Reduction in Secretion of Very Low Density Lipoprotein–Triacylglycerol by a Matrix Metalloproteinase Inhibitor in a Rat Model of Diet-Induced Hypertriglyceridemia

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ABSTRACT

Matrix metalloproteinase inhibitors (MMPIs) reduced serum triacylglycerol (TAG) levels in streptozotocin-induced diabetic rats and Zucker fa/fa rats in our previous study. However, the mechanisms underlying TAG reduction by MMPIs remain unclear. The present study aimed to elucidate the mechanism by which F81-1144b, an MMPI, lowers serum TAG levels in an animal model of high-sucrose diet (HSD)-induced hypertriglyceridemia. F81-1144b was repeatedly administered to rats fed HSD, and its effects were evaluated on TAG levels in serum and the liver, very low density lipoprotein (VLDL) secretion, de novo fatty acid (FA) synthesis in the liver, and the expression of genes regulating the metabolism of FA, TAG, and VLDL in the liver and serum. F81-1144b lowered serum TAG levels in serum and the liver, VLDL-TAG secretion, de novo FA synthesis in the liver, and serum levels of insulin and glucose. F81-1144b suppressed the expression of genes related to the de novo synthesis of FA and TAG, key proteins (lipin 1 and apolipoprotein CIII) responsible for VLDL metabolism, and sterol regulatory element-binding protein-1c and carbohydrate response element-binding protein. F81-1144b little affected the expression of genes related directly to the degradation of TAG or FA, but it upregulated that of gene for uncoupling protein 2 in the liver. These results suggest that MMPIs are a novel type of therapeutic agent for the treatment of hypertriglyceridemia, because the metabolic effects of F81-1144b expected from changes in the expression of genes regulating lipid metabolism would alter metabolism differently from those induced by fibrates, niacin, or n-3 FAs.

Introduction

Low density lipoprotein (LDL) has been firmly established as the primary atherogenic lipoprotein. However, even at low plasma-LDL levels among patients treated with statins, the high residual risk of cardiovascular disease (CVD) is not abrogated (Fruchart et al., 2008; Kearney et al., 2008). The independent relationship between plasma triacylglycerol (TAG) and the risk of future CVD events has remained controversial; however, recent studies demonstrated that elevated plasma TAG levels are strongly and independently associated with the risk of CVD (Harchaoui et al., 2009; Neil et al., 2010; Sarwar et al., 2010; Langsted et al., 2011; Nordestgaard and Varbo, 2014; Nordestgaard, 2016). A number of metabolic conditions are frequently associated with hypertriglyceridemia. The factors associated with increases in plasma TAG levels are a genetic predisposition (such as familial hypertriglyceridemia and familial combined hyperlipidemia), diseases (such as insulin resistance, metabolic syndrome, type 2 diabetes, type 1 diabetes, and renal diseases), lifestyle factors (such as obesity, alcohol consumption, and a diet enriched with simple carbohydrates), and medications (such as estrogens) (Boullart et al., 2012). Very low density lipoprotein (VLDL) overproduction appears to be central to the pathophysiology of hypertriglyceridemia in an insulin-resistant state (Taskinen, 2003; Adiels et al., 2008). Hypertriglyceridemic subjects with more visceral fat storage have been reported to have larger TAG-enriched VLDL particles (Wang et al., 2011). In patients with hepatic steatosis, VLDL overproduction results from compensatory mechanisms for hepatic lipid overload, which ultimately increases the risk of developing hypertriglyceridemia and atherosclerosis (Chatrath et al., 2012). The aberrant metabolism of TAG-rich lipoproteins (TRL) (VLDL and chylomicrons) results in increased plasma TRL remnants and others...
elevated small dense LDL, lipoprotein particles that are strongly associated with an increased risk of CVD (Miller et al., 2011). Under conditions of TRL overproduction and/or delayed clearance, TRL and/or TRL remnants formed during lipolysis are more likely to enter artery walls, causing foam cell formation and activating vascular cell inflammation (Nordestgaard et al., 1995; Schwartz and Reaven, 2012). Moreover, VLDL particles are larger and LDL particles are smaller in insulin-resistant states; LDL size is inversely associated with plasma TAG levels (Berneis and Krauss, 2002). Small dense LDL particles are retained for longer period of time in the circulation, are glycated and oxidized, and are prone to cross the endothelial surface, contributing to plaque formation. Thus, increased TRL remnants play a pivotal role in the pathophysiology of atherosclerosis, and thereby contribute to CVD (Chapman et al., 2011; Nordestgaard, 2016).

In this context, the prevalence of elevated TRL is a major concern (Miller et al., 2011). Fibrates, niacin, and n-3 fatty acids (FAs) are currently the first choice treatments for hypertriglyceridemia (Ito, 2015). The mechanistic actions of these agents markedly differ from each other. Considering the diversity in the pathogenesis of hypertriglyceridemia, the wide variety of agents available for selection to reduce plasma TAG is a therapeutic benefit. We previously demonstrated that among various metabolic conditions that are known to be associated with increases in serum TAG, the administration of matrix metalloproteinase inhibitors (MMPIs), the structures of which are similar to that of F81-1144b, reduced the serum levels of TAG, VLDL-TAG, and LDL-cholesterol, but not those of high density lipoprotein-cholesterol in streptozotocin-induced diabetic rats (a model of type 1 diabetes) and genetically obese Zucker fa/fa rats (a model of genetic obesity owing to a dysfunction in the action of leptin) (Morikawa et al., 2007). In comparison with a population with hypertriglyceridemia attributed to these two metabolic disorders, however, in a population with hypertriglyceridemia that results from changes in dietary habits, a marked increase in dietary intake of fructose from sucrose and fructose-enriched sweeteners is considered to be much greater. Epidemiologic studies have shown that the consumption of sucrose or a mixture of glucose and fructose is associated with the occurrence of metabolic disorders including hypertriglyceridemia (Ouyang et al., 2008; Tappy and Lê, 2010). Nevertheless, no information is currently available on whether MMPIs reduce serum TAG levels in animal models with diet-induced hypertriglyceridemia, but not diabetes or obesity. Moreover, the mechanisms underlying MMPI-induced reductions in serum TAG remain unclear. In this context, the present study aimed 1) to investigate whether MMPIs suppress serum TAG levels in animals with high-sucrose diet (HSD)-induced hypertriglyceridemia and, if this is the case, 2) to elucidate the metabolic mechanism by which MMPIs reduce serum TAG levels.

**Materials and Methods**

**Chemicals.** The following materials were obtained from the indicated commercial sources: [1-14C]acetic acid (2.035 TBq/mol), [U-14C]-glycerol-3-phosphate (5.55 TBq/mol), and [1,3-3H]glycerol (1480 TBq/mol) (American Radiolabeled Chemicals, Inc., St. Louis, MO); acetyl-CoA, palmityl-CoA, glycerol-3-phosphate, Triton WR-1339, and bovine serum albumin (BSA) (MilliporeSigma, St. Louis, MO). F81-1144b, the structure of which is shown in Fig. 1, was kindly provided by Daiichi Fine Chemical Co., Ltd. (Takaoka, Japan); F81-1144b is a subcutaneously active hydroxyamic acid MMP inhibitor that exhibits potent activity against a number of MMPs, including MMP-1 (IC50 = 5 nM) and MMP-3 (IC50 = 29 nM) (Pujisawa et al., 2001).

**Animals.** All animal procedures were approved by the Institutional Animal Care Committee of Josai University in accordance with the Guidelines for the Proper Conduct of Animal Experiments (Science Council of Japan). Six-week-old male Wistar rats were purchased from SLC Inc. (Hamamatsu, Japan). Rats were acclimated for 1 week on a standard rodent chow (CE-2; Clea Japan Inc., Tokyo, Japan).

**Study Design.** After acclimatization for 1 week, 7-week-old rats were divided into four groups. Group 1 was fed the standard diet for an additional 21 days [normal diet (ND)-fed rats]. Group 2 was fed HSD for 21 days. HSD (catalog No 960403; ICN Pharmaceuticals, Costa Mesa, CA) was purchased from Oriental Yeast Co. (Tokyo, Japan) and contained (by weight): 68% sucrose, 8% cottonseed oil, 18% milk casein, 2% beer yeast, and the required vitamins and minerals. Group 3 was maintained on HSD for 21 days and subcutaneously received F81-1144b at a dose of 50 mg/kg once a day for 7 days before being sacrificed. Group 4 was fed HSD for 21 days and subcutaneously received F81-1144b at a dose of 100 mg/kg once a day for 7 days before being sacrificed. F81-1144b was suspended in saline at a concentration of 50 mg/ml. At the age of 10 weeks, rats were sacrificed in the fed state. Rats were anesthetized with diethyl ether, and blood was withdrawn from the inferior vena cava. The liver was rapidly removed, washed with ice-cold saline, and weighed. Two portions of the liver were frozen in liquid nitrogen and stored at −80°C until the analysis of mRNA and acetyl-CoA. The remainder of the liver was used for a lipid analysis and the preparation of homogenates and microsomes. The parts of the liver that were used to prepare homogenates and microsomes were perfused with ice-cold saline.

**Serum Clinical Chemistry.** Serum was obtained from blood by centrifugation at 1200g for 15 minutes. Serum TAG, total cholesterol, non-esterified fatty acids (NEFA), and glucose were measured using colorimetric enzymatic assay kits from Wako Pure Chemicals (Osaka, Japan). Serum insulin was measured using a rat RIA kit from MilliporeSigma (Billerica, MA).

**Hepatic Lipid Analysis.** After the addition of a known amount of triheptadecanoin as an internal standard, total lipids were extracted from liver homogenates by the reported method (Bligh and Dyer, 1959). Cholesterol ester, TAG, diacylglycerol, free FA, and phospholipid were separated by thin-layer chromatography on silica gel G plates, which were developed with n-hexane/diethyl ether/acetic acid (80:30:1, v/v/v). After visualization by spraying 0.001% (w/v) primuline in 80% acetone, each region that corresponded to TAG and phospholipids on the plates was scraped off and transferred to tubes. A known amount of methyl heptadecanoate was added to the tubes containing phospholipids as an internal standard. TAG and phospholipids were extracted from silica gel as described previously (Imai
were prepared by centrifugation at 3000 and 120 minutes after the Triton WR1339 injection. Plasma samples lipase (LPL) (Millar et al., 2005). Blood samples were collected into (600 mg/3 ml saline per kilogram) was injected via the tail vein per kilogram) via the portal vein, and Triton WR1339 solution of 50 or 100 mg/kg for 7 days were intraperitoneally injected with [14C]acetic acid was dissolved in saline (370 kBq/ml). In brief, [14C]acetic acid was dissolved in saline (370 kBq/ml). FA methyl esters were prepared using sodium methoxide/methanol. FA methyl esters were measured by gas-liquid chromatography (Shimadzu GC-2014; Shimadzu, Kyoto, Japan) as described (Kawahashi et al., 1999). FA methyl esters were measured using a liquid scintillation counter (Aloka LSC6100; Hitachi and then extracted from silica as described above. Radioactivity was measured using a liquid scintillation counter. 

**Measurement of In Vivo Synthesis of FA in the Liver.** Hepatic FA synthesis was estimated by measuring the in vivo incorporation of [1-14C]acetate into FA in the liver according to the previously reported method (Sakurai et al., 1978) with some modifications. In brief, [14C]acetate was dissolved in saline (370 kBq/ml). Under anesthesia with diethyl ether, HSD-fed rats were treated daily with F81-1144b at a dose of 1.48 MBq/10 μmol/4 ml saline per kilogram body weight. Ten minutes after the injection, livers were immediately isolated and snap-frozen in liquid nitrogen. Lipids were extracted from the liver (Bligh and Dyer, 1959). Extracted lipids were saponified with 10% methanolic KOH at 80°C for 60 minutes under a nitrogen atmosphere. After being diluted with water, unsaponifiable matter was extracted with n-hexane three times. The aqueous phase was acidified with 6 M HCl, and FAs were extracted with n-hexane three times. FAs were mixed with scintillation fluid after drying down, and radioactivity was measured using a liquid scintillation counter.

**Real-Time Quantitative PCR.** Total RNA was isolated from liver tissues using the QIAzol reagent and RNeasy kit (QIAGEN, Hilden, Germany). cDNA was synthesized from 500 ng of total RNA with avian myeloblastosis virus reverse transcriptase (Takara Bio Inc., Otsu, Japan). PCR amplification was conducted using SYBR Premix EX Taq (Takara). Amplification and detection were performed with the StepOneplus Real-Time PCR System (Thermo Fisher Scientific, Carlsbad, CA). The thermal cycling program was as follows: 10-second denaturation steps at 95°C, followed by 50 cycles of 5-second denaturation steps at 95°C, and 34-second annealing steps at 60°C. After the reaction, dissociation curve analyses were performed to confirm the amplification of a single PCR product. Changes in gene expression were calculated using the comparative threshold cycle (Ct) method. Ct values were first normalized by subtracting the Ct value obtained from β-actin (control). The sequences of primers used in the present study are listed in Table 1.

**Assay for Glycerol-3-Phosphate Acyltransferase.** One portion of the perfused liver was homogenized in four volumes of 0.25 M sucrose/1 mM EDTA per 10 mM Tris–HCl buffer (pH 7.4). Hepatic microsomes were prepared by centrifugation at 30000 g for 20 minutes. Microsomes were extracted from plasma by the method of Bligh and Dyer (1959). Radioactivity was measured using a liquid scintillation counter.

**Measurement of VLDL-TAG Secretion in HSD-Fed Rats in the Fed State.**

**Acetyl-CoA Measurement in the Liver.** Acetyl-CoA was extracted from one portion of the liver and measured enzymatically using a reported method (Decker, 1985).

**Measurement of In Vivo Synthesis of FA in the Liver.** Hepatic FA synthesis was estimated by measuring the in vivo incorporation of [1-14C]acetate into FA in the liver according to the previously reported method (Sakurai et al., 1978) with some modifications. In brief, [14C]acetate was dissolved in saline (370 kBq/ml). Under anesthesia with diethyl ether, HSD-fed control rats and HSD-fed rats that had been treated daily with F81-1144b at a dose of 50 or 100 mg/kg for 7 days were intraperitoneally injected with [14C]acetate at a dose of 1.48 MBq/10 μmol/4 ml saline per kilogram body weight. Ten minutes after the injection, livers were immediately isolated and snap-frozen in liquid nitrogen. Lipids were extracted from the liver (Bligh and Dyer, 1959). Extracted lipids were saponified with 10% methanolic KOH at 80°C for 60 minutes under a nitrogen atmosphere. After being diluted with water, unsaponifiable matter was extracted with n-hexane three times. The aqueous phase was acidified with 6 M HCl, and FAs were extracted with n-hexane three times. FAs were mixed with scintillation fluid after drying down, and radioactivity was measured using a liquid scintillation counter.

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were set at multiple range test was used as a post-hoc test. Levels of significance of variance. When a difference was significant (homogeneity of variance was established using a one-way analysis after acidification of the incubation mixture with 0.1 M HCl. Extracted CoA, 0.5 mg/ml of BSA (essentially FA free), 0.1 M Tris-HCl (pH 7.5), 2 mM (9.26 kBq/ml) [U-14C]glycerol-3-phosphate, 40 (Yamada and Okuyama, 1978). In brief, the assay mixture consisted of 2 mM (9.26 kBq/ml) [U-14C]glycerol-3-phosphate, 40 μM palmitoyl-CoA, 0.5 mg/ml of BSA (essentially FA free), 0.1 M Tris-HCl (pH 7.5), and 200 μg/ml of microsomal proteins. Incubations were performed at 25°C for 2 minutes. Lipids were extracted with chloroform/methanol after acidification of the incubation mixture with 0.1 M HCl. Extracted lipids were mixed with scintillation fluid after drying down, and radioactivity was measured.

**Statistical Analysis.** Data are presented as the mean ± S.D. The homogeneity of variance was established using a one-way analysis of variance. When a difference was significant (P < 0.05), Scheffé multiple range test was used as a post-hoc test. Levels of significance were set at P < 0.05. The significance of differences between two groups was analyzed using the Student t test.

**Results**

**Effects on Serum TAG Levels in HSD-Fed Rats.** It was initially confirmed that feeding HSD causes hyperglyceridemia. When rats were fed HSD, serum TAG levels gradually increased by as much as 4-fold on day 21 (Fig. 2A). To estimate the effects of F81-1144b on serum TAG levels, rats maintained on HSD for 21 days were subcutaneously administered F81-1144b once a day for 7 days at doses of 50 or 100 mg/kg before being killed, and biochemical and physiologic parameters were measured. Elevations in serum TAG levels by the feeding of HSD significantly decreased in a dose-dependent manner following the treatment of rats with F81-1144b, and TAG levels in the F81-1144b dosing group at a dose of 100 mg/kg were similar to those in ND-fed control rats (Fig. 2B). The feeding of HSD did not affect body weight, and the treatment with F81-1144b at a dose of 100 mg/kg insignificantly reduced body weight (Table 2). No significant changes were noted in the overall health of rats following the administration of F81-1144b (100 mg/kg). The feeding of HSD slightly increased liver weights and relative liver weights. Serum levels of cholesterol were markedly elevated by the feeding of HSD, and these increases were reduced by F81-1144b in a dose-dependent manner. Serum levels of glucose slightly increased upon the feeding of HSD; these levels returned to normal values following the treatment of rats with F81-1144b. The treatment with F81-1144b at a dose of 50 or 100 mg/kg significantly suppressed the serum levels of TAG, but not glucose, in rats that were maintained on the normal diet (Supplemental Fig. 1, A and B).

**Effects on the Expression of Genes Related to VLDL Formation and Degradation.** Regarding VLDL degradation, HSD feeding tended to downregulate the expression of genes for LPL (Student t test, P = 0.0075, ND vs. HSD-fed control), and significantly upregulated that for apolipoprotein (apo) CIII (Fig. 3, A and B). The administration of F81-1144b to rats maintained on HSD significantly increased the expression of Lpl in a dose-dependent manner, and markedly reduced the elevated levels of mRNA for Apoc3 in a dose-dependent manner to the levels of ND-fed control rats (Fig. 3, A and B). When ND-fed rats were treated with F81-1144b, the expression of Lpl was upregulated and that of Apoc3 was downregulated (Supplemental Fig. 1, C and D). Regarding the formation of VLDL, HSD feeding markedly upregulated the expression of gene encoding lipin 1, and significantly elevated the levels of mRNAs for microsomal triglyceride transfer protein (MTP) and Apoc-CIII (Fig. 3, B–D). F81-1144b dosing little affected the expression of Mtp, but markedly lowered that of Apoc3 and Lipin1 in a dose-dependent manner to the levels of ND-fed control rats (Fig. 3, B–D). The expression of gene for Apo-AI, the main component of high density lipoprotein, was little affected by HSD feeding, and markedly elevated by F81-1144b dosing (Fig. 3E).

**Effects on VLDL-TAG Secretion in HSD-Fed Rats in the Fed State.** To estimate the effects of F81-1144b on the secretion of VLDL-TAG that formed in the liver, [3H]glycerol was injected into the portal vein and Triton WR-1339 was injected into the tail vein of HSD-fed control rats and in HSD-fed rats treated with F81-1144b. [3H]TAG that appeared in serum was then measured (Fig. 4). It is important to note that this experiment was performed in the fed state to avoid the influence of fasting. No marked differences were observed in diet intake between control and F81-1144b-treated rats (Supplemental Fig. 2A). [3H]TAG that appeared in the circulation was markedly less in F81-1144b-treated rats than in HSD-fed control rats (Fig. 4A). The [3H]TAG secretion rate, which was calculated between 0 and 60 minutes, was reduced by 45% with F81-1144b dosing (Fig. 4B).

**Effects on TAG Metabolism in the Liver of HSD-Fed Rats.** The administration of F81-1144b to HSD-fed rats significantly reduced hepatic TAG levels without affecting hepatic phospholipid contents (Fig. 5, A and B). F81-1144b dosing markedly changed the FA profile (mol %) of TAG and phospholipids in the liver (Supplemental Table 1). F81-1144b at a dose of 100 mg/kg reduced the proportions of palmitic, palmitoleic, stearic, and octadecenoic acids, but increased those of linoleic, 8, 11, 14-eicosatrienoic, and arachidonic acids in TAG. However, the treatment with F81-1144b reduced the proportions of octadecenoic, α-linolenic, and 5, 8, 11-eicosatrienoic acids in phospholipids. When these results were compared by the differences in the content (micromoles per gram liver) of a particular FA between HSD-fed control...
rats and HSD-fed rats receiving 100 mg/kg of F81-1144b (Fig. 5, C and D), the differences in the masses of de novo synthesized FAs (palmitic, octadecenoic, palmitoleic, and stearic acids) in TAG were significantly decreased (Fig. 5C); changes in the differences in the masses of these FAs in phospholipids were less prominent than those in TAG (Fig. 5D). The changes caused by 50 mg/kg of F81-1144b in profiles (mol %) and differences in the masses of de novo synthesized FAs were less than those induced by 100 mg/kg of F81-1144b.

To gain insights into the molecular basis for understanding the reduction in hepatic TAG by F81-1144b, the mRNA levels of key enzymes and proteins involved in the synthesis of FA and TAG were measured (Fig. 6; Supplemental Table 2). The expression of genes for fatty acid synthase (FAS) was markedly upregulated by the feeding of HSD, and the elevated expression was suppressed by F81-1144b dosing in a dose-dependent manner (Fig. 6A). The treatment of ND-fed rats with F81-1144b tended to suppress hepatic expression of \( \text{Fas} \) (Scheffé multiple range test, \( P < 0.05 \), control vs. group receiving 100 mg/kg) (Supplemental Fig. 1E). The levels of mRNA of \( \text{Fas} \) in HSD-fed rats receiving 100 mg/kg of F81-1144b were similar to those in ND-fed control rats receiving F81-1144b at a dose of 100 mg/kg.

### TABLE 2

<table>
<thead>
<tr>
<th>ND</th>
<th>HSD</th>
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<tbody>
<tr>
<td><strong>Dosage of F81-1144b (mg/kg)</strong></td>
<td>0</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>266.2 ± 8.8 (^a)</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>10.79 ± 0.48 (^a)</td>
</tr>
<tr>
<td>Relative liver weight (% body weight)</td>
<td>4.05 ± 0.11 (^a)</td>
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<tr>
<td>Serum cholesterol (mg/dl)</td>
<td>47.39 ± 2.66 (^a)</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>143.6 ± 8.2 (^a)</td>
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ND, high sucrose diet; ND, normal diet. \(^a\), \(^b\), \(^c\) Differences in the mean without a common superscript are significant (\( P < 0.05 \)). In the absence of a superscript, the difference in the means is not significant (\( P > 0.05 \)).

Fig. 3. Effects of F81-1144b on the expression of genes related to VLDL formation and degradation. Rats were maintained on ND or HSD for 21 days. HSD-fed rats subcutaneously received F81-1144b at a dose of 0, 50, or 100 mg/kg once a day for 7 days before being killed. (A) \( \text{Lpl} \) (lipoprotein lipase), (B) \( \text{Apoc3} \) (apolipoprotein CIII), (C) \( \text{Lipin1} \) (lipin 1), (D) \( \text{Mtp} \) (microsomal triglyceride transfer protein), (E) \( \text{Apoa1} \) (apolipoprotein AI). The levels of mRNA in liver of HSD-fed rats relative to those in liver of ND-fed control rats are presented as the mean ± S.D. (\( n = 8 \)/group). \(^a\), \(^b\), \(^c\) Differences in the mean without a common superscript are significant (\( P < 0.05 \)).
Regarding the other enzymes related to de novo FA synthesis, the expression of genes encoding acetyl-CoA carboxylase 1 (ACC1), glucose-6-phosphate dehydrogenase (G6PD), ATP-citrate lyase (ACLY), malic enzyme 1 (ME1), and stearoyl-CoA desaturase 1 (SCD1) were all significantly downregulated by the treatment of HSD-fed rats with F81-1144b (Supplemental Table 2). The effects of F81-1144b on de novo FA synthesis in the liver were estimated in vivo by...

Fig. 4. Effects of F81-1144b on VLDL-TAG secretion in HSD-fed rats in the fed state. Rats that were maintained on HSD for 21 days were subcutaneously administered F81-1144b at a dose of 0 or 100 mg/kg once a day for 7 days before measuring VLDL-TAG secretion. Rats were administered $[^{3}H]$glycerol via the portal vein, and Triton WR1339 was injected via the tail vein 15 minutes later. A blood sample was collected 1 minute before and 30, 60, and 120 minutes after the Triton WR1339 injection. The incorporation of radioactivity into plasma TAG was measured. (A) Time-course of the appearance of $[^{3}H]$TAG in plasma. (B) The rate of $[^{3}H]$TAG secretion between 0 and 60 minutes. Values are presented as the mean ± S.D. ($n = 4$ or 5/group). *Significantly different from HSD-fed control ($^*P < 0.05$).

Fig. 5. Effects of F81-1144b on contents and fatty acid composition of TAG and phospholipid in liver of HSD-fed rats. Rats were maintained on ND or HSD for 21 days. HSD-fed rats subcutaneously received F81-1144b at a dose of 0, 50, or 100 mg/kg once a day for 7 days before being killed. (A) Effects of F81-1144b on TAG contents in liver of HSD-fed rats ($n = 8$/group). (B) Effects of F81-1144b on phospholipid contents in liver of HSD-fed rats ($n = 8$/group). (C) Differences in contents (micromoles per gram liver) of fatty acids in hepatic TAG between HSD-fed rats receiving F81-1144b at a dose of 50 mg/kg and HSD-fed control rats. Regarding each fatty acid, differences in contents between the two groups were calculated from the data in Supplemental Table 1 ($n = 8$/group). (D) Differences in contents (micromoles per gram liver) of fatty acids in hepatic phospholipids between HSD-fed rats receiving F81-1144b at a dose of 50 mg/kg and HSD-fed control rats and between HSD-fed rats receiving F81-1144b at a dose of 100 mg/kg and HSD-fed control rats ($n = 8$/group). Regarding each fatty acid, differences in contents between the two groups were calculated from the data in Supplemental Table 1. Values are presented as the mean ± S.D. a, b, cDifferences in the mean without a common superscript are significant ($P < 0.05$). In the absence of a superscript, the difference in the means is not significant ($P > 0.05$). Fatty acids are designated by the numbers of carbon atoms and double bonds: palmitic acid, 16:0; palmitoleic acid, 16:1n-7; stearic acid, 18:0; octadecenoic acid, 18:1; linoleic acid, 18:2n-6; α-linolenic acid, 18:3n-3; 5,8,11-eicosatrienoic acid, 20:3n-9; 8,11,14-eicosatrienoic acid, 20:3n-6; arachidonic acid, 20:4n-6; 5,8,11,14,17-eicosapentaenoic acid, 20:5n-3; 7,10,13,16,19-docosapentaenoic acid, 22:5n-3; 4,7,10,13,16,19-docosahexaenoic acid, 22:6n-3.
measuring the incorporation of $[^{14}C]$acetate, which was intraperitoneally injected, into FAs; the incorporation of $[^{14}C]$acetate into hepatic FA was significantly suppressed by the treatment with F81-1144b at a dose of 100 mg/kg (Fig. 7A). Hepatic concentrations of acetyl-CoA in rats treated with F81-1144b were slightly greater than those in HSD-fed control rats; however, the extent of the increase was only 1.27-fold (Fig. 7B). Therefore, the decrease observed in the formation of $[^{14}C]$-labeled FAs by F81-1144b did not appear to be the result of isotopic dilution of acetyl-CoA in the liver. F81-1144b dosing markedly affected the expression of genes encoding enzymes related to glycerolipid synthesis (Fig. 6, B and C; Supplemental Table 2). The expression of gene for Gpat4 was significantly upregulated by HSD feeding; the elevated levels were suppressed by F81-1144b dosing (Fig. 6C). The activity of microsomal GPAT was increased by the feeding of HSD, and the elevated activity was significantly decreased by the treatment of rats with F81-1144b (Fig. 7C). The expression of Gpat1 was little affected by HSD feeding, whereas that in HSD-fed rats was significantly downregulated by F81-1144b dosing (Fig. 6B). The expression of genes encoding diacylglycerol acyltransferase (DGAT) 2 was suppressed by F81-1144b dosing, whereas the levels of mRNA for DGAT1 were unchanged (Supplemental Table 2). Among FA
transporters in the liver, the expression of gene encoding fatty acid translocase (CD36) tended to be downregulated by the feeding of HSD (Student t test, \( P = 0.0234, \text{ND vs. HSD-fed control} \)), and that was markedly upregulated by the treatment of HSD-fed rats with F81-1144b in a dose-dependent manner (Fig. 6D). Fatty acid transport protein 2 was also significantly increased by the administration of F81-1144b (Supplemental Table 2). F81-1144b dosing did not change the serum levels of NEFA (Fig. 7D), and lowered the relative weight of epididymal fat (Supplemental Fig. 2B). The treatment of rats with F81-1144b significantly decreased the levels of mRNAs for long-chain acyl-CoA synthetase (ACSL) 1 and ACSL5 (Supplemental Table 2).

Regarding the effects of F81-1144b dosing on TAG catabolism in the liver, HSD feeding did not affect the expression of genes for adipose triglyceride lipase (ATGL) or carnitine palmitoyltransferase 1a (CPT1a) (Fig. 6, E and F), and the administration of F81-1144b did not upregulate the expression of genes encoding the key enzymes and proteins related to either TAG degradation (ATGL and comparative gene identification-58) or FA catabolism (CPT1a, medium-chain acyl-CoA dehydrogenase, and long-chain acyl-CoA dehydrogenase) (Fig. 6, E and F; Supplemental Table 2). The levels of mRNA encoding uncoupling protein 2 (UCP2) in the liver were not affected by the feeding of HSD, and those were markedly augmented by the treatment of HSD-fed rats with F81-1144b (Fig. 6G).

The effects of F81-1144b on the expression of genes encoding transcription factors regulating the metabolism of FA and TAG in the liver of HSD-fed rats were estimated (Fig. 6, H and I; Supplemental Table 2). The expression of gene encoding sterol regulatory element-binding protein-1c (SREBP-1c) was significantly upregulated by the feeding of HSD, and F81-1144b dosing markedly downregulated it (Fig. 6H). Likewise, the expression of gene for carbohydrate response element-binding protein (ChREBP) was significantly upregulated by HSD feeding, and the elevated levels were markedly decreased by F81-1144b dosing (Fig. 6I); the mRNA levels for L-type pyruvate kinase, a typical ChREBP target gene (Thompson and Towle, 1991), were reduced by F81-1144b dosing (Supplemental Table 2). The administration of F81-1144b to ND-fed rats markedly downregulated the expression of genes for these two transcription factors (Supplemental Fig. 1, F and G). The expression of the gene for peroxisome proliferator-activated receptor α (PPARα) was suppressed by the treatment of HSD-fed rats with F81-1144b (Supplemental Table 2); nevertheless, the levels of mRNAs for acyl-CoA thioesterase 1, CPT1a, and ATGL, typical PPARα target genes (Rakhshandehroo et al., 2010; Karahashi et al., 2013a), were unchanged (Supplemental Table 2). The feeding of HSD significantly increased serum concentrations of insulin, and the elevated levels were markedly reduced by the treatment of HSD-fed rats with F81-1144b (Fig. 7E). However, the administration of F81-1144b to streptozotocin-induced diabetic rats significantly lowered the serum levels of TAG without changing glucose concentrations (Supplemental Fig. 3).

**Discussion**

Overfeeding of sucrose or fructose leads to several adverse metabolic effects, including hypertriglyceridemia, hepatic steatosis, and insulin resistance (Tappy and Lê, 2010). Fructose increases the expression of lipogenic enzymes by inducing SREBP-1c and activating ChREBP, and suppresses FA oxidation by reducing PPARα expression (Nagai et al., 2002; Koo et al., 2008; Softic et al., 2016), thereby favoring FA...
esterification and VLDL formation in the liver. Moreover, fructose deteriorates VLDL-TAG clearance (Hirano et al., 1989). Collectively, these metabolic changes cause hepatic steatosis and hypertriglyceridemia. The present study showed that F81-1144b reduced serum TAG levels in rats with HSD-induced hypertriglyceridemia. Two reasons have been proposed for this reduction. F81-1144b enhanced TAG degradation by LPL in plasma or suppressed VLDL-TAG secretion. Regarding VLDL-TAG metabolism in plasma, F81-1144b upregulated Lpl expression and downregulated Apoc3 expression in the liver. Since apo-CIII is attached to VLDL and delays VLDL breakdown by inhibiting LPL (Jong et al., 1999), the increase in Lpl expression and decrease in Apoc3 expression by F81-1144b appears to result in LPL activation, thereby reducing VLDL-TAG. Besides the potential effects of F81-1144b on VLDL-TAG degradation suggested by gene expression data, the present study showed that F81-1144b suppressed VLDL-TAG secretion from the liver to the circulation in rats in the HSD-fed state. The secretion rate is considered to depend not only on hepatic TAG availability, but also on the capacity for VLDL assembly. Regarding factors related to VLDL assembly, the present study showed that the expression of Lipin1 and Apoc3 in the liver was markedly downregulated by F81-1144b, but that of Mtp was not. Lipin1 positively affects the secretion pathway of TAG and apoB (Bou Khalil et al., 2010), and apo-CIII is involved in the TAG loading of VLDL (Qin et al., 2011). Moreover, the present study revealed that intrahepatic TAG was reduced by F81-1144b. Therefore, the decreased expression of Lipin1 and Apoc3 in concert with the reduced TAG supply within hepatocytes by F81-1144b may have reduced VLDL formation and secretion.

We then focused on elucidating the metabolic mechanisms underlying reductions in the hepatic TAG supply by F81-1144b. Changes in hepatic TAG concentrations occur as a result of an imbalance between lipid acquisition (de novo lipogenesis and the uptake of circulating FA) and disposal (TAG degradation and TAG export as a component of VLDL). Regarding its effects on lipogenesis, F81-1144b significantly downregulated the expression of genes encoding key enzymes (Pfas, Acc1, G6pd, Acly, and Me1) related to de novo FA synthesis, strongly suggesting the suppression of de novo FA synthesis by F81-1144b. As expected, the in vivo incorporation of [14C]acetate into hepatic FAs was decreased by F81-1144b. Moreover, F81-1144b reduced the expression of Gpat1, Gpat4, Dgat2, and Scd1, but not Dgat1, all of which are directly or indirectly involved in TAG synthesis. GPAT 1 and 4 are rate-limiting enzymes of TAG synthesis (Coleman and Mashek, 2011). The decrease observed in hepatic TAG levels by F81-1144b appears to be attributable to the suppressed expression of Gpat1 and/or Gpat4. GPAT1 and DGAT2 are responsible for incorporating de novo synthesized FAs into TAG (Qi et al., 2012; Wendel et al., 2013). DGAT2 appears to be linked with the esterification of monounsaturated FAs endogenously formed by SCD1 to produce TAG (Man et al., 2006). Therefore, the decreased supply of FAs formed de novo (particularly palmitic, octadecenoic, and palmitoleic acids) by fatty acid synthase and SCD1, in concert with the reduced expression of Gpat1 and Dgat2 by F81-1144b, appears to reduce TAG formation in the liver. Regarding FAs from the circulation, since F81-1144b markedly upregulated expression of Cd36, a transporter that mediates the uptake of circulating FAs (Glatz et al., 2010), and did not change serum NEFA levels, the supply of exogenous FAs potentially available for TAG synthesis and/or β-oxidation within hepatocytes may have been increased by F81-1144b. GPAT4 is prone to use exogenous FAs for TAG formation (Wendel et al., 2013), and resides on the endoplasmic reticulum (Beigneux et al., 2006). Consistent with reduced Gpat4 expression by F81-1144b, it significantly suppressed microsomal GPAT activity, potentially suppressing TAG formation from circulating FAs. DGAT1 plays a greater role in esterifying exogenous FAs (Qi et al., 2012). F81-1144b markedly increased Ucp2 expression in the liver. This may ultimately help to limit TAG accumulation within hepatocytes, because TAG synthesis is an ATP-dependent process and the upregulation of Ucp2 provides hepatocytes with a mechanism to constrain ATP production (Cortez-Pinto et al., 1999). Regarding lipid degradation, ACSL5 and GPAT1 are considered to mediate FA channeling between anabolic and catabolic pathways; namely, the knockdown of Acs5 decreased hepatic TAG secretion and increased FA oxidation (Bu and Mashek, 2010). GPAT1 is localized on outer mitochondrial membranes and channels FAs toward glycolipids such as TAG, thereby diverting them away from CPT1a-mediated entry into mitochondria (Wendel et al., 2013). Therefore, the present result that F81-1144b significantly downregulated the expression of Acs5 and Gpat1 suggests that F81-1144b enhanced FA degradation by elevating the supply of FAs available for β-oxidation, even though F81-1144b did not upregulate the expression of genes for the enzymes related directly to TAG degradation (Atg1) and FA β-oxidation (Cpt1a and Acs1) (Coleman and Mashek, 2011; Lee et al., 2011).

The changes induced in the hepatic expression of key regulatory genes related to gluconeogenesis and de novo lipogenesis by fructose overfeeding are largely similar to those induced by a leptin deficiency (Shimomura et al., 2000; Koo et al., 2008). This state produces a vicious cycle of coexisting insulin resistance (overproduction of glucose owing to resistance to the normal actions of insulin through the down-regulation of insulin receptor substrate (IRS)-2) and insulin sensitivity (elevated lipogenesis owing to the continuous stimulation of SREBP-1c production by insulin signals through IRS-1). Since HSD causes hepatic insulin resistance (Tappy and Lê, 2010) and the elevated expression of many lipogenic genes (Softic et al., 2016), HSD-fed rats may be in a state of coexisting insulin resistance and insulin sensitivity, as reported in leptin-deficient mice. F81-1144b dosing to HSD-fed rats decreased the hepatic expression of Srebp1c and serum insulin levels; these changes subsequently suppressed the expression of genes (Fas, Acc1, G6pd, Acly, Me1, Srebp1c, Scd1, Gpat1, Acs1, and Acs5) related to FA and TAG metabolism, because these genes are known to be regulated by insulin (Ning et al., 2011; Xu et al., 2013; Grevengoed et al., 2014). These results imply that F81-1144b ameliorates dysfunctions in responses to insulin in HSD-fed rats. It is noteworthy that F81-1144b decreased serum TAG levels without changing serum glucose levels in streptozotocin-induced diabetic rats. Moreover, SREBP-1c induction and subsequent elevations in the expression of genes for key lipogenic enzymes in the liver were shown to be independent of insulin in streptozotocin-treated mice (Matsuzaka et al., 2004). Therefore, the effects of F81-1144b on TAG metabolism in the liver and plasma in HSD-fed rats may be secondary, at least in part, to its effects on insulin, and another
mechanism, which is independent of insulin, may operate for the metabolic changes induced in lipids by F81-1144b.

Fibrates, n-3 FAs are typical tools in interventions to reduce serum TAG (Ito, 2015). Fibrates facilitate lipid degradation (increased expression of Atgl and Cpt1a) in the liver by stimulating PPARα (Staels et al., 1998; Rakshandehroo et al., 2010; Karahashi et al., 2013a), thereby decreasing VLDL secretion; fibrates also promote lipid degradation (increased expression of Atgl and Cpt1a) in the liver by stimulating PPARα (Shimizu (Daiichi Fine Chemical Co., Ltd.) for helpful suggestions in previous study (Morikawa et al., 2007) are a novel type of results suggest that F81-1144b and its analogs reported in our study design and continuous encouragement. They are grateful to Dr. Takashi Tanikawa and Shingo Yamada (Josi University) for technical assistance.

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Authorship Contributions

Participated in research design: Kudo, Kawa, Kawashima. Conducted experiments: Eguchi, Yamazaki, Karahashi. Performed data analysis: Kudo, Karahashi, Kawashima. Wrote or contributed to the writing of the manuscript: Kudo, Kawashima.

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Fujisawa T, Yoko T, Oto H, Otani M, Yasuda J, and Morikawa T (2007) Fatty acids. These results suggest that F81-1144b and its analogs reported in our previous study (Morikawa et al., 2007) are a novel type of therapeutic agent for the treatment of hypertriglyceridemia.

Participated in research design: Kudo, Kawa, Kawashima. Conducted experiments: Eguchi, Yamazaki, Karahashi. Performed data analysis: Kudo, Karahashi, Kawashima. Wrote or contributed to the writing of the manuscript: Kudo, Kawashima.