Basic Study

Pregnane X receptor and constitutive androstane receptor modulate differently CYP3A-mediated metabolism in early- and late-stage cholestasis

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We demonstrated that early- and late-stage cholestasis affects CYP3A-mediated metabolism differently, probably as consequence of the differential activation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR). As a consequence, cholestatic patients may have an altered drug metabolism: in the early stage due to the induction of CYP3A enzymes; and in the late stage due to the high deposition of fibrotic liver and consequent hepatocyte loss. Secondly, since PXR activation is known to induce alternative hepatic export routes and detoxification enzymes, the induction of these cellular pathways with PXR and/or CAR agonists could be exploited as a therapeutic strategy for the management of cholestatic diseases.

**Abstract**

**AIM**

To ascertain whether cholestasis affects the expression of two CYP3A isoforms (CYP3A1 and CYP3A2) and of pregnane X receptor (PXR) and constitutive androstane receptor (CAR).

**METHODS**

Cholestasis was induced by bile duct ligation in 16 male Wistar rats; whereas 8 sham-operated rats were used as controls. Severity of cholestasis was assessed on histological examination of liver sections, and serum concentrations of albumin, AST, ALT, GGT, ALPK and bilirubin. Gene and protein expressions of PXR, CAR, CYP3A1 and CYP3A2 were assessed by means of qRT-PCR and Western blot, respectively. Alterations in CYP3A activity were measured by calculating the kinetic parameters of 4-OH- and 1'-OH-midazolam hydroxylation, marker reactions for CYP3A enzymes.

**RESULTS**

The mRNA and protein expression of CYP3A1 increased significantly in mild cholestasis ($P < 0.01$). At variance, mRNA and protein expression of CYP3A2 didn’t change in mild cholestasis, whereas the expression and activity of both CYP3A1 and CYP3A2 decreased dramatically when cholestasis became severe. Consistently with these observations, the nuclear expression of both PXR and CAR, which was measured because they both translocate into the cell nucleus after their activation, virtually disappeared in the late stage of cholestatic injury, after an initial increase. These results indicate that early- and late-stage cholestasis affects CYP3A-mediated drug metabolism differently, probably as consequence of the different activation of PXR and CAR.

**CONCLUSION**

Early- and late-stage cholestasis affects CYP3A-mediated drug metabolism differently. PXR and CAR might be targeted therapeutically to promote CYP3A-mediated liver detoxification.

**Key words:** Cholestasis; CYP3A; Drug metabolism; Pregnane X receptor; Constitutive androstane receptor

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**Core tip:** We demonstrated that early- and late-stage cholestasis affects CYP3A-mediated metabolism differently, probably as consequence of the differential activation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR). As a consequence, cholestatic patients may have an altered drug metabolism: in the early stage due to the induction of CYP3A enzymes; and in the late stage due to the high deposition of fibrotic liver and consequent hepatocyte loss. Secondly, since PXR activation is known to induce alternative hepatic export routes and detoxification enzymes, the induction of these cellular pathways with PXR and/or CAR agonists could be exploited as a therapeutic strategy for the management of cholestatic diseases.
drug-metabolizing enzymes. PXR and CAR act as xenobiotic sensors, as one of their main functions is to regulate the expression of enzymes and transporters involved in xenobiotic elimination. These NRs are also involved in controlling hepatic processes closely related to the progression of cholestatic diseases, such as BA homeostasis, lipid metabolism, fibrosis, and inflammation\(^5\). Changes in CAR and PXR expression have already been identified in the course of cholestatic liver disease, but different effects have been documented depending on the etiology of the cholestasis. In particular, a pronounced increase in PXR and CAR expression was documented in patients with obstructive cholestasis\(^5\), as opposed to a moderate reduction of their expression in primary biliary cholangitis\(^6\). In an animal model of cholestasis, it was demonstrated that PXR has a protective effect, reducing inflammation and fibrosis\(^7\).

There is general consensus that liver disease impairs various pathways of hepatic drug metabolism. Both in vitro and in vivo studies have shown a decrease in CYP3A activity in cholestatic liver disease, but a clear demonstration of the mechanism(s) responsible for it is still lacking\(^8-13\). To our knowledge, no studies published to date simultaneously analyzed CYP3A enzyme expression and activity and the activation of NRs responsible for their transcriptional control in different stages of cholestatic disease. CYP3A1 and CYP3A2 are regarded as the metabolically most important CYP3A isoforms in male rats. The former is the isoform mainly susceptible to induction, while the latter is the isoform expressed at the highest constitutive level\(^14,15\). CYP3A enzymes are the most abundant CYPs in human beings, and the most important enzymes in terms of drug metabolism, because they have a key role in the first-pass and systemic metabolism of many drugs\(^16,17\).

The aim of the present study was to analyze the gene and protein expression, and the enzymatic activity of CYP3A1 and CYP3A2, as well as the nuclear expression of CAR and PXR. For this purpose, we used a validated animal model of cholestasis based on the bile duct ligation technique in animals rigorously stratified by degree of liver injury.

**MATERIALS AND METHODS**

**Reagents**

Midazolam, 4-hydroxymidazolam (4OH-MDZ), nicotinamide adenine dinucleotide 2' -phosphate (NADPH), dimethylsulfoxide (DMSO), 40% acrylamide solution, sodium dodecyl sulfate (SDS), and Tween 20 were purchased from Sigma-Aldrich Italy (Milan, Italy), 1'-hydroxymidazolam (1OH-MDZ) was purchased from SPIBio Bertin Pharma (Montigny le Bretonneux, France). Ultrapure water was obtained with PureLab Option Q apparatus (Elga Lab Water, High Wycombe, United Kingdom). Sucrose, Tris and MgCl\(_2\) were purchased from Applichem (Chicago, IL, United States). HPLC-grade methanol was purchased from Scharlau (Barcelona, Spain). Rabbit polyclonal anti-PXR, rabbit polyclonal anti-CAR, mouse anti-CYP3A1, rabbit anti-CYP3A2 and HRP-conjugated anti-mouse IgG antibodies were obtained from Abcam (Cambridge, United Kingdom). HRP-conjugated anti-rabbit IgG were obtained from Millipore (Billerica, MA, United States), and HRP-conjugated anti-goat IgG from Jackson ImmunoResearch (West Grove, PA, United States). Rabbit polyclonal anti-calnexin, rabbit polyclonal anti-GAPDH and goat polyclonal anti-acetyl histone H3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States).

**Animal care and use statement**

The procedures involving the animals were managed in accordance with national and international laws and policies (Directive 2010/63/EU on the protection of animals used for scientific purposes). The study design was approved by the Ethics Committee of University of Padova, and by the Italian Ministry for the care and use of laboratory animals (Prot. no. 24, 2015). Rats were kept in standard conditions (24°C, 12 h/12 h day/night cycle), and they have ad libitum access to food and water. Protocols involving animals were designed to minimize their pain, and each treatment was performed under isoflurane anesthesia.

**Animal treatments**

Cholestasis was induced in 16 male Wistar Kyoto rats (Charles River, Boston, MA, United States) by bile duct ligation and resection, as previously described\(^18,19\). Eight sham-operated rats were used as control animals. Eight bile-duct-ligated (BDL) animals were sacrificed two weeks after surgery to obtain a model of mild cholestasis, and the other 8 four weeks after surgery to obtain a model of severe liver injury. Blood was collected at the time of their sacrifice by cardiac puncture to perform biochemical liver function tests: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALKP), \(\gamma\)-glutamyl transferase (GGT), serum albumin, total and conjugated bilirubin. After sacrificing the animals, their livers were promptly removed and weighed, and a piece was excised, fixed in 4% formalin and embedded in paraffin for histological examination. Bile acid concentration was measured on liver tissue omogenates by means of the Bile Acid Assay kit obtained from Sigma-Aldrich Italy (Milan, Italy), following manufacturer’s instructions. The remaining liver tissue was washed in a buffer containing 50 mmol/L Tris, 150 mmol/L KCl, 2 mmol/L EDTA, (pH 7.4), frozen and stored at -80°C.

**Histological examination**

Two 4-micron sections were cut from the paraffin-
embedded liver tissue, and stained with hematoxylin-
eosin and fuchsin-picric acid (van Gieson staining), as
previously described[20,21]. Images were obtained with
a Leica SCN400 slide scanner. The Ishak system was
adopted to quantify the extent of liver damage, scoring
livers from 0 to 6 by severity of fibrosis[21].

Determination of mRNA levels by qRT-PCR
Total RNA was extracted from liver tissue after
homogenization and purified with the SV Total RNA
Isolation System (Promega Corporation, Madison,
WI, United States). Liver tissue was homogenized in
lysis buffer and mRNA was purified by means of silica-
gel-based columns, according to the manufacturer’s
instructions. A DNase treatment was used during RNA
extraction to prevent genomic DNA contamination.
Purified RNA was eluted in a final volume of 100 μL
RNAase-free water. Aliquots were stored at -80 °C until
use. qRT-PCR was carried out with the commercial
One Step SYBR PrimeScript RT-PCR Kit (Takara,
Mountain View, CA, United States). The reaction was
run as follows: 2 min at 50 °C and 2 min at 95 °C for the reverse
transcription, 40 cycles of 15 s at 95 °C and 60 s at
60 °C for the PCR reaction, and then 15 s at 95 °C,
15 s at 55 °C and 15 s at 95 °C for the dissociation
curve. During the exponential phase, the fluorescence
signal threshold was calculated, and the cycle threshold
(Ct) was ascertained. The Ct values were used to
calculate the relative mRNA expression, according to
the mathematical quantification model proposed by
Pfaff[23]. All samples were tested in triplicate and β-actin
was used as the housekeeping gene.

Preparation of hepatic nuclear fraction
Nuclear and cytoplasmic fractions were obtained
according to the method described by Dimarco et al[24]
with minor modifications. Briefly, 150 mg of liver tissue
was allowed to thaw in STM buffer containing 250
mmol/L sucrose, 50 mmol/L Tris-HCl pH 7.4, 5 mmol/L
MgCl₂ and Complete Protease Inhibitor Cocktail (Roche,
Milan, Italy). The tissue was then homogenized for
1 min at 1000 rpm with an IKA T25 Ultra-Turrax
dispenser. The homogenate was maintained in ice for
30 min, vortexed and centrifuged at 800 g for 15 min.
The pellet was resuspended in STM buffer, vortexed
and centrifuged at 1500 g for 15 min. The supernatant
was centrifuged at 10000 g for 10 min and
the supernatant was centrifuged at 45000 g for 30 min. The resulting supernatant was the “nuclear
fraction”.

Preparation of hepatic microsomal fraction
Microsomal fractions were obtained as reported
previously by Floreani et al[21]. The pellet containing the
microsomal fraction was resuspended in 250 mmol/L
sucrose, aliquoted and stored at -8 °C. The protein
content of the nuclear and microsomal fractions was
measured with a commercially available kit (Thermo
Fisher BCA Protein Assay kit, Rockford, IL, United
States) using bovine serum albumin for the calibration
curve.

Western blot analyses
Western blot analyses to ascertain the protein
expression of CYPs and the nuclear protein expression
of PXR and CAR were performed using 30 μg per lane
of nuclear or microsomal fractions, as previously
described[20]. Sodium dodecyl sulfate polyacrylamide
gel electrophoresis (SDS-PAGE) was performed on
8% polyacrylamide gels in reducing-denaturing
conditions, and proteins were transferred to a 0.45
μm nitrocellulose membrane (BioRad, Hercules, CA,
United States). Anti-PXR, anti-CAR, anti-CYP3A1
and anti-CYP3A2 primary antibodies were used to
detect PXR, CAR, CYP3A1 and CYP3A2 proteins in the
hepatic fractions, as previously described by De Martin
et al[20]. The signal intensity of the immunoreactive
bands was analyzed with the Quantity One software
(Bio-Rad Laboratories, Hercules, CA, United States),
and normalized to that of the acetyl-histone H3 or
the calnexin bands, for the nuclear or microsomal
fractions, respectively.

Measurements of 4- and 1’-midazolam hydroxylation
To analyze the formation of 4OH-MDZ and 1’OH-
MDZ, microsomal preparations were incubated with
increasing concentrations of midazolam (between
0.5 pmol/L and 20 pmol/L, n = 9) in a total volume
of 200 μL, as previously described[20]. Ten μg of
microsomal proteins obtained from sham and rats
with mild cholestasis were used, and 80 μg obtained
from rats with severe cholestasis. The reaction was
run for 5 or 10 min at 37 °C in 0.1 mol/L KH₂PO₄,
adding 0.5 mmol/L of NADPH, and then the reaction
was stopped by adding 100 μL of cold methanol. The
samples were centrifuged at 20000 g for 10 min and
the supernatants were analyzed by HPLC using a Shimadzu system equipped with a UV detector. The
two metabolites were separated chromatographically
by means of a Kinetex EVO C18 column (5 μm, 150
mm × 4.6 mm, Phenomenex, Castel Maggiore, Italy).
The mobile phase was water and acetonitrile (67:33).
Isocratic elution was conducted with a constant flow of
1 mL/min for 15 min. The UV detector was set up
at 220 nm, eluting 4OH-MDZ after 4.7 min, and
1'OH-MDZ after 5.2 min. The 4OH-MDZ and 1'OH-MDZ were quantified with standard calibration curves obtained with authentic 4OH-MDZ and 1'OH-MDZ at concentrations ranging from 1 to 50 nmol/mL (n = 8), processed in exactly the same way as the samples obtained from the kinetic experiments. The calibration curves were linear in this concentration range (r² = 0.99), the lowest value in the range representing the quantification limit of the assay. Both inter- and intra-assay CVs for the 4OH-MDZ and 1'OH-MDZ determinations (n = 5) were lower than 5% at 1 nmol/mL, and lower than 3% at 50 nmol/mL. MDZ 4- and 1'-hydroxylase activity was expressed as pmol of 4OH-MDZ and 1'OH-MDZ produced per mg of protein per min.

**Kinetic and statistical analyses**

The F-test was used to judge the best-fitting kinetic model for 4- and 1'-hydroxylation of midazolam (hyperbolic Michaelis-Menten model or Michaelis-Menten model with substrate inhibition). Kinetic parameters were estimated by non-linear regression analysis of untransformed initial velocity data (GraphPad Prism software) using the appropriate equation. The following kinetic parameters were calculated: Vmax (maximum velocity of the reaction), and Km (substrate concentration yielding 50% of Vmax).

Statistical analyses were performed with the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, United States). Unless specified otherwise, the data are presented as mean values ± SD. The experimental results were compared by one-way analysis of variance (ANOVA) or Student’s t-test, as appropriate. In the case of statistically significant differences (α = 0.05), the analysis of variance was followed by the Newman-Keuls post-hoc test. A P value < 0.05 was considered statistically significant.

**RESULTS**

**Biochemical findings and histology of rat livers**

The animal model of cholestasis was obtained by ligating the common bile duct[25]. The presence of cholestasis was confirmed after sacrificing the animal on the basis of plasma biochemical tests and histological examination. The latter revealed a normal liver architecture in the sham-operated animals (Figure 1A). The livers from rats sacrificed after 2 wk showed mild cholestasis (Figure 1B, Ishak score 1-3), and fibrous septa. Rats sacrificed after 4 wk showed severe cholestasis with bridging fibrosis surrounding cirrhotic nodules, and the original liver structure completely destroyed (Figure 1C, Ishak score 4-6). The degree of liver dysfunction was also assessed on the basis of serum biochemistry (albumin, AST, ALT, total and conjugated bilirubin, alkaline phosphatase and γGT), as shown in Table 2. The rats with mild cholestasis had the same serum albumin levels as the healthy control animals, whereas the levels were significantly lower in the rats with severe cholestasis (P < 0.05 vs control and mildly cholestatic rats). All other serum markers of liver injury were significantly increased in rats with severe cholestasis by comparison with either the controls or the animals with mild cholestasis. Liver bile acid concentration increased as liver function worsens (252 ± 32 nmol/g in sham-operated vs 404 ± 51 nmol/g in mildly cholestatic vs 515 ± 62 nmol/g liver tissue in severely cholestatic rats).
mRNA and protein expression of CYP3A1 and CYP3A2
The mRNA expressions of CYP3A1 and CYP3A2 are shown in Figure 2A and B. We found CYP3A1 gene expression significantly increased in mild cholestasis ($P < 0.01$ vs controls), while CYP3A2 gene expression tended to increase with respect to controls, but not to any significant degree. In contrast, there was a significant drop in the gene expression of both CYP3A1 ($P < 0.05$ vs controls) and CYP3A2 ($P < 0.05$ vs controls) when cholestasis became severe.

Figure 3 shows the protein expression of the two CYP3A isoforms (assessed on liver microsomal fractions). Like the mRNA, CYP3A1 protein expression increased significantly in mild cholestasis ($P < 0.01$ vs controls), then decreased significantly in severe cholestasis ($P < 0.05$ vs controls, and $P < 0.001$ vs rats with mild cholestasis). CYP3A2 protein expression only decreased when cholestasis became severe without first showing any significant increase in mild cholestasis ($P < 0.05$ vs controls, and $P < 0.01$ vs rats with mild cholestasis).

Kinetic analysis of 4- and 1'-hydroxylation activities
Midazolam hydroxylation in positions 1' and 4 was used as a marker reaction to assess CYP3A1 and CYP3A2 enzyme activity in the rat liver microsomal fractions$^{26,27}$. As shown in Figure 4, both reactions followed the classical Michaelis-Menten kinetic model. Table 3 shows the kinetic parameters of the midazolam hydroxylation reactions in the three groups of rats. For both reactions, there was a slight, statistically insignificant increase in Vmax in the rats.
with mild cholestasis, while the Vmax of both reactions decreased in the animals with severe cholestatic injury ($P < 0.001$ vs controls and rats with mild cholestasis).

In both hydroxylation reactions, there were no significant inter-group differences in the $K_m$ values.

**Expression of nuclear receptors PXR and CAR**

To see whether cholestasis influences the expression of CYP3A enzymes by modifying the nuclear levels of the two NRs mainly responsible for regulating their transcription, we measured PXR and CAR mRNA and protein nuclear expression (because they both translocate into the cell nucleus after their activation). As shown in Figures 5 and 6, a significant increase in PXR mRNA and protein expression was observed in the rats with mild cholestasis ($P < 0.001$), whereas a significant reduction was evident in those with severe cholestasis ($P < 0.05$ vs sham-operated rats; $P < 0.05$; $P < 0.01$; $P < 0.001$ vs mildly cholestatic rats).

**DISCUSSION**

In this study we investigated whether cholestasis affects the gene and protein expression, and the enzymatic activity of CYP3A1 and CYP3A2 enzymes, as well as the activation of CAR and PXR, the nuclear receptors controlling their transcription. For this purpose, we used a validated animal model of cholestasis induced by bile duct ligation, and rigorously stratified the animals on the basis of the severity of their liver injury. We found that mRNA and protein expression of CYP3A1 increased significantly in mild cholestasis ($P < 0.01$), whereas the expression and activity of both CYP3A1 and CYP3A2 decreased dramatically when cholestasis became severe. Alterations of the $V_{\text{max}}$ of 4-OH and 1'-OH-midazolam hydroxylation (marker reactions of CYP3A activity) were also identified. Consistently with these findings, the nuclear expression of both PXR and CAR rose initially, then virtually disappeared in the late stage of cholestatic injury.
Liver disease is known to impair various pathways of hepatic drug metabolism. Animal and clinical *in vivo* studies have shown that drug metabolism due to the 3A subfamily of CYP enzymes is significantly altered in severe liver disease.[28,29]. *In vitro* studies have shown a decrease in CYP3A activity in cholestatic liver disease,[30] though the mechanism(s) behind it have not been clarified.

Various studies, as reviewed in Chen *et al.*[31] for instance, focused on the NRs controlling the expression of drug-metabolizing enzymes, analyzing their regulation of gene transcription. Since these events are crucial in the detoxification and elimination of the potentially toxic biliary constituents accumulating in cholestasis, NRs represent attractive targets of pharmacotherapy for cholestatic disorders. CAR and PXR control the expression of CYP3A, and are known to facilitate adaptation to the higher intracellular bile acid concentrations of cholestasis by upregulating alternative hepatic export routes (MRP3 and MRP4), and inducing detoxification enzymes (SULTs, UGTs and CYPs).[5] In the present study, we examined how different degrees of cholestasis influenced the expression of CYP3A1 and CYP3A2, the main CYP3A isoforms involved in drug metabolism in the rat liver. We demonstrated that cholestasis-induced changes in their expression levels correlate with the observed changes in nuclear PXR and CAR expression, which are probably due in turn to the fact that they are activated by compounds, such as BAs, which increase in the cholestatic liver.[5,7,32]. Our finding that only the mRNA and protein levels of CYP3A1, but not CYP3A2, increased significantly in mild cholestasis is consistent with the significant increase in nuclear PXR and CAR expression, and probably due to CYP3A1 being much more susceptible to induction.[15]. The significant increase in CYP3A1 mRNA and protein expressions in the early stages of cholestasis prompts a slight, but statistically insignificant increase in the CYP3A1-mediated metabolism of midazolam, probably because the constitutive expression of CYP3A1 in the rat liver is much lower than that of CYP3A2,[20], and its protein expression is only doubled in rats with mild cholestasis. It is worth noting that a protective role of PXR had already been identified in an animal model of cholestasis, in which this NR was able to modulate hepatic damage.[7]. Indeed, hepatic damage from bile acid accumulation was increased in PXR−/− mice, and, on the basis of gene expression analyses, it has been suggested that, in response to cholestasis, PXR repressed and induced the expression of the hepatic membrane transporters OATP-C and OATP2 (OATP1B1), respectively.[33]. Accordingly, Xie *et al.*[34], demonstrated that the PXR agonist PCN could not reduce lithocholic acid (LCA)-induced toxicity in PXR−/− mice. Indeed, Saini and collaborators[35] have demonstrated that CAR activation is both necessary and sufficient to confer resistance to the hepatotoxicity of LCA. Our findings support these observations, suggesting that this protective role of PXR is lost in the late stages of

### Table 3 Kinetic parameters for 4- and 1’-hydroxylation activities of microsomal preparations obtained from sham-operated and cholestatic rats

|              | 4-OH MDZ | Vmax (pmol/mg/min) | Km (μmol/L) | 1'-OH MDZ | Vmax (pmol/mg/min) | Km (μmol/L) |
|--------------|----------|--------------------|-------------|-----------|--------------------|-------------|
| Sham-operated rats | 1009.9 ± 269.4 | 3.4 ± 1.1 | 946.7 ± 292.2 | 3.5 ± 1.4 | 1116.0 ± 333.7 | 2.8 ± 1.6 |
| Mildly cholestatic rats | 1116.0 ± 333.7 | 6.1 ± 4.7 | 1017.0 ± 317.4 | 6.1 ± 4.7 | 1009.9 ± 269.4 | 2.8 ± 1.6 |
| Severely cholestatic rats | 218.4 ± 88.11| 2.5 ± 0.9 | 200.1 ± 68.7 | 2.5 ± 0.9 | 1116.0 ± 333.7 | 2.5 ± 0.9 |

The results are reported as mean ± SD of 8 rats per group. *P < 0.001 vs sham-operated rats; P < 0.001 vs mildly cholestatic rats.

**Figure 5** mRNA expression of nuclear receptors in rat livers. Pregnane x receptor (A) and constitutive androstane receptor (B) gene expression in sham-operated and cholestatic rats reported as fold variations compared with sham rats. Results are mean ± SEM obtained from 8 rats in each group. *P < 0.05, *P < 0.001 vs sham rats; *P < 0.05, *P < 0.01 vs mildly cholestatic rats.
CYP3A1, to promote the elimination of endogenous and exogenous substances. This compensatory mechanism is lost when liver function deteriorates because the associated reduction in NRs gives rise to a dramatic decrease in both CYP3A isoforms, with a consequent further accumulation of hepatic BAs. There is a well-known cross-talk operating between these NRs[36,37], but the molecular mechanisms behind it remain to be clarified. It has recently been observed that some NRs share the same response elements in transactivation of their target genes. This is the mechanistic molecular base for the so called “cross-talk of NRs”[38]. We know that PXR cross-talks with other NRs (in particular, CAR and FXR) to regulate intermediate metabolism through the trans-activation and trans-repression of genes controlling cholesterol, bile acid, bilirubin, glucose and lipid homeostasis[38]. FXR, CAR and PXR share many regulatory effects and their functionality in the context of regulation of liver detoxification and bile acid metabolism is largely overlapping. Studies performed in animal models of cholestasis have already shown that FXR and PXR agonists show overlapping activities[39]. On the other hand, it has been shown that FXR functions as a CAR antagonist in regulating the expression of the basolateral transporter MRP4[40]. It has also been suggested that PXR may play a role in the regulation of CYP7A1, the rate-limiting enzyme of cholesterol catabolism and bile acid formation, which is transcriptionally regulated by FXR[41].

On the basis of the results obtained in this study, we can hypothesize that PXR and CAR work synergistically to maintain CYP3A induction in the early stages of cholestasis, and their detoxification function fails when the liver dysfunction becomes severe. This finding can have two clinical consequences. For start, cholestatic patients may have an altered drug metabolism: in the early stage due to the induction of CYP3A enzymes; and in the late stage due to the high deposition of fibrotic liver and consequent hepatocyte loss. This effect could be particularly relevant in humans because a single isoform, i.e., CYP3A4, is responsible for metabolizing more than 50% of the drugs used in medical practice[42]. Secondly, since PXR activation is known to induce alternative hepatic export routes (MRP3 and MRP4) and detoxification enzymes (SULTs, UGTs and CYPs), the induction of these cellular pathways with PXR and/or CAR agonists could be exploited as a therapeutic strategy for the management of cholestatic diseases.

In conclusion, our findings clearly demonstrate that early- and late-stage cholestasis affects CYP3A-mediated drug metabolism differently. Since changes in the BA detoxification routes play a pivotal part in cholestatic liver injury, the regulation of PXR and CAR could be targeted therapeutically with the aim of ameliorating cholestatic liver injury by regulating...
CYP3A-mediated metabolism of BA.

ARTICLE HIGHLIGHTS

Research background
Primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are the two main classes of cholestatic disease, and chronic cholestasis and liver inflammation are their main pathophysiological components\[1\]. To escape the deleterious effects of high concentrations of bile acids (BAs), a peculiar feature of cholestatic disease, the liver triggers an adaptive response to cholestasis, activating a complex network of nuclear receptors (NRs) that tightly regulate the BA transporters to maintain proper BA homeostasis. Among liver NRs, both the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR), which control the expression of CYP3A drug-metabolizing enzymes, play a major role in these adaptive responses. PXR and CAR act as xenobiotic sensors, as one of their main functions is to regulate the expression of enzymes and transporters involved in xenobiotic elimination. These NRs are also involved in controlling hepatic processes closely related to the progression of cholestatic diseases, such as BA homeostasis, lipid metabolism, fibrosis and inflammation\[2\]. Changes in CAR and PXR, and CYP3A expression have already been identified in the course of chronic liver disease, but different effects have been documented depending on the etiology and severity of the cholestasis. The aim of this study was to analyze expression and enzymatic activity of CYP3A1 and CYP3A2, as well as the nuclear expression of CAR and PXR in a validated animal model of cholestasis rigorously stratified on the basis of the degree of liver dysfunction.

Research motivation
Both in vitro and in vivo studies have shown a decrease in CYP3A activity in cholestatic liver disease, but a clear demonstration of the mechanism(s) responsible for it is still lacking. To our knowledge, no studies published to date simultaneously analyzed CYP3A enzyme expression and activity and the activation of NRs responsible for their transcriptional control in different stages of cholestatic disease. CYP3A enzymes are the most abundant CYPs in human beings, and the most important enzymes in terms of drug metabolism, because they have a key role in the first-pass and systemic metabolism of many drugs. On the basis of these considerations, the aim of this study was to analyze expression and enzymatic activity of CYP3A1 and CYP3A2, as well as the nuclear expression of CAR and PXR. For this purpose, we used a validated animal model of cholestasis based on the bile duct ligation technique in animals rigorously stratified by degree of liver injury.

Research objectives
In this study we investigated whether cholestasis affects the gene and protein expression, and the enzymatic activity of CYP3A1 and CYP3A2 enzymes, as well as the activation of CAR and PXR, the nuclear receptors controlling their transcription. Our results let us hypothesize that PXR and CAR work synergistically to maintain CYP3A induction in the early stages of cholestasis, and their detoxification function fails when the liver dysfunction becomes severe.

Research methods
The procedures involving the animals were managed in accordance with national and international laws and policies (Directive 2010/63/EU on the protection of animals used for scientific purposes). The study design was approved by the Ethics Committee of University of Padova, and by the Italian Ministry for the care and use of laboratory animals (Prot. no. 24, 2015). Gene and protein expressions of PXR, CAR, CYP3A1 and CYP3A2 were assessed by means of qRT-PCR and Western blot, respectively. Alterations in CYP3A activity were measured by calculating the kinetic parameters of marker reactions for CYP3A enzymes. Statistical analyses were performed with the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, United States). The experimental results were compared by one-way analysis of variance or Student’s t-test, as appropriate. In the case of statistically significant differences (p < 0.05), the analysis of variance was followed by the Newman-Keuls post-hoc test. A P value < 0.05 was considered statistically significant.

Research results
On the basis of the results obtained in this study, we could hypothesize that PXR and CAR work synergistically to maintain CYP3A induction in the early stages of cholestasis, and their detoxification function fails when the liver dysfunction becomes severe. The mechanism by which cholestasis affects “cross-talk” between PXR, CAR and other NRs in the liver remains to be described in detail.

Research conclusions
The findings of this study can have two clinical consequences. For start, cholestatic patients may have an altered drug metabolism: in the early stage due to the induction of CYP3A enzymes; and in the late stage due to the high deposition of fibrotic liver and consequent hepatocyte loss. This effect could be particularly relevant in humans because a single isofrom, i.e., CYP3A4, is responsible for metabolizing more than 50% of the drugs used in medical practice. Secondly, since PXR activation is known to induce alternative hepatic export routes (MRP3 and MRP4) and detoxification enzymes (SULTs, UGTs and CYPs), the induction of these cellular pathways with PXR and/or CAR agonists could be exploited as a therapeutic strategy for the management of cholestatic diseases.

Research perspectives
On the basis of the results obtained in this study, we hypothesized that cholestatic patients may have an altered drug metabolism and suggested the induction of liver detoxification by means of PXR and/or CAR agonists as a therapeutic strategy for the management of cholestatic diseases. To test the first hypothesis, clinical studies analyzing the pharmacoekinetik parameters of cholestatic patients stratified by degree of liver injury can be performed after the administration of selected drugs. Preclinical studies analyzing the effects of the administration of PXR/CAR agonists on the cholestatic liver are currently in progress in our laboratory.

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