Induction of Phase I and Phase II Drug-Metabolizing Enzyme mRNA, Protein, and Activity by BHA, Ethoxyquin, and Oltipraz

Timo M. Buettler, Evan P. Gallagher, Changhong Wang, Dana L. Stahl, John D. Hayes, and David L. Eaton

Department of Environmental Health and Institute for Environmental Studies, University of Washington, Seattle, Washington 98195; and Biomedical Research Center, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, United Kingdom

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Various natural and synthetic compounds are known to protect against cancer by elevating phase II detoxification enzymes. Generally classified as monofunctional, these inducers are believed to trigger the expression of genes that activate metabolism through an antioxidant or electrophile response element (ARE/EpRE) in responsive genes. In contrast, the phase I enzymes of drug metabolism (cytochrome P450s) are not believed to be induced by monofunctional inducers and P450 genes have not been found to contain functional ARE/EpREs. In this study, rats were treated with the monofunctional inducers tert-butylation hydroxynisole, ethoxyquin, and oltipraz to study the induction of individual glutathione S-transferase isozymes, NADP(H)quinone oxidoreductase, 7-glutamylcysteine synthetase, UDP-glucuronosyltransferase, and cytochrome P450 enzymes. Hepatic mRNAs were analyzed on Northern blots using gene-specific oligonucleotide probes for GST Ya1, Ya2, Yc1, Yc2, Yb1, Yb2, and Yf, for UGT 1*06, and for P450 1A1, 1A2, 2B1, 2C11, 3A2, and 4A1. NADP(H)quinone oxidoreductase and 7-glutamylcysteine synthetase mRNAs were detected using cDNA probes. All the phase II detoxification enzymes analyzed, except GST Yf, were induced by the three monofunctional inducers, suggesting that these genes may be regulated by a mechanism involving an ARE/EpRE element in their promoter region. Interestingly, it was found that ethoxyquin was particularly a good inducer for both members of the P450 2B family, 2B1 and 2B2, and both ethoxyquin and oltipraz were also capable of modestly inducing P450 1A2 and 3A2. Oltipraz was found to slightly induce P450 2B2, but not 2B1, at the dose and time analyzed. Induction of mRNA generally correlated well with induction of protein levels determined by Western blot and/or enzyme activity measurements for selected enzymes. The results of this study suggest that many phase II enzymes may contain ARE/EpRE elements in addition to those confirmed to be regulated by a mechanism involving ARE/EpRE elements. In addition, it was found that several P450 enzymes were induced by monofunctional inducers, suggesting a possibility that some phase I enzymes may also be regulated by a mechanism involving ARE/EpRE elements.

Several natural and synthetic compounds have protective activity against certain carcinogens. Among the natural compounds with apparent anticarcinogenic activity are diallyl sulfide and related compounds found in garlic (Gudi and Singh, 1991; Lee et al., 1994; Ludeke et al., 1992; Marks et al., 1992), polyphenols from green tea (Agarwal et al., 1992; Bhimani et al., 1993; Huang et al., 1992; Wang et al., 1992), myristicin, a volatile component from parsley leaf (Zheng et al., 1992), and components in cruciferous vegetables such as broccoli and brussels sprouts (Bogaards et al., 1990; Prochaska and Fernandez, 1993; Prochaska et al., 1992; Ramsdell and Eaton, 1988; Zhang et al., 1992). In addition, numerous reports have provided evidence that the synthetic chemicals tert-butylation hydroxyisole (BHA), tert-butylation hydroxytetanolene (BHT), ethoxyquin (EQ), and 1,2-dithiole-3-thiones can protect animals against carcinogenic substances both in vivo and in vitro (Ahmad et al., 1992; Benson, 1993; Buettler and Eaton, 1992b; Ellis et al., 1995).

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2 Current address: University of Kansas Medical Center, Dept. of Pharmacology, Toxicology and Therapeutics, 3901 Rainbow Boulevard, Kansas City, KS 66160-7417.
3 To whom correspondence should be addressed at University of Washington, Dept. of Environmental Health, 4225 Roosevelt Way N.E., No. 100, Seattle, WA 98195-6089.

4 Abbreviations used: 3MC, 3-methylcholanthrene; ADI, Δ5-androstene-3,17-dione; ARE, antioxidant response element; BHA, tert-butylation hydroxyisole; BNF, β-naphthoflavone; BROD, benzo(α)pyrene-7,12-diol-9,10-epoxide; CDNB, 1-chloro-2,4-dinitrobenzene; CHP, cumene hydroperoxide; CF, ciprofibrate; DCNB, 3,4-dichloro-1-nitrobenzene; ECA, ethacrynic acid; EQ, ethoxyquin; EROD, ethoxyresorufin-O-deethylase; GCS, γ-glutamylcysteine synthetase; MRDOD, methylresorufin-O-deethylase; OPZ, oltipraz; PCN, pregnenolone 16α-carbonitrile; QR, NADP(H)quinone oxidoreductase; SOD, superoxide dismutase; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; UGT, UDP-glucuronomyl transferases; XRE, xenobiotic response element.
1993; Hayes et al., 1991b,c, 1993; Kessler et al., 1985, 1992; Mandel et al., 1987; Monroe and Eaton, 1987; Moon et al., 1994; Pett et al., 1991; Rao et al., 1993; Roebuck et al., 1991; Rogers et al., 1990; Verhagen et al., 1991). These compounds have been shown to elevate phase II detoxification enzymes, resulting in increased detoxification of carcinogens and a net decrease in DNA-bound carcinogen. Detailed analysis of the mechanism of elevated enzyme levels has revealed that increased gene transcription can account for most of this increase in enzyme activity (Bergelson et al., 1994; Friling et al., 1992; Nguyen et al., 1994; Rushmore and Pickett, 1990). Phase I enzymes are generally considered to represent an activating system for many natural and synthetic carcinogens while phase II enzymes often, but not always, serve to detoxify harmful natural or activated carcinogens. Thus, inducers that preferentially elevate phase II enzymes are generally considered to be beneficial because they stimulate the detoxification of potentially carcinogenic chemicals. This concept is currently being tested in cancer treatment and prevention strategies using selected monofunctional inducers.

Talalay and co-workers have defined monofunctional inducers as electrophilic compounds that can react with glutathione and selectively induce the phase II detoxification enzymes (Prochaska and Talalay, 1988; Talalay et al., 1988). Bifunctional inducers, on the other hand, are capable of inducing both phase I and phase II enzymes, and include most classical cytochrome P450 inducers.

Pickett and co-workers, along with other investigators, have identified several genetic elements in the promoter regions of phase I and II enzymes that confer responsiveness to distinct groups of inducers (Li and Jaiswal, 1992; Rushmore et al., 1991; Rushmore and Pickett, 1991, 1993). A specific element, termed the “antioxidant response element” (ARE), which is found in the promoter region of the rat GST Ya and QR genes, appears to confer inductibility by the monofunctional phenolic antioxidant and/or their metabolite(s) (Favreau and Pickett, 1991; Li and Jaiswal, 1992; Rushmore et al., 1991; Rushmore and Pickett, 1991, 1993). A similar element, termed the “electrophile response element” (EpRE), has been identified in the mouse GST Ya gene by Daniel and co-workers (Bergelson et al., 1994; Friling et al., 1990, 1992).

A different element, termed the “xenobiotic response element” (XRE) or “dioxin response element” (DRE), identified in CYP 1A genes, was shown to confer inductibility by bifunctional inducers such as 2,3,7,8-tetrachlorodibenzo-\(\alpha\)-dioxin (TCDD), 3MC, and \(\beta\)NF (Rushmore and Pickett, 1991, 1993). A functional XRE was subsequently identified in the promoter region of the GST Ya and QR genes (Favreau and Pickett, 1991; Rushmore and Pickett, 1991, 1993). Inducers such as TCDD, that act via the XRE, appear to be dependent on the presence of a functional XRE-binding arylhydrocarbon (Ah) receptor. In contrast, the bifunctional inducer 5,6-benzoflavone (\(\beta\)NF) is not dependent on the Ah receptor for induction of the GST Ya gene but requires a functional CYP 1A gene product, suggesting that \(\beta\)NF requires metabolism in order to transcriptionally activate the GST Ya gene. The response element for the \(\beta\)NF metabolite in the GST Ya gene has been shown to be the ARE (Rushmore et al., 1991; Rushmore and Pickett, 1993). Recently, another bifunctional inducer, phenobarbital, was shown to induce the mouse GST Ya gene through involvement of the EpRE (Pinkus et al., 1993). Thus, XREs are found in both phase I and phase II enzymes, while AREs are found predominantly in phase II enzymes. Therefore, it can be hypothesized that, on a mechanistic basis, monofunctional inducers increase gene transcription by producing intracellular signals that converge on the ARE/EpRE, which have been identified in several phase II drug-metabolizing genes (Table 1), whereas bifunctional inducers may act through the XRE.

Phase II enzymes play a role in acquired resistance to chemotherapeutic drugs in cancer treatment. GSTs and other enzymes of glutathione metabolism are often elevated in resistant tumors and tumor cell lines and contribute to the multiple drug-resistance phenotype. Therefore, GST activity is often measured in resistant tumors or tumor cell lines using the generic GST substrate 1-chloro-2,4-dinitrobenzene (CDNB). However, glutathione S-transferases are a multigene family with almost 100 members characterized to date in many different species, and at least 13 GST isoforms have been identified in rat (Butler and Eaton, 1992a; Manervik and Danielson, 1988). At least 7 of the GST isoforms are constitutively expressed at relatively high and inducible levels in rat liver (Ya1, Ya2, Yc1, Yk, Yb1, Yb2, and 7) while at least two isoforms (Yc2 and Yf) are inducible but are either not constitutively expressed (Yf) or expressed at very low levels (Yc2) in rat liver (Meyer et al., 1993). Each isoform has unique biochemical and enzymatic characteristics which results in distinct substrate specificities (Manervik and Danielson, 1988). Although the major liver GST isoforms all conjugate CDNB with GSH, they have distinct kinetic characteristics. Therefore, relative changes in CDNB activity may not be representative of the actual changes in individual GST isoforms. Furthermore, because two GST isoforms have little or no constitutive expression in liver, elevation of one of these isoforms in drug resistance may not result in a significant change in CDNB activity but may actually have a large impact on the resistant phenotype if it is capable of detoxifying the anticancer drug used.

In the present study we investigated the effect of the monofunctional enzyme inducers BHA, ethoxyquin, and ol-tipraz on individual GST isoforms using gene-specific oligo-

\(^3\) The GST subunits are identified by the nomenclature for rat isoforms proposed by Bass et al. (1977). For further reference on nomenclature see Mantle et al. (1987).
TABLE 1

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Functionality of ARE*</th>
<th>Induction by</th>
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<tbody>
<tr>
<td></td>
<td>MA*</td>
<td>BHA</td>
</tr>
<tr>
<td>Rat glutathione S-transferase Ya2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rat glutathione S-transferase Yc2</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Mouse glutathione S-transferase Ya</td>
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<td>+</td>
</tr>
<tr>
<td>Rat NADPH:quinone reductase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Human NADPH:quinone reductase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Human dihydrolipo dehydrogenase</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Rat aldehyde reductase</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Mouse metallothionein I + II</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rat metallothionein I + II</td>
<td>?</td>
<td>+</td>
</tr>
<tr>
<td>Human metallothionein IIa</td>
<td>?</td>
<td>+</td>
</tr>
</tbody>
</table>

* Functionality was tested either by promoter deletion analysis or analysis of element proper in reporter constructs.

* Abbreviations used MA, Michael acceptors; BHA, tert-butylated hydroxyanisole; EQ, ethoxyquin; OPZ, oltipraz.

nucleotide probes for GST Ya1, Ya2, Yc1, Yc2, Yb1, Yb2, and Yf, as well as isozyme-selective antibodies for GST Ya, Yc1, Yc2, Yb1, Yb2, and Yf and isozyme-selective GST substrates. While this work was in progress, a similar study was published by Waxman and co-workers (Waxman et al., 1992) who also used gene-specific oligonucleotides to detect individual GST mRNAs in rat liver. Although our results using bifunctional P450 inducers closely match those observed by Waxman et al. (1992), we also present data on the induction of phase II enzymes after treatment with monofunctional inducers. In addition to the genes analyzed by Waxman et al., our study included analysis of the GST Yc2 isozyme, which is critical for detoxification of aflatoxin B1 (Hayes et al., 1994), as well as analysis of γ-glutamylcysteine synthetase, NADPH:quinone reductase, and the UDP-glucuronosyl transferase UGT 1*06 isozyme. UGT 1*06 was selected because of reports that showed induction of the naphthol-metabolizing UDP-glucuronosyltransferase UGT 1*06 in rat liver by BHA treatment (Goon and Klaassen, 1992) and under conditions of glutathione depletion (Manning and Franklin, 1990). In addition to the analysis of mRNA expression, we also investigated the induction of protein by Western blot, as well as induction of enzyme activity. We found that all the phase II drug-metabolizing enzymes, except GST Yf, were induced by the monofunctional inducers at the mRNA, protein, and enzyme activity level. Surprisingly, we also found that ethoxyquin was a very potent inducer of both P450 2B enzymes (2B1 and 2B2). Ethoxyquin and oltipraz also caused a two- to fivefold induction of the mRNA encoding CYP 1A2 and 3A2, suggesting a possibility of a mechanism involving ARE/EpRE regulatory elements in the regulation of phase I P450 genes.

MATERIALS AND METHODS

Chemicals and antibodies. Pregnenolone-16α-carboxinitrile (PCN) was obtained from Upjohn Co. (Kalamazoo, MI). 3-Methylcholanthrene (3MC) and 3,4-dichloro-1-nitrobenezene (DCNB) were from Aldrich Co. (Milwaukee, WI). Oltipraz (5-(2-purazinyl)-4-methyl-1,2-dithiole-3-thione) was generously provided by Dr. Thomas Kensler (Department of Environmental Health Sciences, The Johns Hopkins University, Baltimore, MD). Ciprofibrate (CF) was provided by Sterling Drug Inc. (Rensselaer, NY). Tris base, glutathione (GSH), NADPH, glucose-6-phosphate dehydrogenase, 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid, cumene hydroperoxide, 2,3-tet-buty1-4-hydroxyanisole (BHA, mixed isomers), ethoxyquin, diethyl pyrocarbonate, formamide, and β-mercaptopethanol were from Sigma (St. Louis, MO). Emulphor EL-719 was from GAF Co. (New York, NY). Ethanol was obtained from Midwest Grain Products, Co. (Pekin, IL). Agaro-ose (ultrtrapure electrophoresis grade), guanidinium isothiocyanate (enzyme grade), acid phenol (redissolved nucleic acid grade), and some other molecular biology reagents were obtained from Gibco/BRL (Gaithersburg, MD). T4 polynucleotide kinase was purchased from Stratagene (La Jolla, CA). [γ-32P]ATP was purchased from DuPont NEN (Boston, MA). Δ5-Androstene dione (ADI) was purchased from Steroids Inc. (Wilton, NH). GST isozyme-specific antibodies either were purchased from Biotrin International (Dublin, Ireland) or were prepared as described previously (Hayes et al., 1991a). HPLC-grade solvents were obtained from J. T. Baker (Phillipsburg, NJ). Other reagents were of analytical reagent grade and were obtained from various commercial sources.

Animal treatments. Adult male Sprague-Dawley rats, obtained from Tyler Laboratorios (Bellevue, WA), were housed in plastic cages on wire-mesh covered corn-cob bedding. Rats were maintained for 1 week on Wayne Rodent Blix chow diet, and then switched to AIN-76A diet without addition of synthetic antioxidants (Teklad Research Diets, Madison, WI) for 1 week. Food and water was available ad libitum. A weight log was kept to monitor weight gain indicative of adequate food consumption. Administration of the test compounds by intraperitoneal injections or the diet ensured first-pass metabolism in the liver and optimal induction of the liver enzymes. In the first experiment four animals per treatment group were injected ip with PCN (75 mg/kg) in vehicle. Three animals received an injection of 3MC (20 mg/kg) in vehicle, and six control animals received vehicle (75% saline, 25% corn oil, and 2% Emulphor EL-719) injections only. The doses were selected based on common dosing regimens for CYP induction found in the literature. Treatment involved a single injection, and animals were euthanized by CO2; narcosis and exsanguination 24 hr later. Livers were removed, rinsed in ice-cold phosphate-buffered saline (PBS), partially minced, immediately frozen in liquid nitrogen, and stored at −80°C.

In a second experiment, the P450 4A inducer ciprofibrate (CF, 0.025%
TABLE 2

Sequences of Oligonucleotide Probes Used

<table>
<thead>
<tr>
<th>Oligonucleotide probes specific for GST isozymes</th>
<th>GST</th>
<th>Oligo sequence</th>
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<tr>
<td>α</td>
<td>Yα1 (1a)</td>
<td>5'-ATGCCCTCGGTTGACATGTC-3'</td>
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<tr>
<td></td>
<td>Yα2 (1b)</td>
<td>5'-ATACCCCTGAAATGTC-3'</td>
</tr>
<tr>
<td></td>
<td>Yγc (2)</td>
<td>5'-GGGAGGTTGGCAGGCGT-3'</td>
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<tr>
<td></td>
<td>Yγ2</td>
<td>5'-GGTCCGACTATTGGAATTCG-3'</td>
</tr>
<tr>
<td>μ</td>
<td>Yβ1 (3)</td>
<td>5'-CTCTAGATGCGATAGCTGA-3'</td>
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<tr>
<td></td>
<td>Yβ2 (4)</td>
<td>5'-CAGAGATTGCGAAGACCT-3'</td>
</tr>
<tr>
<td>π</td>
<td>Yf (7)</td>
<td>5'-GCGGACTATGGGGACAGCCT-3'</td>
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Sequences of CYP oligomer probes obtained from Dr. Curt Omiecki in -80°C.

CYP

<table>
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<th>Sequence</th>
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<td>1A1</td>
<td>5'-AGCTGGAGATGCTAGCAGC-3'</td>
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<td>1A2</td>
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<td>2B1</td>
<td>5'-GGTGTATAGCGGTTGTA-3'</td>
</tr>
<tr>
<td>2B2</td>
<td>5'-GGATGGTGTGCTCTGAAGAA-3'</td>
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<tr>
<td>2C1</td>
<td>5'-TTTCCGCAAGAAGTGTTCGAC-3'</td>
</tr>
<tr>
<td>3A2</td>
<td>5'-CCGCTTGGCTTGGATGACAC-3'</td>
</tr>
<tr>
<td>4A1</td>
<td>5'-CCGCTTGGCTTGGATGACAC-3'</td>
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Sequences of other oligonucleotide probes used

<table>
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<tr>
<td>UGT1*06</td>
<td>5'-GTCGCCGCTTCTAGCGGTAAGCA-3'</td>
</tr>
<tr>
<td>Albumin</td>
<td>5'-GCTGTGCTTGGGTGTGGTTCAC-3'</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>5'-CAGACTTACGGGCGCAATAC-3'</td>
</tr>
</tbody>
</table>

* GST subunit nomenclature of Mannervik and Danielson (1988) in parentheses.

w/w) and the monofunctional inducers BHA (0.75% w/w), EQ (0.5% w/w), and OPZ (0.075% w/w) were administered in the AIN-76 diet for 3 days with four animals per treatment group and six animals in the control group. The doses were selected based on common dosing regimens for phase II enzyme induction found in the literature. Animals were euthanized by CO₂ narcosis and exsanguination. Livers were removed, rinsed in ice-cold PBS, partially minced, immediately frozen in liquid nitrogen, and stored at -80°C.

Oligonucleotide and cDNA probes. The amino acid sequences of the known rat GST isozymes were aligned using the Chastal software program (Buettler and Eaton, 1992a). Unique amino acid stretches were identified and the corresponding cDNA sequences served as templates for the design of antisense oligonucleotides of 20 nucleotides in length. Each GST-specific probe contained at least 3 mismatches to other known non-target GST sequences present in the GenBank/EMBL databases using the University of Wisconsin Genetics Computing Group software (GCC). Oligonucleotides were synthesized on a Cyclone DNA synthesizer (Millipore/Biosearch, Burlington, MA) and purified by HPLC. The sequences of the gene-specific oligonucleotides are shown in Table 2. Gene-specific oligonucleotides recognizing the cytochrome P450 enzymes were obtained from Dr. Curt Omiecki (University of Washington, Seattle, WA). Oligonucleotides for UGT1*06 and albumin were designed based upon sequences available in the GenBank/EMBL databases. An oligonucleotide directed against 18S ribosomal RNA was used as an internal control for RNA loading. cDNA probes were used for GCS and QR. Construction of the GCS probe was described previously (Boroz et al., 1994) and the QR cDNA (Fayneau and Pickett, 1991) probe was a gift from Dr. Thomas Rushmore at Merck Frost Center of Therapeutic Research, Pomona Clare, Dorval, Quebec, Canada.

RNA isolation and Northern blots. RNA was isolated by standard procedures (Chomczynski and Sacchi, 1987). Briefly, 0.1 to 0.3 g of frozen liver was thawed in 3 ml solution D (14 mM guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, 5% sarcosyl, 0.2% v/v mercaptoethanol) and homogenized with a Polytron tissueizer. Sodium acetate (300 μg, 0.2 M, pH 4), water-saturated acid phenol (3 ml), and CHCl₃/isoamyl alcohol (24:1, 600 μl) were added and samples were vortexed. Mixtures were kept on ice for 15 min and then centrifuged at 10,000 g for 30 min at 4°C. The aqueous phase was precipitated with isopropanol and precipitates were collected by centrifugation at 10,000 g for 30 min at 4°C. Pellets were washed with 75% ethanol, air-dried, and resuspended in 0.5 ml solution D. RNA samples were again precipitated with isopropanol (0.5 ml) and precipitates were pelleted, washed with 75% ethanol, and dried. RNA pellets were suspended in 200 μl RNAse-free water, and yields were determined by uv spectrophotometry. RNA samples were stored at -80°C. Ten or twenty micrograms of total RNA was separated on a 1.25% agarose/formaldehyde gel, blotted onto Nyntran membranes (Schleicher & Schuell, Keene, NH) and hybridized with a 3²-P-endabeled oligonucleotide or ³²-P-random-labeled cDNA probes. Following overnight incubation in a hybridization oven (Roche Scientific Corp., Nutley, CA), blots were washed once at room temperature and twice at 2°C below hybridization temperature for 15 min in 5 x standard sodium citrate (SSC) containing 0.1% SDS. Autoradiographs were scanned and quantitatively analyzed using a Bioquant 3000 densitometric scanner and Millipore's Bioscope whole-band analysis software (Millipore/Biosearch, Burlington, MA).

Tissue preparations. Three to five grams of frozen liver tissue was used to prepare cytosolic and microsomal fractions as described previously (Ramsdell and Eaton, 1988) for Western blot and enzyme activity analyses. The tissue fractions were stored at -80°C until use.

Western blot procedures. Cytosolic proteins (100,000g supernatant) were separated on a 1.0 mm SDS-polyacrylamide gel (16% acrylamide; 0.09% N,N'-bis acrylamide) and transferred onto Immobilon PVDF membranes (Millipore, Bedford, MA). Nonspecific binding was blocked with 0.5% 1-block (Tropix, Bedford, MA) in PBS. Primary antibodies to rat GST isozymes were incubated overnight in 0.1% 1-block in PBS containing 0.1% Tween 20. Anti-rabbit and anti-goat alkaline phosphatase-conjugated secondary antibodies were used from Bio-Rad (Richmond, CA) and Bio-Ranger Mannheim (Indianapolis, IN), respectively. Blots were developed with 0.5 mg/ml BCIP/NBT development kit (Bio-Rad) for alkaline phosphatase.

Enzyme assays. Enzyme activities of glutathione S-transferases were assayed using CDNB (broad specificity), DCNB (selective for Yβ), Δ⁴-AD (selective for the α class isozymes Yα and Y2a) and cumene hydroperoxide (CHP, selective for the α class isozymes Yα, Yc, and Yk), and ethacrynic acid (ECA, selective for Y1, with activity toward GST Yc and Yk) as substrates according to standard procedures (Habig and Jakoby, 1981). Cytochrome P450 enzyme activities were measured using methoxy- or ethoxyresorufin for CYP 1A and benzoyl-Mexresorufin for CYP 2B enzymes (Nerurkar et al., 1993). GST content and γ-glutamylcysteine synthetase (GCS) activity were determined using the HPLC method described previously (Hamel et al., 1992). NADPH:quinone oxidoreductase activity was determined by analyzing the dicoumarol-inhibitable QR activity using 2,6-dichlorodinophenol as substrate (Benson et al., 1980).

Statistical analysis. Statistical analysis was performed by single-factor ANOVA and Dunnett's t test using the statistical analysis extension of Microsoft Excel (Microsoft Corp., Redmond, WA).

RESULTS

Induction of cytochrome P450 mRNA and catalytic activity. Messenger RNA from individual control animals or
Treatment with EQ and OPZ, but not BHA, resulted in an increase in CYP 1A2 mRNA which, at the enzyme level, was reflected by a small, but significant increase in MROD activity (Fig. 3A). Treatment with BHA, EQ, and OPZ resulted in a significant increase in BROD activity (12-, 135-, and 3-fold induction, respectively, Fig. 3B) which is consistent with the induction of mRNA levels in animals treated with BHA, EQ, and OPZ (Fig. 2) with the largest increase observed with EQ for both mRNA and enzyme activity.

Induction of mRNAs encoding glutathione S-transferase isozymes. Figure 4 shows that BHA, EQ, and OPZ induced the mRNA encoding the GST Ya2 isozyme approximately 5-, 10-, and 3-fold, respectively, whereas the mRNA encoding the GST Ya1 isozyme was only induced by BHA (2-fold) and EQ (3-fold), but not by OPZ under our experimental conditions. The GST Ya1 oligonucleotide detected an unidentified, slower migrating band which always correlated with that of the GST Ya1 band in signal intensity. The mRNA encoding the constitutively expressed rat GST Yc isozyme, GST Yc1, was induced 2-, 3-, and 3-fold by BHA, EQ, and OPZ, respectively, whereas all three treatments resulted in significant induction of the mRNA encoding the low- or nonconstitutively expressed GST Yc2 subunit (5-, 20-, and 5-fold, respectively). The mRNA encoding the GST Yb1 subunit was induced 6-, 10-, and 4-fold by BHA, EQ, and OPZ, respectively, whereas the mRNA encoding the GST Yb2 isozyme was only induced by EQ (3-fold), but

<table>
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<th>OLIGO</th>
<th>CONTROL</th>
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<th>TREATMENT</th>
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<td>CYP 4A1</td>
<td><img src="image17.png" alt="Image" /></td>
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<td>18S rRNA</td>
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FIG. 1. Effect of cytochrome P450-inducing agents (3MC, PCN, CF) on the expression of mRNA encoding P450 enzymes. Ten micrograms of total RNA was loaded per well and hybridized with gene-specific oligonucleotides. Each lane represents RNA of an individual animal with the amount of RNA loaded per slot detected by hybridization with an oligonucleotide specific for 18S ribosomal RNA shown at the bottom.

animals treated with each inducer are shown in Figs. 1 and 2. Northern blots were hybridized with an oligonucleotide directed against 18S rRNA as control for RNA loading. Whole band densitometric analysis was used to quantitate mRNA expression relative to 18S rRNA expression and represents the sum of new synthesis and breakdown of RNA. The constitutive levels of expression were low for many of the CYP mRNAs analyzed and considerable interanimal variation in expression levels was observed for certain genes. As positive controls, the CYP enzymes were induced by known, family-selective inducers (Fig. 1). The mRNA encoding the two CYP 1A enzymes were induced approximately 25- to 40-fold by 3MC, the mRNA encoding CYP 3A2 enzyme was induced approximately 5-fold by PCN, while the mRNA encoding the CYP 4A1 enzyme was induced more than 35-fold with CF.

Dietary BHA treatment had little effect on the mRNA expression levels of P450 enzymes (Fig. 2). The slight induction of CYP 1A2, 2B2, and 3A2 mRNA in one or two BHA-treated animals was not statistically significant. However, EQ and OPZ treatment resulted in a statistically significant 3-fold elevation of CYP 1A2 mRNA and a significant 2- to 2.5-fold increase in CYP 3A2 mRNA. EQ caused an unexpected 30- to 40-fold induction of the two members of CYP 2B family (Fig. 2). None of the treatments resulted in induction of CYP 1A1, 2C11, or 4A1 at the dose and time analyzed in this study.

FIG. 2. Effect of monofunctional inducers (BHA, EQ, OPZ) on the expression of mRNA encoding cytochrome P450 enzymes. Ten micrograms of total RNA was loaded per well and hybridized with gene-specific oligonucleotides. Each lane represents RNA of one individual animal. Because more than two blots loaded with identical RNA samples were used, two representative blots hybridized with an oligonucleotide specific for 18S ribosomal RNA are shown at the bottom.
FIG. 3. Effect of inducers on catalytic activities of selected cytochrome P450 enzymes. (A) Microsomal fractions of animals treated with BHA, EQ, and OPZ were analyzed using the CYP 1A2-selective substrate methoxyresorufin (MROD). (B) The substrate benzylxresorufin (BROD) was used to measure CYP 2B enzyme activity following treatment with the various inducers. Activity was measured in microsomal fractions of each individual animal and bars represents the mean activity with standard deviation indicated for each treatment group. Statistical analysis included ANOVA and Dunnett's t test. Significant differences are indicated: *p < 0.005 and **p < 0.05.

not by BHA or OPZ under our experimental conditions. The mRNA encoding the Yf subunit is not constitutively expressed in rat liver and was induced significantly only in 2 of 4 BHA-treated animals. Furthermore, a slight, but nonsignificant elevation of GST Yf was observed in animals treated with EQ under our experimental conditions.

Induction of GST proteins. The induction of GST proteins was also analyzed using specific antibodies directed against GST Ya1/2, Yc1, Yc2, Yb1, and Yb2 (Fig. 5). The constitutive levels of protein expression appeared to be high in untreated animals (Fig. 5), whereas the constitutive levels of mRNA expression were quite low in untreated animals (Fig. 4). The induction of GST proteins generally followed the induction of messenger RNA, although the magnitude of induction was considerably lower (Fig. 5). The maximal induction at the protein level appeared to be 3- to 5-fold.

FIG. 4. Effect of monofunctional inducers (BHA, EQ, OPZ) on the expression of mRNA encoding GST isozymes. Ten micrograms of total RNA was loaded per well and hybridized with gene-specific oligonucleotides. Each lane represents RNA of one individual animal. Because more than two blots loaded with identical RNA samples were used, two representative blots hybridized with an oligonucleotide specific for 18S ribosomal RNA are shown at the bottom.

The antibody directed against GST Yc2 was raised against the constitutively expressed mouse GST Yc isozyme, which corresponds to the rat GST Yc2 protein (Hayes et al., 1991a). This antibody strongly cross-reacts with the rat GST Yc1 and Yc2 subunits and also weakly with the rat GST Ya subunit(s). However, because the relative mobilities of these three subunits are different in SDS–polyacrylamide gels, the Yc2 subunit was easily identified as the middle band of the three proteins in Fig. 5.

FIG. 5. Western blot results of cytosolic proteins of animals treated with the different monofunctional inducers. One to five micrograms of cytosolic proteins was separated electrophoretically, blotted onto Immobilon membranes and probed with isozyme-selective polyclonal anti-rat GST antibodies as described under Materials and Methods. Each lane represents cytosolic proteins of one individual animal. The fourth lane labeled REF contains mouse liver cytosol as positive control for GST Yc2 (the antibody was raised against mouse GST Yc).
Induction of GST enzyme activity. Induction of enzymatic activity (Fig. 6) generally followed the changes observed at the protein level detected by Western blot (Fig. 5) but was less in magnitude than the induction observed for message levels (Fig. 4). The phenolic antioxidants BHA and EQ significantly increased CDNB activity, with a maximum 2-fold induction observed with EQ. Olitraz increased CDNB activity approximately 20% under the conditions of this study (significant at $p < 0.05$). Using ADI as an $\alpha$-class-selective substrate for the GST Yg subunits, a significant 2.2- and 2.5-fold increase was observed in BHA- and EQ-treated animals, respectively, whereas OPZ had little effect. When the GST Yh1-class-selective substrate DCNB was used, a significant increase in activity was observed following treatment with BHA and EQ (2-fold), while OPZ had only a small, but still significant inducing effect (30% increase, Fig. 6). BHA and EQ induced the expression of the Yf subunit gene, resulting in concomitant significant increases in Yf-associated enzyme activities toward ECA of 45 and 63%, respectively. OPZ had little effect on the expression of the GST Yf subunit gene and activity under the conditions of this study. It should be noted that ECA is also conjugated by GST Yc and Yk (Mannervik and Danielsen, 1988).

Induction of mRNAs encoding other phase II drug-metabolizing enzymes. The constitutive mRNA expression levels of the three other phase II enzymes analyzed, GCS, QR, and UGT1*06, were very low in untreated animals maintained on the synthetic antioxidant-free AIN-76A diet (Fig. 7). The mRNA levels encoding all three enzymes were significantly induced by BHA (4-, 3-, and 40-fold, respectively) and EQ (7-, 8-, and 60-fold, respectively). However, OPZ treatment resulted only in significant elevation of mRNA levels encoding QR (3-fold) and UGT 1*06 (8-fold), whereas GCS was not significantly induced by OPZ treatment under our experimental conditions. Albumin was analyzed as a hepatic ‘housekeeping’ gene and the mRNA expression levels encoding albumin did not change with any of the treatments.

Induction of GCS and QR enzyme activity and hepatic GSH levels. The QR enzyme activity was induced 4- and 7.5-fold by BHA and EQ, respectively, whereas OPZ had no effect on QR activity under the conditions of this study (Fig. 8). The increase in QR mRNA appears to be directly translated into elevated enzyme activity because, unlike observed with GSTs, the magnitude of induction of both mRNA and activity were very similar. GCS, the rate-limiting enzyme in glutathione biosynthesis, was induced by both
BHA and EQ at the mRNA level, which resulted in a significant 2-fold increase in GCS activity. In BHA-treated animals, this increase in GCS activity was followed by an apparent increase in GSH levels, although the changes in GSH levels were not significant at $p < 0.05$ (Fig. 8). EQ treatment did not significantly raise hepatic GSH levels despite the significant 2-fold increase in GCS activity. OPZ had little effect on GCS activity and GSH levels under the conditions of this study (Fig. 8).

**DISCUSSION**

To quantitatively assess the induction of individual gene products by various enzyme inducers, we used densitometric scanning of autoradiographs of Northern blots hybridized with gene-specific oligonucleotide probes. The whole-band analysis software used in this study is among the most accurate tools available to quantitate relative intensities of scanned autoradiographs because it integrates the entire area of the band, rather than a single "slice" through the band. All values given for densitometric scans are the average of hybridization intensities of individual animals in a treatment group normalized to the respective hybridization intensities of the 18S ribosomal RNA and represent the sum of new synthesis and breakdown of RNA. We believe that our quantitative data analysis is relatively accurate and representative of the intensities of the bands on the autoradiographs. However, it should be noted that intensities of marks on X-ray films are linear only over a short range, thus hampering the quantitative analysis of very strongly induced mRNAs. Likewise, nonconstitutively expressed or otherwise weak signals in control animals may prevent accurate measurement of baseline signals necessary to evaluate the extent (fold) of induction.

**Induction of Cytochrome P450 Enzymes**

Analysis of cytochrome P450 mRNA and/or protein and activity after treatment with known P450 inducers (3MC, PCN, and CF) served as control for normal induction responses of P450 enzymes. In our study, PCN resulted in a fivefold induction of the CYP 3A2 gene, which is consistent with the PCN-induced fourfold increase in CYP 3A2 protein observed by Cooper et al. (1993). Although isosafrole (ISF), used in the study by Waxman et al. (1992), is generally considered to be a CYP 1A-inducer with selectivity for the CYP 1A2 isozyme (Sesardec et al., 1990), it was also shown to induce the CYP 2B genes (Horbach et al., 1990), which are generally recognized to be inducible by phenobarbital (Nerurkar et al., 1993; Nims et al., 1993; Omiecinski et al., 1985). In the Waxman et al. (1992) study, ISF induced all GST isozymes analyzed, as was found in this study for EQ. Like ISF, EQ also induced the CYP 1A2 and CYP 2B1 and 2B2 genes. Thus, these two structurally related compounds may also share some functional relationship.

Although BHA, EQ, and OPZ have similar effects as monofunctional inducers toward phase II enzymes, we found that their effect on phase I enzymes was strikingly different. CYP 1A2 was induced to a similar extent by OPZ and EQ, but BHA had no effect. EQ was a very efficient inducer of CYP 2B, whereas BHA had only a very minor inducing effect and OPZ had no effect at the dose and time analyzed. It is interesting to note that sequence evaluation of the promoter regions of the two CYP 2B genes for the presence of an ARE (TGACnnnGC) identified such an element at approximately $-1$ kb in the CYP 2B1 gene (personal observation). The significance of this finding for induction of the CYP 2B1 gene by phenolic antioxidants is unknown at the present time. Recently, Pan et al. reported that both the CYP 2B1 and 2B2 genes were induced by diallyl sulfide,
an organosulfur compound and component of garlic known to induce GSTs, in both whole animals and primary hepatocyte cultures (Pan et al., 1993a,b). Although not described by Talalay et al. (1988) as a monofunctional inducer, diallyl sulfide has been considered a monofunctional inducer because of its strong induction of the phase II GST enzymes in mice (Maurya and Singh, 1991). In addition, Pinkus et al. showed that phenobarbital induced the mouse GST Ya gene promoter via the EpRE which previously was implicated in induction by phenolic antioxidants (Pinkus et al., 1993). Thus, there is a possibility that the regulation of the CYP 2B genes may, among other mechanisms, involve ARE/EpRE elements, although the fact that the CYP 2B genes were not induced by BHA or OPZ would argue against such a mechanism.

In summary, we found that, while the P450 enzymes were induced by their known inducers, the monofunctional inducers used in this study at the dose and time analyzed were capable of inducing several of the P450 enzymes. The level of induction was different for each inducer and enzyme. The exact mechanism by which these monofunctional inducers induce the phase I CYP genes is currently unknown, but our results suggest that ARE/EpRE elements may be involved in their regulation.

**Induction of Glutathione S-Transferases**

Whereas large increases in mRNA levels encoding GST subunits were detected with the three inducers used in this study, the increase in associated protein and enzyme activity levels were significantly less pronounced. One possible explanation for this discrepancy may be the relatively long half-lives of GST proteins. In a different study, we observed that the half-lives of GST proteins appeared to be 4–6 days (unpublished observations). Because regular rodent chow diet contains natural components that can induce phase II drug-metabolizing enzymes, it is likely that untreated animals kept on a regular chow diet express elevated levels of GST proteins. In fact, unpublished data from this laboratory show that rats kept on the AIN-76A diet express lower levels of mRNA encoding GSTs than rats fed a regular rodent chow diet. Thus, even though the animals used in this study were kept on the synthetic antioxidant-free AIN-76A diet for 1 week prior to application of the test chemicals, the levels of GST proteins may still be detectable due to the long half-lives of the proteins. Because the half-life of mRNAs encoding GST isozymes appears to be shorter than that of the encoded proteins, their levels may have reached their minima after 1 week on the synthetic antioxidant-free AIN-76A diet, whereas the protein levels had not. Thus, treatment of these animals with the inducers apparently resulted in a large increase in mRNA levels but only a moderate increase in protein levels.

The two dietary antioxidants BHA and EQ were very effective inducers of several phase I and phase II drug-metabolizing enzymes; EQ was the more potent inducer of the two at the dose and time analyzed. As with the P450 enzymes, several differences were apparent in the induction properties of GST isozymes between these two inducing agents. Using the GST Yf substrate EACA, a twofold increase in GST activity was detected. The fact that ECA is not very selective for the GST Yf isoform and is also metabolized by GST Yk may explain such discordance. In addition, ECA is also an effective inhibitor of the GST Yf enzyme (Phillips and Mantle, 1993; van Bladeren and van Ommen, 1991). Thus, ECA may not be a good substrate to accurately assess changes in GST Yf activity because of substrate inhibition and poor specificity. Curiously, the BHA induction response of the GST Yf gene was high in those animals that responded least to BHA for the other GST isozymes, and vice versa (Fig. 4). The GST Yf (π class) subunit appears to be regulated by a TPA-responsive element (TRE), which is distinct from, but very similar to, the ARE/EpRE (Diciccianni et al., 1992; Nguyen et al., 1994; Okuda et al., 1987, 1990; Sakai et al., 1988). It appears that different transcription factors bind to the TRE and ARE (Nguyen et al., 1994; Rushmore et al., 1991; Rushmore and Pickett, 1990). In support of these in vitro findings, we found in our in vivo study that the regulation of the GST Yf subunit gene was clearly different from that of the other GST subunit genes analyzed.

Under our experimental conditions we found that OPZ was much less effective than the dietary antioxidants BHA and EQ for GST induction which could be due to the fact that the dose used was only approximately 10% that of BHA or EQ. However, the induction levels for OPZ were consistent with published data using similar doses of OPZ (Davidson et al., 1990; Meyer et al., 1993). The protective effect of OPZ against AFB carcinogenesis can probably be ascribed to the induction of the GST Yc2 isozyme which has been shown to have high activity to detoxify activated aflatoxin B1 (Hayes et al., 1991a, 1994). In our study we observed a fivefold increase in steady-state GST Yc2 mRNA levels by OPZ, while other GST genes were induced two- to fourfold. GST Ya1 and Yb2 were not significantly induced by OPZ. BHA and EQ are also known to protect against aflatoxin B1 genotoxicity in vivo (Eaton and Gallagher, 1994; Kensler et al., 1985) and both are shown here to induce the GST Yc2 gene. This observation is consistent with the reported inducibility of GST Yc2 by EQ (Hayes et al., 1991a).

Determination of CDN activity is often used to assess induction of GST enzymes in cells, tissues and tumors. Most GST enzymes in rat can utilize CDN as a substrate in the conjugation reaction with GSH; however, the specific activity and enzyme kinetic parameters of individual isozymes with CDN may vary (Mannervik and Danielson, 1988). Furthermore, the relative abundance of individual cytosolic rat GST isozymes varies considerably; GST Yb1 and Yb2
account for approximately 65% of all GST isozymes expressed in rat liver cytosol, GST Yα1 and Yα2 account for about 11%, GST Yε1 accounts for 18%, and GST Yκ accounts for 3%, with GST Yε2 and Yb3 making up the remaining 3% (data taken from Meyer et al., 1993). Thus, using CDNB as substrate, an increase in the levels of GST Yb subunits will be readily detected while a similar increase in GST Yε2 or Yf may go unnoticed in assays of total cytosolic GST activity toward CDNB. The results of our study are consistent with this concept. CDNB activity is increased 1.8-, 2.2-, and 1.2-fold for BHA, EQ, and OPZ, respectively. Activities measured with substrates more selective for individual GST isozymes revealed a similar pattern of enzyme induction. However, analysis of the mRNA levels revealed that certain isozymes were preferentially induced by a specific inducer (GST Yf induction by BHA, GST Yε2 induction by OPZ), whereas other GST isozymes were not induced (GST Yb2 induction by BHA or OPZ). Thus, whereas CDNB activity in livers of animals treated with OPZ did not substantially increase (1.2-fold), the mRNA of one specific GST isozyme (Yε2) was induced 5-fold. Because GST Yε2 is the isozyme capable of conjugating GSH to the activated aflatoxin B1, 8,9-epoxide (Hayes et al., 1991a), and this activity is elevated 2-fold (data not shown), measuring changes in CDNB, or even α-class-selective GST activity toward ADI, did not reveal the true changes in GST Yε2 isozyme expression.

Collectively, these data show that all GST subunits analyzed in this study were induced by the monofunctional inducers BHA, EQ, and OPZ at the mRNA, protein, and activity level, with the exception of the GST Yf subunit. Because there is strong evidence to suggest that the inducing agents used in this study increase the rate of gene transcription via the ARE/EpRE promoter element, we postulate that the GST subunit genes encoding Yα1, Yα2, Yε1, Yε2, Yb1, and Yb2 contain ARE/EpREs in their promoter regions, thus conferring inducibility to monofunctional inducing agents. However, additional promoter elements may also contribute to the regulation of these genes and further analysis of the regulation of these genes is needed.

Induction of GCS and GSH

Both BHA and EQ were found to be effective inducers of GCS mRNA under the conditions used in this study. Increases in GCS mRNA were paralleled by a 2-fold increase in GCS enzyme activity. Interestingly, only treatment with BHA resulted in an increased hepatic GSH levels, whereas EQ-treated animals exhibited levels of hepatic GSH similar to those of control animals. Considering the extensive effects of EQ, it is possible that the greater elevation of total GST-CDNB activity by EQ, compared to BHA, may have negated the increase in newly synthesized GSH due to increased utilization of GSH through GST-mediated GSH conjugation and export. Alternatively, EQ may have induced an active transport system for GSH in hepatocytes or formed an electrophile capable of oxidizing GSH. Treatment with BHA induced GCS mRNA and catalytic activity, and also tended to increase GSH which, however, was not significant. OPZ did not have any effect on GCS mRNA and activity or on GSH levels. Recently, we showed that the mRNA encoding the GCS large subunit in mouse liver was increased 5-fold by dietary treatment with BHA (Borroz et al., 1994). Similar to the results shown here, the BHA-induced increase in GCS mRNA in mouse liver was followed by only a 1.5-fold increase in GCS activity and GSH levels. Feedback inhibition of GCS by GSH has been shown to regulate the intracellular levels of GSH (Meister, 1984, 1991) which may account for this observation. The fact that GCS large subunit mRNA is induced by BHA and EQ may suggest that the expression of the GCS large subunit gene may also be regulated via a mechanism involving ARE/EpRE regulatory element(s) in the promoter region.

Induction of NADPH:Quinone Oxidoreductase and UDP-Glucuronosyl Transferase UGT1*06

Both QR and UGT1*06 mRNAs were induced by the phenolic antioxidants BHA and EQ. In contrast, OPZ induced QR mRNA threefold without a measurable elevation of QR activity. In the absence of information on posttranslational modifications or mRNA and protein stability for QR, we can only speculate on the discrepancy between RNA induction and lack of QR activity induction. As for the GST enzymes, QR may be a very stable protein and 1 week on the AIN-76A diet may not have been sufficient to decrease the protein to minimal levels, whereas mRNA levels may have decreased and subsequently responded to OPZ induction. The induction of the mRNA levels encoding QR and UGT1*06 by BHA and EQ suggests that these two genes may also contain ARE/EpRE regulatory elements in their promoter regions. Our results are consistent with a recent study by Kashfi et al. (1994) who reported induction of rat liver, kidney, and small intestine UDP-GT mRNA by phenolic antioxidants. In the preceeding study, the authors used a nonspecific oligonucleotide probe that recognized the common 3'-domain of bilirubin/phenol UDP-glucuronosyl transferase mRNAs.

In conclusion, the results of this study indicate that the expression of a variety of phase I and phase II enzymes is increased on exposure to monofunctional inducers. Substantial differences were noted with regard to potency and extent of mRNA and protein induction among the various chemicals tested. Further studies involving direct promoter analysis or indirect analysis of DNA-binding activities to the ARE/EpRE in nuclear extracts of treated animals, as recently reported by Ciaccio et al. (1994), will be needed to confirm the presence and functionality of ARE/EpRE regulatory elements in the genes analyzed in this study.
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