Changes in the Expression of Cytochromes P450 and Nuclear Receptors in the Liver of Genetically Diabetic db/db Mice

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Physiological and pathophysiological conditions often affect the expression of drug metabolizing enzymes such as cytochromes P450 (P450s). Diabetes is one such factor and it is of great interest to understand its effects on drug metabolism, since diabetic patients generally have increased need for pharmacotherapy. We have recently reported the coordinated reduction of CYP2B1/2 and their transcriptional regulator constitutive androstane receptor (CAR), a member of the nuclear receptor superfamily, in the liver of genetically obese/diabetic Zucker fatty rats (Xiong, H., Yoshinari, K., et al., Drug Metab. Dispos., 30, 918—923, 2002). In this study, we investigated the expression of P450s and liver-enriched nuclear receptors in the liver of genetically diabetic db/db mice. Surprisingly, both CYP2B10 and CAR levels were increased in db/db mice. CYP4A expression was also increased at both mRNA and protein levels in db/db mice, while those of peroxisome proliferator-activated receptor α, a key regulator for the transcriptional activation of CYP4As, were comparable to those in age-matched C57BL/6 mice. Our results demonstrate that db/db mice and Zucker fatty rats exhibit different expression profiles of P450s and nuclear receptors despite their similar characteristics for obesity and diabetes resulting from a defect in the leptin signaling pathway.

Key words cytochrome P450; nuclear receptor; db/db mouse; phenobarbital; constitutive androstane receptor; peroxisome proliferator-activated receptor

The capacity of organisms to eliminate xenobiotics such as pharmaceutical drugs and environmental pollutants from their body is subject to change. One of the best-known factors to affect is the genetic variation of drug metabolizing enzymes and transporters. Numerous genetic polymorphisms have been reported with cytochromes P450 (P450s) CYP2D6 and CYP2C19 studied most intensively.1) In addition to the genetic background, xenobiotic-induced transcriptional activation (i.e. induction) has been documented in large numbers and drawing many researchers’ attention in order to avoid unfavorable drug–drug interactions and side effects of therapeutic drugs. After the discovery of constitutive androstane receptor (CAR) and pregnane X receptor (PXR), members of the nuclear receptor superfamily, the molecular mechanisms of the transcriptional activation of genes encoding drug metabolizing enzymes and transporters have been revealed in detail. In rodents liver prototypes of P450 inducers, phenobarbital (PB) and dexamethasone, activate CAR and PXR, respectively, and the activated receptors form a heterodimer with retinoid X receptor (RXR) and transcriptionally activate the expression of CYP2B and CYP3A genes.2,3) Another member of the nuclear receptor superfamily, peroxisome proliferator-activated receptor α (PPARα), mediates CYP4A induction by fibrates as a heterodimer with RXR.2,3)

Physiological and pathophysiological conditions also affect the activity of P450s and other enzymes.4) Obesity is a worldwide concern as a risk factor for metabolic syndrome including type 2 diabetes, and is one such factor modulating drug metabolism in the liver. Since obese and diabetic patients tend to have increased opportunities for pharmacotherapy compared to slim subjects, it is of great interest to understand the effect of these conditions on the expression of drug-metabolizing enzymes. Using genetically obese/diabetic model animals such as Zucker fatty rats and ob/ob mice, and nutritionally obese mice, it has been reported that the expression profiles of P450s and phase II enzymes in the liver are different from those in the corresponding controls5—10); however, these changes vary with model animals, and little is known about their molecular mechanisms.

In this study, we have investigated the expression of P450s and nuclear receptors in the liver of genetically obese/diabetic db/db mice, which have a mutation in the leptin receptor gene.11,12) Here, we show that db/db mice exhibited an increased expression of CYP2Bs and CYP4As in the liver compared to control C57BL/6 (C57) mice. The amount of nuclear receptors including CAR, RXRα and HNF-4α also increased in db/db mice.

MATERIALS AND METHODS

Materials PB was purchased from Wako Pure Chemical Industries (Osaka, Japan). Antibodies against rat CYP1A2, CYP2B1, CYP2C6, rat CYP2E1, CYP4A1 and rat NADPH cytochrome P450 reductase (POR) were from Daiichi Pure Chemicals (Tokyo, Japan). Anti-CYP3A4 antiserum was a generous gift from Dr. Yasushi Yamazoe (Tohoku University, Sendai, Japan). Anti-PPARα (A-20), anti-hepatocyte nuclear factor-4α (HNF-4α; C-19), anti-RXRα and anti-PPARα (H-98) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-PPARγ and anti-histone H1 antibodies were from Affinity BioReagents (Golden, CO, U.S.A.) and Stressgen (Victoria, Canada), respectively. Anti-CAR antiserum was produced and purified as described previously.13) Oligonucleotides were synthesized by Kurabo (Osaka, Japan) and their sequences are shown in Table 1.

Animals Age-matched male C57 and db/db mice were obtained from Japan SLC (Hamamatsu, Japan), and experiments were carried out at 10 weeks old after acclimation. They were housed under a constant dark/light cycle (12 h each) and given standard chow and water ad libitum. Mice were treated with PB (100 mg/kg) or saline (vehicle) 12 h before killing. Under ether anesthesia, blood was collected

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from the inferior vena cava in the fed state between 10:00 and 11:30 a.m. The animals were then killed and the liver was excised for further analyses. All animal experiments were approved by the Laboratory Animal Committee of University of Shizuoka.

**Western Blotting** Hepatic microsomal fractions were prepared individually and total nuclear extracts were prepared from the pooled livers as described previously. Portions of the microsomes (5 μg) and the nuclear extract (50 μg) were separated by SDS-polyacrylamide gel (9%, 12%, respectively) electrophoresis and transferred onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Billerica, MA, U.S.A.). The membrane was incubated with primary antibodies and appropriate horseradish peroxidase-labeled secondary antibodies, and signals were detected with Lumi GLO reagent (Cell Signaling Technology, Beverly, MA, U.S.A.).

**Reverse Transcription–Polymerase Chain Reaction** Total RNAs were prepared by the acid guanidine–phenol-chloroform method using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.), and quantitative reverse transcription–polymerase chain reaction (RT-PCR) experiments were carried out as described previously by using SYBR Premix Ex Taq (Takara Bio) with 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.).

**Other Methods** Protein concentrations were determined with bovine serum albumin as the standard. Serum levels of glucose, total cholesterol, free fatty acid and triacylglycerol were measured with NIH image software (Fig. 1). Since antibodies against CYP2C6, CYP3A4 and CYP4A1 used in this study could not distinguish mouse P450 forms in the corresponding subfamilies (i.e. CYP2C, CYP3A and CYP4A, respectively), we hereafter describe the sum of immunoreactive proteins as CYP2Cs, CYP3As and CYP4As, respectively. With CYP2B1 antibody, we measured the intensity of the immunoreacted band indicated by an arrowhead in Fig. 1A, which corresponds to CYP2B10. Among P450s examined, basal levels of CYP2B10 and CYP4As were significantly higher in db/db mice than C57 mice, while CYP1A2 tended to decrease in db/db mice (see open columns). CYP2Cs, CYP2E1 and CYP3As did not demonstrate significant differences between saline-treated C57 and db/db mice. PB treatment increased the amount of CYP2B10 in db/db mice as well as in C57 mice.

We next performed quantitative RT-PCR analyses to determine P450 mRNA levels. Although many enzymes are reported in CYP2B, CYP2C, CYP3A and CYP4A subfamilies, we amplified CYP2B10, CYP2C29, CYP3A7 and CYP4A10 mRNAs as respective representatives of each subfamily in this study (Fig. 2). Consistent with changes in protein levels, CYP2B10 and CYP4A10 mRNA levels in saline- or PB-treated db/db mice were significantly higher than those in corresponding C57 mice. In addition, CYP2C29 and POR mRNA levels were also increased significantly in db/db mice regardless of treatment. In contrast, CYP1A2, CYP2E1 and CYP3A4 mRNA levels were not reached. Interestingly, PB treatment decreased serum glucose levels (Table 2).

**RESULTS** In this study, we determined the expression levels of P450s and nuclear receptors to understand their relationship in genetically obese db/db mice. The average body weight of db/db mice was 1.6-fold higher than that of control C57 mice (41.7 ± 5.1 g versus 26.4 ± 2.1 g; the mean ± S.D., n = 7). These mice were treated with PB (100 mg/kg) or saline (vehicle) 12 h before killing. As shown in Table 2, serum levels of glucose, total cholesterol and free fatty acid in both saline- and PB-treated db/db mice were significantly higher than those in C57 mice, and triacylglycerol also seemed higher in obese mice than in C57 mice, although statistical significance was not reached. Interestingly, PB treatment decreased serum glucose levels (Table 2).

Hepatic microsomal fractions were prepared from livers, and Western blot analyses were performed and band intensities were measured with NIH image software (Fig. 1). Since antibodies against CYP2C6, CYP3A4 and CYP4A1 used in this study could not distinguish mouse P450 forms in the corresponding subfamilies (i.e. CYP2C, CYP3A and CYP4A, respectively), we hereafter describe the sum of immunoreactive proteins as CYP2Cs, CYP3As and CYP4As, respectively. With CYP2B1 antibody, we measured the intensity of the immunoreacted band indicated by an arrowhead in Fig. 1A, which corresponds to CYP2B10. Among P450s examined, basal levels of CYP2B10 and CYP4As were significantly higher in db/db mice than C57 mice, while CYP1A2 tended to decrease in db/db mice (see open columns). CYP2Cs, CYP2E1 and CYP3As did not demonstrate significant differences between saline-treated C57 and db/db mice. PB treatment increased the amount of CYP2B10 in db/db mice as well as in C57 mice.

Table 1. Sequences of Primers Used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>ATGAGAAGCTGCTAGACGCATGAGTC</td>
<td>TCCACTGCTTTCTATACATGAG</td>
</tr>
<tr>
<td>CYP2B10</td>
<td>AAAAGTCTGCTGGCAATTTCTCT</td>
<td>CATCCTAGTGTCTTATTCTGAG</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>GCTGACTGAGCAATGAGATTCTCT</td>
<td>TOATGAAGACCATACACAGAG</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>AGACAGGAGACGACACACCCT</td>
<td>AGGCTGAGTGTCAATATC</td>
</tr>
<tr>
<td>CYP3A11</td>
<td>AACACTGAGAAGATGCTTTCTC</td>
<td>TACAGCAGAAGGAGACGAG</td>
</tr>
<tr>
<td>CYP4A10</td>
<td>TATACCTGCGTACCGTATG</td>
<td>AGAAGTACTGAGTCACTTGTG</td>
</tr>
<tr>
<td>POR</td>
<td>GAGACATCAAGCAAGCAATG</td>
<td>TGGTACAGTCCACCTTTC</td>
</tr>
<tr>
<td>RPS9a</td>
<td>TTGCTGACAAAGCAGAGGA</td>
<td>TTGCTGACAAAGCAGAGGA</td>
</tr>
<tr>
<td>HNF-4a</td>
<td>ATGAGAAGACGCTGCTTGG</td>
<td>TCGACCTGTCATCTTGG</td>
</tr>
<tr>
<td>PXR</td>
<td>ATTTACGTGCTGTAGATG</td>
<td>TGCTGACATGACAGCATC</td>
</tr>
<tr>
<td>CAR</td>
<td>GTGAGTGCAGAAGACGAGGATG</td>
<td>TTGAGCAGATGAGCAGGATG</td>
</tr>
<tr>
<td>RXRα</td>
<td>CCGAGTCCACAAAGGTAC</td>
<td>GGAGCAGTCCACAAAGGTAC</td>
</tr>
<tr>
<td>PPARβ/γ</td>
<td>TGACAGAAGACGAGGATG</td>
<td>TTGACTGCTGCATGAGGATG</td>
</tr>
<tr>
<td>RPS9b</td>
<td>GATGGTGGACTGGACGAGGATG</td>
<td>GATGGTGGACTGGACGAGGATG</td>
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</tbody>
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a) All sequences are shown in 5’ to 3’ direction. b) RPS9, ribosomal protein S9.

Table 2. Serum Concentrations of Glucose and Lipids in the Fed State

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C57 (Saline)</th>
<th>PB (4)</th>
<th>Saline (3)</th>
<th>PB (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dl)</td>
<td>242 ± 14</td>
<td>173 ± 17</td>
<td>700 ± 108a</td>
<td>512 ± 172a*</td>
</tr>
<tr>
<td>Triacylglycerol (mg/dl)</td>
<td>101 ± 15</td>
<td>122 ± 66</td>
<td>310 ± 90</td>
<td>527 ± 328a*</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>68 ± 8</td>
<td>92 ± 17</td>
<td>137 ± 10a</td>
<td>151 ± 18a</td>
</tr>
<tr>
<td>Free fatty acid (μEq/l)</td>
<td>518 ± 225</td>
<td>564 ± 265</td>
<td>1587 ± 82a</td>
<td>1572 ± 657a</td>
</tr>
</tbody>
</table>

a) Serum parameters were determined in duplicate for each mouse as described in MATERIALS AND METHODS. Numbers in parentheses indicate the number of mice in the group. * p < 0.05 versus corresponding controls (saline-treated mice); † p < 0.01 versus C57 mice with the same treatment (one-way ANOVA with Fisher’s post-hoc test).
CYP3A11 mRNA levels in db/db mice treated with saline or PB were comparable to those in corresponding C57 mice. As expected from the results of Western blotting, PB treatment increased CYP2B10 mRNA levels in both mouse strains.

Nuclear receptors play important roles in the transcriptional regulation of P450 genes. We thus also determined hepatic mRNA and nuclear protein levels of liver-enriched nuclear receptors including CAR, PXR, PPARα, PPARγ, RXRα and HNF-4α. Quantitative RT-PCR analyses demonstrated that mRNA levels of PXR, RXRα and PPARγ in saline- or PB-treated db/db mice were significantly higher than those in corresponding C57 mice (Fig. 3A). CAR and HNF-4α mRNA levels in db/db mice were also higher than those in controls, although statistical significance was observed only for PB-treated animals (Fig. 3A). No significant differences were observed for PPARα mRNA levels between C57 and db/db mice regardless of treatment (Fig. 3A). Nuclear extracts were then prepared from the liver of C57 and db/db mice and were subjected to Western blotting (Fig. 3B). The amounts of RXRα and HNF-4α (bands indicated by dots) in db/db mice were significantly higher than those of C57 mice regardless of treatment. One of the bands immunoreacted with anti-PXR.1 antibody, indicated by an arrowhead, was also increased slightly in db/db mice compared to C57 mice. In saline-treated mice, the CAR protein level in db/db mice was dramatically higher than that in C57 mice, while no significant difference was detected between PB-treated C57 and db/db mice. PB treatment increased nuclear CAR levels only in C57 mice. In contrast, the amount of PPARα in the liver of db/db mice was comparable that in C57 mice. PPARγ was not detected with the antibody (data not shown).

DISCUSSION

Changes in physiological and pathophysiological conditions including obesity and diabetes often affect P450 expression, although the mechanisms remain unclear. In this study, we have examined the expression of P450s and nuclear receptors in the liver of genetically obese db/db mice. Our results demonstrate that CYP2B and CYP4A levels were increased in db/db mice compared to C57 mice. Since nuclear receptors CAR and PPARα activate the transcription of CYP2B and CYP4A genes, respectively, we also deter-
mRNA and nuclear protein levels of these nuclear receptors, and found that the amount of nuclear CAR was increased in \( db/db \) mice with a trend of increasing mRNA levels, while no significant differences were observed between \( db/db \) and C57 mice for PPAR\( \alpha \) mRNA and nuclear protein levels. These results suggest that P450 expression changes seem to result in the reduced expression of CYP2Bs in the liver of genetically obese Zucker fatty rats,\(^{15} \) which may be due, at least in part, to increased mRNA levels. In this study, we observed an increase in CYP2B mRNA levels in \( db/db \) mice, which contrasts with the concomitant increase in CYP2B and CAR levels. Although a precise reason remains to be identified in future studies, there are at least two nonexclusive possibilities. First, the amount of endogenous ligands for PPAR\( \alpha \) might be increased in the liver of \( db/db \) mice. PPAR\( \alpha \) is activated by various fatty acids such as palmitic acid, linoleic acid and \( 8(S) \)-hydroxyeicosatetraenoic acid as well as fatty acids,\(^{23} \) and \( db/db \) mice show hyperlipidemia and fatty liver.\(^{16,24} \) mRNA levels of other PPAR\( \alpha \)-responsive genes including acyl-CoA oxidase 1 and carnitine palmitoyltransferase 1 were higher in \( db/db \) mice than C57 mice (data not shown). The other possibility is that PPAR\( \gamma \) might be involved in the transcriptional regulation of CYP4A genes. It is generally believed that PPAR\( \alpha \) and PPAR\( \gamma \) regulate different sets of genes, although they recognize similar DNA response elements.\(^{31} \) Recent studies, however, have demonstrated that PPAR\( \gamma \)-specific antagonists including rosiglitazone and troglitazone up-regulated the expression of PPAR\( \alpha \)-responsive genes such as acyl-CoA oxidase and peroxisomal bi-functional enzyme.\(^{25–27} \) Although it cannot be ruled out that rosiglitazone and troglitazone activated PPAR\( \alpha \), these data suggest that some PPAR\( \alpha \)-responsive genes can be regulated by PPAR\( \gamma \) as well. In this study, we observed an increase in PPAR\( \gamma \) mRNA levels in \( db/db \) mice, consistent with a previous study.\(^{26} \) Since a couple of endogenous compounds reportedly act as a PPAR\( \gamma \) ligand,\(^{23} \) the up-regulation of CYP4A genes in \( db/db \) mice may be due, at least in part, to increased PPAR\( \gamma \) levels.

CYP2E1 has been identified as an enzyme that increased in the liver of obese and diabetic humans as well as some experimental animals.\(^{28–31} \) However, we found no differences in either CYP2E1 mRNA or protein levels between \( db/db \) and C57 mice, consistent with previous reports demonstrating that these expressions were unchanged or reduced in the liver of \( db/ob \) mice and Zucker fatty rats.\(^{8,9,22,32} \) The induc-
tion of CYP2E1 is thought to be mainly regulated at post-transcriptional levels, and it was demonstrated that reduced insulin levels were associated with the increased stability of CYP2E1 mRNA in rat hepatoma cells and primary cultures of rat hepatocytes.\textsuperscript{13–15} These observations are in agreement with the result that CYP2E1 levels were unchanged or decreased in these genetically diabetic animals with hyperinsulinemia.

In conclusion, we have determined the hepatic levels of P450s and nuclear receptors of genetically diabetic \textit{db/db} mice. Since the expression profile of P450s and nuclear receptors varies with model animals, these expressions do not seem to depend entirely on metabolic changes. It will be of great interest to investigate in future studies the involvement of signal transduction pathways, including leptin, in the transcriptional control of P450 and nuclear receptor genes.

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REFERENCES