Nrf2-Keap1 Signaling Pathway Regulates Human UGT1A1 Expression in Vitro and in Transgenic UGT1 Mice*

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The formation of β-D-glucopyranosides (glucuronides) by the UDP-glucuronosyltransferases (UGTs) is a significant metabolic pathway that facilitates the elimination of small hydrophobic molecules such as drugs, dietary constituents, steroids, and bile acids. We elucidate here that an anti-oxidative response leads to induction of UGT1A1 through the Nrf2-Keap1 pathway. When human HepG2 cells were treated with the prooxidants tert-butylhydroquinone and β-naphthoflavone, cellular UGT1A1 glucuronidation activities were increased. The induction of UGT1A1 proceeded following the overexpression of Nrf2 and was blocked following overexpression of Keap1, demonstrating that Keap1 suppresses Nrf2 activation of the UGT1A1 gene. Loss of function analysis for Nrf2 conducted by small interfering RNA revealed that induction of UGT1A1 was not seen in Nrf2 knock-out cells. To examine the contribution of oxidants toward the regulation of human UGT1A1 in vivo, transgenic mice bearing the human UGT1 locus (Tg-UGT1) were treated with tert-butylhydroquinone. Human UGT1A1 was markedly increased in small and large intestines as well as in liver. Gene mapping experiments including transfections of UGT1A1 reporter gene constructs into HepG2 cells coupled with functional analysis of Nrf2 expression and binding to anti-oxidant-response elements (ARE) resulted in identification of an ARE in the phenobarbital-response enhancer module region of the UGT1A1 gene. The ARE flanks the recently identified Ah receptor xenobiocresponsive element. The results suggest that Nrf2-Keap1-dependent UGT1A1 induction by prooxidants might represent a key adaptive response to cellular oxidative stress that defends against a variety of environmental insults, including electrophile attacks and chemical carcinogenesis.

UDP-glucuronosyltransferases (UGTs)2 generate β-D-glucopyranosidic acids (glucuronides) utilizing bile acids, steroids, phenolic neurotransmitters, bilirubin, and a host of xenobiotics, including dietary substances, therapeutics, and environmental compounds (1). Glucuronide formation plays a central role in cellular protection by transforming the substrates into more water-soluble forms, thus facilitating the excretion of the metabolites into the urine and bile. This highly efficient detoxification process is carried out by 19 functional UGTs in humans, 9 of which are encoded by the UGT1 locus on chromosome 2 and the remainder by individual UGT2 genes on chromosome 4 (2). The expression of these genes in human tissue is highly organized, with each tissue expressing its own unique complement of the individual UGTs (1, 3). We have recently demonstrated through the use of transgenic mice that express the human UGT1 locus (Tg-UGT1) that the pattern of expression of the UGT1A genes in mouse tissues is comparable with what has been found for expression of the locus in human tissues (4). Based upon clinical studies and experiments conducted in transgenic mouse models, liver and gastrointestinal tract are major organs where the UGT genes are expressed (4).

Previous studies have shown that regulation of UGT expression is targeted by a number of xenobiotic and steroid receptors in response to xenobiotics, carcinogens, stress signals, and hormones. The xenobiotic receptors pregnane X receptor (5), the constitutive androstane receptor (6), the peroxisome proliferator-activated receptors (7, 8), the liver X receptor (9) as well as the Ah receptor (10, 11) have been shown in tissue culture and Tg-UGT1 mice studies to regulate expression of the UGT genes (4). Recently, the transcription factor Nrf2 has been identified as the major regulator of cytoprotective genes encoding detoxification and antioxidant enzymes (12, 13). The induction of proteins following exposure to electrophiles and oxidants has been termed the antioxidant response and has been linked to Nrf2 activation (14). The induction of UGT activity in mice treated with natural or synthetic chemopreventive agents represents an initial indication that UGT activity may be among the cytoprotective proteins induced by this signaling pathway (15). This has been supported in Nrf2 knock-out mice where treatment with the antioxidant tBHQ led to a reduction in glucuronidation capacity when compared with wild type mice (16), indicating a physiological role for Nrf2 in the regulation of UGT expression.

The transcriptional factor Nrf2 is a 66-kDa ubiquitous protein (17) that belongs to the small family of basic leucine zipper transcription factors. It binds to an AP1-NF-E2 tandem repeat sequence, originally identified in regulating globin gene expression in hematopoietic cells (17). The oxidative stress signals are conveyed to Nrf2 through the actin-associated kelch-domain
protein called Keap1, which acts as an upstream regulator of Nrf2 (18). Keap1 has been shown to bind to both Nrf2 and the actin cytoskeleton to retain Nrf2 in the cytoplasm. Under basal conditions, Nrf2 is sequestered in the cytoplasm by the Keap1 protein, which negatively regulates Nrf2 by facilitating its proteasomal degradation (19). When oxidative or xenobiotic stimuli occur, Nrf2 and Keap1 dissociate, and Nrf2 migrates to the nucleus where it functions as a strong transcriptional activator of selected target genes (20). Therefore, agents that inhibit the interaction between Keap1 and Nrf2 lead to an accumulation of the Nrf2 transcription factor in the nucleus followed by enhanced expression of target genes (16, 21, 22).

In tissue culture and Tg-UGT1A1 mice, UGT1A1 has been shown to be induced by activators of the Ah receptor, constitutive androstane receptor, pregnane X receptor, and the glucocorticoid receptor (4, 11, 23, 24). The DNA binding domain of the UGT1A1 promoter for all of these activated transcriptional factors resides approximately 3300 bp from the start of transcription within a 300-bp region identified as the phenobarbital-responsive enhancer module. In this study, we demonstrate that UGT1A1 is regulated in response to oxidative stress and that the Nrf2-Keap1 complex has a central role in this process.

**EXPERIMENTAL PROCEDURES**

Reagents—tert-Butylhydroquinone (tBHQ), β-naphthoflavone (BNF), and hydroperoxide were purchased from Sigma. l-Sulforaphane (SULF) was purchased from LKT Laboratories (St. Paul, MN). Restriction enzymes and T4 DNA ligase for subcloning were from New England Biolabs (Boston, MA). Omniscript reverse transcriptase and Hot Start Mastermix were purchased from Qiagen (Valencia, CA). The Bradford assay for protein concentration analysis was purchased from Bio-Rad. Genescreen membrane for Northern blot analysis was obtained from PerkinElmer Life Sciences. DNA Taq polymerase, the dual luciferase reporter assay system and reporter plasmids, pGL3-basic vector, pGL3 promoter vector, and pRL-SV40 vector were from Promega (Madison, WI). Expression vectors for Nrf2 (pcDNA3-mNrf2) and Keap1 (pcDNA3-mKeap1) were the kind gift provided by Dr. Yamamoto at the University of Tsukuba, Japan. Lipofectamine 2000, Opti-MEM for transfection, pcDNA3 vectors, TRIZol for RNA extractions, and medium for cell culture were from Invitrogen. Radionucleotides [γ-32P]ATP (3000 Ci/ml/mmol) and [α-32P]dCTP (3000 Ci/mmol) were purchased from Amersham Biosciences, and uridine diphospho-[U-14C]glucuronic acid (225 mCi/ml/mmol ammonium salt) was obtained from ICN Radiochemicals (Irvine, CA). Thin layer chromatography plates for enzyme analysis were from Whatman. Custom oligonucleotides used in PCR cloning, subcloning, DNA sequencing, and gel shift assays were purchased from Value Gene (San Diego, CA) or Genbase (San Diego, CA). Sequence service was provided by CFAR at the University of California, San Diego. The preparation of polyclonal anti sera recognizing residues 29–159 of the human UGT1A1 protein has been described (25). The antibodies for Western blot analyses of Keap1, Keap1 (N-19), and Nrf2, Nrf2 (H-300), were purchased from Santa Cruz Biotechnology. Real time quantitative PCR was performed using the QuantiTect™ SYBR Green PCR kit (Qiagen, Valencia, CA), and the reactions were run in an MX400 Multiplex QPCR machine (Stratagene, La Jolla, CA).

**Tissue Culture**—Human hepatoma-derived HepG2 cells, obtained from American Type Culture Collection (Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Chemicals for HepG2 treatments were dissolved in dimethyl sulfoxide (Me2SO) with Me2SO concentrations in media never exceeding 0.1% (v/v). For transient transfection experiments, 2 × 10^5 cells/well were seeded on 12-well plates a day before transfection, with transfections carried out using Lipofectamine 2000 according to the manufacturer’s instructions.

**Glucuronidation Activity by TLC Assay**—UDP-glucuronosyltransferase activities were determined using 1-naphthol and 17α-ethynylestradiol as substrates by TLC assay according to the method of Bansal and Gessner (26) with modifications. Briefly, HepG2 cell lysates were prepared by washing and scraping with ice-cold phosphate-buffered saline after 24–72 h of treatment. Cell pellets were then homogenized in a 5-fold volume of 50 mM Tris-HCl, pH 7.6, and 10 mM MgCl2. Each UGT assay was run in a total volume of 100 µl and contained 50 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 100 µM substrate, 500 µM uridine 5-diphosphoglucuronic acid, 0.04 µCi of UDP[14C]glucuronic acid, 8.5 mM saccharolactone, and 100 µg of total cell extract. The reactions were performed at 37 °C in a shaking water bath for 60 min. At the end of incubation, 100 µl of cold ethanol was added, and the resulting cell debris was removed by centrifugation. The remaining supernatant was applied to TLC plates, and chromatography performed in a mixture of (35:35:10:20 v/v) of n-butanol:acetone:acetic acid:water. The location of the glucuronides in the TLC plates was visualized with a Storm 820 PhosphorImager (Amersham Biosciences), and resident glucuronides were removed and placed in scintillation fluid for quantification with a liquid scintillation counter (Beckman Instruments, Palo Alto, CA).

**Quantitation of UGT1A1, Nrf2, Keap1, and NQO1 Gene Transcripts**—Total RNA was isolated from treated HepG2, mouse primary hepatocytes, or mouse tissues using TRIzol as recommended by the supplier. Northern blot analysis was conducted by applying RNA on 1% formaldehyde-agarose gels followed by electrophoresis with the resolved RNA being transferred to Genescreen membrane by capillary diffusion. An oligonucleotide probe specific for UGT1A1 (34-mers: 5′-aaactaggttggactagactaaagactcttt) was 5′-end-labeled using γ-32P]ATP in the presence of polynucleotide kinase. Following labeling, the probe was purified by filtering through MicroSpin G-25 columns (Amersham Biosciences). The RNA blot was prehybridized with ULTRAhyb-Oligo hybridization buffer (Ambion) at 42 °C for 1 h, followed by hybridization with the radioisotope-labeled oligonucleotide probe at 42 °C overnight. The membrane was then washed twice in 0.5% SDS, 2× SSC for 30 min, dried, and exposed to x-ray film. For detection of gene transcripts by RT-PCR, total RNA (2 µg) was used for the generation of cDNA with Omniscript reverse transcriptase in a 20-µl reaction according to the manufacturer’s protocol. Following cDNA synthesis, 2 µl of the cDNA reaction was used in 20 µl of PCR containing 0.4 µM UGT1A1-specific primer pair.
Prooxidants Induce UGT1A1 Both in Vitro and in Vivo

(27), 0.4 μm mouse or human β-actin primers, and 10 μl of Hotstart Mastermix. For Nqo1 gene expression analysis, the following primers were used: forward, 5'-atggtgcaagagtcatgtgc-3', and reverse, 5'-ttttctgattgtctggctactg-3'. The mouse (mouse forward, 5'-atggcactcgcctcttc, and reverse, 5'-gggtcatgggtgaccacc) or human (27) β-actin RNA was amplified with sequence-specific primers and used as an internal control gene. Real time PCRs were conducted with a QuantiTect SYBR Green PCR kit (Qiagen) by the MX4000 Multiplex QPCR as described previously (4). Briefly, a 2-μl reaction of cDNA synthesis with total RNA by reverse transcriptase was used in a real time PCR. The polymerase was activated at 95 °C for 10 min followed by 40 cycles of amplification that consisted of the following: 95 °C for 30 s and variable annealing temperature, ranging from 57 to 63 °C and 1 min followed by 72 °C for 45 s. Amplification was followed by DNA denaturation at 95 °C for 1 min and a 41-cycle dissociation curve starting at 55 °C and ramping 1 °C every 30 s. Three fluorescent data points at the end of each annealing plateau were taken, and each sample was performed in triplicate. The dissociate curve was performed, and the quantity for each sample was derived from ΔΔCt = Ct(target gene) - Ct(β-actin reference). The data were log-transformed, and the difference of each sample relative to their concomitant Me2SO control sample was calculated. For the detection of human UGT1A1 RNA, the forward primer was 5′-acagagctatcatgcatcgc-3′ and the reverse primer was 5′-tccgctgaaggtggcc. For quantitation of RNA levels of Nrf2 and Keap1, the primers were as follows: Nrf2 forward, 5′-tgccctgaaggtgcaaca, and Nrf2 reverse, 5′-ccagggaggtaggtaatgatt; Keap1 forward, 5′-ctcactcctaaagctggata, and Keap1 reverse, gacagttgaagattcgatggtcg. For quantitation of RNA levels of Nrf2 or Keap1, the primers were as follows: Nrf2 forward, 5′-ttttctgattgtctggctactg-3′, and reverse, 5′-ttttctgattgtctggctactg-3′. For quantitation of RNA levels of human UGT1A1, the forward primer was 5′-ttttctgattgtctggctactg-3′, and the reverse primer was 5′-ttttctgattgtctggctactg-3′. For quantitation of RNA levels of human Nqo1, the forward primer was 5′-atggtgcaagagtcatgtgc-3′, and reverse, 5′-ttttctgattgtctggctactg-3′. For quantitation of RNA levels of human Nqo1, the forward primer was 5′-atggtgcaagagtcatgtgc-3′, and reverse, 5′-ttttctgattgtctggctactg-3′.

Protein Preparation and Immunoblot Analysis—Following exposure by chemical treatment, HepG2 cells were harvested by washing with cold phosphate-buffered saline. Cell lysates were prepared for detection of Nrf2-Keap1 expression by dis- solving cell pellets in 1x RIPA lysis buffer (Upstate Biotechnol- ogy, Inc., Lake Placid, NY) on ice for 30 min, and the supernatant was collected by centrifuging at 14,000 rpm for 20 min. Microsomal proteins were isolated by homogenizing cell sus- pensions in 4 volumes of ice-cold 1.15% KCl solution 20 times. The microsomal pellet was resuspended in buffer containing 10% glycerol, 100 mM phosphate-buffered saline, 1 mM EDTA, 20 μM butylated hydroxytoluene, and 1 mM phenylmethylsulfonyl fluoride. Protein concentra- tions were determined and 20 μg heated at 70 °C in loading buffer. Electrophoresis was conducted at 200 V for 50 min. The resolved protein was transferred onto a nitrocellulose membrane by using an electrotransfer apparatus (Invitrogen) at 30 V for 60 min. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline solution (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h. The membrane was then incubated with a specific primary antibody prepared in Tris-buffered saline with 5% bovine serum albumin (Sigma) overnight at 4 °C. The membrane was washed for 30 min in Tris-buffered saline solution followed by incubation with horseradish peroxidase-conjugated secondary antibody (Cell Signaling) for 1 h at room temperature. Protein was visualized using Renaissance Western blot chemiluminescence reagent (PerkinElmer Life Sciences) followed by exposure to the x-ray film.

Nrf2 Gene Knockdown by Short Hairpin RNA—To decipher the role of Nrf2 for up-regulation of UGT1A1, Nrf2 siRNAs were generated to silence Nrf2 at the post-transcriptional level (28). The Nrf2 siRNAs were a generous gift from Dr. Karin (University of California, San Diego). Preparation of recombinant lentivirus was carried out by transient transfections with a packaging cell line. Brieﬂy, human 293T cells were cotrans- fected with packaging plasmids together with a lentivirus expression plasmid containing siRNA insertion by using Lipo- fectamine 2000. Infectious recombinant lentviruses were har- vested at 72 h post-transfection and ﬁltered through a 0.45-μm syringe ﬁlter to get rid of cell debris. HepG2 cells were plated in 6-well plates, and recombinant lentviruses were directly used in infection of cells with Polybrene for 3 h. The culture medium was changed after infection. Western blot analysis was per- formed to monitor Nrf2 gene knockdown. RNA analysis was performed for UGT1A1 expression in response to tBHQ treatment.

Studies with Transgenic UGT1A (Tg-UGT1) Mouse—Generation of the Tg-UGT1 mouse is described previously (4). Wild type or Tg-UGT1 mice (n = 3) were treated by intraportal injection every 24 h with either Me2SO or tBHQ (40 mg/kg). After 48 h, the tissues, including liver, lung, kidney, and small and large intestines, from each treatment group were combined and pulverized in liquid nitrogen and used for preparation of microsomes and total RNA. Microsomal fractions were pre- pared as described previously (4) for quantitation of UGT1A1 protein with Western blot analysis. Total RNA for Northern blot analysis and RT-PCR was isolated using TRIzol.

Isolation of Primary Hepatocytes from Tg-UGT1 Mice—Primary hepatocytes were isolated from both wild type and Tg- UGT1 mice. Mice were anesthetized by isoflurane inhalation. The needle containing the catheter was inserted slowly into the portal vein, and the tubing was placed into the catheter to allow Hanks’ balanced salt solution (without Ca2+/Mg2+) containing 0.2 mM EGTA and 10 mM Hepes, pH 7.4, to ﬂow through the liver for 2 min followed by a perfusion with 40 ml of Hanks’ balanced salt solution (with Ca2+/Mg2+) containing 35 μg/μl Liberase Blendzyme and 10 mM Hepes, pH 7.4, at 4 ml/min. The liver was dissected into a Petri dish and washed with Dulbecco’s modiﬁed Eagle’s medium. The hepatocytes were ﬁltered through a sterile 70-μm ﬁlter and washed twice by centrifugation at 50 × g for 5 min. The cells were cultured in 6-well plates with Dulbecco’s modiﬁed Eagle’s medium containing 10% fetal bovine serum. The medium was replaced by fresh medium to eliminate dead cells 4 h after plating. The hepatocytes were treated with 100 μM tBHQ for 48 h after seeding for further studies.

Identification of the Response Element in the UGT1A1 Promoter Region—A 11-kb region of the UGT1A1 promoter region was ampliﬁed by PCR from a human BAC library, as described previously (11), and subcloned into either PGL basic vector or PGL promoter vectors. For transient transfections, HepG2 cells
were seeded in a 12-well plate (2 × 10^5 cells/well) 1 day before transfections and grown overnight to 50% confluence. Luciferase reporter constructs containing the UGT1A1 promoter (UGT1A1 −2512/−7), UGT1A1 enhancers, or 5′- or 3′-deletion mutants were mixed with pRL-SV40 DNA as an internal control for DNA transfection efficiency. Using DNA fragments spanning −3430 to −3201 (forward, 5′-aaattagcttacaaggttctaaa; reverse, 5′-tttaactccgagaggctctggatcc), site-directed mutagenesis was carried out, altering two bases (underlined) on the ARE core sequences (ARE-1 mutant, forward, 5′-ctcattgcg). Transient transfections were carried out by Lipofectamine 2000 based on the manufacturer’s instructions. Following 48 h of treatment with tBHQ or Me2SO, cells were harvested with lysis buffer (Promega), and the supernatant was collected by brief centrifugation. Transcription activity was determined by the expression of firefly luciferase and was normalized to the Renilla luciferase levels by using a dual luciferase reporter assay kit (Promega).

**Electrophoretic Mobility Shift Assay (EMSA)**—Nuclear extract preparation and mobility shift assays were performed as described previously (29), with the exception that all buffers contained the protease inhibitors aprotinin (10 μg/ml), leupeptin (10 μg/ml), and phenylmethylsulfonyl fluoride (200 μM). Nuclear proteins were isolated from Me2SO- and tBHQ-treated HepG2 cells. The cell pellet was washed three times by resuspending in 25 mM Hepes, pH 7.5, 100 mM KCl, 3 mM MgCl2 and centrifuging at 5,000 rpm at 4 °C for 5 min. Preparation of nuclear protein was carried out by resuspending the cells in 25 mM Hepes, pH 7.5, containing 400 mM KCl, and incubating on ice for 30 min. Nuclei were pelleted for 15 min at 14,000 rpm at 4 °C, and the supernatant was adjusted to a glyc erol concentration of 10%. This solution was then centrifuged at 50,000 rpm for 1 h at 4 °C, and the nuclear protein was aliquoted and stored at −80 °C until further use. Complementary pairs of oligonucleotides containing the core sequence of the UGT1A1 ARE were synthesized (sense, 5′-gatcaaccgccgaagcttacacgacg-3′, and antisense, 5′-gatccgcttacaaggttctaaa; reverse, 5′-aaaactcgagccattctggatcc), site-directed mutagenesis was carried out, altering two bases (underlined) on the ARE core sequences (ARE-1 mutant, forward, 5′-ctcattgcg). Transient transfections were carried out by Lipofectamine 2000 based on the manufacturer’s instructions. Following 48 h of treatment with tBHQ or Me2SO, cells were harvested with lysis buffer (Promega), and the supernatant was collected by brief centrifugation. Transcription activity was determined by the expression of firefly luciferase and was normalized to the Renilla luciferase levels by using a dual luciferase reporter assay kit (Promega).

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17α-ethynylestradiol UGT activity. The induction of 17α-ethynylestradiol UGT activity by BNF and tBHQ indicated that these chemicals were inducing UGT1A1 in HepG2 cells. A similar induction in 17α-ethynylestradiol UGT activity was also seen in cells exposed to H2O2 (data not shown). Using specific oligonucleotide primers, induced UGT1A1 gene transcripts were detected in response to these chemicals when total cellular RNA was used for reverse transcription followed by PCR for detection of UGT1A1 transcripts (Fig. 1C). The induction of UGT1A1 in HepG2 cells by these diverse prooxidants, which have been shown previously to activate transcription factor Nrf2, implies the involvement of Nrf2 in UGT1A1 up-regulation.

Nrf2- and Keap1-dependent Expression of UGT1A1—Nrf2 has been identified as a target transcriptional factor linked to altered gene expression in response to various oxidants and reactive oxygen species challenges (13, 15, 16). To determine whether induction of UGT1A1 by tBHQ is linked to activation of the Nrf2-Keap1 signaling pathway, we examined the effect of tBHQ on Nrf2. Cell lysates were collected following tBHQ treatment and analyzed for Nrf2 expression by immunoblot analysis. Activation of Nrf2 leads to cytoplasmic stabilization, a result that is directly dependent upon the dissociation in the cytosol of Keap1 (19, 20). As shown in Fig. 2A, basal Nrf2 protein levels were difficult to detect, whereas Nrf2 was induced in a concentration- and time-dependent fashion following exposure to tBHQ. Conversely, Keap1 expression was inhibited as early as 30 min after tBHQ treatment (Fig. 2B), implicating that Nrf2 accumulation results from the targeted knockdown of Keap1 in response to oxidative stress generated by tBHQ. This result is consistent with previous studies showing that Keap1 negatively regulates Nrf2 through rapid degradation of Nrf2, whereas induction of an anti-oxidant response disrupts the coupling of Nrf2 and Keap1 leading to the stabilization of Nrf2 (30).

To ascertain whether Keap1 has a role in UGT1A1 regulation, we examined the expression of UGT1A1 following overexpression of Keap1 (Fig. 3). Nrf2 and Keap1 expression levels were enhanced by transfection of expression plasmids by 2.0- and 35.7-fold, respectively, as detected by real time PCR (Fig. 3A). Following tBHQ treatment of HepG2 cells, UGT1A1 mRNA levels were induced. Constitutively activated Nrf2 following transfection of an Nrf2 expression plasmid led to mild induction of UGT1A1 (Fig. 3B). However, the overexpression of Keap1 repressed the transactivation seen with tBHQ. When HepG2 cells were transfected with a plasmid that expressed Keap1, the treatment of these cells with tBHQ led to complete reduction of UGT1A1 expression when compared with HepG2 cells treated with Me2SO or tBHQ (100 μM) and transfected with Nrf2 or Keap1 expression vectors. Western blots were performed using 25 μg of protein and blotted using an anti-UGT1A1 antibody. C, total RNA extracted from different treatment groups was used for real time RT-PCR analysis of UGT1A1. Ctrl, control.

FIGURE 2. Expression of Nrf2 and Keap1 in response to tBHQ. A and B, HepG2 cells were treated with various concentrations of tBHQ for 4 h or 100 μM tBHQ for various time points as indicated. Cell lysates were collected, and Nrf2 and Keap1 protein levels were analyzed with anti-Nrf2 and anti-Keap1 antibodies by Western blot analysis using 30 μg of total cell lysates.

FIGURE 3. Nrf2- and Keap1-dependent expression of UGT1A1. A, Nrf2 and Keap1 were overexpressed by transient transfection of HepG2 with expression vector pcDNA3-mNrf2 and pcDNA-mKeap1, respectively. The mRNA levels of overexpressed Nrf2 and Keap1 were quantitated by real time PCR using pcDNA3 vector transfection as control. B, effect of overexpression of Nrf2 and Keap1 on UGT1A1 expression was detected by Western blot analysis (bottom panel). HepG2 cells were treated with tBHQ or transfected with expression vectors for Nrf2 and Keap1 proteins. Microsomal fractions were collected from HepG2 cells treated with Me2SO or tBHQ (100 μM) and transfected with Nrf2 or Keap1 expression vectors. Western blots were performed using 25 μg of protein and blotted using an anti-UGT1A1 antibody. C, total RNA extracted from different treatment groups was used for real time RT-PCR analysis of UGT1A1. Ctrl, control.
Nrf2, whereas induction of an anti-oxidant response disrupts the coupling of Nrf2 and Keap1 leading to the stabilization of Nrf2 (30). This implicates an important role for Keap1 in the regulation of UGT1A1 and indicates that the steady accumulation of Nrf2 in response to tBHQ acts as a direct transcriptional regulator of UGT1A1 gene response.

To confirm that Nrf2 activation modulates the expression of UGT1A1, Nrf2 was silenced by siRNA expression (Fig. 4). Two lentiviral hairpin constructs targeting Nrf2 and one control hairpin containing random sequences were subcloned into a lentiviral expression vector. Subconfluent HepG2 cells were infected with control lentivirus or siNrf2-lentivirus. The cells were then cultured for 3 days. After an additional incubation of 4 h with tBHQ (100 μM), cells were harvested and lysed for immunoblot analysis using an anti-Nrf2 antibody as described under “Experimental Procedures.” After 48 h of tBHQ treatment, the cells were harvested, and total RNA was isolated for determination of NQO1 and UGT1A1 mRNA levels by RT-PCR using gene-specific primers. Real time PCR was performed to quantitate UGT1A1 mRNA levels. DMSO, Me2SO.

**FIGURE 4. Lentiviral siRNA-mediated Nrf2 knockdown that blocked tBHQ-directed UGT1A1 induction.** A, HepG2 cells were infected with control lentivirus or siNrf2-lentivirus. The cells were then cultured for 3 days. After an additional incubation of 4 h with tBHQ (100 μM), cells were harvested and lysed for immunoblot analysis using an anti-Nrf2 antibody as described under “Experimental Procedures.” After 48 h of tBHQ treatment, the cells were harvested, and total RNA was isolated for determination of NQO1 and UGT1A1 mRNA levels by RT-PCR using gene-specific primers. B, real time PCR was performed to quantitate UGT1A1 mRNA levels. DMSO, Me2SO.

Assessment of tBHQ-mediated Regulation in Tg-UGT1 Mice—Having demonstrated that tBHQ is a potent inducing agent among a number of oxidants, it was used to determine the regulation of human UGT1A1 in vivo in Tg-UGT1 mice (4). Tg-UGT1 were treated with either tBHQ (40 mg/kg) or Me2SO for 48 h with two intraperitoneal injections every 24 h. Three mice representing each treatment group along with tBHQ-treated wild type mice were treated, and liver, large intestine, small intestine, kidney, and lung microsomes were prepared along with total RNA. To assess the expression of UGT1A1 in microsomal preparation from mouse tissues, Western blot analysis was performed with antibody against human UGT1A1 (25). The polyclonal antibody to UGT1A1 has been shown previously not to recognize mouse UGT protein (4). The constitutive expression of human UGT1A1 was detected in the small intestine by Western blot analysis and was induced in both the small and large intestines by tBHQ treatment (Fig. 5A). The tBHQ-activated UGT1A1 expression appeared to be tissue-specific, since a negligible level was observed in the other tissues (data not shown). Induction of UGT1A1 was also evident when primary hepatocytes isolated from Tg-UGT1 mice were treated with tBHQ (Fig. 5C).

**FIGURE 5. Human UGT1A1 induction in Tg-UGT1 mice following tBHQ treatment.** A, wild type (WT) or Tg-UGT1 mice (n = 3) were treated by intraperitoneal injection every 24 h with either Me2SO (DMSO) or tBHQ (40 mg/kg). After 48 h, the tissues from each treatment group were combined and pulverized. The expression of UGT1A1 protein is shown in small and large intestine. Also shown is expression of UGT1A1 RNA as determined by Northern blot analysis. B, UGT catalytic activities in liver microsomes toward 17α-ethynyl estradiol are compared with levels of UGT1A1 detected in liver microsomes by Western blot analysis. C, hepatocytes from Tg-UGT1 mice were isolated and treated for 24 h with 100 μM tBHQ. Microsomal UGT1A1 levels and Northern blot analysis of UGT1A1 are shown.
Identification of a tBHQ-responsive Region in the UGT1A1 Promoter—The significant induction of UGT1A1 gene transcripts and the contribution of Nrf2 activation following tBHQ treatment would suggest that the UGT1A1 gene is regulated by Nrf2 in response to tBHQ treatment. To determine the ability of the 5′-flanking region of the UGT1A1 gene to confer transcriptional activation in response to tBHQ, DNA fragments that span the entire UGT1A1 promoter were amplified and subcloned into pGL3 reporter plasmids. Transient transfections of HepG2 cells with these reporter constructs were performed followed by tBHQ treatment. As shown in Fig. 6, an enhancer segment of the UGT1A1 gene spanning −3712/−2068 is responsible for tBHQ-mediated induction of the UGT1A1 gene. Further dissection of this fragment identified an enhancer region that spanned bases −3529 to −3143 relative to the transcriptional start site. This formed the basis for additional 5′ mutations that eventually led to a loss of tBHQ induction when the enhancer sequence deletions were performed between −3529 and −3296 (Fig. 6). Nucleotide sequence analysis revealed that there are three imperfect ARE motifs within these 60 nucleotides. To characterize the contribution of each ARE to tBHQ responsiveness, ARE mutant constructs were created by introducing a 2-bp mutation within each ARE on the UGT1A1 promoter template using PCR followed by transient transfection and analysis of luciferase activity. Following tBHQ treatment, induced luciferase activity was compromised significantly when the ARE-1 sites were mutated in comparison with mutation of the ARE-2 and ARE-3 sites. It is noted that this 60-bp fragment also consists of an XRE, an enhancer element responsible for Ah receptor-mediated transcription activation (11). We further examined the involvement of the Ah receptor in tBHQ-mediated transcription activation. A TV101 stable cell line containing the CYP1A1 promoter that harbors multiple XREs was generated previously by stable transfection of HepG2 cells (32) and has been used as a convenient in vitro biomarker for detecting Ah receptor ligands, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). tBHQ was incapable of inducing CYP1A1-luciferase in TV101 cells as shown in Fig. 7A indicating that it is not a candidate as an Ah receptor ligand. In contrast, tBHQ was able to activate the UGT1A1 −3712/−7 promoter-luciferase construct (11) that covers the
tBHQ-responsive region with three imperfect ARE sites and an XRE-response element in MH1A1 cells (11), confirming that transcriptional activation by tBHQ may occur through an Ah receptor-independent mechanism (Fig. 7A). This response is not synergistic when both TCDD and tBHQ are used in combination (data not shown). Based on the core XRE motif located in the UGT1A1 promoter region, the UGT1A1-XRE concatamers containing three replicated XRE sites were synthesized, constructed into pGL3 promoter vector, and transfected into HepG2 cells. When transfected cells were treated with TCDD, luciferase expression was markedly induced. However, tBHQ failed to activate luciferase expression (Fig. 7B). When the XRE site was mutated in the promoter region −3430/−3201, as expected, TCDD lost the ability to induce UGT1A1, whereas tBHQ still conferred significant promoter induction (Fig. 7C). Taken together, the segment between −3354 and −3296 is a cis-regulatory module responsible for tBHQ-mediated transactivation of the UGT1A1 gene via the Ah receptor-independent signaling pathway.

To address whether the ARE sequence found in the UGT1A1 promoter has the ability to bind Nrf2, oligonucleotides were synthesized to the UGT1A1-ARE. The UGT1A1-ARE oligonucleotides do not contain the XRE core sequence; therefore, the possible contribution of the Ah receptor binding would be excluded. When nuclear extracts were prepared from HepG2 treated with tBHQ, EMSAs were performed as described under “Experimental Procedures.” UGT1A1-ARE probes were incubated with nuclear proteins from Me2SO (DMSO) or tBHQ-treated HepG2 cells. For control, a 100-fold molar excess of the respective unlabeled oligonucleotide was added during the binding procedure. For supershift, nuclear proteins were incubated with anti-Nrf2 antibody (sc-722, Santa Cruz Biotechnology).
NQO1-ARE element showed similar results to that of the UGT1A1-ARE (data not shown). Taken together, these studies indicate that the UGT1A1 promoter contains one functional ARE that is responsible for the Nrf2-dependent induction by tBHQ.

**DISCUSSION**

The induction of phase II detoxification and antioxidant enzymes in response to chemical carcinogens, electrophiles, and oxidants has been termed the antioxidant response. The ability of cells to up-regulate the synthesis of these enzymes is an important cellular defense mechanism that is mechanistically distinct from induction of the phase I battery of enzymes. Studies on the Cap and Collar family of transcription factors such as Nrf2 provide compelling evidence that Nrf2 is a master regulator of the antioxidant transcriptional response. Mice that lack the Nrf2 basic region leucine-zipper transcription factor are more susceptible than wild type animals to the cytoxic and genotoxic effects of foreign chemicals and oxidants (33–36). The cellular and molecular mechanisms leading to Nrf2 activation are being elucidated. Several models have implicated a convergence of cellular signaling pathways implicating mitogen-activated protein kinases both in up-regulation and suppression of Nrf2 activation. Recent evidence indicates that phosphorylation of tyrosine 568 on Nrf2 controls nuclear export of Nrf2, where it associates with Keap1 and is eventually targeted for ubiquitin-dependent degradation (37). In the absence of antioxidant response, Keap1 constitutively targets Nrf2, and this complex serves as a biological sensor in the cytosol for oxidative or electrophilic stress. The current thinking is that reactive oxygen species generated during xenobiotic exposure act as SH-modifying electrophiles leading to oxidation of protein thiols in Keap1 (20). However, recent findings have suggested a phosphorylation event perturbs the oligomerization of Nrf2-Keap1, initiating the nuclear uptake of Nrf2 (37–39). Thus, modification of Nrf2 by phosphorylation may underlie the mechanism of antioxidant response leading to activation of AREs.

One of the current paradigms of the antioxidant response is the activation of the family of phase II drug-metabolizing enzymes. Because the role of these proteins favor detoxification of chemicals and xenobiotics, the UGTS have always been implicated as target genes that respond to oxidative and electrophilic stress. However, a direct link for the role of activated Nrf2 in UGT gene activation has never been demonstrated. In this study, we have treated cells with a hydroquinone (tBHQ), hydroperoxide, a dietary disulfide antioxidant (sulfuraphane), and a bifunctional (phase I and phase II) enzyme-inducing agent (BNF). Exposure of HepG2 cells with these agents led to induction of UGT1A1 mRNA levels. These agents have been shown to activate Nrf2 and up-regulate target genes such as NQO1. Several lines of evidence indicate that induction of UGT1A1 was linked to Nrf2 activation. For example, the overexpression of Nrf2 increased the steady-state levels of UGT1A1 gene transcripts. At concentrations of tBHQ that initiated UGT1A1 induction, the overexpression of Keap1 was able to inhibit this response, demonstrating that the antioxidant response is highly dependent on the cellular concentration of Keap1. A direct role implicating Nrf2 in the induction of UGT1A1 was demonstrated in Nrf2 knockdown experiments by siRNA that ablated the effect of tBHQ on UGT1A1 induction. Induction of UGT1A1 by antioxidants resulted directly from binding of Nrf2 to an ARE sequence on the UGT1A1 gene that was located between −3328 and −3323 bases from the transcriptional start site. The results here provide a molecular mechanism for transcriptional activation of UGT1A1 by electrophilic xenobiotics and provide the first direct evidence that Nrf2 targets the family of UGT genes as part of the antioxidant response.

It is noteworthy that the promoter region responsive to tBHQ and Nrf2 contains a functional binding site for the Ah receptor. It adds another layer of complexity in that compounds activating Nrf2 are chemically diverse and overlap those that induce the Ah receptor-dependent pathway. They include oxidants, polycyclic aromatic hydrocarbons, redox-cycling agents, and electrophiles (40, 41). The treatment of mouse hepatic cells with TCDD led to induction of Nqo1, a response that was lost in mouse Nrf2−/− cells (42). This finding indicated the possibility of cross-talk between the Ah receptor and Nrf2 in controlling the anti-oxidant response. Interestingly, when AhR−/− and wild type cells were treated with tBHQ, a complete Nqo1 response was observed, a finding which indicates that hydroquinones elicit transcriptional activation independent of the Ah receptor. When we treated Tg-UGT1 mice with tBHQ, there was no induction of mouse CYP1A1 (data not shown), although we observed significant induction of UGT1A1. Clearly, anti-oxidants are capable of eliciting induction of UGT1A1 independent of those mechanisms that lead to transcriptional activation through the Ah receptor.

The abundant expression of UGT1A1 in liver and the gastrointestinal tract clearly points to an important role for this enzyme in xenobiotic metabolism in these target organs. In Tg-UGT1 mice, the expression of UGT1A1 and the other UGT1A gene products closely resembles the expression pattern that has been documented for most human tissues. An important role for Nrf2 in these tissues has been implicated in these studies by demonstrating that Tg-UGT1 mice exposed to tBHQ leads to prominent induction of UGT1A1 in the gastrointestinal tract. In addition, the results generated in Tg-UGT1 mice support the findings generated in HepG2 cells, indicating that mechanisms outlined for Nrf2-Keap1 regulation can be extrapolated as a potential mode of regulation in vivo. These preliminary findings suggest that Tg-UGT1 mice may be useful in future experiments to examine the influence of dietary constituents as possessing potential antioxidant properties.

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