0022-3565/09/3291-141–149$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 329:141–149, 2009

Vol. 329, No. 1
149690/3451835
Printed in U.S.A.

Pregnane X Receptor (PXR) Regulates P-Glycoprotein at the Blood-Brain Barrier: Functional Similarities between Pig and Human PXR

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Received December 10, 2008; accepted January 14, 2009

ABSTRACT

Pharmacotherapy of central nervous system (CNS) disorders is impaired by the drug efflux transporter, P-glycoprotein, which limits drug penetration across the blood-brain barrier into the CNS. One strategy to increase brain drug levels is to modulate P-glycoprotein regulation. This approach requires understanding of the mechanisms that control transporter expression and function. One mechanism through which P-glycoprotein is regulated is the nuclear receptor, pregnane X receptor (PXR). Xenobiotics including drugs activate PXR and induce P-glycoprotein, which potentially affects pharmacokinetics/pharmacodynamics of coadministered drugs. Because rodent models are not suitable to predict xenobiotic interactions with human PXR, in a porcine model, we studied functional similarities between pig and human PXR. We used brain capillary endothelial cells from pig to study the effect of PXR activation on P-glycoprotein. To activate PXR, we used the PXR ligands, rifampicin, hyperforin, and pregnenolone-16α-carbonitrile (PCN), and measured *abcb1* mRNA with quantitative polymerase chain reaction, P-glycoprotein expression with Western blotting, and P-glycoprotein transport activity with a calcein assay. We provide first proof of principle that the human PXR ligands, rifampicin and hyperforin, but not the rodent PXR ligand, PCN, activate pig PXR at the blood-brain barrier and induce mRNA, protein expression, and transport activity of P-glycoprotein. Our data indicate functional similarities between human and pig PXR that suggest the pig model could be useful for predicting xenobiotic-PXR interactions in humans. Because PXR is crucial in controlling drug efflux transporters, our findings will contribute to a better understanding of the regulation of blood-brain barrier function, which could potentially have important clinical implications for the treatment of CNS disorders.

Pharmacotherapy of CNS disorders is greatly impaired by the blood-brain barrier. In this barrier, ATP-driven efflux transporters limit a large number of drugs from getting into the brain. P-glycoprotein (*ABCB1*, *MDR1*), the most prominent drug efflux transporter at the blood-brain barrier, plays an essential role in barrier function (Schinkel, 1999). For example, brain penetration of a large number of drugs is increased by 10- to 100-fold in P-glycoprotein-null mice compared with wild-type controls (Schinkel et al., 1994). Likewise, specific inhibition of P-glycoprotein in mice increases brain levels of paclitaxel, resulting in a 90% reduction of brain tumor volume (Fellner et al., 2002). On the other hand, up-regulation of P-glycoprotein, e.g., in drug-resistant epilepsy, CNS inflammation, or through oxidative stress selectively tightens the barrier for drugs that are P-glycoprotein substrates (Löscher and Potschka, 2005; Bauer et al., 2007; Hartz et al., 2008). In this regard, we have previously shown expression of the ligand-activated nuclear receptor, pregnane X receptor (PXR), in isolated rat brain capillaries and demonstrated PXR-mediated up-regulation of P-glycoprotein (Bauer et al., 2004). Using transgenic mice expressing human PXR (hPXR), we showed that activation of hPXR in vivo tightens the barrier to the CNS-acting P-glycoprotein substrate, methadone, reducing the drug's central effect (Bauer et al., 2006). Moreover, we have shown recently that multidrug resistance-associated protein 2 (Mrp2) and the phase-II metabolizing enzyme, glutathione transferase, are coordinately regulated in the brain capillary endothelium in vivo (Bauer et al., 2008). This is an interesting finding that suggests, like in liver, a regulatory network consisting of nuclear

This work was supported by the ESF Baden Württemberg [Schlieben-Lange Program Grant]; the Deutsche Forschungsgemeinschaft [Grant FR1211/8-1]; and the University of Minnesota [GIA Award 20919].
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.108.149690.

ABBREVIATIONS: CNS, central nervous system; PXR, pregnane X receptor; hPXR, human PXR; Mrp2, multidrug resistance-associated protein 2; PCN, pregnenolone-16α-carbonitrile; pgPXR, pig PXR; FITC, fluorescein isothiocyanate; AM, acetoxymethyl ester; RT, reverse transcriptase; PBEC, porcine brain capillary endothelial cell; PCR, polymerase chain reaction; PBGD, porphobilinogen deaminase; PSC833, 3'-oxo-4-butenyl-4-methyl-threonine(1), (valine(2)) cyclosporin.

receptors, metabolizing enzymes, and efflux transporters (Rosenfeld et al., 2003).

PXR is a xenobiotic-activated nuclear transcription factor that is expressed in barrier and excretory tissues, including intestine, liver, and kidney (Kliewer et al., 2002; Francis et al., 2003). In these tissues, PXR controls metabolism and efflux transport of xenobiotics and, therefore, is also referred to as the "master regulator" of xenobiotic defense (Geick et al., 2001; Dussault and Forman, 2002). On a molecular level, ligand binding initiates PXR movement from the cytoplasm into the nucleus, where it activates transcription of phase I and phase II enzymes and phase III transporters (Geick et al., 2001; Synold et al., 2001; Kast et al., 2002; Wang and LeCluyse, 2003). This affects numerous drugs that are substrates for metabolizing enzymes and efflux transporters, which can have severe clinical consequences. For example, several groups reported transplant rejections in patients taking the herbal remedy, St. John's Wort, alongside the immunosuppressant, cyclosporine A (Breidenbach et al., 2000; Karliova et al., 2000; Ruschitzka et al., 2000). Hyperforin, the main constituent of St. John's Wort, is a potent PXR ligand that leads to increased expression of metabolizing enzymes and efflux transporters and, thus, alters the pharmacokinetics of the immunosuppressant, cyclosporin A (Moore et al., 2000). As a consequence, in these patients, cyclosporin A blood levels dropped below the therapeutically effective concentration, which resulted in transplant rejection.

Given the clinical relevance of PXR, efforts are aimed at identifying potential drug candidates as PXR ligands early in the drug discovery process to avoid problems in later phases of drug development. However, because of a low degree of sequence identity in the PXR ligand binding domains, rodent models are not suitable to predict xenobiotic interactions with hPXR (Kliewer et al., 2002; Moore et al., 2002; Orans et al., 2005). As a consequence, xenobiotics activating rodent PXR, such as the prototypical PXR ligand, pregnenolone-16α-carbonitrile (PCN), do not activate hPXR; conversely, compounds activating hPXR, such as rifampicin and hyperforin, do not activate rodent PXR.

Moore et al. (2002) recently cloned the pig PXR (pgPXR) ligand binding domain and found a 87% sequence identity with the hPXR ligand binding domain. They further characterized xenobiotic activation of pgPXR in transfection assays and found a similar activation profile to that of hPXR. However, except for these data from an artificial expression system, it is unknown whether pgPXR can be activated in a more physiological model, whether such activation up-regulates PXR target genes, and whether these induction patterns are similar to those in a human or rodent model. In the present study, we demonstrate functional expression of PXR in a blood-brain barrier cell culture model from pig and show that activation of pgPXR with rifampicin and hyperforin up-regulated protein expression and transport function of the drug efflux transporter, P-glycoprotein. Our data indicate functional similarities between human and pig PXR that suggest the pig model could be a useful tool for predicting xenobiotic-PXR interactions in humans.

Materials and Methods

Chemicals. Hyperforin, rifampicin, PCN, and CellLytic MT mammalian tissue lysis buffer were purchased from Sigma Chemie (Deisenhofen, Germany). Calcein-AM and FITC-conjugated rabbit anti-mouse IgG were purchased from Invitrogen (Carlsbad, CA). Complete protease inhibitor cocktail, FastStart DNA Master PLUS SYBR Green I Kit, Dispase II, collagenase-dispase, and rat tail collagen were from Roche Diagnostics (Mannheim, Germany). C219 monoclonal antibody against P-glycoprotein and monoclonal antibody against Na^+/K^+-ATPase were obtained from Alexis Corporation (Lausen, Switzerland). Polyclonal PXR H-160 antibody was purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). FITC-conjugated goat secondary anti-rabbit IgG was from Biozol GmbH (Eching, Germany), and horseradish peroxidase-conjugated secondary IgG was from Dako Deutschland GmbH (Hamburg, Germany). Lightning Chemiluminescence Reagent was from PerkinElmer Life and Analytical Sciences (Waltham, MA). RNeasy Mini Kit and QIAGEN GmbH RNase-Free DNase were purchased from QIAGEN GmbH (Hilden, Germany). iScriptcDNA Synthesis Kit was purchased from Bio-Rad (Hercules, CA). All cell culture materials and supplies were obtained from Biochrom (Berlin, Germany). PSC833 was a kind gift from Novartis (Basel, Switzerland). All other materials and supplies were of highest grade from commercial sources.

Isolation and Culture of Porcine Brain Capillary Endothelial Cells. Porcine brain capillary endothelial cells (PBCECs) were isolated as described previously (Bauer et al., 2003; Bubik et al., 2006). In brief, brains from freshly slaughtered pigs (male and female, 3–6 months, approximately 200 pounds) were cleaned from meninges and choroid plexus. Cortical gray matter was removed, collected in ice-cold artificial cerebrospinal fluid (103 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 10 mM D-glucose, 10 mM HEPES, 1 mM sodium pyruvate, 100 μg/ml penicillin/streptomycin, 100 μg/ml gentamycin, pH 7.4), and minced. The brain homogenate was digested in 0.5% Dispase II in preparation medium (Earle's Medium 199, 0.8 mM L-glutamine, 10 mM HEPES, 100 μg/ml penicillin/streptomycin, 100 μg/ml gentamycin) for 2 h at 37°C and afterward centrifuged at 1000*g* for 10 min at 4°C. The supernatant was discarded, and the pellet was resuspended in dextran (15% final dextran concentration). Brain capillaries were separated from the brain homogenate by density centrifugation (5800*g*, 10 min, 4°C) and afterward digested with 0.1% collagenase/dispase in preparation medium at 37°C for 1.5 h. The resulting brain capillary endothelial cell suspension was filtered through a 150-μm nylon mesh, centrifuged (130g, 10 min, 4°C), resuspended, and added on a discontinuous Percoll gradient (1.03 and 1.07 g/ml). Density centrifugation was performed at 1250*g* for 10 min at 4°C. PBCECs enriched at the interface between the two Percoll solutions were collected, washed, and seeded onto collagen-G-coated cell culture dishes at a density of 250,000 cells/cm^2 in medium containing 10% horse serum. Cells were cultured in Earle's Medium 199 containing 1.5 mM L-glutamine, 100 μg/ml penicillin/streptomycin, 10 mM HEPES, and 10% horse serum. PBCECs were exposed to PXR activators for up to 48 h and used for experiments after a total of 7 days in culture.

Western Blotting. To isolate PBCEC crude plasma membranes, cells after a total of 7 days in culture were scraped from cell culture dishes and homogenized in CellLytic MT mammalian tissue lysis buffer containing Complete protease inhibitor cocktail. Homogenized samples were centrifuged at 10,000*g* for 30 min at 4°C, and denucleated supernatants were centrifuged at 100,000*g* for 90 min at 4°C. The resulting pellets (crude plasma membranes) were suspended in lysis buffer containing protease inhibitors, and protein concentrations were determined. Crude plasma membrane proteins (15 μg/lane) were separated on a 7.5% SDS-polyacrylamide gel and blotted on polyvinylidene difluoride membranes. After blocking with 1% bovine serum albumin/1% milk powder/0.05% Tween 20 in phosphate-buffered saline for 1 h, membranes were incubated overnight at 4°C with 1 μg/ml (1:100) monoclonal C219 antibody to P-glycoprotein or (1:250) monoclonal antibody to Na^+/K^+-ATPase-α. Afterward, membranes were washed and then incubated with 1.3 μg/ml (1:1000) horseradish peroxidase-conjugated secondary IgG for 1 h.

Protein bands were detected using Western Lightning Chemiluminescence Reagent and visualized with a ChemiDoc XRS imaging system (Bio-Rad).

Calcein Assay. Calcein assays to determine P-glycoprotein transport activity were performed as described previously (Bauer et al., 2003). In brief, confluent PBCECs were exposed to hyperforin, rifampicin, or PCN for up to 48 h before experiments after 7 days of cell culture. After washing with Krebs-Ringer bicarbonate (142 mM NaCl, 3 mM KCl, 1.5 mM K_2HPO_4, 10 mM HEPES, 4 mM D-glucose, 1.2 mM $MgCl_2$, 1.4 mM $CaCl_2$), 2 μM calcein-AM was added to the cells for 15 min at 37°C. Cells were washed subsequently two times with ice-cold Krebs-Ringer bicarbonate and lysed with 1% Triton X-100. Calcein fluorescence was measured with a Fluoroskan Ascent plate reader (Thermo Fisher Scientific, Waltham, MA).

Immunochemistry. PBCECs grown for a total of 7 days on collagen G-coated chamber slides were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 30 min, and blocked with 5% goat serum for 30 min. Cells were incubated subsequently overnight at 4°C with 10 μg/ml (1:20) polyclonal PXR (H-160) antibody to PXR. Cells were washed with phosphate-buffered saline and then incubated at 37°C with FITC-conjugated goat anti-rabbit IgG (1:20) for 60 min. Nuclei were counterstained with 1 μg/ml propidium iodide. Immunofluorescence was visualized by confocal microscopy (Leica, Wetzlar, Germany) using the 488-nm line of an argon laser and a 40× oil immersion objective (numerical aperture, 1.2).

RT-PCR. Total RNA was isolated from 7-day-old PBCECs using the RNeasy Mini Kit according to the manufacturer's protocol. First, contaminating DNA was removed from samples by digestion with the QIAGEN RNase-Free DNase Set. One microgram of total RNA was reverse-transcribed using the iScriptcDNA Synthesis Kit. A 524-bp sequence, specific to the porcine PXR gene, *NR1I2* (GenBank accession no. AB214980), was detected with the following primer set: 5′-TGCCAGCTGAGATTCAACAC-3′ (forward primer, bases 1084–1103) and 5′-GACCTTTGGGCATTGTCAGT-3′ (reverse primer, bases 1588–1607). PCR was carried out at 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s for 40 cycles. PCR products were separated on a 1.5% ethidium bromide-stained agarose gel, purified, and sequenced.

Quantitative Real-Time PCR. *Abcb1* mRNA expression was quantified by real-time PCR using porphobilinogen deaminase (*PBGD*) as the housekeeping gene. Total RNA was isolated from 7-day-old PBCECs and reverse-transcribed as described above; 50 ng of RT product (cDNA) was used as template. Real-time PCR was carried out using the LightCycler System with the FastStart DNA Master PLUS SYBR Green I Kit and the following primers: *Abcb1* (GenBank accession no. AY825267), 5′-CCTGTTTGACTGCAGCATTG-3′ (forward primer, bases 2561–2580) and 5′-GAGAGCTGCGTTCCTTTGTC-3′ (reverse primer, bases 2709–2728); and *PBGD* (GenBank accession no. AY148221), 5′-AACGCAGCCGCAACGGC-3′ (forward primer, bases 1–17) and 5′-AGCGTTGCCACCACACTG-3′ (reverse primer, bases 93–110). The four-step PCR protocol was as follows: 1) denaturation (10 min, 95°C), 2) amplification (10 s, 95°C/10 s, 55°C/9 s, 72°C/5 s, 82°C/single fluorescence measurement), 3) melting curve program (60–99°C; heating rate: 0.1°C/s), and 4) cooling program (40°C). The crossing point was determined by the Second Derivate Maximum Method using LightCycler Software 3.5. *Abcb1* and pgPXR expression levels were normalized to PBGD using the relative expression software tool, REST (Pfaffl et al., 2002).

Statistics. Data are given as mean ± S.E.M. Means were significantly different when $p < 0.05$ using the appropriate Student's t test.

Results

pgPXR Expression in Porcine Brain Capillary Endothelial Cells. To show expression of PXR in pig, we isolated total RNA from porcine brain capillary endothelial cells (PBCECs), brain, kidney, and liver and performed RT-PCR. In liver and kidney, which were used as positive controls for pgPXR expression, we detected a 524-bp amplicon identical to a sequence of the porcine PXR gene, *NR1I2* (Fig. 1A). We also detected pgPXR mRNA in PBCECs and total brain. pgPXR protein expression in PBCECs was demonstrated by immunostaining. Figure 1B shows immunoreactive pgPXR (green staining in left image) in 7-day-old PBCEC cultures. pgPXR immunofluorescence was evenly distributed over the spindle-shaped endothelial cells, with no indication of nuclear or perinuclear accumulation.

PXR is a ligand-activated transcription factor that resides within the cytoplasm and moves into the nucleus upon ligand binding. To test pgPXR activation, we exposed PBCECs to the hPXR ligands, rifampicin or hyperforin, or to the prototypical rodent PXR ligand, PCN, for 24 h. Figure 2A shows that rifampicin and hyperforin both increased PXR immunofluorescence (green) compared with the control, whereas the rodent PXR ligand, PCN, had no effect on pgPXR immunofluorescence. It is important that cells exposed to rifampicin or hyperforin showed accumulation of immunoreactive pgPXR in the perinuclear region and the nucleus (arrows in Fig. 2A), which is consistent with pgPXR activation and movement into the nucleus.

To study the effect of PXR up-regulation through PXR ligands in more detail, we performed quantitative PCR on pgPXR mRNA levels in PBCECs exposed to rifampicin, hyperforin, or PCN. Figure 2B shows that both rifampicin and hyperforin increased pgPXR mRNA in a time-dependent manner. With 1 μM hyperforin pgPXR mRNA was increased 2.3-, 4.4-, 5.5-, and 5.4-fold over controls after 4, 6, 12, and 24 h, respectively. Exposing cells to 10 μM rifampicin increased pgPXR mRNA by 3.5-fold after 4 h and 4.7-fold after 6 h. After 12 h, pgPXR mRNA levels had declined from the previous time point to 2.3-fold of controls and was 2.9-fold of controls after 24 h. Viability assays showed this was not due to toxic rifampicin effects (data not shown). It is important that although both rifampicin and hyperforin increased pgPXR mRNA levels, PCN had no effect. PgPXR mRNA expres-

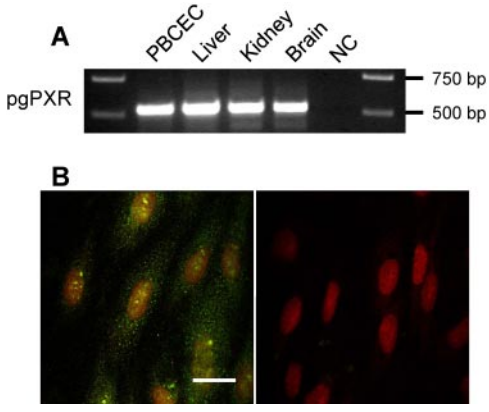

Fig. 1. PXR expression in PBCECs. A, RT-PCR of a 524 b- sequence from pgPXR mRNA in PBCECs. pgPXR mRNA was also detected in kidney, brain, and liver (used as positive control). NC, negative control. B, representative confocal image of a confluent PBCEC monolayer immunostained for pgPXR (green) and counterstained for nuclei with propidium iodide (red). Left, green PXR immunostaining and red nuclei staining. Right, negative control (no primary antibody added) with red nuclei staining. Scale bar, 20 μm.

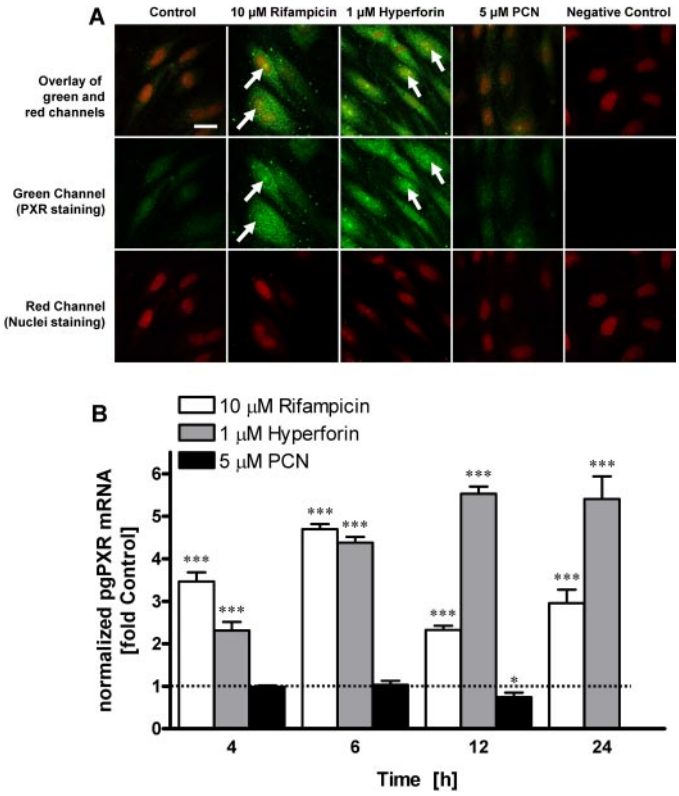

Fig. 2. PXR mRNA and protein expression in PBCECs exposed to PXR ligands. A, representative confocal images of PBCECs immunostained for pgPXR (green) after 24-h exposure to rifampicin, hyperforin, or PCN. Rifampicin and hyperforin increased immunoreactive PXR compared with controls. Note that pgPXR accumulated in the perinuclear region and the nucleus (arrows). PCN had no effect on pgPXR immunofluorescence. Negative control, no primary PXR antibody; nuclei are shown in red; scale bar, 20 μm. B, time course of pgPXR mRNA expression in PBCECs exposed to rifampicin, hyperforin, or PCN (dashed line, control = 1). Data are expressed as pgPXR/*PBGD* ratio relative to controls. Each data point represents mean ± S.E.M. from three independent experiments. For rifampicin and hyperforin: ***, significantly greater than control, $p < 0.001$; for PCN: *, significantly lower than control, $p < 0.05$.

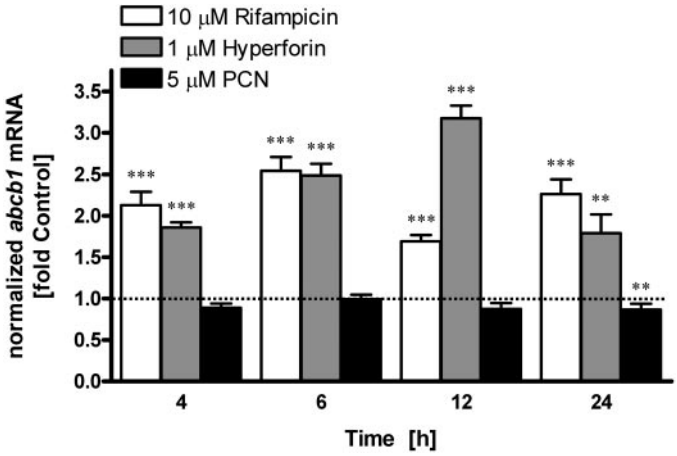

Fig. 3. *Abcb1* mRNA expression in PBCECs exposed to PXR ligands. Time course of *abcb1* mRNA expression in PBCECs exposed to rifampicin, hyperforin, or PCN (dashed line, control = 1). Data are expressed as *abcb1*/*PBGD* ratio relative to controls. Each data point represents mean ± S.E.M. from three independent experiments. For rifampicin and hyperforin: **, significantly greater than control, $p < 0.01$; ***, significantly greater than control, $p < 0.001$; for PCN: **, significantly lower than control, $p < 0.01$.

sion levels relative to control were 0.98 at 4 h, 1.03 at 6 h, and 0.74 at 12 h. The drop in PXR mRNA levels after 12 h was probably due to PCN toxicity, which we have observed previously (Bauer et al., 2004).

Activation of pgPXR up-Regulates P-Glycoprotein Expression in PBCECs. One important PXR target gene is *abcb1*, which encodes the most prominent blood-brain barrier drug efflux transporter, P-glycoprotein (Geick et al., 2001; Synold et al., 2001; Bauer et al., 2004, 2006). In a time course experiment, we determined whether *abcb1* mRNA levels increased in PBCECs exposed to rifampicin, hyperforin, or PCN. Figure 3 shows time-dependent increases in *abcb1* mRNA when PBCECs were exposed to rifampicin or hyperforin. Rifampicin (10 μM) increased *abcb1* mRNA by 2.1- and 2.5-fold over controls after 4 and 6 h, respectively. However, after 12 and 24 h, *abcb1* mRNA levels decreased and were 1.7- and 2.3-fold of control levels, respectively. Again, data from viability assays showed this was not due to toxic effects on PBCECs exposed to rifampicin for longer periods of time (data not shown). With 1 μM hyperforin, *abcb1* mRNA in PBCECs continuously increased over 12 h to levels 3.2-fold higher than observed in controls (4 h, 1.9-fold; 6 h, 2.5-fold relative to control). After 24 h, *abcb1* mRNA levels also dropped with hyperforin to a level of 1.8-fold of the control. Experiments testing cell viability showed that this drop in mRNA levels was not caused by hyperforin toxicity (data not shown). Over the entire time course, 5 μM PCN, a concentration that induces *mdr1* in rodents, did not up-regulate *abcb1* mRNA in porcine cells. It is important that this induction pattern in PBCECs resembles the one observed in human tissue, where only hPXR activators such as rifampicin and hyperforin induce PXR target genes, whereas rodent PXR activators such as PCN are without effect (Jones et al., 2000).

We further tested whether pgPXR activation induced protein expression of P-glycoprotein in PBCECs. Western blots of plasma membranes from 7-day-old PBCECs exposed to rifampicin or hyperforin for 24 h showed a concentration-dependent increase of P-glycoprotein (Fig. 4, A and B). This effect was detectable at rifampicin concentrations as low as 0.5 μM and at hyperforin concentrations as low as 0.05 μM and was stronger with increasing concentration of PXR ligand. Note that hyperforin induced comparable effects at a 10-fold lower concentration than rifampicin, indicating that it was approximately 10 times more potent than rifampicin in activating pgPXR.

Rifampicin- and hyperforin-induced P-glycoprotein up-regulation was also time-dependent, whereas 6-h exposure of PBCECs to 10 μM rifampicin had only a minor effect on P-glycoprotein expression (Fig. 4C), and with longer exposure time, P-glycoprotein expression increased and reached a plateau after 12 h. Pooled data from membrane density measurements of 16 Western blots showed that protein levels of P-glycoprotein were maximal after 12-h rifampicin exposure (Fig. 4D). In contrast, hyperforin had a more pronounced effect on P-glycoprotein levels (Fig. 4, E and F). Six-hour exposure of PBCECs to 1 μM hyperforin significantly up-regulated P-glycoprotein, and this effect further increased with time. Maximal P-glycoprotein expression levels were observed after 48 h (Fig. 4, E and F). It is important that consistent with the quantitative mRNA data, 5 μM PCN had

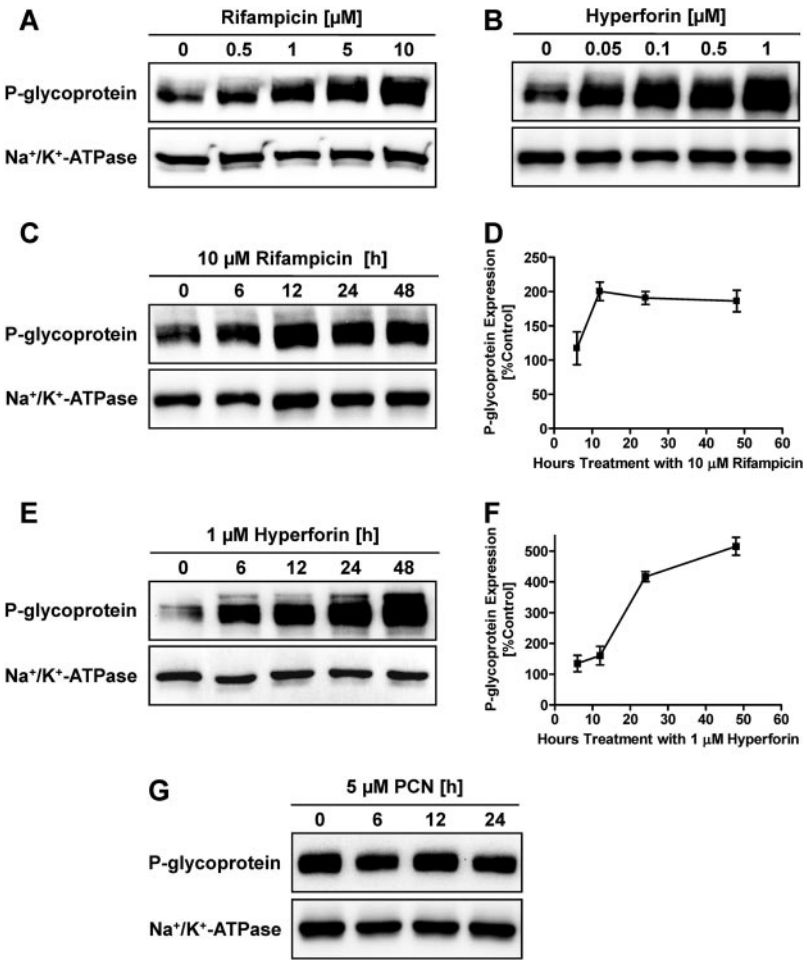

Fig. 4. P-glycoprotein expression in PBCECs exposed to PXR ligands. A, rifampicin dose response showing increased P-glycoprotein expression after 24 h. Na^+/K^+-ATPase was used as protein loading control. B, hyperforin dose response showing increased P-glycoprotein expression after 24 h. C, time course showing increased P-glycoprotein expression with 10 μM rifampicin. D, average of P-glycoprotein Western blot density analyses from time courses with 10 μM rifampicin (control = 100%; each data point represents mean ± S.E.M. from three to four independent experiments). E, time course showing increased P-glycoprotein expression with 1 μM hyperforin. F, average of P-glycoprotein Western blot density analyses from time courses with 1 μM hyperforin (control = 100%; each data point represents mean ± S.E.M. from three to four independent experiments). G, 5 μM PCN had no effect on P-glycoprotein expression in PBCECs over 24 h.

no effect on protein expression levels of P-glycoprotein in PBCECs over a 24-h time period (Fig. 4G).

Activation of pgPXR up-Regulates P-Glycoprotein Transport Activity in PBCECs. To measure P-glycoprotein transport activity in PBCEC cultures, we used a fluorometric assay as described previously (Bauer et al., 2003). This assay utilizes lipophilic, nonfluorescent calcein-AM, which penetrates into the cell, where it is immediately cleaved by esterases to highly fluorescent calcein. Because calcein-AM is a high-affinity substrate for P-glycoprotein, intracellular calcein fluorescence inversely correlates with P-glycoprotein activity and, therefore, is a measure of P-glycoprotein-mediated transport. Thus, an increase in P-glycoprotein activity results in reduced intracellular calcein fluorescence and vice versa. In this assay, exposing PBCECs to rifampicin or hyperforin decreased intracellular calcein fluorescence in a concentration- and time-dependent manner (Fig. 5). Ten micromolar rifampicin or 1 μM hyperforin reduced intracellular calcein fluorescence by more than 30% (Fig. 5, A and B); this reduction was maximal after 48 h (Fig. 5, C and D). It is important that rifampicin and hyperforin effects in the calcein assay were abolished at each time point with the P-glycoprotein-selective blocker, PSC833 (data not shown), indicating that the reduction in intracellular calcein fluorescence was specifically mediated by P-glycoprotein.

In accordance with *abcb1* mRNA and P-glycoprotein expression, PCN had no effect on intracellular calcein fluorescence, indicating that it did not affect P-glycoprotein transport activity (Fig. 5E). Moreover, in a control experiment for transporter inhibition, PSC833 increased intracellular calcein fluorescence more than 2.5-fold compared with controls (Fig. 5F).

Rifampicin and Hyperforin Maximally up-Regulate P-Glycoprotein through pgPXR Activation. In a next series of experiments, we tested whether simultaneous exposure of PBCECs to rifampicin and hyperforin for 24 h would have a synergistic effect on P-glycoprotein expression and transport activity. Figure 6A shows that exposing PBCECs to 10 μM rifampicin plus 1 μM hyperforin, concentrations under which each compound elicited maximal P-glycoprotein up-regulation (Figs. 4 and 6), did not have an additional, synergistic effect on P-glycoprotein expression. Consistent with this finding, Fig. 6B shows that rifampicin or hyperforin alone reduced intracellular calcein fluorescence to 71 ± 7% and 69 ± 8% of controls, respectively, indicating increased P-glycoprotein transport activity. Simultaneous exposure of PBCECs to rifampicin and hyperforin reduced intracellular calcein fluorescence to 55 ± 8% of controls; however, this effect was statistically not greater than that of either drug alone. These findings suggest that at the concentrations and exposure times used here, rifampicin and hyperforin had a maximal effect on P-glycoprotein expression and transport activity. This is also shown in Fig. 7, which summarizes data from 12 independent experiments for each rifampicin and hyperforin and four independent experiments for PCN. Note that P-glycoprotein transport activity was calculated as the

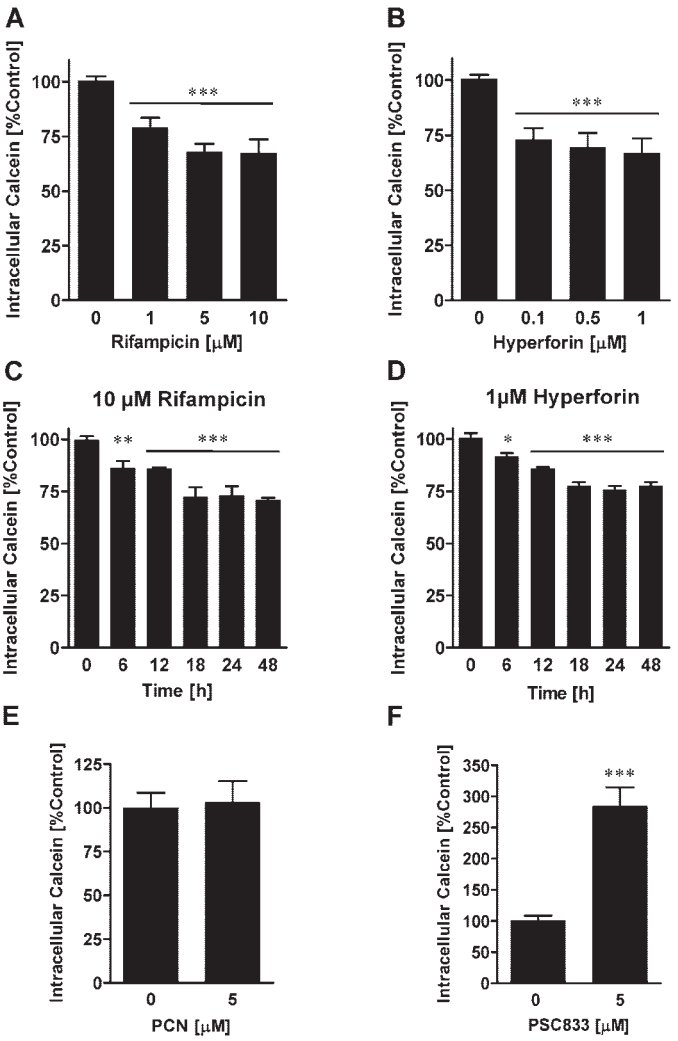

Fig. 5. P-glycoprotein transport activity in PBCECs exposed to PXR ligands. A, rifampicin dose response showing decreased intracellular calcein fluorescence relative to controls after 24 h, indicating an increase in P-glycoprotein transport activity. B, dose response showing decreased intracellular calcein fluorescence after 24-h exposure to hyperforin. C, 10 μM rifampicin reduced intracellular calcein levels in a time-dependent manner, indicating an increase in P-glycoprotein transport activity. D, 1 μM hyperforin reduced intracellular calcein in a time-dependent manner. E, P-glycoprotein-specific inhibitor, PSC833 (valspodar), increased intracellular calcein fluorescence. F, PCN had no effect on P-glycoprotein-mediated intracellular calcein fluorescence. Data are expressed as percentage of control; each data point represents mean ± S.E.M. ($n = 6$). For rifampicin and hyperforin: *, significantly lower than control, $p < 0.05$; ***, significantly lower than control, $p < 0.001$; for PSC833: ***, significantly greater than control, $p < 0.001$.

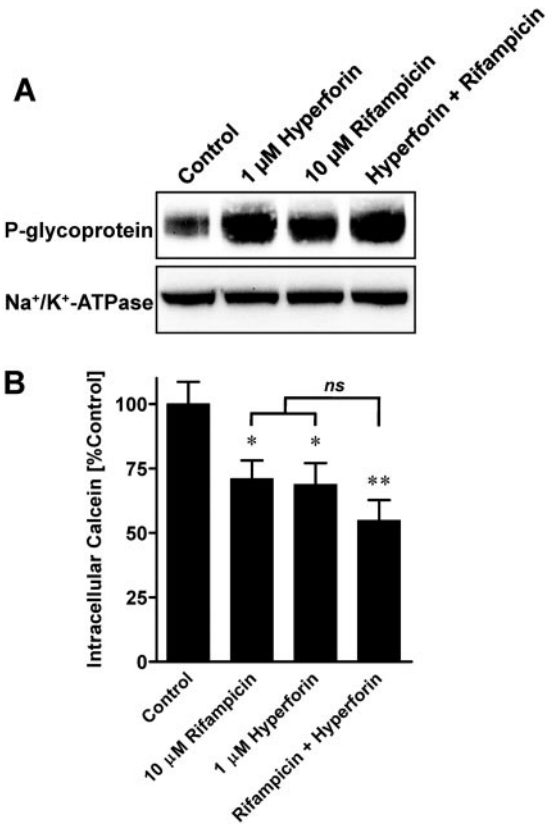

Fig. 6. Effect of simultaneous exposure of PBCECs to rifampicin and hyperforin on P-glycoprotein. A, Western blot showing that exposure of PBCECs to rifampicin and hyperforin at the same time did not have a synergistic effect on P-glycoprotein expression levels. B, simultaneous exposure of PBCECs to rifampicin plus hyperforin had no synergistic effect of P-glycoprotein function. The slight drop in intracellular calcein fluorescence was not statistically significant compared with either drug alone. Data are expressed as percentage of control; each data point represents mean ± S.E.M. from three independent experiments. *, significantly lower than control, $p < 0.05$; **, significantly lower than control, $p < 0.01$.

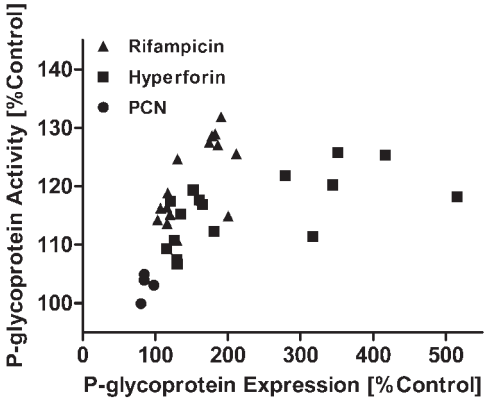

Fig. 7. Correlation between P-glycoprotein transport activity and P-glycoprotein expression. P-glycoprotein transport activity was calculated as difference of intracellular calcein fluorescence between control cells and treated cells and expressed relative to controls (control = 100%). P-glycoprotein expression was analyzed by Western blot density measurements and also expressed relative to controls (control = 100%). Each data point represents the mean from three independent experiments; error bars were omitted for clarity.

difference of intracellular calcein fluorescence between control cells and treated cells and expressed relative to controls and that P-glycoprotein expression was analyzed by Western blot density measurements and also expressed relative to controls. The data demonstrate that P-glycoprotein expression and transport activity correlated and increased in parallel with increasing dose of PXR ligand. P-glycoprotein activity steadily increased up to about 130% of that of controls, which was reached at a transporter expression of about 200% of control expression. It is interesting that a further increase in P-glycoprotein expression above 200% of controls did not result in a further increase in transport activity, but in a plateau. This may indicate that intracellular calcein fluorescence does not relate one-to-one to P-glycoprotein transport activity.

Discussion

PXR controls metabolism and efflux transport of xenobiotics in barrier and excretory tissues, thereby affecting the pharmacokinetics and pharmacodynamics of a large number of drugs, which can have severe clinical consequences (Geick et al., 2001; Synold et al., 2001; Kast et al., 2002; Wang and LeCluyse, 2003). Thus, efforts are aimed at identifying drug candidates as PXR ligands early in the drug discovery process to avoid problems in later phases of drug development. However, rodent models are not suitable to predict xenobiotic interactions with hPXR because of species differences in the ligand binding domains of the receptor (Kliewer et al., 2002; Moore et al., 2002; Orans et al., 2005). Here, we demonstrate functional expression of PXR in a blood-brain barrier cell culture model from pig and show that activation of pgPXR with the hPXR ligands, rifampicin and hyperforin, but not the rodent PXR ligand, PCN, up-regulated protein expression and transport function of the drug efflux transporter, P-glycoprotein.

Several points of this study require further discussion. First, PXR expression has been demonstrated in several tissues, including liver, intestine, kidney, and lung of several species, such as human, monkey, pig, dog, rabbit, rat, mouse, and flounder (Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998; Moore et al., 2002). In addition, we previously detected PXR at the blood-brain barrier of rat and mouse (Bauer et al., 2004, 2006, 2008). In the present study, we demonstrate mRNA and protein expression of PXR in brain capillary endothelial cells from pig. This finding underlines that PXR could be an important player in regulating P-glycoprotein and other transporters and metabolizing enzymes at the blood-brain barrier. We recently have demonstrated coordinated PXR regulation of Mrp2 and glutathione transferase π (Bauer et al., 2008). In addition to PXR, other nuclear receptors are also expressed at the blood-brain barrier, where they control regulation of several transporters. Narang et al. (2008) demonstrated that dexamethasone activation of the glucocorticoid receptor increased P-glycoprotein, Mrp2, and BCRP in rat brain endothelial cells and showed that PXR activation with PCN increased BCRP expression. In this context, the present study adds another mosaic to the picture that is emerging for the blood-brain barrier, that is, like in liver (Rosenfeld et al., 2003), a complex network of nuclear receptors controlling metabolizing enzymes and drug efflux transporters. Because a large number of drugs are PXR ligands, understanding this regulatory network at the blood-brain barrier could have important clinical implications for the pharmacotherapy of CNS disorders.

Second, we show here induction and movement of PXR into the nucleus of brain capillary endothelial cells, suggesting functional activation of this transcription factor at the blood-brain barrier. Induction of nuclear receptor expression has been described previously for liver and intestine (Pascussi et al., 2000; Maglich et al., 2002; Cooper et al., 2008; Haslam et al., 2008; Martin et al., 2008). On the other hand, literature on the cellular localization of PXR provides conflicting findings. Based on immunostaining data, some groups have reported that hPXR is exclusively localized in the nucleus (Kawana et al., 2003; Koyano et al., 2004), but other groups provided contradictory results (Kawana et al., 2003; Squires et al., 2004). For example, Squires et al. (2004) showed in mouse liver that PXR was localized in the cytoplasm and only translocated into the nucleus when PXR was activated. The discrepancies between these studies may be due to differences in species or the nature of the experiments. Thus, at present, PXR induction, activation, and localization is a matter of debate, and it remains to be seen what data future studies will provide to answer some of these questions for PXR at the blood-brain barrier.

Third, we demonstrate here that *abcb1* mRNA and P-glycoprotein expression and function increased in parallel, indicating that activation of pgPXR with rifampicin or hyperforin increased transcription and translation of the functionally active transporter protein. Maximal *abcb1* mRNA levels were reached at slightly earlier time points compared with maximal protein and activity levels, suggesting a chronologically connected process where mRNA up-regulation was followed by an increase in transporter protein and functional activity, as would be expected. In our experiments, both rifampicin and hyperforin caused a drop in PXR and *abcb1* mRNA in PBCECs after longer exposure times (12–24 h). However, cell viability experiments showed that both compounds were nontoxic at the concentrations used (data not shown). Moreover, even though *abcb1* mRNA levels decreased in our experiments, we observed an increase in P-glycoprotein expression and function. Previous studies showed that P-glycoprotein activity does not always increase with *abcb1* mRNA and vice versa (Haslam et al., 2008). Furthermore, Maglich et al. (2002) reported that PXR is under the control of a complex autoregulatory mechanism. Thus, complex transcriptional, post-transcriptional, and/or translational mechanisms that are currently unknown could be involved in regulating PXR and P-glycoprotein.

Fourth, we measured P-glycoprotein function with an assay that was used in previous studies to screen for P-glycoprotein-drug interactions (Bauer et al., 2003; Bubik et al., 2006). In these studies, compounds blocking P-glycoprotein activity increased penetration of nonfluorescent calcein-AM into cells, where it was rapidly cleaved to fluorescent calcein, resulting in increased intracellular fluorescence. In our study, however, we used this assay to determine PXR-induced increases in P-glycoprotein function, which resulted in decreased intracellular calcein. Although rifampicin and hyperforin induced P-glycoprotein to more than 200 and 500% of controls, respectively, intracellular calcein fluorescence was reduced at most by about 30% relative to controls, which has been observed before by other groups (Perloff et al., 2007). In this regard, it is important to distinguish between two factors that affect intracellular calcein fluorescence: 1) P-glycoprotein activity and 2) calcein-AM penetration. Because calcein-AM is a highly lipophilic compound, it easily penetrates cell membranes independently from P-glycoprotein-mediated efflux transport, resulting in high intracellular calcein fluorescence. Therefore, a high increase in P-glycoprotein transport activity prevents calcein-AM cell penetration only to a small degree and, consequently, does not reduce intracellular calcein fluorescence to a large extent. This lack of sensitivity of the calcein assay might explain the discrepancies observed in intracellular calcein levels when P-glycoprotein expression was increased versus when P-glycoprotein function was inhibited, where intracellular calcein fluorescence was high. It might also explain the discrepancy

between high P-glycoprotein expression after pgPXR activation and modest decreases in intracellular calcein fluorescence. It is important that P-glycoprotein expression correlated with transport activity, indicating that increased expression resulted in increased function. Moreover, reduced intracellular calcein fluorescence was PSC833-sensitive indicating this effect was due to P-glycoprotein. These findings in pig are consistent with our data from mouse and rat (Bauer et al., 2004, 2006, 2007; Hartz et al., 2008).

Finally, our experiments show that hyperforin was about an order of magnitude more potent than rifampicin in inducing *abcb1* mRNA and P-glycoprotein expression. This is in agreement with reports stating that hyperforin is the most potent activator of hPXR (Moore et al., 2000). We previously observed similar effects in brain capillaries using dexamethasone and PCN, where dexamethasone was 5- to 10-fold more potent in inducing P-glycoprotein (Bauer et al., 2004). It is important that in contrast to our previous studies in rodents where PCN induced P-glycoprotein, no such PCN effect was observed in the present study. PCN activates rodent PXR, but not hPXR, and rifampicin and hyperforin activate hPXR, but not rodent PXR (Kliewer et al., 2002; Moore et al., 2002; Orans et al., 2005). Our data are consistent with this induction pattern and with data showing that the ligand binding domains of pgPXR and hPXR have a higher sequence identity than pgPXR and rodent PXR (87 versus 76%) (Moore et al., 2002). In addition, Moore et al. (2002) found a similar activation profile of pgPXR to that of hPXR, which further underlines our findings.

Together, we provide first proof of principle that the hPXR ligands, rifampicin and hyperforin, but not the rodent PXR ligand, PCN, induce and activate pgPXR at the blood-brain barrier and induce *abcb1* mRNA, P-glycoprotein expression, and transport activity. This indicates functional similarities between human and pig PXR and suggests that porcine models are useful for predicting xenobiotic-PXR interactions in humans. It is important that because PXR is a key player in controlling metabolizing enzymes and efflux transporters, our findings will contribute to a better understanding of blood-brain barrier function, which could potentially have important clinical implications for the treatment of CNS disorders.

Acknowledgments

We thank Dr. Anika Hartz and Dr. David S. Miller for helpful discussions and critical proofreading of the manuscript.

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