Differential Effect of Liver Cirrhosis on the Pregnan X Receptor–Mediated Induction of CYP3A1 and 3A2 in the Rat

Sara De Martin, Daniela Gabbia, Giovanna Albertin, Maria Martina Sfriso, Claudia Mescoli, Laura Albertoni, Giovanna Paliuri, Sergio Bova, and Pietro Palatini

Department of Pharmaceutical and Pharmacological Sciences (S.D.M., D.G., G.P., S.B., P.P.), Department of Molecular Medicine, Section of Anatomy (G.A., M.M.S.), and Department of Medicine, Surgical Pathology and Cytopathology Unit (C.M., L.A.), University of Padova, Padova, Italy

ABSTRACT

Conflicting results have been obtained by clinical studies investigating the effect of liver cirrhosis on enzyme induction. Because ethical concerns do not allow for methodologically rigorous studies in humans, we addressed this question by examining the effect of the prototypical inducer dexamethasone (DEX) on the pregnane X receptor (PXR)–mediated induction of CYP3A1 and 3A2 in a validated animal model of liver cirrhosis obtained by exposure of rats to carbon tetrachloride. For this purpose, we assessed mRNA levels, protein expressions, and enzymatic activities of both CYP3A enzymes, as well as mRNA and protein expressions of PXR in rat populations rigorously stratified according to the severity of liver insufficiency. Constitutive mRNA and protein expressions of CYP3A1 and CYP3A2 and their basal enzyme activities were not affected by liver dysfunction. DEX treatment markedly increased steady-state mRNA level, protein content, and enzymatic activity of CYP3A1 in healthy and cirrhotic rats, irrespective of the degree of liver dysfunction. On the contrary, the inducing effect of DEX on gene and protein expressions and enzyme activity of CYP3A2 was preserved in moderate liver insufficiency, whereas it was greatly curtailed when liver insufficiency became severe. mRNA and protein expressions of PXR were neither reduced by liver dysfunction nor increased by DEX treatment. These results indicate that even the inducibility of cytochrome P450 isoforms under the transcriptional control of the same nuclear receptor may be differentially affected by cirrhosis and may partly explain why conflicting results were obtained by human studies.

Introduction

Metabolic interactions, that is, inhibition and induction of drug-metabolizing enzymes, especially those of the cytochrome P450 (P450) superfamily, are the main cause of serious drug-drug interactions encountered in clinical practice (Lin and Lu, 1998). Although inhibition-mediated drug interactions are generally considered more dangerous than those due to induction (Bachmann and Lewis, 2005), serious or even fatal consequences of P450 induction have been reported, such as transplant rejection and exacerbation of liver failure caused by acetaminophen overdose (Park et al., 1996; Fuhr, 2000).

In principle, drug interactions can be managed by dose adjustment. However, the wide interindividual variability in the magnitude of metabolic drug interactions makes this task difficult. Identification of the factors responsible for this variability is therefore crucial for an appropriate dose adjustment in clinical practice. The influence of liver functional status on the magnitude of metabolic interactions has long been investigated. However, whereas it has been clearly established that liver dysfunction markedly decreases the magnitude of inhibitory drug interactions (Orlando et al., 2004, 2006, 2009; Palatini et al., 2010), the effect of liver insufficiency on the inducibility of drug-metabolizing enzymes is still a controversial matter. It has been pointed out that two main factors are probably responsible for the conflicting results obtained by studies on human beings (Hoyumpa and Schenker, 1982; Palatini et al., 2008): 1) the variable nature and degree of hepatic dysfunction in the clinical studies thus far performed; and 2) the possible differential susceptibility to liver insufficiency of the diverse types of inducing effects examined, which are mediated by different mechanisms.

Early animal studies (Marshall and McLean, 1969; Farrell and Zaluzny, 1984; Wu et al., 1991) found no cirrhosis-associated modification of the degree of induction of P450-mediated metabolic reactions. However, they examined rats with essentially mild liver cirrhosis, which generally causes minor alterations in the regulation of drug metabolism (Palatini et al., 2008). Thus, they left unanswered the question of whether inducibility may be compromised in severe liver insufficiency.

Because ethical constraints do not give consent for methodologically rigorous studies in human beings, we recently tried to clarify this question by investigating the aryl hydrocarbon receptor (AhR)–mediated induction of CYP1A1 and CYP1A2 in rats with experimentally induced cirrhosis, rigorously stratified according to the severity of liver dysfunction (Floreani et al., 2013). That study showed that the inducibility of CYP1A enzymes is strictly related to the degree of liver impairment, as it is not affected by liver dysfunction.
essentially preserved in the compensated state of cirrhosis, whereas it becomes severely curtailed when decompensation occurs. However, these results cannot a priori be extended to the induction of other P450 isoforms, because different nuclear receptors are involved in the transcription of their P450 genes.

The aim of the present study was to assess the influence of liver dysfunction on the pregnane X receptor (PXR)–mediated induction of CYP3A enzymes, the P450 subfamily most abundantly expressed in the liver (Shimada et al., 1994), by dexamethasone (DEX), which is the most effective inducer of rat CYP3A enzymes (Ghosal et al., 1996). For this purpose, we evaluated the effect of DEX on control and cirrhotic rats obtained by exposure to carbon tetrachloride (CCl₄), which produces animals with compensated or decompensated cirrhosis depending on the length of treatment (Floreani et al., 2013).

Although four CYP3A enzymes are expressed in rat liver (Mahnke et al., 1997), CYP3A2 and CYP3A1 are regarded as the most metabolically relevant isoforms in male rats; the former, which is male specific, is the isoform expressed at the highest constitutive level, and the latter is the isoform most susceptible to induction (Ghosal et al., 1996; Jan et al., 2006). Therefore, we studied the inducing effect of DEX on these two CYP3A enzymes by means of the three techniques previously used for the assessment of the AhR-mediated CYP1A induction, as follows: 1) determination of CYP3A1 and CYP3A2 mRNA steady-state levels by quantitative real-time reverse transcription–polymerase chain reaction (PCR); 2) measurement of the protein expressions of CYP3A enzymes by means of Western blot analysis; and 3) determination of the kinetic parameters of CYP3A1 and CYP3A2 enzyme reactions by means of kinetic analysis of testosterone (TST) 6β-hydroxylase activity. Because in vitro experiments (Huss and Kasper, 2000; Pascussi et al., 2000) have shown that PXR-mediated induction of CYP3A enzymes by DEX is partly due to a positive regulation of PXR expression by the glucocorticoid receptor (GR), we also assessed the effects of cirrhosis and DEX on the mRNA and protein expressions of these two nuclear receptors.

Materials and Methods

Reagents. NADPH was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Dimethylsulfoxide, 40% acrylamide solution, SDS, and Tween 20 were purchased from Sigma-Aldrich Italy (Milan, Italy). Phenobarbital was obtained from Bracco S.p.A. (Milan, Italy). Acetonitrile (high-performance liquid chromatography grade) was from Carlo Erba Reagents (Milan, Italy). Ultrapure water was obtained by means of a Pure-Laboratory Option Q apparatus (Elga Laboratory Water, High Wycombe, UK).

Microsomes prepared from baculovirus-infected insect cells expressing rat CYP3A1 or CYP3A2 (Supersomes) were purchased from BD Gentest (Woburn, MA). The expression levels of CYP3A1 and CYP3A2 were provided by the manufacturer’s data sheets.

Rabbit polyclonal anti-CYP3A1 antibody, sheep polyclonal anti-CYP3A2 antibody, mouse polyclonal anti-PXR and anti-GR antibodies, and rabbit anti-mouse IgG, used as secondary antibody, were obtained from Abcam (Cambridge, UK); goat anti-rabbit and rabbit anti-sheep IgG, also used as secondary antibodies, were obtained from Millipore (Billerica, MA). Mouse monoclonal anti-β-actin antibody was purchased from Santa Cruz Biotechnology.

Animals and Treatments. The procedures involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies (European Economic Community Council Directive 86/609, OJ L 358, 1, December 12, 1987; National Institutes of Health Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication 85-23, 1985). The study design was approved by the Ethics Committee of the University of Padova for the care and use of laboratory animals (Protocol 18758; March 26, 2010).

Study Design. The investigation was performed on male Wistar rats according to the experimental protocol shown in Fig. 1, which was previously described in detail (Floreani et al., 2013). Briefly, cirrhosis was induced by

![Fig. 1. Study design. PB, phenobarbital.](image-url)
exposing animals to CCl₄ in an inhalation chamber twice per week, following a method previously described (Jimenez et al., 1992). Exposure time increased from 30 seconds to 4 minutes during the first 5 weeks, and was then 5 minutes from the sixth week to the end of the treatment. Phenobarbital (0.3 g/l) was added to the drinking water of rats, because it greatly increases the formation rate of the reactive metabolites of CCl₄ responsible for the development of cirrhosis, thereby drastically reducing the cirrhosis induction time (Jimenez et al., 1992; Rosa et al., 2010). After a 2-week washout period, which was shown to be sufficient to allow full withdrawal of the inducing effect of phenobarbital on hepatic P450s (Marshall and McLean, 1969; Farrell and Zaluzny, 1984), healthy, nonascitic, and ascitic rats were divided into two subgroups, as follows: induced and control rats. The former received 100 mg/kg DEX dissolved in 3 ml corn oil once daily for 3 consecutive days by the oral route (Zaluzny, 1984), healthy, nonascitic, and ascitic rats were divided into two subgroups, as follows: induced and control rats. The former received 100 mg/kg DEX dissolved in 3 ml corn oil once daily for 3 consecutive days by the oral route.

Total RNA Isolation System (Promega, Madison, WI). During RNA extraction, mRNA expression of CYP3A1, CYP3A2, PXR, and GR, hepatic tissue was homogenized, and total RNA was extracted according to the severity of cirrhotic alterations (Ishak et al., 1995).

Histogram Evaluation. Immediately after excision, a piece (3–4 g) of each liver was fixed in 4% neutral buffered formalin for histologic examination, as previously described (Floreani et al., 2013). Images were obtained by means of a Leica SCN400 slide scanner. To evaluate the degree of the CCl₄-induced liver damage, the Ishak scoring system was adopted, which scores livers from 0 to 6 according to the severity of cirrhotic alterations (Ishak et al., 1995).

Preparation of Liver Microsomal Fractions and Total Protein Extracts. Microsomal fractions were prepared exactly as previously described (Floreani et al., 2013).

Western blot analysis of PXR expression in hepatic tissue was performed on total liver protein extracts. For this purpose, frozen hepatic tissue (about 0.2 g) was allowed to thaw in a modified ice-cold radioimmunoprecipitation assay buffer consisting of 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and a Complete Protease Inhibitor Cocktail (Roche, Milan, Italy). Hepatic tissue was then homogenized in 10 volumes (w/v) of the same buffer in ice for two 5-second intervals with an IKA T25 Ultra-Turrax disperser and processed, as previously described (Floreani et al., 2012). Protein concentrations of both microsomal fractions and total protein extracts were determined by means of a commercially available kit (Novagen BCA protein assay kit; Merck Millipore, Merck Millipore S.p.A., Milan, Italy) using a standard calibration curve obtained with known amounts of bovine serum albumin.

Determination of mRNA Levels by Real-Time PCR. To determine the mRNA expression of CYP3A1, CYP3A2, PXR, and GR, hepatic tissue was homogenized and total RNA was extracted and purified by means of the SV Total RNA Isolation System (Promega, Madison, WI). During RNA extraction, a DNase treatment was carried out to remove genomic DNA contamination. Total RNA was then reverse-transcribed to cDNA. Negative controls, used to verify the absence of contaminating DNA, were obtained by processing samples of RNA in the absence of reverse transcriptase. Real-time PCR was carried out in an I-Cycler iQ system (Bio-Rad Laboratories, Milan, Italy), as described in detail previously (Albertin et al., 2005), using the primers reported in Table 1. The PCR program included a denaturation step at 95°C for 3 minutes, 40 cycles of two amplification steps (95°C for 15 seconds and annealing extension at 60°C for 30 seconds) and a melting curve (60–90°C with a heating rate of 0.5°C/10 seconds). During the exponential phase, the fluorescence signal threshold was calculated, and the fractional cycle number at which each sample crossed the threshold (cycle threshold) was determined. Cycle threshold values were used to calculate the relative mRNA expression, according to the mathematical quantification model proposed by Pfaffl (2001).

Table 1: Primer sequences used in this study, National Center for Biotechnology Information reference sequences, and amplicon sizes (base pairs)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>RefSeq</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A1</td>
<td>5'-cca-tca-ccg-cag-aag-tg-3'</td>
<td>5'-ctt-tcc-cca-taa-tcc-cca-et-3'</td>
<td>NM013105</td>
<td>102</td>
</tr>
<tr>
<td>CYP3A2</td>
<td>5'-agt-agg-gat-ttt-gga-aag-3'</td>
<td>5'-ca-tgg-gga-aca-tct-cc-3'</td>
<td>NM155312</td>
<td>119</td>
</tr>
<tr>
<td>PXR</td>
<td>5'-cg-cag-tgc-gtt-ttg-3'</td>
<td>5'-ggt-tca-tgg-ccc-ttc-tga-a-3'</td>
<td>NM052980</td>
<td>97</td>
</tr>
<tr>
<td>GR</td>
<td>5'-gtt-gag-gaa-cga-gaa-3'</td>
<td>5'-ca-sag-agg-gga-aca-tct-cc-3'</td>
<td>NM012576</td>
<td>136</td>
</tr>
<tr>
<td>HMBS</td>
<td>5'-ctc-ttg-tgc-tgc-ttc-3'</td>
<td>5'-ggt-tga-gtt-ttc-ccc-caa-ta-3'</td>
<td>NM013168</td>
<td>161</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5'-ccc-agg-gta-a-3'</td>
<td>5'-ggt-tca-tgg-ccc-ttc-tga-a-3'</td>
<td>NM031144</td>
<td>163</td>
</tr>
</tbody>
</table>

HMBS, hydroxymethylbilane synthase.
The inhibitory effect of the selective CYP3A inhibitor clotrimazole (Turan et al., 2001) on TST 6-
OH-TST production per mg protein per minute.

**Results**

**Serum Chemistry and Histology of Rat Livers.** The results of biochemical tests of liver function and histologic examination of healthy and cirrhotic rats are shown in Table 2. Apart from serum albumin, there were only minor, statistically nonsignificant differences between healthy and nonascitic cirrhotic rats. All indexes of liver function were significantly altered in rats with ascites, indicating advanced hepatocellular insufficiency. The significantly higher aspartate aminotransferase and alanine aminotransferase values in DEX-treated rats of all three groups can be ascribed to the inducing effect of DEX on these hepatic enzymes (Ennulat et al., 2010). Histologic examination revealed normal liver architecture in healthy animals (Fig. 2A), whereas nonascitic (Fig. 2B) and ascitic (Fig. 2C) cirrhotic rats showed progressive alterations of liver structure, with increasing presence of fibrous septa, which were never present in healthy animals. Quite similar cirrhosis-associated alterations were observed in control and DEX-treated rats of each group.

**mRNA and Protein Expressions of CYP3A1 and CYP3A2.** Figure 3 shows the values of CYP3A1 mRNA and protein expressions relative to those of control healthy rats. The level of CYP3A1 mRNA concentrations causing 50% inhibition of control enzyme activities) were determined by the use of nonlinear regression of relative reaction velocities at increasing inhibitor concentrations (Eagling et al., 1998).

Statistical analyses were also performed by means of the GraphPad Prism software. Unless otherwise indicated, the data are presented as mean values ± S.D. Comparison of the experimental data obtained from healthy, nonascitic, and ascitic cirrhotic groups was made by one-way analysis of variance. In case of significant differences (α = 0.05), the analysis of variance was followed by the Newman-Keuls post hoc test. Comparison of the results obtained from control and induced animals within each group was performed by means of Student’s t test, P < 0.05 was considered statistically significant.

**Results**

**Serum Chemistry and Histology of Rat Livers.** The results of biochemical tests of liver function and histologic examination of healthy and cirrhotic rats are shown in Table 2. Apart from serum albumin, there were only minor, statistically nonsignificant differences between healthy and nonascitic cirrhotic rats. All indexes of liver function were significantly altered in rats with ascites, indicating advanced hepatocellular insufficiency. The significantly higher aspartate aminotransferase and alanine aminotransferase values in DEX-treated rats of all three groups can be ascribed to the inducing effect of DEX on these hepatic enzymes (Ennulat et al., 2010). Histologic examination revealed normal liver architecture in healthy animals (Fig. 2A), whereas nonascitic (Fig. 2B) and ascitic (Fig. 2C) cirrhotic rats showed progressive alterations of liver structure, with increasing presence of fibrous septa, which were never present in healthy animals. Quite similar cirrhosis-associated alterations were observed in control and DEX-treated rats of each group.

**mRNA and Protein Expressions of CYP3A1 and CYP3A2.** Figure 3 shows the values of CYP3A1 mRNA and protein expressions relative to those of control healthy rats. The level of CYP3A1 mRNA concentrations causing 50% inhibition of control enzyme activities) were determined by the use of nonlinear regression of relative reaction velocities at increasing inhibitor concentrations (Eagling et al., 1998).

Statistical analyses were also performed by means of the GraphPad Prism software. Unless otherwise indicated, the data are presented as mean values ± S.D. Comparison of the experimental data obtained from healthy, nonascitic, and ascitic cirrhotic groups was made by one-way analysis of variance. In case of significant differences (α = 0.05), the analysis of variance was followed by the Newman-Keuls post hoc test. Comparison of the results obtained from control and induced animals within each group was performed by means of Student’s t test, P < 0.05 was considered statistically significant.
did not decrease significantly in cirrhotic with respect to healthy animals (Fig. 3A). DEX treatment caused a significant increase in CYP3A1 mRNA expression in all three groups of rats, although to a somewhat lesser extent in ascitic animals. A representative Western blot (Fig. 3B) shows that the specificity of the anti-rat CYP3A1 antibody was not absolute, because it cross-reacted, although to a very limited extent, with CYP3A2. Quantification of the immunodetectable CYP3A1 protein (Fig. 3C) gave results in substantial agreement with those of real-time PCR, because it revealed that protein expression was similar in the three groups of control rats and increased significantly in all groups of DEX-treated animals.

In contrast to CYP3A1, the constitutive mRNA expression of CYP3A2 (Fig. 4A) tended to increase with the decline of liver function, although to a nonsignificant extent. At further variance with CYP3A1, significant DEX induction of CYP3A2 mRNA was only observed in healthy and nonascitic rats, whereas it was absent in ascitic animals. The results of Western blot analysis (Fig. 4, B and C) were in accordance with those of real-time PCR experiments, regarding both the effects of cirrhosis and those of DEX treatment, because there was a slight, nonsignificant cirrhosis-associated increase in CYP3A2 protein expression in control animals, and a significant inducing effect of DEX in healthy and nonascitic animals, but not in ascitic ones.

**mRNA and Protein Expressions of PXR and GR.** Because DEX has been reported to induce CYP3A enzymes through both direct activation of PXR and GR-mediated increase in PXR expression (Huss and Kasper, 2000; Pascussi et al., 2000), we measured the mRNA levels and the protein contents of both PXR and GR. Figure 5A shows that PXR gene expression was affected neither by cirrhosis nor by DEX treatment. The results of Western blot analysis (Fig. 5, B and C) were in agreement with those of real-time PCR, because no significant difference could be observed between healthy and cirrhotic rats or control and induced ones. Similarly, neither cirrhosis nor DEX treatment caused any statistically significant modification of mRNA or protein expression of GR (Fig. 6).

**Kinetic Analysis of Testosterone 6β-Hydroxylase Activity.** Although TST 6β-hydroxylation is currently used as a marker reaction of CYP3A enzymes in both humans and rats (Kobayashi et al., 2002;
Walsky and Obach, 2004), its specificity in the rat has been questioned (Chovan et al., 2007). Thus, to assess the specificity of TST 6-b-hydroxylation, we tested the effect of clotrimazole, a potent and selective inhibitor of rat CYP3A enzymes (Turan et al., 2001), on microsomal preparations of all six groups of rats. Figure 7 shows that clotrimazole-induced inhibition of TST 6-b-hydroxylation was always complete at 50 nM, a concentration that does not appreciably affect the activity of other major P450 isoforms (Turan et al., 2001). IC50 values were very similar for all microsomal preparations (6–7 nM and 2–3 nM for microsomes obtained from control and induced rats, respectively), and similar to those observed with recombinant CYP3A1 (4.6 ± 0.5 nM) and CYP3A2 (2.0 ± 0.2 nM).

In preliminary experiments, we also measured midazolam 4- and 1'-hydroxylations, alternative marker reactions for rat CYP3A enzymes (Kobayashi et al., 2002). Both of these reactions exhibited Michaelis-Menten kinetics with substrate inhibition, which could not be fitted to a two-enzyme kinetic model. In the concentration range used in this study (5–800 μM), the TST 6-b-hydroxylation activity of rat liver microsomes was best fitted to a two-enzyme Michaelis-Menten equation, which is consistent with the presence of two main CYP3A isoforms: CYP3A1 and CYP3A2. To distinguish between the activities of these two enzymes, we determined the kinetic parameters of the reaction using rat recombinant CYP3A1 and CYP3A2 enzymes. The results (shown in Table 3) indicate that, whereas the Vmax values of the two enzymes were similar, the mean Km value was 13 times lower for CYP3A2. Best fitting of the kinetic data obtained with microsomal preparations yielded two Km values differing by an order of magnitude and similar to those observed with recombinant CYP3A1 (4.6 ± 0.5 nM) and CYP3A2 (2.0 ± 0.2 nM).

In Fig. 5, PXR mRNA and protein expressions in control and DEX-treated rats are shown. Experimental data showing PXR mRNA levels are reported as fold variation compared with healthy control rats. (B) Representative Western blots showing the separation of total liver protein extracts obtained from control and DEX-treated rats. Lanes 1–3, healthy, non-ascitic, and ascitic control rats; lanes 4–6, healthy, non-ascitic, and ascitic DEX-treated rats. (C) Amount of PXR protein in livers from control and DEX-treated rats normalized to the signal intensity of the corresponding β-actin band (43 kDa) and shown as fold variation compared with healthy control rats. All results are means ± S.E. of data obtained from eight rats per group.

Fig. 5. PXR mRNA and protein expressions in control and DEX-treated rats. (A) Experimental data showing PXR mRNA levels are reported as fold variation compared with healthy control rats. (B) Representative Western blots showing the separation of total liver protein extracts obtained from control and DEX-treated rats. Lanes 1–3, healthy, non-ascitic, and ascitic control rats; lanes 4–6, healthy, non-ascitic, and ascitic DEX-treated rats. (C) Amount of PXR protein in livers from control and DEX-treated rats normalized to the signal intensity of the corresponding β-actin band (43 kDa) and shown as fold variation compared with healthy control rats. All results are means ± S.E. of data obtained from eight rats per group.

In Fig. 6, GR mRNA and protein expressions in control and DEX-treated rats are shown. Experimental data showing GR mRNA levels are reported as fold variation compared with healthy control rats. (B) Representative Western blots showing the separation of total liver protein extracts obtained from control and DEX-treated rats. Lanes 1–3, healthy, non-ascitic, and ascitic control rats; lanes 4–6, healthy, non-ascitic, and ascitic DEX-treated rats. (C) Amount of GR protein in livers from control and DEX-treated rats normalized to the signal intensity of the corresponding β-actin band (43 kDa) and shown as fold variation compared with healthy control rats. All results are means ± S.E. of data obtained from eight rats per group.

Fig. 6. GR mRNA and protein expressions in control and DEX-treated rats. (A) Experimental data showing GR mRNA levels are reported as fold variation compared with healthy control rats. (B) Representative Western blots showing the separation of total liver protein extracts obtained from control and DEX-treated rats. Lanes 1–3, healthy, non-ascitic, and ascitic control rats; lanes 4–6, healthy, non-ascitic, and ascitic DEX-treated rats. (C) Amount of GR protein in livers from control and DEX-treated rats normalized to the signal intensity of the corresponding β-actin band (43 kDa) and shown as fold variation compared with healthy control rats. All results are means ± S.E. of data obtained from eight rats per group.
caused a nonsignificant increment, particularly in nonascitic cirrhotic animals, as previously observed with nonspecific P450 probes in the same rat model of cirrhosis (Wu et al., 1991). In full agreement with mRNA and protein expression data, DEX treatment significantly increased the $V_{\text{max}}$ of the CYP3A1-catalyzed reaction in all three groups of rats, although to a somewhat lower extent in ascitic animals, whereas it increased significantly the $V_{\text{max}}$ of CYP3A2 only in healthy and nonascitic cirrhotic rats. The $K_m$ values of both CYP3A1 and CYP3A2 activities were similar in all groups of rats. As a consequence, the modifications of $\text{CL}_{\text{int}}$ (the ratio of $V_{\text{max}}$ to $K_m$) paralleled those of $V_{\text{max}}$. The absolute values of $\text{CL}_{\text{int}}$ of the recombinant enzymes were very similar to those previously obtained by Carr et al. (2006) (3.3 and 0.1 $\mu$L/min per pmol CYP for rCYP3A2 and rCYP3A1, respectively), thus confirming the greater catalytic efficiency of CYP3A2.

Fig. 7. Inhibitory effect of clotrimazole on TST 6β-hydroxylase activities of pooled microsomal preparations obtained from control (left column) and DEX-treated (right column) rats. Results are the means ± S.E. of three separate determinations performed in duplicate. S.E. is not shown where the size of data points is larger than the S.E. bar.

Discussion

Induction in human beings has been assessed by measuring modifications of the pharmacokinetic parameters (half-life or systemic clearance) of a metabolic probe or of the enzyme activities of liver tissues obtained at biopsy. Although such determinations provide a reliable measure of the extent of induction, they give no information regarding the mechanism by which liver cirrhosis may impair enzyme induction. We have recently shown that the use of an animal model of liver cirrhosis produced by exposure to CCl4 makes it possible to study the mechanisms by which cirrhosis may affect enzyme induction. We have recently shown that the use of an animal model of liver cirrhosis produced by exposure to CCl4 makes it possible to study the mechanisms by which cirrhosis may affect enzyme induction.
Previous studies using Long-Evans (Cooper et al., 1993) or Sprague-Dawley (Jan et al., 2006) male rats found no detectable mRNA level or negligible protein expression of CYP3A1, respectively. The results of our real-time PCR determinations and Western blot analyses show that both CYP3A1 and CYP3A2 are constitutively expressed in male Wistar rats, and their mRNA and protein levels are not significantly modified by liver cirrhosis. The latter finding contrasts with the previous observation regarding CYP1A2 expression in the same animal model of cirrhosis, because the basal mRNA and protein levels of CYP1A2 were found to decrease significantly (up to 80%) in proportion to the severity of liver dysfunction (Floreani et al., 2013). That result was in agreement with the observations of human studies, which have consistently found a profound fall in CYP1A2 expression in liver cirrhosis (reviewed in Murray et al., 2001; Floreani et al., 2012), PXR mRNA and protein levels are upregulated following the administration of various AhR ligands (Franc et al., 2001; Floreani et al., 2012), PXR mRNA and protein levels are neither appreciably reduced by liver cirrhosis nor increased by DEX treatment. The former finding is consistent with the observation that the constitutive expression of both CYP3A enzymes is unaffected by liver dysfunction, and is also consistent with the observation that CYP3A1 induction is not compromised in liver cirrhosis, but leaves unanswered the question of why the induction of CYP3A2, which is transcriptionally activated by the same nuclear receptor, is compromised in decompensated cirrhosis. Although no certain explanation can be provided on the basis of the present knowledge of CYP3A2 transcription machinery, it may be hypothesized that, in addition to mRNA translation, the gene transcription of CYP3A2 is also under the control of a different regulatory mechanism involving either coactivators or a RNA polymerase more sensitive than those of CYP3A1 to the modifications associated with severe liver cirrhosis. The observation that the mRNA level of CYP3A1 is increased to a far greater extent in response to DEX treatment than that of CYP3A2 is consistent with a different regulatory control of their transcription. Evidence that the mRNA expressions of CYP3A1 and CYP3A2 are not coordinately regulated in response to DEX treatment has also been obtained by Choudhuri et al. (1995).

Although real-time PCR and Western blot analyses provide information relevant to the mechanism (altered transcription or translation) underlying changes in P450 expression, determination of P450 activity provides the unique quantitative measure of changes in P450-mediated drug metabolism (Murray et al., 2001) and is the methodological approach recommended by US Food and Drug Administration (Hewitt et al., 2007). In previous kinetic analyses of TST 6-hydroxylation by rat liver microsomes, velocity data were fitted to a one-enzyme hyperbolic kinetic model (Jimenez et al., 1992; Murray and Butler, 1996; Jiko et al., 2005; Velenosi et al., 2012) or to a sigmoidal kinetics with low degree of autoactivation [Hill coefficients of 1.2–1.4 (Carr et al., 2006)]. This did

### TABLE 3

Kinetic parameters for testosterone 6β-hydroxylase activities of recombinant cytochrome P450 and microsomal preparations obtained from control and dexamethasone-treated rats

<table>
<thead>
<tr>
<th>mRNA translation</th>
<th>CYP3A1</th>
<th>CYP3A2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V_max</strong> (pmol/min per mg protein)</td>
<td>5.3 ± 1.0</td>
<td>7.4 ± 0.3</td>
</tr>
<tr>
<td><strong>K_m</strong> (μM)</td>
<td>34 ± 1</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td><strong>CL_{int}</strong> (l/min per mg protein)</td>
<td>0.15 ± 0.04</td>
<td>2.8 ± 0.1</td>
</tr>
</tbody>
</table>

*Results for recombinant enzymes are the means ± S.D. of three separate determinations performed in duplicate; those for liver microsomes are the means ± S.D. of data obtained from eight rats per group.*

---

*V_max is expressed as pmol/min per mg protein for liver microsomes and pmol/min per pmol P450 for recombinant enzymes.*

*CL_{int} is expressed as μl/min per mg protein for liver microsomes and μl/min per pmol P450 for recombinant enzymes.*

*P < 0.05, **P < 0.01, ***P < 0.001 versus control rats of the same group; $P$ < 0.05 versus healthy rats; $P$ < 0.05 versus nonascitic cirrhotic rats.*

---

Because the expression of CYP3A enzymes is under the transcriptional control of PXR, and PXR upregulation, mediated by DEX activation by GR, has been observed in vitro with both human (Pascussi et al., 2000) and rat hepatic cell lines (Huss and Kasper, 2000), we assessed the effects of liver dysfunction and DEX on the mRNA and protein expressions of both PXR and GR. However, we could not confirm in vivo the results previously obtained with cultured hepatocytes, because we observed no significant modification of the mRNA or protein expression of these nuclear receptors. Thus, unlike AhR, whose transcription has been found to be reduced in vivo in the presence of liver cirrhosis (Floreani et al., 2013), and upregulated by GR, has been observed in vitro with both human (Pascussi et al., 2000) and rat hepatic cell lines (Huss and Kasper, 2000), we assessed the effects of liver dysfunction and DEX on the mRNA and protein expressions of both PXR and GR.
not consent a determination of the separate contributions of the two main CYP3A isoforms (CYP3A1 and CYP3A2) to the measured enzymatic activity. Based on velocity curves obtained with 11 substrate concentrations in rigorously controlled initial rate conditions, we could show that the kinetic data were best described by a two-enzyme Michaelis-Menten model, and that the $K_m$ values obtained with liver microsomes were in good agreement with those of recombinant CYP3A1 and CYP3A2. This has enabled us to determine for the first time the change in activity of each of these two P450 isoforms. The results of these determinations confirm the indications of real-time PCR and Western blot analyses that both CYP3A1 and CYP3A2 are constitutively expressed in Wistar rats. The observed changes in $V_{max}$ are also in full agreement with those in mRNA and protein expressions because of the following: 1) the constitutive activities of both CYP3A1 and CYP3A2 are not significantly modified by liver dysfunction; and 2) the inducibility of CYP3A1 activity is preserved in both nonasitic and asitic cirrhotic rats, whereas that of CYP3A2 is preserved in rats with moderate liver dysfunction, but significantly decreased when liver dysfunction becomes severe.

In conclusion, the results of our real-time PCR, Western blot, and kinetic analyses all concur in showing that the induction of CYP3A enzymes is differentially affected by liver function impairment because, unlike CYP3A1, CYP3A2 is not significantly induced in severe liver dysfunction. The induction of the latter enzyme is compromised at a pretranslational level, because proportional reductions in induced mRNA level, protein expression, and enzyme activity are observed in asitic rats. The effect of liver dysfunction on both the constitutive expression and the PXR-mediated induction of CYP3A enzymes differs from that on the basal expression and the AhR-mediated induction of CYP1A enzymes, observed in the same animal model of liver cirrhosis (Floreani et al., 2013). That study showed that the constitutive expression of CYP1A2 (the sole CYP1A isoform constitutively expressed in the liver) is decreased in cirrhosis, and the induction of both CYP1A enzymes is well preserved in compensated, but markedly reduced in decompensated, cirrhosis. These effects on both constitutive and induced enzyme expressions could be ascribed to a decreased transcription of CYP1A genes as a consequence of an AhR-reduction. Unlike AhR, PXR expression is not reduced in liver dysfunction, and the precise mechanism responsible for the selective impairment of CYP3A2 transcription remains to be determined. Whatever the explanation may be for this selective effect, the present results make it clear that no general conclusion can be drawn from the study of any particular P450 isoform, because even the induction of enzymes under the transcriptional control of the same nuclear receptor may be differentially affected by liver function impairment. These findings may also partly explain why conflicting results have been obtained by human studies assessing the effect of liver dysfunction on the induction of drug metabolism.

Acknowledgments

The authors thank Mauro Berto for skilful technical assistance.

Authorship Contributions

Participated in research design: Palatini.

Conducted experiments: Gabbia, Sfriso, Mescoli, Albertoni, Paliuri.

Performed data analysis: De Martin, Albertini, Mescoli, Bova, Palatini.

Wrote or contributed to the writing of the manuscript: De Martin, Palatini.

References


Address correspondence to: Dr. Pietro Palatini, Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Largo Meneghetti 2, 35131 Padova, Italy. E-mail: pietro.palatini@unipd.it