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Nutritional status affects fluvastatin-induced hepatotoxicity and myopathy in rats

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B) ABBREVIATIONS: ALT, alanine aminotransferase; AST, aspartate aminotransferase; C, vehicle; CAR, constitutive androstane receptor; CPR, NADPH-cytochrome P450 reductase; CK, creatine kinase; F, fluvastatin; γ-GTP, γ-glutamyltranspeptidase; HF, high fat and high sucrose diet; HNF, hepatocyte nuclear factor; Mdr, multidrug resistance; Mrp, multidrug resistance- associated protein; OATP (in humans)/ Oatp (in rodents), organic anion transporter polypeptide; P450, cytochrome P450; PXR, pregnane X receptor; PCR, polymerase chain reaction; RXR, retinoid X receptor ; SD, standard diet; UGT, UDP-glucuronosyltransferase.

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Abstract

Rats that consumed a high-fat and high-sucrose (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 on HF diet led to an increase in systemic exposure, suggesting altered metabolism and elimination. In fact, although hepatic Mrp2 and Mdr1b protein levels were not significantly changed by fluvastatin treatment for 8 days of rats on HF diet, the Oatp1, Mrp3, CYP1A, CYP2C, UGT1A1, 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 between 4 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.

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Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (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. While statins appear to be relatively safe and well tolerated, considerable attention has recently been paid to their adverse effects including muscular toxicity and hepatotoxicity (MacDonald and Halleck, 2004; Pasternak et al., 2002). 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 (Noe 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 (Goosen et al., 2007; Kyrklund et al., 2001; Backman et al., 2002), 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 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 (SD) diet. Treatment with fluvastatin at 8 mg/kg for 8 days caused an elevation in serum aspartate aminotransferase (AST) and creatine kinase (CK) levels and severe hind leg muscle damage, which suggest hepatotoxicity and myopathy, in animals on the HF 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

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, since 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 Yolk, CN). 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; since hepatic Oatp2 protein levels in female rats decreased to 44% of those in male rats (Sugatani et al., 2006), we used male rats in order to examine whether nutritional status affects adverse effects of fluvastatin. Animals were acclimatized for one week prior to 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 one week on either diet, each group was divided into three subgroups and given 0, 4, or 8 mg fluvastatin/kg/day with the diet for 4, 8, or 14 days, since the lethal doses of fluvastatin in SD diet- and HF diet-fed male rats were 16 mg/kg/day 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 [kJ]. The SD diet (Table 1) consisted of 12.9% lipid, 60.4% carbohydrate and 26.7% protein [kJ]. 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^{o}C prior to 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%

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paraformaldehyde in 0.12 M sodium phosphate (pH 7.3) for histological examination. Sections were stained with hematoxylin-eosin.

Biochemical analyses: Blood and tissue sampling were done as described previously (Sugatani et al., 2006). Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltranspeptidase (γ-GTP), and creatine kinase (CK) levels were measured using kits from Wako Pure Chemicals (Osaka, Japan). Frozen livers (about 0.5 g) were homogenized in 20 volumes (SD-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 9,000g for 15 min followed by 105,000g for 60 min at 4^{o}C and stored at –80^{o}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 (about 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 PMSF with 20 strokes of a Dounce homogenizer. After centrifugation at 1,500 g for 10 min, the supernatant was centrifuged at 100,000 g for 30 min. The precipitate was suspended in 6 ml of the buffer and again centrifuged at 100,000 g for 30 min. The crude membrane fraction was resuspended in 0.1 M Tris-HCl buffer (pH 7.4) containing the above proteinase 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 protein 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 sodium dodecyl sulfate-12.5% polyacrylamide gel, and electroblotted onto a polyvinylidene difluoride membrane (Millipore Corporation, Bedford 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 CYP2C11, rat CYP3A2, and rat NADPH P450 reductase (CPR) (Daiichi Pure Chemicals Co., Tokyo, Japan), human MRP2 (sc-5770), human MRP3 (sc-5776), mouse CAR (sc-13065), mouse PXR (sc-7737), human RXRα (sc-551), human HNF4α (sc-6556), human PPARα (sc-9000), and human histone H1 (sc-8030) (Santa Cruz Biotechnology, Santa Cruz, CA), rat Oatp1 and rat Oatp2 (Alpha Diagnostic International, San Antonio, TX), rat Mdr1b (Milipore Co., Billerica, MA) and chicken α-tubulin (Oncogen Research Products, Boston, MA), and rabbit antibodies against rat UGT1A1-, rat UGT1A5-, and rat UGT2B1-specific peptides (Ikushiro et al., 1995, 1997). Antigen-antibody complexes were detected using the appropriate secondary antibody conjugated with horseradish peroxidase and visualized with an enhanced chemiluminescence system (GE Healthcare Bio-Sciences, Piscataway, NJ).

Determination of mRNA levels. Total RNA was prepared from the liver using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA). Samples were quantitated by spectrophotometry, and 1 μg of total RNA was used to generate cDNA by reverse transcription (RT) using a Prime Script RT reagent kit (Takara Bio. Inc., Otsu, Japan) according to the manufacturer's protocol. cDNA synthesized from 50 ng of total RNA was subjected to a quantitative real-time polymerase chain reaction (PCR) as described previously (Sugatani et al., 2006) with an ABI PRISM 7000 Sequence Detector instrument (Applied Biosystems, Foster City, CA) using Premix Ex Taq reagent (TaKaRa Bio Inc.) for the TaqMan probe-based method or SYBR Premix Ex Taq reagent (TaKaRa Bio Inc.) for the intercalation reaction with SYBR Green I according to the manufacturer's specifications. The TaqMan probes and primers for rat *CAR* (NM_022941), rat *UGT1A1* (NM_012683), rat *UGT1A6* (NM_057105) and rat *UGT2B1* (NM_173295) were as reported (Sugatani et al., 2006; Osabe et al., 2008). Those for rat *UGT1A2*

(NM_201423) (assay identification number Rn02749814_s1), rat *UGT1A3* (NM_201424) (assay identification number Rn02749831_s1), rat *UGT1A5* (NM_001039549) (assay identification number Rn01427785_m1), and rat *β-actin* (NM_031144) (assay identification number Rn00667869_m1) were assay-on-demand gene expression products (Applied Biosystems). The gene-specific probes were labeled using the reporter dye FAM, and the β-actin internal control probe was labeled with a different reporter dye, VIC, at the 5' end. The primers for rat *Oatp2* (AF426312), rat *CYP2C11* (NM_019184), rat *CYP3A1* (NM_173144), rat *CYP3A2* (NM_153312), rat *PXR* (NM_052980), and rat *HNF4α* (NM_022180) were as reported (Sugatani et al., 2006; Osabe et al., 2008). The thermal cycle conditions were as follows: hold for 10 sec at 95°C, then a two-step PCR for 40 cycles of 95°C for 5 sec followed by 60°C for 30 sec. β-Actin was used to normalize gene expression in all samples. Fold-induction values were calculated by subtracting the mean difference of gene and β-actin cycle threshold Ct numbers for each treatment group from the mean difference of gene and β-actin Ct numbers for the vehicle-treated group and raising the difference to the power of 2 ($2^{-\triangle\triangle Ct}$).

HPLC-mass spectrometric analysis of fluvastatin. Fluvastatin levels in plasma over 4 h after removal of the diets containing fluvastatin were determined by liquid chromatography/tandem mass spectrometry (LC/MS/MS) at Sumika Chemical Analysis Service LTD (Osaka, Japan). The LC/MS/MS system consisted of a API4000 mass spectrometer (Applied Biosystems/MDS SCIEX, Toronto, Ontario, Canada) equipped with an electrospray ionization interface and a Agilent 1200 Series HPLC system (Agilent Tecnologies Inc., San Jose, CA). To 50 μl of plasma were added 10 μl of atorvastatin (1 ng) as an internal standard and 0.5 ml of 100 mM acetate buffer (pH 5.0). After extraction with 3 ml of methyl *t*-butylether, the eluents were evaporated dry under nitrogen, the dry extracts were reconstituted in 0.2 ml of acetonitrile - ammonium acetate (pH 4.0; 10 mM) (2 : 3) and then 10 μl of the reconstituted sample was injected onto a HPLC column (Symmetry Shield RP8, 3.5-μm particle size, 50 x 2.1 mm). Fluvastatin and atorvastatin were separated by isocratic HPLC on a reverse phase column with water:

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formic acid (1000 : 1) (Eluent A) and acetonitrile : formic acid (1000 : 1) (Eluent B) (Eluent A : Eluent B, 1 : 1) at a flow rate of 0.2 ml/min at 40°C. The mass spectrometer was operated in the electrospray ionization mode with positive ion detection. The analytes were monitored by selected reaction monitoring of the collision-induced dissociation of the precursor ion (fluvastatin, m/z 412; atorvastatin, m/z 559) to its corresponding product ion (fluvastatin, m/z 224; atorvastatin, m/z 440). Standard curves for fluvastatin in plasma of rats fed a standard diet (0.5 – 500 ng/ml) and a high-fat and high-sucrose diet (0.5 – 2000 ng/ml) were linear ($r \geq 0.9999$ and $r \geq 0.9939$, respectively).

Glucuronidation of fluvastatin. Liver microsomes were pretreated with alamethicin at the concentration of 200 μg/mg protein on ice for 30min to diminish the latency of UGT activity. Fluvastatin (100 μM) was incubated in reaction mixture consisted of 0.1 mg liver microsomal protein, 4mM UDP-glucuronic acid, 1mM $MgCl_2$ and 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 100 μl. After preincubation for 5min at 37°C, the reaction was started by adding UDP-glucuronic acid. All reactions were incubated at 37°C for 12hr, and terminated by the addition of an equal volume of cold acetonitrile, followed by centrifugation at 15,000g for 15min to obtain the supernatants. Aliquots of the supernatant were directly analyzed by a Hitachi UPLC system. Fluvastatin and its metabolites were separated on a COSMOSIL 2.5C18-MS-II column (2.5 μm particle size, 100 × 2.0 mm; NACARAI 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 fluavastatin with its metabolites was achieved 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 returned to 20% B for another 2 min of equilibration. The column temperature was 40°C and UV detection was achieved at 245 nm. The fluvastatin glucuronide were splited by several peaks with retention time around 4.9 to 5.2 min because of acylglucuronide formation. The glucuronide were confirmed by experiment showed in supplementary Figure 3B.

Cell culture conditions and treatments. Chang liver human cells from the American Type Culture

Collection (0.5~1 x 10^5 cells/ml) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and antibiotics (100 μg of streptomycin and 10 U of penicillin/ml) at 37^{o}C in the presence of 5% CO_2 unless otherwise stated.

Statistics. Values are expressed as the mean ± standard error. All data were analyzed using a 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

Fluvastatin causes severe hepatic injury and myopathy in rats fed a HD diet. Treatment with fluvastatin at 4 mg/kg for 14 days, given as a component of the diet, suppressed the hepatic total cholesterol level in rats fed a HD diet, but not in those fed a SD diet (Table 2). Treatment with fluvastatin 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, fluvastatin 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 $\pm$ 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 fluvastatin (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, in order to evaluate skeletal muscle toxicity, we examined the histological appearance of the hind leg skeletal muscle. As shown in Figure 2, muscle loss was observed in HF diet-fed rats treated with fluvastatin (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 fluvastatin in HF diet-fed rats compared to SD diet-fed rats, the peripheral plasma concentration of unchanged fluvastatin was determined in rats treated with fluvastatin (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 to those in the SD diet-fed rats, while the plasma levels in SD diet-fed rats treated with fluvastatin for 8 days were slightly elevated compared to 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-metabolising 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 gulucuronidation. Since 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 to SD diet-fed rats. As shown in Figure 4, in SD diet-fed rats, while Mdr1b 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 $\pm$ 0.7% and 41.6 $\pm$ 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 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 CYP1As (mainly CYP1A2), CYP2Cs (mainly CYP2C11), CYP3As (mainly CYP3A1), and CPR in SD diet-fed rats (Fig. 5). In HF diet-fed rats, while fluvastatin treatment resulted in a significant decrease in the protein levels of CYP1As and CYP3As, to 45.2 $\pm$ 14.2% and 33.5 $\pm$ 6.6% of the control value, respectively, the levels of the major fluvastatin-metabolizing enzyme CYP2Cs were only slightly decreased, to 75.7 $\pm$ 6.6% (Fig. 5). Furthermore, while the UGT isoenzymes metabolizing

fluvastatin remain to be elucidated, we examined 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, while 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 $\pm$ 14% and 29 $\pm$ 4% of the control SD rats, respectively, but no significant change in UGT1A3, UGT1A5, UGT1A6, and UGT1A7 mRNA levels. In addition, we examined 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. While 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 (Figs. 6A, 6B, and 6C). 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. Since the glucuronidation activities in UGT1As-deficient Gunn rat liver microsomes were not detectable (Supplementary 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 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 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 on 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 the decreased influx of fluvastatin into hepatocytes, while 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 a heterodimer 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; Chen et al., 2005; Tirona et al., 2003; 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 $\pm$ 16.1%, 72.5 $\pm$ 11.1%, and 59.4 $\pm$ 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 on 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, in order to investigate whether fluvastatin at a concentration near 10 μM can cause hepatocellular damage since 4.1

μM of 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 (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.

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Discussion

Statins are powerful cholesterol-lowering drugs used throughout the world in clinical practice. They are well tolerated, but occasionally associated with severe side effects such as hepatotoxicity and skeletal muscle toxicity. This study 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 ~ 4 h. Even over 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 appears 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 hydroxylation of fluvastatin by human CYP2C9 produces 5-hydroxy-fluvastatin, while that by human CYP2C9, CYP3A4, CYP2C8, CYP2D6, and CYP1A1 produces 6-hydroxy-fluvastatin and *N*-deisopropyl-fluvastatin. CYP2C9 is the major enzyme eliminating fluvastatin in humans, while simvastatin, lovastatin, and atorvastatin are considered to be metabolized by CYP3A4, on the basis of drug-drug interactions with CYP inhibitors. Since 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 UGT1A1, UGT1A3, UGT1A4, UGT1A8, UGT1A9, and UGT2B7 (Prueksaritanont et al., 2002; Goosen et al., 2007). Riedmaier et al. (2010) have demonstrated that lactonization of atorvastatin is catalyzed mainly by 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 (Backman et al., 2002; Spence et al., 1995) 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, though 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 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 (Lecleroq et al., 2000), intake of the HF diet did not increase the protein levels of CYP2E1 and CYP4A (Sugatani et al., 2006). Previously, we 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.

While 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, CYP2B2's 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 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 HF diet-fed groups following fluvastatin treatment. Oyadomari et al. (2000) demonstrated that HNF4α gene expression in rat liver was activated by glucocorticoids and glucagon and repressed by insulin, that is, expression changed in response to hormones controlling glucose homeostasis. The reduction in HNF4α expression in the nucleus caused by a high-sucrose diet may be associated with the regulation of glucose metabolism and/or biological programs linked to energy homeostasis. On the other hand, liver injury was found at day 4 after fluvastatin treatment. Cross-talk between transcription factors has been demonstrated to be markedly increased in liver from patients with severe liver disease, particularly between CAR, HNF4α, and PXR (Congiu et al., 2009). Our results support these observations in diseased liver.

In conclusion, this study provides a comprehensive examination of the expression of P450 isoforms, UGT isoforms, 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 fluvastatin's elimination. 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 to elevation in serum CK levels and muscle damage, serum AST levels were elevated in HF diet-fed rats by fluvastatin treatment. In order to prevent severe adverse effects in patients carrying hepatic steatosis, we should be careful in elevation in serum AST levels as the

sign, although whether AST elevation with statin therapy constitutes true hepatotoxity has not been determined (Pasternack et al., 2002). Furthermore, we should examine whether statin therapy associated with dietary cure would be effective to prevent the severe adverse effects.

References

Backman JT, Kyrklund C, Neuvonen M, Neuvonen PJ (2002) Gemfibrozil greatly increases plasma concentration of cerivastatin. *Clin Pharmacol Ther* **68**: 122-129.

Beaudet M-J, Desrochers M, Lachaud AA, Anderson A (2005) The *CYP2B2* phenobarbital response unit contains binding sites for hepatocyte nuclear factor 4, PBX-PREP1, the thyroid hormone receptor β and the liver X receptor. *Biochem J* **388**: 407-418.

Bogan AA, Dallas-Yang Q, Ruse Jr MD, Maeda Y, Jiang G, Nepomuceno L, Scanlan TS, Cohen FE, Sladek FM (2000) Analysis of protein dimerization and ligand binding of orphan receptor HNF4α. *J Mol Biol* **302**: 831-851.

Bugianesi E, Leone N, Vanni E, Marchesini G, Brunello F, Carucci P, Musso A, De Paolis P, Capussotti L, Salizzoni M, Rizzetto M (2002) Expanding the natural history of nonalcoholic steatohepatitis: From cryptogenic cirrhosis to hepatocellular carcinoma. *Gastroenterology* **123**: 134-140.

Chen Y, Kissling G, Negishi M, Goldstein JA (2005) The nuclear receptors constitutive androstane receptor and pregnane X receptor cross-talk with hepatic nuclear factor 4α to synergistically activate the human *CYP2C9* promoter. *J Pharmacol Exp Ther* **314**: 1125-1133.

Congiu M, Mashford ML, Slavin JL, Desmond PV (2009) Coordinate regulation of metabolic enzymes and transporters by nuclear transcription factors in human liver disease. *J Gastroenterol Hepatol* **24**: 1038-1044.

Ding X, Lichti K, Kim I, Gonzalez FJ, Staudinger JL (2006) Regulation of constitutive androstane receptor and its target genes by fasting, cAMP, hepatocyte nuclear factor α, and the coactivator peroxisome proliferators-activated receptor γ coactivator-1α. *J Biol Chem* **281**:26540-26551.

Emery M, Fisher JM, Chien JY, Kharasch ED, Dellinger EP, Kowdley KV, Thummel KE (2003) CYP2E1 activity before and after weight loss in morbidly obese subjects with nonalcoholic fatty liver disease. *Hepatology* **38**: 428-435.

Fischer V, Johanson L, Heitz F, Tullman R, Graham E, Baldeck J-P, Robinson WT (1999) The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor fluvastatin: Effect on human cytochrome P-450 and implications for metabolic drug interactions. *Drug Metab Dispos* **27**: 410-416.

Goosen TC, Bauman JN, Davis JA, Yu C, Hurst SI, Williams JA, Loi C-M (2007) Atorvastatin glucuronidation is minimally and nonselectively inhibited by the fibrates gemfibrozil, fenofibrate, and fenofibric acid. *Drug Metab Dispos* **35**: 1315-1324.

Hartman HA, Myers LA, Evans M, Robison RL, Engstrom RG, Tse FL (1996) The safety evaluation of fluvastatin, an HMG-CoA reductase inhibitor, in beagle dogs and rhesus monkeys. *Fundam Appl Toxicol* **29**: 48-62.

Hedenmalm K, Alvan G, Ohagen P, Dahl ML (2010) Muscle toxicity with statins. *Pharmacoepidemiol Drug Saf* **19**: 223-231.

Honkakoski P, Zelko I, Sueyoshi T, Negishi M (1998) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the *CYP2B* gene. *Mol Cell Biol* **18**: 5652-5658.

Ikushiro S, Emi Y, Iyanagi T (1995) Identification and analysis of drug-responsive expression of UDP-glucuronosyltransferase family (UGT1) isozyme in rat hepatic microsomes using anti-peptides antibodies. *Arch Biochem Biophys* **324**:267-272.

Ikushiro S, Emi Y, Iyanagi T (1997) Protein-protein interactions between UDP-glucuronosyltransferase isozymes in rat hepatic microsomes. *Biochemistry* **36**:7154-7161.

Jover R, Bort R, Gomez-Lechon MJ, Castell JV (2001) Cytochrome P450 regulation by hepatocyte nuclear factor 4 in human hepatocytes: A study using adenovirus-mediated antisense targeting. *Hepatology* **33**: 668-675.

Kromer A, Moosmann B (2009) Statin-induced liver injury involves cross-talk between cholesterol and selenoprotein biosynthetic pathway. *Mol Pharmacol* **72**: 1421-1429.

Kyrklund C, Backman JT, kivisti KT, Neuvonen M, Laitila J, Neuvonen PJ (2001) Plasma concentrations of active lovastatin and are markedly increased by gemfibrozil but not by bezafibrate. *J Clin Pharmacol Ther* **69**: 340-345.

Leclercq IA, Farrell GC, Field J, Bell DR, Gonzalez FJ, Robertson GR (2000) CYP2E1 and CYP4A as microsomal catalysts of lipid peroxides in murine nonalcoholic steatohepatitis. *J Clin Invest* **105**: 1067-1075.

Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* **102**: 1016-1023.

MacDonald JS, Halleck MM (2004) The toxicology of HMG CoA reductase inhibitors: Prediction of human risk. *Toxicol Pathol* **32**: 26-41.

Masuda N, Akasaka I, Ohtawa M (1995) Metabolic fate of fluvastatin, an inhibitor of HMG-CoA reductase (1): Absorption, distribution and excretion of [^{14}C]fluvastatin after single administration in rats. *Yakubutsudoutai* **10**: 513-528.

Noe J, Portmann R, Brun ME, Funk C (2007) Substrate-dependent drug-drug interactions between gemfibrozil, fluvastatin and other organic anion-transporting peptide (OATP) substrates on OATP1B1, OATP2B1, and OATP1B3. *Drug Metab Dispos* **35**: 1308-1314.

Ogawa K, Suzuki H, Hirohashi T, Ishikawa T, Meier PJ, Hirose K, Akizawa T, Yoshioka M, Sugiyama Y (2000) Characterization of inducible nature of MRP3 in rat liver. *Am J Physiol* **278**: G438-G446.

Osabe M, Sugatani J, Fukuyama T, Ikushiro S, Ikari A, Miwa M (2008) Constitutive androstane receptor and peroxisome proliferator-activated receptor a are activated in liver of male rats fed a high-fat and high-sucrose diet and the hepatic expression of UGT1A1 and UGT1A6 is enhanced. *Drug Metab. Dispos* **36**: 294-302.

Oyadomari S, Matsuno F, Chowdhury S, Kimura T, Iwase K, Araki E, Shichire M, Mori M, Takiguchi M

(2000) The gene for hepatocyte nuclear factor (HNF)-4α is activated by glucocorticoids and glucagon, and repressed by insulin in rat liver. *FEBS Lett* **478**: 141-146.

Pasternak RC, Smith SC, Bairey-Merz CN, Grundy SM, Cleeman JI, Lenfant C (2002) ACC/AHA/NHLBI clinical advisory on the use and safety of statins. *J Am Coll Cardiol* **40**: 567-572.

Prueksaritanont T, Tang C, Qiu Y, Mu L, Subramanian R, Lin JH (2002) Effects of fibrates on metabolism of statins in human hepatocytes. *Drug Metab Dispos* **30**: 1280-1287.

Riedmaier S, Klein K, Hofmann U, Keskitalo JE, Neuvonen PJ, Schwab M, Niemi M, Zanger UM (2010) UDP-glucuronosyltransferase (UGT) polymorphisms affect atorvastatin lactonization *in vitro* and *in vivo*. *Clin Pharmacol Ther* **87**: 65-73.

Sakamoto K, Mikami H, Kimura J (2008) Involvement of organic anion transporting polypeptides in the toxicity of hydrophilic pravastatin and lipophilic fluvastatin in rat skeletal myofibres. Br J Pharmacol 154: 1482-1490.

Seithel A, Eberi S, Singer K, Auge D, Heinkele G, Wolf NB, Dorje F, Fromm MF, Konig J (2007) The influence of macrolide antibiotics on the uptake of organic anions and drugs mediated by OATP1B1 and OATP1B3. *Drug Metab Dispos* **35**: 779-786.

Shitara Y, Hirano M, Sato H, Sugiyama Y (2004) Gemfibrozil and its glucuronide inhibit the organic anion transporting polypeptide 2 (OATP2/OATP1B1:*SLC21A6*)-mediated hepatic uptake and CYP2C8-mediated metabolism of cerivastatin: Analysis of the mechanism of the clinically relevant drug-drug interaction between cerivastatin and gemfibrozil. *J Pharmacol Exp Ther* **311**: 228-236.

Spence JD, Munoz CE, Hendricks L, Latchinian L, Khouri HE (1995) Pharmacokinetics of the combination of fluvastatin and gemfibrozil. *Am J Cardiol* **76**: 80A-83A.

Sugatani J, Nishitani S, Yamakawa K, Yoshinari K, Sueyoshi T, Nagishi M, Miwa M (2005) Transcriptional regulation of human UGT1A1 gene expression: Activated glucocorticoid receptor enhances constitutive androstane receptor/pregnane X receptor-mediated

UDP-glucuronosyltransferase 1A1 regulation with glucocorticoid receptor-interacting protein 1. *Mol Pharmacol* **67**: 845-855.

Sugatani J, Wada T, Osabe M, Yamakawa K, Yoshinari K, Miwa M (2006) 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. *Drug Metab Dispos* **34**:1677-1687.

Triona RG, Lee W, Leake BF, Lan L-B, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y, Gonzalez FJ, Schuetz EG, Kim RB (2003) The orphan nuclear receptor HNF4α determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nature Med* **9**:220-224.

Watanabe T, Kusuhara H, Maeda K, Kanamaru H, Saito Y, Hu Z, Sugiyama Y (2010) Investigation of the rate-determining process in the hepatic elimination of HMG-CoA reductase inhibitors in rats and humans. *Drug Metab Dispos* **38**: 215-222.

Wortham M, Czerwinski M, He L, Parkinson A, Wan Y-J Y (2007) Expression of constitutive Androstane receptor, hepatic nuclear factor 4α, and P450 oxidoreductase genes determines interindividual variability in basal expression and activity of a broad scope of xenobiotic metabolism genes in the human liver. *Drug Metab Dispos* **35**:1700-1710.

Weltman MD, Farrell GC, Liddle C (1996) Increased hepatocyte CYP2E1 expression in a rat nutritional model of hepatic steatosis with inflammation. *Gastroenterology* **111**: 1645-1653.

Zhang L, Reynolds KS, Zhao P, Huang SM (2010) Drug interactions evaluation: an integrated part of risk assessment of therapeutics. *Toxicol Appl Pharmacol* **243**: 134-145.

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Footnote

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Legends for figures

Fig. 1 Effects of fluvastatin treatment on serum levels of AST, ALT, γ-GTP, and CK in rats fed a SD- or HF-diet and administered fluvastatin. Rats (7 weeks of age) were fed a SD or HF diet for 1 week, and then administered fluvastatin (0, 4, or 8 mg/kg) with the diet daily. The rats were sacrificed 4, 8, or 14 days after starting the administration of fluvastatin. Values are the mean ± S.E. (n=4). *p<0.05, **p<0.01, ***p<0.001 versus SD diet-fed control rats. #p<0.05, ##p<0.01, ###p<0.001 versus HF diet-fed control rats. n.d.: not determined

Fig. 2 Histological examination of rat hind leg skeletal muscle after administration of fluvastatin. Rats were fed a SD or HF diet for 1 week, and then administered fluvastatin (8 mg/kg) with the diet daily. The rats were sacrificed 8 days after starting the administration of fluvastatin. Hematoxylin and eosin-stained slides of rat hind leg skeletal muscle samples were analyzed histologically. In comparison with the hind leg muscle from SD (A) or HF (B) diet-fed control animals, myofibrillar necrosis in the hind leg muscles from animals with elevated CK levels (C) is apparent. Scale bar indicates 50 μm.

Fig. 3 Plasma levels of fluvastatin in rats fed a SD or HF diet 4 and 8 days after stating the administration of fluvastatin. Rats were fed a SD or HF diet for 1 week, administered fluvastatin (8 mg/kg) with the diet for another 4 or 8 days, and then sacrificed. Values are the mean ± S.E. (n= 4-6). *p<0.05 for fluvastatin-treated rats versus the control SD diet-fed rats at day 8.

Fig. 4 Effects of fluvastatin treatment on expression of hepatic drug transporter proteins in rats fed a SD or HF diet. Rats were fed a SD or HF diet for 1 week, administered fluvastatin (8 mg/kg) with the diet for another 8 days, and then sacrificed. A plasma membrane fraction (20 μg/lane) was 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. Values are the mean

± S.E. (n=3). ***p*<0.01, ****p*<0.001 for fluvastatin-treated HF diet-fed rats versus the control SD diet-fed rats. + *p*<0.10, #*p*<0.05, ##*p*<0.01 for fluvastatin-treated HF diet-fed rats versus the control HF diet-fed rats.

Fig. 5 Effects of fluvastatin treatment on expression of hepatic drug-metabolizing phase I enzymes in rats fed a SD or HF diet. Rats were fed a 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. Values are the mean ± S.E. (n=3). ***p*<0.01 for fluvastatin-treated HF diet-fed rats versus the control SD diet-fed rats.

Fig. 6 Effects of fluvastatin treatment on expression of hepatic drug-metabolizing phase II enzymes in rats fed a SD or HF diet (A, B, C) and glucuronidation activities in liver microsomes (D). (A-C) Rats were fed a 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 condition under described in Materials & 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.

Fig. 7 Hepatic gene expression of *Oatp2*, *CYP2C11*, *CYP3A1*, and *CYP3A2* in rats fed a SD or HF diet 4, 8, and 14 days after stating the administration of fluvastatin. Rats were fed a SD or HF diet for 1 week, administered fluvastatin (0, 4, or 8 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

Fig. 8 Effects of fluvastatin treatment on expression of transcription factors in the liver cell nuclei of rats fed a SD or HF diet. Rats were fed a 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. 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.

Fig. 9 Hepatic gene expression of transcription factors in rats fed a SD or HF diet 4, 8, or 14 days after stating the administration of fluvastatin. Rats were fed a SD or HF diet for 1 week, administered fluvastatin (0, 4, or 8 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

Fig. 10 Effects of fluvastatin treatment on AST levels released from Chang liver cells and cell growth. (A) Chang liver cells (2 x 10^5 cells/2ml/well) were cultured for 48 h and then treated with fluvastatin at the indicated concentrations or vehicle (dimethylsulfoxide) for another 72 h. The AST activity in the medium was determined as described in Materials and Methods. (B) The cells (5 x 10^4 cells/ml/well) were cultured for 24 h and then treated with fluvastatin at the indicated concentrations for another 72 h. Relative cell number was calculated by taking values obtained from control cells as 100. Values are the mean ± S.E. (n=3). *p<0.05, **p<0.01, ***p<0.001 versus the vehicle-treated group.

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Table 1. Composition of the high-fat and high-sucrose (HF) and standard (SD) diets

HF Diet	g	SD Diet (MF chow)	g
Casein	19.70	Crude Protein	23.80
Soybean oil	1.0	Crude fat	5.10
Lard	10.00	Crude fiber	3.20
Mineral mixture	4.00	Ash	6.1
Vitamin mixture	1.00	Nitorogen-free extract	54.0
Choline chloride	0.15	Humidity	7.8
Cholesterol	0.50		
Sodium cholate	0.25		
Cellulose	3.40		
Sucrose	60.00		
Total	100		100

Table 2. Effects of fluvastatin on biomarkers of metabolic disease in rats fed 3-week SD or HF diets. Rats (7 weeks of age) were fed a SD- or HF diet for 1 week, administered fluvastatin (0, 4, or 8 mg/kg) with the diet for another 8 or 14 days, and then sacrificed. Values are the mean ± S.E. (n=4). *p<0.05, **p<0.01, ***p<0.001 versus SD diet-fed animals; #p<0.05, ###p<0.001 versus HF diet-fed animals.

	Day	SD			HF		
		Control	Fluvastatin (4 mg/kg)	Fluvastatin (8 mg/kg)	Control	Fluvastatin (4 mg/kg)	Fluvastatin (8 mg/kg)
Initial body weight (g)	0	235 ± 5	229 ± 6	229 ± 6	230 ± 2	219 ± 2	231 ± 2
Final body weight (g)	14	336 ± 7	322 ± 5	297 ± 8***	345 ± 7	330 ± 16	n.d.
Epididymal white adipose tissue weight (g)	14	3.72 ± 0.16	3.29 ± 0.22	2.98 ± 0.20*	4.21 ± 0.42	3.97 ± 0.57	n.d.
Liver weight (g)	8	12.81 ± 0.11	n.d.	12.21 ± 0.78	16.81 ± 0.48**	n.d.	15.96 ± 0.76
	14	13.42 ± 0.40	13.06 ± 0.44	12.00 ± 0.52	19.59 ± 1.12**	19.38 ± 2.62	n.d.
Liver triacylglycerol (mg/g)	8	10.33 ± 1.51	n.d.	6.48 ± 0.44	44.48 ± 6.62**	n.d.	49.45 ± 8.32
	14	7.76 ± 1.14	5.09 ± 0.63*	5.09 ± 0.17*	47.98 ± 2.92***	35.1 ± 5.24$^{\#}$	n.d.
Liver total cholesterol (mg/g)	8	n.d.	n.d.	n.d.	34.69 ± 2.54	n.d.	29.11 ± 2.47$^{\#}$
	14	8.47 ± 1.83	8.04 ± 0.98	3.66 ± 0.19*	79.44 ± 9.00***	46.44 ± 10.45$^{\#\#\#}$	n.d.
Serum triacylglycerol (g/l)	14	0.97 ± 0.11	0.55 ± 0.10**	0.33 ± 0.06***	2.07 ± 0.59***	0.96 ± 0.35$^{\#\#\#}$	n.d.
Serum total cholesterol (g/l)	14	0.72 ± 0.03	0.68 ± 0.05	0.73 ± 0.08	2.01 ± 0.26***	1.73 ± 0.29	n.d.

n.d.: not determined

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Table 3. Survival of rats administered fluvastatin with a standard or high-fat and high-sucrose diet. Four rats per group were administered indicated doses of fluvastatin.

Diet	Fluvastatin (mg/kg)	Survivor (head/group)	Survival (day)
Standard diet	4	4	>14
	8	4	>14
High-fat and high-sucrose diet	4	4	>14
	8	0	10.8 $\pm$ 1.9

Table 4. Hepatic gene expression of drug-metabolizing phase II enzymes in rats fed a SD or HF diet 8 days after starting the administration of fluvastatin. Rats were fed a 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 sacrificed. Values are the means ± S.E. (n=4). *p<0.05, **p<0.01, ***p<0.001 versus SD diet-fed animals; #p<0.05, ##p<0.01, ###p<0.001 versus HF diet-fed animals.

Gene	Accession number	SD (fold induction)		HF (fold induction)	
		Control	Fluvastatin	Control	Fluvastatin
UGT1A1	NM_012683	1.00 ± 0.09	0.68 ± 0.06	1.36 ± 0.23**	0.63 ± 0.14##
UGT1A2	NM_201423	1.00 ± 0.26	1.42 ± 0.33	2.50 ± 0.37	6.89 ± 1.27***、###
UGT1A3	NM_201424	1.00 ± 0.19	1.06 ± 0.26	1.95 ± 0.39*	1.19 ± 0.15
UGT1A5	NM_001039549	1.00 ± 0.07	0.84 ± 0.14	1.51 ± 0.35	1.08 ± 0.12
UGT1A6	NM_057105	1.00 ± 0.14	0.89 ± 0.11	3.76 ± 0.66*	2.02 ± 1.19
UGT1A7	NM_130407	1.00 ± 0.12	1.26 ± 0.16	1.21 ± 0.18	1.22 ± 0.17
UGT2B1	NM_173295	1.00 ± 0.19	0.89 ± 0.09	0.92 ± 0.18	0.29 ± 0.04**,##

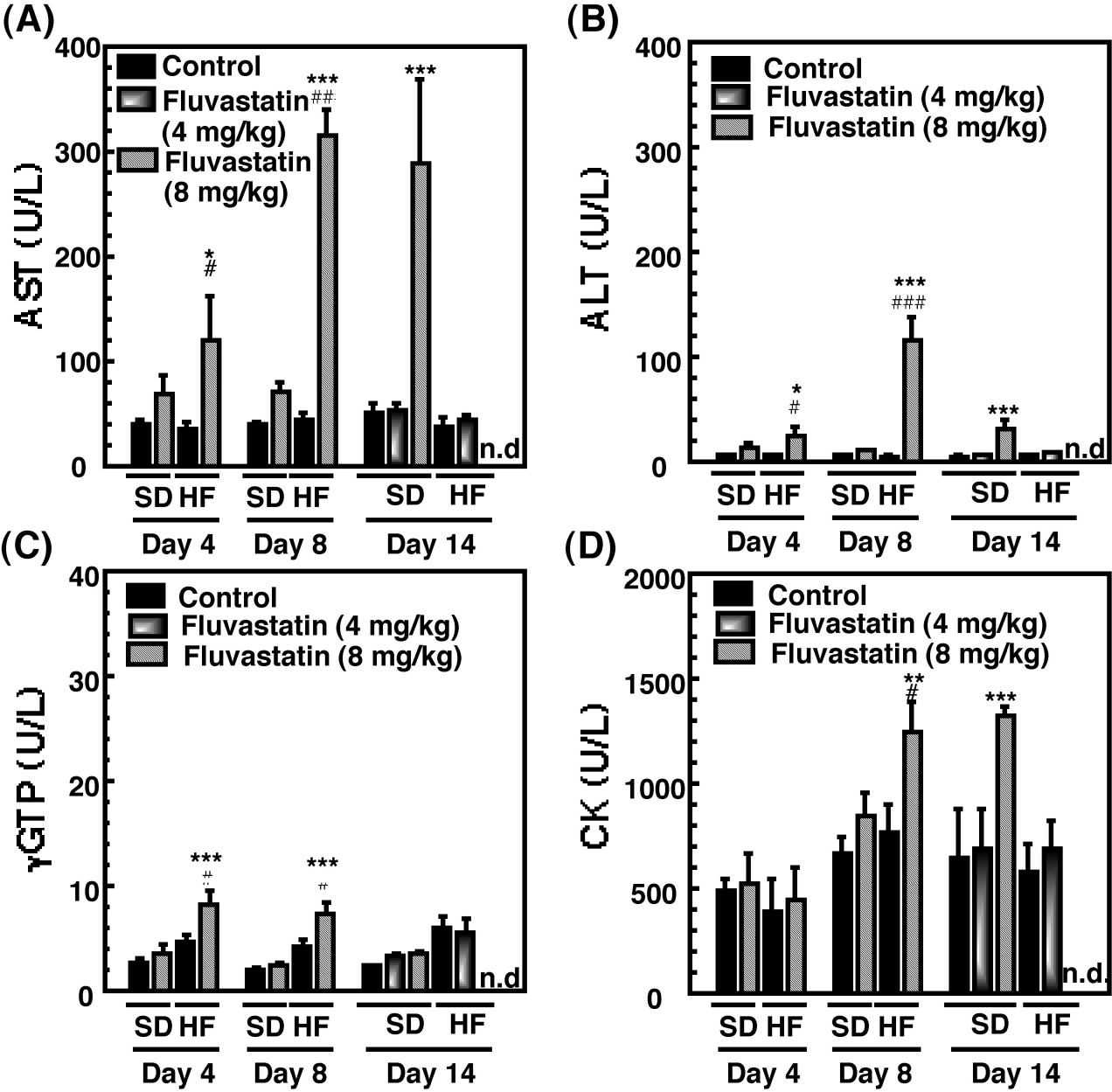
(A)
AST (U/L)
Control
Fluvastatin (4 mg/kg)
Fluvastatin (8 mg/kg)
n.d
SD HF SD HF SD HF
Day 4 Day 8 Day 14
(B)
ALT (U/L)
Control
Fluvastatin (4 mg/kg)
Fluvastatin (8 mg/kg)
n.d
SD HF SD HF SD HF
Day 4 Day 8 Day 14
(C)
γGTP (U/L)
Control
Fluvastatin (4 mg/kg)
Fluvastatin (8 mg/kg)
n.d
SD HF SD HF SD HF
Day 4 Day 8 Day 14
(D)
CK (U/L)
Control
Fluvastatin (4 mg/kg)
Fluvastatin (8 mg/kg)
n.d.
SD HF SD HF SD HF
Day 4 Day 8 Day 14

Fig. 1

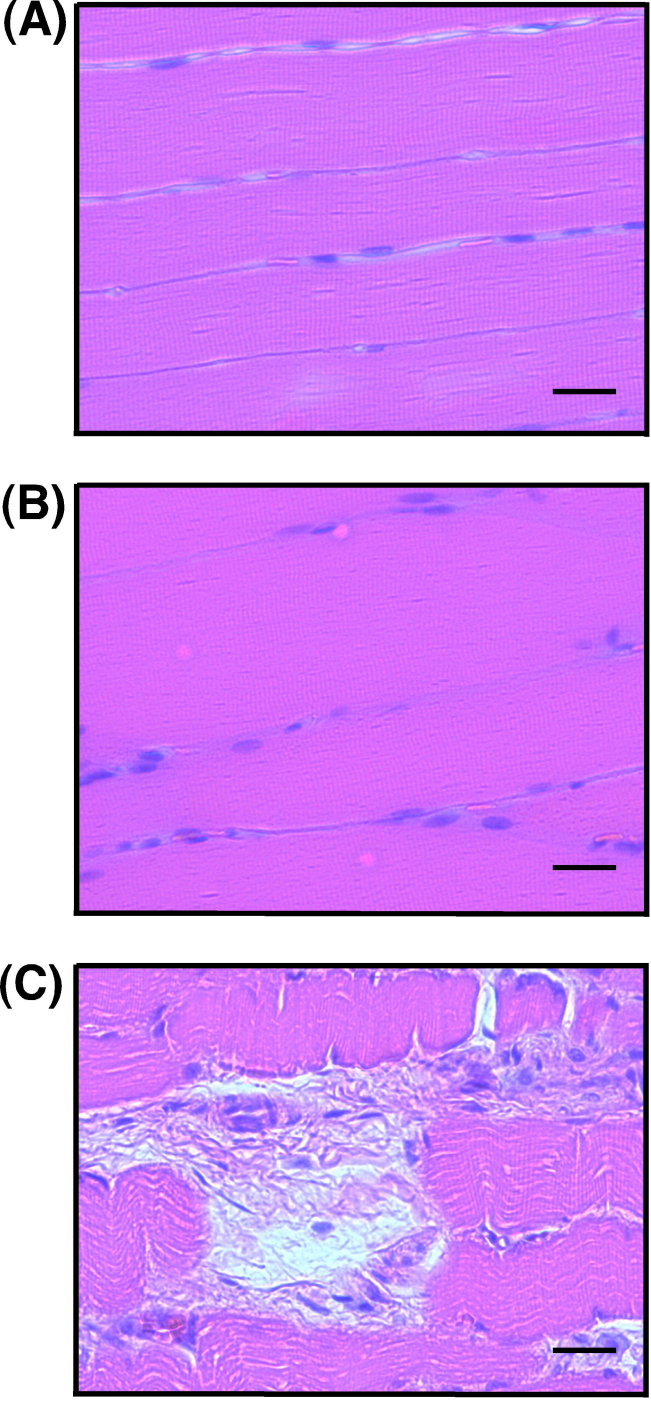

Fig. 2

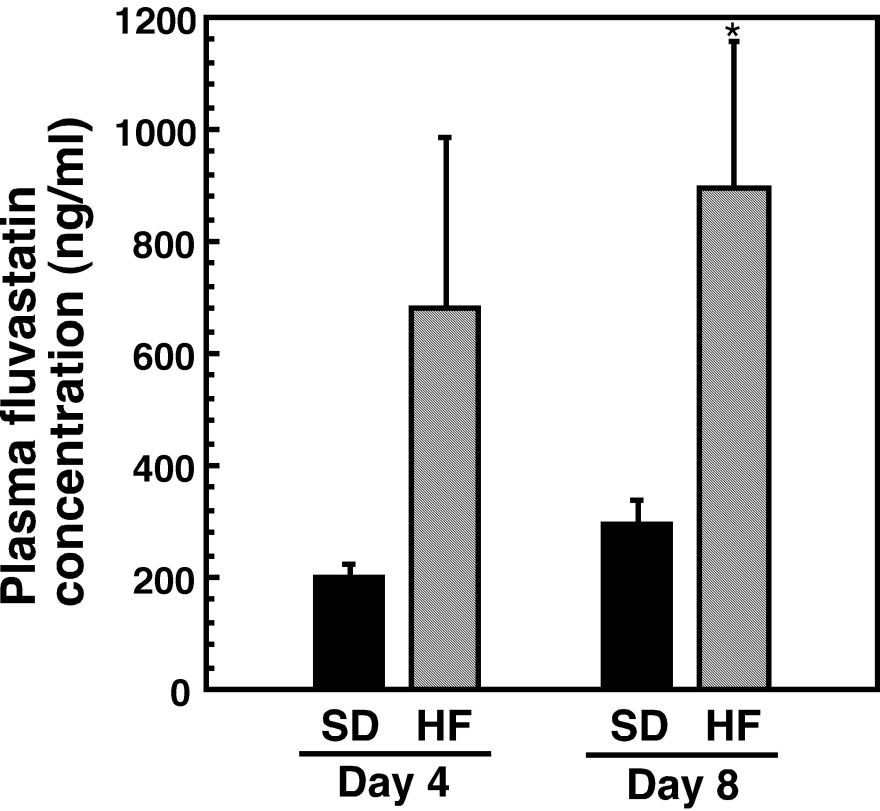
Plasma fluvastatin concentration (ng/ml)
1200
1000
800
600
400
200
0
*
SD
HF
SD
HF
Day 4
Day 8

Fig. 3

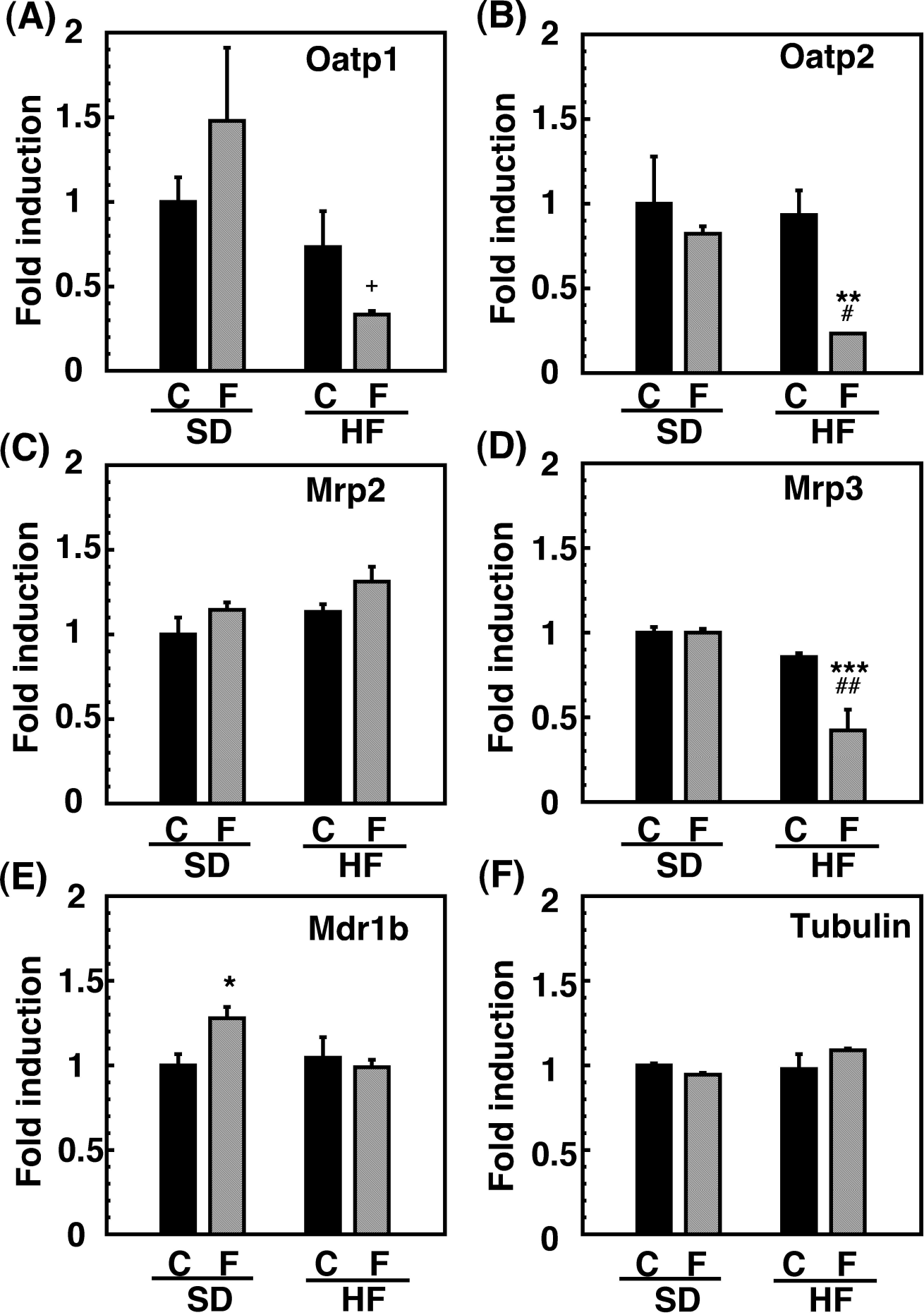
(A)
Oatp1
Fold induction
2
1.5
1
0.5
0
+
C F
SD
C F
HF
(B)
Oatp2
Fold induction
2
1.5
1
0.5
0
** #
C F
SD
C F
HF
(C)
Mrp2
Fold induction
2
1.5
1
0.5
0
C F
SD
C F
HF
(D)
Mrp3
Fold induction
2
1.5
1
0.5
0
*** ##
C F
SD
C F
HF
(E)
Mdr1b
Fold induction
2
1.5
1
0.5
0
*
C F
SD
C F
HF
(F)
Tubulin
Fold induction
2
1.5
1
0.5
0
C F
SD
C F
HF

Fig. 4

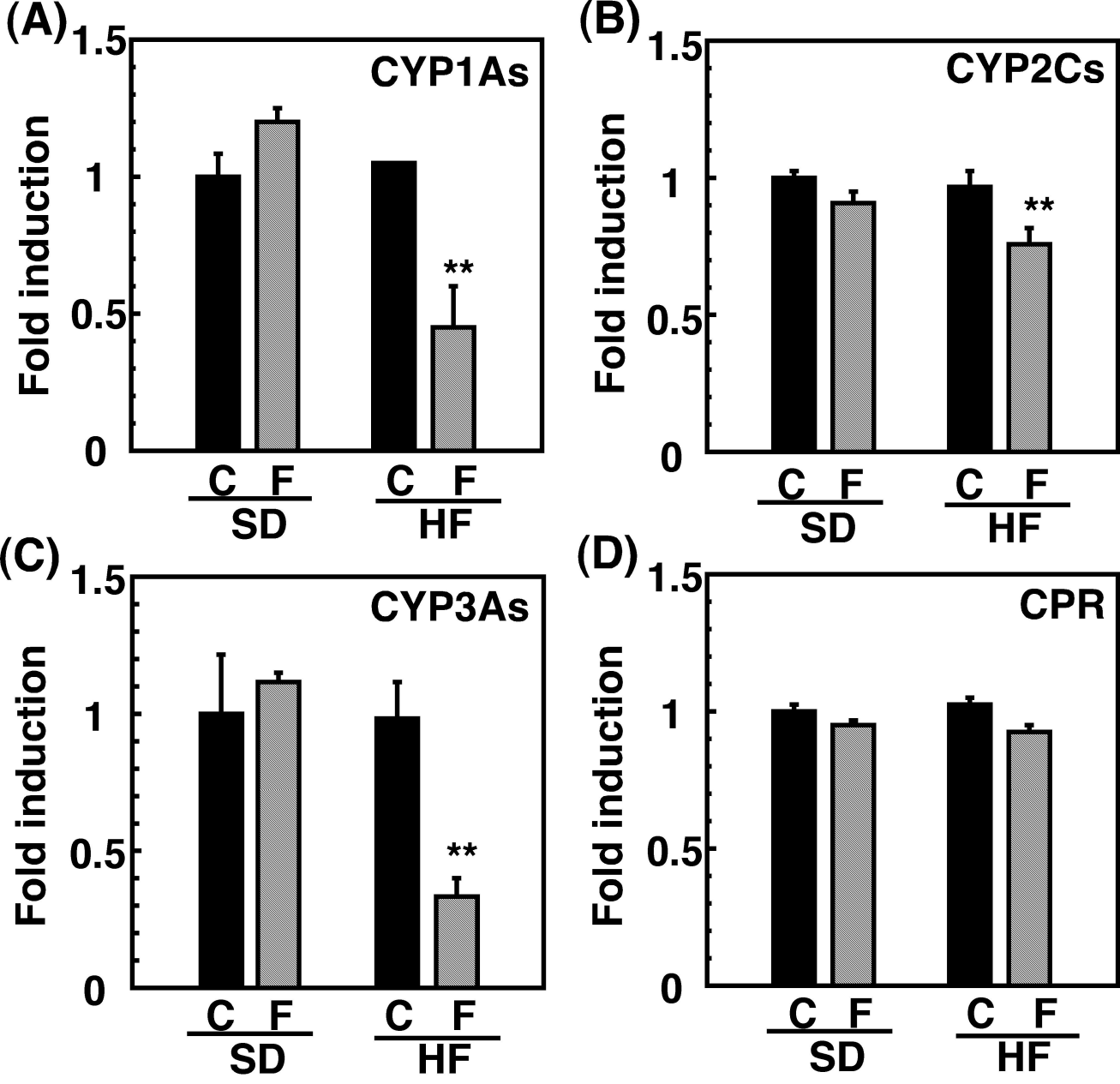
(A)
CYP1As
Fold induction
1.5
1
0.5
0
**
C F
SD
C F
HF
(B)
CYP2Cs
Fold induction
1.5
1
0.5
0
**
C F
SD
C F
HF
(C)
CYP3As
Fold induction
1.5
1
0.5
0
**
C F
SD
C F
HF
(D)
CPR
Fold induction
1.5
1
0.5
0
C F
SD
C F
HF

Fig. 5

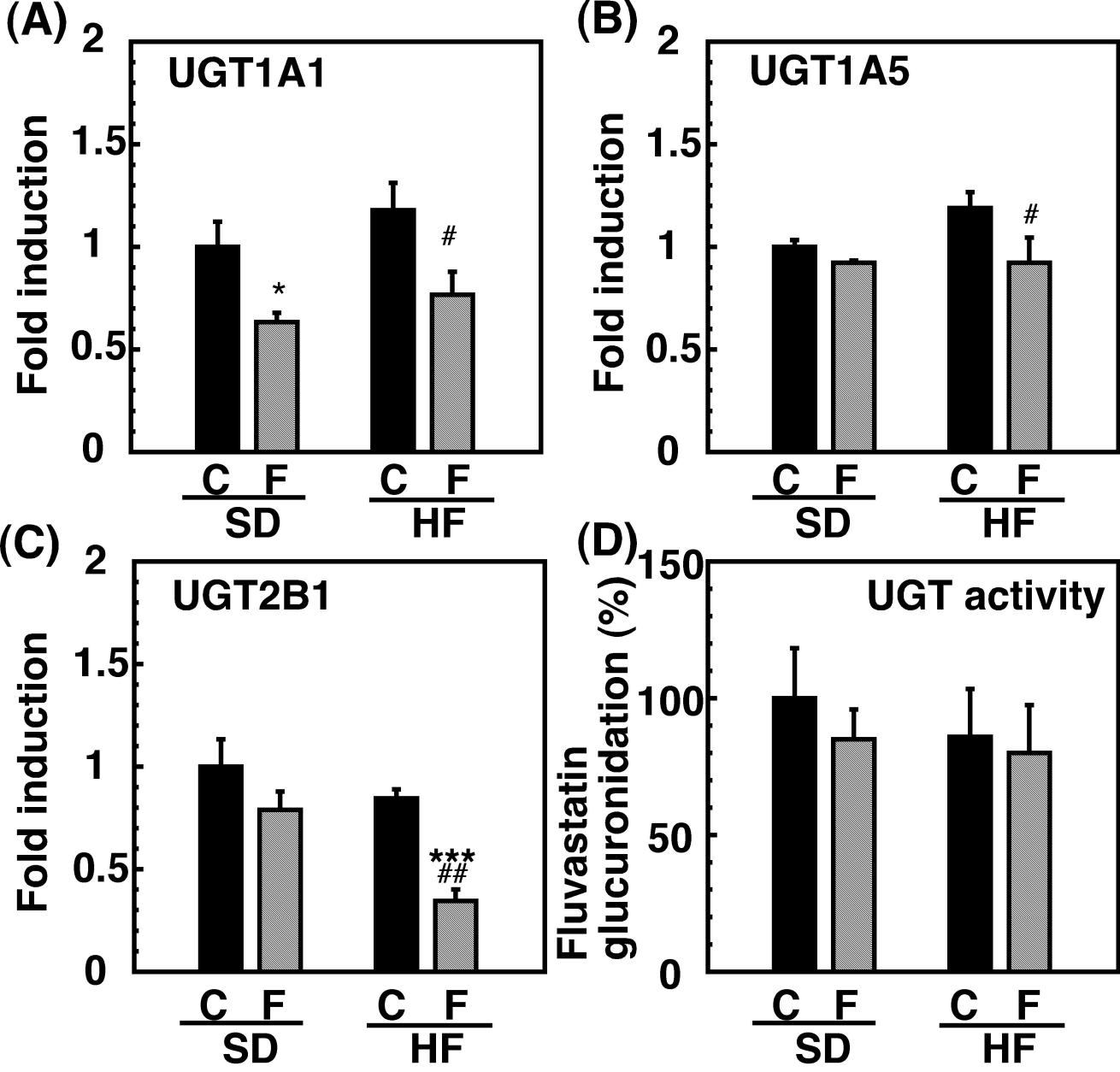
(A)
UGT1A1
Fold induction
2
1.5
1
0.5
0
*
#
C F
SD
C F
HF
(B)
UGT1A5
Fold induction
2
1.5
1
0.5
0
#
C F
SD
C F
HF
(C)
UGT2B1
Fold induction
2
1.5
1
0.5
0
***
##
C F
SD
C F
HF
(D)
UGT activity
Fluvastatin glucuronidation (%)
150
100
50
0
C F
SD
C F
HF

Fig. 6

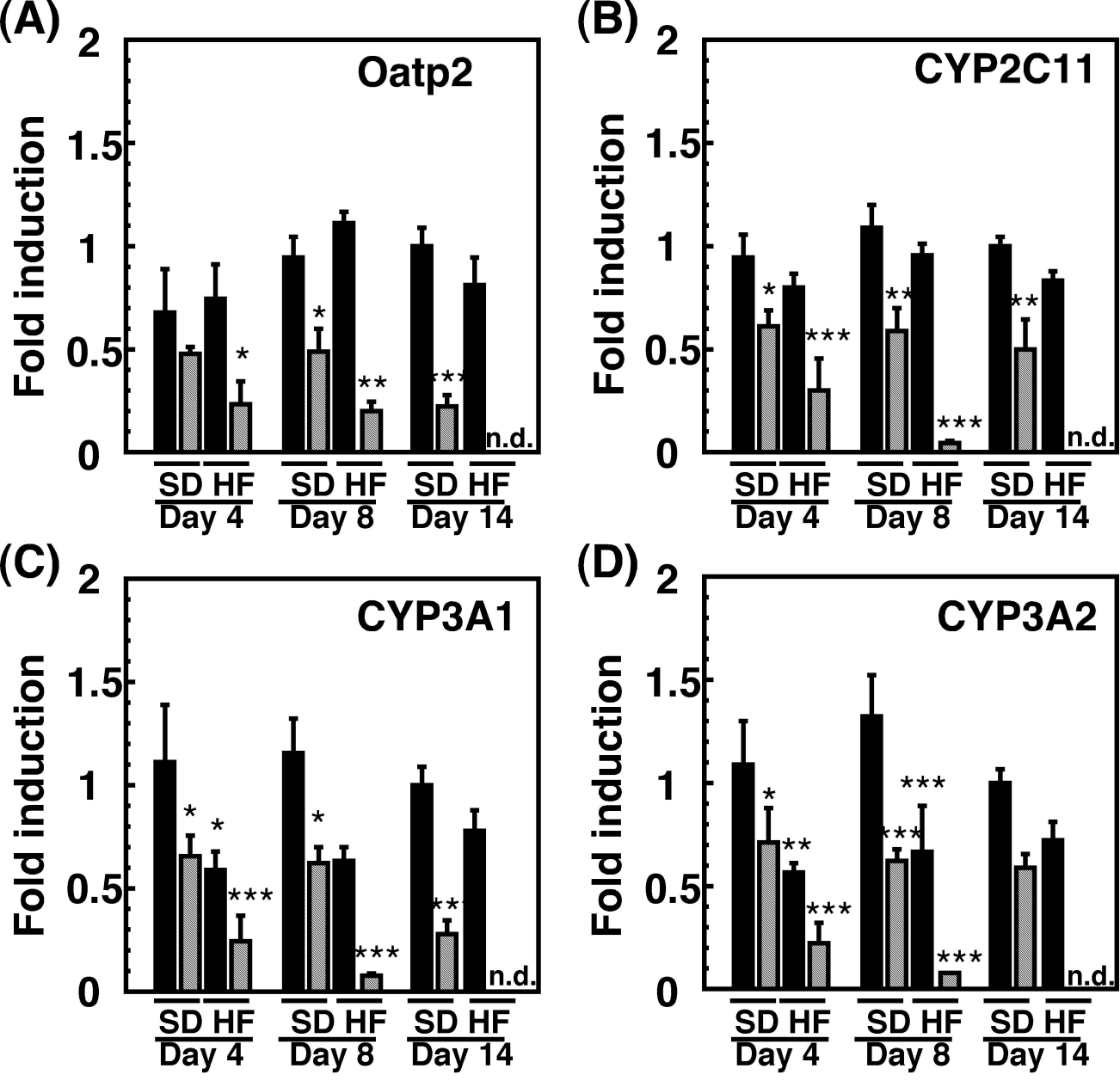
(A)
Oatp2
Fold induction
2
1.5
1
0.5
0
n.d.
SD HF
Day 4
Day 8
Day 14
(B)
CYP2C11
(C)
CYP3A1
(D)
CYP3A2

Fig. 7

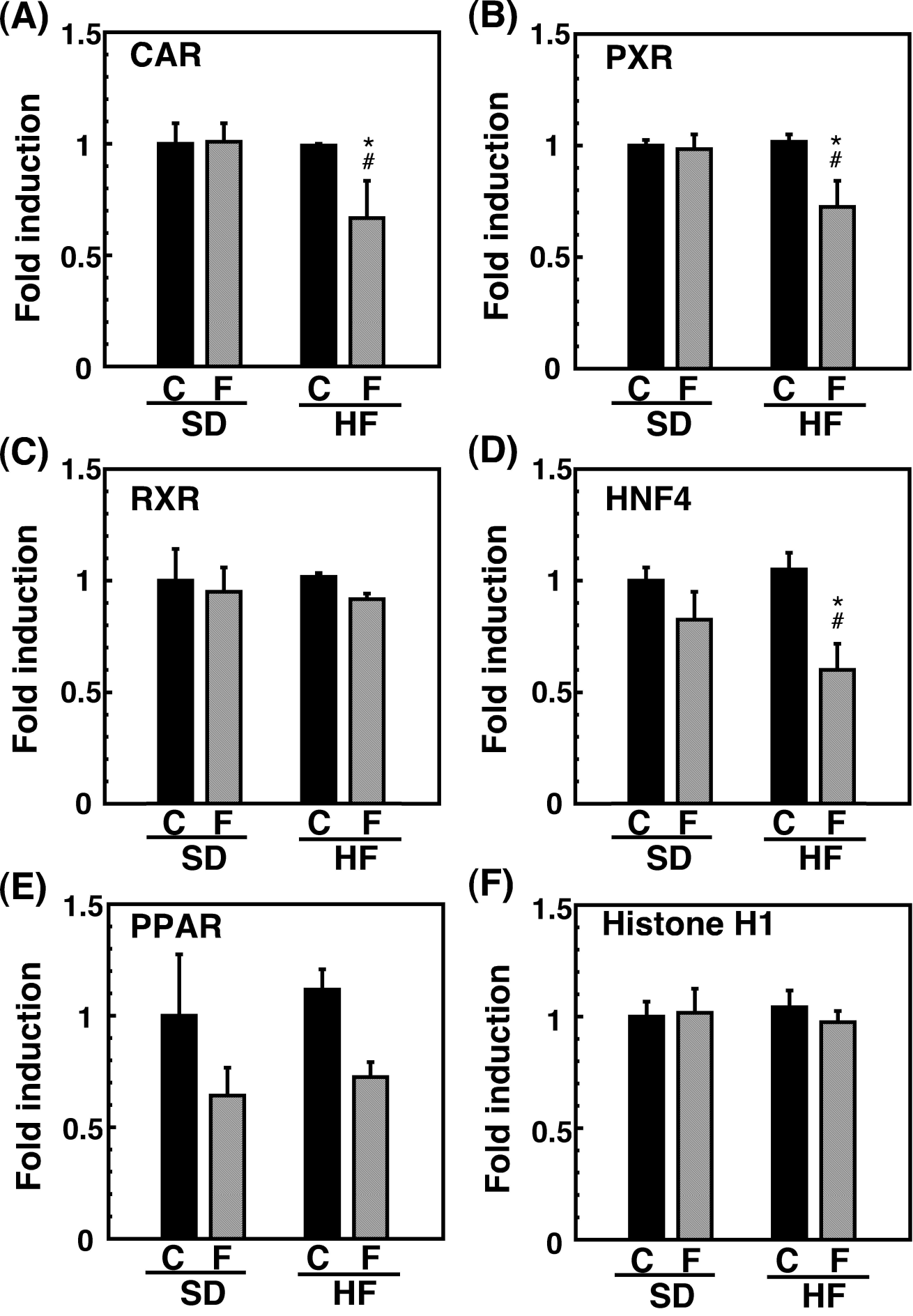
(A)
CAR
Fold induction
1.5
1
0.5
0
C F C F
SD HF
*
#
(B)
PXR
Fold induction
1.5
1
0.5
0
C F C F
SD HF
*
#
(C)
RXR
Fold induction
1.5
1
0.5
0
C F C F
SD HF
(D)
HNF4
Fold induction
1.5
1
0.5
0
C F C F
SD HF
*
#
(E)
PPAR
Fold induction
1.5
1
0.5
0
C F C F
SD HF
(F)
Histone H1
Fold induction
1.5
1
0.5
0
C F C F
SD HF

Fig. 8

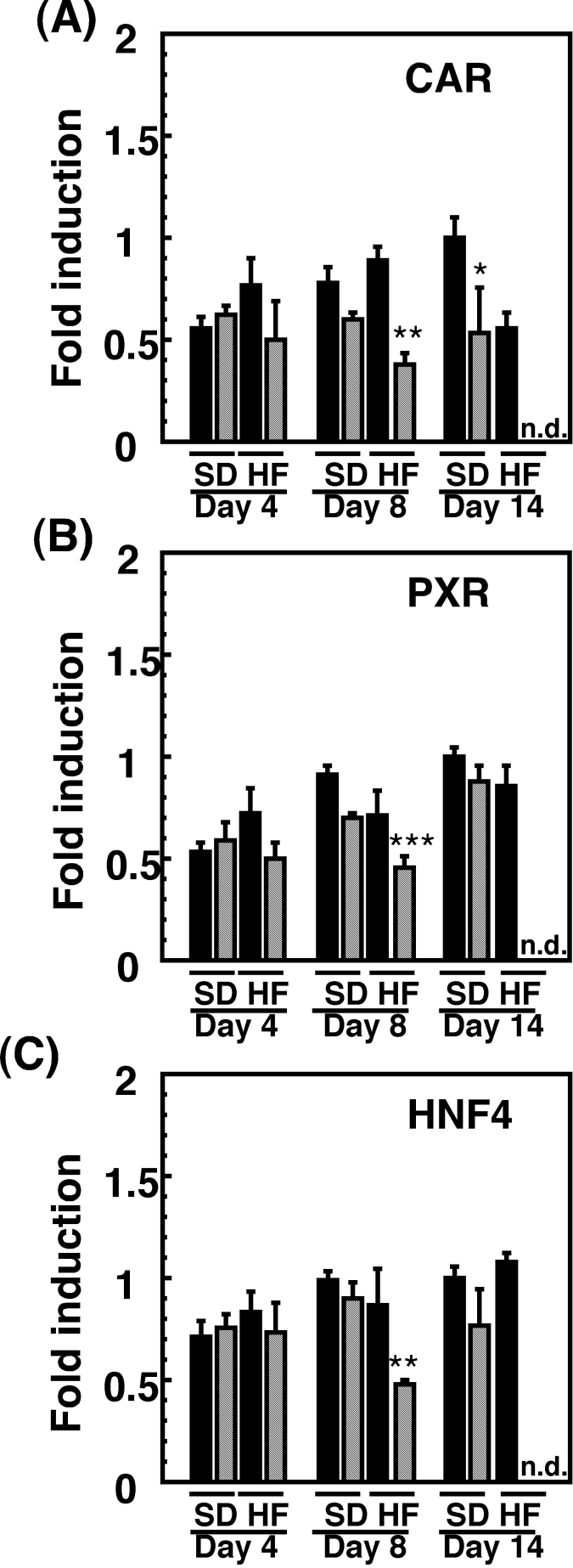
(A)
CAR
Fold induction
2
1.5
1
0.5
0
**
*
n.d.
SD HF
Day 4
SD HF
Day 8
SD HF
Day 14
(B)
PXR
Fold induction
2
1.5
1
0.5
0
***
n.d.
SD HF
Day 4
SD HF
Day 8
SD HF
Day 14
(C)
HNF4
Fold induction
2
1.5
1
0.5
0
**
n.d.
SD HF
Day 4
SD HF
Day 8
SD HF
Day 14

Fig. 9

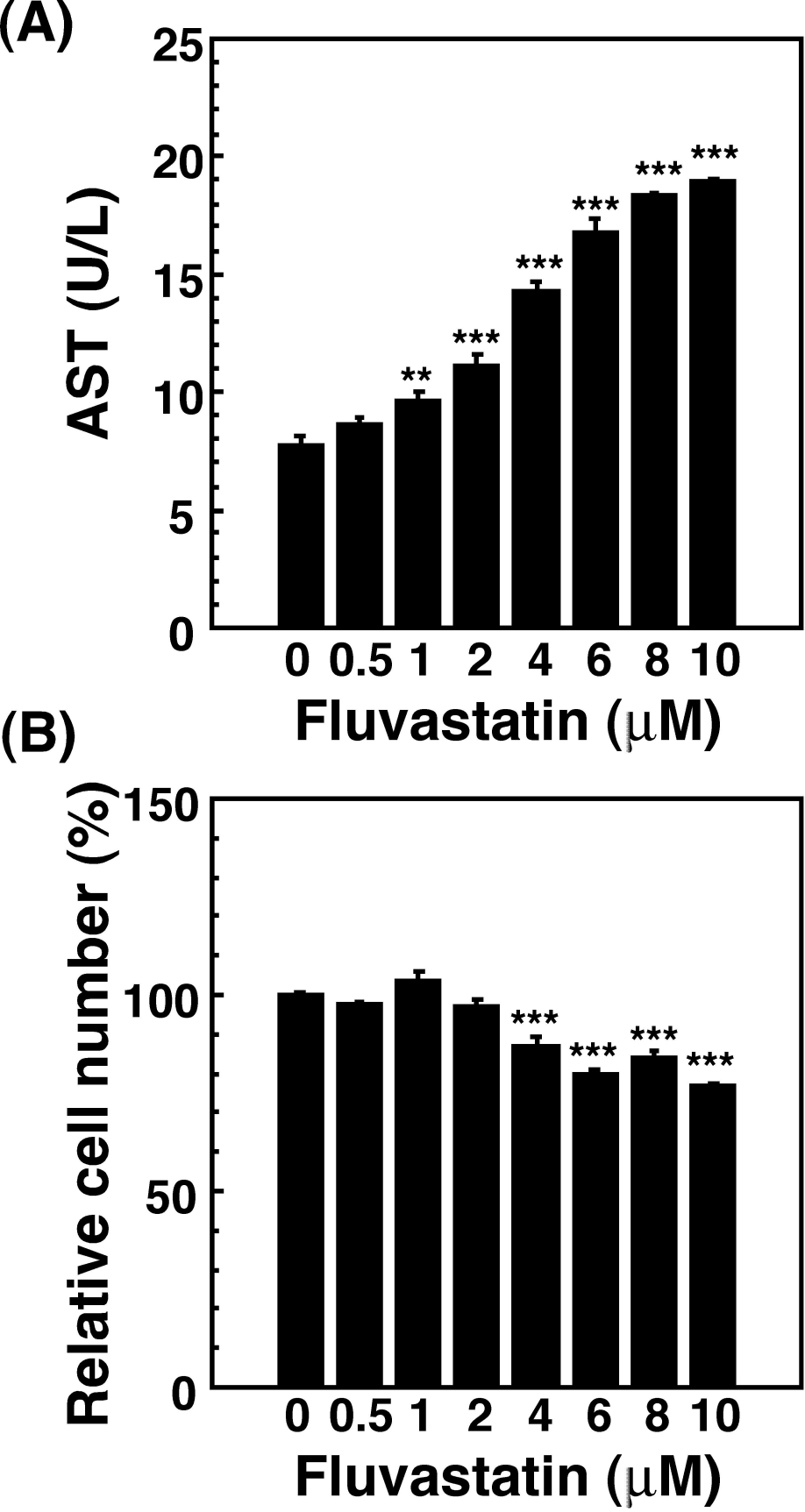
(A)
AST (U/L)
25
20
15
10
5
0
**
***
***
***
***
***
0 0.5 1 2 4 6 8 10
Fluvastatin (μM)
(B)
Relative cell number (%)
150
100
50
0
***
***
***
***
0 0.5 1 2 4 6 8 10
Fluvastatin (μM)

Fig. 10