carrier organic anion transporter family-1b2 (Slco1b2) gene, it was induced by BHA at 3 h. The induction patterns of both MRP1 and Slco1b2 appear to be similar kinetically as GST-p1 and UGT1A6 (Fig. 2A-right panel). We had also examined the transcript levels of MRP2 and Slco2b1 and found that their transcript levels were not changed by BHA treatments in the duration of this study (data not shown).

MAPK activation

To investigate the potential signal transduction events induced by BHA that could contribute to its induction of various genes including phase II enzymes, we studied the activation of MAPK pathway with pooled mouse liver samples. We found that the oral treatments of BHA resulted in a time-dependent phosphorylation and activation of all three major MAPKs, JNK, ERK and p38 in the mouse livers. Activation of JNK was evident as early as 30 min after 200 mg/kg BHA treatment and persisted for the duration of the experiment (Fig. 3). Phosphorylation of ERK was detected at only 3 h after the BHA treatment, while p38 activation was observed between 2 to 4 h and peaked at 3 h. These results showed for the first time in the mouse liver that BHA activated all three MAPKs and activation of JNK by BHA was rapid and persistent, whereas activations of ERK and p38 were relatively delayed and transient. This result suggests that in the *in vivo* mouse liver, the MAPK pathway may play an important role in the early signal transduction event leading to the transcriptional activation of many genes including ARE-mediated phase II genes expression, analogous to the *in vitro* cell lines situation (Yu *et al.*, 1999).

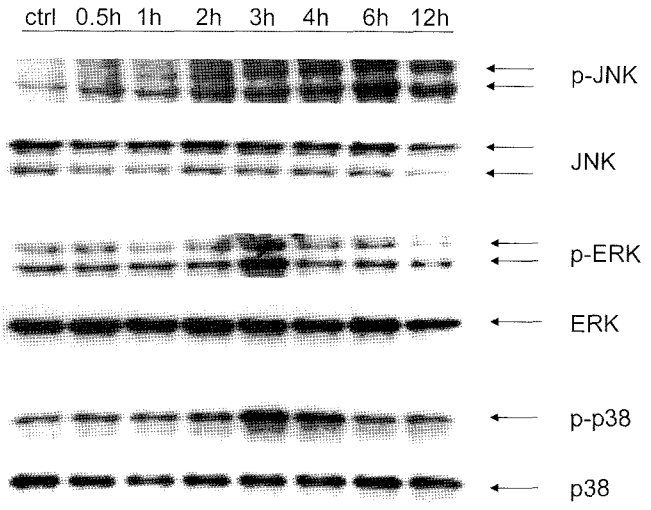

Fig. 3. Activation of MAPKs (ERK, JNK and p38) in mouse liver after BHA treatment. The data shown are representative blots of four mouse liver samples per time point.

DISCUSSION

BHA and its metabolite tBHQ were first reported to reduce the incidence of tumors in rodents (Wattenberg, 1973, 1983, 1985). Subsequently, many other studies have also found that phenolic compounds/antioxidants including BHA are effective cancer preventive (chemopreventive) agents against for carcinogenesis/tumorigenesis induced by a variety of carcinogens at many organ sites (Talalay *et al.*, 1978; King and McCay, 1983) including colon cancer model induced by methylazoxymethanol acetate in female CF1 mice (Reddy and Maeura, 1984). BHA significantly reduced the genotoxic effects, such as the frequency of 6-thioguanidine resistant mutations and micronuclei, induced by *N*-methyl-*N*9-nitro-*N*-nitrosoguanidine (MNNG) in Chinese hamster V79 cells (Horvathova *et al.*, 1999). BHA also induced apoptosis by oxidative stress in neurons (Ratan *et al.*, 1994) and human monocytes and macrophages (Hayashi *et al.*, 1997). In contrast to the beneficial effects of BHA in animals or cultured cells, adverse effects have also been reported. At the dose of 0.5 or 2% (w/w) in the diet, BHA induced papillomas and squamous cell carcinoma in the forestomach of rodents (Ito *et al.*, 1983a; Ito *et al.*, 1983b). At doses that induce phase II detoxifying enzymes, 15-60 μM, it induced apoptosis in human colon carcinoma cells (Kirlin *et al.*, 1999). Cytotoxic effects have been observed in human dermal fibroblasts, keratinocytes, melanocytes, and melanoma tumor cells (Babich and Borenfreund, 1990). Its cytotoxicity in rat hepatocytes was attributed to the induction of apoptosis through a molecular mechanism of direct release of cytochrome C and subsequent activation of caspases (Yu *et al.*, 2000). These beneficial effects versus adverse effects of BHA might be dependent on its concentrations and or on the nature of cells or tissues.

Detailed pharmacokinetics analysis of BHA in mice is not our primary objective, since some of these findings had been previously reported by other investigators (Taylor *et al.*, 1984; Verhagen *et al.*, 1989; Vora *et al.*, 1999). Our goal here is to selectively quantify the plasma concentrations profiles after different routes of administration, so that we can compare the *in vivo* plasma concentrations to that of *in vitro* cell culture concentrations. The peak plasma concentration of BHA achieved in our current study after oral administration was around 2 μg/mL, which can be converted to approximately 10 μM. The peak *in vivo* concentrations of BHA offers some insights for the numerous *in vitro* cell culture studies, where between 10 to 500 μM BHA were used for various signal transduction studies as well as phase II gene induction studies. In addition, it has been previously reported that the AUC in the rat liver was 49 times higher than that observed in plasma (Della Corte *et al.*, 1989). The oral bioavailability

of BHA was 42.8% in the mice. The relatively low bioavailability could be potential due to poor oral absorption. In addition, the calculation of oral bioavailability was based on the concentrations of the parent compound, without including its potential metabolites such as tBHQ.

Since BHA was introduced as a food preservative in the 1960s, it has attracted a lot of attention and debate because of its potential beneficial as well as adverse effects on the potential health of humans. Although extensive studies have been conducted to define the biological activities of BHA in many animal model systems and in humans, the mechanisms of action of BHA are not fully understood. It has been shown to be potent cancer chemopreventive agent (Wattenberg, 1973, 1983; Benson *et al.*, 1978; King and McCay, 1983) and it is a potent inducer of phase II detoxifying enzymes in animals (Benson *et al.*, 1978; Lam *et al.*, 1980; Sparnins *et al.*, 1982a, 1982b). In addition, we have previously shown that BHA and its metabolite tBHQ strongly stimulated MAPK cascades (ERK and JNK) in human HepG2, and HeLa cells (Yu *et al.*, 1997), and demonstrated the possible involvement of MAPK pathway in the induction of phase II enzymes by tBHQ (Yu *et al.*, 1999). The potential role of ERK and JNK in the expression of MRP1 gene were suggested by previously studies (Cripe *et al.*, 2002; Guan *et al.*, 2004).

In our current study, we showed that certain phase II genes were induced by the treatment of BHA in the mouse liver, and they followed two kinetic expression patterns. GST-pi and UGT-1A6 were up-regulated shortly after BHA administration and peaked around 2-3 h, whereas NQO-1, HO-1 and γ-GCS were up-regulated at relatively later time points between 6 to 12 h. This may be due to the differences in the requirement of transcription factors such as Fos, Jun, Nrf2 and others (Kong *et al.*, 2001). We showed that the phase III drug transporter genes including MRP-1 and Slco1b2 were induced by the treatment of BHA in the mouse liver with earlier kinetics similar to that of GST-p1 and UGT1A6. We have also demonstrated for the first time that BHA activates the important signaling kinases, ERK, JNK and p38 *in vivo* mouse liver. The activation of ERK was a little delayed and transient, similar to that reported previously for some growth factors (Wood *et al.*, 1992; Ohmichi *et al.*, 1994). In contrast, JNK activation was relatively early and sustained, following a pattern similar to that shown by many stress stimuli, such as UV C (Chen *et al.*, 1996), protein synthesis inhibitors (Cano *et al.*, 1994), and arsenite (Liu *et al.*, 1996). The phase II and phase III tranporter mRNA levels somewhat correlated with and followed the changes of the MAPK activities, which might suggest a potential role of MAPK pathway in the activation of these genes *in vivo* obviously working in concert with the pivotal Nrf2 transcription factor (Shen *et al.*, 2004). To acertain the role of MAPK in the induction of phase II and phase III transporter genes and explore the involvement of additional and/or alternative signaling pathways *in vivo*, further studies involving knockout mice would be needed.

In summary, our current study shows that BHA has relatively poor oral bioavailability in the mice (~43%), with a terminal $t_{1/2}$ of 0.8 and 2 h for i.v. and oral administration in the mice. The CL values after the i.v. administration of BHA was 17.2 L/h/kg; and the Vss of 43.3 L/kg for mice. Despite its relatively low bioavailability, the peak plasma concentration after oral dosing reached about 2 μg/mL (10 μM) in the mice. Additionally, in the target tissue (such as the liver, although not measured in our current study), the concentrations of BHA could be higher than that of the plasma, and therefore would explain some of the pharmacological effects such as activation of MAPK and induction of phase II drug metabolizing and phase III drug transporter genes expression between the *in vitro* cell line studies and *in vivo* animal models. To our knowledge, this is the first study to demonstrate this *in vivo* pharmacokinetics of BHA, *in vivo* activation of MAPK signaling proteins, as well as the *in vivo* induction of Phase II/III drug metabolizing enzymes/transporters in the mouse livers.

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