Nutritional Status Affects Fluvastatin-Induced Hepatotoxicity and Myopathy in Rats

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ABSTRACT:

Rats that consumed a high-fat and high-sucrose diet (HF diet) developed hepatic steatosis. Treatment of HF diet-fed rats with fluvastatin (8 mg/kg) was lethal, followed by an elevation in levels of plasma aspartate aminotransferase and creatine kinase activities and skeletal muscle toxicity. This study was conducted to determine whether nutritional status affects statin-induced adverse effects in rats. Fluvastatin treatment of rats fed the HF diet led to an increase in systemic exposure, suggesting altered metabolism and elimination. In fact, although hepatic multidrug resistance-associated protein (Mrp) 2 and multidrug resistance (Mdr) 1b protein levels were not significantly changed by fluvastatin treatment for 8 days of rats fed a HF diet, the organic anion-transporting protein (Oatp) 1, Mrp3, CYP1A, CYP2C, UDP-glucuronosyltransferase (UGT) 1A1, and UGT1A5 protein levels were moderately decreased and the Oatp2, CYP3A, and UGT2B1 protein levels were markedly suppressed. No significant difference in the baseline level of Oatp1, Oatp2, Mrp2, Mrp3, Mdr1b, CYP1A, CYP2C, CYP3A, UGT1A1, UGT1A5, or UGT2B1 protein was found between the standard diet- and HF diet-fed groups. In addition, the mRNA levels of Oatp2, CYP2C11, and CYP3A1/2 were markedly decreased in HF diet-fed and fluvastatin-treated rats. There was no significant difference in the glucuronidation activities against fluvastatin among the four groups. In liver cell nuclei, levels of constitutive androstane receptor, pregnane X receptor, and hepatocyte nuclear factor 4α proteins were decreased in fluvastatin-treated HF diet-fed rats, which correlated with the decrease in Oatp2, CYP2C, and CYP3A. Taken together, these results indicate that nutritional status may influence adverse effects of fluvastatin by increasing systemic exposure through modulation of hepatic uptake and elimination.

Introduction

HMG-CoA reductase inhibitors (statins), whose competitive inhibition of HMG-CoA reductase reduces the amount of HMG-CoA converted to mevalonate, the rate-limiting step of cholesterol biosynthesis, are members of an important class of lipid-lowering drugs. Although statins seem to be relatively safe and well tolerated, considerable attention has recently been paid to their adverse effects including muscular toxicity and hepatotoxicity (Pasternak et al., 2002; MacDonald and Halleck, 2004). These side effects are a major concern in that they can lead to severe myopathy, rhabdomyolysis, renal damage, and even death. The risks posed by statins are considered to be elevated by drug-drug interactions and pharmacogenetic factors that increase the concentration of statins in plasma.

The liver is the major site of clearance for most statins in the body. The hepatic elimination involves metabolic enzymes and drug transporters. Organic anion-transporting polypeptide (OATP in humans; Oatp in rodents) 2 plays a role in the hepatic uptake of statins such as pravastatin, pitavastatin, atorvastatin, and fluvastatin (Noé et al., 2007; Watanabe et al., 2010). OATP2/Oatp2 inhibitors can decrease the hepatic uptake of statins, which is associated with enhanced systemic exposure to the drugs. OATP2/Oatp2 is also important in fluvastatin-induced muscle toxicity (Sakamoto et al., 2008). The metabolic processes include phase I oxidation by cytochrome P450 (P450) isoenzymes and phase II glucuronidation by UDP-glucuronosyltransferase (UGT) isoenzymes. Atorvastatin, lovastatin, and simvastatin are oxidized mainly by CYP3A4 (Fischer et al., 1999) and their glucuronidation is catalyzed mainly by human UGT1A1 and UGT1A3 (Prueksaritanont et al., 2002). Although a potent inhibitor of CYP3A4 can significantly increase the plasma concentrations of the
active forms of atorvastatin, lovastatin, and simvastatin, which are oxidized mainly by CYP3A4 (Kyrklund et al., 2001; Backman et al., 2002; Goosen et al., 2007), fluvastatin is oxidized mainly by CYP2C9 (Spence et al., 1995).

Nutritional status such as starvation, fasting, and a high-lipid diet and pathophysiological factors such as diabetes have been reported to affect liver drug-metabolizing phase I enzymes, leading to the altered hepatic metabolism of drugs, carcinogens, steroid hormones, and fatty acids (Ding et al., 2006; Sugatani et al., 2006; Osabe et al., 2008). The accumulation in the liver of triacylglycerols, defined as hepatic steatosis, is proposed to be the first stage of more severe liver diseases such as nonalcoholic steatohepatitis, which shows histological signs of fibrosis and necroinflammation, through cirrhosis, terminal liver failure, and hepatocellular carcinoma (Bugianesi et al., 2002). The elevated expression of CYP2E1, a microsomal oxidase involved in fatty acid ω-oxidation, as well as CYP4A, has been shown to be largely responsible for the pathogenesis of liver disease in patients with nonalcoholic steatohepatitis (Weltman et al., 1996; Emery et al., 2003). In a previous study (Sugatani et al., 2006), we demonstrated that feeding a high-lipid and high-sucrose diet (HF diet) to rats produces hepatic steatosis, which is associated with the induction of liver injury by xenobiotics such as phenobarbital and dexamethasone but is not associated with liver injury via the alteration of hepatic CYP2E1 and CYP4As. A number of metabolic food-drug interactions involving drug-metabolizing phase I and II enzymes and drug transporters have been documented; for example, the interaction of St. John’s wort with cyclosporin, and pharmacokinetic-related alterations in the expression of drug-metabolizing phase I and II enzymes and drug transporters dependent on nutritional status such as fasting and hepatic steatosis have been reported (Zhang et al., 2010). However, little is known about the role of nutrition on the adverse effects of statins.

In the present study, it was determined whether nutritional status affects statin-induced adverse effects using rats fed a HF diet or a standard diet (SD diet). Treatment with fluvastatin at 8 mg/kg for 8 days caused an elevation in serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which suggest hepatotoxicity and myopathy, in animals fed a HF diet but not SD diet. Accordingly, to determine whether nutritional status affected the adverse effects of fluvastatin by increasing systemic exposure through modulation of hepatic uptake and elimination, we examined the effects of fluvastatin on the expression of drug transporters and drug-metabolizing phase I and II enzymes in the SD and HF diet-fed rats. This study provides evidence that Oatp2 played an important role in the elimination of fluvastatin in rats, because the hepatic uptake of fluvastatin through Oatp2 was decreased, followed by severe hepatotoxicity and myopathy.

**Materials and Methods**

**Chemicals.** Fluvastatin sodium salt was purchased from Toronto Research Chemicals, Inc. (North York, ON, Canada). All other chemicals were of reagent grade.

**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 Charles River (Tokyo, Japan) at 6 weeks of age; because hepatic Oatp2 protein levels in female rats decreased to 44% of those in male rats (Osabe et al., 2008), we used male rats to examine whether nutritional status affects adverse effects of fluvastatin. Animals were acclimatized for 1 week before the experiment, housed in hanging stainless steel cages with free access to food and water, and maintained on a 12-h light/dark cycle. All animals were randomly assigned to SD and HF diets. After 1 week of either diet, each group was divided into three subgroups and given 0, 4, or 8 mg of fluvastatin/kg/day with the diet for 4, 8, or 14 days, because the lethal doses of fluvastatin in SD diet- and HF diet-fed male rats were 16 and 8 mg/kg/day, respectively. The rats were weighed three times per week, and food intake in grams was monitored.

**Diets.** The HF diet (Table 1) consisted of 23.9% lipid, 56.8% carbohydrate, and 19.3% protein (kilojoules). The SD diet (Table 1) consisted of 12.9% lipid, 60.4% carbohydrate, and 26.7% protein (kilojoules). The HF and SD diets were purchased from Oriental Yeast Co. (Tokyo, Japan).

**Blood and Tissue Sampling.** Rats were anesthetized with diethyl ether, the abdominal cavity was rapidly opened, and blood was drawn from the abdominal vena cava into syringes between 11:00 AM and 12:00 PM. 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 −80°C before analysis. Livers were rapidly excised and weighed. The liver median lobe was excised for the preparation of microsomes, plasma membranes, and nuclear extracts and the extraction of RNA. Hind leg skeletal muscle was fixed in 4% paraformaldehyde in 0.12 M sodium phosphate (pH 7.3) for histological examination. Sections were stained with hematoxylin and eosin.

**Biochemical Analyses.** Blood and tissue sampling was done as described previously (Sugatani et al., 2006). AST, alanine aminotransferase (ALT), γ-glutamyltranspeptidase (γ-GTP), and CK levels were measured using kits from Wako Pure Chemicals (Osaka, Japan). Frozen livers (approximately 0.5 g) were homogenized in 20 volumes (SD diet-fed group) or 100 volumes (HF-fed group) of 0.9% NaCl containing 0.1% Triton X-100, and triacylglycerol and total cholesterol concentrations in serum and livers were estimated with kits from Shino Test (Tokyo, Japan).

**Preparation of Microsomes.** Liver microsomes were prepared by differential centrifugation at 9000g for 15 min followed by 105,000g for 60 min at 4°C and stored at −80°C (Sugatani et al., 2006). 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 Membranes.** Crude liver membranes were prepared as described previously (Ogawa et al., 2000). Liver (approximately 1.5 g) was homogenized in 5 volumes of 0.1 M Tris-HCl buffer (pH 7.4) containing 1 μg/ml leupeptin and pepstatin A and 50 μg/ml phenylmethylsulfonyl fluoride with 20 strokes of a Dounce homogenizer. After centrifugation at 1500g for 10 min, the supernatant was centrifuged at 100,000g for 30 min. The precipitate was suspended in 6 ml of the buffer and again centrifuged at 100,000g for 30 min. The crude membrane fraction was resuspended in 0.1 M Tris-HCl buffer (pH 7.4) containing the above protease inhibitors using five strokes of a Potter homogenizer.

**Preparation of Nuclear Extracts.** Liver nuclear extracts were prepared as described previously (Sugatani et al., 2006). The protein concentration was determined with a Bradford assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard.

**Immunoblot Analysis.** Microsomal proteins (20 μg), membrane proteins (20 μg), or nuclear extracts (30 μg) were resolved on a SDS-12.5% polyacrylamide gel, and electroblotted onto a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA). The immunoblots were incubated with primary antibodies against rat CYP1A1 (the antibody also recognizes rat CYP1A2, whose molecular weight is lower than that of rat CYP1A1), rat

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**Table 1**

<table>
<thead>
<tr>
<th>TABLE 1 Composition of the HF and SD diets</th>
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<tbody>
<tr>
<td>HF Diet</td>
</tr>
<tr>
<td>Casein</td>
</tr>
<tr>
<td>Soybean oil</td>
</tr>
<tr>
<td>Lard</td>
</tr>
<tr>
<td>Mineral mixture</td>
</tr>
<tr>
<td>Vitamin mixture</td>
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</tr>
<tr>
<td>Sodium cholate</td>
</tr>
<tr>
<td>Cellulose</td>
</tr>
<tr>
<td>Sucrose</td>
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<tr>
<td>Total</td>
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diminish the latency of UGT activity. Fluvatatin (100 μM) was incubated in a reaction mixture consisting of 0.1 mg of liver microsomal protein, 4 mM UDP-glucuronic acid, 1 mM MgCl₂, and 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 100 μl. After preincubation for 5 min at 37°C, the reaction was started by adding UDP-glucuronic acid. All reactions were incubated at 37°C for 12 h, and terminated by the addition of an equal volume of ice-cold acetonitrile, followed by centrifugation at 15,000g for 15 min to obtain the supernatants. Aliquots of the supernatant were directly analyzed by a Hitachi ultra-performance liquid chromatography system. Fluvatatin and its metabolites were separated on a COSMOSIL 2.5C18-MS-II column (2.5-μm particle size, 100 × 2.0 mm; Nacala Tesque Inc., Kyoto, Japan). The mobile phase consisted of 0.1% (v/v) trifluoroacetic acid in water (A) and acetonitrile (B) at a flow rate of 0.4 ml/min. The separation of fluvatatin with its metabolites was achieved by using the following elution gradient: linear gradient from 20% B to 60% B (0–7 min), hold at 60% B (7–9 min), and then return to 20% B for another 2 min of equilibration. The column temperature was 40°C, and UV detection was achieved at 245 nm. The fluvatatin glucuronide was split by several peaks with a retention time of approximately 4.9 to 5.2 min because of acyl glucuronide formation. The glucuronide was confirmed by an experiment as shown in Supplemental Fig. 3B.

**Results**

Fluvastatin Causes Severe Hepatic Injury and Myopathy in Rats Fed a HF Diet. Treatment with fluvatatin at 4 mg/kg for 14 days, given as a component of the diet, suppressed the hepatic total cholesterol level in rats fed a HF diet but not in those fed an SD diet (Table 2). Treatment with fluvatatin at 8 mg/kg for 14 days resulted in a decrease in the hepatic triacylglycerol and total cholesterol levels, body weights, and epididymal white adipose tissue weights in rats fed the SD diet, but not in the total cholesterol level in serum (Table 2). In contrast, fluvatatin at 8 mg/kg was lethal to rats fed the HF diet. The survival time of HF-fed rats treated with the 8 mg/kg dose was 10.8 ± 1.9 days (Table 3). Hepatic levels of triacylglycerols were significantly elevated in rats fed the HF diet compared with those fed the SD diet, and fluvatatin (8 mg/kg) suppressed the accumulation of hepatic cholesterol but not triacylglycerols at day 8 (Table 2). At this time point, there were no significant differences among the groups in food intake or body weight (data not shown).

Fluvastatin treatment (8 mg/kg) for 4 days in HF diet-fed rats resulted in a significant rise in serum AST activity but only a slight elevation in serum ALT and γ-GTP activities and no change in serum CK activity, indicating hepatocellular damage (Fig. 1). Fluvastatin treatment (8 mg/kg) for 8 days resulted in a remarkable increase in serum CK activity in addition to AST activity in HF diet-fed rats, but no change in serum AST or CK activity in SD diet-fed rats (Fig. 1). Furthermore, to evaluate skeletal muscle toxicity, we examined the histological appearance of the hind leg skeletal muscle. As shown in Fig. 2, muscle loss was observed in HF diet-fed rats treated with fluvatatin (8 mg/kg) for 8 days. The damage was characterized by myofibrillar necrosis, interstitial edema, and cellular infiltration (Fig. 2). To examine whether the risk of myopathy was related to systemic exposure to higher concentrations of fluvatatin in HF diet-fed rats compared with SD diet-fed rats, the peripheral plasma concentration of unchanged fluvatatin was determined in rats treated with fluv-
Effects of fluvastatin on biomarkers of metabolic disease in rats fed 3-week SD or HF diets

TABLE 2
Effects of fluvastatin on serum levels of AST (A), ALT (B), γ-GTP (C), and CK (D) in rats fed an SD or HF diet and administered fluvastatin. Rats (7 weeks of age) were fed an SD or HF diet for 1 week, administered fluvastatin (0, 4, or 8 mg/kg) with the diet daily. The rats were sacrificed 4, 8, or 14 days after administration of fluvastatin. Four rats per group were administered the indicated doses of fluvastatin. *p < 0.05, versus SD diet-fed animals. **p < 0.01, versus HF diet-fed control rats. ***p < 0.001.

<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>Fluvastatin (4 mg/kg)</th>
<th>Fluvastatin (8 mg/kg)</th>
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<tbody>
<tr>
<td>4</td>
<td>0</td>
<td>235 ± 5</td>
<td>229 ± 6</td>
</tr>
<tr>
<td>8</td>
<td>14</td>
<td>336 ± 7</td>
<td>322 ± 5</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>372 ± 0.16</td>
<td>329 ± 0.22</td>
</tr>
<tr>
<td>14</td>
<td>8</td>
<td>12.81 ± 0.11</td>
<td>12.21 ± 0.78</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>13.42 ± 0.40</td>
<td>13.06 ± 0.44</td>
</tr>
<tr>
<td>14</td>
<td>8</td>
<td>10.33 ± 1.51</td>
<td>6.48 ± 0.44</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>7.76 ± 1.14</td>
<td>5.09 ± 0.63*</td>
</tr>
<tr>
<td>14</td>
<td>8</td>
<td>8.47 ± 1.83</td>
<td>8.04 ± 0.98</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>0.97 ± 0.11</td>
<td>0.55 ± 0.010**</td>
</tr>
<tr>
<td>14</td>
<td>8</td>
<td>0.72 ± 0.03</td>
<td>0.68 ± 0.05</td>
</tr>
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N.D., not determined

Statin (8 mg/kg) for 4 or 8 days. In HF diet-fed rats, the plasma levels of fluvastatin even over 4 h after removal of the diets containing fluvastatin were relatively high on day 4 and significantly elevated on day 8, compared with those in the SD diet-fed rats, whereas the plasma levels in SD diet-fed rats treated with fluvastatin for 8 days were slightly elevated compared with those in rats treated with fluvastatin for 4 days (Fig. 3). The plasma concentrations of fluvastatin at day 8 were between 0.75 and 4.1 μM.

Effects of Fluvastatin on Hepatic Expression of the Protein and mRNA of Drug Transporters and Drug-Metabolizing Phase I and II Enzymes in Rats Fed the Two Diets. Fluvastatin has been considered to be metabolized primarily in the liver and its metabolites eliminated in feces. Liver drug transporters (Oatp2, Mdr1b, Mrp2, and Mrp3) and drug-metabolizing phase I (CYP2C, CYP3A, and CYP1A) and phase II (UGT1A and UGT2B) enzymes contribute to the hepatic influx and efflux of statin and its metabolites, oxidized metabolism, and glucuronidation. Because serum AST and CK activities were increased by fluvastatin treatment for 8 days, we examined effects of the 8-day treatment on the hepatic expression of the membrane proteins Oatp1, Oatp2, Mdr1b, Mrp2, and Mrp3 in HF diet-fed rats compared with SD diet-fed rats. As shown in Fig. 4, in SD diet-fed rats, although Mrd1b protein levels were slightly increased by fluvastatin, there were no significant changes in the levels of Oatp1, Oatp2, Mrp2, and Mrp3. In contrast, in HF diet-fed rats, Oatp2 and Mrp3 protein levels in hepatic membranes were significantly decreased to 22.8 ± 0.7 and 41.6 ± 13.0% of the control value, respectively, by fluvastatin treatment. The baseline and post-treatment levels of Mrp2 and Mdr1b protein were not significantly changed.

Next, we examined the effects of fluvastatin treatment for 8 days on hepatic expression of the microsomal proteins CYP1A1/2, CYP2C11, and CYP3A1/2 in rats fed the two diets. There was no significant change in the levels of CYP1A1s (mainly CYP1A2), CYP2Cs (mainly CYP2C11), CYP3As (mainly CYP3A1), and NADPH-cytochrome P450 reductase in SD diet-fed rats (Fig. 5). In HF diet-fed rats, although fluvastatin treatment resulted in a significant decrease in the protein levels of CYP1As and CYP3As, to 45.2 ± 14.2 and 33.5 ± 6.6% of the control value, respectively, the levels of the major fluvastatin-metabolizing enzyme CYP2Cs were only slightly decreased, to 75.7 ± 6.6% (Fig. 5). Furthermore, although the UGT isoenzymes metabolizing fluvastatin remain to be elucidated, we examined the effects of fluvastatin (8 mg/kg) treatment for 8 days on the hepatic expression of UGT1A1, UGT1A2, UGT1A3, UGT1A5, UGT1A6, UGT1A7, and UGT2B1 mRNAs in rats fed the two diets.
There was no significant change in the baseline mRNA levels of UGT1A2, UGT1A5, UGT1A7, and UGT2B1 in HF diet-fed rats, whereas the baseline mRNA levels of UGT1A1, UGT1A3, and UGT1A6 were increased (Table 4). Fluvastatin treatment in HF diet-fed rats resulted in a decrease in UGT1A1 and UGT2B1 mRNA levels to 63\% and 29\% of that of the control SD rats, respectively, but no significant change in UGT1A3, UGT1A5, UGT1A6, and UGT1A7 mRNA levels. In addition, we examined the effects of fluvastatin (8 mg/kg) treatment for 8 days on the hepatic expression of UGT1A1, UGT1A5, and UGT2B1 proteins in rats fed the two diets. Although the baseline levels of UGT1A1 and UGT1A5 were slightly but not significantly increased by the HF diet, treatment with fluvastatin in the HF diet-fed rats resulted in a moderate decrease in UGT1A1 and UGT1A5 protein levels and a marked decrease in UGT2B1 protein levels (Fig. 6, A–C). Rat UGT isoforms involved in the metabolism of statins have not been elucidated, although human UGT1A1 and UGT1A3 have been characterized as the isoforms involved in the metabolism (Prueksaritanont et al., 2002). Therefore, we have further examined effects of fluvastatin (8 mg/kg) treatment on glucuronidation of fluvastatin in rat liver microsomes. Because the glucuronidation activities in UGT1A1-deficient Gunn rat liver microsomes were not detectable (Supplemental Fig. 3B), UGT1As may mainly contribute to the glucuronidation of fluvastatin (Ikushiro et al., 1995). As shown in Fig. 6D, there was no significant alteration
between the glucuronidation activities in the liver microsomes of control SD diet-fed rats, fluvastatin-treated SD diet-fed rats, control HF diet-fed rats, and fluvastatin-treated HF diet-fed rats.

Furthermore, we compared the mRNA levels of Oatp2, CYP2C11, CYP3A1, and CYP3A2 in the two groups at days 4, 8, and 14 after fluvastatin treatment (Fig. 7). The extent of the decrease for CYP2C11, CYP3A1, and CYP3A2 was greater in the HF diet-fed rats than in the SD diet-fed rats. The mRNA levels of Oatp2 in the HF diet-fed rats decreased at day 4 and those in the SD diet-fed rats were almost the same at day 14. In the rats fed the HF diet, mRNA levels of CYP2C11 also were decreased at day 4.

These observations indicate that the decrease in hepatic Oatp2 mRNA and protein levels may result in decreased influx of fluvastatin into hepatocytes, whereas the decrease in mRNA and protein levels of the metabolizing enzymes may be partly associated with the decreased elimination (Figs. 4–7).

**Effect of Fluvastatin on Hepatic Expression of the Protein and mRNA of Transcription Factors in Rats Fed the Two Diets.** CAR and PXR function as heterodimers with RXRα, whereas HNF4α functions as a homodimer, and these nuclear receptors are considered to regulate genes encoding Oatp2, CYP2C11, CYP3A1, and CYP3A2 (Honkakoski et al., 1998; Lehmann et al., 1998; Bogan et al., 2000; Tirona et al., 2003; Chen et al., 2005; Wortham et al., 2007). We next analyzed the effects of the SD and HF diets and fluvastatin treatment on the expression of CAR, PXR, RXRα, HNF4α, and PPARα proteins in the liver nucleus. There was no significant change in the baseline levels of CAR, PXR, RXRα, and PPARα proteins in the two diet groups (Fig. 8). On day 8 after fluvastatin treatment, the levels of CAR, PXR, and HNF4α in the hepatic nucleus of the HF diet-fed group were decreased to 66.7 ± 16.1, 72.5 ± 11.1, and 59.4 ± 11.7%, respectively, but no significant change was observed in the SD diet-fed group. We further investigated the effect of the diet and fluvastatin treatment on the expression of CAR, PXR, and HNF4α mRNAs in the liver. No significant change was observed at day 4 after fluvastatin treatment in the rats fed either diet (Fig. 9). The mRNA levels of CAR, PXR, and HNF4α at day 8 were significantly decreased in the HF diet-fed rats but not in the SD diet-fed rats (Fig. 9). In SD diet-fed rats at day 14, the mRNA levels of CAR were significantly decreased and those of HNF4α tended to be reduced (Fig. 9). These results indicate that alterations in the hepatic expression of Oatp2, P450s, and UGTs after fluvastatin treatment may be attributed to reduced levels of CAR, PXR, and HNF4α proteins in the nucleus of hepatocytes.

**Effects of Fluvastatin Treatment on AST Release and Cell Growth in Chang Liver Cells.** Next, to investigate whether fluvastatin at a concentration near 10 μM can cause hepatocellular damage because 4.1 μM unchanged fluvastatin was detected in the peripheral plasma (Fig. 3), we measured AST activity in the medium released from human Chang liver cells. The AST activity was significantly elevated at 72 h after exposure to fluvastatin at the concentrations of 1 to 10 μM (Fig. 10A). Furthermore, fluvastatin suppressed cell growth at concentrations of 4 to 10 μM (Fig. 10B). These results indicate that fluvastatin at the concentration of 0.75–4.1 μM detected in the peripheral plasma of HF diet-fed rats caused hepatocellular damage to Chang liver cells such as the release of AST.

**Discussion**

Statins are powerful cholesterol-lowering drugs used throughout the world in clinical practice. They are well tolerated but are occasionally associated with severe side effects such as hepatotoxicity and skeletal muscle toxicity. We demonstrated that rats fed a high-lipid and high-sucrose diet developed severe hepatic steatosis, accompanied by hepatic injury and severe myositis, after treatment with a xenobiotic, fluvastatin. Therefore, this study was conducted to determine whether nutritional status affects statin-induced adverse effects in rats. Masuda et al. (1995) reported that after a single oral administration of fluvastatin at 1 and 20 mg/kg to rats, the concentration of unchanged fluvastatin in plasma reached a maximum at 0.5 h and decreased with a half-life of 3 to 4 h. Even more than 4 h after the oral administration of fluvastatin (8 mg/kg), a high concentration of unchanged fluvastatin (0.75–4.1 μM) was detected in the peripheral plasma of HF diet-fed rats. In fact, 1 to 10 μM fluvastatin caused hepatocellular damage to Chang liver cells such as the release of AST. Although how statins increase the risk of myopathy is still not clear, the risk of rhabdomyolysis with statin treatment is considered to increase with an increase in systemic exposure to the statin (MacDonald and Halleck, 2004; Hedenmalm et al., 2010).

In the overall hepatic elimination of statins in rats and humans, the uptake has been demonstrated to be the rate-determining process (Watanabe et al., 2010). In fact, it has been reported that coadministration of statins and drugs such as gemfibrozil and macrolide antibiotics that inhibit Oatp2-mediated hepatic uptake but not CYP3A4 markedly increased the area under plasma concentration-time curve (Shitara et al., 2004; Seithel et al., 2007). In the present study, the expression of mRNA and protein of a transporter for the influx of fluvastatin into hepatocytes, Oatp2, was markedly suppressed in HF diet-fed rats by fluvastatin (8 mg/kg) treatment. In contrast, the expression of efflux transport proteins, Mdr1b and Mrp2, was not significantly changed by fluvastatin (8 mg/kg) treatment. Therefore, inhibition of transporter-mediated hepatic uptake seems to be the main mechanism leading to this increase in systemic exposure to fluvastatin.

Fluvastatin is almost entirely eliminated via metabolism in the liver. The hydroxylolation of fluvastatin by human CYP2C9 produces 5-hydroxyfluvastatin, whereas that by human CYP2C9, CYP3A4, CYP2C8, CYP2D6, and CYP1A1 produces 6-hydroxyfluvastatin and N-desisopropyl-fluvastatin. CYP2C9 is the major enzyme eliminating fluvastatin in humans, whereas simvastatin, lovastatin, and atorvastatin are considered to be metabolized by CYP3A4, on the basis of drug-drug interactions with P450 inhibitors. Because in HF diet-fed
rats treated with fluvastatin for 8 days, the decrease in hepatic levels of CYP2Cs was moderate but that in CYP3As was extensive, the administration of other statins such as simvastatin, lovastatin, and atorvastatin to HF diet-fed rats might cause more severe adverse effects. The dihydroxy heptanoic or heptenoic acid side chain of statin hydroxy acids is a structural feature common to all statins, which undergo glucuronidation to form an acyl glucuronide conjugate and lactonization to form a statin hydroxy acid lactone by human UGT1A3 in humans and affected by polymorphisms at the UGT1A locus. Hepatic expression of UGT2B1 but not UGT1A3 was reduced after fluvastatin (8 mg/kg) treatment in HF diet-fed rats. Studies (Spence et al., 1995; Backman et al., 2002) have demonstrated that a combination of gemfibrozil and statins results in increases in the plasma statin area under the curve and inhibition of the glucuronidation of statins is postulated as a potential mechanism of interaction, although similar pharmacokinetic interaction is not expected with fluvastatin. However, fluvastatin (8 mg/kg) in the HF diet-fed rats did not influence the glucuronidation activities against fluvastatin in liver microsomes. These observations suggest that alterations in levels of phase I and phase II enzymes were associated with the reduced elimination of fluvastatin but only to a limited extent.

Liver injury was found at day 8 after fluvastatin treatment in the HF diet-fed groups but not in the SD diet-fed groups, along with a markedly greater accumulation of hepatic triacylglycerols in the former groups. This study indicates for the first time that a high-fat and high-sucrose diet was associated with hepatocellular liver injury and myopathy caused by fluvastatin; in other words, nutritional state influenced the side effects of statins such as hepatotoxicity and myotoxicity. Although the expression of CYP2E1 and CYP4A is up-regulated in nonalcoholic steatohepatitis and plays a key role in the development of liver injury by initiating lipid peroxidation (Leclercq

**TABLE 4**

Hepatic gene expression of drug-metabolizing phase II enzymes in rats fed an SD or HF diet 8 days after starting the administration of fluvastatin

Rats were fed an SD or HF diet for 1 week, continued to consume each diet containing fluvastatin (0 or 8 mg/kg) for another 8 days, and then were sacrificed. Values are the means ± S.E. (n = 4).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>SD Diet Control</th>
<th>Fluvastatin</th>
<th>HF Diet Control</th>
<th>Fluvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>fold induction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UGT1A1</td>
<td>NM_012683</td>
<td>1.00 ± 0.09</td>
<td>0.68 ± 0.06</td>
<td>1.36 ± 0.23**</td>
<td>0.63 ± 0.14##</td>
</tr>
<tr>
<td>UGT1A2</td>
<td>NM_201423</td>
<td>1.00 ± 0.26</td>
<td>1.42 ± 0.33</td>
<td>2.50 ± 0.37</td>
<td>6.89 ± 1.27***##</td>
</tr>
<tr>
<td>UGT1A3</td>
<td>NM_201424</td>
<td>1.00 ± 0.19</td>
<td>1.06 ± 0.26</td>
<td>1.95 ± 0.39**</td>
<td>1.19 ± 0.15##</td>
</tr>
<tr>
<td>UGT1A5</td>
<td>NM_001039549</td>
<td>1.00 ± 0.07</td>
<td>0.84 ± 0.14</td>
<td>1.51 ± 0.35</td>
<td>1.08 ± 0.12##</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>NM_057105</td>
<td>1.00 ± 0.14</td>
<td>0.89 ± 0.11</td>
<td>3.76 ± 0.66##</td>
<td>2.02 ± 1.19##</td>
</tr>
<tr>
<td>UGT1A7</td>
<td>NM_130407</td>
<td>1.00 ± 0.12</td>
<td>1.26 ± 0.16</td>
<td>1.21 ± 0.18</td>
<td>1.22 ± 0.17##</td>
</tr>
<tr>
<td>UGT2B1</td>
<td>NM_173295</td>
<td>1.00 ± 0.19</td>
<td>0.89 ± 0.09</td>
<td>0.92 ± 0.18</td>
<td>0.29 ± 0.04**##</td>
</tr>
</tbody>
</table>

*p < 0.05, versus SD diet-fed animals.

**p < 0.01.

###p < 0.001.

#p < 0.05, versus HF diet-fed animals.

##p < 0.01.

###p < 0.001.

Fig. 6. Effects of fluvastatin treatment on expression of hepatic drug-metabolizing phase II enzymes in rats fed an SD or HF diet (A, B, and C) and glucuronidation activities in liver microsomes (D). A–C, rats were fed an SD or HF diet for 1 week, administered fluvastatin (8 mg/kg) with the diet for another 8 days, and then sacrificed. Microsomal proteins (20 µg/lane) were prepared and subjected to an immunoblot analysis. Relative levels were expressed by taking the control values obtained from the control SD diet-fed rats as 100. D, glucuronidation activities toward fluvastatin in liver microsomes were assayed in the conditions described under Materials and Methods. Relative levels of the activity were expressed by taking the control values obtained from the control SD diet-fed rats as 100. Values are the mean ± S.E. (n = 3). **, p < 0.01; ***, p < 0.001, for HF diet-fed rats versus the control SD diet-fed rats. ##, p < 0.01 for fluvastatin-treated HF diet-fed rats versus the control HF diet-fed rats. C, vehicle; F, fluvastatin.

Fig. 7. Hepatic gene expression of Oatp2 (A), CYP2C11 (B), CYP3A1 (C), and CYP3A2 (D) in rats fed an SD or HF diet 4, 8, and 14 days after administration of fluvastatin was started. Rats were fed an SD or HF diet for 1 week, administered fluvastatin (0 (closed bar) or 8 (hatched bar) mg/kg) with the diet for another 4, 8, or 14 days, and then sacrificed. Relative levels were expressed by taking the control values obtained from SD diet-fed rats at day 14 as 100. Values are the means ± S.E. (n = 4). *, p < 0.05; **, p < 0.01; ***, p < 0.001, for fluvastatin-treated rats versus the control SD diet-fed rats. n.d., not determined.
et al., 2000), intake of the HF diet did not increase the protein levels of CYP2E1 and CYP4A (Sugatani et al., 2006). We previously showed that feeding rats the HF diet for 8 weeks produced severe hepatic steatosis that was associated with hepatic injury caused by xenobiotics such as phenobarbital and dexamethasone via induction of CYP2B and CYP3A (Sugatani et al., 2006). Although the exact mechanism responsible for the liver injury in HF diet-fed rats is unclear, Kromer and Moosmann (2009) have reported that statins inhibit the expression of inducible selenoproteins by preventing the mevalonate-dependent maturation of human selenocysteine-tRNA and may thereby increase the vulnerability of the liver to secondary toxins such as peroxides. However, it remains to be elucidated whether fluvastatin-induced liver injury in HF diet-fed rats results from increased production of peroxides and/or increased sensitivity to peroxides due to cross-talk between the pathways of cholesterol and selenoprotein biosynthesis.

Whereas CAR and PXR regulate the expression of rat and human CYP2B and CYP3A and rat UGT2B1, HNF4α synergistically enhanced CAR- and PXR-mediated xenobiotic induction of human CYP2C9 (Chen et al., 2005), human CYP3A (Tirona et al., 2003), and human OATP2 (Wortham et al., 2007) expression. Although there is no direct evidence that HNF4α synergistically enhances the CAR- and PXR-mediated xenobiotic induction of CYP2B expression, the CYP2B2 phenobarbital response unit contains an HNF4-binding site (Beaudet et al., 2005). A study using HNF4 antisense RNA 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). There was no significant change based on diet in the baseline expression of the CAR, PXR, RXRα, or HNF4α protein, but CAR, PXR, and HNF4α protein levels were...

![Fig. 8](image-url) Effects of fluvastatin treatment on expression of transcription factors in the liver cell nuclei of rats fed an SD or HF diet. Rats were fed an SD or HF diet for 1 week, administered fluvastatin (8 mg/kg) with the diet for another 8 days, and then sacrificed. Nuclear proteins (30 μg/lane) were prepared and subjected to an immunoblot analysis [CAR (A), PXR (B), RXR (C), HNF4α (D), PPARα (E), and histone H1 (F)]. Relative levels were expressed by taking the control values obtained from the control SD diet-fed rats as 100. Values are the mean ± S.E. (n = 3). *, p < 0.05 for fluvastatin-treated HF diet-fed rats versus the control SD diet-fed rats. #, p < 0.05 for fluvastatin-treated HF diet-fed rats versus the control HF diet-fed rats. C, vehicle; F, fluvastatin.

![Fig. 9](image-url) Hepatic gene expression of transcription factors [CAR (A), PXR (B), and HNF4α (C)] in rats fed an SD or HF diet 4, 8, or 14 days after administration of fluvastatin was started. Rats were fed an SD or HF diet for 1 week, administered fluvastatin [0 (closed bar) or 8 (hatched bar) mg/kg] with the diet for another 4, 8, or 14 days, and then sacrificed. Relative levels were expressed by taking the control values obtained from SD diet-fed rats at day 14 as 100. Values are the mean ± S.E. (n = 4). *, p < 0.05; **, p < 0.01; ***, p < 0.001 for fluvastatin-treated rats versus the control SD diet-fed rats. n.d., not determined.
significantly reduced in the nucleus of liver cells in HF diet-fed rats treated with fluvastatin. The mechanism by which hepatic lipid accumulation was associated with the suppression of the nuclear expression is unknown. However, the decline in the expression of CAR, PXR, and HNF4α proteins in the liver nucleus may contribute to the reduced hepatic expression of CYP2C11, CYP3A1, and Oatp2 in the hepatic lipid accumulation. Preceding the elevation in serum CK levels and muscle damage, serum AST levels were elevated in HF diet-fed rats by fluvastatin treatment. To prevent severe adverse effects in patients with hepatic steatosis, we should be mindful of an elevation in serum AST levels as a sign, although whether AST elevation with statin therapy constitutes true hepatotoxicity has not been determined (Pasternak et al., 2002). Furthermore, we should examine whether statin therapy associated with a dietary cure would be effective to prevent severe adverse effects.

In conclusion, this study provides a comprehensive examination of the expression of P450 isofoms, UGT isofoms, drug transporters, and transcription factors in the fatty liver of rats relevant to statins. The results indicate that 1) nutritional status affected fluvastatin-induced liver failure and 2) reduced expression of Oatp2 after fluvastatin treatment in HF diet-fed rats may be attributable to the reduced expression and/or activation of CAR, PXR, and HNF4α and cross-talk, resulting in suppression of elimination of fluvastatin. Although the data cannot be directly extrapolated to human OATP2, they indicate that the reduced expression of Oatp2 in rat liver could reduce the overall hepatic elimination of fluvastatin. In particular, these results provide a clue for regulating drug-induced liver failure associated with lipid accumulation. Preceding the elevation in serum CK levels and muscle damage, serum AST levels were elevated in HF diet-fed rats by fluvastatin treatment. To prevent severe adverse effects in patients with hepatic steatosis, we should be mindful of an elevation in serum AST levels as a sign, although whether AST elevation with statin therapy constitutes true hepatotoxicity has not been determined (Pasternak et al., 2002). Furthermore, we should examine whether statin therapy associated with a dietary cure would be effective to prevent severe adverse effects.

References


Goosen TC, Bauman JN, Davis JA, Yu C, Hurst SI, Williams JA, and Loi CM (2007) Statin-induced liver failure and 2) reduced expression of Oatp2 after fluvastatin treatment. To prevent severe adverse effects in patients with hepatic steatosis, we should be mindful of an elevation in serum AST levels as a sign, although whether AST elevation with statin therapy constitutes true hepatotoxicity has not been determined (Pasternak et al., 2002). Furthermore, we should examine whether statin therapy associated with a dietary cure would be effective to prevent severe adverse effects.

The references are cited in the document.

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