Squalene synthase inhibitors suppress triglyceride biosynthesis through the farnesol pathway in rat hepatocytes

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Abstract **We recently demonstrated that squalene synthase (SQS) inhibitors reduce plasma triglyceride through an LDL receptor-independent mechanism in Watanabe heritable hyperlipidemic rabbits (Hiyoshi et al. 2001. *Eur. J. Pharmacol.* 431: 345–352). The present study deals with the mechanism of the inhibition of triglyceride biosynthesis by the SQS inhibitors ER-27856 and RPR-107393 in rat primary cultured hepatocytes. Atorvastatin, an HMG-CoA reductase inhibitor, had no effect on triglyceride biosynthesis, but reversed the inhibitory effect of the SQS inhibitors. A squalene epoxidase inhibitor, NB-598, affected neither triglyceride biosynthesis nor its inhibition by ER-27856 and RPR-107393. The reduction of triglyceride biosynthesis by ER-27856 and RPR-107393 was potentiated by mevalonolactone supplementation. Treatment of hepatocytes with farnesol and its derivatives reduced triglyceride biosynthesis. In addition, we found that ER-27856 and RPR-107393 significantly reduced the incorporation of [1-^{14}C]acetic acid into oleic acid, but not the incorporation of [1-^{14}C]oleic acid into triglyceride. Though ER-27856 and RPR-107393 increased mitochondrial fatty acid β-oxidation, the inhibition of β-oxidation by *RS*-etomoxir had little effect on their inhibition of triglyceride biosynthesis. These results suggest that SQS inhibitors reduce triglyceride biosynthesis by suppressing fatty acid biosynthesis via an increase in intracellular farnesol and its derivatives.**—Hiyoshi, H., M. Yanagimachi, M. Ito, N. Yasuda, T. Okada, H. Ikuta, D. Shinmyo, K. Tanaka, N. Kurusu, I. Yoshida, S. Abe, T. Saeki, and H. Tanaka. **Squalene synthase inhibitors suppress triglyceride biosynthesis through the farnesol pathway in rat hepatocytes. *J. Lipid Res.* 2003.** 44: **128–135.**

Supplementary key words ER-27856 • RPR-107393 • HMG-CoA reductase • squalene epoxidase • isoprenoid pathway

The isoprenoid metabolic pathway is involved in the synthesis of a wide range of cellular products (1). Farnesyl pyrophosphate (FPP) is at the key branch point of the pathway with the potential to be incorporated into either sterols or other non-sterol products, such as ubiquinone, dolichol, heme A, prenylated proteins, and farnesol. Squalene synthase (SQS, EC 2.5.1.21) reductively dimerizes FPP to form squalene, which is the first committed intermediate in the pathway to cholesterol. Selective inhibitors of SQS are thus of interest, because they should inhibit cholesterol biosynthesis without any deleterious effect on the branching pathways of isoprenoid metabolism (2–6).

We previously reported that ER-27856, a potent SQS inhibitor, lowered plasma cholesterol more potently, and with less adverse effects, than did HMG-CoA reductase inhibitors in rhesus monkeys (6). Inhibitors of SQS were also reported to be potent hypotriglyceridemic agents (4, 5, 7). Because elevated plasma triglyceride has attracted increased attention as a risk factor for coronary heart disease (CHD) in recent years (8–11), agents that potently lower both plasma cholesterol and triglyceride, such as SQS inhibitors, are expected to contribute to the primary, as well as secondary, prevention of CHD events.

Although the lipid-lowering mechanism of HMG-CoA reductase inhibitors has been intensively studied (12–16), it remains unknown how SQS inhibitors lower plasma triglyceride levels. Recently, we have reported that ER-27856, but not atorvastatin (an HMG-CoA reductase inhibitor), reduced plasma triglyceride through an LDL receptor-independent mechanism in Watanabe heritable hyperlipidemic (WHHL) rabbits (7). SQS inhibitors also reduced triglyceride biosynthesis in hepatocytes isolated from WHHL rabbits.

SQS inhibitors have been reported to increase farnesol and farnesol-derived dicarboxylic acids owing to the in-

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Abbreviations: CHD, coronary heart disease; CPT I, carnitine palmitoyltransferase I; SQS, squalene synthase; FPP, farnesyl pyrophosphate; MVL, mevalonolactone; SD rats, Sprague-Dawley rats; WHHL rabbit, Watanabe heritable hyperlipidemic rabbit.

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crease of FPP utilization in other branching pathways of isoprenoid metabolism (4, 17–20). Farnesol is produced from FPP by farnesyl pyrophosphatase (21). It exhibits a wide variety of biological activities, including cell growth inhibition (22, 23), induction of apoptosis (24, 25), and vascular tone regulation (26–28). In the lipid metabolism, farnesol accelerates the degradation of HMG-CoA reductase (29–32). Farnesol also acts as an agonist for orphan nuclear receptors, designated farnesoid X-activated receptor (33) and peroxisome proliferator-activated receptor α (34), both of which play important roles in lipid metabolism.

Here, we report that SQS inhibitors suppressed triglyceride biosynthesis in rat primary cultured hepatocytes, and that an increase in farnesol and its derivatives contributed to the effect by suppressing fatty acid biosynthesis. The role of farnesol and its derivatives in fatty acid metabolism is discussed.

MATERIALS AND METHODS

Chemicals

ER-27856 (4-[*N*-[(2*E*)-3-(2-Methoxyphenyl)-2-butenyl]-*N*-methylamino]-1,1-butylidenebisphosphonic acid tris (pivaloyloxymethyl) ester), RPR-107393, atorvastatin (35, 36), NB-598, farnesyl acetate, 3,7,11-trimethyl-2,6,10-dodecatriene-1,12-dioic acid methylester, 3,7-dimethyl-2,6-decadiene-1,10-dioic acid methylester, 3,7-dimethyl-2,6-octadiene-1,8-dioic acid methylester, and 3-methyl-2-hexamonoene-1,6-dioic acid methylester were synthesized in our laboratories. *RS*-Etomoxir was a gift from L. Agius (Department of Diabetes and Metabolism, University of Newcastle upon Tyne, UK). Insulin, dexamethasone, farnesol, squalene, and mevalonolactone (MVL) were purchased from Sigma (St. Louis, MO). Suberic acid was purchased from Nacalai tesque (Kyoto, Japan). Penicillin and streptomycin were from Life Technologies (Rockville, MD).

Animals

Five-week-old male Sprague-Dawley (SD) rats were obtained from Japan SLC (Shizuoka, Japan). Animals were housed in a well-ventilated (10–15 changes/h), temperature-controlled (23°C ± 3°C) room with constant humidity (55% ± 15%) under a reversed 12 h light/dark (22:00/10:00) cycle. Animals were fed with normal diet.

Preparation of rat primary hepatocytes

Primary hepatocytes were prepared from male SD rats (200–300 g) grown under an inverse light/dark cycle, as previously described (37). In the night phase, rats were anesthetized and heparinized intravenously. The liver was perfused with Liver Perfusion Medium (Life Technologies Inc., MD) at 37°C for 15 min at 20 ml/min., then with Liver Digest Medium (Life Technologies Inc.) for another 15 min. Liver cells were dispersed in Williams' E medium (pH 7.4) supplemented with 10% FBS, 0.1 μM insulin, 1 μM dexamethasone, 100 U/ml penicillin, and 100 μg/ml streptomycin by dissection and gentle shaking. After filtration through 70 μm nylon mesh filter, hepatocytes were isolated by repeated centrifugation (3–5 times) at 50 *g* for 2 min. Hepatocytes with >90% viability were cultured in Type I collagen-coated 24-well plates (Iwaki) at a cell density of 1×10^5 cells/well. After a 2-h incubation at 37°C in a 5% CO_2 atmosphere, non-attached cells were removed by washing with culture medium. Test compounds were added immediately after washing, and incubation was continued for 24 h. Hepatocytes were incubated overnight (18 h) before use in 6-h experiments.

Measurement of lipid biosynthetic activity of rat hepatocytes

Inhibition of lipid biosynthesis was determined by measuring the conversion of [1-^{14}C]acetic acid (185 kBq/ml, 1.85–2.29 GBq/mmol, Amersham Pharmacia Biotech, Little Chalfont, UK) or BSA-bound [1-^{14}C]oleic acid (2.47 kBq/ml, 16.7 MBq/mmol, PerkinElmer Life Sciences, Boston, MA) into cellular lipids.

For cholesterol and triglyceride biosynthesis, cells were incubated with or without compounds for the indicated time periods. [1-^{14}C]acetic acid or [1-^{14}C]oleic acid was added to the cells 2 h prior to harvest. Cells were washed twice and lipids were extracted by incubating the cells with 750 μl of hexane-2-propanol (3:2, v/v) for 30 min at room temperature. Aliquots were transferred to glass tubes and evaporated under a nitrogen stream. Samples were resuspended in 30 μl of chloroform, applied onto TLC plastic sheets (Silica gel 60 F_{254}, Merck, Darmstadt, Germany), and developed twice, first with toluene-isopropyl ether (1:1, v/v) for 10 min and then with heptane for 15 min. The radioactivities in the cholesterol and triglyceride fractions were analyzed by means of a BAS 2000 imaging plate system (Fuji Film, Tokyo, Japan).

To determine fatty acid biosynthesis, cells were incubated with a test compound for 4 h and with [1-^{14}C]acetic acid for an additional 2 h. The cells were then dissolved in 500 μl of 4 N KOH. Ethanol (1 ml) was added, and an aliquot of the mixture was saponified at 80°C for 30 min. Non-saponifiable lipids were removed by extracting with 3 ml of petroleum ether. The aqueous phase was mixed with 1 ml of 6 N HCl, and acid-insoluble fatty acids were extracted with 3 ml of petroleum ether. The extract was evaporated, and the residue was resuspended in 25 μl of chloroform, applied onto high-performance TLC sheets (RP-8 F_{254S}, Merck), and developed with a solvent (CH_3CN-H_2O-MeOH-HCOOH, 95:3:2:0.5, v/v/v/v). The radioactivities in the oleic acid fractions were analyzed by BAS 2000.

Measurement of β-oxidation activity of hepatocytes

β-Oxidation activity was determined by measuring ^3H_2O produced from [^{3}H]palmitic acid (200 μM, 167 MBq/mmol, Amersham Pharmacia Biotech) as described previously (38), with some modifications. Cells were incubated with or without a test compound for 4 h. Bovine serum albumin-bound 9,10-[^{3}H]palmitic acid was added, and the cells were incubated for an additional 2 h. An aliquot of the medium (300 μl) was taken and excess [^{3}H]palmitic acid was removed by precipitation with an equal volume of 10% perchloric acid. The supernatant (500 μl) was transferred to a microtube, which was placed uncapped in a scintillation vial containing 1 ml of unlabeled water. The vial was tightly capped and incubated at 50°C for 18 h to equilibrate ^3H_2O in the aliquot with water in the vial. Radioactivity in the vial was then measured by liquid scintillation counting.

Cellular protein determination

The amount of cellular protein was determined using BCA Protein Assay Reagent Kit (Pierce, IL).

Statistical analysis

Statistical analysis was conducted using the software package SAS 6.12 (SAS Institute Japan Ltd., Tokyo, Japan). The IC_{50} values were calculated by nonlinear regression analysis. Statistical evaluation was performed by means of a one-way ANOVA, followed by Dunnett's *t*-test for comparison with the control, unless otherwise specified.

RESULTS

Inhibitory effect of cholesterol biosynthesis inhibitors on lipid biosynthesis in rat hepatocytes

In order to study the effect of cholesterol biosynthesis inhibitors on lipid biosynthesis, rat hepatocytes were exposed to ER-27856 and PRR-107393 (SQS inhibitors), atorvastatin (an HMG-CoA reductase inhibitor), or NB-598 (a squalene epoxidase inhibitor), and the incorporations of [1-^{14}C]acetic acid into cholesterol and triglyceride were determined (**Fig. 1**). In a 6-h experiment, all the test compounds concentration-dependently inhibited cholesterol biosynthesis (Fig. 1A). The IC_{50} values were 4.3 nM for ER-27856, 880 nM for RPR-107393, 20 nM for atorvastatin, and 89 nM for NB-598. In contrast, inhibition of triglyceride biosynthesis was specific to SQS inhibitors (Fig. 1B). ER-27856 and RPR-107393 decreased the incorporation of [1-^{14}C]acetic acid into triglyceride in a concentration-dependent manner. The IC_{50} values for ER-27856 and RPR-107393 were 4.6 nM and 410 nM, respectively, which are comparable to the IC_{50} values for cholesterol biosynthesis. However, atorvastatin and NB-598 had no effect on the incorporation of [1-^{14}C]acetic acid into triglyceride, even at 10 μM.

In the time-course study, cells were treated with ER-27856 (1 μM), RPR-107393 (10 μM), atorvastatin (1 μM), or NB-598 (1 μM) for 2–24 h, and lipid biosynthesis during the last 2 h of the incubation was determined (**Fig. 2**). Again, all the test compounds inhibited cholesterol biosynthesis, but only the SQS inhibitors reduced triglyceride biosynthesis. The inhibition of cholesterol biosynthesis was maximal at 2 h, when [1-^{14}C]acetic acid was added to the cells immediately after the addition of the test compounds (Fig. 2A). On the other hand, the reduction of triglyceride biosynthesis by the SQS inhibitors gradually increased as the incubation time was extended, becoming maximal after 12 h to 24 h (Fig. 2B). In contrast to the case of cholesterol biosynthesis, SQS inhibitors did not completely halt triglyceride biosynthesis, and more than 10% of the total triglyceride biosynthetic activity was retained. These findings indicated a possibility that the decrease of triglyceride biosynthesis by squalene synthase inhibitors was a secondary response to the enzyme inhibition. Since neither atorvastatin nor NB-598 affected triglyceride biosynthesis, some factor induced by SQS inhibitors, but not by atorvastatin or NB-598, may suppress triglyceride biosynthesis.

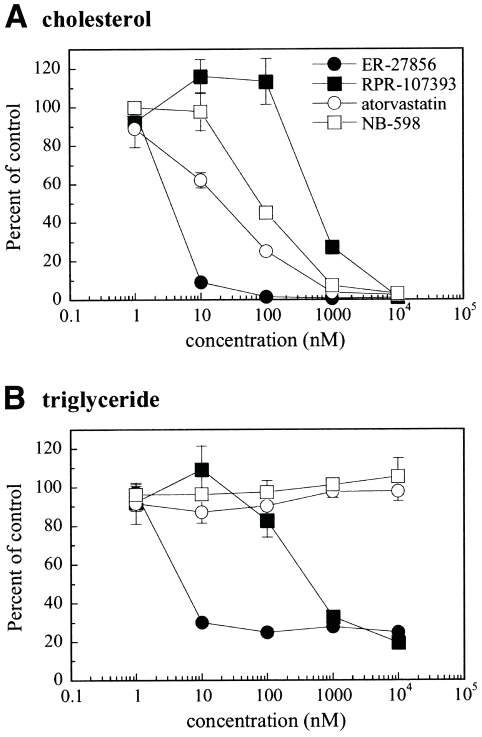

Fig. 1. Dose-dependent reduction of lipid biosynthesis in SQS inhibitor-treated rat hepatocytes. Rat primary cultured hepatocytes were treated with the indicated concentrations of ER-27856 (black circle), RPR-107393 (black square), atorvastatin (white circle), or NB-598 (white square) for 4 h. Cells were then incubated in the presence of [1-^{14}C]acetic acid (185 kBqml) for 2 h. Cellular lipids were extracted, and the radioactivities in cellular cholesterol (A) and triglyceride (B) were determined. Each point represents the mean ± SEM as percent of the control (n = 3).

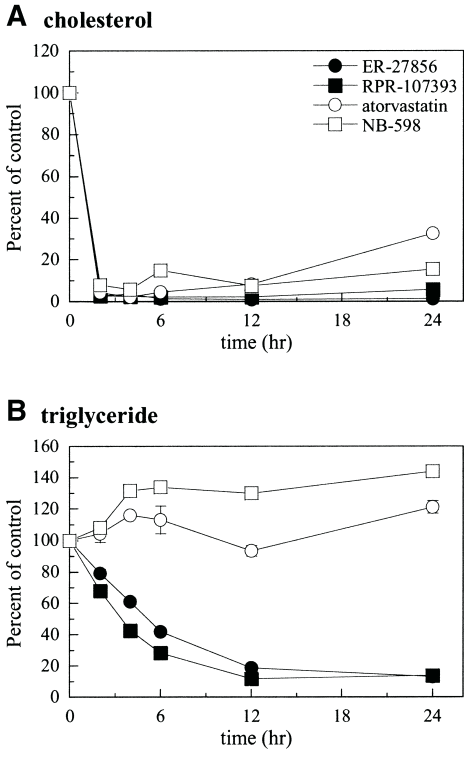

Fig. 2. Time-course of the reduction of lipid biosynthesis in SQS inhibitor-treated rat hepatocytes. Rat primary cultured hepatocytes were treated with 1 μM ER-27856 (black circle), 10 μM RPR-107393 (black square), 1 μM atorvastatin (white circle), or 1 μM NB-598 (white square) for 2–24 h, during the last 2 h of which the medium contained 1-[^{14}C]acetic acid. Cellular lipids were extracted, and the radioactivities in cellular cholesterol (A) and triglyceride (B) were determined. Each value represents the mean ± SEM as percent of the control (n = 3).

Effects of atorvastatin, NB-598, and MVL on the inhibition of triglyceride biosynthesis by SQS inhibitors

As SQS inhibitors suppress one of the branching pathways of FPP metabolism, changes in the amounts of other non-sterol products may affect the triglyceride biosynthetic activity. Therefore, we examined the effect of SQS inhibitors on triglyceride biosynthesis in the presence of atorvastatin or NB-598.

ER-27856 at 0.1 μM inhibited the biosynthesis of cholesterol and triglyceride by 98.2% and 71.1%, respectively (**Fig. 3A**). Although atorvastatin did not influence the cholesterol biosynthesis inhibition by ER-27856, it caused triglyceride biosynthesis to revert to the control level. NB-598 had no effect on the inhibition of either cholesterol or triglyceride biosynthesis by ER-27856. Similarly, 1 μM RPR-107393 inhibited cholesterol and triglyceride biosynthesis by 82.4% and 70.0%, respectively (Fig. 3B). Atorvastatin potentiated the inhibition of cholesterol biosynthesis by RPR-107393, but diminished the inhibition of triglyceride biosynthesis. NB-598 potentiated the inhibitory activity of RPR-107393 on cholesterol biosynthesis, but had no effect in the case of triglyceride biosynthesis.

Next, cells were treated with ER-27856 and RPR-107393 in the presence of 2 mM or 10 mM MVL to increase the levels of FPP derivatives. We found that the reduction of triglyceride biosynthesis was potentiated by MVL supplementation (**Fig. 4**). In the absence of MVL, the decreases of triglyceride biosynthesis by 0.1 μM ER-27856 and 1 μM RPR-107393 amounted to 33.3% and 36.2% of the control, respectively. The decreases were further potentiated by 2 mM (25.0% and 28.0%, respectively) and 10 mM MVL (19.5% and 21.1%, respectively). MVL alone, at 2 or 10 mM, did not decrease triglyceride biosynthesis under these conditions (data not shown). These results suggest that the reduction of triglyceride biosynthesis was mediated by an increase of FPP derivatives.

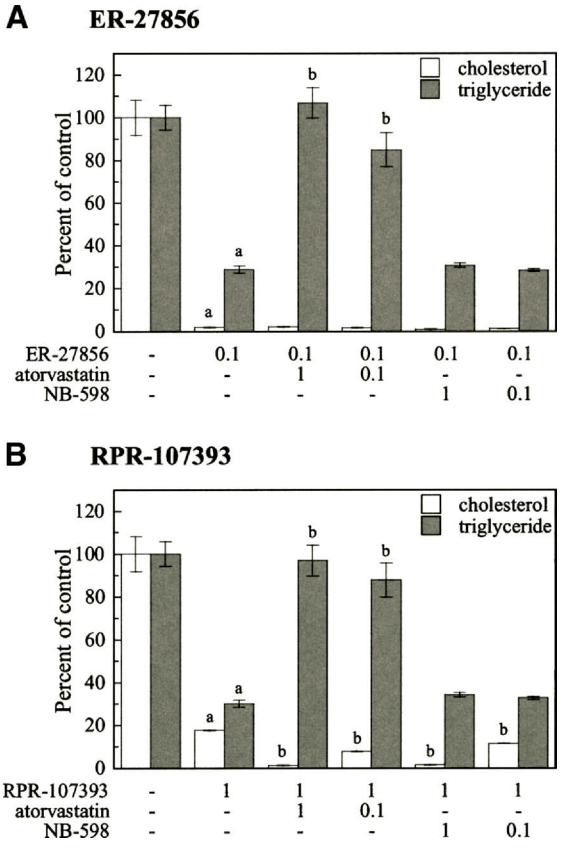

Fig. 3. Effects of atorvastatin and NB-598 on the lipid biosynthesis reduction in SQS inhibitor-treated hepatocytes. Rat hepatocytes were treated with 0.1 μM ER-27856 (A) or 1 μM RPR-107393 (B) in the presence of 1 μM atorvastatin, 0.1 μM atorvastatin, 1 μM N B-598, or 0.1 μM NB-598. After a 4-h treatment, [1-^{14}C]acetic acid (185 kBq/ml) was added to the medium and incubation was continued for 2 h. Cellular lipids were extracted, and the radioactivities in cellular cholesterol (open columns) and triglyceride (closed columns) were determined. Each value represents the mean ± SEM (n = 3) as percent of the control. A: Significantly different from the corresponding control with $P < 0.01$ by Student's *t*-test. B: Significantly different from the corresponding value of SQS inhibitor alone with $P < 0.01$ by Dunnett's *t*-test.

Effect of farnesol and its derivatives on lipid biosynthesis

SQS inhibitors increase farnesol and farnesol-derived dicarboxylic acids in vitro (17), and an increase was also reported in mice, rats, and beagle dogs (4, 18–20). To study the effects of farnesol and its derivatives on triglyceride biosynthesis, we added 100 μM farnesol, squalene, farnesyl acetate, 3,7,11-trimethyl-2,6,10-dodecatriene-1,12-dioic acid methylester, 3,7-dimethyl-2,6-decadiene-1,10-dioic acid methylester, 3,7-dimethyl-2,6-octadiene-1,8-dioic acid methylester, 3-methyl-2-hexamonoene-1,6-dioic acid methylester, and suberic acid to hepatocytes, and evaluated their ability to inhibit triglyceride biosynthesis (**Fig. 5**). Although squalene and suberic acid did not affect triglyceride biosynthesis, farnesol and its derivatives significantly reduced it. Farnesol and its derivatives also decreased the rate of cholesterol biosynthesis, but to a lesser extent,

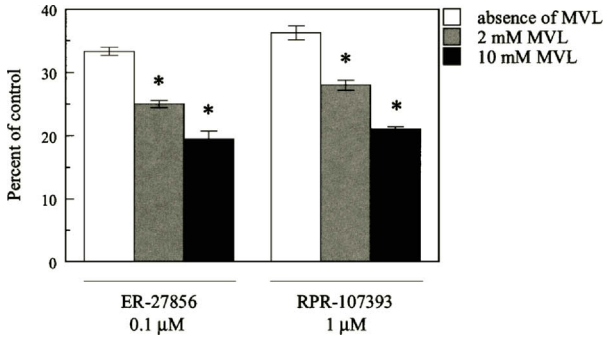

Fig. 4. Effect of MVL on triglyceride biosynthesis reduction in SQS inhibitor-treated hepatocytes. Rat hepatocytes were treated with 0.1 μM ER-27856 or 1 μM RPR-107393 in the absence (open columns), or in the presence of 2 mM (gray columns) or 10 mM (solid columns) of MVL. After 4 h treatment, [1-^{14}C]acetic acid (185 kBq/ml) was added to the medium and incubation was continued for 2 h. Cellular lipids were extracted, and the radioactivity in cellular triglyceride was determined. Each value represents the mean ± SEM (n = 3) as percent of the control (not shown). An asterisk indicates a significant difference from the control with $P < 0.01$ by Dunnett's *t*-test.

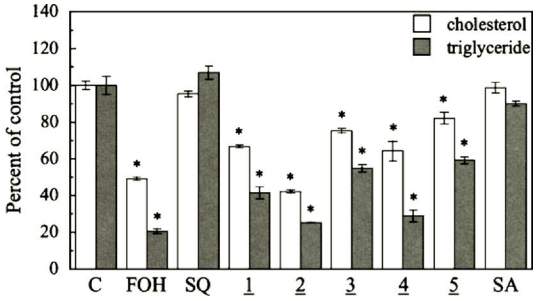

Fig. 5. Effect of FPP derivatives on lipid biosynthesis. Rat hepatocytes were treated with farnesol (FOH, 100 μM), squalene (SQ, 100 μM), farnesyl acetate (1, 100 μM), 3,7,11-trimethyl-2,6,10-dodecatriene-1, 12-dioic acid methylester (2, 100 μM), 3,7-dimethyl-2,6-decadiene-1,10-dioic acid methylester (3, 100 μM), 3,7-dimethyl-2,6-octadiene-1,8-dioic acid methylester (4, 100 μM), 3-methyl-2-hexamonoene-1,6-dioic acid methylester (5, 100 μM), or suberic acid (SA, 100 μM). After 4-h treatment, [1-^{14}C]acetic acid (185 kBq/ml) was added to the medium and incubation was continued for 2 h. Cellular lipids were extracted, and the radioactivities in cellular cholesterol (open columns) and triglyceride (gray columns) were determined. Each value represents the mean ± SEM (n = 3) as percent of the corresponding controls (open columns). An asterisk indicates a significant difference from the corresponding controls with $P < 0.01$ by Dunnett's *t*-test.

though the effects were well correlated with those on triglyceride biosynthesis. Because farnesol and its derivatives did not affect the rate of protein biosynthesis (data not shown), the effects cannot be attributed to cytotoxicity. The results suggest that the SQS inhibitors decreased triglyceride biosynthesis via an increase of farnesol and its derivatives, and that a common mechanism may regulate triglyceride and cholesterol biosynthesis.

Effect of SQS inhibitors on mitochondrial fatty acid β-oxidation

Changes of fatty acid β-oxidation affect the rate of triglyceride biosynthesis (39). In order to determine whether or not SQS inhibitors affected the fatty acid metabolic pathway, we first studied the effect of cholesterol biosynthesis inhibitors on fatty acid β-oxidation (**Fig. 6A**). In a 6-h incubation, ER-27856 increased the rate of overall β-oxidation by 23.6% at 10 nM, 25.2% at 100 nM, and 34.6% at 1 μM, and RPR-107393 did so by 26.5% at 1 μM, and 39.5% at 10 μM. However, atorvastatin and NB-598 had no effect at concentrations up to 1 μM. *RS*-Etomoxir, a carnitine palmitoyltransferase I (CPT I) inhibitor, reduced the overall β-oxidation activity by 70% at 1 μM. *RS*-Etomoxir suppressed the increase of β-oxidation by ER-27856 and RPR-107393, indicating that SQS inhibitors increase carnitine-dependent mitochondrial β-oxidation. However, *RS*-etomoxir had no effect on triglyceride biosynthesis or its reduction by ER-27856 and RPR-107393 (Fig. 6B). The data indicate that SQS inhibitors reduce overall triglyceride biosynthesis through a β-oxidation-independent pathway.

Effects of SQS inhibitors on fatty acid biosynthesis and on triglyceride synthesis from fatty acid

We next examined the effect of SQS inhibitors on fatty acid synthesis and fatty acid esterification. The overall inhibitory activity of SQS inhibitors on triglyceride biosynthesis was measured in terms of the incorporation of [1-^{14}C]acetic acid into triglyceride (**Fig. 7A**). ER-27856 (1 μM) and RPR-107393 (10 μM) decreased [1-^{14}C]acetic acid incorporation into triglyceride by 59.8% and 68.5%, respectively. The changes of incorporation of [1-^{14}C]acetic acid into tri-

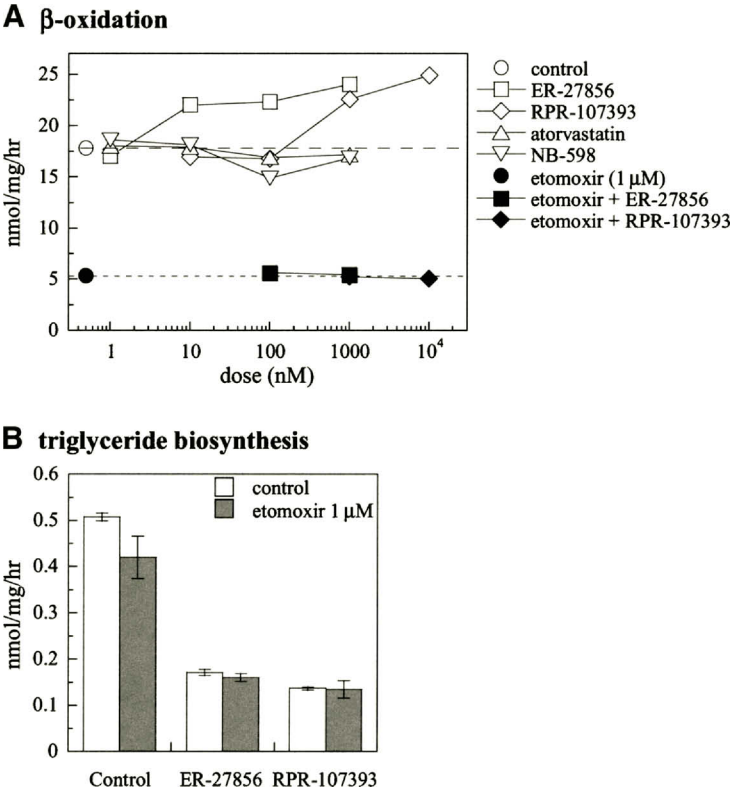

Fig. 6. Increase of fatty acid β-oxidation and decrease of triglyceride biosynthesis by SQS inhibitors. A: The activity of β-oxidation was measured by quantifying [^{3}H]H_2O produced from [9,10-^{3}H]palmitic acid as a result of β-oxidation. Rat hepatocytes were treated with the indicated concentrations of ER-27856 (white square), RPR-107393 (white diamond), atorvastatin (white triangle), or NB-598 (inverted white triangle) for 4 h. Hepatocytes were also incubated with ER-27856 (black square) or RPR-107393 (black diamond) in the presence of 1 μM *RS*-etomoxir (circle). [9,10-^{3}H]palmitic acid (33.4 kBq/ml) was added to the medium, and incubation was continued for 2 h. [^{3}H]H_2O released in the medium was determined. Each point represents nmol of palmitic acid β-oxidized/mg protein/h. B: Rat hepatocytes were treated with 1 μM ER-27856 or 10 μM RPR-107393 and incubated for 4 h. Cells were then incubated in the presence of [1-^{14}C]acetic acid (185 kBq/ml) for 2 h. Cellular lipids were extracted, and the radioactivity in cellular triglyceride was determined. Each point represents the mean value ± SEM as nmol of acetic acid incorporated into triglyceride/mg protein/h (n = 3).

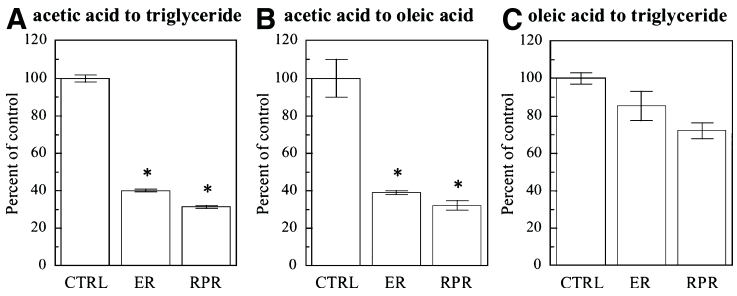

Fig. 7. Effect of SQS inhibitors on fatty acid biosynthesis. Rat hepatocytes were treated with 1 μM ER-27856 (ER) or 10 μM RPR-107393 (RPR) and incubated for 4 h. Incubation was then continued in the presence of [1-^{14}C]acetic acid (A, B) or [1-^{14}C] oleic acid (C) for 2 h. Cellular triglyceride (A, C) or oleic acid (B) was separated by TLC, and the radioactivity was determined. Each value represents the mean ± SEM (n = 3) as percent of the control (CTRL). An asterisk indicates a significant difference from the control with $P < 0.01$ by Dunnett's *t*-test.

glyceride were well correlated with those of fatty acid biosynthesis, detected as the incorporation of [1-^{14}C]acetic acid into oleic acid (Fig. 7B). ER-27856 decreased fatty acid biosynthesis by 60.9%, and RPR-107393 did so by 67.7%. On the other hand, ER-27856 and RPR-107393 did not affect later stages of triglyceride metabolism, detected as the incorporation of [1-^{14}C]oleic acid into triglyceride (Fig. 7C). Since fatty acid biosynthesis is a key step in the formation of triglyceride, these results clearly indicate that SQS inhibitors reduced triglyceride biosynthesis by suppressing fatty acid biosynthesis.

DISCUSSION

SQS inhibitors have been reported to reduce plasma cholesterol and plasma triglyceride more potently than HMG-CoA reductase inhibitors in experimental animal models (4, 5, 7). We have recently reported that ER-27856, but not atorvastatin, reduced plasma triglyceride through an LDL receptor-independent mechanism in WHHL rabbits, and that ER-27856 reduced triglyceride biosynthesis in hepatocytes isolated from WHHL rabbits (7).

In the present study, we further investigated the effect of SQS inhibitors on triglyceride biosynthesis in hepatocytes isolated from SD rats. The potency of each SQS inhibitor in reducing triglyceride biosynthesis was well correlated with that in reducing cholesterol biosynthesis (Fig. 1). The results suggest that SQS plays a role in the regulation of triglyceride biosynthesis. Neither atorvastatin nor NB-598 reduced the rate of triglyceride biosynthesis. These observations are consistent with previous findings obtained with simvastatin in rat hepatocytes (40), atorvastatin, NK-104, or NB-598 in HepG2 cells (41–43), and the open acid form of simvastatin and NB-598 in CaCo2 cells (44).

Our results indicate that SQS inhibitors reduce triglyceride biosynthesis via an increase of farnesol and its derivatives. The effect of SQS inhibitors on triglyceride biosynthesis was blocked by atorvastatin, but not by NB-598 (Fig. 3). The effect was potentiated by MVL supplementation (Fig. 4). Moreover, farnesol and its derivatives significantly reduced the rate of triglyceride biosynthesis (Fig. 5). It was reported that inhibition of SQS increased farnesol levels in cultured cells (17). In animal experiments, SQS inhibitors increased farnesol and farnesol-derived dicarboxylic acids in liver tissues (4, 18, 19). Bostedor and colleagues identified common dicarboxylic acids that were increased in urine of rats and dogs treated with either zaragozic acid A, a SQS inhibitor, or farnesol (20). The identified diacids included 3,7-dimethyl-2,6-octadiene-1,8-dioic acid (free acid form of compound 4 in Fig. 5), and the CoA ester of 3,7,11-trimethyl-2,6,10-dodecatriene-1,12-dioic acid (free acid form of compound 2) and 3,7-dimethyl-2,6-decadiene-1,10-dioic acid (free acid form of compound 3), all of which (as the methyl esters) decreased triglyceride biosynthesis in our study (Fig. 5). The results of these studies are therefore consistent with our hypothesis. Although the effect of SQS inhibitors on triglyceride biosynthesis was detected at 2 h after the start of incubation, the maximum reduction was not attained until 12 h. This observation indicates that the accumulation of farnesol and its derivatives is necessary for the maximum suppression of triglyceride biosynthesis.

Overall triglyceride biosynthetic activity in hepatocytes, measured in terms of the incorporation of [1-^{14}C]acetic acid into triglyceride, can be decreased by *1*) reduction of fatty acid biosynthesis and esterification, *2*) increase of fatty acid β-oxidation, and *3*) increase of lipolysis or triglyceride secretion as VLDL. Our studies clearly demonstrate that SQS inhibitors reduced triglyceride biosynthesis by decreasing fatty acid biosynthesis (Fig. 7). Interestingly, SQS inhibitors increased the rate of mitochondrial fatty acid β-oxidation (Fig. 6). In rat hepatocytes, fatty acid oxidation is reciprocally related to the rate of fatty acid biosynthesis and the malonyl-CoA content (45). Therefore, the decrease in cellular malonyl-CoA, following a reduction of fatty acid biosynthesis at acetyl-CoA carboxylase, the rate-limiting enzyme in fatty acid biosynthesis, might increase the CPT I-dependent β-oxidation in SQS inhibitor-treated hepatocytes. The increase, however, did not seem to play a major role in the inhibition of triglyceride biosynthesis by SQS inhibitors because a CPT I inhibitor, *RS*-etomoxir, did not influence the reduction of triglyceride biosynthesis.

Although the molecular mechanism through which farnesol and its derivatives reduce fatty acid biosynthesis remains to be elucidated, several mechanisms are potentially involved in the effect. The increase of farnesol and its derivatives may regulate fatty acid biosynthesis at the transcriptional level. Farnesol is known to act as an agonist for nuclear receptors such as farnesoid X-activated receptor and peroxisome proliferator-activated receptor α, both of which play important roles in lipid metabolism (33, 34). In fatty acid biosynthesis, expression of acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase is controlled at the transcriptional level by sterol regulatory element binding proteins (46–49). However, in our preliminary study (6 h incubation), 10 nM actinomy-

cin D, which reduced [^{3}H]uridine uptake by 70.1%, affected neither the rate of triglyceride biosynthesis (92.6% vs. control) nor the inhibitory effect of 0.1 μM ER-27856 on triglyceride biosynthesis (68.7% and 71.6% inhibition in the presence and absence of actinomycin D, respectively). These results indicate that the regulation of gene expressions was not involved in the reduction of fatty acid biosynthesis.

While farnesol and its derivatives reduced the rate of triglyceride biosynthesis, they also suppressed cholesterol biosynthesis, but to a lesser extent than triglyceride biosynthesis (Fig. 5). Although we do not know why triglyceride biosynthesis showed a higher susceptibility to farnesol and its derivatives than cholesterol biosynthesis, there may be a common mechanism underlying the regulation of cholesterol and triglyceride biosynthesis by isoprenoids. Farnesol and its derivatives may regulate the activities of enzymes in the fatty acid biosynthetic pathway through enzyme phosphorylation. Both HMG-CoA reductase and acetyl-CoA reductase are inactivated through phosphorylation by AMP-activated kinase and activated through dephosphorylation by type-2A protein phosphatases (50, 51). A common kinase pathway may therefore regulate the activity of these enzymes. Citrate and long-chain fatty acids are known to allosterically modify the acetyl-CoA carboxylase polymerization, which controls the phosphorylation state of the enzyme (52). Recently, glutamate was reported to act not only as an activator of type-2A protein phosphatases, but also as a direct allosteric ligand for dephosphorylated acetyl-CoA carboxylase (53). It is also of interest to know whether or not farnesol and its derivatives affect the allosteric control of acetyl-CoA carboxylase activity.

In addition, the activity of these enzymes may be regulated by their degradation. In case of cholesterol biosynthesis, farnesol and its derivatives have been reported to accelerate the degradation of HMG-CoA reductase (29–32). In yeast, the Hmg2P isozyme of HMG-CoA reductase is regulated through ubiquitination-dependent degradation by proteasome, and an FPP-derived signal increased the ubiquitination and consequently the degradation of HmgP2 (54, 55). In mammalian cells, both 25-hydroxycholesterol and mevalonic acid accelerated the degradation of HMG-CoA reductase, and the process was inhibited by lactacystin, a proteasome inhibitor, even though ubiquitination is not involved in sterol-induced HMG-CoA reductase degradation (56). It is possible that farnesol and its derivatives also induce the degradation of enzymes that are related to fatty acid biosynthesis.

In conclusion, our results indicate that SQS inhibitors reduce fatty acid biosynthesis by increasing the levels of farnesol and its derivatives, which consequently play a key role in the triglyceride-lowering effect of SQS inhibitors.

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