Inducibility of Drug-Metabolizing Enzymes by Xenobiotics in Mice with Liver-Specific Knockout of Ctnnb1

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ABSTRACT:

Basal as well as xenobiotic-induced expression of the main enzymes from phase I and phase II of drug metabolism is confined to the perivenous areas of the mammalian liver lobule. Whereas signaling transduction pathways that govern xenobiotic-induced expression of these enzymes via ligand-activated transcription factors such as constitutive androstane receptor (CAR) or the aryl hydrocarbon receptor (AhR) have been intensively studied, the mechanisms regulating zone-specific basal expression of genes related to drug metabolism and preferential response of perivenous hepatocytes to xenobiotics inducers are still largely unknown. Recent publications by our and other groups point to an important role for the Wnt/β-catenin pathway in the maintenance of the perivenous hepatocyte gene expression profile including the main hepatic detoxification enzymes, and β-catenin signaling was recently implicated in the expression of several cytochrome P450 isoenzymes.

To analyze, whether the β-catenin pathway would also affect inducible expression of drug-metabolizing enzymes, mice with liver-specific knockout of the Ctnnb1 gene (encoding β-catenin) were treated with different model inducers of xenobiotic metabolism. Knockout of β-catenin led to alterations in basal expression of most drug metabolism-related genes analyzed and resulted in strongly diminished responses to agonists of CAR-, AhR-, and nuclear factor erythroid-related factor 2-dependent transcription. Taken together, the data presented in this study indicate that β-catenin not only regulates basal expression of drug-metabolizing enzymes but also determines the magnitude and hepatic localization of response to xenobiotic inducers in vivo.

In the absence of xenobiotic inducers of drug metabolism, expression of most drug-metabolizing enzymes is restricted to the perivenous hepatocyte compartment. This applies especially to many cytochrome P450 (P450) isoforms from families 1 to 3, which consist of the main detoxification enzymes in mammalian liver (Jungermann and Katz, 1989; Gebhardt, 1992; Lindros, 1997; Oinonen and Lindros, 1998). Expression of these enzymes is induced to high levels by substances that bind to nuclear receptors that act as ligand-activated transcription factors, such as the constitutive androstane receptor (CAR) and the aryl hydrocarbon receptor (AhR) (for reviews, see Swales and Negishi (2004) and Bock (1994)). Once activated, receptors trigger the induction of distinct subsets of drug-metabolizing enzymes. In most cases, this process occurs preferentially in perivenous hepatocytes. However, the number of hepatocytes expressing the respective enzymes increases with increasing doses of enzyme inducers, and enzyme expression is extended to the adjacent, more periporal hepatocytes without altering overall zonation of the proteins (Wolf et al., 1984; Tritscher et al., 1992; Oinonen and Lindros, 1998).

Extensive research over the last decades has elucidated the mechanisms that govern xenobiotic-induced expression of mRNAs and proteins related to drug metabolism. The mechanisms that regulate zone-specific basal expression of these enzymes in perivenous hepatocytes remained largely unknown. However, expression of various P450 isoforms has been linked to the hypothalamic-pituitary axis, which seems to be involved in the repression of P450 transcription in periporal hepatocytes and in the regulation of gender-specific P450 isoforms (Oinonen and Lindros, 1998). Furthermore, effects of the insulin/glucagon system on P450 expression in cultured hepatocytes were reported (Saad et al., 1994).

More recently, β-catenin has been established as a new player in the regulation of zonal gene expression in mouse liver. For reviews on β-catenin signaling, see Willert and Nusse (1998) and Lustig and Behrens (2003). In brief, the so-called “canonical” Wnt/β-catenin pathway is physiologically activated by Wnt molecules that bind to receptors at the cell surface. Wnt binding leads to the inhibition of a multiprotein complex that, in the absence of Wnt signaling, phosphorylates β-catenin thus marking the protein for proteasomal degradation.

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ABBREVIATIONS: P450, cytochrome P450; CAR, constitutive androstane receptor; AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene; PB, phenobarbital; 3MC, 3-methylcholanthrene; PCN, pregnenolone-α-carbonitrile; BHA, butylated hydroxyanisole; RT-PCR, reverse transcription-polymerase chain reaction; GST, glutathione S-transferase; G6P, glucose-6-phosphatase; Ugt, UDP-glucuronosyl-transferase; PXR, pregnane-X-receptor; PPAR, peroxisome proliferator-activated receptor; Nrf2, nuclear factor erythroid-related factor 2; Ephx1, microsomal epoxide hydrolase; Nqo, NAD(P)H-quinone-oxidoreductase.
When that phosphorylating complex is inhibited upon Wnt binding, β-catenin accumulates in the cytosol and translocates to the nucleus, where it binds to transcription factors such as T cell factor-1 and initiates transcription of target genes.

Based on the observation that mouse hepatomas with mutational activity of β-catenin express high levels of various “periveneous” marker mRNAs and proteins including several P450 iso-enzymes (Loepfen et al., 2005; Stahl et al., 2005; Braeuning et al., 2007a), we postulated that β-catenin-dependent signaling determines the periveneous hepatocyte gene expression profile including the main drug-metabolizing enzymes (Hailfinger et al., 2006). Accordingly, treatment of cultured hepatocytes with inducers of β-catenin signaling was able to induce the expression of Cyp1a1, Cyp2b10, and Cyp2e1 mRNAs (Hailfinger et al., 2006). More evidence for an important contribution of β-catenin signaling to the basal expression of various P450 enzymes comes from recent studies with transgenic mice: Sekine et al. (2006) described the loss of Cyp1a2, Cyp2c29, and Cyp2e1 expression in mice with liver-specific knockout of the Ctnnb1 gene (encoding β-catenin), whereas levels of Cyp1a1 and Cyp3a11 were not altered. These findings were largely confirmed shortly thereafter by Tan et al. (2006), who detected down-regulation of Cyp1a2, Cyp2e1, Cyp2a5, and Cyp2d9 isoforms in the Ctnnb1 knockout mice by microarray analysis, whereas up-regulation was reported for Cyp1a1, Cyp2b9, and Cyp2b10 upon disruption of the Ctnnb1 gene.

Thus, it is beyond dispute that signaling through the β-catenin signaling pathway influences hepatic basal expression of different P450s. However, there is also some evidence that β-catenin might be involved in the regulation of inducible expression of P450s and other drug-metabolizing enzymes mediated by xenobiotic-sensing receptors: 1) the AhR is a target of β-catenin signaling (Chesire et al., 2004; Stahl et al., 2005; Hailfinger et al., 2006); 2) cotransfection of activated β-catenin facilitates activation of an AhR-responsive luciferase reporter plasmid in mouse hepatoma cells by the AhR ligand 2,3,7,8-tetrachloro-p-dibenzodioxin (TCDD) (Loepfen et al., 2005). In this work, we analyzed the expression and hepatic localization of representative drug-metabolizing enzymes in response to different model inducers of xenobiotic metabolism in mice with hepatocyte-specific Ctnnb1 knockout in vivo.

Materials and Methods

Animal Breeding. Mice with hepatocyte-specific knockout of Ctnnb1 (encoding β-catenin) were obtained as follows: transgenic mice withloxP sites inserted in the introns flanking exons 3 and 6 of the Ctnnb1 gene (Huelsken et al., 2001) were interbred with Alb-Cre mice expressing Cre recombinase under control of the hepatocyte-specific albumin promoter (purchased from The Jackson Laboratory, Bar Harbor, ME). Genotyping was performed by standard PCR using the primer pairs Ctnnb1loxP sense, 5’-ACTGCTTTGTGTCTCT- TCCCTCTG3’ and Ctnnb1loxP antisense, 5’-CAGCCCAAGGAACGAC -GTGAGG-3’; and Cre sense, 5’-TCTAGTGAAGCAACCCCTGGTCG-3’ and Cre antisense, 5’-TTTGCTGCTATTACGGTGTACG3’. Homozygous Ctnnb1loxP/loxP mice carrying a Cre allele, resulting in hepatocyte-specific knockout of the Ctnnb1 gene, are referred to as “Ctnnb1 knockout” mice in the following text. Mice were kept on a 12-h dark/light cycle and had access to food and tap water ad libitum. Mice were sacrificed between 9:00 and 11 AM to avoid circadian influences. Animals received humane care and protocols were followed according to institutional guidelines.

Animal Experiments. Groups (n = 5) of Ctnnb1 knockout or age- and gender-matched wild-type animals were treated at 8 weeks of age with different inducers of drug-metabolizing enzymes as follows: 1) a single i.p. injection of 3 mg/kg body weight 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene (TCPOBOP) (Sigma-Aldrich, Taufkirchen, Germany) 72 h before killing; 2) a single i.p. injection of 90 mg/kg body weight phenobarbital (PB) (Geyer, Renningen, Germany) followed by 3 days of PB-containing (0.05% w/v) diet (Sniff, Soest, Germany); 3) 2 i.p. injections of 50 mg/kg body weight 3-methylcholanthrene (3MC) at 48 and 24 h before killing; 4) a single i.p. injection of 200 mg/kg body weight pregnenolone-α-carbonitrile (PCN) 72 h before killing; 5) a single i.p. injection of 350 mg/kg body weight butylated hydroxyanisole (BHA) 72 h before killing; 6) a single i.p. injection of 500 mg/kg body weight clofibrate (substances 3–6 were purchased from Sigma-Aldrich) 72 h before killing. Control groups received injections with the respective solvents, i.e., corn oil (3MC, TCPOBOP, PCN, BHA, clofibrate) or phosphate-buffered saline. Isopropanol (2 g/kg body weight) was administered by gavage in a 25% aqueous solution.

Isolation of Hepatocytes. Periportal and perivenous subpopulations of hepatocytes were isolated and enriched by combined digitorin/collagenase perfusion of the liver as described previously (Braeuning et al., 2006; Hailfinger et al., 2006). To obtain periporal hepatocyte subpopulations, 5 mM digitoron solution was infused through the venous cava and then immediately flushed out from the opposite direction. To obtain perivenous hepatocytes, digitoron solution was infused through the portal vein. After digitorin treatment, liver was perfused with collagenase solution. Subsequently, viable hepatocytes were separated by density gradient centrifugation. Viability of the resulting hepatocyte fractions was always ~80 to 90% as determined by trypan blue staining.

Real-Time Reverse Transcription-Polymerase Chain Reaction. Total RNA was isolated using TRIzol reagent (Invitrogen, Karlsruhe, Germany) and reverse transcribed by using the Quantitect Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions. Real-time reverse transcription-polymerase chain reaction (RT-PCR) was carried out on a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) using the LightCycler FastStart DNA Master SYBR Green I Kit (Roche) and the primer pairs listed in Table 1. Relative expression ratio of the target genes in the samples was computed based on the crossing point difference of a sample versus a control and its real-time PCR efficiency according to Pfaffl (2001). 18s rRNA expression was used for normalization.

Immunostaining. Slices from frozen (10-μm thick) or paraffin-embedded (5-μm thick) liver samples were stained by standard methods as recently described (Hailfinger et al., 2006; Braeuning et al., 2007b) using antisera against overall-Cyp1A (1:1000 dilution; a gift from Dr. R. Wolf, Biochemical Research Centre, University of Dundee, Dundee, UK), overall-Cyp2C1 (1:300; a gift from Dr. R. Wolf), overall-Cyp3A (1:1000 dilution; Biotrend, Cologne, Germany), and overall-GSTM1 (1:500; a gift from Dr. R. Wolf), in combination with appropriate horseradish peroxidase-conjugated secondary antibodies (1: 20; Dako Denmark A/S, Glostrup, Denmark) with 3-aminio-9-ethylcarbazole/ H2O2 as substrates.

Glucose-6-Phosphatase Staining. Activity of glucose-6-phosphatase (G6P) in slices from glutaraldehyde-fixed liver samples was determined by using a method described by Wachtstein and Meisel (1957).

Western Blotting. Whole cell extracts were separated by SDS-polyacryl- amide gel electrophoresis (30 μg/lane) and transferred to polyvinylidene difluoride membranes using standard methods. Protein detection was carried out as recently described (Hailfinger et al., 2006) using antibodies against overall-Cyp1A (1:5000 dilution; a gift from Dr. R. Wolf, Biochemical Research Centre, University of Dundee, Dundee, UK), overall-Cyp2C1 (1:300; a gift from Dr. R. Wolf), overall-Cyp3A (1:1000 dilution; Biotrend, Cologne, Germany), and overall-GSTM1 (1:500; a gift from Dr. R. Wolf), in combination with appropriate horseradish peroxidase-conjugated secondary antibodies (1: 20; Dako Denmark A/S, Glostrup, Denmark) with 3-aminio-9-ethylcarbazole/ H2O2 as substrates.

Statistical Analysis. For statistical analysis of genotype differences, a Student’s t test was used. Differences were considered significant when p < 0.05. Statistical probability is indicated by asterisks as follows: *p < 0.05, **, p < 0.01, and ***, p < 0.001. Data were also subjected to two-way ANOVA analysis for genotype and treatment effects. These results are shown in Supplemental Table 1.

Results

Mice with hepatocyte-specific knockout of the Ctnnb1 gene (referred to as Ctnnb1 knockout mice) and age- and gender-matched controls (n = 5 per group) were treated with different inducers of
drug-metabolizing enzymes to elucidate the role of β-catenin signaling in xenobiotic-induced expression of drug metabolism-related genes in vivo. Due to limited availability of animals, most inducers were tested with either males or females. Induction of CAR-dependent gene expression by the differentially acting CAR agonists TCPOBOP and phenobarbital was studied with mice from both genders, because gender differences in response to CAR agonists have been reported in the literature (Ledda-Columbano et al., 2003). Livers were isolated, and hepatic levels and zonation of various mRNAs and proteins were analyzed in vivo. Due to limited availability of animals, most inducers were tested with either males or females. Induction of CAR-dependent gene expression by the differentially acting CAR agonists TCPOBOP and phenobarbital was studied with mice from both genders, because gender differences in response to CAR agonists have been reported in the literature (Ledda-Columbano et al., 2003). Livers were isolated, and hepatic levels and zonation of various mRNAs and proteins were analyzed.

### Basal Expression of CAR Target Genes

In the absence of known inducers of CAR-dependent transcription, striking alterations in the amount and hepatic localization of various drug-metabolizing enzymes from both phase I and phase II were observed in male and female Ctnnb1 knockout mice: mRNAs for the CAR targets Cyp1a2, Cyp2c, GSTm2, GSTm3, and GSTm6 were expressed at significantly lower levels in Ctnnb1 knockout mice from both genders. Inversely, Cyp2b10 and Cyp2f2 mRNAs were more abundantly expressed in these animals with Cyp2b10 showing the more pronounced effect. Cyp3a mRNA levels were significantly increased in male Ctnnb1 knockout mice. A trend for higher expression of this isoform in the knockout was also observed in females, but it missed the criteria of significance in our analysis (Fig. 1, left bars of the diagrams). The loss of Cyp2c and GSTm expression in the livers of Ctnnb1 knockout mice was confirmed at the protein level by Western blotting, whereas only minor effects were seen for Cyp2c (Fig. 2A). Immunohistochemical analysis demonstrated an induction of Cyp3a mRNA in perivenous hepatocytes. Zonation of all other mRNAs analyzed (Cyp1a2, Cyp2b10, Cyp2c, Cyp2e1, Cyp2f2, GSTm2, GSTm3, GSTm6) was either lost or at least strongly reduced in the Ctnnb1 null mice (Table 2).

### Effects of TCPOBOP

In the presence of the CAR activator TCPOBOP, strong induction of mRNAs encoding Cyp1a2, Cyp2b10, Cyp2c, Cyp3a, GSTm2, and GSTm3 was observed in male wild-type mice, whereas no remarkable effects were observed for GSTm6 and the “peripheral” P450 isoform Cyp2f2 (Fig. 1A). Cyp2b10 and Cyp3a expression was significantly higher in livers of wild-type mice after treatment with TCPOBOP, despite higher basal mRNA levels in Ctnnb1 knockout mice. Likewise, Cyp1a2, Cyp2c, GSTm2, and GSTm3 mRNAs were also induced to significantly higher levels by TCPOBOP in mice from the wild-type group. At the protein level, induction of Cyp3a and GSTm was also clearly detectable by Western blotting, whereas only minor effects were seen for Cyp2c (Fig. 2A). Immunohistochemical analysis demonstrated an induction of Cyp2c, Cyp3a, and GSTm proteins in wild-type mice associated with the expected broadening of the perivenous hepatocyte subpopulation expressing these enzymes (Fig. 3A). Induction of the aforementioned proteins was also visible in Ctnnb1 knockout livers, but without remarkable zonation. However, there were obvious differences in Cyp3a and GSTm (and, to a lesser extent, Cyp2c) expression between individual cells in the response to TCPOBOP: a scattered pattern of strong and weak responders was observed (Fig. 3A).

When TCPOBOP was administered to female mice, an induction pattern of Cyp1a2, Cyp2b10, Cyp2c, Cyp3a, and GSTm2 was detected, which was similar (i.e., up to higher levels in the wild-type animals) to what had been observed in males (Fig. 1B). However, genotype differences between TCPOBOP-stimulated expression of Cyp2c, Cyp3a, and GSTm2 missed our criteria of significance. GSTm3 was induced to approximately equal levels in female mice from both genotypes, whereas Cyp2f2 and GSTm6 were slightly repressed after TCPOBOP treatment. The lower -fold induction of
Cyp2b10 in female mice (approximately 100-fold, compared with >1000-fold in males) was due to higher basal levels of the respective mRNA in these animals.

**Effects of PB.** An additional experiment was performed using an indirect activator of CAR signaling, PB. After treatment with PB, results were comparable with those of the TCPOBOP experiment. Significantly higher cellular levels of Cyp1a2, Cyp2c, GSTm2, GSTm3, and GSTm6 mRNAs were detected in male wild-type mice compared with their Ctnnb1-deficient counterparts (Fig. 1C). A tendency for higher PB-induced levels of Cyp2b10 and Cyp3a was also observed in the wild-type group but was not statistically significant. In contrast to TCPOBOP, PB was able to induce Cyp2f2 in wild-type mice, whereas Ctnnb1 null mice, which exhibited higher basal levels of this P450 isoform, did not respond with further induction of the enzyme (Fig. 1C). Again, the results obtained with female mice largely resembled those seen with males. PB-induced expression of Cyp1a2, Cyp2b10, Cyp2c, GSTm2, and GSTm6 was significantly higher in the wild-types compared with PB-treated Ctnnb1 knockout mice, and the (nonsignificant) trend for higher PB-induced Cyp3a expression in wild-type mice was also seen in this experiment (Fig. 1D). As in the TCPOBOP experiment, PB induced GSTm3 up to equal levels in female mice from both genotypes, contrasting the situation in males.

In summary, activation of CAR led to the expression of higher levels of Cyp1a2, Cyp2b10, Cyp2c, GSTm2, GSTm6, and—to a lesser extent—Cyp3a in male and female wild-type mice compared with their littermates with hepatocyte-specific knockout of Ctnnb1. CAR agonist-induced GSTm3 expression was also higher in wild-type males, whereas no differences regarding this particular enzyme were observed in females.

**Expression of AhR Target Genes: Effects of 3MC.** In an analogous experiment, 3MC/AhR-dependent enzyme induction was analyzed in male wild-type and Ctnnb1 knockout mice. In the absence of

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**Fig. 1.** Basal and inducible expression of CAR target mRNAs in livers from wild-type and Ctnnb1 knockout mice, as determined by real-time RT-PCR. Gene expression in response to treatment with TCPOBOP in male (A) and female (B) mice and in response to phenobarbital treatment in males (C) and females (D) is shown relative to vehicle controls. Mean ± S.D. (n = 5 per group) are given relative to the values obtained with the wild-type controls. Significant differences between genotypes are indicated as follows: *, p < 0.05; **, p < 0.01, and ***, p < 0.001 (Student’s t test). TCP, 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene; wt, wild-type; ko, Ctnnb1 knockout.

**Fig. 2.** Western analysis of protein expression in livers from male wild-type and Ctnnb1 knockout mice. A, expression of CAR targets Cyp2C, Cyp3A, and GSTm in TCPOBOP-treated animals and the respective controls. Successful knockout of Ctnnb1 is demonstrated by Western analysis for β-catenin protein. B, expression of AhR-dependent Cyp1A in 3MC-treated animals. In all experiments, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as a loading control. β-cat, β-catenin; wt, wild-type; ko, Ctnnb1 knockout.
3MC, mRNA for Cyp1a1 was barely detectable and did not show significant differences in expression between the two genotypes. The more constitutively expressed Cyp1a isoform Cyp1a2, two different transcript variants of Ugt1a6, Ugt1a7, and GSTa were analyzed as additional AhR target genes. Cyp1a2 and GSTa were more abundantly expressed in wild-type mice. A similar trend was also visible for the Ugts. However, statistical significance was only achieved in the case of one of the Ugt1a6 variants (Fig. 4).

3MC led to a strong increase in Cyp1a1 mRNA in both genotypes. Resulting Cyp1a1 mRNA levels were significantly higher in the wild-types (Fig. 4). A similar effect was also observed for Cyp1a2, but in this case the differences were not statistically significant. One of the Ugt1a6 transcripts, Ugt1a7, and GSTa were also affected by 3MC treatment, whereas the second Ugt1a6 transcript (containing an alternative exon 1) was unsusceptible to AhR activation. 3MC-induced Ugt1a6 mRNA levels in Ctnnb1 knockout mice remained slightly lower than in wild-type mice. However, statistical significance was only achieved in the case of one of the Ugt1a6 variants (Fig. 4).

At the protein level, the highest levels of Cyp1A were detected by Western blotting in 3MC-treated wild-type mice, whereas much less of the protein was detected in liver lysates from their Ctnnb1-deficient littermates. Resembling the findings of the mRNA expression analysis, basal Cyp1A expression was also lower in the knockout animals (Fig. 2B). Immunohistochemical staining demonstrated an almost complete loss of noninduced hepatic Cyp1A expression in the knockout mice. After injection with 3MC, increased amounts of Cyp1A protein were detectable in mice from both genotypes. In agreement with previous studies of AhR ligand-induced Cyp1A expression (see the Introduction), the number of Cyp1A-expressing cells of wild-type mice increased toward the portal vein. However, no clear zonation was visible in the knockout animals, which showed a rather uniform-like expression (Fig. 3B).

Expression of Pregnane-X-Receptor Target Genes: Effects of PCN. Stimulation of pregnane-X-receptor (PXR)-dependent transcription was performed in male mice by intraperitoneal injection of PCN. Quantification of mRNAs from the PXR target genes Cyp2b10 and Cyp3a demonstrated considerable differences between the genotypes (Fig. 5A): Cyp2b10 was strongly induced by PCN in wild-type mice. PCN did not exert remarkable effects on Cyp2b10 in mice with hepatocyte-specific knockout of the Ctnnb1 gene, which possessed significantly higher basal levels of this P450 isoform. Cyp3a mRNA levels were strongly stimulated by PCN in both groups. Whereas basal

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<tr>
<td><strong>Hepatic zonation of mRNAs encoding drug-metabolizing enzymes in Ctnnb1 wild-type (wt) and knockout (ko) mice</strong></td>
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<tr>
<th>Gene</th>
<th>Ctnnb1 Wild-Type</th>
<th>Ctnnb1 Knockout</th>
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<tbody>
<tr>
<td>Cyp1a2</td>
<td>821 ± 72***</td>
<td>160 ± 24</td>
</tr>
<tr>
<td>Cyp2b10</td>
<td>319 ± 82*</td>
<td>121 ± 39</td>
</tr>
<tr>
<td>Cyp2c</td>
<td>964 ± 44***</td>
<td>302 ± 113</td>
</tr>
<tr>
<td>Cyp2e1</td>
<td>1736 ± 151***</td>
<td>204 ± 11**</td>
</tr>
<tr>
<td>Cyp2f2</td>
<td>4 ± 1**</td>
<td>83 ± 10</td>
</tr>
<tr>
<td>Cyp3a</td>
<td>169 ± 36</td>
<td>35 ± 9*</td>
</tr>
<tr>
<td>GSTm2</td>
<td>906 ± 117**</td>
<td>105 ± 23</td>
</tr>
<tr>
<td>GSTm3</td>
<td>1579 ± 350**</td>
<td>92 ± 29</td>
</tr>
<tr>
<td>GSTm6</td>
<td>162 ± 22</td>
<td>86 ± 26</td>
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Mean [n = 3 (wt); n = 4 (ko)] ± S.E.M. are given. Significant differences in zonal gene expression between wild-type and Ctnnb1 knockout mice are indicated by asterisks: *** p < 0.001, * p < 0.05, and ** p < 0.01 (Student’s t test).
Cyp3a expression was higher in Ctnnb1 knockout mice (see above), no significant differences were detectable between the two genotypes after treatment with PCN. Results of the mRNA quantification were confirmed by Western analysis (data not shown) and immunostaining for Cyp3a. It is interesting to note that a reversal of Cyp3a zonation was seen in wild-type mice, which displayed higher levels of the protein in periportal hepatocytes after PCN treatment (Fig. 3C).

Expression of Peroxisome Proliferator-Activated Receptor Target Genes: Effects of Clofibrate. Cellular mRNA levels of the peroxisome proliferator-activated receptor (PPAR)α-regulated Cyp4a14 isoform were quantified in female wild-type and Ctnnb1 knockout mice. Basal Cyp4a14 levels were reduced by ∼75% in Ctnnb1 knockout mice. Induction of PPARα signaling with clofibrate led to moderately (∼2-fold, compared with the wild-type control group) elevated levels of Cyp4a14, which did not differ between both genotypes (Fig. 5B).

Expression of Nuclear Factor Erythroid-Related Factor 2 Target Genes: Effects of BHA. Basal and BHA-induced levels of the nuclear factor erythroid-related factor 2 (Nrf2)-dependent mRNAs encoding microsomal epoxide hydrolase (Ephx1), GSTα, and NAD(P)H-quinone-oxidoreductase (Nqo1) were analyzed (Fig. 5C): basal Ephx1 expression was slightly higher in Ctnnb1 knockout mice than in their wild-type littermates, but without being statistically significant. GSTα mRNA levels were significantly higher in the wild-type animals. No remarkable effect of genotype on basal Nqo1 levels was found. After BHA injection, an approximately 2.2-fold increase in Ephx1 mRNA was observed in wild-type mice, whereas their knockout counterparts did not show an induction of the enzyme (Fig. 5C). Levels of GSTα mRNA were also significantly elevated by BHA in the wild-type animals (∼2-fold), whereas mice with knockout of Ctnnb1 did not respond to BHA with a significant induction of GSTα. Comparable effects were observed for Nqo1, which was exclusively inducible by BHA in Ctnnb1 wild-type mice (Fig. 5C). In summary, Ctnnb1 null mice were not responsive to BHA-mediated induction of Nrf2-dependent transcription.

Induction of Cyp2e1. In the absence of known inducers, Cyp2e1 was almost completely absent in livers from male and female knockout mice, whereas high levels of Cyp2e1 mRNA and protein were detectable in wild-type hepatocytes located in the perivenous zone of the liver lobule (data not shown). After intragastric administration of 2 g/kg body weight isopropanol, slightly elevated Cyp2e1 protein contents were detected by immunohistochemistry and Western blotting in wild-type animals, whereas the enzyme was still absent in Ctnnb1 knockout mice (data not shown).

Expression of Nuclear Receptors Involved in Drug Metabolism. Levels of mRNAs encoding nuclear receptors involved in xenobiotic-mediated induction of drug-metabolizing enzymes, namely AhR, CAR, PXR, PPARα, retinoid-X-receptor α, and Nrf2, were analyzed by real-time RT-PCR (Fig. 6). Both AhR and CAR were significantly down-regulated by ∼60% in male and in female Ctnnb1 knockout mice. No further significant differences in receptor mRNA expression were observed in males. However, female Ctnnb1 knockout mice expressed significantly reduced levels of PXR (∼20% lower) and also displayed a tendency toward a reduction of PPARα and retinoid-X-receptor α mRNAs. However, the latter effects missed the criteria of significance in our analysis. Nrf2 levels were not affected by knockout of the Ctnnb1 gene.

Discussion
This study gives an overview of basal and inducible expression of drug-metabolizing enzymes from phase I and phase II in mice with hepatocyte-specific knockout of Ctnnb1 (encoding β-catenin). It
should be noted that physiological activation of the \( \beta \)-catenin pathway in healthy adult liver is restricted to perivenous hepatocytes, as it has been recently demonstrated (Benhamouche et al., 2006; Sekine et al., 2007).

The significance of the presented data can be subdivided into two major aspects. First, the present work confirms previous reports describing a loss of basal P450 expression (especially Cyp1a2 and Cyp2e1) in mice with liver-specific knockout of \( \text{Ctnnb}1 \) (Sekine et al., 2006; Tan et al., 2006). However, it goes far beyond these studies by demonstrating striking alterations in overall expression, especially in hepatic localization of several drug-metabolizing enzymes from phase I and II of xenobiotic metabolism, at the mRNA and protein levels in the knockout and wild-type mice, as determined by real-time RT-PCR. Significant differences between genotypes are indicated as follows: *, \( p < 0.05 \) and **, \( p < 0.01 \) (Student’s \( t \) test). RXR, retinoid-X-receptor.

Second—and this is the major novelty of this study—response of \( \text{Ctnnb}1 \) knockout mice to a broad spectrum of enzyme inducers was analyzed. For the first time, an important role of \( \beta \)-catenin signaling in the regulation of inducible expression of drug-metabolizing enzymes mediated by different ligand-activated transcription factors was demonstrated. In particular, remarkable differences were observed in response to CAR, AhR, and Nrf2 activators. The situation with the xenobiotic-induced expression presents itself more consistently, compared with basal expression: at large, xenobiotic-induced levels of the respective target enzymes were generally lower in \( \text{Ctnnb}1 \) knockout mice. On the other hand, PXR- and PPAR\( \alpha \)-dependent gene expression was, if at all, much less affected. Diminished response to the AhR agonist 3MC in mice lacking hepatic \( \beta \)-catenin is in line with previous in vitro studies from our group demonstrating increased response of mouse hepatoma cells to the AhR agonist TCDD after activation of \( \beta \)-catenin signaling (Loeppen et al., 2005).

After treatment with CAR activators, a paradoxical inversion of the original wild-type/knockout Cyp2b10 expression ratio was observed: whereas basal expression of this gene was strongly elevated after knockout of \( \text{Ctnnb}1 \), the differences were inverted after treatment with inducers. Thus, \( \beta \)-catenin seems to play a negative-regulatory role in basal Cyp2b10 expression. Irrespective of this repressive role, \( \beta \)-catenin seems to play a directly or indirectly repressive role in basal Cyp2b10 expression. It is tempting to speculate that \( \beta \)-catenin indirectly diminishes the expression of mouse hepatoma cells to the AhR agonist TCDD after activation of \( \beta \)-catenin signaling.

It is not surprising that \( \beta \)-catenin not only promotes perivenous gene expression, but it also induces the release of a yet unknown soluble factor repressing Cyp3a expression in periportal hepatocytes of wild-type but not of \( \beta \)-catenin-deficient mice.
Hepatic localization of the induction of drug-metabolizing enzymes—a process largely taking place in the perivenous hepatocytes of wild-type mice—was substantially changed in Ctnnb1 knockout mice. These mice displayed a mostly uniform-type induction of the respective proteins in all hepatocytes. In the case of treatment with TCPOBOP, weaker- and stronger-responding hepatocytes were distributed in a scattered pattern throughout the liver lobule. Thus, β-catenin not only influences the magnitude of response but also regulates the zone specificity of gene expression after treatment with xenobiotic inducers.

It cannot be ruled out that diminished levels of CAR and AhR are, at least in part, responsible for the overall smaller receptor-mediated response seen in the Ctnnb1 knockout mice. However, in consideration of the fact that 1) TCPOBOP and PB induced the expression of the CAR target gene GSTm3 to equal levels in female wild-type and knockout mice, irrespective of appreciable differences observed for other CAR targets, and that 2) 3MC-induced levels of α-class GSTs were higher in knockout than in the wild-type mice, whereas other AhR targets were induced to significantly higher levels in wild-type mice by 3MC, AhR/CAR depletion cannot bear the sole responsibility for the observed genotype differences in enzyme induction. Moreover, no alterations in cellular Nr2f2 mRNA levels were detected, whereas BHA-mediated induction of Nr2f2 targets was largely abrogated in Ctnnb1 knockout mice. As in the case of basal expression, these facts point toward isoenzyme-specific mechanisms by which β-catenin differentially regulates the response of individual genes to various xenobiotic stimuli.

In our experiments, we observed an inversion of Cyp3a zonation in wild-type mice after treatment with PCN. This was unexpected, because PXR-dependent enzyme induction was reported to preferentially occur in perivenous hepatocytes of Sprague-Dawley rats (Wolf et al., 1984). This discrepancy might be explained by species differences in enzyme induction patterns. However, preferential induction of a perivenous P450 isoform in peripoortal hepatocytes has been reported previously: 3MC induced the expression of Cyp1A, whereas administration of another AhR agonist, β-naphthoflavone, induces Cyp1A expression in peripoortal hepatocytes (Wolf et al., 1984; Foster et al., 1986; Bars and Elcombe, 1991; Oinonen et al., 1994). The mechanisms that govern this particular type of enzyme induction are still unknown. It is noteworthy that β-naphthoflavone induction of isolated hepatocytes from different zones of the liver could not reproduce the zonal differences observed in vivo (Oinonen et al., 1994). It is therefore likely that regionally acting factors, possibly some not yet identified zonal-specific expressed AhR-associated proteins, modulate peripoortal-and perivenous-specific responses to different AhR agonists, rather than inherent features of certain acinar hepatocyte subpopulations.

In summary, the present data clearly show that β-catenin participates in the coordinate regulation of xenobiotic-induced expression of phase I and phase II enzymes of drug metabolism, where, on the whole, β-catenin supports enzyme induction via AhR, CAR, and Nr2f2. β-Catenin is also involved in the regulation of basal expression of many drug-metabolizing enzymes. In this particular context, β-catenin seems to have both positive- and negative-regulatory functions, depending on the individual gene. Moreover, the nearby complete loss of zonation of basal and drug induced in the Ctnnb1 knockout mice provides further evidence that β-catenin functions as a central regulator of perivenous drug metabolism in mouse liver.

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References


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