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EFFECT OF PHENOBARBITAL ON THE EXPRESSION OF BILE SALT AND ORGANIC ANION TRANSPORTERS OF RAT LIVER

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Abstract

Background/Aims. The hepatic clearance of drugs and cholephilic organic anions is stimulated by phenobarbital (PB). Our aim was to analyze the effects of PB on the expression of hepatocellular bile salt and organic anion transporters. *Methods.* Male Sprague-Dawley rats were treated intraperitoneally with PB (80 mg/kg/d) or saline for 5 days. Transporter expression was quantified by northern and western blot analysis and initial uptake rates of bromosulphophthalein (BSP) and digoxin were measured in isolated hepatocytes. *Results.* Compared to control rats, PB treatment increased expression of the organic anion transporting polypeptide 2 (Oatp2) more than 2fold on the RNA ($P<0.05$) and protein ($P<0.001$) levels. Expression of Oatp1, Oatp4 and the Na^+-taurocholate cotransporting polypeptide (Ntcp) was unaltered. At the canalicular pole, expression of the bile salt export pump (Bsep) and of the multidrug resistance proteins 2 (Mrp2) and 6 (Mrp6) was not significantly changed. Whereas hepatocellular BSP uptake was unaffected by PB, digoxin uptake was stimulated 4fold. *Conclusions.* The induction of digoxin uptake by PB correlates with Oatp2 expression. In contrast, the lack of increase of Oatp1 and Oatp4 expression is in accordance with unchanged BSP uptake. These data challenge the previously held view that PB induces hepatocellular BSP uptake systems.

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Key words: membrane proteins – drug transport – gene expression – liver transport – bile formation – cholestasis

Introduction

Bile formation is driven by the continuous vectorial secretion of bile salts and other cholephilic organic anions from blood into bile. The transport pathways across hepatocytes are mediated by transport proteins at the basolateral and canalicular domains of hepatocytes. On the basolateral side, the Na^+-taurocholate cotransporting polypeptide (Ntcp) represents the chief uptake system for conjugated bile salts (1). For sodium-independent bile salt transport, at least three members of the family of organic anion transporting polypeptides, Oatp1, Oatp2 and Oatp4, are expressed at the basolateral membrane of hepatocytes (2,3). Oatp1 represents a major uptake system for the cholephilic dye bromosulphophthalein (BSP) but also transports other organic compounds including bile salts and numerous drugs (4,5). Oatp2 is a close homologue of Oatp1 and transports bile salts, cardiac glycosides and cyclic peptides, however BSP transport via Oatp2 is negligible (6-8). Oatp4 transports a variety of organic compounds including BSP and bile salts (3,9).

At the canalicular domain of the hepatocyte, two members of the ATP-binding cassette (ABC) superfamily of transporters mediate bile salt and organic anion secretion. The bile salt export pump (Bsep) is a major canalicular bile salt transporter of rat liver (10). Non-bile salt organic anions such as glutathione and glucuronic acid conjugates are substrates of the canalicular multidrug resistance protein Mrp2 (11-13).

Studies in humans have shown that phenobarbital (PB) increases the net hepatic clearance of the organic anions BSP and bilirubin from plasma (14). In rat studies, PB treatment enhanced the transport maximum for the excretion of bilirubin and BSP into bile (15,16). In isolated rat hepatocytes, the maximum uptake velocity of BSP was found to be stimulated more than

6fold following treatment of rats with PB (17). This effect was attributable neither to the induction of the intracellular BSP binding protein ligandin nor to the induction of UDP-glucuronyltransferase, which increased to 260% and 200% of controls, respectively, indicating a direct effect on basolateral BSP transport (17). A direct effect of PB – known to regulate expression of phenobarbital-responsive genes in the liver (18,19) – on basolateral BSP transporter expression was postulated. Since Oatp1 can account for approximately 50% of total hepatocellular BSP uptake (20,21) and Oatp4 is the second major BSP uptake system in rat hepatocytes (3), we investigated whether these carriers are induced by PB. To study whether additional, as yet unidentified basolateral BSP uptake systems are induced by PB, we measured initial BSP uptake rates in isolated hepatocytes. Our data show that the known increase in hepatic BSP clearance that is induced by PB is not a result of increased hepatocellular uptake.

Materials and Methods

Chemicals

All chemicals were of the highest degree of purity available and were available from commercial sources. Phenobarbital was obtained from Hänseler AG (Herisau, Switzerland).

Animals

Male Sprague-Dawley rats (150-250 g) were purchased from RCC Ltd. (Füllinsdorf, Switzerland) and kept under routine laboratory conditions with free access to standard laboratory chow and water. All studies were performed in accordance with the Swiss Federal regulations concerning animal care.

Treatments

Rats were treated with phenobarbital (PB) injected intraperitoneally at a dosage of 80 mg/kg body weight for five consecutive days. PB was freshly dissolved in 0.9% (wt/vol) NaCl at a concentration of 40 mg/mL prior to each treatment at 11 a.m. The control group was injected with 0.9% NaCl alone. Rats were starved on day 5 of the treatment period and were sacrificed on day 6 in the morning.

Northern blots

Total RNA was extracted from rat liver by the acid guanidinium phenol-chloroform procedure. Poly (A^+) RNA was isolated from total RNA using the PolyATtract System (Promega Corporation, Madison, WI). 2.5 µg poly (A^+) RNA were separated by denaturing formaldehyde gel electrophoresis and transferred to a Hybond-NX nylon membrane (Amersham International PLC, Buckinghamshire, UK). Blots were hybridized with an [α-

^{32}P]dCTP labelled DNA probe at 60°C over two hours using ExpressHyb solution (Clontech Laboratories Inc., Palo Alto, CA). The following cDNA probes were used: Oatp1 (20), Oatp2 (6), Mrp2 (22), Bsep (10), Ntcp (23), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and cytochrome P450 (CYP) II B1. The Oatp4 probe was obtained by PCR amplification of a fragment corresponding to nt 535-790 of the cloned rlst-1 (9) from rat liver cDNA. The resulting 0.255 kb amplicon was 100% identical with the published sequence on the nucleotide level.

Blots were washed 2 x 10 minutes in 2xSSC/0.1%SDS at room temperature and 1x15 minutes in 0.1xSSC/0.1%SDS at 60°C. Membranes were exposed to autoradiography film (Biomax, Kodak) and the autoradiographs were quantitated densitometrically with a CAMAG TLC Scanner II (Muttenz, Switzerland).

Isolation of microsomes

Rat liver microsomes were isolated according to Meier et al. (24) and extracted at pH 8.0 as described by Kast et al. (25). Protein was determined by a modification of the Lowry procedure (26).

Western Blot Analysis

Microsomes (75-150 µg) were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (27) and processed by standard Western blotting (28). The blots were probed with rabbit antisera raised against Oatp1 (5,29), Oatp2 (8), Ntcp (30), Bsep (10), Mrp2 (31) and Mrp6 (31) at appropriate dilutions. Bound antibodies were visualized with iodinated protein A and subsequent autoradiography. For comparison of expression levels, autoradiographs were scanned with a CAMAG TLC Scanner II (Muttenz, Switzerland).

Immunofluorescence studies

Rat livers were fixed by perfusion and semithin cryosections (0.5-1 μm) were incubated with antisera as described (30). Micrographs were taken with a Zeiss Axiophot epifluorescence microscope (Zeiss, Oberkochen, Germany).

Transport assays in isolated rat hepatocytes

Hepatocytes from PB treated rats (n=2) and control rats (n=2) were isolated by the two step collagenase perfusion method (19,20). Viability was > 85% as assessed by trypan blue exclusion. Cells (2 x 10^6) were seeded onto collagen coated 60 mm dishes in 3 ml Williams Medium E. After 3 hours, cells were prewashed in 3 x 2 ml NaCl uptake buffer (33). Subsequently uptake of [^{35}S]-BSP (1 μmol/L) and [^{3}H]-digoxin (10 nmol/L) was measured at 37° C. Uptakes were stopped by removal of the uptake solution and washing of cell monolayers in 4 x 3 ml ice-cold uptake buffer. Cells were lysed in 2 ml 1% Triton-X100. Protein concentration was measured with the bicinoic acid method as described (34). Triton-X100 was present in samples and standards at identical concentrations. 1 ml cell lysate was dissolved in 10 ml scintillation fluid (Optifluor; Packard Instruments, Meriden, CA) and radioactivity was determined in a Packard Tri-Carb 2200 CA liquid scintillation analyzer.

Statistical analysis

Transporter expression on the RNA and protein levels is shown as the mean ± one standard deviation for each treatment group. Expression levels between PB treated and saline treated control rats were compared using the unpaired Student *t* test (35). Statistical significance was assumed at $P < 0.05$.

Results

The body mass of the PB treated rats averaged 202 ± 18 g (n.s. vs. controls) with a liver mass of 9.6 ± 0.8 g (P<0.002 vs. controls) compared to a body mass of 203 ± 9 g and a liver mass of 7.6 ± 0.1 g in the control group. Expression of cytochrome P450 II B1, known to be induced by PB (36), was induced 35fold (Fig. 1).

Effect of phenobarbital on basolateral transport protein expression. For Oatp1, the ratio of Oatp1/GAPDH mRNA levels in the PB treated group decreased to 68 ± 33% compared to controls (n.s.) (Fig. 1). In western blot analyses, Oatp1 protein levels in the PB group amounted to 73 ± 26% of controls (n.s.) (Fig. 2), which is in the range of the PB-induced reduction of mRNA levels. A similar decrease was observed for the Na^+-dependent bile salt transporter Ntcp. PB treatment decreased the Ntcp/GAPDH ratio to 47 ± 11% of controls on the RNA (n.s.) (Fig. 1) and 51 ± 29% on the protein level (n.s.) (Fig. 2). Hence, although these changes did not reach statistical significance, there was a tendency towards downregulation of both Oatp1 and Ntcp expression secondary to PB treatment. For Oatp1, this tendency was also evident in morphological studies (Fig. 3). No effect of PB treatment was found on the expression of Oatp4 (Fig. 1).

In contrast to Oatp1, Ntcp and Oatp4, expression of Oatp2 was stimulated by PB treatment. Oatp2 mRNA was increased to 224 ± 91% of controls (P<0.05) relative to GAPDH (Fig. 1). On the protein level, an identical degree of Oatp2 stimulation was found in the PB group (223 ± 26% compared to controls, P<0.001) (Fig. 2). This PB induced upregulation of Oatp2 was most pronounced in the pericentral region of the hepatic lobules. Thus, while in control liver basolateral Oatp2 expression was weak or even absent in the innermost layer of perivenous

hepatocytes (8), Oatp2 expression was strong throughout zone 3 hepatocytes in the PB treated animals (Fig. 3). These results support preferential PB induction in centrilobular hepatocytes and indicate a differential effect of PB treatment on the expression of individual basolateral organic anion transporters.

Effect of PB on canalicular transport protein expression. Expression of the bile salt export pump Bsep was not significantly changed by PB treatment on both the RNA (Bsep/GAPDH ratio 180 ± 83% of controls, n.s., Fig. 4) and the protein (82 ± 17% of controls, n.s., Fig. 5) levels. For Mrp2, there was a tendency towards increased expression levels on the RNA level, although the changes did not reach statistical significance. The Mrp2/GAPDH mRNA ratio in the PB group amounted to 152 ± 65% of controls (Fig. 4), whereas Mrp2 protein levels averaged 108 ± 23% of controls (Fig. 5). For Mrp6, which has been localized on both the lateral and canalicular membrane of rat hepatocytes (31,37), protein levels were not significantly different in PB treated rats and amounted to 59 ± 48% of controls (n.s., Fig. 5).

Initial uptake rates of BSP and digoxin in isolated hepatocytes. To correlate expression levels of Oatps with transport function, initial uptake rates of BSP and digoxin were measured in isolated hepatocytes. As shown in Fig. 6, BSP uptake was unaffected by PB pretreatment (controls vs. PB: 72 ± 9 vs. 70 ± 11 pmol/mg protein/30 seconds). In contrast, net digoxin uptake over 30 seconds, after subtraction of nonspecific binding at 0 seconds, was stimulated 4fold in the PB group (controls vs. PB: 83 ± 9 vs. 337 ± 60 fmol/mg protein, $P<0.0001$).

Discussion

This study analyzes the effect of PB treatment of rats upon the expression of organic anion transport proteins of the basolateral and canalicular hepatocyte membrane. Expression of Oatp1 was moderately downregulated on both the RNA (Fig. 1) and protein (Fig. 2) levels, although the changes were not statistically significant. In contrast, expression of Oatp2, a major uptake system for xenobiotics, was upregulated 2fold (Figs. 1 and 2) and most pronounced in the perivenous region of the hepatic lobules (Fig. 3). The expression of Oatp4 was not significantly altered by PB treatment (Fig. 1). The Na^+-dependent bile salt transporter Ntcp was moderately downregulated on both the RNA and protein levels (Figs. 1 and 2). At the canalicular domain, expression of the multispecific organic anion transporters Mrp2 and Mrp6 and of the bile salt export pump Bsep was not significantly influenced by PB treatment (Figs. 4 and 5).

To correlate expression levels of Oatp1, Oatp2 and Oatp4 with transport function, we measured initial uptake rates of BSP and digoxin in isolated hepatocytes. Whereas digoxin uptake was stimulated 4fold, which was in agreement with increased Oatp2 expression, BSP uptake was unaffected by PB (Fig. 6). Thus BSP transport data correlated with Oatp1 and Oatp4 expression, indicating the predominant role of these two carriers in hepatocellular BSP uptake. Importantly, we were unable to reproduce the 6fold increase in BSP uptake reported by Potter et al. (17). We conclude that the known increase in hepatic BSP clearance that is induced by PB (38-42) is not a result of increased hepatocellular uptake.

The induction of Oatp2 expression could explain the increase in the biliary excretion of xenobiotic compounds such as ouabain and digoxin, and of the endogenous hormone

thyroxine, that is induced by PB (43-49). Whether Oatp2 accounts for the reported 8fold increase in the Vmax of ditekiren uptake in hepatocytes from PB treated rats (50) is unknown at present. Because ditekiren uptake was inhibitable by BSP, the involved transporter probably also transports BSP and may thus not be identical with Oatp2, which exhibits negligible BSP transport activity (8).

Numerous studies have shown an increase in the bile salt-independent fraction of bile flow following PB treatment (51,52). A major determinant of bile salt-independent bile flow is the canalicular secretion of anionic glutathione and glucuronic acid conjugates (53), substrates of the canalicular multispecific organic anion transporter Mrp2. PB treatment did not significantly affect Mrp2 expression, confirming previous functional data obtained in cLPM vesicles, that showed no effect of PB treatment on the ATP-dependent transport of BSP and leukotriene C_4 (54). Furthermore, PB has previously been shown to induce Mrp3 but not Mrp1 or Mrp2 to a significant level (55,56). Considering the lack of Mrp2 stimulation by PB, it is of interest that in the isolated perfused rat liver model the biliary excretion of anionic conjugates such as BSP-GSH, bilirubin glucuronide, bromcresol green, eosine and thyroxine glucuronide was enhanced following PB treatment (43,48,57). Although speculative at present, it is conceivable that the canalicular membrane possesses additional efflux systems for organic anions that are selectively induced by PB. This concept is supported by studies in cLPM vesicles, that have indicated selective induction of the electrogenic transport of glutathione and BSP by PB (54). Furthermore, normal canalicular secretion of bilirubin ditaurate has been shown in Mrp2-deficient TR^- rats, suggesting the existence of additional canalicular organic anion transporters (58). The molecular identity of these additional canalicular transporters is unknown at present.

In conclusion, this study shows that the previously reported PB induced stimulation of hepatic BSP clearance is not attributable to an increase in hepatocellular BSP uptake. Accordingly, expression of the two major BSP uptake systems Oatp1 and Oatp4 is unchanged. In contrast, Oatp2 expression is stimulated by PB, which is reflected by increased hepatocellular uptake of the Oatp2 specific substrate digoxin. At the canalicular membrane, the known stimulation of organic anion secretion that is induced by PB is not attributable to increased expression of Bsep and Mrp2. It could be a result of protein activation, e.g. phosphorylation, via second messenger pathways, or of selective induction of an as yet unidentified canalicular transport system for anionic conjugates.

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Figure legends

Fig. 1: Northern blot analysis of basolateral transporter expression in phenobarbital treated rats compared to saline treated control rats. 2.5 μg poly (A^+) RNA from rat livers was separated by denaturing formaldehyde electrophoresis and transferred to Nylon membranes (see "Materials and Methods"). Following hybridization, the blots were exposed to autoradiography film and band intensities were quantitated densitometrically. Transporter expression was normalized for GAPDH expression and the densitometric ratios are shown in the bar graph (X ± SD for each treatment group). Stimulation of the expression of cytochrome P450 II B1 by phenobarbital was documented for control purposes. *Statistically significantly different from controls

Fig. 2: Western blot analysis of basolateral transporter expression in phenobarbital treated rats compared to saline treated control rats. 75 μg of rat liver microsomes were separated by SDS-PAGE and transferred to nitrocellulose. Blots were incubated with rabbit antibodies (see "Materials and Methods") and bound antibodies were visualized with iodinated protein A and subsequent autoradiography. Densitometric quantification of the band intensities is shown in the bar graph. *Statistically significant from controls.

Fig. 3: Distribution of basolateral organic anion transporters in control (left) and PB treated (right) rat liver as demonstrated by indirect immunofluorescence on semithin cryosections. Oatp1 (top) displayed a basolateral distribution in control liver with no detectable intensity differences throughout the liver acinus. This distribution pattern was not altered by PB treatment. In contrast, the basolateral Oatp2 (middle) displayed an uneven distribution of immunoreactivity in control liver. Signal strength was high in zone 3 (around the central vein,

CV) and decreased towards the portal tracts *(PV*, portal vein), while the centralmost layer of cells was immunonegative. PB treatment resulted in an increased immunoreactivity in zone 3 but did not alter signal intensity in zone 1 of the liver acinus. Close up views of zone 3 (pericentral) hepatocytes (bottom) demonstrate that immunoreactivity was increased predominantly in the centralmost layer of hepatocytes.

Fig. 4: Northern blot analysis of canalicular transporter expression in phenobarbital treated rats compared to saline treated control rats. The densitometric ratios of Mrp2/GAPDH and Bsep/GAPDH expression are shown in the bar graph. The PB induced changes did not reach statistical significance.

Fig. 5: Western blot analysis of canalicular transporter expression in phenobarbital treated rats compared to saline treated control rats. No significant changes of Bsep, Mrp2 and Mrp6 expression were observed secondary to PB treatment.

Fig. 6: Initial uptake rates of (A) BSP and (B) digoxin in isolated hepatocytes from phenobarbital treated (▲) and control (■) rats. Data points represent the mean ± 1 SD of triplicate measurements from two separate hepatocyte preparations.