Dietary Inulin Alleviates Hepatic Steatosis and Xenobiotics-Induced Liver Injury in Rats Fed a High-Fat and High-Sucrose Diet: Association with the Suppression of Hepatic Cytochrome P450 and Hepatocyte Nuclear Factor 4α Expression

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ABSTRACT:

Inulin enzymatically synthesized from sucrose is a dietary component that completely escapes glucose digestion. Supplementing inulin to a high-fat and high-sucrose diet (HF) ameliorated hypertriglyceremia and hepatic steatosis in 8-week-fed rats by suppressing elevated levels of serum triacylglycerols, fatty acids, and glucose, and the accumulation of hepatic triacylglycerols and fatty acids. Inulin intake prevented phenobarbital (PB)- and dexamethasone-induced liver injuries in the HF group. No significant alteration in the baseline expression of CYP2B, CYP2C11, CYP3A, and NADPH-cytochrome P450 (P450) reductase mRNAs and proteins was found. In contrast, baseline and PB-treated expressions of CYP2E1 mRNA were reduced in HF-fed rats. The induction of P450s in response to PB was affected by the nutritional status of the rats; mRNA levels of CYP2B1 and CYP3A1 after PB treatment, as assessed by quantitative real-time polymerase chain reaction analysis were reduced in the inulin-supplemented HF (HF+i) group, compared with those in the HF group. Western blot analysis detected the corresponding changes of CYP2B and CYP3A proteins. These alterations were correlated with changes in hepatic thiobarbituric acid-reactive substances. Furthermore, no significant difference in the expression of nuclear receptors constitutive androstane receptor, pregnane X receptor, and retinoid X receptor α and coactivator peroxisome proliferator-activated receptor-γ coactivator 1α proteins was found in the hepatic nucleus between the HF and HF+i groups, but the expression of hepatocyte nuclear factor α (HNF4α) protein was significantly reduced in the HF+i group. Taken together, these results indicate that inulin intake ameliorates PB-induced liver injury, associated with a decline in lipid accumulation and PB-induced expression of CYP2B and CYP3A, which may be related by a reduction in the nuclear expression of HNF4α.

The liver plays a central role in metabolizing therapeutic drugs and environmental contaminants. The activities of drug-metabolizing enzymes in our body are affected by the genotypes of the translating gene and also by nongenetic factors including environmental factors. Nutritional status such as starvation, fasting, and high-lipid diet, and pathophysiological alterations such as diabetes have been reported to modulate liver microsomal cytochrome P450 (P450) composition, leading to altered hepatic metabolism of drugs, carcinogens, steroid hormones, and fatty acids. Uncontrolled diabetes enhances hepatic CYP2E1, CYP2B, CYP3A, and CYP4A expression (Woodcroft and Novák, 1999). An increase in the hepatic production and accumulation of triacylglycerols is frequently associated with metabolic syndromes such as hypertriglyceridemia, obesity, and insulin resistance. The accumulation of triacylglycerols, defined as hepatic steatosis, is proposed to be the setting for more severe liver diseases such as nonalcoholic steatohepatitis, possessing histologic signs of fibrosis and necroinflammation through to cirrhosis, terminal liver failure, and hepatocellular carcinoma (Bugianesi et al., 2002). CYP2E1, a microsomal oxidase involved with fatty acid ω-oxidation as well as CYP4A, is up-regulated in nutritional statuses such as starvation, fasting, obesity, and hyperlipidemia (Hong et al., 1987; Johansson et al., 1988; Weltman et al., 1996; Leclercq et al., 2000). The elevated expression of CYP2E1 has been largely attributed to the pathogenesis of liver...

ABSTRACT:

Inulin enzymatically synthesized from sucrose is a dietary component that completely escapes glucose digestion. Supplementing inulin to a high-fat and high-sucrose diet (HF) ameliorated hypertriglyceremia and hepatic steatosis in 8-week-fed rats by suppressing elevated levels of serum triacylglycerols, fatty acids, and glucose, and the accumulation of hepatic triacylglycerols and fatty acids. Inulin intake prevented phenobarbital (PB)- and dexamethasone-induced liver injuries in the HF group. No significant alteration in the baseline expression of CYP2B, CYP2C11, CYP3A, and NADPH-cytochrome P450 (P450) reductase mRNAs and proteins was found. In contrast, baseline and PB-treated expressions of CYP2E1 mRNA were reduced in HF-fed rats. The induction of P450s in response to PB was affected by the nutritional status of the rats; mRNA levels of CYP2B1 and CYP3A1 after PB treatment, as assessed by quantitative real-time polymerase chain reaction analysis were reduced in the inulin-supplemented HF (HF+i) group, compared with those in the HF group. Western blot analysis detected the corresponding changes of CYP2B and CYP3A proteins. These alterations were correlated with changes in hepatic thiobarbituric acid-reactive substances. Furthermore, no significant difference in the expression of nuclear receptors constitutive androstane receptor, pregnane X receptor, and retinoid X receptor α and coactivator peroxisome proliferator-activated receptor-γ coactivator 1α proteins was found in the hepatic nucleus between the HF and HF+i groups, but the expression of hepatocyte nuclear factor α (HNF4α) protein was significantly reduced in the HF+i group. Taken together, these results indicate that inulin intake ameliorates PB-induced liver injury, associated with a decline in lipid accumulation and PB-induced expression of CYP2B and CYP3A, which may be related by a reduction in the nuclear expression of HNF4α.

The liver plays a central role in metabolizing therapeutic drugs and environmental contaminants. The activities of drug-metabolizing enzymes in our body are affected by the genotypes of the translating gene and also by nongenetic factors including environmental factors. Nutritional status such as starvation, fasting, and high-lipid diet, and pathophysiological alterations such as diabetes have been reported to modulate liver microsomal cytochrome P450 (P450) composition, leading to altered hepatic metabolism of drugs, carcinogens, steroid hormones, and fatty acids. Uncontrolled diabetes enhances hepatic CYP2E1, CYP2B, CYP3A, and CYP4A expression (Woodcroft and Novák, 1999). An increase in the hepatic production and accumulation of triacylglycerols is frequently associated with metabolic syndromes such as hypertriglyceridemia, obesity, and insulin resistance. The accumulation of triacylglycerols, defined as hepatic steatosis, is proposed to be the setting for more severe liver diseases such as nonalcoholic steatohepatitis, possessing histologic signs of fibrosis and necroinflammation through to cirrhosis, terminal liver failure, and hepatocellular carcinoma (Bugianesi et al., 2002). CYP2E1, a microsomal oxidase involved with fatty acid ω-oxidation as well as CYP4A, is up-regulated in nutritional statuses such as starvation, fasting, obesity, and hyperlipidemia (Hong et al., 1987; Johansson et al., 1988; Weltman et al., 1996; Leclercq et al., 2000). The elevated expression of CYP2E1 has been largely attributed to the pathogenesis of liver...
disease in patients with nonalcoholic steatohepatitis (Weltman et al., 1996, 1998; Leclercq et al., 2000; Emery et al., 2003; Chalasani et al., 2003).

Biochemical processes that generate oxidative stress can initiate hepatocellular injury, and CYP2B, CYP2C, and CYP3A, as well as CYP2E1 and CYP4A, are microsomal sources producing prooxidants in liver cells, which create oxidative stress under uncoupling with antioxidants. Here we found that the intake of a high-fat and high-sucrose diet (HF) for a long period (8 weeks) produced marked accumulation of hepatic and serum triacylglycerols in rats, leading to hypertriglyceremia and hepatic steatosis but not hepatic necroinflammatory lesions. Also, there was no significant influence in the baseline levels of hepatic CYP2B, CYP2C, and CYP3A proteins, but levels of CYP2E1 and CYP4A proteins were reduced. In contrast, the administration of phenobarbital (PB) to HF-fed rats more promptly caused liver injury, leading to cell necrosis and inflammation, compared with the standard diet (SD)-fed rats and was associated with changes in PB-inducible P450 expression or via the constitutive androstane receptor (CAR)-mediated pathway associated with hepatic lipid accumulation.

Some dietary components that completely escape glucide digestion, such as resistant starch and oligofructose, have been demonstrated to exert systemic effects by modifying lipid metabolism (Younes et al., 1995; Daubioul et al., 2000). In contrast to starch, inulin is fermentable dietary fiber, resistant to hydrolysis by pancreatic amylase and saccharidases in the upper gastrointestinal tract. Previous studies by our laboratory have demonstrated that inulin was produced enzymatically from sucrose, and that supplementing inulin to the HF diet for 12 weeks reduced elevated body weight and serum and hepatic levels of triacylglycerols in rats (Wada et al., 2005). Interestingly, this study has demonstrated that PB-induced liver failure was suppressed in rats fed an inulin-supplemented HF diet. It is important to investigate whether the reduction in elevated levels of blood glucose and serum and hepatic lipids by supplementing inulin to the HF diet was associated with the down-regulation of P450 induction by PB, consequently leading to the suppression of liver injury. Hence, in this study, we characterized PB-induced P450 mRNA and protein profiles in connection with profiles of blood glucose and serum and hepatic lipids. Treatment of inulin-supplemented HF diet-fed rats with PB resulted in lower levels of CYP2B mRNA and protein than with only the SD or HF diet. PB induces CYP2B, CYP2C, and CYP3A via the activation of nuclear receptors, CAR and pregnane X receptor (PXR), which regulate the transactivation of those genes. Previous studies have demonstrated that hepatocytocyte nuclear receptor-α (HNF4α) is an important regulator of coordinate nuclear receptor (such as CAR and PXR)-mediated response to xenobiotics (Triona et al., 2003), and that peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α) modulates the function of nuclear receptors such as CAR as a common coactivator (Puiuviarner and Spiegelman, 2003; Shiraki et al., 2003). Little is known about the effects of nutritional status on baseline and xenobiotic-mediated expression of nuclear receptors (CAR, PXR, RXRα forming a heterodimer with CAR or PXR) and coactivator PGC1α. Thus, we examined the effect of supplementing inulin to SD and HF diets on the hepatic expression of CAR, PXR, RXRα, HNF4α, and PGC1α mRNAs and nuclear expression of those proteins in rats.

Materials and Methods

Materials. Phenobarbital sodium (PB) was purchased from Wako Pure Chemicals (Osaka, Japan). Dexamethasone (DEX), pentoxyresorufin, and resorufin were obtained from Sigma-Aldrich (St. Louis, MO). Inulin, which consists of a linear polymer (average ratio of glucose/fructose, 1:17) having β(2-1) linkages of α-fructose with one terminal glucose, was synthesized enzymatically from sucrose by inulin-producing enzyme as described previously (Wada et al., 2005). All other chemicals and solvents were of analytical grade and were obtained from commercial sources.

Experimental Animals. All studies followed protocols approved by the Institutional Animal Care and Life Committee, University of Shizuoka. Male Wistar rats were obtained from Japan SLC (Hamamatsu, Japan) at 4 weeks of age and were acclimatized for 1 week before the experiment. Animals were housed in stainless steel hanging cages with free access to food and water and maintained on a 12-h light/dark cycle. All animals were randomly assigned to SD, 5% inulin-supplemented standard diet (SD+1), HF, or 5% inulin-supplemented high-fat and high-sucrose diet (HF+1) for 4 and 8 weeks. The HF diet consisted of 19.7% casein, 1% soybean oil, 10%lard, 4%mineral mixture, 1% vitamin mixture, 0.15% chloride, 0.5% cholestrol, 0.25% sodium cholate, 3.4% cellulose, and 60% sucrose [23.9% lipid, 56.8% carbohydrate, and 19.3% protein (kj), and the SD diet consisted of 23.8% crude protein, 5.1% crude fat, 3.2% crude fiber, 6.1% ash, 54% nitrogen-free extract, and 7.8% humidity [12.9% lipid, 60.4% carbohydrate, and 26.7% protein (kj)]. Three times per week, all rats were weighed, and food intake in grams was monitored. At 9 or 13 weeks of age, the rats in each group were administered PB (80 mg/kg) or vehicle (0.9% sodium chloride) by intraperitoneal injection once daily for 4 days, and dexamethasone (50 mg/kg) or vehicle (0.1 M citric acid in 0.5% methylcellulose) once daily for 3 days between 8:00 AM and 9:00 AM.

Blood and Tissue Sampling. The experiments were run 4 h after the last administration of PB. Rats were anesthetized with diethyl ether; then, the abdominal cavity was rapidly opened, and blood was rapidly and simultaneously drawn from the abdominal vena cava into syringes. Plasma samples were separated from blood collected into heparinized tubes by centrifugation, and serum samples were separated from blood by centrifugation after standing for 30 min at room temperature. The resulting plasma/serum was stored at −30°C until analysis. Livers were rapidly excised and weighed. The liver median lobe was excised for the preparation of microsomes and nuclear extracts and RNA extraction, or was fixed in 4% paraformaldehyde in 0.12 M sodium phosphate (pH 7.3) for histological examination. Aliquots were snap-frozen and stored at −80°C until analysis. Sections were stained with hematoxylin-eosin (HE) or Oil Red O.

Preparation of Microsomes. Liver microsomes were prepared by differential centrifugation, first at 9,000 g for 15 min, followed by 105,000 g for 60 min at 4°C, and stored at −80°C. The microsomal protein concentration was determined with a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard.

Preparation of Nuclear Extracts. Livers (about 1 g) were homogenized in 5 volumes of 10 mM HEPES-NaOH buffer (pH 7.6) containing 25 mM KCl, 2 M sucrose, 10% glycerol, 0.15 M spermine, 0.5 M spermidine, 1 mM EDTA, 1 mM dithiothreitol, 1 µg of leupeptin per ml, 1 µg of pepstatin per ml, 1 µg of aprotinin per ml, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4 with a Teflon-glass homogenizer with three strokes. The homogenate was carefully layered onto 5 ml of the same buffer in a centrifuge tube, and this was followed by centrifugation at 25,000 rpm and 4°C for 60 min with an SW28 rotor (Himac CP56GII; Hitachi Koki, Hitachinaka, Japan). The precipitate was homogenized in 1 ml of lysis buffer (10 mM HEPES-NaOH buffer [pH 7.6] containing 0.1 M KCl, 3 mM MgCl2, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 1 µg of leupeptin per ml, 1 µg of pepstatin per ml, 1 µg of aprotinin per ml, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4) with a Dounce homogenizer 10 times. Sodium chloride solution was added to the homogenate to bring the final concentration to 0.4 M, and the homogenate was incubated at 4°C for 30 min with gentle shaking. The supernatant of the centrifugation at 105,000 g for 20 min was dialyzed against 1 L of dialysis buffer (20 mM HEPES-NaOH buffer, pH 7.6, containing 0.1 M NaCl, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol, 1 µg of leupeptin per ml, 1 µg of pepstatin per ml, 1 µg of aprotinin per ml, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM Na3VO4) at 4°C overnight and stored at −80°C. The protein concentration was determined by the Bradford assay (Bio-Rad protein assay kit; Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard.

Immunoblot Analysis. Microsomal proteins or nuclear extracts (20 µg) were resolved on a sodium dodecyl sulfate-12.5% polyacrylamide gel, elec-
TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SD</th>
<th>SD+I</th>
<th>HF</th>
<th>HF+I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>C 289.7±5.3</td>
<td>279.2±8.3</td>
<td>287.1±4.4</td>
<td>258.5±4.9***</td>
</tr>
<tr>
<td></td>
<td>PB 285.9±7.0</td>
<td>272.9±6.6</td>
<td>283.6±7.2</td>
<td>256.7±7.0***</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>C 9.80±0.22</td>
<td>9.27±0.23</td>
<td>14.07±0.33***</td>
<td>12.51±0.39***</td>
</tr>
<tr>
<td></td>
<td>PB 11.63±0.15***</td>
<td>11.55±0.42***</td>
<td>16.53±0.63***</td>
<td>15.14±0.42***</td>
</tr>
<tr>
<td>Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver weight/body weight</td>
<td>C 0.032±0.001</td>
<td>0.034±0.001</td>
<td>0.051±0.002</td>
<td>0.047±0.002</td>
</tr>
<tr>
<td></td>
<td>PB 0.040±0.001***</td>
<td>0.042±0.001***</td>
<td>0.059±0.001***</td>
<td>0.059±0.001***</td>
</tr>
<tr>
<td>Liver triacylglycerol (mg/g liver)</td>
<td>C 12.93±0.82</td>
<td>14.27±0.34</td>
<td>72.55±8.67***</td>
<td>44.80±5.18***</td>
</tr>
<tr>
<td></td>
<td>PB 21.25±2.62</td>
<td>13.89±0.43</td>
<td>103.57±6.48***</td>
<td>94.29±6.63***</td>
</tr>
<tr>
<td>Liver total cholesterol (mg/g liver)</td>
<td>C 4.73±0.92</td>
<td>3.29±0.49</td>
<td>32.47±3.74***</td>
<td>27.88±5.52***</td>
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<tr>
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<td>PB 5.95±0.82</td>
<td>4.69±0.70</td>
<td>26.78±3.12***</td>
<td>31.21±7.06***</td>
</tr>
<tr>
<td>Liver free fatty acid (µEq/g liver)</td>
<td>C 8.97±0.58</td>
<td>8.89±0.69</td>
<td>49.86±2.55***</td>
<td>43.76±2.46***</td>
</tr>
<tr>
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<td>PB 11.08±1.02</td>
<td>9.22±0.48</td>
<td>36.50±3.62***</td>
<td>41.33±4.83***</td>
</tr>
<tr>
<td>Serum triacylglycerol (g/l)</td>
<td>C 1.54±0.12</td>
<td>1.47±0.17</td>
<td>1.73±0.25</td>
<td>1.11±0.10*</td>
</tr>
<tr>
<td></td>
<td>PB 0.94±0.07</td>
<td>0.91±0.05</td>
<td>2.10±0.28***</td>
<td>1.83±0.25**</td>
</tr>
<tr>
<td>Serum total cholesterol (g/l)</td>
<td>C 0.60±0.02</td>
<td>0.57±0.02</td>
<td>2.17±0.29***</td>
<td>2.21±0.17***</td>
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<tr>
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<td>PB 0.90±0.08</td>
<td>0.82±0.04</td>
<td>1.78±0.01***</td>
<td>1.92±0.20***</td>
</tr>
<tr>
<td>Serum free fatty acid (µEq/l)</td>
<td>C 462.6±19.3</td>
<td>454.1±33.4</td>
<td>831.1±93.9***</td>
<td>651.9±84.1***</td>
</tr>
<tr>
<td></td>
<td>PB 451.3±36.3</td>
<td>462.5±40.7</td>
<td>905.5±67.8***</td>
<td>827.6±81.1***</td>
</tr>
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<td>Serum glucose (g/l)</td>
<td>C 1.34±0.07</td>
<td>1.34±0.09</td>
<td>1.60±0.11*</td>
<td>1.35±0.05*</td>
</tr>
<tr>
<td></td>
<td>PB 1.21±0.08</td>
<td>1.26±0.04</td>
<td>1.41±0.11</td>
<td>1.25±0.07</td>
</tr>
<tr>
<td>Microsome protein (mg/g liver)</td>
<td>C 25.4±1.3</td>
<td>22.5±1.6</td>
<td>26.9±1.1</td>
<td>26.0±1.0</td>
</tr>
<tr>
<td></td>
<td>PB 24.7±1.5</td>
<td>23.7±0.8</td>
<td>25.9±2.7</td>
<td>27.8±0.7</td>
</tr>
<tr>
<td>Pentoxysorufin O-dealkylation (pmol/mg microsome protein/min)</td>
<td>C 10.0±1.9</td>
<td>12.7±2.3</td>
<td>8.0±1.2</td>
<td>7.1±0.6</td>
</tr>
<tr>
<td></td>
<td>PB 813.5±51.5***</td>
<td>786.8±81.6***</td>
<td>751.5±61.3***</td>
<td>640.6±49.8***</td>
</tr>
</tbody>
</table>

Values are means ± S.E. of six to seven determinants.

- C, vehicle
- *p < 0.05, **p < 0.01, ***p < 0.001 for SD + I, HF, or HF + I-fed and vehicle-administered animals versus SD-fed and vehicle-administered animals, or SD + I, HF, or HF + I-fed and PB-administered animals versus SD-fed and PB-administered animals.
- ++p < 0.01, +++p < 0.001 for PB-administered animals versus vehicle-administered animals.
- #p < 0.05, ##p < 0.01 for HF + I-fed and vehicle-administered animals versus HF-fed and vehicle-administered animals or HF + I-fed and PB-administered animals versus HF-fed and PB-administered animals.
FIG. 1. Histologic analysis of liver from rats fed SD, SD+I, HF, or HF+I for 8 weeks and then administered PB (80 mg/kg, i.p.) or saline as vehicle once daily for 4 days. The livers were stained with HE (original magnification 200×) (A) and Oil Red O (original magnification 200×) (B). Blurred trabecular structure of the liver lobule with distinctly enlarged clear and vacuolated hepatocytes and numerous Oil Red O-positive hepatocytes can be seen throughout the hepatic lobule in rats fed HF. The lower intensity of Oil Red O staining can be seen in rats fed HF+I. Mixed inflammatory infiltrate with lymphocyte and polymorphonuclear neutrophil necroinflammation is dispersed in the liver lobule of rats administered PB, but rare necroinflammation can be seen in the liver lobule of rats administered vehicle. Scale bar indicates 100 μm.
plate reader (PerkinElmer Inc., Wellesley, MA) with excitation and emission wavelengths at 550 nm and 590 nm, respectively. The incubation mixtures contained Tris-HCl (100 mM, pH 7.4), MgCl₂ (3.3 mM), EDTA (1 mM), glucose 6-phosphate (3.3 mM), NADP (1.3 mM), glucose-6-phosphate dehydrogenase (0.4 U/ml), pentoxyresorufin (5 µM), and micosomal protein from control rats and PB-treated rats (25 and 2.5 µg/ml, respectively). NADPH was produced using the Gentest NADPH regenerating system (BD Biosciences, Woburn, MA). The mixtures were incubated for 10 min at 37°C. The formation rate of resorufin was determined by comparison to the fluorescence of known amounts of resorufin.

Statistics. Values are expressed as the mean ± standard error. All data were analyzed using one-way analysis of variance. The difference between the means of the groups was tested for significance using Student’s t test. The level of statistical significance was set at p < 0.05.

Results

Dietary Inulin Ameliorates the Development of Fatty Livers in Rats Fed a High-Fat- and High-Sucrose-Supplemented Diet for 8 Weeks. We previously reported that supplementing inulin for 12 weeks reduced the elevation in body weight, liver weight, and serum levels of lipids and glucose in rats fed the SD diet (Table 1). These observations indicate that inulin intake suppressed the further development of hepatic steatosis. In addition, we measured serum levels of insulin and β-hydroxybutyrate in 8-week-fed rats, there was no significant change in either level in the four diet groups (data not shown). We interpret these data as indicating that feeding the HF diet for a long period (8 weeks) produced severe hepatic steatosis but did not induce diabetes.

Histological analysis of livers with HE and Oil Red O staining showed that steatosis was significantly improved after HF diet feeding for 8 weeks. As shown in Fig. 1, A and B, lipid droplets accumulated in the cytoplasm of hepatocytes in both periportal and pericentral areas in the HF group. In comparison, lipid droplet accumulation in hepatocytes was significantly reduced in the HF-I group.

Dietary Inulin Ameliorates Drug-Induced Liver Injury in Rats with Fatty Liver. PB administration has been reported to induce oxidative changes in rat liver, detected as elevated hydroxyl radical levels (Kinoshita et al., 2002). We compared drug-induced liver injury in rats fed the SD and HF diets. Treatment of 8-week-fed rats with PB at a dose of 80 mg/kg once daily for 4 days resulted in increased liver weights and liver-to-body weight ratios in the SD, SD+I, HF, and HF+I groups, and increased hepatic triacylglycerol levels in the HF and HF+I groups (Table 1). Staining of HF-fed rat liver with Oil Red O confirmed the lipid content of clear macrovacuoles (Fig. 1B), but there were few areas in the SD-fed rat liver stained with Oil Red O.
In addition to the further development of hepatic steatosis, there were areas of mixed inflammatory cell infiltration and hepatocyte necrosis dissecting the liver parenchyma after PB treatment (Fig. 1, A and B). Consistent with morphological changes, PB treatment of HF-fed rats resulted in significant increases in serum AST and ALT levels, but slight increases in serum \( \text{GTP} \), compared with those in the SD group (Fig. 2). At this time, the liver injury in the HF group was not accompanied by high levels of serum and hepatic TNF-\( \alpha \) and interleukin 6, inasmuch as there was no significant difference of the TNF-\( \alpha \) and interleukin 6 levels in the four diet groups (data not shown). Furthermore, treatment of rats with dexamethasone at a dose of 40 mg/kg once daily for 3 days (p.o.) resulted in significant increases in serum levels of AST and ALT in the SD, HF, and HF+I groups (Fig. 2). In contrast, there was no significant change between hepatic TBARS levels in rats fed SD and SD+I diets (Fig. 2). These observations indicate that lowered lipid accumulation by inulin intake may be associated with the reduction of lipid peroxides produced by PB administration in the HF diet group (Fig. 4).

To examine whether the development of hepatic steatosis was associated with the setting of PB-induced liver injury, we compared serum AST and ALT levels after PB treatment in rats fed the SD and HF diets for 4 and 8 weeks. Figure 3 shows that treatment of 8-week-fed rats with PB at a dose of 80 mg/kg once daily for 4 days caused elevated levels of AST and ALT, but treatment of 4-week-fed rats with PB did not elevate the levels. These results suggest that hepatic steatosis further developed by feeding the HF diet for 8 weeks may be given to produce PB- and DEX-induced liver injury, and that inulin intake may ameliorate PB- and DEX-induced liver injury by reducing accumulated lipids such as triacylglycerols and free fatty acids in rat hepatocytes.

To assess oxidative changes induced by PB administration in rat liver, we measured TBARS. As indicated by the accumulation of TBARS, the amount of total lipid peroxides was slightly higher in the livers of rats fed the HF diet and administered PB than in the livers of rats fed the inulin-supplemented HF diet and administered PB (Fig. 4). In contrast, there was no significant change between hepatic TBARS levels in rats fed SD and SD+I diets (Fig. 4). These observations indicate that lowered lipid accumulation by inulin intake may be associated with the reduction of lipid peroxides produced by PB administration in the HF diet group (Fig. 4).

**Hepatic Expression and Induction by PB of Enzyme Activities, mRNAs, and Proteins of P450s in Rats Fed the Four Diets.** Since PB particularly induces CYP2B1/2B2 expression in rat liver, we first compared the enzyme activity of pentoxyresorufin \( \text{O-dealkylation} \) (PROD) activities in the four diet groups. As shown in Table 1, the HF and HF+I diets did not influence baseline or induced levels of microsomal protein. Although the HF diet did not influence the baseline and induced levels of PROD activity, lower PROD activity in the HF+I group than in the SD group after PB treatment was detected (Table 1).

To assess the effects of the SD, SD+I, HF, and HF+I diets on the
TABLE 2
Liver gene expression in rats fed SD-, SD <sup>I</sup>-H11001-, HF-, or HF <sup>I</sup>-H11001 for 8 weeks and administered PB (80 mg/kg, i.p.) once daily for 4 days. Values are means ± S.E. of three to five determinations.

<table>
<thead>
<tr>
<th>Gene Accession</th>
<th>Vehicle</th>
<th>PB</th>
<th>Vehicle</th>
<th>PB</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2B1</td>
<td>1.00 0.19</td>
<td>231.21 33.92</td>
<td>12.1 0.88</td>
<td>2.18 0.17</td>
</tr>
<tr>
<td>CYP2B2</td>
<td>1.00 0.12</td>
<td>75.03 23.03</td>
<td>21.51 0.56</td>
<td>4.89 0.15</td>
</tr>
<tr>
<td>CYP2C11</td>
<td>101.03 91.03</td>
<td>11 0.89</td>
<td>3.41 0.27</td>
<td>0.82 0.07</td>
</tr>
<tr>
<td>CYP2C12</td>
<td>101.03 91.03</td>
<td>11 0.89</td>
<td>3.41 0.27</td>
<td>0.82 0.07</td>
</tr>
<tr>
<td>CYP3A1</td>
<td>101.03 91.03</td>
<td>11 0.89</td>
<td>3.41 0.27</td>
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</tr>
<tr>
<td>HNF4α</td>
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<td>11 0.89</td>
<td>3.41 0.27</td>
<td>0.82 0.07</td>
</tr>
<tr>
<td>RXRα</td>
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<td>11 0.89</td>
<td>3.41 0.27</td>
<td>0.82 0.07</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01, ***p < 0.001 for PB-administered animals versus vehicle-administered animals. 

There was no significant change in mRNA levels of CYP2B1 in the SD group following PB administration. However, mRNA levels of CYP2B1 were significantly increased in the HF group compared to the SD group. The mRNA levels of CYP2C11 in the HF group were decreased compared to those in the SD group. The mRNA levels of CYP3A1 in the HF group were decreased compared to those in the SD group.

As shown in Table 2, there was no significant change in baseline mRNA levels of CYP2B1 in the SD group following PB administration. However, mRNA levels of CYP2B1 were significantly increased in the HF group compared to the SD group. The mRNA levels of CYP2C11 in the HF group were decreased compared to those in the SD group.

Next, we compared protein levels of P450s in the four diet groups. The baseline and treated protein levels of CYP4A4 and PB-treated levels of CYP2E1 were lower than those in the HF group. There was no significant change in the baseline protein levels of CYP2B1 and CYP2C11 in the four diet groups. PB-induced protein levels of CYP2B1 and CYP2C11 in the four diet groups were further decreased compared to those in the SD group.

EFFECT OF INULIN ON EXPRESSION OF PB-INDUCED P450 AND HNF4α

Inulin supplementation to the HF diet reduced hepatic expression of baseline and PB-treated P450 mRNAs, but not to the same extent. Unexpectedly, fatty liver production by feeding the diet down-regulated CYP2E1 mRNA expression to 69% ± 2% of the control (Table 2). The reduced levels were maintained even after PB treatment for 4 days. Supplementing inulin to the HF diet did not reverse the mRNA levels of CYP2E1. In the HF+I group, there was no significant increase in the levels of CYP4A1, CYP4A2, and CYP4A3 in the vehicle- and PB-treated groups.

As shown in Table 2, there was no significant change in baseline mRNA levels of CYP2B1 in the SD group following PB administration. However, mRNA levels of CYP2B1 were significantly increased in the HF group compared to the SD group. There was no significant change in the baseline protein levels of CYP2B1 and CYP2C11 in the four diet groups. PB-induced protein levels of CYP2B1 and CYP2C11 in the four diet groups were further decreased compared to those in the SD group.

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EFFECT OF PB TREATMENT ON HEPATIC EXPRESSION OF mRNAs AND PROTEINS OF TRANSCRIPTION FACTORS IN RATS FED THE FOUR DIETS

CAR and PXR function as a heterodimer with RXRα, and HNF4α functions as a homodimer, regulating genes encoding CYP2B2, CYP2C2, and CYP3A (Honkakoski et al., 1998; Lehmann et al., 1998; Bogan et al., 2000; Tirona et al., 2003; Chen et al., 2005). We next analyzed the effects of the SD, SD+I, HF, and HF+I diets and PB treatment on the expression of CAR, PXR, RXRα, and HNF4α mRNAs in the liver. There was no significant change in the baseline mRNA levels of CAR and PXR, but RXRα in the four diet groups except that the levels of CAR were slightly increased in the HF and HF+I groups (Table 2). The mRNA levels of CAR and PXR after PB treatment exhibited a trend toward reduced levels in the four diet groups. No significant difference was observed in the mRNA levels of RXRα after PB treatment in the four diet groups. The baseline mRNA levels of HNF4α in the HF and HF+I groups and the treated levels in the HF group exhibited a trend toward reduced levels, and PB-treated levels in the HF+I group were reduced and lower than those in the HF group.
We further investigated the effect of the diets and PB treatment on the protein levels of nuclear receptors in the liver nucleus. As shown in Fig. 6, the protein levels of CAR in the nucleus were increased after PB treatment, but no difference was observed between the levels of the HF and HF\textsuperscript{I} groups. No significant difference was found in the baseline and PB-treated protein levels of PXR and RXR\textsubscript{\alpha} in the four diet groups. In contrast, the baseline and PB-treated protein levels of HNF4\textsubscript{\alpha} in the HF group were decreased to 86% and 77% of the ones in the SD group, and the baseline and PB-treated levels in the HF\textsuperscript{I} group were 78% and 46%, respectively of those in the SD\textsuperscript{I} group. In addition, the treated protein levels in the HF\textsuperscript{I} group were significantly lower than in the HF group.

Transcriptional cofactor PGC1\textsubscript{\alpha} is considered to regulate biological programs linked to energy homeostasis through specific interaction with a variety of transcription factors such as nuclear hormone receptors (Puigserver and Spiegelman, 2003). Here, we investigated whether the expression of hepatic PGC1\textsubscript{\alpha} mRNA and protein is influenced by the four diets and PB treatment. As shown in Table 2, no significant change in the baseline and treated levels of the four diet groups was detected, whereas a trend toward reduced PGC1\textsubscript{\alpha} mRNA levels after PB treatment was observed in the four diet groups. Correspondingly, the baseline and treated levels of PGC1\textsubscript{\alpha} protein were not significantly changed in the liver nucleus of rats fed each of the four diets except that PB-treated levels of PGC1\textsubscript{\alpha} protein in the SD group were decreased (Fig. 6). These observations indicate that alterations in the PB induction of P450s by feeding the HF and HF\textsuperscript{I} diets may not be attributed to reduced levels of PGC1\textsubscript{\alpha} protein in the nucleus of hepatocytes.

Discussion

This study demonstrates that feeding the high-lipid and high-sucrose diet for 8 weeks produces severe hepatic steatosis, which is associated with hepatic injury produced by the administration of xenobiotics such as PB and DEX (Table 1; Figs. 1 and 2). Although large amounts of lipid deposition were evident in the liver sections of the rats, there was no inflammation and fibrosis in the liver of control rats (Fig. 1). This model is not an experimental model of nonalcoholic steatohepatitis, but is beneficial for investigating the effects of lipid accumulation on xenobiotic-induced liver injury. Liver injury by PB treatment was found in the HF group of 8-week-fed rats but not 4-week-fed rats (Fig. 3), consistent with the accumulation of hepatic triacylglycerol in the HF group of 8-week-fed rats being markedly higher than that of 4-week-fed rats. As found in liver sections (Fig. 1) and serum levels of AST and ALT (Fig. 2), inulin intake attenuated lipid droplets and PB- and DEX-induced hepatotoxic injury. These results suggest that 1) marked lipid accumulation in hepatocytes may be associated with the induction of liver injury by xenobiotics, and 2) potential preventive effects of dietary inulin on blood glucose and lipid profiles, and hepatic lipid profiles may lead to the prevention of
liver injury produced by xenobiotics. Steatosis produced by diets deficient in lipotropes such as choline and methionine (Weltman et al., 1996) and that induced by chemicals such as orotic acid (Su et al., 1999) are established as experimental models of nonalcoholic steatohepatitis and drug-mediated liver injury, respectively. Those models have been reported to be associated with liver injuries via the alteration of hepatic expression of drug-metabolizing enzymes such as CYP2E1, CYP4A, and CYP2C11. In addition, a pathophysiological status such as chemically induced and spontaneous diabetes has been demonstrated to influence the hepatic expression of CYP2B, CYP2C11, CYP3A, and CYP4A (Bellward et al., 1988; Dong et al., 1988; Favreau and Schenkman, 1988; Woodcroft et al., 1999). Nevertheless, the detailed effects of lipid accumulation on the expression of transcriptional factors, which regulate the gene expression of drug-metabolizing enzymes, remain to be clarified. This study establishes that dietary manipulation such as high lard and high sucrose produces hepatic steatosis associated with xenobiotic-induced liver injury (Figs. 1 and 2; Table 1). Thus, in this study, we examined the effects of lipid accumulation on baseline and xenobiotic-treated expression of CYP2E1, CYP2B, CYP2C11, CYP3A, and CYP4A mRNAs and proteins, and the association between alterations in the expression of P450s and transcript factors and xenobiotic-induced liver injury.

Leclercq et al. (2000) have demonstrated that CYP2E1 in wild-type mice but CYP4A1 in CYP2e1−/− mice is up-regulated in nonalcoholic steatohepatitis and plays a key role in the development of liver injury by initiating lipid peroxidation. In contrast, feeding a high-fat and high-sucrose diet down-regulated CYP2E1 and CYP4A mRNAs and proteins, and even after treatment with PB, the enzymes remained down-regulated (Table 2; Fig. 5). These results indicate that CYP2E1 and CYP4A4s seemed not to play a central role in this PB-induced liver injury.

Rats fed the HF+I diet exhibited reduced induction in response to PB treatment as evidenced by PROD activities and CYP2B mRNA and protein levels (Tables 1 and 2; Fig. 5). Zannikos et al. (1993) have demonstrated that rats fed the energy-dense diet for 32 weeks as compared with rats fed the control diet exhibit a lower increase in PROD activities after PB treatment (20-fold increase versus 45-fold increase), whereas similar amounts of protein and mRNA of CYP2B1/2B2 are found. Hepatic nutritional status in this experimental model appears to be rather different from the energy-dense diet model; lipid accumulation in the liver of rats fed the HF diet for 8 weeks was more than 2-fold higher than that of rats fed the energy-dense diet for 32 weeks. As pointed out by Zannikos et al. (1993), the change in the microsomal lipid environment based on diet and/or aging may influence enzyme activity.

PB activates CAR and PXR, which bind to and transactivate CYP2B and CYP3A promoters (Honkakoski et al., 1998; Lehmann et al., 1998). HNF4α synergistically enhanced CAR- and PXR-mediated xenobiotic induction of human CYP2C9 (Chen et al., 2005) and CYP3A (Tirona et al., 2003). Although there is no direct evidence that HNF4α synergistically enhances the CAR- and PXR-mediated xenobiotic induction of CYP2B, the CYP2B2 phenobarbital response unit contains an HNF4 binding site (Beaudet et al., 2005). In addition, single nucleotide polymorphism in a putative HNF4 binding site in the phenobarbital response element of CYP2B6 has been demonstrated to be correlated with lowered CYP2B6 expression (Lamba et al., 2003). By a study using HNF4 antisense RNA, it has been demonstrated that HNF4 is a general regulator of major drug-metabolizing P450s such as CYP3A4, CYP3A5, CYP2A6, CYP2B6, CYP2C9, and CYP2D6 (Jover et al., 2001). Thus, in this study, we assessed whether the reduced PB induction of CYP2B and CYP3A mRNAs and proteins in the HF+I group may be attributable to a decline in the expression of transcription factors. Although PGC1α has been demonstrated to mediate the ligand-independent activation of CAR (Shiraki et al., 2003), the levels of PGC1α mRNA and protein in the HF+I group were similar to those of the control of the SD+I and HF groups (Table 2; Fig. 6), suggesting that PGC1α may not play a central role in the preventive action of dietary inulin. There was no significant change.

![Figure 6](image-url)
based on HF and HF+I diets in the baseline and PB-treated expression of CAR, PXR, and RXRα mRNAs and proteins except for increased levels of CAR mRNA in the vehicle-treated HF+I group (Table 2; Fig. 6). In contrast, HNF4α protein significantly declined in the liver nucleus of rats fed the HF and HF+I diets (Fig. 6). Thus, the reduced induction of hepatic CYP2B and CYP3A in the PB-treated HF+I group may be attributable to a decline in the expression of HNF4α protein in the liver nucleus. Serum glucose levels were increased in HF-fed and vehicle-treated rats, and inulin-supplemented diet suppressed the increase (Table 1). Furthermore, the levels in HF-fed rats after PB treatment exhibited a tendency to remain increased. Glucose itself has been demonstrated to induce the expression of HNF4α mRNA in primary-cultured rat hepatocytes (Oyadomari et al., 2000). Thus, the lower levels of HNF4α mRNA and protein in the PB-treated HF+I group than those in the HF group may be associated with suppression of increase in serum glucose by inulin. Dhe-Paganon et al. (2002) presume that fatty acids or related molecules might be the endogenous ligands for HNF4α; that is, HNF4α may function as a biosensor for fatty acids within the cell. However, it remains to be addressed whether the decreased expression of HNF4α protein is influenced by the liver lipid environment; that is, marked accumulation of liver fatty acids as found in the HF and HF+I groups. Analysis of the HNF4α gene expression is currently progressing in our laboratory.

We proposed the scheme of PB-induced liver injury associated with lipid accumulation and the suppression by inulin. Absorption of a high-fat and high-sucrose diet was concomitant with increases in blood glucose and liver lipids (Table 1). Glucose has been demonstrated to induce transcription factors such as sterol regulatory element binding protein-1c, which enhances transcription of genes involved in de novo lipogenesis such as fatty acid synthase, leading to increased synthesis of fatty acids and triacylglycerols. Kinoshita et al. (2002) have demonstrated that a significant increase of hydroxy radical levels by day 4 of high-dose treatment with PB accompanied the accumulation of 8-hydroxydeoxyguanosine in the nucleus. In fact, as indicated by the accumulation of TBARS, lipid peroxides were slightly increased in the HF groups after PB treatment (Fig. 4). It is possible that CYP2B and CYP3A induced by PB exposure may contribute to produce reactive oxygen species or that changed microsomal lipid environment may prevent the elimination of oxygen radicals leading to lipid peroxidation. On the other hand, Zhang et al. (2002) have demonstrated that overdoses of acetaminophen cause significant hepatoxicity via a pathway mediated by CAR, because PB increases susceptibility to acetaminophen hepatotoxicity, PXR appears to be an important modulator (Wolf et al., 2005). In addition, Dai et al. (2005) have presented that RXRα regulates the expression of glutathione S-transferase genes and modulates acetaminophen-glutathione conjugation in mouse liver, because RXRα null mice are protected from acetaminophen-induced hepatotoxicity and exhibit greater levels of acetaminophen-gluthathione one in the liver. Thus, a potential hepatotoxic compound in fatty liver may cause acute liver failure after PB treatment via a CAR- and/or PXR-mediated pathway or associated with an RXRα-glutathione S-transferase regulatory network (Fig. 7). Together, we speculate that inulin suppresses increase in blood glucose, which leads to a coordinate suppression of lipogenic gene transcription, causing, in turn, a decrease in excessive accumulation of liver lipids, and reduces expression of HNF4α, causing a decrease in xenobiotic-induced expression of CYP2B and CYP3A. The model may explain the inhibitory effect of inulin on xenobiotic-induced liver failure.

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References


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