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Phenobarbital Alters Hepatic Mrp2 Function by Direct and Indirect Interactions

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ABSTRACT

Phenobarbital (PB) treatment impairs the biliary excretion of some organic anions. One mechanism may involve direct competition for biliary excretion by PB and/or a PB metabolite. Alternatively, PB may alter the expression and/or function of hepatic organic anion transport proteins. The role of multidrug resistance-associated protein 2 (Mrp2) in the biliary excretion of PB and metabolites was studied using isolated perfused livers (IPLs) from Wistar and Mrp2-deficient TR$^-$ rats. In normal livers, 4.19 ± 0.53% of the PB dose was recovered in bile as PB metabolites [2.21 ± 0.69% as 5-ethyl-5-(4-OH phenyl) barbituric acid (PBOH)-glucuronide; 1.98 ± 0.09% as PBOH-sulfate]. In TR$^-$ livers, only PBOH-sulfate was recovered in bile (0.35 ± 0.16% of dose) during the 2-h perfusion. Mrp2 message was increased (2.3-fold) by PB pretreatment (80 mg/kg i.p. × 4 days) but decreased to control values after a 48-h washout. Mrp2 protein was increased slightly in PB-treated livers and remained slightly elevated after a 24-h washout, but it was decreased significantly to 62 ± 7% of control values after a 48-h washout. The 120-min cumulative biliary excretion of the Mrp2 substrate 5-(and-6)-carboxy-2′,7′-dichlorofluorescein in IPLs from PB-treated rats after a 48-h washout was significantly lower than in vehicle-treated livers (66.3 ± 9.2% versus 83.4 ± 2.4% of the dose, respectively). These data support two mechanisms for impaired biliary excretion of some organic anions by PB treatment: 1) PBOH-glucuronide is a substrate for Mrp2 and may compete with other organic anions for biliary excretion and 2) Mrp2 protein expression and functional capacity is decreased 48 h after PB treatment.

Phenobarbital (PB), a prototypical inducer of cytochrome P450 mixed-function oxidase and UDP-glucuronosyltransferase systems enhances metabolite formation (Sher, 1971; Bock et al., 1973) and alters biliary excretion of some organic anions (Brouwer and Jones, 1990; Gregus et al., 1990). The influence of PB on the hepatobiliary disposition of xenobiotics is dependent in part on the chemical nature of the substrate and its biotransformation products. In rats, PB treatment significantly impaired the biliary excretion of the glucuronide metabolites of morphine (Roerig et al., 1974), valproic acid (Watkins and Klaassen, 1982), and acetaminophen (Brouwer and Jones, 1990; Studenberg and Brouwer, 1992). Impaired biliary excretion of acetaminophen glucuronide was demonstrated in vivo at 24 h and in isolated perfused rat livers at 48 h after a standard 5-day in vivo enzyme-inducing regimen of PB (Brouwer and Jones, 1990; Studenberg and Brouwer, 1992). Impaired biliary excretion of acetaminophen glucuronide also was observed when a bolus dose of acetaminophen was coadministered with a bolus dose of PB in the isolated perfused rat liver (Studenberg and Brouwer, 1992).

In addition to metabolic interactions, two explanations for PB-impaired biliary excretion of glucuronide conjugates have been proposed. First, hepatocellular PB and/or a PB metabolite may directly impair biliary excretion of organic anions at a canalicular transport site. PB metabolites, specifically PBOH-glucuronide and PBOH-sulfate, undergo significant biliary excretion (Cooper et al., 1979). Given the long in vivo half-life of PB (approximately 9 h in rats) (Brouwer et al., 1984), circulating PB concentrations in serum of 13.4 ± 4.2 μg/ml have been reported up to 24 h after PB administration (Brouwer and Jones, 1990). Thus, it is conceivable that im-

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ABBREVIATIONS: PB, phenobarbital; HRP, horseradish peroxidase; CDF, 5-(and-6)-carboxy-2′,7′-dichlorofluorescein; DTT, dithiothreitol; LDH, lactate dehydrogenase; Mrp, multidrug resistance-associated protein; Oatp, organic anion transporting polypeptide; PBOH, 5-ethyl-5-(4-OH phenyl) barbituric acid; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidine difluoride; TBST, Tris-buffered saline/Tween 20; IPL, isolated perfused liver.

paired biliary excretion of certain organic anions may be caused by competition between a substrate and PB or a PB metabolite for a common carrier into bile. The canalicular multidrug resistance-associated protein 2 (Mrp2) (AbcC2; canalicular multispecific organic anion transporter) exports glutathione *S*-conjugates, leukotriene C4, steroid conjugates, and glucuronidated and sulfated bile salt conjugates into bile, and it may be a common transporter for glucuronides (Oude Elferink et al., 1995; Jedlitschky et al., 1996; Konig et al., 1999a).

A second explanation for the impaired biliary excretion of glucuronide conjugates is that PB treatment may cause changes in the expression and/or function of transport proteins that alter their capacity to excrete substrates into the blood or bile. Thus, PB-associated perturbations in the expression of canalicular or basolateral transport proteins may alter the disposition of glucuronide conjugates. The basolateral efflux transporter Mrp3 (AbcC3) has a substrate specificity similar to that of Mrp2, and its expression and function increase when Mrp2-mediated biliary excretion is compromised (Ortiz et al., 1999; Ogawa et al., 2000; Xiong et al., 2000). PB treatment (80 mg/kg/day × 5 days) markedly induced Mrp3 protein after a 24-h washout, and the basolateral clearance of the Mrp3 substrate acetaminophen glucuronide was increased in proportion to Mrp3 expression while biliary clearance was impaired (Xiong et al., 2002). Also, in cholestasis, the canalicular efflux of organic anions by Mrp2 is impaired, and the expression of the organic anion transporting polypeptide 2 (Oatp2) which may function as a bidirectional transporter, is increased, thus enhancing the sinusoidal efflux of potentially toxic bile salts (Trauner et al., 1997, Oswald et al., 1998). Hagenbuch et al. (2001) reported that the hepatic expression of Oatp2 increased to 256 ± 68% at the RNA level and 223 ± 26% at the protein level in PB-treated rats (80 mg/kg/day × 5 days) after a 24-h washout period.

Elucidation of the mechanisms of interaction between PB and hepatic transport proteins would help to delineate potential sites of drug-drug interactions in hepatobiliary transport. This study investigated the role of Mrp2 in the biliary excretion of PB and metabolites with the use of Mrp2-deficient TR$^-$ rats (Jansen et al., 1985). In addition, the effect of PB treatment on the mRNA and protein expression of Mrp2 was determined in rats at several time points after PB treatment. Mrp2 function also was assessed by quantifying the biliary excretion of 5-(and-6)-carboxy-2′,7′-dichlorofluorescein in isolated perfused livers (IPLs) from vehicle- and PB-treated rats under the conditions in which the most significant alteration in Mrp2 protein was noted.

Materials and Methods

Chemicals. The sodium salt of PB, β-glucuronidase (from *Helix pomatia*), D-saccharic acid-1,4-lactone, phenylmethylsulfonyl fluoride (PMSF), EDTA, dithiothreitol (DTT), caffeine, and the lactate dehydrogenase (LDH) assay kit were obtained from Sigma Chemical Co. (St. Louis, MO). 5-Ethyl-5-(4-OH phenyl) barbituric acid (PBOH) was purchased from Aldrich Chemical Co. (Milwaukee, WI). 5-(and-6)-Carboxy-2′,7′-dichlorofluorescein (CDF) was obtained from Molecular Probes (Eugene, OR). Rabbit antisera raised against Mrp2 (Madon et al., 2000) were used. The specificity of the antisera was confirmed previously in vesicles derived from Sf9 cells expressing the proteins and isolated plasma membrane vesicles (data not shown). Anti-mouse actin antibody was purchased from Chemicon International (Temecula, CA). Bis-Tris (4 to 12% gradient gels) and electrophoresis reagents were obtained from Invitrogen (Carlsbad, CA). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Corporation (Bedford, MA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody was purchased from Amersham Biosciences Inc. (Piscataway, NJ) and HRP-conjugated rabbit anti-mouse antibody was purchased from Zymed Laboratories (South San Francisco, CA). LumiGlo chemiluminescent peroxidase substrate kit was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

Animals. Male Wistar rats, obtained from Charles River (Raleigh, NC), or Mrp2-deficient (TR$^-$) male Wistar rats (breeding colony of the Academic Medical Center, Amsterdam, the Netherlands) were used as liver donors. Male Wistar retired breeders (Charles River) were used as blood donors. All animals were housed on wood-chip bedding, maintained on a 12-h light/dark cycle with access to rodent chow and water ad libitum. Rats (275–290 g) were treated with PB (80 mg/kg i.p.) or saline vehicle for 4 days followed by a 0-, 24-, or 48-h washout period.

Isolated Perfused Liver Studies. Liver donor rats were anesthetized with ketamine/xylazine (60/20 mg/kg i.p.), bile ducts were cannulated, and the livers were isolated and perfused initially with Krebs-Ringer bicarbonate buffer (118 mM NaCl, 5 mM KCl, 1.1 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 25 mM $CaCl_2$, and 55.5 mM glucose, pH 7.4). The isolated livers were transferred to a temperature-controlled chamber, and perfusion was continued with 80 ml of recirculating Krebs-Ringer-bicarbonate buffer containing 20% whole Wistar rat blood. Taurocholate (30 μmol/h) was infused into the reservoir throughout the 2-h perfusion to maintain bile flow. Perfusate samples for LDH determination were collected at 30-min intervals, kept at room temperature, and analyzed within 24 h. Livers remained viable during the first 120 min of the perfusion as measured by portal vein pressure (60–80 mm H_2O), bile flow, gross liver morphology, and LDH leakage into perfusate (<20 IU/l) (Hong et al., 1998).

After equilibration of the liver and collection of baseline bile and perfusate samples, a bolus dose of PB (5 μmol in saline) was added to the perfusate in the reservoir. Perfusate and bile samples were collected continuously from control and TR$^-$ livers at 15-min intervals for 2 h. The stability and extent of binding of PB to the perfusion tubing and apparatus were determined in a control experiment in the absence of a liver. Bile flow was determined gravimetrically, assuming a bile density of 1 g/ml. Perfusate and bile samples for high performance liquid chromatography analysis of PB and metabolites were stored at −80°C. CDF (10 mM in dimethyl sulfoxide; <0.05% v/v) was infused for 35 min at a rate of 0.1 μmol/min into the perfusate reservoir of IPLs obtained from vehicle- or PB-treated rats after a 48-h washout period. Perfusate and bile samples were collected at 15-min intervals, frozen on dry ice upon collection, and stored at −80°C until further analysis. Preliminary studies indicated that CDF was stable in perfusate and bile under these conditions.

Assay Methodology for PB and PB Metabolites. The glucuronide and sulfate moieties of PBOH were cleaved enzymatically with β-glucuronidase containing sulfatase activity to yield PBOH. The amount of PBOH generated from PBOH-sulfate in the incubation medium was determined in separate incubations by the addition of D-saccharic acid-1,4-lactone, a β-glucuronidase inhibitor. Bile (10 μl) or perfusate samples (40 μl) were incubated in 100 μl of 0.2 M sodium acetate buffer, pH 5, containing β-glucuronidase/sulfatase from *H. pomatia* (7500 U/ml) at 37°C for 24 h. An identical set of samples was incubated in the presence of D-saccharic acid-1,4-lactone (1.8 mg/ml). Standard curves containing PB (10–200 μg/ml) and PBOH (5–250 μg/ml) in bile and perfusate samples were generated in parallel.

Ice-cold acetonitrile (100 μl) containing 4 μg of caffeine (internal standard) was added to incubation samples and vortexed, and precipitated proteins were removed by centrifugation. The supernatant was evaporated to dryness, and the samples were reconstituted in

150 μl of mobile phase. A volume of 50 μl was injected onto an isocratic high performance liquid chromatography system, equipped with a 150 × 4.6 mm column (Adsorbosphere-HS C_{18}, 3 μm; Alltech Associates, Deerfield, IL) connected to a UV detector set at 254 nm. The mobile phase used for bile sample analysis was 50 mM sodium phosphate buffer, pH 7.65, with 20% methanol (retention times: PBOH, 8.3 min; PB, 9.4 min; caffeine, 11.1 min), and that used for perfusate samples was 50 mM sodium phosphate buffer, pH 8.0, with 30% methanol (retention times: PBOH, 4.9 min; PB, 8.3 min; caffeine, 13.9 min). The intraday CV was <5%, and the interday CVs for PB and PBOH were less than 5 and 7%, respectively.

Assay for CDF in Bile. The amount of CDF in bile samples was determined using a spectrofluorometric assay (excitation at 505 nm; emission at 523 nm). Standard curves containing CDF (0.5 to 100 nM) were prepared in phosphate-buffered saline, pH 7.4. Background fluorescence in CDF-free bile and perfusate samples was negligible.

Liver Membrane Preparations. Livers from vehicle- or PB-treated male Wistar ether-anesthetized rats were excised after portal vein perfusion with 20 ml of phosphate-buffered saline. The expression of transporter proteins after ether, ketamine/xylazine, or decapitation was shown to be identical as measured by Western blot analysis in preliminary studies. Vehicle- or PB-treated livers were processed to enrich integral membrane proteins in the preparation by extraction with 0.1 M Na_2CO_3 (Bergwerk et al., 1996). Briefly, excised livers were minced with scissors, rinsed, and homogenized with 20 strokes using a motor-driven Potter-Elvehjem homogenizer in 4 volumes (w/v, wet liver weight) of ice-cold buffer A (1 mM $NaHCO_3$ containing 50 μM PMSF, 1 μg/ml of aprotinin, and 1 μg/ml of leupeptin). The homogenate was brought to a final volume of 10.6 ml/g of wet liver with addition of buffer B (buffer A with 1 mM EDTA). Aliquots (2 ml) of the resulting mixture were extracted in 40 ml of buffer C (0.1 M Na_2CO_3 containing 50 μM PMSF and 1 μg/ml each of aprotinin and leupeptin) with gentle agitation for 15 min at 4°C followed by centrifugation at 100,000*g* for 60 min. The resulting pellets were reconstituted in buffer B and stored in suspension at −80°C. Protein concentrations in the membrane preparations were measured by the method described by Lowry et al. (1951).

RNA Isolation and Northern Analysis. Total RNAs from PB- or vehicle-treated livers were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. For Northern blot analysis, 20 μg of total RNA was denatured and separated in a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to Hybond-N membrane (Amersham Biosciences). Blots were prehybridized in hybridization solution (Rapid-hyb buffer; Amersham Biosciences) for 1 h at 65°C followed by hybridization with Mrp2 cDNA fragments labeled with [α-^{32}P]dCTP by random primer extension (Ambion, Austin, TX). After 4 h of incubation at 65°C, the blots were washed once with 2× standard saline citrate/0.1% SDS at room temperature and twice with 0.1× standard saline citrate/0.1% SDS at 65°C. Equivalent loading of RNA samples was confirmed by hybridizing the same blot with a 28S ribosomal RNA. Membranes were exposed to phosphor image film for 12 h. Northern blot densitometric analysis was expressed as the ratio of the Mrp2/28S rRNA signals in the phenobarbital-treated group to the Mrp2/28S rRNA signals in the control group for the relevant washout period, expressed as a percentage.

Gel Electrophoresis and Western Blotting. Isolated membrane protein preparations were suspended in sample buffer containing 50 mM DTT, heated for 5 min at 70°C, and loaded (20–50 μg/well) onto 10-well 4 to 12% Bis-Tris gels. Molecular weight standards, as well as isolated canalicular and basolateral liver plasma membrane preparations were loaded as positive control markers for individual proteins. Proteins were resolved under reducing conditions at a constant voltage (150 V) for 2 h. At the end of the run, proteins were electrophoretically transferred onto PVDF membranes at a constant current (160 mA) for 1 h. The blots were blocked overnight at room temperature with 5% dry milk in Tris-buffered saline/Tween 20 (TBST; pH 7.4) and incubated with Mrp2 (1:4000 dilution) antisera in TBST for 1 h. After several washes with TBST, the blots were incubated with HRP-conjugated goat anti-rabbit antibody (1:4000) in TBST for 1 h and then washed several times again. Antigens were detected using a LumiGlo chemiluminescent reagent kit according to the manufacturer's directions. Membranes were stripped according to the manufacturer's directions (LumiGlo chemiluminescent reagent kit), reprobed with anti-mouse actin antisera (1:1500 dilution), and actin bands were detected with HRP-conjugated rabbit anti-mouse antibody (1:2000 dilution). Basolateral and canalicular membrane preparations also were loaded onto gels as positive controls for the identification of domain-specific protein bands. Densitometric analysis of the blots was linear for Mrp2 over a range of 5 to 15 μg of loaded canalicular liver plasma membrane protein. Exposure of blots to film was minimized to avoid saturation of the signal. Western blot densitometric analysis was expressed as the ratio of the Mrp2/actin signals in the phenobarbital-treated group to the Mrp2/actin signals in the control group for the relevant washout period, expressed as a percentage.

Data Analysis. The Student's two-tailed *t* test was used to determine statistically significant differences between normal and TR^- livers and between vehicle- and PB-treated livers. In all cases, data were presented as mean ± S.D., and the criterion for statistical significance was $p < 0.05$.

Results

Biliary Excretion of PBOH-Glucuronide by Mrp2. As expected, initial bile flow in TR^- rat livers (0.28 ± 0.13 μl/min/g of liver) was lower than in normal rat livers (0.75 ± 0.10 μl/min/g of liver); livers remained viable throughout the 2-h perfusion. Approximately 100% of the PB dose was recovered in the perfusate in the absence of a liver (data not shown). In the presence of a liver, perfusate concentrations of PB rapidly declined within the first 15 min of administration; 55.1 ± 15.6 and 42.7 ± 2.6% of the dose was recovered in perfusate at the end of the 2-h perfusion as unchanged PB in normal and TR^- livers, respectively. The majority of the remainder of the dose was recovered as PBOH-sulfate in perfusate at the end of the 2-h perfusion. The accumulation of PBOH equivalents in normal and TR^- bile derived from PBOH-glucuronide and PBOH-sulfate is plotted in Fig. 1, A and B, respectively. In normal livers, 4.19 ± 0.53% of the PB dose was recovered in bile as PB metabolites (2.21 ± 0.69% as PBOH-glucuronide, 1.98 ± 0.09% as PBOH-sulfate). In TR^- livers, only 0.35 ± 0.16% of the PB dose was recovered as PBOH-sulfate in bile; negligible PBOH-glucuronide was excreted in TR^- bile. PB and PBOH were not detected in normal or TR^- bile before incubation with β-glucuronidase/sulfatase.

Effects of PB Treatment on Mrp2 mRNA. Northern Blot analysis (Fig. 2A) indicated that Mrp2 mRNA was significantly increased after 4 days of PB treatment to 227 ± 81% of control values. The message was still significantly increased (1.6-fold) in the PB-treated group after a 24-h washout. Mrp2 mRNA had returned to control levels (97 ± 25% of control) after a 48-h washout period after 4 days of PB treatment.

Effects of PB Treatment on Mrp2 Protein. Gel electrophoretic resolution of Mrp2 was achieved under reduced conditions [addition of DTT to the samples and NuPAGE antioxidants (Biocompare, Inc., South San Francisco, CA) to electrophoresis buffers], which was favorable over nonreduced conditions. The reduced conditions gave a single sharp

band at 180 kDa for Mrp2; with nonreduced conditions, band broadening, splitting, and smearing were noted in preliminary studies. The use of liver membranes prepared by the Na_2CO_3 extraction method used by Bergwerk et al. (1996) was preferred over liver homogenates or membranes from differential centrifugation methods (preliminary work in our laboratory). The carbonate extraction procedure enabled a clear enrichment of the desired membrane proteins and gave stronger immunoblot signals for transport proteins compared with protein signals from membranes obtained with other methods (data not shown).

Densitometric analysis of Western blots for Mrp2 (30 μg of protein loaded per lane) indicated that Mrp2 expression was increased to 109 ± 13% of control values after a 0-h washout period and 121 ± 15% of control values after a 24-h washout period in PB-treated livers, although these differences were not statistically significant (Fig. 2, B and C). The most notable difference was the significant decrease in Mrp2 expression to 62 ± 7% of control values in PB-treated livers after a 48-h washout period.

Effects of PB on CDF Biliary Excretion. Initial bile flow in IPLs from vehicle- and PB-treated rats after a 48-h washout period was comparable (1.02 ± 0.07 and 0.85 ± 0.10 μl/min/g of liver, respectively), and livers remained viable

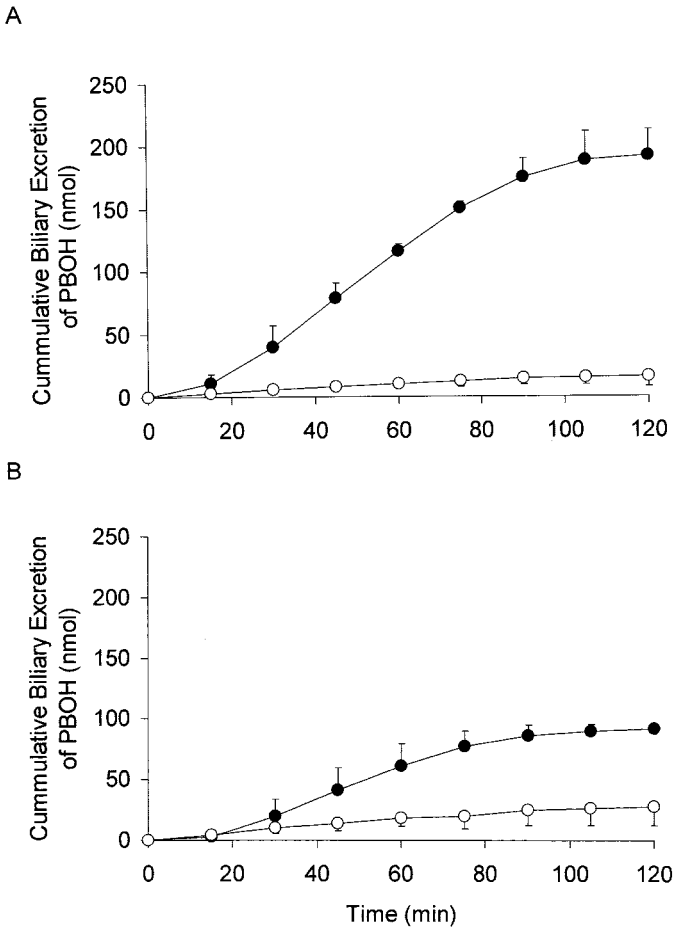

Fig. 1. Cumulative biliary excretion of PBOH-glucuronide plus PBOH-sulfate (A) and PBOH-sulfate (B) in isolated perfused livers from normal (●) and TR^- (○) rats after administration of 5 μmol PB to the recirculating perfusate reservoir (mean ± S.D.; n = 3). PBOH-glucuronide and PBOH-sulfate were measured as PBOH after enzymatic hydrolysis of metabolites as described under *Materials and Methods*.

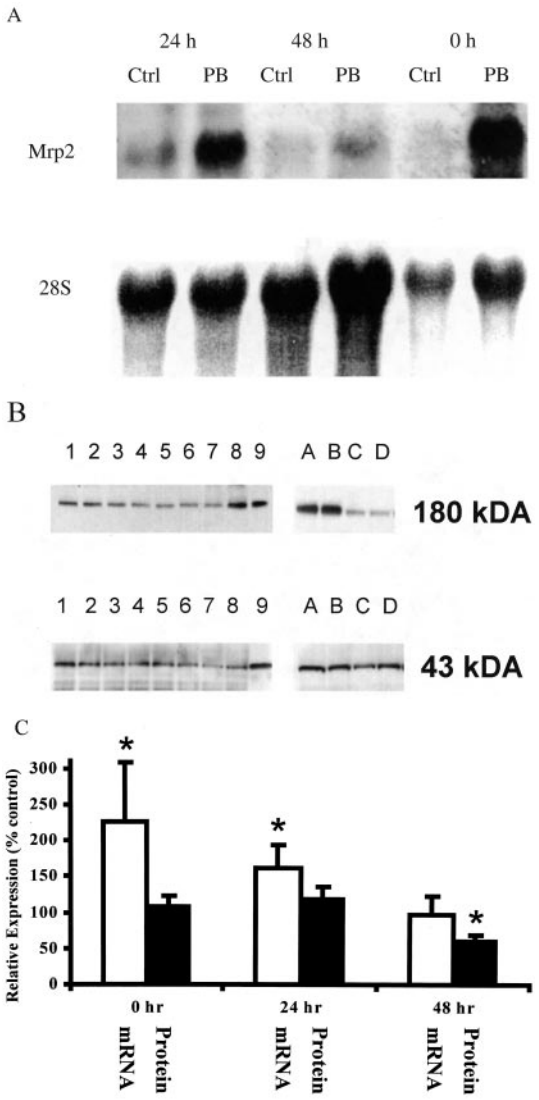

Fig. 2. A, Northern blot detection of Mrp2 mRNA in livers obtained from PB- or vehicle-treated rats after a 24- and 48-h washout or no washout. A total of 20 μg of RNA was denatured and separated in a 1.2% formaldehyde agarose gel and transferred to a Hybond-N membrane. After hybridization with ^{32}P-labeled Mrp2 cDNA, bands were detected by exposure to film. Equivalent loading of RNA samples was confirmed by hybridizing the same blot with a 28S ribosomal RNA. B, effect of PB treatment on expression of Mrp2 and actin in rat liver preparations. Membrane protein-enriched rat liver homogenates (30 μg per lane) were subjected to 4 to 12% gradient SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto PVDF membranes and probed with antisera (1:4000 dilution) raised against the C terminus of Mrp2. Single 180-kDa protein bands were visualized by chemiluminescence in membrane preparations obtained 0 h after vehicle treatment (lanes 1 and 2), 0 h after PB treatment (lanes 3 and 4), 24 h after vehicle treatment (lanes 5 and 6), 24 h after PB treatment (lanes 7 and 8), purified canalicular liver plasma membrane preparation (lane 9), 48 h after vehicle treatment (lanes A and B), and 48 h after PB treatment (lanes C and D). Bands 1 to 9 and A to D were run on separate gels; the latter was exposed longer to film to better present the difference between control and PB-treated livers. Blots were stripped and reprobed for actin (43 kDa). C, densitometric analysis of Mrp2 and actin expression in membrane preparations from control and PB-treated rat livers 0-, 24-, and 48-h after PB or vehicle (control) treatment. Band densities associated with Mrp2 from PB-treated liver preparations, expressed as the percentage of control, were normalized to lane-specific actin signal (■; mean ± S.D., n = 7). Densitometric analysis of Mrp2 mRNA and 28S rRNA in liver preparations from control and PB-treated rat livers 0-, 24-, and 48-h after PB or vehicle (control) treatment. Band densities associated with Mrp2 mRNA from PB-treated liver preparations, expressed as the percentage of control, were normalized to lane-specific 28S rRNA signal (□; mean ± S.D., n = 3) (*, $p <$ 0.05, PB versus control).

during the 2-h perfusion. The infusion vehicle (<0.5% v/v dimethyl sulfoxide in 80 ml of perfusate) did not compromise bile flow or liver viability in this study. After infusion of 3.5 μmol of CDF over 35 min to isolated perfused vehicle-treated livers ($n = 4$), 83.4 ± 2.4% of the dose was excreted into bile as CDF by 120 min (Fig. 4); the 120-min cumulative biliary excretion of CDF in PB-treated livers ($n = 4$) was significantly lower (66.3 ± 9.2% of the dose, $p = 0.011$). Perfusate concentrations of CDF at 120 min were approximately 2-fold higher in PB-treated compared with vehicle-treated livers (16.4 ± 9.2% versus 7.96 ± 2.85% of the dose). The results of this study demonstrate that PB treatment of rats (80 mg/kg i.p. for 4 days) followed by a 48-h washout period impaired the extent of biliary excretion of CDF, a Mrp2 substrate.

Discussion

PB impairs the biliary excretion of some organic anions, particularly glucuronide conjugates of acetaminophen (Brouwer and Jones, 1990; Gregus et al., 1990; Studenberg and Brouwer, 1992), valproic acid (Watkins and Klaassen, 1982), and morphine (Roerig et al., 1974). Mutual competition between PB or a PB metabolite with organic anions for a common canalicular transport protein may be one mechanism of these interactions. Mrp2 generally recognizes glucuronides as substrates for biliary excretion (Oude Elferink et al., 1995; Konig et al., 1999a). Results of the present study demonstrate that biliary excretion of PBOH-glucuronide was absent in Mrp2-deficient TR^- rats, a finding which indicates that this metabolite of PB is a substrate for Mrp2. Furthermore, the biliary excretion of PBOH-sulfate was impaired approximately 60% in TR^- rats. PBOH-sulfate may be excreted into bile by Mrp2 as well as one or more mechanisms that is/are preserved in TR^- rats. Collectively, these data suggest that impaired biliary excretion of glucuronide metabolites noted after PB treatment may be caused, in part, by direct competition between PBOH-glucuronide and anionic metabolites for Mrp2 transport. Alternately, competition could also occur at the level of glucuronidation by UDP glucuronosyltransferases. However, glucuronidation is a low-affinity and high-capacity pathway; hence, at the relatively low concentrations of phenobarbital achieved in this study, competitive inhibition seems unlikely.

A second mechanism that could explain the impaired biliary excretion of organic anions by PB is altered expression of hepatic transport proteins resulting in functional changes in hepatobiliary transport. This mechanism could explain the 5-fold impairment in biliary excretion of acetaminophen glucuronide in PB-treated rats after a 48-h washout period, when hepatic concentrations of PB and PB metabolites are expected to be low (Studenberg and Brouwer, 1992). To further evaluate this hypothesis, the expression of Mrp2 was examined after a 0-, 24-, and 48-h washout period after PB treatment. Mrp2 mRNA was increased markedly after PB treatment, but it declined during the 48-h washout period to control levels. Mrp2 protein was slightly increased after PB treatment and remained modestly elevated, although these differences failed to reach statistical significance. These results are in agreement with the findings of Hagenbuch et al. (2001), who reported a modest increase in Mrp2 expression at the RNA level based on Northern blot analysis after PB treatment without a washout, and of Ogawa et al. (2000), who reported a nonsignificant (1.4-fold) increase in Mrp2 protein measured by Western blot in PB-treated rats after a 24-h washout period. Elevated Mrp2 expression immediately after long-term PB treatment has been implicated in increased dibromosulfophthalein plasma and biliary clearance (Johnson and Klaassen, 2002). Interestingly, the expression of Mrp2 was significantly decreased after PB treatment and a 48-h washout period. The lack of correlation between Mrp2 transcription and translation has been reported previously (Johnson et al., 2002). Xiong et al. (2002) demonstrated that Mrp3 protein, a basolateral organic anion efflux transporter (Konig et al., 1999b; Kool et al., 1999), was significantly increased in PB-treated rats (80 mg/kg/day i.p. × 4 days) after a 24-h washout period. Likewise, Rausch-Derra et al. (2001) reported that PB significantly induced transcription and expression of Oatp2, a basolateral bidirectional transporter. Increased basolateral egress of organic anions observed after in vivo PB treatment and a 48-h washout (Brouwer and Jones 1990; Studenberg and Brouwer, 1992) may be attributed, in part, to up-regulation of one or more of these basolateral organic anion transporters, whereas Mrp2 protein expression and function is decreased.

CDF, a model Mrp2 substrate that is selectively transported into bile by Mrp2 (Kitamura et al., 1990), was used to examine the functional effect of PB on Mrp2-mediated biliary excretion 48 h after PB treatment when circulating concentrations of PB and metabolites are negligible. The observed decrease in CDF biliary excretion (21% decrease) (Fig. 3) corresponds to the decrease in Mrp2 protein observed by Western blot analysis 48 h after PB treatment (Fig. 2, B and C). The PB-associated decrease in Mrp2 expression (38% decrease) was expected to represent a decrease in the capacity of the transporter. Functionally, this effect should result in reduced CDF excretion into bile. A PB-induced increase in Mrp3 protein also would be expected to contribute to increased CDF perfusate concentrations because CDF is a Mrp3 substrate and Mrp3 is up-regulated after PB pretreatment (Xiong et al., 2002). These results further support the hypothesis that the modulation of hepatobiliary transport proteins by direct and indirect interactions can influence the extent of biliary excretion and route of elimination of some substrates.

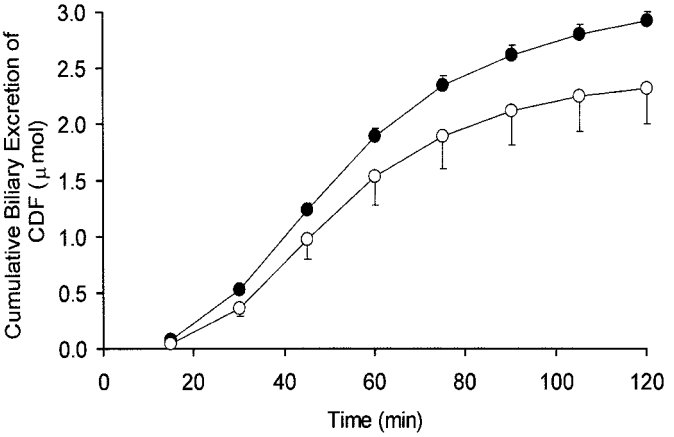

Fig. 3. Cumulative biliary excretion of CDF after infusion of CDF into the perfusate reservoir over 35 min at a rate of 0.1 μmol/min 48 h after vehicle (●; $n = 4$) or PB treatment (○; $n = 4$) (80 mg/kg i.p. × 4 days). Bile fluorescence associated with CDF was measured at $Ex_{505\ nm}$ and $Em_{523\ nm}$ (mean ± S.D., $n = 4$).

Studies aimed at investigating the hepatobiliary disposition of organic anions during PB coadministration or after PB treatment should be designed carefully with consideration of the effects of PB on the expression and function of Mrp2 and potentially other transport proteins. This study clearly demonstrates that PB may alter the hepatobiliary disposition of drugs and metabolites by interacting with the transport proteins in one or more ways. First, a direct competition for biliary excretion at the Mrp2 transport site between PBOH-glucuronide and other glucuronide conjugates or organic anions may exist because PBOH-glucuronide is an Mrp2 substrate. Second, PB treatment alters the expression of Mrp2, resulting in functional changes in the hepatobiliary disposition of Mrp2 substrates such as CDF. This study emphasizes the complexities of PB-associated alterations in the hepatobiliary disposition of Mrp2 substrates and identifies potential mechanisms of drug interactions in the hepatobiliary system.

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