Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 471 (2003) 69–76
www.elsevier.com/locate/ejphar

Anti-hyperlipidemic action of a newly synthesized benzoic acid derivative, S-2E

Koichi Ohmori[a], Haruo Yamada[b], Akio Yasuda[c], Akira Yamamoto[b], Naosuke Matsuura[b], Mamoru Kiniwa[a,*]

[a] *Pharmacobioregulation Research Laboratory, Taiho Pharmaceutical Co., Ltd., 1-27 Misugidai, Hanno, Saitama 357-8527, Japan*
[b] *Pharmacology Research Laboratory, Taiho Pharmaceutical Co., Ltd., 224-2 Ebisuno, Hiraishi, Kawauchi-cho, Tokushima 771-0194, Japan*
[c] *OTC Products Research Laboratory, Taiho Pharmaceutical Co., Ltd., 224-2 Ebisuno, Hiraishi, Kawauchi-cho, Tokushima 771-0194, Japan*

Received 11 February 2003; received in revised form 24 April 2003; accepted 29 April 2003

Abstract

A newly synthesized benzoic acid derivative, (+)-(*S*)-*p*-[1-(*p*-*tert*-butylphenyl)-2-oxo-4-pyrrolidinyl]methoxybenzoic acid (S-2E), has the capacity to inhibit the biosynthesis of both sterol and fatty acids. Here, we report the mechanism by which S-2E lowers blood cholesterol and triglyceride levels. In the liver, S-2E was converted into its active metabolite, S-2E-CoA. S-2E-CoA noncompetitively inhibited the enzymatic activities of both 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase and acetyl-CoA carboxylase at $K_i = 18.11$ μM and $K_i = 69.2$ μM, respectively. Interestingly, pharmacokinetic experiments in rats showed that the concentration of S-2E-CoA in the liver was sufficient to inhibit the activities of HMG-CoA reductase and acetyl-CoA carboxylase, for example, when orally given to rats at 10 mg/kg. Indeed, S-2E (3–30 mg/kg) given orally suppressed the secretion rate of very-low-density lipoprotein (VLDL)-cholesterol and triglyceride in Triton WR-1339-injected rats. Furthermore, S-2E lowered the blood total cholesterol and triglyceride levels simultaneously in Zucker fatty rats. Collectively, S-2E may be useful in the treatment of familial hypercholesterolemia and mixed hyperlipidemia.

Keywords: VLDL (very-low-density lipoprotein) particle production; Sterol synthesis; Fatty acid synthesis; HMG-CoA reductase; Acetyl-CoA carboxylase; VLDL (very low density lipoprotein) secretion

1. Introduction

Hypercholesterolemia and hypertriglyceridemia are independent major risk factors that, alone or together, can accelerate the development of coronary artery disease and the progression of atherosclerotic lesions (McKenney, 2001). The cause of hyperlipidemia has been thought to be related to increased lipid synthesis, decreased lipid clearance from the blood or a combination of these two processes. Consequently, one method to lower blood lipid levels would be to inhibit the synthesis of cholesterol or triglyceride. Such agents have been developed and currently serve as therapeutics for hyperlipidemia. Inhibitors of the rate-limiting enzyme of sterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase, include pravastatin-Na, simvastatin and atorvastatin: These drugs effectively lower the serum cholesterol levels and are widely used to treat patients with hypercholesterolemia (Moghadasuan et al., 2000). These drugs lower blood cholesterol by decreasing its hepatic synthesis. Moreover, they up-regulate the hepatic low-density lipoprotein (LDL) receptor, thus increasing cholesterol clearance (Goldstein and Brown, 1984). However, the effects of HMG-CoA reductase inhibitors on blood triglyceride levels in mixed hyperlipidemia are still less effective (McKenney, 2001). Bezafibrate, a fibrate derivative, has been shown to lower the blood triglyceride level mainly by up-regulation of lipoprotein lipase and then degradation of fatty acids (Staels et al., 1998), while exerting no inhibition on increased levels of cholesterol (Monk and Todd, 1987). It is interesting, therefore, to note the efficacy of therapeutics capable of lowering both blood cholesterol and triglyceride. In the lowering of blood total cholesterol and triglyceride, the production and secretion of very low-density lipoprotein (VLDL) particles, which are rich in lipid components such as cholesterol and

* Corresponding author. Tel.: +81-429-71-0010; fax: +81-429-72-0034.
E-mail address: makiniwa@taiho.co.jp (M. Kiniwa).

0014-2999/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0014-2999(03)01793-X

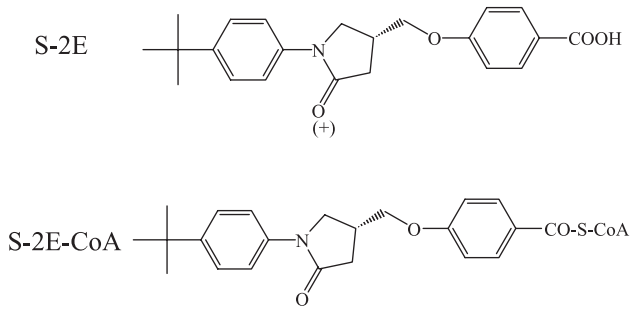

Fig. 1. Chemical structure of S-2E. Upper: S-2E [(+)-(S)-*p*-[1-(*p*-*tert*-butylphenyl)-2-oxo-4-pyrrolidinyl]methoxybenzoic acid; molecular weight 367.445]. Lower: S-2E-CoA.

triglyceride, are expected to be suppressed. As such an agent, an inhibitor of microsomal triglyceride transfer protein (MTP) shows remarkable properties. It has been reported that an inhibitor of MTP, compound 9, shows remarkable lowering of the blood total cholesterol and triglyceride levels in the blood in Watanabe heritable hyperlipidemic rabbits (Wetterau et al., 1998). In that study, the blockage of MTP by this inhibitor was caused by an accumulation of hepatic lipid levels such as triglyceride. Such an accumulation may increase the risk of hepatic dysfunction after long-term therapy. Therefore, an antihyperlipidemic agent to lower blood total cholesterol and triglyceride simultaneously is required that has the capacity to inhibit synthesis of the lipid components such as sterols and fatty acids of VLDL particles.

It has previously been reported that a newly synthesized benzoic acid derivative, (+)-(*S*)-*p*-[1-(*p*-*tert*-butylphenyl)-2-oxo-4-pyrrolidinyl]methoxybenzoic acid (S-2E, Fig. 1), inhibits the biosynthesis of both sterols and fatty acids (Watanabe et al., 1994; Ohno et al., 1999). Although sterols and fatty acids are synthesized through independent pathways, the cholesterol and triglyceride produced by these pathways form VLDL particles, which are the principal vehicles involved in the transport of lipids from the liver to the bloodstream (Olofsson et al., 1999). In the present study, the mechanism of action of S-2E was examined using in vitro and in vivo methods. The results indicate that S-2E taken up by the liver may be efficiently converted to its active metabolite, which allows S-2E to lower the blood cholesterol and triglyceride levels simultaneously by suppressing the production of VLDL particles.

2. Materials and methods

2.1. Chemicals

S-2E was synthesized at Taiho Fine Chemical (Japan). Pravastatin-Na was extracted from commercial tablets (Mevalotin, Sankyo, Japan). S-2E-CoA was synthesized by Taiho Pharmaceutical Co. Ltd. (Tokyo, Japan). For in vitro assay, S-2E was dissolved in dimethylsulfoxide (DMSO) or 0.1 N NaOH. Pravastatin-Na was dissolved in DMSO or distilled water.

S-2E ground in an agate mortar was suspended in 0.5% hydroxypropylmethylcellulose (HPMC, code 60SH50, Shin-Etsu Chemical, Japan) aqueous solution, and a uniform suspension was obtained by ultrasonication. Triton WR-1339 (Tyloxapol, Sigma, USA) was dissolved in physiological saline at a concentration of 80 mg/ml.

2.2. Animals

Male Sprague–Dawley rats (4–6 weeks old) were purchased from Clea Japan. Male Zucker fatty rats (fa/fa) were purchased from Charles River (USA). Diet and water were provided ad libitum under a 12-h light–dark cycle (light period: 6:00–18:00) throughout the experimental period.

2.3. Measurement of sterol and fatty acid biosyntheses in rat liver slices

The biosyntheses of sterol and fatty acid were assayed by the method of Endo et al. (1977). Briefly, the rats were sacrificed by decapitation, and then the liver was excised and perfused with Krebs–Ringer bicarbonate buffer (118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 10 mM $NaHCO_3$, 10 mM HEPES, 5 mM glucose, pH 7.4). Liver slices (100 mg) were incubated with [^{14}C]acetic acid (74 kBq/2 μmol/50 μl, Amersham, UK) in Krebs–Ringer bicarbonate buffer at 37 °C for 2 h with shaking (140 rpm). Saponification was performed by the addition of 15% KOH–ethanol solution (w/v) at 75 °C for 2 h.

The sterol and fatty acid fractions were extracted by the method of Kuroda and Endo (1977). After drying under a nitrogen gas stream, sterol fractions were dissolved in 1 ml of acetic acid, and fatty acid fractions were dissolved in 1 ml of chloroform–methanol (2:1, v/v). Aliquots of 0.5 ml from these fractions were transferred into scintillation vials, and mixed with 10 ml of scintillator (ACS II, NACS204, Amersham). The incorporated radioactivity of sterol and fatty acid was determined using a liquid scintillation counter (2000 Tri-CARB Liquid Scintillation Analyzer, Packard, USA). The biosynthetic activities of sterol and fatty acid were estimated as the percentages relative to control values. The 50% inhibition concentration (IC_{50}) was calculated from the inhibition rate (%) for each compound.

2.4. Measurement of HMG-CoA reductase activity

The rats were given a powder diet (CE-2, Clea Japan) for 2 weeks, and then HMG-CoA reductase activity was induced by powder diets (CE-2, Clea Japan) containing 5% (w/w) cholestyramine resin for 3.5 days. After decapitation, the livers were excised and perfused with physiological saline. The HMG-CoA reductase enzyme solution was prepared as microsomes (holoenzymes) by the method of Vermilion and Schroepfer (1990). The enzyme was

suspended in 50 mM Tris–HCl buffer (250 mM sucrose, 50 μM leupeptin, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) and stored at −80 °C until use. The protein contents of the microsomes were determined with a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Tokyo, Japan).

The assay mixture contained 100 μg of HMG-CoA reductase protein, 0.2% (v/v) of polyoxyethylene ether W-1, 50 mM phosphate buffer (pH 6.8) and alkaline phosphatase (10 U) to obtain maximum HMG-CoA reductase enzyme activity (Brown et al., 1979). After the addition of HMG-CoA as a substrate and NADPH, the reaction mixture was incubated for 3 min at 37 °C with shaking at 50 rpm. The enzyme activity was estimated as the decrease in NADPH content (Darnay et al., 1992). After preincubation with the enzyme solution for 3 min in the presence of test compounds, their inhibitory activities were estimated.

The Michaelis constant (K_m) and maximum reaction rate (V_{max}) were estimated from Lineweaver–Burk plots. In the case of the noncompetitive inhibition, the inhibition constant of enzyme-inhibitor complex (K_i) was estimated by the method of Cornis-Boweden (1974).

2.5. Measurement of acetyl-CoA carboxylase activity

Acetyl-CoA carboxylase was induced according to the method of Thampy and Wakil (1985). The rats were given commercial chow (CE-2, Clea Japan) for 1 week. After fasting for 48 h, they were given a low fat diet (Clea Japan) for 3 days to induce acetyl-CoA carboxylase enzyme. After decapitation, the livers were excised, and perfused with ice-cold physiological saline.

The acetyl-CoA carboxylase solutions were prepared from rat livers by the method of Winder and Hardie (1996). The acetyl-CoA carboxylase-containing fractions were recovered by avidin-Sepharose affinity chromatography (Promega SoftLink Soft Release, Fisher Scientific) by production of [^{14}C]malonyl-CoA from acetyl-CoA and [^{14}C]$NaHCO_3$ (NEN, USA). After salting out by addition of an equal volume of saturated ammonium sulfate, the precipitate was recovered and dialyzed against 100 mM Tris–HCl buffer (0.5 M NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol, pH 7.5) overnight. This acetyl-CoA carboxylase enzyme solution was stored at −80 °C until use.

The enzyme activity was assayed by the method of Thampy and Wakil (1985) with some modifications. The assay mixture contained 2.8 μg of acetyl-CoA carboxylase protein, 50 mM Tris–HCl buffer, pH 7.5, 1.5 mM $MgSO_4$, 2 mM dithiothreitol, 0–0.1235 mM acetyl-CoA (Sigma), 4 mM ATP, 12.5 mM [^{14}C]$NaHCO_3$ (74 kBq), 0.75 mg/ml fatty acid-free bovine serum albumin (Sigma), 10 mM citrate and 10 mM Mg-acetate in a total volume of 200 μl. After addition of acetyl-CoA carboxylase, the assay was carried out at 37 °C for 3 min. The reaction was stopped by the addition of 50 μl of 5 N HCl. After centrifugation (1800 × g, 5 min), 150 μl of supernatant was transferred into a scintillation vial and evaporated at 80 °C. After dissolution in 400 μl of distilled water, 10 ml of scintillator (ACS II, NACS204, Amersham) was added, and the radioactivity was determined in a liquid scintillation counter (1500 Tri-CARB Liquid Scintillation Analyzer, Packard).

K_m, V_{max} and K_i were estimated by the same method as HMG-CoA reductase.

2.6. Estimation of S-2E and S-2E-CoA in plasma and rat liver

S-2E at 10 mg/kg was given orally by gavage to the rats. Blood was collected from the inferior vena cava under diethylether anesthesia 0.5, 1, 2, 4, 8 and 24 h after S-2E administration (each point: $n = 5$). After decapitation, the livers were excised and perfused with cold physiological saline. Plasma and livers were stored at −80 °C until use. Next, S-2E was extracted from the plasma (250 μl) by addition of 500 μl of acetonitrile and 250 μl of distilled water. S-2E and S-2E-CoA were extracted from the livers (250 mg) by addition of 500 μl of acetonitrile–acetone (450:50, v/v) and 250 μl of distilled water. The supernatants were filtrated, and S-2E and S-2E-CoA were measured by high-performance liquid chromatography (Shimadzu LC-6B, Inertsil ODS-2 column) at 254 nm. A TBA (0.5 mM tetra-*n*-butylammonium hydroxide, pH 7.0)/acetonitrile gradient (45–55%) was used as the eluent solution. The area under the curve (AUC) was calculated with Multistaff (version 5.61).

2.7. Estimation of VLDL-cholesterol and triglyceride secretion rate

Rats were fasted throughout this experiment period, although water was provided ad libitum. The rats were divided into five groups (each group: $n = 8$) by body weight: nontreated group, control (Triton WR-1339-treated) group and S-2E-treated group (3, 10 and 30 mg/kg). Blood from the nontreated group was collected from the inferior vena cava under diethylether anesthesia. Immediately after oral administration of S-2E to rats by gavage, Triton WR-1339 at 400 mg/kg was injected intravenously except in the nontreated group. Four hours after the injection of Triton WR-1339, blood was collected from the inferior vena cava under diethylether anesthesia. Serum was obtained by centrifugation.

2.8. Evaluation of blood total cholesterol and triglyceride levels in Zucker fatty rats

For Zucker fatty rats, there was a pretreatment period of 3 weeks. During treatment, to monitor the blood lipid levels, 150 μl of blood was collected from the caudal vein with a capillary blood collection tube (Terumo, Japan) by venisection once a week under diethylether anesthesia, and diluted with 300 μl of physiological saline to recover blood effectively. Then the diluted plasma was obtained by centrifugation. Based on the diluted plasma total cholesterol values

and triglyceride values during the pretreatment period, the rats were divided into two groups (control group, S-2E 5 mg/kg group). S-2E was given orally by gavage to rats for 4 weeks and the dosing time was set at 16:00–17:00. To evaluate the efficacy of S-2E, at 16 h after the final administration, blood was collected from the inferior vena cava vein under diethylether anesthesia with heparinized syringes. Then plasma was obtained by centrifugation.

2.9. Fractionation of lipoproteins by the ultracentrifugation

Lipoprotein fractionation in the plasma was performed by ultracentrifugation (optima TLX Ultracentrifuge, Beckman, USA) following the method of Hatch and Less (1968). According to their method, solution 1 (density = 1.006 g/l) was prepared with NaCl. Then, according to the specific gravity of each, VLDL fractions were isolated at 20 °C as described below. That is, 490 μl of solution 1 was layered over the surface of 980 μl of plasma in a Quick seal 11 × 25 mm ultracentrifuge tube (Beckman), using a TLA 120.2 rotor (Beckman). After ultracentrifugation (26,000 × *g*, 30 min), the chylomicron fraction, which floated to the top of the tube, was removed. Then, the 980-μl fraction solutions were again overlaid with 490 μl of solution 1 and centrifuged for 3 h at 436,000 × *g*. The 490-μl top fraction was separated as the VLDL fraction (density < 1.006 g/l). The cholesterol level in its lipoprotein fraction was measured by the same method with an automatic analyzer (7170, Hitachi, Japan), as described below.

2.10. Measurement of blood lipids

Total cholesterol, low-density lipoprotein (LDL)-cholesterol, HDL-cholesterol and triglyceride in the serum and plasma were measured by enzymatic methods [total cholesterol: L-CHO S, Wako (Japan); LDL-cholesterol: cholestest LDL-C, Fujirebio (Japan); HDL-cholesterol: cholestest HDL-C, Fujirebio; triglyceride: L-TG H, Wako] with an automatic analyzer (7170, Hitachi).

VLDL-cholesterol, VLDL-cholesterol secretion rate (VCSR) and triglyceride secretion rate (TGSR) were calculated as follows:

VLDL-cholesterol = total cholesterol − (LDL-cholesterol + HDL-cholesterol)

VCSR=[(VLDL-cholesterol 4 h after the injection of Triton WR-1339) − (mean VLDL-cholesterol in the nontreated group)]/4 h

TGSR=[(triglyceride 4 h after the injection of Triton WR-1339) − (mean triglyceride in the nontreated group)]/4 h.

2.11. Statistical analysis

Data are basically expressed as the mean value (mean) and standard deviation (S.D.). Statistical analysis was performed with the Dunnett's test (JMP, SAS Institute). Asterisks are used to denote a significant difference at the level of $p < 0.05$. Results are expressed as follows: $*p < 0.05$, $**p < 0.01$.

3. Results

3.1. Effects of S-2E on the synthesis of sterols and fatty acids in rat liver slices

As reported previously, S-2E was selected due to its capacity to inhibit the synthesis of sterols and fatty acids in rat liver slices (Ohno et al., 1999). First, to confirm the ability of S-2E to inhibit the synthesis of sterols and fatty acids, rat liver slices (a part of the liver) were incubated for 2 h in the presence of various concentrations of S-2E (1, 3, 10, 30 and 100 μM) (each point: $n = 3$). The extracts from the assay tube, which contained both the liver slices and incubation medium, were analyzed for sterols and fatty acids. S-2E inhibited the synthesis of both sterols and fatty acids (Table 1). IC_{50} of S-2E on the synthesis of sterols and fatty acids were 5.32 and 3.05 μM, respectively. Note that the HMG-CoA reductase inhibitor pravastatin inhibited the synthesis of sterols (IC_{50}: 0.03 μM), but not that of fatty acid in a concentration range of 0.01–100 μM (each point: $n = 3$).

3.2. Inhibition of HMG-CoA reductase activity

To determine the mechanism by which S-2E inhibited sterol synthesis, we first investigated the effects of S-2E on the catalytic activity of enzymes including HMG-CoA

Table 1
Inhibition of in vitro synthesis of sterols and fatty acids by S-2E and pravastatin in rat liver slices

Content compound		Concentration (μM)	Synthetic activity (%)	
			Sterols	Fatty acids
Experiment 1	Control		100 ± 15.2	100 ± 27.8
	S-2E	1	78.3 ± 6.3	61.4 ± 1.2
		3	62.9 ± 11.8	67.0 ± 7.6
		10	35.0 ± 3.4	13.8 ± 1.9
Experiment 2	Control		100 ± 2.0	100 ± 19.8
	Pravastatin	0.01	70.1 ± 12.3	89.9 ± 11.5
		0.03	46.0 ± 10.3	91.4 ± 11.5
		0.1	26.4 ± 6.2	80.2 ± 15.2

The synthetic activity of sterols and fatty acids in rat liver slices was determined by incorporation of [^{14}C]acetic acid. In each assay (each point: $n = 3$), S-2E was used at a concentration range of 1–100 μM, and pravastatin was at 0.01–100 μM. In experiment 1, the synthetic activities on sterol and fatty acids in the absence of the compounds were 22,165 ± 3775 and 138,778 ± 38,512 dpm/100 mg liver, respectively. In experiment 2, they were 10,751 ± 217, 21,997 ± 4365 dpm/100 mg liver, respectively. Data are indicated as means ± S.D. of the percentage relative to the values in the absence of the compound. In this table, S-2E is indicated at a concentration of 1–10 μM, and pravastatin is at 0.01–0.03 μM.

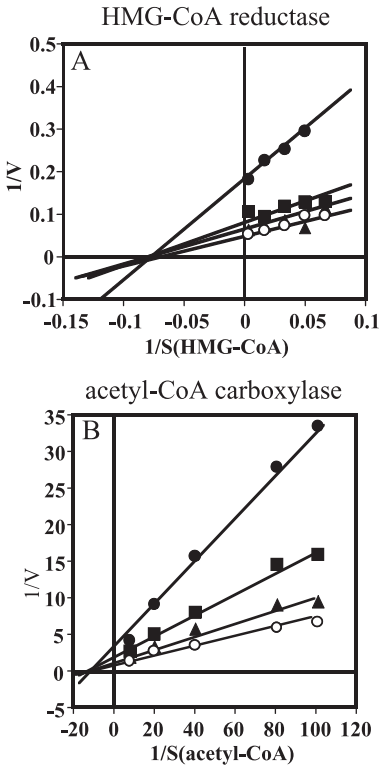

Fig. 2. Lineweaver–Burk plots for HMG-CoA reductase and acetyl-CoA carboxylase inhibition by S-2E-CoA. As described below, data are indicated as mean (each point: $n=3$). For reasons of clarity, S.D. is not given. Panel A: Inhibition of HMG-CoA reductase. K_m and V_{max} for HMG-CoA reductase were 10.32 μM and 18.32 nmol/min/mg protein, respectively. In the presence of S-2E-CoA at 0 (○), 4.721 (▲), 9.44 (■) and 47.21 μM (●), HMG-CoA reductase activity was determined. S-2E showed noncompetitive inhibition of HMG-CoA reductase activity. K_i was 18.11 μM. Panel B: Inhibition of acetyl-CoA carboxylase. K_m and V_{max} were 36.7 μM and 0.693 μmol/min/mg protein, respectively. In the presence of S-2E-CoA at 0 (○), 50.3(▲), 100.7 (■) and 201.3 μM (●), acetyl-CoA carboxylase activity was determined. S-2E showed noncompetitive inhibition of acetyl-CoA carboxylase activity. K_i was 69.2 μM.

reductase, which is the rate-limiting enzyme in sterol synthesis. In this experiment, we used the membrane-bound enzyme of HMG-CoA reductase as the enzyme, because S-2E may bind to the catalytic domain, regulatory domain or both. S-2E, however, showed no inhibitory effect even at 100 μM (data not shown), suggesting that the active metabolite(s) of S-2E may have an inhibitory effect on sterol synthesis. A series of metabolites of S-2E identified in the liver homogenates from rats that received S-2E administration were tested for their inhibitory effects on the enzymatic activity of HMG-CoA reductase. S-2E-CoA (Fig. 1) was finally found to have an inhibitory effect on HMG-CoA reductase. As shown in Fig. 2, panel A, S-2E-CoA showed noncompetitive inhibition of HMG-CoA reductase; K_i value of HMG-CoA reductase by S-2E-CoA was 18.11 μM. Note that pravastatin showed competitive inhibition of HMG-CoA reductase activity ($K_i=21.05$ nM).

3.3. Inhibition of acetyl-CoA carboxylase activity

We next examined the effects of S-2E on fatty acid synthesis, using the prepared enzymes. Similar to the case of sterol synthesis, S-2E itself had no inhibitory effect on the catalytic activities of enzymes involved in fatty acid synthesis (data not shown). However, as shown in Fig. 2, panel B, S-2E-CoA noncompetitively inhibited the rate-limiting activity of acetyl-CoA carboxylase ($K_i=69.2$ μM).

3.4. Detection of S-2E and its metabolite, S-2E-CoA, in the rat

The results described above indicated that S-2E-CoA may be the active metabolite of S-2E in terms of suppression of sterol and fatty acid synthesis. To determine whether S-2E-CoA is generated in vivo, we performed a pharmacokinetic study in rats. Fig. 3 shows the changes in concentration of S-2E in the plasma and S-2E-CoA in the liver. When S-2E was given orally at 10 mg/kg, their concentrations reached maximum levels after 4 h. The concentration of S-2E-CoA was approximately eightfold higher than that of S-2E (C_{max}: S-2E 11.5 μg/g liver vs. S-2E-CoA 89.2 μg/g liver, Table 2).

3.5. Effect of S-2E on VLDL particle secretion rate

To clarify whether S-2E inhibited the production and secretion of VLDL particles, Triton WR-1339 was injected intravenously. As shown in the study by Goldfarb (1978), the accumulation of VLDL particles was at least linear following injection of Triton WR-1339 up to a 4-h period. Four hours later, the VLDL-cholesterol and triglyceride levels were significantly increased to 135 ± 11 and 1609 ± 154 mg/dl, compared with 29 ± 5 and 152 ± 84 mg/dl in the nontreated group, respectively. VCSR was significantly reduced at doses of 3 and 10 mg/kg ($p<0.05$, $p<0.01$) (Fig. 4). TGSR was also significantly reduced at doses of

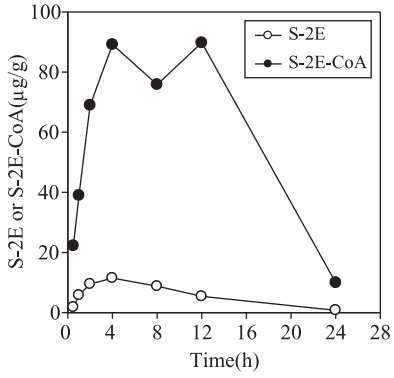

Fig. 3. Time course of changes in the levels of S-2E and S-2E-CoA in rat livers after oral administration at 10 mg/kg. S-2E at 10 mg/kg was given orally by gavage to Sprague–Dawley rats. Livers were sampled at 0.5, 1, 2, 4, 8 and 24 h (each point: $n=5$). S-2E (○) and S-2E-CoA (●, the major metabolite) in the rat liver were measured. Data are expressed as means.

Table 2
Pharmacokinetic parameters of S-2E and S-2E-CoA in rats

Content	$AUC_{0-24\ h}$ (μg·Eq·h/ml)	C_{max} (μg/ml)	T_{max} (h)
S-2E (plasma)	9.0	1.4	2
S-2E (liver)	136.0	11.5	4
S-2E-CoA (liver)	1493.5	89.2	4

S-2E at 10 mg/kg was given orally to Sprague–Dawley rats ($n=5$). S-2E and S-2E-CoA were measured at 0.5, 1, 2, 4, 8 and 24 h in plasma and liver. $AUC_{0-24\ h}$ (area under the curve), C_{max} (maximum concentration) and T_{max} (time of maximum concentration) are indicated.

10 and 30 mg/kg ($p<0.01$). These results showed that S-2E inhibited the production and secretion of VLDL particles.

3.6. Effects of S-2E on the blood total cholesterol and triglyceride levels in Zucker fatty rat

To clarify the hypolipidemic actions of S-2E by inhibition of VLDL particle production, we further investigated whether S-2E improved the blood lipid profiles in Zucker fatty rats, which exhibit the hyperlipidemia by the overproduction of VLDL particles (Bray, 1977). Here, we used 5 mg/kg as dose of S-2E, because in a preliminary results using Zucker fatty rats, S-2E at 5 mg/kg suppressed the blood levels of total cholesterol and triglyceride after a week (data not shown). Here, during the experiment, the food consumption of the control and S-2E-treated groups for 4 weeks was similar (data not shown). In the caudal vein of control group, total cholesterol and triglyceride levels in the diluted plasma were maintained from the start of administration to 4 weeks (total cholesterol: 99.3–120.5 mg/dl, triglyceride: 544.0–599.7 mg/dl as mean, respectively). After 4 weeks, the values in the plasma were 165.4 ± 65.6 and 911.2 ± 434.9 mg/dl, respectively. In the S-2E-treated group after 4 weeks, S-2E significantly lowered the total cholesterol and triglyceride levels against the control group (Fig. 5, panels A and B). Furthermore, S-2E significantly lowered VLDL-cholesterol levels (Fig. 5, panel C). In addition to the results above, these results suggested that S-2E may inhibit the production of VLDL particles.

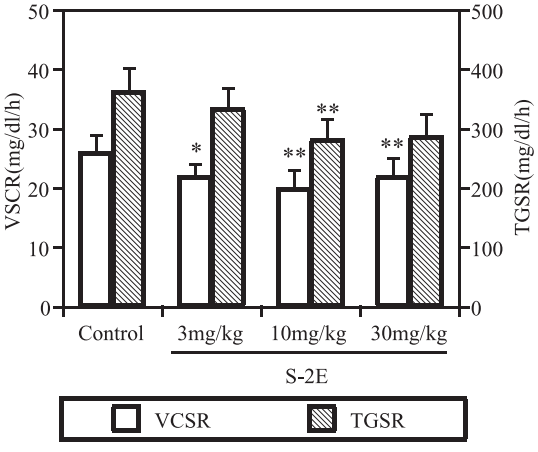

Fig. 4. Effects of S-2E on the VLDL-cholesterol and triglyceride secretion rates. Rats were divided into five groups [non-treated group, control (Triton WR-1339-treated) group, S-2E 3 mg/kg group, S-2E 10 mg/kg group and S-2E 30 mg/kg group] ($n=8$). Immediately after oral administration of S-2E, Triton WR-1339 was injected intravenously at 400 mg/kg. Four hours after Triton WR-1339 injection, blood was collected. Total cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride in the obtained serum were measured. VLDL-cholesterol and triglyceride secretion rates were calculated (VCSR: open column; TGSR: stripped column). Data are expressed as means ± S.D. Significance of differences was determined by Dunnett's test relative to the control group. Results are expressed as follows: $*p<0.05$, $**p<0.01$.

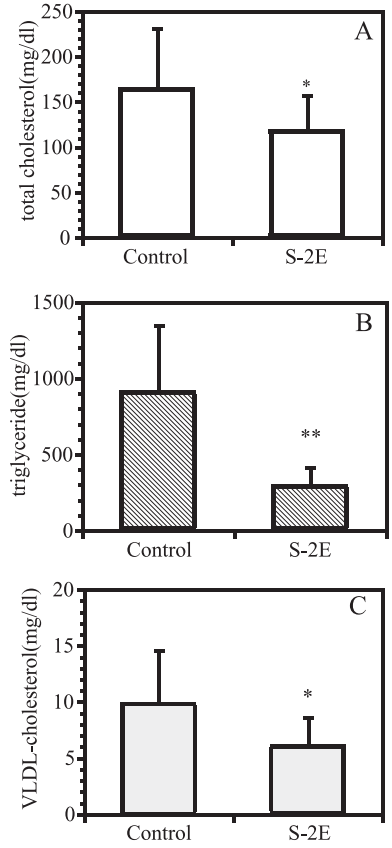

Fig. 5. The anti-hyperlipidemic effects of S-2E on the total cholesterol, triglyceride and VLDL-cholesterol levels in Zucker fatty rats. After monitoring the total cholesterol and triglyceride levels for pretreatment period, the rats were divided into two groups: control ($n=9$) group and S-2E 5 mg/kg group ($n=10$). S-2E was orally given to the rats for 4 weeks. Blood was collected 16 h after the final administration. To evaluate the efficacy of S-2E, total cholesterol and triglyceride in the obtained plasma were measured. Furthermore, cholesterol levels in VLDL fractions were measured after fractionation by ultracentrifugation. Panel A: Total cholesterol (open column); panel B: triglyceride (stripped column); panel C: VLDL-cholesterol (hatched column). Data are expressed as means ± S.D. Significance of differences was determined by Dunnett's test relative to the control group. Results are expressed as follows: $*p<0.05$, $**p<0.01$.

4. Discussion

In the present study, we first confirmed a previous observation that S-2E inhibited the biosynthesis of sterols

and fatty acids in rat liver slices (Watanabe et al., 1994; Ohno et al., 1999). The sterols and fatty acids are synthesized by independent pathways (Gruffat et al., 1996), raising questions about the mechanism by which S-2E inhibits the synthesis of these two factors simultaneously. As for sterol synthesis, a number of compounds have been reported to inhibit it by inhibition of the enzymatic activity of HMG-CoA reductase in a substrate-competitive manner (Ucar et al., 2000). We found in preliminary experiments that S-2E itself at a concentration of up to 100 μM did not inhibit the activity of this enzyme or of other enzymes involved in sterol synthesis (data not shown). It is reasonable, therefore, to assume that S-2E may be converted to active metabolite(s) during incubation with liver slices. In fact, S-2E-CoA was identified as the major metabolite in liver homogenates from rats that had been administered S-2E, and this metabolite inhibited the enzymatic activity of HMG-CoA reductase in a noncompetitive manner. Interestingly, S-2E-CoA also inhibited the enzymatic activity of acetyl-CoA carboxylase, which is the rate-limiting enzyme in fatty acid synthesis, in a noncompetitive manner. These results showed that S-2E-CoA generated in hepatocytes inhibited the rate-limiting enzymes of both sterol and fatty acid synthesis.

HMG-CoA reductase is a transmembrane protein with seven membrane-spanning helices that anchor the amino-terminal portion in the membrane (Vermilion and Schroepfer, 1990). Its catalytic domain (53 kDa) lies in the hydrophilic domain located in the cytosol, and the inhibitors mentioned above were shown to bind to its catalytic site (Moghadasian, 1999). However, little is known about the mechanism by which noncompetitive inhibitors such as S-2E-CoA regulate the enzymatic activity of HMG-CoA reductase. Similar to our observations, Lippe et al. (1985) reported that CoA esters noncompetitively inhibit this enzyme. Recent analysis of crystallized HMG-CoA reductase by Istvan et al. (2000) showed that the ADP moiety of CoA binds to a positively charged pocket near the enzyme surface. CoA derivatives including S-2E-CoA may modulate the enzyme activity of HMG-CoA reductase by interacting with this pocket. On the other hand, acetyl-CoA carboxylase activity has been reported to be inhibited in a noncompetitive manner by final products of fatty acid biosynthesis (Faergeman and Knudsen, 1997). For instance, in the presence of citrate, acetyl-CoA carboxylase monomer is activated and change to the active form, that is, acetyl-CoA carboxylase polymer. This polymer is dissociated by long chain acyl-CoA to acetyl-CoA carboxylase protomer, resulting in a decrease in acetyl-CoA carboxylase activity (Winder and Hardie, 1996). Although we did not determine whether S-2E-CoA causes dissociation of the acetyl-CoA carboxylase polymer, it is reasonable to assume that S-2E-CoA inhibits acetyl-CoA carboxylase activity partly by such a feedback mechanism.

The K_i values of S-2E-CoA for HMG-CoA reductase and acetyl-CoA carboxylase were 18.11 and 69.2 μM, respectively. These values were too low to explain the inhibitory effects of S-2E at the μM order on sterol and fatty acid synthesis in liver slices. However, a pharmacokinetic study using rats showed that the S-2E-CoA concentration in liver was much higher than that of S-2E in plasma. This finding suggested that S-2E taken up into the liver may be easily converted to S-2E-CoA, and that this active metabolite accumulates in the hepatocytes to a greater extent than in the extracellular fluid. When 10 mg/kg of S-2E was given orally, the concentration of S-2E-CoA in the liver reached 78.7 μM after 4 h (T_{max}). Taken together with the above results, it is reasonable to predict that this dose of S-2E may suppress blood total cholesterol and triglyceride by inhibiting the biosynthesis of sterol and fatty acids.

Alternatively, it is thought the possibility that S-2E may express anti-hyperlipidemic effect by modulating the activities of MTP or peroxisome proliferator-activated receptor (PPAR) α. Their modulation influences hepatic triglyceride levels (Wetterau et al., 1998; Shiomi and Ito, 2001; Roglans et al., 2002). However, S-2E treatment did not change hepatic triglyceride levels (data not shown). This suggests that S-2E may not influence the activities of MTP and PPARα. Further studies are required to clear whether S-2E may modulate the activities of MTP or PPARα.

Indeed, S-2E at doses of 3–30 mg/kg significantly suppressed the blood levels of VLDL-cholesterol and triglyceride in rats injected with Triton WR-1339. Triton WR-1339 is known to inhibit the degradation of VLDL particles by blocking lipoprotein lipase (Otway and Robinson, 1967), causing the accumulation of blood VLDL particles formed in the liver. S-2E may interfere with the process of VLDL particle secretion in a similar manner to bezafibrate (Kusaka et al., 1988). However, this is unlikely because no increase in the triglyceride pool was seen in the liver of S-2E-treated rats (data not shown). Alternatively, since the formation of mature VLDL particles requires the supply of cholesterol and triglyceride (Olofsson et al., 1999), S-2E suppression of VLDL particle secretion may be explained by the inhibition of sterol and fatty acid synthesis. In contrast to S-2E, however, it has been reported that pravastatin did not suppress VLDL-cholesterol secretion in Triton WR-1339-injected rats (Fujioka et al., 1995). This can be explained by the findings that a decrease in the cholesterol pool by pravastatin results in the induction of HMG-CoA reductase transcription (Goldstein and Brown, 1984). To clarify the differences in terms of the HMG-CoA reductase-inducible effect between competitive and noncompetitive inhibitors of HMG-CoA reductase, further studies are required to determine whether S-2E treatment induces enzyme activity.

S-2E lowered the blood levels of total cholesterol, triglyceride and VLDL-cholesterol in Zucker fatty rats, which exhibit hyperlipidemia by the overproduction of VLDL particles (Bray, 1977). This finding implies that S-2E expresses the anti-hyperlipidemic effects in in vivo study. Furthermore, S-2E suppression of VLDL-cholesterol in Zucker fatty rats suggests that S-2E may suppress the overproduction of VLDL particles. In addition to the finding

described above, it is reasonable, therefore, to assume that S-2E may inhibit the production of VLDL particles, but not impair the secretion of them.

In conclusion, S-2E may be effectively converted to the active metabolite S-2E-CoA in the liver. S-2E-CoA may suppress the production and secretion of VLDL particles by inhibiting the synthesis of sterols and fatty acids. These abilities may allow S-2E to become a useful agent for the treatment of patients with familial hyperlipidemia and mixed hyperlipidemia.

Acknowledgements

We thank Dr. Hidekazu Miyake for advice regarding this work. The authors are indebted to Professor J. Patrick Barron of the International Medical Communications Center of Tokyo Medical University for his review of this manuscript.

References

Bray, G.A., 1977. The Zucker fatty rat: a review. Fed. Proc. 36, 148–153.

Brown, M.S., Goldstein, J.L., Dietschy, J.M., 1979. Active and inactive forms of 3-hydroxy-3-methlyglutaryl coenzyme A reductase in the liver of the rats. J. Biol. Chem. 254, 5144–5149.

Cornis-Boweden, A., 1974. A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. Biochem. J. 137, 143–144.

Darnay, B.G., Wang, Y., Rodwell, V.W., 1992. Identification of the catalytical important histidine of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. J. Biol. Chem. 267, 15064–15070.

Endo, A., Tsujita, Y., Kuroda, M., Tanzawa, K., 1977. Inhibition of cholesterol synthesis in vitro and in vivo by ML-236A and ML-236B, competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Eur. J. Biochem. 77, 31–36.

Faergeman, N.J., Knudsen, J., 1997. Role of long-chain acyl-CoA esters in the regulation of mechanism and in cell signaling. Biochem. J. 323, 1–12.

Fujioka, T., Nara, F., Tsujita, Y., Fukushige, J., Fukami, M., Kuroda, M., 1995. The mechanism of lack of hypocholesterolemic effects of pravastatin sodium. 3-hydroxy-3methylglutaryl coenzyme A reductase inhibitor, in rats. Biochim. Biophys. Acta 1254, 7–12.

Goldfarb, S., 1978. Rapid increase in hepatic HMG CoA reductase activity and in vivo cholesterol synthesis after Triton WR 1339 injection. J. Lipid Res. 25, 1450–1461.

Goldstein, J.L., Brown, M.S., 1984. Progress in understanding the LDL receptor and HMG-CoA reductase, two membrane proteins that regulate the plasma cholesterol. J. Lipid Res. 25, 1450–1461.

Gruffat, D., Durand, D., Graulet, G., Bauchart, D., 1996. Regulation of VLDL synthesis and secretion in the liver. Reprod. Nutr. Dev. 36, 375–389.

Hatch, F.T., Less, R.S., 1968. Practical method for plasma lipoprotein analysis. Adv. Lipid Res., vol. 6. Academic Press, New York, pp. 1–68.

Istvan, E.S., Palnitlkar, M., Buchanan, S.K., Deisenhofer, J., 2000. Crystal structure of the catalytic portion of human HMG-CoA reductase: insights into regulation of activity and catalysis. EMBO J. 19, 819–830.

Kuroda, M., Endo, A., 1977. Inhibition of in vitro cholesterol synthesis by fatty acid. Biochim. Biophys. Acta 486, 70–81.

Kusaka, H., Nishiyama, M., Matsubara, Y., Ikeda, S., 1988. Pharmacological investigation of bezafibrate, a hypolipidemic agent (2). Mechanism of the hypolipidemic action of bezafibrate in rats. Folia Pharmacol. Jpn. 92, 181–191.

Lippe, G., Denz, R., Cavalini, L., Galzigna, L., 1985. Inhibition of rat liver hydroxymethylglutaryl-CoA reductase by sulfhydryl reagents, coenzyme A ester and synthetic compounds. Biochem. Pharmacol. 34, 3293–3297.

McKenney, J.M., 2001. Pharmacotherapy of dyslipidemia. Cardiovasc. Drugs Ther. 15, 413–422.

Moghadasian, M.H., 1999. Clinical pharmacology of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. Life Sci. 65, 1329–1337.

Moghadasuan, M.H., Mancini, G.B.J., Frohlich, J.F., 2000. Pharmacotherapy of hypercholesterolaemia: statins in clinical practice. Expert Opin. Pharmacother. 1, 683–695.

Monk, J.P., Todd, P.A., 1987. Bezafibrate: a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in hyperlipidaemia. Drugs 33, 539–576.

Ohno, T., Yano, S., Yamada, H., Shirasaka, T., Yamamoto, A., Kobayashi, K., Ogawa, K., 1999. Synthesis of the optical isomers of 4-[1-(4-*tert*-butylphenyl)-2-oxo-4-pyrrolidine-4-yl] methyloxybenzoic acids (S-2) and their biological evaluation as aitilipidemic agent. Chem. Pharm. Bull. 47, 1549–1554.

Olofsson, S.-O., Asp, L., Boren, J., 1999. The assembly and secretion of apolipoprotein B-containing lipoproteins. Curr. Opin. Lipidol. 10, 341–346.

Otway, S., Robinson, D.S., 1967. A non-ionic detergent (Triton WR-1339) to determine rates of triglyceride entry into the circulation of the rat under different physiological conditions. J. Physiol. 190, 321–332.

Roglans, N., Sanguino, E., Peris, C., Alegret, M., Vazquez, M., Adzet, T., Diaz, C., Hernandez, G., Laguma, J.C., Sanchez, R.M., 2002. Atorvastatin treatment induced peroxisome proliferator-activated receptor α expression and decreased plasma nonesterified fatty acids and liver triglyceride in fructosed-fed rats. J. Pharmacol. Exp. Ther. 302, 232–239.

Shiomi, M., Ito, T., 2001. MTP inhibitor decreases plasma cholesterol levels in LDL-receptor-deficient WHHL rabbits by lowering the VLDL secretion. Eur. J. Pharmacol. 431, 127–131.

Staels, B., Dallongeville, J., Aurwerx, J., Schoonjans, K., Leitersdorf, E., Fruchart, J.-C., 1998. Mechanism of action of fibrates on lipid and lipoprotein metabolism. Circulation 98, 2088–2093.

Thampy, K.G., Wakil, S.J., 1985. Activation of acetyl-CoA carboxylase. Purification and properties of a Mn^{2+}-dependent phosphatase. J. Biol. Chem. 260, 6318–6323.

Ucar, M., Mjorndal, T., Dahlqvist, T., 2000. HMG-CoA reductase inhibitors and mytotoxicity. Drug Safety 22, 441–457.

Vermilion, J.L., Schroepfer, G.J., 1990. Solubilization of the 97-kDa native form of liver microsomal 3-hydroxy-3-methylglutaryl-coenzyme A reductase. J. Biol. Chem. 265, 9984–9992.

Watanabe, S., Ogawa, K., Ohno, T., Yano, S., Yamada, H., Shirasaka, T., 1994. Synthesis of 4-[1-(substituted phenyl)-2-oxo-4-pyrrolidin-4-yl] methyloxybenzoic acids and related compounds, and their inhibitory capacities toward fatty-acid and sterol biosyntheses. Eur. J. Med. Chem. 29, 675–686.

Wetterau, J.R., Gregg, R.E., Harrity, T.W., Arbeeby, C., Cap, M., Connolly, F., Chu, C.-H., Geroge, R., Gordon, J.D.A., Jamil, H., Jolibis, K.G., Kunselman, L.K., Lan, S.-J., Maccagnan, T.J., Ricci, B., Yan, M., Young, D., Chen, Y., Fryszman, O., Logan, J.V.H., Musial, C.L., Poss, M.A., Robl, J.A., Simpkins, L.M., Slusararchyk, W.A., Sulsky, R., Taunk, P., Mahnin, D.R., Tino, J.A., Lawrence, R.M., Dikson Jr., J.K., Biller, S.A., 1998. An MTP inhibitor that normalizes atherogenic lipoprotein levels in WHHL rabbits. Science 282, 751–754.

Winder, W.W., Hardie, D.G., 1996. Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in musle during exercise. Am. J. Physiol. 270, E299–E304.