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Research Paper

Pharmacogenomics of Phenolic Antioxidant Butylated Hydroxyanisole (BHA) in the Small Intestine and Liver of Nrf2 Knockout and C57BL/6J Mice

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Purpose. The objective of this study was to investigate the pharmacogenomics and the spatial regulation of global gene expression profiles elicited by cancer chemopreventive agent butylated hydroxyanisole (BHA) in mouse small intestine and liver as well as to identify BHA-modulated nuclear factor-E2-related factor 2 (Nrf2)-dependent genes.
Methods. C57BL/6J (+/+; wildtype) and C57BL/6J/Nrf2(−/−; knockout) mice were administered a single 200 mg/kg oral dose of BHA or only vehicle. Both small intestine and liver were collected at 3 h after treatment and total RNA was extracted. Gene expression profiles were analyzed using 45,000 Affymetrix mouse genome 430 2.0 array and GeneSpring 7.2 software. Microarray results were validated by quantitative real-time reverse transcription-PCR analyses.
Results. Clusters of genes that were either induced or suppressed more than two fold by BHA treatment compared with vehicle in C57BL/6J/Nrf2(−/−; knockout) and C57BL/6J Nrf2 (+/+; wildtype) mice genotypes were identified. Amongst these, in small intestine and liver, 1,490 and 493 genes respectively were identified as Nrf2-dependent and upregulated, and 1,090 and 824 genes respectively as Nrf2-dependent and downregulated. Based on their biological functions, these genes can be categorized into ubiquitination/proteolysis, apoptosis/cell cycle, electron transport, detoxification, cell growth/differentiation, transcription factors/interacting partners, kinases and phosphatases, transport, biosynthesis/metabolism, RNA/protein processing and nuclear assembly, and DNA replication genes. Phase II detoxification/antioxidant genes as well as novel molecular target genes, including putative interacting partners of Nrf2 such as nuclear corepressors and coactivators, were also identified as Nrf2-dependent genes.
Conclusions. The identification of BHA-regulated and Nrf2-dependent genes not only provides potential novel insights into the gestalt biological effects of BHA on the pharmacogenomics and spatial regulation of global gene expression profiles in cancer chemoprevention, but also points to the pivotal role of Nrf2 in these biological processes.

KEY WORDS: butylated hydroxyanisole; chemoprevention; global gene expression profiles; microarray; nuclear Factor-E2-related factor 2.

INTRODUCTION

The phenolic antioxidant butylated hydroxyanisole (BHA) is a commonly used food preservative with broad biological activities (1), including protection against acute toxicity of chemicals, modulation of macromolecule synthesis and immune response, induction of phase II detoxifying enzymes, and, indeed, its potential tumor-promoting activities. Whereas the potential cytotoxicity of BHA has been partially attributed to reactive intermediates (1,2), BHA has also been shown to shift cell death from necrosis to apoptosis (3,4) and to inhibit mitochondrial complex I and lipoxygenases (3). A chemopreventive role for BHA is reiterated by the induction of A5 subunit of GST in rat liver immunoblotting experiments (5). BHA has also been reported to increase the levels of liver glutathione and the activity of hepatic cytosolic gamma-glutamylcysteine synthetase (3). Moreover, BHA has been shown to be an effective inhibitor

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ABBREVIATIONS: ARE, antioxidant response element; BHA, butylated hydroxyanisole; Mapk, mitogen-activated protein kinase; Nrf2, nuclear factor-E2 -related factor 2.

of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis (6) in Sprague–Dawley rats; and is effective in the chemoprevention (7) of 1,2-dimethylhydrazine-induced large bowel neoplasms. In addition, BHA in diet has been demonstrated (8) to inhibit the initiation phase of 2-acetylaminofluorene and aflatoxin B1 hepatocarcinogenesis in rats. We have previously demonstrated (9) that the cytotoxicity of BHA is due to the induction of apoptosis that is mediated by the direct release of cytochrome *c* and the subsequent activation of caspases.

Pivotal to the antioxidant response (10–13) typical in mammalian homeostasis and oxidative stress is the important transcription factor Nrf2 or Nuclear Factor-E2-related factor 2 that has been extensively studied by many research groups (10–13) including this laboratory (14–17). Under homeostatic conditions, Nrf2 is mainly sequestered in the cytoplasm by a cytoskeleton-binding protein called Kelch-like erythroid CNC homologue (ECH)-associated protein 1 (Keap1; (14,18,19). When challenged with oxidative stress, Nrf2 is quickly released from Keap1 retention and translocates to the nucleus (14,20). We have recently identified (14) a canonical redox-insensitive nuclear export signal (NES; ^{537}LKKQLSTLYL^{546}) located in the leucine zipper (ZIP) domain of the Nrf2 protein. Once in the nucleus, Nrf2 not only binds to the specific consensus *cis*-element called antioxidant response element (ARE) present in the promoter region of many cytoprotective genes (15,19,21), but also to other *trans*-acting factors such as small Maf (MafG and MafK; (22) that can coordinately regulate gene transcription with Nrf2. We have previously demonstrated (1,23) that BHA is capable of activating distinct mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated protein kinase 2 (ERK2), and c-Jun N-terminal kinase 1 (JNK1). We have also reported (15) that different segments of Nrf2 transactivation domain have different transactivation potential; and that different MAPKs have differential effects on Nrf2 transcriptional activity with ERK and JNK pathways playing an unequivocal role in positive regulation of Nrf2 transactivation domain activity (15). To better understand the biological basis of signaling through Nrf2, it has also become imperative to identify possible interacting partners of Nrf2 such as coactivators or corepressors apart from *trans*-acting factors such as small Maf (22).

Nrf2 knockout mice are greatly predisposed to chemical-induced DNA damage and exhibit higher susceptibility towards cancer development in several models of chemical carcinogenesis (21). Observations that Nrf2-deficient mice are refractory to the protective actions of some chemopreventive agents (21), indeed, highlight the importance of the Keap1-Nrf2-ARE signaling pathway as a molecular target for prevention. In the present study, we have investigated, by microarray expression profiling, the global gene expression profiles elicited by oral administration of BHA in small intestine and liver of Nrf2 knockout (C57BL/6J/Nrf2−/−) and wild type (C57BL/6J) mice to enhance our understanding of BHA-regulated cancer chemopreventive effects mediated through Nrf2. We have identified clusters of BHA-modulated genes that are Nrf2-dependent in small intestine and liver and categorized them based on their biological functions. The identification of BHA-regulated Nrf2-dependent genes will yield valuable insights into the role of Nrf2 in BHA-modulated gene regulation and cancer chemopreventive effects. This study also enables the identification of novel molecular targets for BHA-mediated chemoprevention that are regulated by Nrf2. The current study is also the first to investigate the global gene expression profiles elicited by BHA in an *in vivo* murine model where the role of Nrf2 is also examined.

MATERIALS AND METHODS

Animals and Dosing. The protocol for animal studies was approved by the Rutgers University Institutional Animal Care and Use Committee (IACUC). Nrf2 knockout mice Nrf2 (−/−) (C57BL/SV129) have been described previously (24). Nrf2 (−/−) mice were backcrossed with C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, USA). DNA was extracted from the tail of each mouse and genotype of the mouse was confirmed by polymerase chain reaction (PCR) by using primers (3′-primer, 5′-GGA ATG GAA AAT AGC TCC TGC C-3′; 5′-primer, 5′-GCC TGA GAG CTG TAG GCC C-3′; and lacZ primer, 5′-GGG TTT TCC CAG TCA CGA C-3′). Nrf2(−/−) mice-derived PCR products showed only one band of ~200 bp, Nrf2 (+/+) mice-derived PCR products showed a band of ~300 bp while both bands appeared in Nrf2(+/−) mice PCR products. Female C57BL/6J/Nrf2(−/−) mice from third generation of backcrossing were used in this study. Age-matched female C57BL/6J mice were purchased from The Jackson Laboratory. Mice in the age-group of 9–12 weeks were housed at Rutgers Animal Facility with free access to water and food under 12 h light/dark cycles. After one week of acclimatization, the mice were put on AIN-76 A diet (Research Diets Inc. New Jersey USA) for another week. The mice were then administered BHA (Sigma-Aldrich, St. Louis, MO) at a dose of 200 mg/kg (dissolved in 50% PEG 400 solution at a concentration of 20 mg/ml) by oral gavage. The control group animals were administered only vehicle (50% PEG 400 solution). Each treatment was administered to a group of four animals for both C57BL/6J and C57BL/6J/Nrf2(−/−) mice. Mice were sacrificed 3 h after BHA treatment or 3 h after vehicle treatment (control group). Livers and small intestines were retrieved and stored in RNA Later (Ambion, Austin, TX) solution.

Sample Preparation for Microarray Analyses. Total RNA from liver and small intestine tissues were isolated by using a method of TRIzol (Invitrogen, Carlsbad, CA) extraction coupled with the RNeasy kit from Qiagen (Valencia, CA). Briefly, tissues were homogenized in trizol and then extracted with chloroform by vortexing. A small volume (1.2 ml) of aqueous phase after chloroform extraction and centrifugation was adjusted to 35% ethanol and loaded onto an RNeasy column. The column was washed, and RNA was eluted following the manufacturer's recommendations. RNA integrity was examined by electrophoresis, and concentrations were determined by UV spectrophotometry.

Microarray Hybridization and Data Analysis. Affymetrix (Affymetrix, Santa Clara, CA) mouse genome 430 2.0 array was used to probe the global gene expression profiles in mice following BHA treatment. The mouse genome 430 2.0 array is a high-density oligonucleotide array comprised

of over 45,101 probe sets representing over 34,000 well-substantiated mouse genes. The library file for the array is available at http://www.affymetrix.com/support/technical/libraryfilesmain.affx. After RNA isolation, all the subsequent technical procedures including quality control and concentration measurement of RNA, cDNA synthesis and biotin-labeling of cRNA, hybridization and scanning of the arrays, were performed at CINJ core expression array facility of Robert Wood Johnson Medical School (New Brunswick, NJ). Each chip was hybridized with cRNA derived from a pooled total RNA sample from four mice per treatment group, per organ, and per genotype (a total of eight chips were used in this study; Fig. 1). Briefly, double-stranded cDNA was synthesized from 5 μg of total RNA and labeled using the ENZO BioArray RNA transcript labeling kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA) to generate biotinylated cRNA. Biotin-labeled cRNA was purified and fragmented randomly according to Affymetrix's protocol. Two hundred microliters of sample cocktail containing 15 μg of fragmented and biotin-labeled cRNA was loaded onto each chip. Chips were hybridized at 45°C for 16 h and washed with fluidics protocol EukGE-WS2v5 according to Affymetrix's recommendation. At the completion of the fluidics protocol, the chips were placed into the Affymetrix GeneChip Scanner where the intensity of the fluorescence for each feature was measured. The expression value (average difference) for each gene was determined by calculating the average of differences in intensity (perfect match intensity minus mismatch intensity) between its probe pairs. The expression analysis file created from each sample (chip) was imported into GeneSpring 7.2 (Agilent Technologies, Inc., Palo Alto, CA) for further data characterization. Briefly, a new experiment was generated after importing data from the same organ in which data was normalized by array to the 50th percentile of all measurements on that array. Data filtration based on flags present in at least one of the samples was first performed, and a corresponding gene list based on those flags was generated. Lists of genes that were either induced or suppressed more than two fold between treated *versus* vehicle group of same genotype were created by filtration-on-fold function within the presented flag list. By use of color-by-Venn-Diagram function, lists of genes that were regulated more than twofold only in C57BL/6J mice in both liver and small intestine were created. Similarly, lists of gene that were regulated over twofold regardless of genotype were also generated.

Quantitative Real-Time PCR for Microarray Data Validation. To validate the microarray data, 13 genes of interest were selected from various categories for quantitative real-time PCR analyses. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) served as the "housekeeping" gene. The specific primers for these genes listed in Table I were designed by using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and were obtained from Integrated DNA Technologies, Coralville, IA. The specificity of the primers was examined by a National Center for Biotechnology information blast search of the mouse genome. Instead of using pooled RNA from each group, RNA

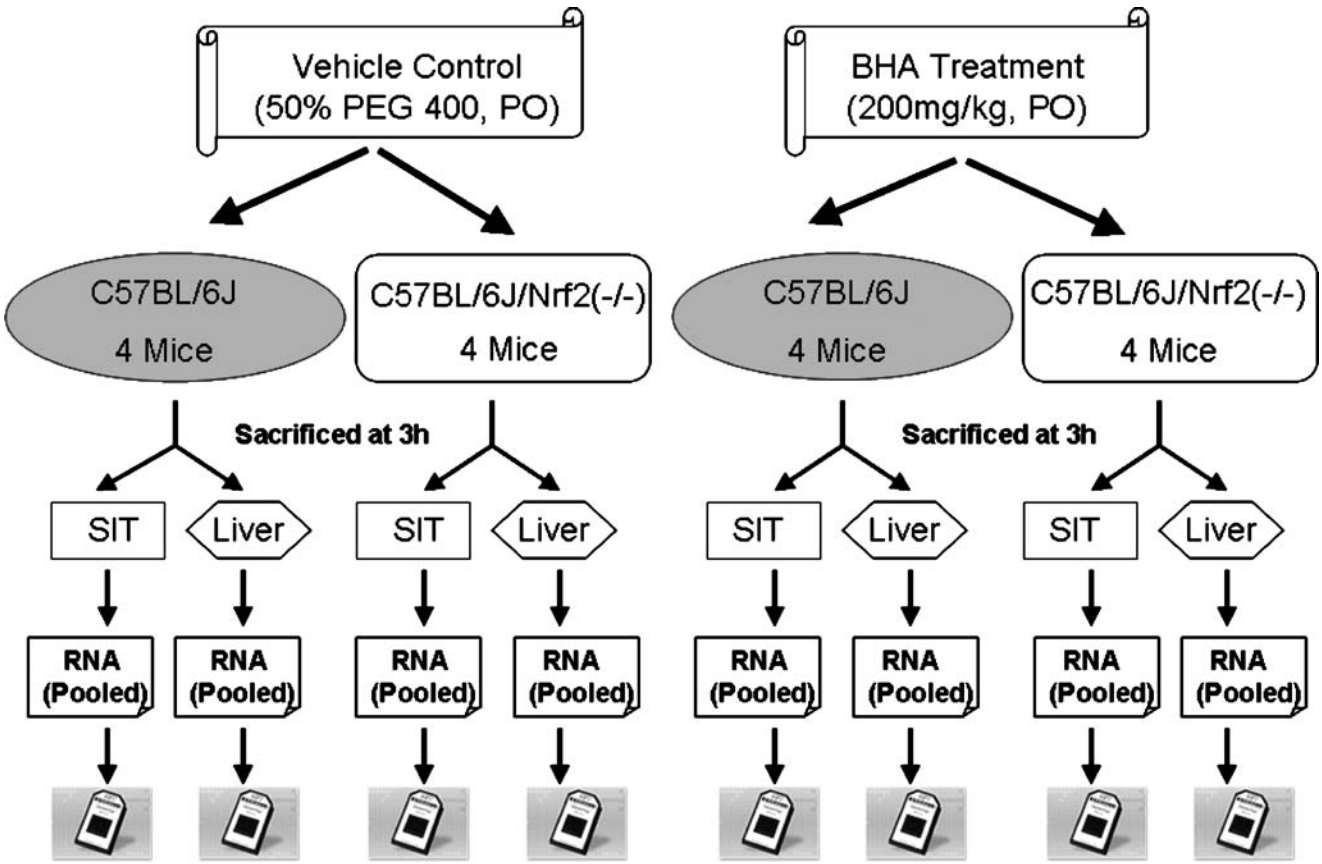

Fig. 1. Schematic representation of experimental design; *SIT*, small intestine.

Table I. Oligonucleotide Primers Used in Quantitative Real-Time PCR

Gene Name	GenBank Accession No.	Forward Primer (5′-3′)	Reverse Primer (3′-5′)
ATP-binding cassette, sub-family B (MDR/TAP), 1A (Abcb1a)	NM_011076	5′-TTCAGGGCTTCACATTTGGC-3′	5′-GGAGTCGCTTGGTGAGGATCT-3′
ATP-binding cassette, sub-family C (CFTR/MRP), 1(Abcc1)	NM_008576	5′-CTCACGATTGCTCATCGGCT-3′	5′-AATCACCCGCGTGTAGTCCA-3′
CASP8 and FADD-like apoptosis regulator (Cflar)	NM_207653	5′-CCAGCTTTTCTTGTTTCCCAAG-3′	5′-CGGCGAACAATCTGGGTTAT-3′
Cytochrome *c* oxidase, subunit VIIa 1(Cox7a1)	NM_009944	5′-CACTTAGAAAACCGTGTGGCAG-3′	5′-ATTGTCGGCCTGGAAGAGCT-3′
Glutathione-S-transferase mu(Gstmu)	NM_010358	5′-GAAGCCAGTGGCTGAATGAGA-3′	5′-GATGGCATTGCTCTGGGTG-3′
Glycogen synthase kinase 3 beta (Gsk3b)	NM_019827	5′-TTTGAGCTGGTACCCTAGGATGA-3′	5′-AGCTGCCCCCTTAACACCAT-3′
Heme oxygenase (decycling) 1(Hmox1)	NM_010442	5′-CCCACCAAGTTCAAACAGCTC-3′	5′-AGGAAGGCGGTCTTAGCCTC-3′
Inhibitor of kappaB kinase gamma (Ikbkg)	NM_010547	5′-CTGAAAGTTGGCTGCCATGAG-3′	5′-GAGTGGTGAGCTGGAGCAGG-3′
Nuclear receptor coactivator 3 (Ncoa3)	NM_013827	5′-GAGGTGTCAGAGACGCCCAG-3′	5′-TTTCTTGTGGCCTTTGCTTTC-3′
Nuclear receptor co-repressor 1 (Ncor1)	NM_011308	5′-GCAGCCTTCTACTTCTACATTCCAA-3′	5′-GTGGATGACAAAGCAGATGGTG-3′
Nuclear receptor interacting protein 1 (Nrip1)	NM_173440	5′-AACAGTGAGCTGCCAACCCT-3′	5′-CTTCGGGACCATGCAGATGT-3′
Protein kinase C, epsilon (Prkce)	NM_011104	5′-ACGCTCCTATCGGCTACGAC-3′	5′-CGAACTGGATGGTGCAGTTG-3′
v-Maf musculoaponeurotic fibrosarcoma oncogene family, protein G (avian; (MafG)	NM_010756	5′-AGAATGGCACCAGCTTGACC-3′	5′-CTCGCACCGACATGGTTACC-3′
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	NM_008084	5′-CACCAACTGCTTAGCCCCC-3′	5′-TCTTCTGGGTGGCAGTGATG-3′

samples isolated from individual mice as described earlier were used in real-time PCR analyses. For the real-time PCR assays, briefly, first-strand cDNA was synthesized using 4 μg of total RNA following the protocol of SuperScript III first-strand cDNA synthesis system (Invitrogen) in a 40 μl reaction volume. The PCR reactions based on SYBR Green chemistry were carried out using 100 times diluted cDNA product, 60 nM of each primer, and SYBR Green master mix (Applied Biosystems) in 10 μl reactions. The PCR parameters were set using SDS 2.1 software (Applied Biosystems) and involved the following stages: 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 s→55°C for 30 s→72°C for 30 s, 40 cycles; and 72°C for 10 min, 1 cycle. Incorporation of the SYBR Green dye into the PCR products was monitored in real time with an ABI Prism 7900 HT sequence detection system, resulting in the calculation of a threshold cycle (C_T) that defines the PCR cycle at which exponential growth of PCR products begins. The carboxy-X-rhodamine (ROX) passive reference dye was used to account for well and pipetting variability. A control cDNA dilution series was created for each gene to establish a standard curve. After conclusion of the reaction, amplicon specificity was verified by first-derivative melting curve analysis using the ABI software and the integrity of the PCR reaction product and absence of primer dimers was ascertained. The gene expression was determined by normalization with control gene GAPDH.

Statistics. In order to validate the results, the correlation between corresponding microarray data and real-time PCR data was evaluated by the 'coefficient of determination', r^2.

RESULTS

BHA-Modulated Gene Expression Patterns in Mouse Small Intestine and Liver

Subsequent to data normalization, 50.5% (22,779) of the probes passed the filtration based on flags present in at least one of four small intestine sample arrays depicted in Fig. 1. Amongst these probes, 13.86 and 11.69% of probes were induced and suppressed over twofold respectively regardless of genotype. Expression levels of 2,580 probes were either elevated (1,490) or suppressed (1,090) over two fold by BHA only in the wild-type mice, while 3,243 probes were either induced (1,669) or inhibited (1,574) over twofold by BHA only in the Nrf2(−/−) mice small intestine (Fig. 2a). Similarly, changes in gene expression profiles were also observed in mice liver. Overall, the expression levels of

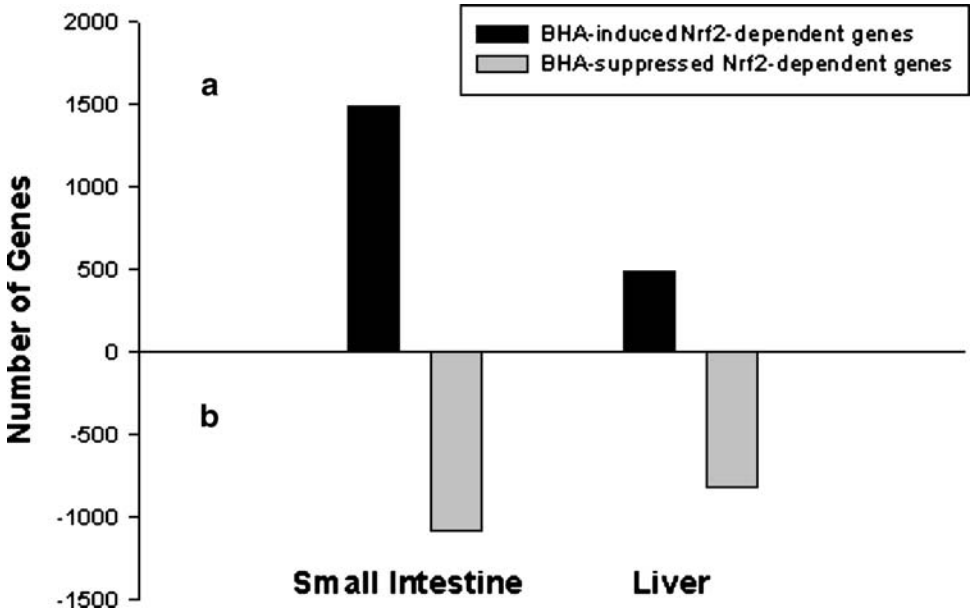

Fig. 2. Regulation of Nrf2-dependent gene expression by BHA in mouse small intestine and liver. Gene expression patterns were analyzed at 3 h after administration of a 200 mg/kg single oral dose of BHA; Nrf2-dependent genes that were either induced or suppressed over twofold were listed. The *positive numbers* on the *y-axis* refer to the number of genes being induced; the *negative numbers* on the *y-axis* refer to the number of genes being suppressed.

52.79% (23,809) probes were detected in least in one of four liver sample arrays depicted in Fig. 1. Amongst these probes, 6.29 and 5.94% of probes were induced and suppressed over twofold respectively regardless of genotype. In comparison with the results from small intestine sample arrays, a smaller proportion (1,317) of well-defined genes were either elevated (493) or suppressed (824) over two fold by BHA in wild-type mice liver alone; whereas 1,596 well-defined genes were induced (1,005) or inhibited (591) in Nrf2(−/−) mice liver (Fig. 2b).

Quantitative Real-Time PCR Validation of Microarray Data

To validate the data generated from the microarray studies, several genes from different categories (Table I) were selected to confirm the BHA-regulative effects by the use of quantitative real-time PCR analyses as described in detail under Materials and Methods. After ascertaining the amplicon specificity by first-derivative melting curve analysis, the values obtained for each gene were normalized by the values of corresponding GAPDH expression levels. The fold changes in expression levels of treated samples over control samples were computed by assigning unit value to the control (vehicle) samples. Computation of the correlation statistic showed that the data generated from the microarray analyses are well-correlated with the results obtained from quantitative real-time PCR (coefficient of determination, $r^2 = 0.89$; Fig. 3).

BHA-Induced Nrf2-Dependent Genes in Small Intestine and Liver

Genes that were induced only in wild-type mice, but not in Nrf2(−/−) mice, by BHA were designated as BHA-induced Nrf2-dependent genes. Based on their biological functions, these genes were classified into categories, including ubiquitination and proteolysis, electron transport, detoxification enzymes, transport, apoptosis and cell cycle control, cell adhesion, kinases and phosphatases, transcription factors and interacting partners, RNA/protein processing and nuclear assembly, biosynthesis and metabolism, cell growth and differentiation, and G protein-coupled receptors (Table II lists a subset of these genes relevant to our interest).

Gene expression in small intestine in response to BHA treatment was more sensitive than that elicited in the liver with a larger number of Nrf2-dependent genes being upregulated in the former. The category of transcription factors and interacting partners predominated the upregulated genes followed by kinases and phosphatases. In the former category, a number of interesting transcription factors were identified as BHA-regulated Nrf2-dependent genes. In the small intestine, these primarily included insulin-like growth factor 2 (Igf2), Jun oncogene (Jun), Notch gene homolog 4 (Drosophila, Notch 4), nuclear receptor co-repressor (Ncor1), nuclear receptor interacting protein 1 (Nrip1), serum response factor binding protein 1 (Srfbp1), Spred-1 (Spred1), suppressor of cytokine signaling 5 (Socs5), thyroid hormone receptor beta (Thrb), transforming growth factor, beta receptor 1(Tgfbr1), transducer of ERBB2, 2 (Tob2), members 19 and 23 of tumor necrosis factor superfamily (Tnfrsf19 and Tnfrsf23), v-maf musculoaponeurotic fibrosacroma oncogene family, protein G (avian, MafG), and wingless-type MMTV integration site 9 B (Wnt9b). Similarly, the major BHA-regulated Nrf2-dependent transcription factors identified in the liver included activating signal cointegrator 1 complex subunit 2 (Ascc2), Eph receptor A3 (Epha3), Eph receptor B1 (Ephb1), fos-like antigen 2 (Fosl2), insulin-like growth factor 2 receptor

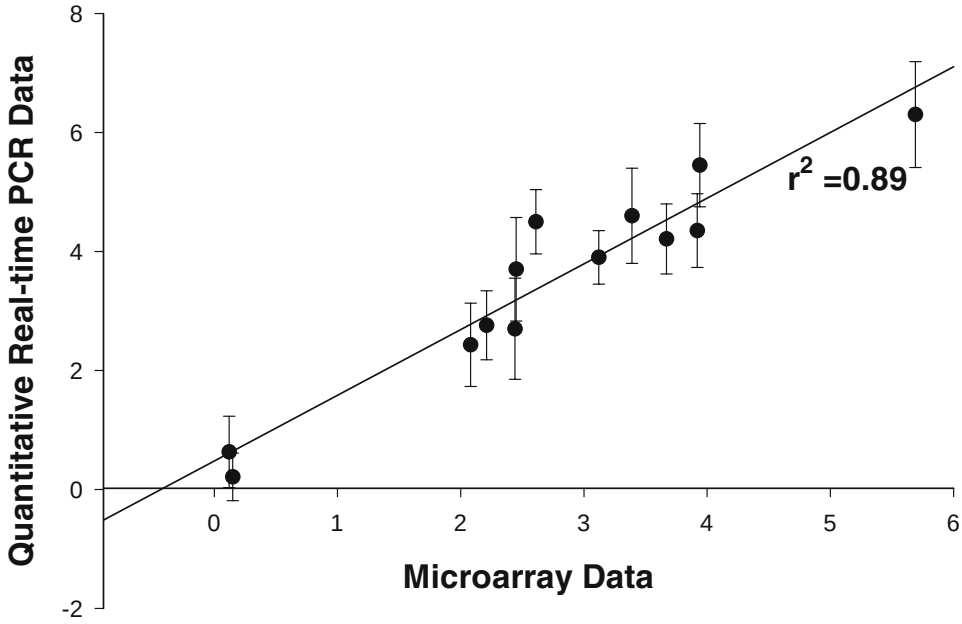

Fig. 3. Correlation of microarray data with quantitative real-time (*qRT*) PCR data. Fold changes in gene expression measured by quantitative real-time PCR for each sample in triplicate ($n = 3$) were plotted against corresponding fold changes from microarray data (coefficient of determination, $r^2 = 0.89$).

(Igf2r), nuclear receptor interacting protein 1 (Nrip1), RAB4A member RAS oncogene family (Rab4a), reticuloendotheliosis oncogene (Rel), and transcription factor AP-2 beta (Tcfap2b). Interestingly, induction of Nrip1 was observed in both small intestine and liver suggesting that the Nrf2/ARE pathway may play a dominant role in BHA-elicited regulation of this gene.

Amongst the kinases and phosphatases in small intestine, the highest expression was seen with microtubule associated serine/threonine kinase-like (Mastl). Several important members of the mitogen-activated protein kinase (Mapk) pathway activating the Nrf2/ARE signaling were also induced in the small intestine including Mapk8, Mapk6, Map3k9, Map4k4 and Map4k5 with strongest induction seen with Mapk8. Moreover, induction was also observed of Janus kinase 2 (Jak2), types 1 and 2 of neurotrophic tyrosine kinase, receptor (Ntrk1 and Ntrk2), and tyrosine kinase, non-receptor, 1 (Tnk1). Comparable expression was noted of different isoforms of protein kinases including epsilon, eta, and cAMP dependent regulatory, type II alpha (Prkce, Prkch and Prkar2a respectively). Regulatory subunits of protein phosphatase 1 (Ppp1r14a) and 2 (Ppp2r5e) and catalytic subunit of protein phosphatase 3, beta isoform (Ppp3cb) were also upregulated in the small intestine as BHA-regulated and Nrf2-dependent genes. In the liver, the genes induced in the same category included diacylglycerol kinase kappa (Dagkκ), inhibitor of kappaB kinase gamma (Iκbkg), protein kinase, cAMP dependent regulatory, type I, alpha (Prkar1a), protein tyrosine phosphatase (Ptp), and putative membrane-associated guanylate kinase 1 (Magi-1) mRNA, alternatively spliced c form (Baiap1).

Representative genes induced by BHA in an Nrf2-dependent manner in the category of apoptosis and cell cycle control genes included members of the caspase cascade as well as cyclins G1 and T2 in small intestine; and growth arrest and DNA-damage-inducible 45 alpha (Gadd45a) in liver. Several important Nrf2-dependent detoxifying genes were also upregulated by BHA including glutamate-cysteine ligase, catalytic subunit (Gclc) in both small intestine and liver, glutathione S-transferase, mu 1 (Gstm1) and mu 3 (Gstm3) isoforms in small intestine and liver respectively, and heme oxygenase (decycling) 1 (Hmox1) in liver. BHA could also modulate many other important categories of genes in an Nrf2-dependent manner. Salient amongst them were the biosynthesis and metabolism genes, G-protein coupled receptors, RNA/protein processing and nuclear assembly, ubiquitination and proteolysis, and transport genes. Major transporters induced included the multidrug resistance associated proteins Mrp1 and 3 and Mdr1. Also induced were the sodium/glucose cotransporter (Slc5a12) and ion channels for sodium, potassium and chloride ions.

BHA-Suppressed Nrf2-Dependent Genes in Small Intestine and Liver

As shown in Table III which lists a subset of genes relevant to our interest, BHA treatment also inhibited the expression of many genes falling into similar functional categories in an Nrf2-dependent manner, although the number of genes was smaller. Notably, the category of transcription factors and interacting partners remained the largest amongst these genes with nuclear receptor coactivator 3 (Ncoa3), and src family associated phosphoprotein 1 (Scap1) being among the genes suppressed by BHA and co-regulated with Nrf2 in small intestine; and epidermal growth factor receptor pathway substrate 15 (Eps15) and hypoxia inducible factor 1, alpha subunit (Hif1a) being suppressed in the liver.

Amongst the kinases and phosphatases, BHA suppressed, in small intestine, the expression of G protein-coupled receptor kinase 5 (Grk5), glycogen synthase kinase 3

Table II. BHA-Induced Nrf2-Dependent Genes in Small Intestine and Liver

Gene Description	Symbol	GenBank Accession	SIT[a]	Liver[b]
Cell Adhesion				
Activated leukocyte cell adhesion molecule	Alcam	NM_009655	2.39	
CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	Cd47	NM_010581	2.02	
Apoptosis and Cell Cycle Control				
Anaphase promoting complex subunit 1	Anapc1	NM_008569	28.6	
BCL2-like 11 (apoptosis facilitator)	Bcl2l11	NM_207680	2.1	
CASP8 and FADD-like apoptosis regulator	Cflar	NM_207653	3.94	
Caspase recruitment domain family, member 6	Card6	XM_139295	2.44	
Cyclin G1	Ccng1	NM_009831	2.61	
Cyclin T2	Ccnt2	NM_028399	2.37	
G1 to S phase transition 1	Gspt1	NM_146066	2.9	
Growth arrest and DNA-damage-inducible 45 alpha	Gadd45a	NM_007836	2.65	3.02
Growth arrest and DNA-damage-inducible 45 beta	Gadd45b	NM_008655	3.19	
Growth arrest specific 1	Gas1	NM_008086	2.17	
Growth arrest-specific 2 like 3	Gas2l3	XM_137276	2.23	
Insulin-like growth factor binding protein 3	Igfbp3	NM_008343	2.26	
Nuclear mitotic apparatus protein 1 (Numa1), mRNA	Numa1	NM_133947	2	
Retinoblastoma binding protein 8	Rbbp8	XM_484703		2.38
Synaptonemal complex protein 1	Sycp1	NM_011516		2.21
Tnf receptor-associated factor 1	Traf1	NM_009421		5.57
Proliferating cell nuclear antigen	Pcna	NM_011045	4.76	9.76
Biosynthesis and Metabolism				
Aldehyde dehydrogenase family 1, subfamily A3	Aldh1a3	NM_053080	2.99	
Acyl-CoA synthetase long-chain family member 1	Acsl1	NM_007981	2.05	
Acyl-Coenzyme A binding domain containing 3	Acbd3	NM_133225	2.01	
Adenylate kinase 3 (Ak3), mRNA	Ak3l1	NM_021299		2.38
Aldehyde dehydrogenase 2, mitochondrial	Aldh2	NM_009656		2.93
Arginine decarboxylase	Adc	NM_172875		2.86
Creatine kinase, mitochondrial 2	Ckmt2	NM_198415		10.07
Creatine kinase, muscle	Ckm	NM_007710	5.17	3.88
Dopamine beta hydroxylase	Dbh	NM_138942		2.45
Ectonucleoside triphosphate diphosphohydrolase 3	Entpd3	NM_178676		5.54
Eukaryotic translation initiation factor 3, subunit 10 (theta)	Eif3s10	NM_010123	2.02	
Galactose-4-epimerase, UDP	Gale	NM_178389		2
Glyoxalase 1	Glo1	NM_025374	2.05	
GTP binding protein 2	Gtpbp2	NM_019581	2.08	2.68
Guanine nucleotide binding protein-like 2 (nucleolar)	Gnl2	NM_145552	2.25	2.16
Guanosine monophosphate reductase	Gmpr	NM_145465	3.79	
Hydroxysteroid (17-beta) dehydrogenase 7	Hsd17b7	NM_010476	2.19	
Nitric oxide synthase 3 antisense	Nos3as	NM_001002897	3.14	
Phosphatidylinositol glycan, class C	Pigc	NM_026078	7.44	
Phosphoglucomutase 2-like 1	Pgm2l1	NM_027629	14.39	
Phospholipase A2, group IIF	Pla2g2f	NM_012045	2.06	
Prostaglandin-endoperoxide synthase 2	Ptgs2	NM_011198		4.9
Stearoyl-coenzyme A desaturase 1	Scd1	NM_009127	3.39	
Tryptophan hydroxylase 1	Tph1	NM_009414		8.64
Very low density lipoprotein receptor	Vldlr	NM_013703		2.24
Cell Growth and Differentiation				
Cysteine rich transmembrane BMP regulator 1 (chordin like)	Crim1	NM_015800	3.44	
FK506 binding protein 12-rapamycin associated protein 1	Frap1	NM_020009	5.35	
Motile sperm domain containing 3	Mospd3	NM_030037	2.21	
Neurotrophin 5	Ntf5	NM_198190		5.42
Tropomodulin 1	Tmod1	NM_021883	2.25	
Tissue inhibitor of metalloproteinase 2	Timp2	NM_011594	3.02	
Detoxification Enzymes				
Carbohydrate sulfotransferase 10	Chst10	NM_145142	2.44	
Esterase D/formylglutathione hydrolase	Esd	NM_016903	4.65	
Esterase D/formylglutathione hydrolase (Esd), mRNA	Esd	NM_016903	2.53	
Fucosyltransferase 4	Fut4	NM_010242	2.02	
Glutamate-cysteine ligase, catalytic subunit	Gclc	NM_010295	2.69	2.14
Glutathione S-transferase, mu 1	Gstm1	NM_010358	3.67	
Glutathione S-transferase, mu 3	Gstm3	NM_010359		3.39
Heme oxygenase (decycling) 1	Hmox1	NM_010442		3.12
Heparan sulfate 6-*O*-sulfotransferase 2	Hs6st2	NM_015819	6.54	

Table II. Continued

Gene Description	Symbol	GenBank Accession	SIT[a]	Liver[b]
ST3 beta-galactoside alpha-2,3-sialyltransferase 2	St3gal2	NM_009179	2.9	
Thioesterase superfamily member 5	Them5	NM_025416	5.53	
UDP glucuronosyltransferase 8A	Ugt8a	NM_011674	5.65	
UDP glucuronosyltransferase 2 family, polypeptide B35	Ugt2b35	NM_172881		2.3
Uronyl-2-sulfotransferase	Ust	NM_177387	2.32	
DNA Replication				
Topoisomerase (DNA) I	Top1	NM_009408	2.08	
Origin recognition complex, subunit 2-like (*S. cerevisiae*)	Orc2l	NM_008765	2.15	
Electron Transport				
Flavin containing monooxygenase 2	Fmo2	NM_018881	8.72	
Monoamine oxidase B	Maob	NM_172778	2.16	
Thioredoxin domain containing 10	Txndc10	NM_198295	3.85	
G-Protein Coupled Receptors				
Angiotensin receptor 1	Agtr1	NM_177322	4.29	
Calmodulin III (Calm3) mRNA, 3′ untranslated region	Calm3	NM_007590		5.59
Chemokine (C-X-C motif) receptor 4	Cxcr4	NM_009911	2.3	
Coagulation factor II (thrombin) receptor-like 2	F2rl2	NM_010170		2.05
G protein-coupled receptor 133	Gpr133	XM_485685		16.59
G protein-coupled receptor 20	Gpr20	NM_173365	2.1	
Guanine nucleotide binding protein (G protein), gamma 3 subunit	Gng3	NM_010316		2.64
Guanine nucleotide binding protein (G protein), gamma 7 subunit	Gng7	NM_010319	2.1	
Guanine nucleotide binding protein, alpha inhibiting 1	Gnai1	NM_010305	2.11	
Regulator of G-protein signaling 9	Rgs9	NM_011268	7.73	13.73
Kinases and Phosphatases				
Protein tyrosine phosphatase, receptor type, T	Ptprt	NM_021464	2.27	
Serine/threonine kinase 24 (STE20 homolog, yeast)	Stk24	NM_145465	3.84	
Diacylglycerol kinase kappa	Dagkk	NM_177914		2.26
Ethanolamine kinase 1	Etnk1	XM_284250	3.46	
FMS-like tyrosine kinase 4	Flt4	NM_008029	2.95	
Inhibitor of kappaB kinase gamma	Ikbkg	NM_010547		3.92
Janus kinase 2	Jak2	NM_008413	3.09	
Microtubule associated serine/threonine kinase-like	Mastl	NM_025979	14.5	
Mitogen activated protein kinase 8	Mapk8	NM_016700	10.39	
Mitogen-activated protein kinase 6	Mapk6	NM_015806	2.11	
Mitogen-activated protein kinase kinase kinase 9	Map3k9	NM_177395	3.06	
Mitogen-activated protein kinase kinase kinase kinase 4	Map4k4	NM_008696	2.46	
Mitogen-activated protein kinase kinase kinase kinase 5	Map4k5	NM_201519	3.94	
Neurotrophic tyrosine kinase, receptor, type 1	Ntrk1	XM_283871	3.88	
Neurotrophic tyrosine kinase, receptor, type 2	Ntrk2	NM_001025074	3.3	
Protein kinase C, epsilon	Prkce	NM_011104	5.69	
Protein kinase C, eta	Prkch	NM_008856	2.87	
Protein kinase, cAMP dependent regulatory, type I, alpha	Prkar1a	AK049832		11.6
Protein kinase, cAMP dependent regulatory, type II alpha	Prkar2a	NM_008924	2.18	
Protein phosphatase 1 (formerly 2C)-like	Ppm1l	NM_178726	2.48	
Protein phosphatase 1, regulatory (inhibitor) subunit 14A	Ppp1r14a	NM_026731	2.1	
Protein phosphatase 2, regulatory subunit B (B56), epsilon isoform	Ppp2r5e	NM_012024	2.12	
Protein phosphatase 3, catalytic subunit, beta isoform	Ppp3cb	NM_008914	2.02	
Protein tyrosine phosphatase 4a1	Ptp4a1	NM_011200	3.79	
Protein tyrosine phosphatase, receptor type Z, polypeptide 1	Ptprz1	XM_620293	2.34	
Putative membrane-associated guanylate kinase 1 (Magi-1), alternatively spliced c form	Baiap1	NM_010367		2.45
TANK-binding kinase 1	Tbk1	NM_019786	2.06	
Ttk protein kinase	Ttk	NM_009445	2.23	
Tyrosine kinase, non-receptor, 1	Tnk1	NM_031880	2.34	
RNA/Protein Processing and Nuclear Assembly				
Heterogeneous nuclear ribonucleoprotein A1	Hnrpa1	NM_010447	3.45	
Histone 1, H4f	Hist1h4f	NM_175655	7.1	
Hypoxia up-regulated 1	Hyou1	NM_021395		3.02
Methionine sulfoxide reductase A	Msra	NM_026322		2.9
Methionine sulfoxide reductase B3	Msrb3	NM_177092	3.41	
Mitochondrial ribosomal protein L52	Mrpl52	NM_026851		5.18
Neuronal pentraxin receptor	Nptxr	NM_030689		3.29
Nucleosome assembly protein 1-like 1	Nap1l1	NM_015781	2.21	
Peptidyl arginine deiminase, type II	Padi2	NM_008812		3.28

Table II. Continued

Gene Description	Symbol	GenBank Accession	SIT[a]	Liver[b]
RNA polymerase 1–2	Rpo1–2	NM_009086	2.99	
RNA polymerase 1–4	Rpo1–4	NM_009088	35.71	
Sarcolemma associated protein	Slmap	NM_032008	2.17	
Tubulin tyrosine ligase-like family, member 4	Ttll4	NM_001014974		2.22
Importin 7	Ipo7	NM_181517	2.51	
karyopherin (importin) alpha 1	Kpna1	NM_008465	29.9	
Transcription Factors and Interacting Partners				
Heat shock protein, alpha-crystalline-related, B6	Hspb6	NM_001012401	3.72	
Bone morphogenetic protein receptor, type 1A	Bmpr1a	NM_009758	2.02	
Metallothionein 1	Mt1	NM_013602	6.24	
Insulin-like growth factor 2	Igf2	NM_010514	2.59	
Activating signal cointegrator 1 complex subunit 2	Ascc2	NM_029291		3.32
Calsequestrin 1	Casq1	NM_009813		2
CBFA2T1 identified gene homolog (human)	Cbfa2t1h	NM_009822	2.42	
CCCTC-binding factor	Ctcf	NM_007794		2.35
C-erbA alpha1 mRNA for thyroid hormone receptor	Thra	NM_178060	2.74	
Checkpoint suppressor 1	Ches1	NM_183186	2.26	
Circadian locomoter output cycles kaput	Clock	NM_007715	2.81	
Eph receptor A3	Epha3	NM_010140		2.23
Eph receptor B1	Ephb1	NM_173447		2.34
Fos-like antigen 2	Fosl2	NM_008037		2.36
Hedgehog-interacting protein	Hhip	NM_020259	6.11	
Hepatoma-derived growth factor, related protein 2	Hdgfrp2	NM_008233	2.22	
Hypermethylated in cancer 2	Hic2	NM_178922		2.82
Insulin-like growth factor 2 receptor	Igf2r	NM_010515		2
Jun oncogene	Jun	NM_010591	2.75	
Kruppel-like factor 7 (ubiquitous)	Klf7	NM_033563	2.21	
Lymphoblastomic leukemia	Lyl1	NM_008535		7.36
MAX dimerization protein 1	Mxd1	NM_010751	2.05	
NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2	Ndufb2	NM_026612	3.43	
Notch gene homolog 4 (Drosophila)	Notch4	NM_010929	2.35	
Nuclear factor, interleukin 3, regulated	Nfil3	NM_017373	17.13	
Nuclear receptor co-repressor 1	Ncor1	NM_011308	3.39	
Nuclear receptor interacting protein 1	Nrip1	NM_173440	2.61	2.63
Nuclear receptor subfamily 2, group C, member 2	Nr2c2	NM_011630	2.32	
Nuclear receptor subfamily 6, group A, member 1	Nr6a1	NM_010264	2.49	
Phosphodiesterase 8A	Pde8a	NM_008803	2.58	
Phosphoprotein associated with glycosphingolipid microdomains 1	Pag1	NM_053182		2.23
Polycystic kidney disease 1 like 3	Pkd1l3	NM_181544	8.44	
Polymerase (RNA) I associated factor 1	Praf1	NM_022811		2.28
Pre B-cell leukemia transcription factor 3	Pbx3	NM_016768	2.05	
Purine rich element binding protein B	Purb	NM_011221	2.04	
RAB4A, member RAS oncogene family	Rab4a	BG080696		16.85
Ras and Rab interactor 1	Rin1	NM_145495		3.11
Ras association (RalGDS/AF-6) domain family 2	Rassf2	NM_175445	8.21	
Ras homolog gene family, member T1	Rhot1	NM_021536	2.28	
Reticuloendotheliosis oncogene	Rel	NM_009044		2.13
Retinol binding protein 1, cellular	Rbp1	NM_011254	2.33	
Serum response factor binding protein 1	Srfbp1	NM_026040	4.47	
Spred1	Spred1	NM_033524	43.87	
Suppressor of cytokine signaling 5	Socs5	NM_019654	19.3	
T-box brain gene 1	Tbr1	NM_009322	28.35	
Thyroid hormone receptor beta	Thrb	NM_009380	2.43	
Transcription factor AP-2 beta	Tcfap2b	NM_009334		3.79
Transducer of ERBB2, 2	Tob2	NM_020507	2.23	
Transforming growth factor beta 1 induced transcript 1	Tgfb1i1	NM_009365	3.36	
Transforming growth factor, beta receptor I	Tgfbr1	NM_009370	14.27	
Tumor necrosis factor receptor superfamily, member 19	Tnfrsf19	NM_013869	3.49	
Tumor necrosis factor receptor superfamily, member 23	Tnfrsf23	NM_024290	2.6	
Tumor necrosis factor, alpha-induced protein 2	Tnfaip2	NM_009396	2.13	
V-abl Abelson murine leukemia viral oncogene 2	Abl2	NM_009595	7.44	
v-Maf musculoaponeurotic fibrosarcoma oncogene family, protein G (avian)	MafG	NM_010756	2.45	
Wingless-type MMTV integration site 9B	Wnt9b	NM_011719	3.35	

Table II. Continued

Gene Description	Symbol	GenBank Accession	SIT[a]	Liver[b]
Yamaguchi sarcoma viral (v-yes) oncogene homolog 1	Yes1	NM_009535	4.21	
Zinc finger homeobox 1b (Zfhx1b), mRNA	Zfhx1b	NM_015753	2.6	
Zinc finger protein 37	Zfp37	NM_009554	3.42	
RAN, member RAS oncogene family	Ran	NM_009391	2	
Transport				
Aquaporin 1	Aqp1	NM_007472	2.37	
ATP-binding cassette, sub-family B (MDR/TAP), member 1A	Abcb1a	NM_011076	2.08	
ATP-binding cassette, sub-family C (CFTR/MRP), member 1	Abcc1	NM_008576	2.23	
ATP-binding cassette, sub-family D (ALD), member 3	Abcd3	NM_008991		2.21
Basic leucine zipper nuclear factor 1	Blzf1	NM_025505	6.45	
Chloride channel calcium activated 4	Clca4	NM_139148	2.64	
Cholinergic receptor, nicotinic, alpha polypeptide 3	Chrna3	NM_145129	2.01	
Glutamate receptor, ionotropic, AMPA4 (alpha 4)	Gria4	NM_019691	6.76	
Multidrug resistance-associated protein 3	Abcc3	NM_029600		2.44
Potassium large conductance calcium-activated channel, subfamily M, beta member 2	Kcnmb2	NM_028231	3.63	
Proton/amino acid transporter 4 (PAT4)	Slc36a4	NM_172289	3.04	
Sideroflexin 2	Sfxn2	NM_053196	2.05	
Signal transducing adaptor molecule (SH3 domain and ITAM motif) 2	Stam2	NM_019667	2.21	
Sodium channel, voltage-gated, type VII, alpha	Scn7a	NM_009135	2.19	
Solute carrier family 16 (monocarboxylic acid transporters), member 10	Slc16a10	NM_028247		2.38
Solute carrier family 35, member A5	Slc35a5	NM_028756		3.32
Solute carrier family 39 (zinc transporter), member 10	Slc39a10	NM_172653	8.55	
Solute carrier family 5 (sodium/glucose cotransporter), member 12	Slc5a12	NM_001003915	3.69	
Solute carrier organic anion transporter family, member 2a1	Slco2a1	NM_033314	2.3	
Type III sodium-dependent phosphate transporter	Slc20a2	NM_011394	2.71	
Ubiquitination and Proteolysis				
A disintegrin and metallopeptidase domain 10	Adam10	NM_007399	3.45	
Cathepsin B	Ctsb	NM_007798	2.06	
Constitutive photomorphogenic protein (Cop1)	Rfwd2	NM_011931		2.05
HECT domain containing 2	Hectd2	NM_172637		2
Serine carboxypeptidase 1	Scpep1	NM_029023	2.05	
SUMO/sentrin specific peptidase 2	Senp2	NM_029457	4.15	
SUMO1/sentrin specific peptidase 1	Senp1	NM_144851	2.62	
Ubiquitin-activating enzyme E1, Chr Y 1	Ube1y1	NM_011667	2.39	
Others				
U1 small nuclear ribonucleoprotein 1C	Snrp1c	NM_011432		3.21
Melanoma antigen, family H, 1	Mageh1	NM_023788	5.84	
HIV TAT specific factor 1	Htatsf1	NM_028242	2.1	
Chemokine (C-X-C motif) ligand 2	Cxcl2	NM_009140		2.1
DEAD (Asp-Glu-Ala-Asp) box polypeptide 10	Ddx10	XM_284494	3.42	
DEAD (Asp-Glu-Ala-Asp) box polypeptide 5	Ddx5	NM_007840		3.05
Golgi phosphoprotein 4	Golph4	NM_175193	2.37	
Kelch-like 7 (Drosophila)	Klhl7	NM_026448	2.95	
Leucine rich repeat containing 48	Lrrc48	NM_029044		9.96
Leucine-rich repeats and immunoglobulin-like domains 3	Lrig3	NM_177152	8.43	
Protein disulfide isomerase associated 3	Pdia3	NM_007952		2
Serine (or cysteine) peptidase inhibitor, clade I, member 1	Serpini1	NM_009250	2.47	
Six transmembrane epithelial antigen of prostate 2	Steap2	XM_284053	2.96	
TCDD-inducible poly(ADP-ribose) polymerase	Tiparp	NM_178892	2.5	
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase, activation protein, zeta polypeptide	Ywhaz	NM_011740	2.17	3.46
Keratin associated protein 6-1	Krtap6-1	NM_010672		5.04
Keratin complex 2, basic, gene 18	Krt2–18	NM_016879		5.85
Seminal vesicle secretion 3	Svs3	NM_021363	8.7	
Antigen p97 (melanoma associated)	Mfi2	NM_013900		6.73

[a] Genes that were induced >2-fold by BHA only in small intestine of Nrf2 wild-type mice but not in small intestine of Nrf2 knockout mice compared with vehicle treatment at 3 h. The relative mRNA expression levels of each gene in treatment group over vehicle group (fold changes) are listed.

[b] Genes that were induced >2-fold by BHA only in liver of Nrf2 wild-type mice but not in liver of Nrf2 knockout mice compared with vehicle treatment at 3 h. The relative mRNA expression levels of each gene in treatment group over vehicle group (fold changes) are listed.

Table III. BHA-Suppressed Nrf2-Dependent Genes in Small Intestine and Liver

Gene Description	Symbol	GenBank Accession	SIT[a]	Liver[b]
Cell Adhesion				
Camello-like 2	Cml2	NM_053096	0.03	
Catenin (cadherin associated protein), delta 2	Ctnnd2	NM_008729		0.36
Intercellular adhesion molecule	Icam1	NM_010493	0.48	
Protocadherin 7	Pcdh7	NM_018764		0.43
Apoptosis and Cell Cycle Control				
B-cell leukemia/lymphoma 2	Bcl2	NM_009741		0.32
Breast cancer 1	Brca1	NM_009764		0.49
CASP2 and RIPK1 domain containing adaptor with death domain	Cradd	NM_009950		0.39
Cell division cycle 37 homolog (S. cerevisiae)-like 1	Cdc37l1	NM_025950	0.28	
Cell division cycle and apoptosis regulator 1	Ccar1	NM_026201		0.4
Centrin 4	Cetn4	NM_145825	0.21	
Cyclin I	Ccni	NM_017367		0.28
G0/G1 switch gene 2	G0s2	NM_008059		0.44
Integrin beta 1 (fibronectin receptor beta)	Itgb1	NM_010578	0.18	
Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	Nfatc1	NM_016791	0.09	
Rho GTPase activating protein 4	Arhgap4	NM_138630	0.38	
WW domain-containing oxidoreductase	Wwox	NM_019573	0.47	
Biosynthesis and Metabolism				
Abhydrolase domain containing 9	Abhd9	XM_128553	0.22	
Acetyl-Coenzyme A carboxylase beta	Acacb	NM_133904		0.39
Alanine-glyoxylate aminotransferase	Agxt	NM_016702	0.48	
B-cell CLL/lymphoma 9-like	Bcl9l	XM_620743	0.1	
Butyrobetaine (gamma), 2-oxoglutarate dioxygenase 1 (gamma-butyrobetaine hydroxylase)	Bbox1	NM_130452	0.42	
Methylmalonyl-Coenzyme A mutase	Mut	NM_008650		0.17
Mitochondrial ribosomal protein L27	Mrpl27	NM_053161	0.47	
Mitochondrial ribosomal protein S14	Mrps14	NM_025474		0.35
Mitochondrial ribosomal protein S21	Mrps21	NM_078479	0.49	
Nicotinamide nucleotide adenylyltransferase 2	Nmnat2	NM_175460	0.33	
Pantothenate kinase 1	Pank1	NM_023792	0.18	
Phosphoglucomutase 2-like 1	Pgm2l1	NM_027629		0.4
Phosphopantothenoylcysteine decarboxylase	Ppcdc	NM_176831	0.05	
Phosphoribosyl pyrophosphate synthetase 2	Prps2	NM_026662		0.47
Propionyl-Coenzyme A carboxylase, alpha polypeptide	Pcca	NM_144844	0.47	
UDP-glucose ceramide glucosyltransferase	Ugcg	NM_011673		0.37
Ureidopropionase, beta	Upb1	NM_133995	0.45	
Uroporphyrinogen III synthase	Uros	NM_009479	0.36	
Very low density lipoprotein receptor	Vldlr	NM_013703		0.43
Nitric oxide synthase 1, neuronal	Nos1	NM_008712		0.4
Cell Growth and Differentiation				
Helicase, lymphoid specific	Hells	NM_008234	0.33	
Male enhanced antigen 1	Mea1	NM_010787	0.48	
Myosin, light polypeptide 7, regulatory	Myl7	NM_022879	0.46	
DNA Replication				
Origin recognition complex, subunit 4-like (S. cerevisiae)	Orc4l	NM_011958	0.1	
Electron Transport				
Cytochrome c oxidase, subunit VIIa 1	Cox7a1	NM_009944	0.42	0.4
Cytochrome P450, family 21, subfamily a, polypeptide 1	Cyp21a1	NM_009995		0.29
Cytochrome P450, family 3, subfamily a, polypeptide 44	Cyp3a44	NM_177380	0.38	
Cytochrome P450, family 7, subfamily a, polypeptide 1	Cyp7a1	NM_007824		0.37
Dual oxidase 1	Duox1	XM_130483	0.39	
NADH dehydrogenase (ubiquinone) 1 beta subcomplex 4	Ndufb4	NM_026610	0.41	
Thioredoxin reductase 2	Txnrd2	NM_013711		0.21
Ubiquinol-cytochrome c reductase (6.4kD) subunit	Uqcr	NM_025650	0.4	
G-Protein Coupled Receptors				
Adrenergic receptor, beta 1	Adrb1	NM_007419		0.45
Bombesin-like receptor 3	Brs3	NM_009766	0.35	
Calmodulin 3	Calm3	NM_007590	0.45	
Cholecystokinin A receptor	Cckar	NM_009827		0.19
G protein-coupled receptor 1	Gpr1	NM_146250	0.45	
G protein-coupled receptor 37	Gpr37	NM_010338		0.1
GNAS (guanine nucleotide binding protein, alpha stimulating) complex locus	Gnas	NM_010309	0.46	

Table III. Continued

Gene Description	Symbol	GenBank Accession	SIT[a]	Liver[b]
Regulator of G-protein signaling 2	Rgs2	NM_009061	0.37	
Vomeronasal 1 receptor, B1	V1rb1	NM_053225	0.5	
Kinases and Phosphatases				
Calcium/calmodulin-dependent protein kinase II, delta	Camk2d	NM_001025439		0.38
G protein-coupled receptor kinase 5 (GRK5)	Gprk5	NM_018869	0.46	
Glycogen synthase kinase 3 beta	Gsk3b	NM_019827	0.15	
Inositol polyphosphate multikinase	Ipmk	NM_027184	0.47	
Inositol polyphosphate-1-phosphatase	Inpp1	NM_008384		0.37
MAP kinase-activated protein kinase 5	Mapkapk5	NM_010765	0.42	
Microtubule associated serine/threonine kinase family member 4	Mast4	XM_283179	0.12	0.48
Mitogen activated protein kinase kinase 7	Map2k7	NM_011944		0.37
Mitogen-activated protein kinase associated protein 1	Mapkap1	NM_177345	0.34	
Multiple substrate lipid kinase	Mulk	NM_023538	0.48	
p21 (CDKN1A)-activated kinase 3	Pak3	NM_008778	0.42	
Phosphoinositide-3-kinase, regulatory subunit 5, p101	Pik3r5	NM_177320		0.46
Protein kinase ATR (Atr)	Prk	NM_028533		0.49
Protein kinase, AMP-activated, alpha 1 catalytic subunit	Prkaa1	NM_001013367		0.35
Protein phosphatase 3, catalytic subunit, alpha isoform	Ppp3ca	NM_008913	0.37	
Protein tyrosine phosphatase, non-receptor type 4	Ptpn4	NM_019933	0.5	
Receptor tyrosine kinase-like orphan receptor 1	Ror1	NM_013845		0.29
Ribosomal protein S6 kinase, polypeptide 4	Rps6ka4	NM_019924	0.45	
Ribosomal protein S6 kinase, polypeptide 5	Rps6ka5	NM_153587		0.3
RNA/Protein Processing and Nuclear assembly				
Aspartate-beta-hydroxylase	Asph	NM_023066		0.17
ATP synthase mitochondrial F1 complex assembly factor 2	Atpaf2	NM_145427	0.48	
Chromatin accessibility complex 1	Chrac1	NM_053068	0.49	
Down syndrome critical region gene 3	Dscr3	NM_007834	0.49	
Fucosyltransferase 9	Fut9	NM_010243		0.05
Histone 3, H2a	Hist3h2a	NM_178218	0.43	
Neuronal pentraxin receptor	Nptxr	NM_030689	0.37	
Nucleoporin 133	Nup133	NM_172288		0.34
Protein-O-mannosyltransferase 2	Pomt2	NM_153415	0.46	
Zinc finger, matrin-like	Zfml	NM_008717	0.44	
Transcription factors and interacting partners				
Activating transcription factor 7 interacting protein 2	Atf7ip2	XM_148109		0.35
Angiotensin II, type I receptor-associated protein	Agtrap	NM_009642		0.49
Bone morphogenetic protein 6	Bmp6	NM_007556		0.12
Breast carcinoma amplified sequence 3	Bcas3	NM_138681		0.4
E2F transcription factor 5	E2f5	NM_007892	0.38	
EGF-like module containing, mucin-like, hormone receptor-like sequence 4	Emr4	NM_139138	0.19	
Epidermal growth factor receptor pathway substrate 15	Eps15	NM_007943		0.19
Fc receptor, IgG, high affinity I	Fcgr1	NM_010186	0.48	
FEV (ETS oncogene family)	Fev	NM_153111	0.48	
Growth hormone receptor	Ghr	NM_010284		0.31
GTP binding protein 7 (putative)	Gtpbp7	NM_199301	0.49	
Heat shock factor 2	Hsf2	NM_008297		0.08
HRAS-like suppressor	Hrasls	NM_013751	0.23	
Human immunodeficiency virus type I enhancer binding protein 2	Hivep2	NM_010437	0.48	
Hypoxia inducible factor 1, alpha subunit	Hif1a	NM_010431		0.4
Insulin-like growth factor 1	Igf1	NM_010512	0.43	
Insulin-like growth factor I receptor	Igf1r	NM_010513	0.49	
Interleukin 2 receptor, gamma chain	Il2rg	NM_013563		0.44
Kruppel-like factor 1 (erythroid)	Klf1	NM_010635	0.43	
Lysosomal trafficking regulator	Lyst	NM_010748		0.43
Metal response element binding transcription factor 2	Mtf2	NM_013827	0.12	
Myc target 1	Myct1	NM_026793		0.43
Nuclear receptor coactivator 3	Ncoa3	NM_013827	0.12	
Nuclear receptor subfamily 2, group F, member 1	Nr2f1	NM_010151		0.4
Peroxisome biogenesis factor 7	Pex7	NM_008822	0.5	
Protein inhibitor of activated STAT 4	Pias4	NM_021501	0.45	
RAB28, member RAS oncogene family	Rab28	NM_027295	0.46	
Rho GTPase activating protein 29	Arhgap29	NM_172525		0.26
SH2 domain containing 4A	Sh2d4a	XM_134197		0.23
Sp2 transcription factor	Sp2	NM_030220		0.45

Table III. Continued

Gene Description	Symbol	GenBank Accession	SIT[a]	Liver[b]
Sphingosine kinase 2	Sphk2	NM_203280	0.45	
Src family associated phosphoprotein 1	Scap1	NM_001033186	0.41	
Suppressor of cytokine signaling 2	Socs2	NM_007706		0.27
Tax1 (human T-cell leukemia virus type I) binding protein 3	Tax1bp3	NM_029564		0.39
Thrombopoietin	Thpo	NM_009379	0.19	
Thyroid hormone receptor associated protein 1	Thrap1	XM_109726		0.37
Topoisomerase I binding, arginine/serine-rich	Topors	NM_134097		0.49
v-raf murine sarcoma 3611 viral oncogene homolog	Araf	NM_009703		0.41
Transport				
Aquaporin 3	Aqp3	NM_016689		0.35
Aquaporin 8	Aqp8	NM_007474	0.33	
ATPase, Ca++ transporting, plasma membrane 4	Atp2b4	NM_213616	0.1	
ATPase, Na+/K+ transporting, alpha 1 polypeptide	Atp1a1	NM_144900		0.39
Calcium channel, voltage-dependent, gamma subunit 6	Cacng6	NM_133183		0.41
Chloride intracellular channel 5	Clic5	NM_172621		0.16
Fatty acid binding protein 6, ileal (gastrotropin)	Fabp6	NM_008375	0.31	
Sodium channel, voltage-gated, type III, beta	Scn3b	NM_153522		0.31
Ubiquitination and Proteolysis				
F-box and leucine-rich repeat protein 8	Fbxl8	NM_015821	0.43	
Granzyme B	Gzmb	NM_013542	0.41	
Matrix metalloproteinase 24	Mmp24	NM_010808	0.38	
Protease, serine, 19 (neuropsin)	Prss19	NM_008940	0.48	
Ubiquitin specific peptidase 16	Usp16	NM_024258	0.26	
Others				
Melanoma antigen, family A, 5	Magea5	NM_020018	0.23	
Hornerin	Hrnr	NM_133698	0.39	
Cystatin E/M	Cst6	NM_028623	0.43	

[a] Genes that were suppressed >2-fold by BHA only in small intestine of Nrf2 wild-type mice but not in small intestine of Nrf2 knockout mice compared with vehicle treatment at 3 h. The relative mRNA expression levels of each gene in treatment group over vehicle group (fold changes) are listed.

[b] Genes that were suppressed >2-fold by BHA only in liver of Nrf2 wild-type mice but not in liver of Nrf2 knockout mice compared with vehicle treatment at 3 h. The relative mRNA expression levels of each gene in treatment group over vehicle group (fold changes) are listed.

beta (Gsk3β), Mapk-activated protein kinase 5 (Mapkapk5), Mapk associated protein 1 (Mapkap1), p21-activated kinase 3 (Pak3), and ribosomal protein S6 kinase, polypeptide 4 (Rps6ka4). In the liver, BHA suppressed, in an Nrf2-dependent manner, genes such as Map2k7, phosphoinositide-3-kinase, regulatory subunit 5, p101 (Pik3r5), Ribosomal protein S6 kinase, polypeptide 5 (Rps6ka5) and microtubule associated serine/threonine kinase family member 4 (Mast4) amongst others.

Major genes down-regulated by BHA in an Nrf2-dependent manner in the category of apoptosis and cell cycle control included B-cell leukemia/lymphoma 2 (Bcl2), breast cancer 1 (Brca1), and Cyclin I in liver. Among the transport genes, the fatty acid binding protein 6, ileal (gastrotropin; Fabp6) was suppressed in small intestine by BHA. In the category of electron transport genes, representative genes included the cytochrome *c* oxidase, subunit VIIa 1 (Cox7a1) which was inhibited in both small intestine and liver, and thioredoxin reductase 2 (Txnrd2) which was suppressed in liver. Besides, members of the cytochrome P450 family such as Cyp3a44 in small intestine, and Cyp21a1 and Cyp7a1 in liver were also down-regulated by BHA in an Nrf2-dependent manner in the same category. Other categories of BHA-suppressed genes including ubiquitination and proteolysis, RNA/protein processing and nuclear assembly, biosynthesis and metabolism, and cell growth and differentiation were also identified as regulated through Nrf2.

DISCUSSION

Since BHA was first introduced as a food preservative back in the 1960s, it has attracted a lot of attention and debate because of its potentially diverse biological effects on the health of humans including its potential cancer chemopreventive effects. Although extensive studies have been conducted to define the biological activities of BHA, and a growing body of evidence has been accumulated, a comprehensive definition of the potential cellular targets of BHA that trigger important signal transduction pathways remains a challenge that has yet to be undertaken. Indeed, the molecular basis and the mechanisms of action of BHA are not quite yet fully understood (1). Transcription factor Nrf2 or Nuclear Factor-E2-related factor 2 is indispensable to cellular defense against many chemical insults of endogenous and exogenous origin (21), which play major roles in the etiopathogenesis of many cancers. Pivotal to this role of Nrf2 is the antioxidant response element (ARE) present in the promoter regions of many cytoprotective genes (19,21). Indeed, Nrf2 (−/−; deficient) mice, which are highly susceptible to cancer development, are known to be refractory to the protective actions of some cancer chemopreventive agents (21). It is, therefore, of interest to investigate the role of Nrf2 in BHA-elicited global gene expression profiles *in vivo* in order to extend the latitude of current understanding on the Nrf2-ARE signaling pathway that has emerged as

an important molecular target for cancer chemoprevention. To our knowledge, this is the first attempt to elucidate, by microarray expression profiling, the gestalt genomic basis of BHA-regulated Nrf2-dependent cancer chemoprevention *in vivo* in an Nrf2-deficient murine model.

In the continuing quest to unravel the complex secrets of the biology of Nrf2 in cancer chemoprevention, there is renewed interest in dissecting the interacting partners of Nrf2 such as coactivators and corepressors which are co-regulated with Nrf2. In a recent microarray study (25), we have reported that CREB-binding protein (CBP) was upregulated in mice liver on treatment with (−)epigallocatechin-3-gallate (EGCG) in an Nrf2-dependent manner. We have also demonstrated (15) previously, using a Gal4-Luc reporter co-transfection assay system in HepG2 cells, that the nuclear transcriptional coactivator CBP, which can bind to Nrf2 transactivation domain and can be activated by extracellular signal-regulated protein kinase (ERK) cascade, showed synergistic stimulation with Raf on the transactivation activities of both the chimera Gal4-Nrf2 (1–370) and the full-length Nrf2. In the current study, we observed the upregulation of the *trans*-acting factor v-maf musculoaponeurotic fibrosarcoma oncogene family, protein G, avian (MafG), nuclear receptor co-repressor 1 (Ncor1) and nuclear receptor co-repressor interacting protein (Nrip1); as well as downregulation of the nuclear receptor co-activator 3 (Ncoa3) in an Nrf2-dependent manner. Although microarray expression profiling cannot provide evidence of binding between partners, this is the first investigation to potentially suggest that co-repressors Ncor1 and Nrip1 and co-activator Ncoa3, such as CBP in our previous studies, may serve as putative BHA-regulated nuclear interacting partners of Nrf2 in eliciting the cancer chemopreventive effects of BHA. Furthermore, induction of Nrip1 was observed in both small intestine and liver suggesting that the Nrf2/ARE pathway may play an important role in BHA-elicited regulation of this gene. Taken together, it is tempting to speculate that the BHA-regulated chemopreventive effects through the ARE may be regulated by a multimolecular complex, which involves Nrf2 along with the transcriptional co-repressors Ncor1 and Nrip1 and the transcriptional co-activator Ncoa3, in addition to the currently known trans-acting factors such as MafG (22), with multiple interactions between the members of the putative complex as we have shown recently with the p160 family of proteins (27). Further studies of a biochemical nature would be needed to substantiate this hypothesis and extend our current understanding of Nrf2 regulation in chemoprevention with BHA.

The major detoxification genes induced in this study included glutamate-cysteine ligase, catalytic subunit (Gclc) in both small intestine and liver, glutathione S-transferase, mu 1 (Gstm1) and mu 3 (Gstm3) isoforms in small intestine and liver respectively, and heme oxygenase (decycling) 1 (Hmox1) in liver. Since Nrf2 binds to the *cis*-acting ARE and induces the expression of many important Phase II detoxification and antioxidant genes (10,19–21), and since BHA is known to induce Phase II genes, the current study on spatial regulation in small intestine and liver of BHA-regulated chemopreventive effects via Nrf2 showing the upregulation of Phase II detoxification genes is, indeed, consistent with previous reports (11, 28–31) and also validates our results from a biological perspective. Indeed, we were able to detect the presence of NAD(P)H:quinone oxidoreductase (NQO1) gene induction in qRT-PCR experiments performed in another study at a 12 h time-point (data not shown) suggesting that there may be a relatively delayed induction of the NQO1 gene compared with the other Phase 2 genes in response to BHA and possible differential kinetics of BHA-regulated Phase 2 gene response. Interestingly, genes involved in Phase I drug metabolism as well as Phase III drug transport were also regulated by BHA via Nrf2. The Phase I drug-metabolizing enzymes (DMEs) identified here included cytochrome P450 family members Cyp7a1 and Cyp21a1 suppressed in liver and Cyp3a44 suppressed in small intestine. The roles played by these Phase I DMEs in BHA-elicited cancer chemopreventive effects, however, remain presently unknown. Several transport-related genes were also identified in this study for the first time as both BHA-regulated and Nrf2-dependent. Amongst the upregulated transporters were the ATP-binding cassette superfamily members belonging to MDR/TAP, MRP or ALD subfamilies such as MDR1a/P-glycoprotein (Abcb1a) and MRP 1 (Abcc1) in small intestine; and MRP3 (Abcc3) in the liver. The ALD member responsible for peroxisomal import (32) of fatty acids (Abcd3) was upregulated in the liver, whereas the fatty acid binding protein 6, ileal (gastrotropin; Fabp6) was suppressed in small intestine, suggesting a putative role for BHA and Nrf2 co-regulation of lipid pathways in chemoprevention that has never been examined. Also identified for the first time as transport genes upregulated by BHA via Nrf2 were members of the solute carrier family such as genes encoding for zinc transport, sodium/glucose cotransport, sodium-dependent phosphate transport and organic anion transport (Slc39a10, Slc5a12, Slc20a2 and Slco2a1 respectively). Although the involvement of water channels (aquaporins) in cell migration, fat metabolism, epidermal biology and neural signal transduction point to a role in the pathophysiology of cancers (33), the current study is the first to show BHA-elicited regulation via Nrf2 of aquaporins 1, 3 and 8 which may play a role in the overall cancer chemopreventive effects of BHA. Taken together, the current study suggests that BHA could coordinately regulate the Phase I, II, and III xenobiotic metabolizing enzyme genes as well as antioxidative stress genes through Nrf2-dependent pathways *in vivo*. The regulation of these genes could have significant effects on prevention of tumor initiation by enhancing the cellular defense system, preventing the activation of procarcinogens/reactive intermediates, and increasing the excretion/efflux of reactive carcinogens or metabolites (26).

Bone morphogenetic proteins (BMPs) are multifunctional signaling molecules regulating growth, differentiation, and apoptosis in various target cells (34,35). BMP receptor 1A (Bmpr1a) is a serine/threonine kinase receptor that mediates the osteogenic effects of the BMPs and is coordinately regulated with transforming growth factor, beta (Tgfβ) *in vitro* (36). Here, we show that Bmpr1a was upregulated along with a strong induction of Tgfβ (>14-fold) in an Nrf2-dependent manner in small intestine. Therefore, the current study is the first to identify a role for Nrf2 in co-regulation of BMP and Tgfβ pathways in BHA-elicited chemopreventive effects *in vivo* which may be, in part, due to induction of apoptosis caused by activation of BMP (34).

Interestingly, Bmp6 was suppressed in the liver suggesting a spatial regulation of BHA-regulated chemoprevention via Nrf2 in the liver and small intestine. A recent study (37) reported that BMP-2 modulates the expression of molecules involved in Wnt signaling, and activates the canonical Wnt pathway in normal human keratinocytes. In our study, we also observed an Nrf2-dependent upregulation of wingless-type MMTV integration site 9 B (Wnt9b) along with a down-regulation of skin hornerin (Hrnr) in small intestine, and an upregulation (greater than fivefold) of stratum corneum (epidermis) development genes such as keratin associated protein 6-1 (Krtap6-1) and keratin complex 2, basic, gene 18 (Krt2-18) in liver. Since BMP-2 and Wnt are involved in the development of skin and skin appendages (37) as morphogens, and since Nrf2 has been implicated in hyperproliferation of keratinocytes (38), our results are the first identification of a putative *in vivo* cross-talk regulated by Nrf2 and modulated by BHA between BMP and Wnt pathway members in the etiopathophysiology and chemoprevention of skin cancers. Further studies in an appropriate *in vivo* model as well as *in vitro* mechanistic studies will be necessary to enhance our current understanding of regulation of skin cancer by Nrf2.

We have demonstrated previously (1) *in vitro* that BHA is capable of activating distinct mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated protein kinase 2 (ERK2), and c-Jun N-terminal kinase 1 (JNK1). The current study elucidates the Nrf2-dependent, BHA-modulated regulation *in vivo* of many members of the MAPK family including Mapk6, Mapk8, Map3k9, Map4k4, Map4k5, Map2k7, Mapkap1, and Mapkapk5; as well as the Jun oncogene, thus, validating the physiological relevance of our results. BHA could also alter the expression of many important signaling biomolecules in discrete signal transduction pathways in an Nrf2-dependent manner including those of the JAK/STAT pathway (Janus kinase 2, *Jak2*, and protein inhibitor of activated Stat4, *Pias4*). In mammalian cells, insulin-induced PI3K (phosphoinositide 3-kinase) activation, generates the lipid second messenger PtdIns (P_3), which is thought (39) to play a key role in triggering the activation of p70 ribosomal S6 protein kinase (S6K). The identification in the current study of phosphoinositide-3-kinase, regulatory subunit 5, p101 (Pik3r5), insulin-like growth factor 1 (Igf1), Insulin-like growth factor 2 receptor (Igf2r), and ribosomal protein S6 kinase, polypeptide 5 (Rps6ka5) as Nrf2-dependent and BHA-regulated genes is interesting as this is the first identification of Igf2r as a target of BHA *in vivo*, and may be another putative mechanism by which BHA elicits its Nrf2-mediated chemopreventive effects. We also observed a BHA-elicited, Nrf2-dependent stimulation of diacylglycerol-kinase kappa, and epsilon and eta isoforms of protein kinase C (Prkce and Prkch respectively) which is consistent with reports of PKC-activation by BHA and diacylglycerol (40,41). In addition, G protein-coupled receptor kinase 5 (GRK5) and glycogen synthase kinase 3 beta (Gsk3β), which were down-regulated in small intestine in an Nrf2-dependent manner, were identified for the first time as putative targets for BHA-mediated chemoprevention.

BHA could also modulate the expression of many genes involved in apoptosis and cell cycle control in an Nrf2-dependent manner including cyclin G1 (Ccng1), cyclin T2 (Ccnt2), cyclin I (Ccni), G0/G1 switch gene 2 (G0s2), growth arrest and DNA-damage-inducible 45 alpha and beta (Gadd45a, Gadd45b), CASP8 and FADD-like apoptosis regulator (Cflar), growth arrest specific 1 (Gas1), G1 to S phase transition 1 (Gspt1), breast cancer 1 (Brca1), and p21 (CDKN1A)-activated kinase 3 (Pak3). Several other important categories of genes were identified as Nrf2-dependent and BHA-regulated such as cell adhesion, biosynthesis and metabolism, ubiquitination and proteolysis, RNA/protein processing and nuclear assembly, cell growth and differentiation, DNA replication and G-protein coupled receptors. The current study, thus, addresses the spatial regulation in mouse small intestine and liver of global gene expression profiles elicited by BHA in exerting its chemopreventive effects via Nrf2. Since a greater number of genes in this study were altered in the small intestine as compared to the liver, and since the phenolic compound BHA (Fig. 4) is a lipophilic molecule with a KowWin (http://www.syrres.com/Esc/est_kowdemo.htm) estimated log octanol/water partition coefficient (log P) as high as 3.50, the spatial regulation of gene expression profiles may be attributed to a complex of physiological factors including partitioning across the gastrointestinal tract, intestinal transit time, uptake into the hepatobiliary circulation, exposure parameters such as Cmax, Tmax and AUC, and pharmacokinetics of disposition after oral administration of BHA. Further studies will be necessary to address the effect(s) of temporal dependence on pharmacokinetic parameters and gene expression profiles to further enhance our current understanding of BHA-mediated chemoprevention mechanisms.

In conclusion, our microarray expression profiling study provides some novel insights into the pharmacogenomics and spatial regulation of global gene expression profiles elicited in the mouse small intestine and liver by BHA in an Nrf2-dependent manner from a gestalt biological perspective. Amongst these BHA-regulated genes, clusters of Nrf2-dependent genes were identified by comparing gene expression profiles between C57BL/6J Nrf2(+/+) and C57BL/6J/Nrf2(−/−) mice. The identification of novel molecular targets that are regulated by BHA via Nrf2 underscores the ineluctable importance of the Nrf2/ARE signaling pathway in cancer chemoprevention. This study clearly extends the current latitude of thought on the molecular mechanisms underlying BHA's cancer chemopreventive effects as well as

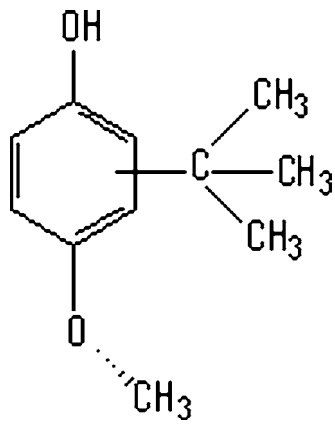

Fig. 4. Chemical structure of butylated hydroxyanisole (*BHA*).

the role(s) of Nrf2 in its biological functions. Future *in vivo* and *in vitro* mechanistic studies exploring the germane molecular targets or signaling pathways as well as Nrf2-dependent genes related to the significant functional categories uncovered in the current study would inexorably extend our current understanding of cancer chemoprevention.

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REFERENCES

1. R. Yu, T. H. Tan, and A. N. T. Kong. Butylated hydroxyanisole and its metabolite *tert*-butylhydroquinone differentially regulate mitogen-activated protein kinases. The role of oxidative stress in the activation of mitogen-activated protein kinases by phenolic antioxidants. *J. Biol. Chem.* **272**:28962–28970 (1997).
2. M. Saito, H. Sakagami, and S. Fujisawa. Cytotoxicity and apoptosis induction by butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). *Anticancer Res.* **23**:4693–4701 (2003).
3. N. Festjens, M. Kalai, J. Smet, A. Meeus, R. Van Coster, X. Saelens, and P. Vandenabeele. Butylated hydroxyanisole is more than a reactive oxygen species scavenger. *Cell Death Differ.* **13**:166–169 (2006).
4. M. Kalai, G. Van Loo, T. Vanden Berghe, A. Meeus, W. Burm, X. Saelens, and P. Vandenabeele. Tipping the balance between necrosis and apoptosis in human and murine cells treated with interferon and dsRNA. *Cell Death Differ.* **9**:981–984 (2002).
5. J. D. Hayes, D. J. Pulford, E. M. Ellis, R. McLeod, R. F. James, J. Seidegard, E. Mosialou, B. Jernstrom, and G. E. Neal. Regulation of rat glutathione S-transferase A5 by cancer chemopreventive agents: mechanisms of inducible resistance to aflatoxin B1. *Chem. Biol. Interact.* **111–112**:51–67 (1998).
6. D. L. McCormick, N. Major, and R. C. Moon. Inhibition of 7,12-dimethylbenz(a)anthracene-induced rat mammary carcinogenesis by concomitant or postcarcinogen antioxidant exposure. *Cancer Res.* **44**:2858–2863 (1984).
7. F. E. Jones, R. A. Komorowski, and R. E. Condon. The effects of ascorbic acid and butylated hydroxyanisole in the chemoprevention of 1,2-dimethylhydrazine-induced large bowel neoplasms. *J. Surg. Oncol.* **25**:54–60 (1984).
8. G. M. Williams, M. J. Iatropoulos, and A. M. Jeffrey. Anticarcinogenicity of monocyclic phenolic compounds. *Eur. J. Cancer Prev.* **11**(Suppl 2):S101–S107 (2002).
9. R. Yu, S. Mandlekar, and A. N. T. Kong. Molecular mechanisms of butylated hydroxylanisole-induced toxicity: induction of apoptosis through direct release of cytochrome c. *Mol. Pharmacol.* **58**:431–437 (2000).
10. J. Alam, D. Stewart, C. Touchard, S. Boinapally, A. M. Choi, and J. L. Cook. Nrf2, a Cap'n'collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J. Biol. Chem.* **274**:26071–26078 (1999).
11. M. McMahon, K. Itoh, M. Yamamoto, A. Chanas, C. J. Henderson, L. I. McLellan, C. R. Wolf, C. Cavin, and J. D. Hayes. The Cap'n'Collar basic leucine zipper transcription factor Nrf2 (NF-E2 p45-related factor 2) controls both constitutive and inducible expression of intestinal detoxification and glutathione biosynthetic enzymes. *Cancer Res.* **61**:3299–3307 (2001).
12. R. K. Thimmulappa, K. H. Mai, S. Srisuma, T. W. Kensler, M. Yamamoto, and S. Biswal. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res.* **62**:5196–5203 (2002).
13. H. J. Prochaska, M. J. De Long, and P. Talalay. On the mechanisms of induction of cancer-protective enzymes: a unifying proposal. *Proc. Natl. Acad. Sci. USA* **82**:8232–8236 (1985).
14. W. Li, M. R. Jain, C. Chen, X. Yue, V. Hebbar, R. Zhou, and A. N. Kong. Nrf2 Possesses a redox-insensitive nuclear export signal overlapping with the leucine zipper motif. *J. Biol. Chem.* **280**:28430–28438 (2005).
15. G. Shen, V. Hebbar, S. Nair, C. Xu, W. Li, W. Lin, Y. S. Keum, J. Han, M. A. Gallo, and A. N. Kong. Regulation of Nrf2 transactivation domain activity. The differential effects of mitogen-activated protein kinase cascades and synergistic stimulatory effect of Raf and CREB-binding protein. *J. Biol. Chem.* **279**:23052–23060 (2004).
16. Y. S. Keum, E. D. Owuor, B. R. Kim, R. Hu, and A. N. Kong. Involvement of Nrf2 and JNK1 in the activation of antioxidant responsive element (ARE) by chemopreventive agent phenethyl isothiocyanate (PEITC). *Pharm. Res.* **20**:1351–1356 (2003).
17. C. Chenand and A. N. Kong. Dietary chemopreventive compounds and ARE/EpRE signaling. *Free Radic. Biol. Med.* **36**:1505–1516 (2004).
18. K. Itoh, N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J. D. Engel, and M. Yamamoto. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* **13**:76–86 (1999).
19. S. Dhakshinamoorthy and A. K. Jaiswal. Functional characterization and role of INrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H: quinone oxidoreductase1 gene. *Oncogene* **20**:3906–3917 (2001).
20. N. Wakabayashi, A. T. Dinkova-Kostova, W. D. Holtzclaw, M. I. Kang, A. Kobayashi, M. Yamamoto, T. W. Kensler, and P. Talalay. Protection against electrophile and oxidant stress by induction of the phase 2 response: fate of cysteines of the Keap1 sensor modified by inducers. *Proc. Natl. Acad. Sci. USA* **101**:2040–2045 (2004).
21. X. Yu and T. Kensler. Nrf2 as a target for cancer chemoprevention. *Mutat. Res.* **591**:93–102 (2005).
22. S. Dhakshinamoorthy and A. K. Jaiswal. Small maf (MafG and MafK) proteins negatively regulate antioxidant response element-mediated expression and antioxidant induction of the NAD(P)H:Quinone oxidoreductase1 gene. *J. Biol. Chem.* **275**: 40134–40141 (2000).
23. A. N. Kong, R. Yu, W. Lei, S. Mandlekar, T. H. Tan, and D. S. Ucker. Differential activation of MAPK and ICE/Ced-3 protease in chemical-induced apoptosis. The role of oxidative stress in the regulation of mitogen-activated protein kinases (MAPKs) leading to gene expression and survival or activation of caspases leading to apoptosis. *Restor. Neurol. Neurosci.* **12**:63–70 (1998).
24. K. Chan, R. Lu, J. C. Chang, and Y. W. Kan. NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proc. Natl. Acad. Sci. USA* **93**:13943–13948 (1996).
25. G. Shen, C. Xu, R. Hu, M. R. Jain, S. Nair, W. Lin, C. S. Yang, J. Y. Chan, and A. N. Kong. Comparison of (−)-epigallocatechin-3-gallate elicited liver and small intestine gene expression profiles between C57BL/6J mice and C57BL/6J/Nrf2 (−/−) mice. *Pharm. Res.* **22**:1805–1820 (2005).
26. G. Shen, C. Xu, R. Hu, M. R. Jain, A. Gopalkrishnan, S. Nair, M. T. Huang, J. Y. Chan, and A. N. Kong. Modulation of nuclear factor E2-related factor 2-mediated gene expression in mice liver and small intestine by cancer chemopreventive agent curcumin. *Mol. Cancer Ther.* **5**:39–51 (2006).
27. W. Lin, G. Shen, X. Yuan, M. R. Jain, S. Yu, A. Zhang, J. D. Chen, and A. N. Kong. Regulation of Nrf2 Transactivation Domain Activity by p160 RAC3/SRC3 and Other Nuclear Co-Regulators. *J. Biochem. Mol. Biol.* **39**(3):304–310.
28. K. I. Borroz, T. M. Buetler, and D. L. Eaton. Modulation of gamma-glutamylcysteine synthetase large subunit mRNA expression by butylated hydroxyanisole. *Toxicol. Appl. Pharmacol.* **126**:150–155 (1994).

29. T. M. Buetler, E. P. Gallagher, C. Wang, D. L. Stahl, J. D. Hayes, and D. L. Eaton. Induction of phase I and phase II drug-metabolizing enzyme mRNA, protein, and activity by BHA, ethoxyquin, and oltipraz. *Toxicol. Appl. Pharmacol* **135**:45–57 (1995).
30. L. I. McLellan, D. J. Harrison, and J. D. Hayes. Modulation of glutathione S-transferases and glutathione peroxidase by the anticarcinogen butylated hydroxyanisole in murine extrahepatic organs. *Carcinogenesis* **13**:2255–2261 (1992).
31. S. A. Chanas, Q. Jiang, M. McMahon, G. K. McWalter, L. I. McLellan, C. R. Elcombe, C. J. Henderson, C. R. Wolf, G. J. Moffat, K. Itoh, M. Yamamoto, and J. D. Hayes. Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase Gsta1, Gsta2, Gstm1, Gstm2, Gstm3 and Gstm4 genes in the livers of male and female mice. *Biochem. J.* **365**:405–416 (2002).
32. A. R. Tanaka, K. Tanabe, M. Morita, M. Kurisu, Y. Kasiwayama, M. Matsuo, N. Kioka, T. Amachi, T. Imanaka, and K. Ueda. ATP binding/hydrolysis by and phosphorylation of peroxisomal ATP-binding cassette proteins PMP70 (ABCD3) and adrenoleukodystrophy protein (ABCD1). *J. Biol. Chem.* **277**:40142–40147 (2002).
33. A. S. Verkman. More than just water channels: unexpected cellular roles of aquaporins. *J. Cell Sci.* **118**:3225–3232 (2005).
34. Y. Shi and J. Massague. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**:685–700 (2003).
35. R. Vittal, Z. E. Selvanayagam, Y. Sun, J. Hong, F. Liu, K. V. Chin, and C. S. Yang. Gene expression changes induced by green tea polyphenol (−)-epigallocatechin-3-gallate in human bronchial epithelial 21BES cells analyzed by DNA microarray. *Mol. Cancer Ther.* **3**:1091–1099 (2004).
36. G. R. Beck Jr., B. Zerler, and E. Moran. Gene array analysis of osteoblast differentiation. *Cell Growth Differ.* **12**:61–83 (2001).
37. L. Yang, K. Yamasaki, Y. Shirakata, X. Dai, S. Tokumaru, Y. Yahata, M. Tohyama, Y. Hanakawa, K. Sayama, and K. Hashimoto. Bone morphogenetic protein-2 modulates Wnt and frizzled expression and enhances the canonical pathway of Wnt signaling in normal keratinocytes. *J. Dermatol. Sci.* (2006).
38. H. Motohashi, F. Katsuoka, J. D. Engel, and M. Yamamoto. Small Maf proteins serve as transcriptional cofactors for keratinocyte differentiation in the Keap1-Nrf2 regulatory pathway. *Proc. Natl. Acad. Sci. USA* **101**:6379–6384 (2004).
39. J. M. Lizcano, S. Alrubaie, A. Kieloch, M. Deak, S. J. Leevers, and D. R. Alessi. Insulin-induced Drosophila S6 kinase activation requires phosphoinositide 3-kinase and protein kinase B. *Biochem. J.* **374**:297–306 (2003).
40. J. Dornand, C. Sekkat, J. C. Mani, and M. Gerber. Lipoxygenase inhibitors suppress IL-2 synthesis: relationship with rise of [Ca++]i and the events dependent on protein kinase C activation. *Immunol. Lett.* **16**:101–106 (1987).
41. M. Ruzzene, A. Donella-Deana, A. Alexandre, M. A. Francesconi, and R. Deana. The antioxidant butylated hydroxytoluene stimulates platelet protein kinase C and inhibits subsequent protein phosphorylation induced by thrombin. *Biochim. Biophys. Acta* **1094**:121–129 (1991).