INDUCTION OF THE MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN FAMILY OF TRANSPORTERS BY CHEMICAL ACTIVATORS OF RECEPTOR-MEDIATED PATHWAYS IN MOUSE LIVER

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ABSTRACT:

The multidrug resistance-associated proteins (Mrp) are ATP-dependent transporters that export a variety of conjugated and unconjugated compounds out of cells. There are nine identified Mrp transporters in humans, with murine orthologs for all except Mrp8. Because nuclear receptors mediate induction of phase I enzymes, Mrp transporter expression might be similarly regulated by these receptors to coordinate metabolism and export of chemicals from liver. To test the hypothesis that Mrp expression may be coordinately regulated with phase I enzyme expression in liver, 15 different compounds were given representing known transcriptionally mediated pathways: aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), peroxisome proliferator-activated receptor α (PPARα), and nuclear factor-E2-related factor 2 (Nrf2). Each of these compounds induced expression of their respective target enzyme in liver, demonstrating that the chemical regimens were effective. The AhR ligands [2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated biphenyl 126 (PCB126), and β-naphthoflavone] induced Mrp2, -3, -5, and -6 mRNA expression. The CAR activator 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) induced Mrp2, -3, -4, -6, and -7 mRNA expression. Mrp3 was also induced by two other CAR activators phenobarbital and dialyl sulfide, two PXR ligands, pregnenolone-16α-carbonitride and spironolactone, and the PPARα ligands clofibrate, ciprofibrate, and diethylhexylphthalate. The Nrf2 activators (butylated hydroxyanisole, oltipraz, and ethoxyquin) induced Mrp2-6. In conclusion, a variety of mechanisms are suggested for Mrp3 induction, including AhR, CAR, PXR, PPARα, and Nrf2, whereas on a whole, a predominant role for AhR and Nrf2 in hepatic induction of the Mrp family was observed. Thus, these specific transcription factors are implicated in regulation of both drug metabolism and efflux transport.

Multidrug resistance-associated proteins (Mrps) are ATP-dependent transporters known to transport a diverse set of substrates. Mrps have an affinity for conjugated compounds, including glutathione-, glucuronide- and sulfate-conjugated metabolites. Mrps as a group are known to efflux these conjugated substrates from cells, including the transport of chemicals such as bilirubin, 2,4-dinitrophenol, 17β-estradiol, taurocholate, and protease inhibitors, such as ritonavir, with high affinity (Jedlitschky et al., 1997; Hirohashi et al., 1999; Keppler, 1999; Huisman et al., 2002). Four Mrp transporters (Mrp2, -3, -4, and -6) are expressed to an appreciable extent in liver (Maher et al., 2005). Mrps (Mrp2 to -6) are expressed to an appreciable extent in liver (Maher et al., 2005).

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Several transcription factors have an important role in modulating the basal and inducible expression of genes involved in phase I drug metabolism. One of the best understood mechanisms of gene induction in liver is the marked induction of Cyp1A1 by dioxin-like compounds, which is mediated through the aryl hydrocarbon receptor (AhR) (Jaiswal et al., 1985). The AhR is known to have a physiological role, although the endogenous ligand has been elusive (Hahn, 2002). Studies in AhR-null mice have revealed that AhR is important in immune function and in hepatic development, and loss of AhR leads to nonresponsiveness to dioxins (Fernandez-Salguero et al., 1995; Schmidt et al., 1996). Furthermore, loss of the AhR protects against benzo-a-pyrene-induced tumors (Shimizu et al., 2000).

The pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) are believed to be “xenosensors” that can cooperate to protect the liver from hepatotoxicity by up-regulating the expression of the Mrp family (Maher et al., 2005).

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of Cyp3a and Cyp2b, which are important drug metabolizing enzymes (Baes et al., 1994; Honokoski et al., 1998; Lehmann et al., 1998; Staudinger et al., 2001). PXR is activated by a diverse number of compounds, including rifampicin, phenobarbital, and mifepristone (RU486) in humans (Kliwer et al., 1998). The dually named constitutively active receptor/constitutive androstane receptor or (CAR) is unique because, as the names suggest, it does not need to have bound ligand to be active, and its activity is repressed by androstenol (Wei et al., 2000).

The peroxisome proliferator-activated receptor α (PPARα) is known to mediate transcription of numerous genes, including those encoding peroxisomal and mitochondrial β-oxidation enzymes, the CYP4A family, and fatty acid-binding proteins (Keller et al., 1993). A class of compounds that include the fibrate class of triglyceride- and cholesterol-lowering drugs has been shown to bind to PPARα and alter transcription and metabolism of other xenobiotics (Lee et al., 1995). PPARα-null mouse livers did not demonstrate the peroxisomal proliferation or the neoplasms that are typically present in rodents after long-term treatment with fibrate drugs (Peters et al., 1997).

Nuclear factor-E2-related factor 2 (Nrf2) has been identified as a transcription factor that is activated by microsomal enzyme inducers such as butylated hydroxyanisole, oltipraz, and ethoxyquin. Nrf2 heterodimerizes with a member of the small muscleaponeuretic fibrosarcoma oncogene family of proteins to bind cognate electrophile response elements in DNA (Motohashi et al., 1998, 2002). Nrf2 is responsible for both constitutive and inducible expression of electrophile response element-regulated genes. For example, the induction of glutathione S-transferase α by butylated hydroxyanisole (BHA) was abolished in Nrf2-null mice (Chanas et al., 2002). Furthermore, oltipraz-induced phase I and II enzymes, such as NAD(P)H:quinone oxidoreductase 1 (Nqo1) and glutathione S-transferase α was completely abolished, and the chemopreventive effect of oltipraz was completely lost in Nrf2-null mice (Ramos-Gomez et al., 2001). Thus, Nrf2 plays a central role in regulating constitutive and inducible expression of some phase I and II enzymes, and the chemoprotective effects of chemicals such as oltipraz are directly due to transcriptional mediation of the antioxidant battery of genes regulated via Nrf2.

Whereas the mechanisms up-regulating certain phase I drug-metabolizing enzymes have been examined in detail, the regulation of efflux transporters is poorly understood, particularly in mice. To determine potential mechanisms for transcriptional regulation of Mrps, 15 compounds that are known activators of nuclear and other transcription factor-mediated pathways were administered to mice. Thus, the aim of this study is to quantitatively determine the expression of mouse Mrps after treatment with structurally distinct types of chemicals to determine whether potential coordinate induction of phase I and II metabolism and efflux transporters exists in livers of mice.

Materials and Methods

Chemicals. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was a gift from Dr. Karl Rozman (University of Kansas Medical Center, Kansas City, KS). Oltipraz was a gift from Dr. Steven Safe (Texas A&M University, College Station, TX). Polychlorinated biphenyl 126 (PCB126) was obtained from AccuStandar (New Haven, CT). All other chemicals, unless otherwise indicated, were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Animals. Male C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) and were exposed to microsomal enzyme inducers at approximately 8 weeks of age (n = 5/treatment). The chemicals, dosing regimen, vehicle, and corresponding control for the treatment are included in Table 1. After dosing each day for 4 days with the microsomal enzyme inducers, livers were collected on the 5th day (96 h after the first dose). Tissues were snap-frozen in liquid nitrogen and stored at −80°C.

RNA Extraction. Total RNA was isolated using the RNA Bee reagent (Tel-test, Friendswood, TX) according to the manufacturer’s instructions. RNA concentrations were determined spectrophotometrically, and the quality of the RNA was determined by gel electrophoresis.

Development of Specific Oligonucleotide Probe Sets for bDNA Analysis. The Mrp gene sequences were accessed from GenBank. The target sequences were analyzed by ProbeDesigner Software Version 1.0. (Genospectra, Fremont, CA). The oligonucleotide probes were specific for only one mRNA transcript. All oligonucleotide probes were designed with a melting temperature of approximately 63°C, enabling optimal hybridization conditions. Each probe set was submitted to the National Center for Biotechnology Information (NCBI) for nucleotide comparison by the basic local alignment search tool (BLASTn) to ensure minimal cross-reactivity with other mouse sequences and expressed sequence tags (Maher et al., 2005).

Branched DNA Assay. The specific Mrp oligonucleotide probes were diluted in Tris-EDTA buffer according to instructions provided by the Quantigene bDNA Signal Amplification Kit (Genospectra). Total RNA (1 μg/μl; 10 μl) was added to each well of a 96-well plate containing 50 μl of capture hybridization buffer and 50 μl of each diluted probe set. Total RNA was allowed to hybridize overnight at 53°C in a hybridization oven. Subsequent hybridization steps were carried out according to the manufacturer’s protocol, and luminescence was measured with a Quantiplex 320 bDNA luminometer interfaced with Quantplex Data Management Software Version 5.02 for analysis of luminescence from 96-well plates.

Statistical Analysis. Data were analyzed by one-way analysis of variance, followed by Duncan’s multiple range post hoc test. Bars represent standard error of the mean. Asterisks (*) represent statistical differences (p ≤ 0.05) in mRNA levels between the control and treated groups.

Results

Validation of Chemical Treatment Regimen by Phase I Inducers. The microsomal enzyme inducers utilized in this experiment can be divided into five distinct mechanisms of activation, those being AhR, CAR, PXR, PPARα, and Nrf2 activators. The chemicals, dosages, and marker gene demonstrating activation of the respective receptor have been summarized in Table 1. The results of the corresponding treatments on Cyp1a1, 2b10, 3a11, 4a14, and Nqo1 in mouse liver are illustrated in Fig. 1. Cyp1a1 mRNA expression was increased by treatment with the Ah ligands TCDD (1230-fold), BNF (1230-fold), and PCB126 (1170-fold). Cyp2B10 was induced by CAR activators PB (15-fold), TCPOBOP (150-fold), and DAS (22-fold). Cyp3A11 was induced by PXR ligands PCN (5-fold), SPR (5-fold), and DEX (5-fold). The mRNA levels of Cyp4A14 were induced by PPARα ligands CLOF (22-fold), CFPF (93-fold), and DEHP (87-
fold). Nqo1 was induced by Nrf2 activators BHA (3-fold), EXQ (5-fold), and OPZ (2-fold).

**Hepatic Expression of Mrp1, -2, and -3 mRNA in Mice Treated with Microsomal Enzyme Inducers.** Hepatic mRNA expression levels of Mrp1 are low in mouse liver and were not significantly induced by any of the treatments (Fig. 2). However, Mrp1 expression in liver was repressed by CPFP (80% decrease), DEHP (80% decrease), and BHA (71% decrease) administration.

Mrp2 mRNA expression in mouse liver was significantly increased by a variety of compounds, including the three AhR ligands TCDD (114%), PCB126 (130%), and BNF (134%); the CAR ligand TCPOBOP (294%); and all three Nrf2 activators BHA (150%), OPZ (119%), and EXQ (129%) (Fig. 2). In previous studies, TCPOBOP also induced Mrp2 in mice in vivo, correlating well with the present data (Guo et al., 2003).

All classes of nuclear receptor activators induced the expression of Mrp3. This includes AhR ligands TCDD (229%), PCB126 (315%), BNF (257%); the CAR activators PB (98%), TCPOBOP (512%), and DAS (82%); the PXR ligands PCN (91%) and SPR (98%); the PPARα ligands CLOF (193%), CPFB (200%), and DEHP (146%); as well as the Nrf2 activators BHA (224%), OPZ (233%), and EXQ (367%) (Fig. 2). These studies correlate well with previous work showing up-regulation of mouse Mrp3 by TCPOBOP and PCN (Staudinger et al., 2003).

**Hepatic Expression of Mrp4, -5, and -6 mRNA in Mice Treated with Microsomal Enzyme Inducers.** Only a few compounds induced Mrp4 (Fig. 3). The CAR activator TCPOBOP induced Mrp4 in liver 338%, and all three Nrf2 activators BHA (133%), OPZ (382%), and EXQ (594%) induced expression of Mrp4. These data correlate with up-regulation of Mrp4 by TCPOBOP by others (Assem et al., 2004).

Mrp5 expression is also low in liver, but was inducible. The three AhR ligands TCDD (641%), PCB126 (515%), and BNF (611%); two of three CAR ligands TCPOBOP (450%) and DAS (184%); and all three Nrf2 activators BHA (413%), OPZ (361%), and EXQ (349%) induced expression of Mrp5 (Fig. 3).

Mrp6, which is highly expressed in liver, was also induced by a similar subset of compounds. The three AhR ligands TCDD (226%), PCB126 (290%), and BNF (248%); two of three CAR ligands TCPOBOP (186%), and PB (105%); and all three of the Nrf2 activators OPZ (239%), EXQ (180%), and BHA (171%) induced Mrp6 expression (Fig. 3).

**Hepatic Expression of Mrp7 and -9 mRNA in Mice Treated with Microsomal Enzyme Inducers.** Mrp7 expression is low in liver, yet was induced by several chemical activators, including all three AhR ligands TCDD (170%), PCB126 (183%), and BNF (201%); all three CAR activators PB (224%), TCPOBOP (230%), and DAS (265%); and one of three Nrf2 activators BHA (188%) (Fig. 3). Mrp9 expression is extremely low in liver, but was repressed by EXQ (99.9%), CPFP (99.9%), DEHP (99.8%) (Fig. 4).

**Discussion**

The purpose of this study was to determine whether Mrps, like phase I and II enzymes, are induced in mouse liver following administration of microsomal enzyme inducers. Five different types of microsomal enzyme inducers with known mechanisms of induction were used to identify potential transcriptional mechanisms that regulate the expression of various Mrps in mouse liver. In this study, all of the compounds used induced their cognate phase I enzyme, indicating that the dosing regimen used was efficacious.

Data from this study demonstrate that Mrp expression is inducible in mouse liver by microsomal enzyme inducers and suggest that...
nuclear receptors regulate Mrp expression. However, the magnitude of induction of Mrps in mouse liver is not as marked as the up-regulation of some cytochromes P450 by administration of microsomal enzyme inducers. Also, some transporters such as Mrp2 and Mrp3 are more highly expressed in mouse liver, and therefore, a two-fold increase in expression may translate into higher effective levels of efflux transport. Mrp1, -5, -7, and -9 are not expressed to an appreciable extent at the mRNA level in mouse liver; nevertheless, Mrp4, whose mRNA is also only minimally expressed, has been detected at the protein level in liver, with localization to the basolateral membrane (Rius et al., 2003; Assem et al., 2004). It is important to note that the expression of many P450s, such as Cyp1a1 and Cyp2b10, is similarly quite low in uninduced liver, but is up-regulated hundred-fold after chemical treatment. Similar induction of these poorly expressed Mrps in liver is not marked and may not correlate to functional changes in hepatic transport. Therefore, the discussion will be limited to the transporters in liver with known expression and function.

Mrp2 is a canalicular Mrp transporter known to transport chemicals into bile (Jansen et al., 1993). Mrp2 has been shown to transport a vast array of organic anions, including monobilirubin glucuronide and 2,4-dinitrophenyl S-glutathione (Jedlitschky et al., 1997; Keppler, 1999). In the present study, AhR ligands, TCPOBOP, and Nrf2 activators increased Mrp2 expression in mouse liver, suggesting that AhR, CAR, and Nrf2 may be important for modulating Mrp2 expression by chemicals.

Whereas few studies have examined the regulation of mouse Mrp2, more is known regarding the regulation of rat and human Mrp2. Induction of rat Mrp2 has been observed with numerous chemicals, such as PCN, SPR, and DEX (PXR ligands), PB (CAR ligand), and OTZ (Nrf2 activator) (Courtois et al., 1999; Johnson and Klaassen, 2002). Similar induction of Mrp2 with indole-3-carbinol and β-naphthoflavone, both AhR ligands, has also been observed in rat liver (Cherrington et al., 2002). Treatment with the chemical carcinogen 2-acetylaminofluorene, the antineoplastic drug cisplatin, and the protein-synthesis inhibitor cycloheximide increased expression of Mrp2 in rat liver (Kauffmann et al., 1997), although the mechanism(s) are not understood. Human Mrp2 is similarly up-regulated by the PXR activators rifampicin and tamoxifen, which differ from known rodent ligands for PXR (Jones et al., 2000). Similarly, Mrp2 is induced by PB (Schrenk et al., 2001) and by tert-butyl hydroquinone in HepG2 cells (Kauffmann et al., 2002), which suggests that CAR and Nrf2, respectively, may regulate expression of the human Mrp2 gene. Taken
together, these experiments suggest that the gene for Mrp2 may be similarly up-regulated by PXR agonists in human and rat, but mouse Mrp2 may not be as sensitive to PXR ligands.

Mrp3 is a basolateral efflux transporter that is up-regulated during cholestatic liver conditions and may serve to protect the liver from bile acid toxicity during cholestasis by transporting bile acids from liver into blood (Soroka et al., 2001). Similarly, Mrp3 can transport a host of conjugated xenobiotics out of the liver. Data from the present study demonstrate that Mrp3 mRNA expression is induced by AhR, CAR, PXR, PPARα, and Nrf2 activators. Previous studies have shown up-regulation of Mrp3 in rodent by PCN, which has been shown to be dependent on PXR, as well as up-regulation of Mrp3 by TCPOBOP and PB (Maglich et al., 2002; Xiong et al., 2002b; Cherrington et al., 2003; Staudinger et al., 2003). The regulation of Mrp3 by TCPOBOP has been described as CAR-dependent (Maglich et al., 2002), whereas induction by PB is CAR-independent (Xiong et al., 2002b; Cherrington et al., 2003). This could be due to differences in activation of CAR, whether by liganded activation as for TCPOBOP, or potentially by cell signaling, leading to activation of CAR (Swales and Negish, 2004). Induction of mouse Mrp3 by AhR, PPARα, and Nrf2 activators has not been previously described; therefore, these may represent new, novel mechanisms of regulation of Mrp3 in mouse liver.

In rats, mice, and humans, Mrp3 has been shown to be regulated by PB, DAS, and polychlorinated biphenyl 99 (PCB99), compounds that induce Cyp2B1/2 and are known or hypothesized CAR activators (Cherrington et al., 2002). Similar to Mrp2, Mrp3 is highly up-regulated by OTZ, suggesting that Nrf2 might be an important transcription factor that regulates Mrp3 (Cherrington et al., 2002). In humans, induction by β-naphthoflavone and rifampicin suggests that Mrp3 might be regulated via AhR or PXR, respectively (Schrenk et al., 2001; Hitzl et al., 2003). Overall, Mrp3 seems to be regulated similarly in rats, mice, and humans, with potential transcriptional regulation by AhR, PXR, CAR, PPARα, and Nrf2.

Mrp4 is localized to the basolateral membrane and can transport chemicals from hepatocytes into blood (Denk et al., 2004). Much like Mrp3, Mrp4 can transport bile acids, as well as many antiviral drugs such as PMEA (Reid et al., 2003; Zelcer et al., 2003). Data from the present study demonstrate that TCPOBOP and Nrf2 activators induce Mrp4 in mouse liver, indicating potential roles for CAR and Nrf2 in the regulation of mouse Mrp4. In rats, Mrp4 is induced in liver by the Nrf2 activators EXQ and OTZ (Chen and Klaassen, 2004). Little data

![Fig. 3. Expression of Mrp4 (above), Mrp5 (middle), and Mrp6 (bottom) mRNA in liver after treatment with prototypical drug-metabolizing enzyme inducers. The abbreviations used are the same as in Fig. 1. Total RNA from liver of chemically treated male C57BL/6 mice (n = 5/treatment) was analyzed by the bDNA assay. Data are presented as mean RLU ± S.E.M. *, statistically significant difference between treated and control mice (p < 0.05).](image-url)
exists on induction of Mrp4 in humans; however, studies in CAR-null mice have definitively shown that induction of Mrp4 by TCPOBOP and PB is via CAR (Assem et al., 2004). Taken together, the most likely means of induction of Mrp4 is by transcriptional activation by CAR and Nrf2.

Mrp6 is thought to be localized on the basolateral membrane in hepatocytes, but may have canalicular localization as well (Madon et al., 2000). The only substrate that has been found to be transported with relatively high affinity by Mrp6 is the endothelin antagonist BQ-123; however, overexpression of Mrp6 does lead to weak resistance to chemotherapeutic drugs (Belinsky et al., 2002). Few chemicals have been observed to modulate expression of Mrp6 in rats or humans, and a battery of inducers similar to those used in this study showed no changes in Mrp6 expression in rat (data not shown). However, in this study, AhR, CAR, and Nrf2 activators induced expression of Mrp6 in mouse liver.

One of the patterns of Mrp expression of note is that AhR and Nrf2 activators often induce the same transporter (i.e., Mrp2, -3, -5, and -6). Several genes known to be regulated by Nrf2 are also regulated in a similar manner compared with these Mrps. Rat UDP-glucuronosyltransferase 1A6 is induced by oltipraz, a classical Nrf2 activator, and oltipraz induction of UDP-glucuronosyltransferase 1A6 is dependent on the binding of AhR to the xenobiotic response element (Auyeung et al., 2003). Furthermore, one of the known target genes of Nrf2 activation, Nqo1, can be induced by the classical AhR ligand TCDD, and that induction was Nrf2-dependent (Ma et al., 2004). Although the mechanism of this cross-activation is not well defined, Mrps may share a similar pattern of inducibility to the phase I and II enzymes known to be regulated by these two receptors. Thus, it is unclear whether the induction of Mrps by some of the microsomal enzyme inducers is mediated through direct mechanisms (transcription factor binding to its cognate response element) or indirect mechanisms that involve some sort of “cross talk” (activation of multiple receptors by a chemical and/or transcriptional up-regulation of another gene or transcription factor that acts on the gene of interest).

In conclusion, these results demonstrate Mrp2, -3, -5, -6, and -7 are induced by AhR ligands (Table 2). The potent and efficacious CAR activator TCPBOP induces mRNA expression of Mrp2–7, although only Mrp3 was induced by all three CAR activators administered (Table 2). PXR ligands PCN and SPR induced mRNA expression of Mrp3 in mouse liver. Mrp3 was induced by all three PPAR agonists administered, whereas the Nrf2 activators induced expression of Mrp2–6. In conclusion, the data demonstrate that a variety of microsomal enzyme inducers increase expression of Mrps in mouse liver and suggest that coordinate regulation mediated by AhR, CAR, PXR, PPARα, and Nrf2 may exist for genes involved in drug metabolism and disposition.

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**TABLE 2**

The number of receptor-activating chemicals in a class that alter mRNA expression of Mrps after treatment

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<th>PPARα</th>
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<td>Mrp2</td>
<td>↑(3/3)</td>
<td>↓(2/3)</td>
<td>↑(1/3)</td>
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<tr>
<td>Mrp3</td>
<td>↑(3/3)</td>
<td>↑(2/3)</td>
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<tr>
<td>Mrp4</td>
<td>↑(3/3)</td>
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<td>Mrp5</td>
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<td>Mrp6</td>
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<td>Mrp7</td>
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<td>Mrp9</td>
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*Statistically significant difference between treated and control mice (p < 0.05).
References


