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Squalene synthase inhibitors reduce plasma triglyceride through a low-density lipoprotein receptor-independent mechanism

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Abstract

Inhibitors of squalene synthase are considered to be candidate drugs to reduce both plasma cholesterol and triglyceride. However, little is known about the mechanism of squalene synthase inhibitor-specific effect on plasma triglyceride. In this study, we confirmed the triglyceride-lowering effect of ER-27856, a potent squalene synthase inhibitor prodrug, in rhesus monkeys. To determine the role of low-density lipoprotein (LDL) receptor in the triglyceride-lowering effect of squalene synthase inhibitors, we intravenously administered ER-28448, the active form of ER-27856, to Watanabe heritable hyperlipidemic (WHHL) rabbits for 4 days. In heterozygotes, ER-28448 reduced plasma cholesterol and triglyceride by 52% and 37%, respectively. In homozygous rabbits, in contrast, ER-28448 lowered plasma triglyceride by 40% but did not lower plasma cholesterol. Orally administered ER-27856 reduced plasma triglyceride in homozygous animals but atorvastatin and bezafibrate did not. In hepatocytes isolated from homozygous WHHL rabbits, squalene synthase inhibitors but not atorvastatin reduced triglyceride biosynthesis. These data demonstrate that squalene synthase inhibitors reduced plasma triglyceride through an LDL receptor-independent mechanism, which was distinct from that of the triglyceride-lowering action of atorvastatin or bezafibrate. The reduction of hepatic triglyceride biosynthesis may play an important role in the hypotrigyceridemic action of squalene synthase inhibitors.

Keywords: Squalene synthase inhibitor; Triglyceride; Low-density lipoprotein (LDL) receptor; WHHL, rabbit; Hypertriglyceridemia

1. Introduction

In the cholesterol biosynthetic pathway, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) catalyzes the rate-limiting reduction of HMG-CoA to mevalonate. HMG-CoA reductase inhibitors such as atorvastatin significantly reduce plasma cholesterol and lower plasma triglyceride to a lesser extent in humans (Brown et al., 1998; Jones et al., 1998).

Squalene synthase (EC 2.5.1.21) catalyzes one of the subsequent reactions in the cholesterol biosynthetic pathway, i.e., it reductively dimerizes two farnesylpyrophosphate molecules to afford squalene, which is the first intermediate committed to cholesterol. Lipid-lowering by administration of squalene synthase inhibitors has been considered to be potentially valuable for the treatment of hyperlipidemia, since the inhibition of squalene synthase did not involve some of the adverse effects of HMG-CoA reductase inhibitors, such as the depletion of non-sterol mevalonate derivatives (Ciosek et al., 1993). We have recently reported that ER-28448 and its prodrug, ER-27856, are potent and selective inhibitors of squalene synthase and that ER-27856 potently reduced plasma cholesterol in rhesus monkeys without severe adverse effects (Hiyoshi et al., 2000).

Besides plasma cholesterol, squalene synthase inhibitors potently decreased plasma triglyceride in several animal models (Amin et al., 1997; Ugawa et al., 2000). There is accumulating evidence that plasma triglyceride is an independent risk factor in coronary heart diseases (Austin et al., 1998; Jeppesen et al., 1998; Sprecher, 1998). A retrospective cohort study suggested that plasma triglyceride levels are predictive of new coronary heart disease events, and that the threshold level for treatment may need to be reassessed as 100 mg/dl (Miller et al., 1998). Therefore, clinical efforts have been made to normalize both plasma

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cholesterol and triglyceride levels in patients with mixed hyperlipidemia. Though atorvastatin and other HMG-CoA reductase inhibitors reduce plasma triglyceride levels (Bakker-Arkema et al., 1996; Stein et al., 1996, 1998; Branchi et al., 1999), they are less effective than peroxisome proliferator-activated receptor (PPAR) α agonists such as bezafibrate (Goa et al., 1996). A combination of an HMG-CoA reductase inhibitor and a fibrate would be a possible approach in these patients, but is not recommended because of the increased prevalence of rhabdomyolysis.

In this study, we report that ER-27856 reduced plasma triglyceride as well as plasma cholesterol in rhesus monkeys. To determine the role of low-density lipoprotein (LDL) receptor in the triglyceride-lowering effect of squalene synthase inhibitors, we studied the hypolipidemic effects of ER-28448 and ER-27856 in Watanabe heritable hyperlipidemic (WHHL) rabbits, an LDL receptor-deficient animal model (Tanzawa et al., 1980; Watanabe, 1980; Havel et al., 1982). The mechanism of reduction of plasma triglyceride by squalene synthase inhibitors is discussed.

2. Materials and methods

2.1. Chemicals

ER-28448 (4-[*N*-[(2*E*)-3-(2-Methoxyphenyl)-2-butenyl]-*N*-methylamino]-1,1-butylidenebisphosphonic acid trisodium salt), ER-27856 (the tris(pivaloyloxymethyl) ester prodrug of ER-28448), RPR-107393, and atorvastatin (Brower et al., 1992; Baumann et al., 1992) were synthesized in our laboratories (Fig. 1). Bezafibrate, insulin, and dexamethasone were purchased from Sigma (St. Louis, MO, USA). Penicillin and streptomycin were from Life Technologies (Rockville, MD, USA).

2.2. Animals

Male rhesus monkeys were purchased from Charles River Laboratories (Key Lois, FL, USA). Male heterozygous (2-month-old) and homozygous (2-month-old) WHHL rabbits were purchased from BMR laboratories (Gifu, Japan). Animals were housed in a ventilated (10–15 times/h), temperature-controlled (23 ± 3 °C) room with constant humidity ($55 \pm 15\%$) under a 12-h light/dark (07:00/19:00) cycle, and were fed with normal diet. All procedures were conducted according to Eisai Animal Care committee's guideline.

2.3. Determination of plasma lipid in rhesus monkeys

Male rhesus monkeys were orally treated with compounds by intragastric intubation for 8 weeks. Blood samples were taken from radial veins of overnight-fasted monkeys at the beginning of the experiments and at 24 h after the last administration. Plasma samples were prepared by centrifugation (3000 rpm, 10 min). Plasma total cholesterol and plasma triglyceride levels were determined with an Olympus AU550. Plasma high-density lipoprotein

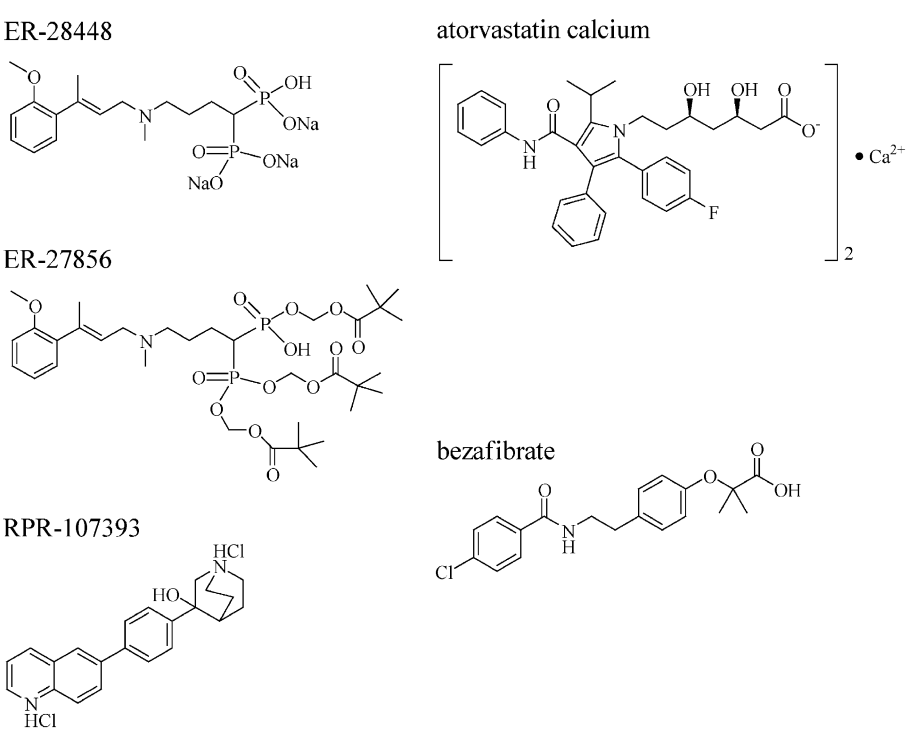

Fig. 1. Structures of SQSIs, atorvastatin, and bezafibrate.

(HDL) cholesterol levels were determined using HDL cholesterol test Wako (Wako, Osaka, Japan). Plasma non-HDL cholesterol was calculated by subtracting HDL cholesterol from plasma total cholesterol.

2.4. Determination of plasma lipid in WHHL rabbits

Heterozygous WHHL rabbits with baseline levels of 39–159 mg/dl of plasma cholesterol and 36–100 mg/dl of plasma triglyceride were used in this study. In homozygous WHHL rabbits, baseline plasma cholesterol and triglyceride were in the range of 451–1064 and 131–764 mg/dl, respectively. Rabbits were allocated to each group and treated with compounds for indicated periods. No decrease in food consumption was observed throughout the experiments. Blood samples were taken from the marginal ear vein of overnight-fasted rabbits. Plasma samples were analyzed as described above.

2.5. Preparation of rat primary hepatocytes

Primary hepatocytes were prepared from homozygous WHHL rabbits as previously described (Moldeus et al., 1978) with some modifications. Briefly, a rabbit was anesthetized by intravenous injection of sodium pentobarbital and 300 unit/kg of sodium heparin was intravenously injected. The liver was perfused with Liver Perfusion Medium (Life Technologies Oriental, Tokyo, Japan) at 37 °C for 15 min at 60 ml/min. The liver was then perfused with Liver Digest Medium (Life Technologies Oriental) supplemented with 1000 unit/ml dispase (Godo Shusei, Tokyo, Japan) for another 15 min. It was carefully transferred to a plastic dish and gently torn apart in Williams' E medium (pH 7.4) supplemented with 10% fetal bovine serum, 0.1 μM insulin, 1 μM dexamethasone, 100 units/ml penicillin, and 100 μg/ml streptomycin, and cells were dispersed by gentle shaking. After filtration through 70-μm nylon mesh filter, hepatocytes were isolated by repeated washing and centrifugation at $50 \times g$ for 2 min. Hepatocytes with $>90\%$ viability were plated in Type I collagen-coated 24-well plates (Iwaki Glass, Tokyo, Japan) at 1×10^5 cells/well. After 2 h incubation at 37 °C in a 5% CO_2 incubator, non-attached cells were removed by washing with the medium.

2.6. Measurement of inhibitory activity on lipid biosynthesis in hepatocytes

Inhibitory activity on lipid biosynthesis was determined by measuring the conversion of [1-^{14}C]acetic acid (185 kBq/ml, 1.66–2.22 GBq/mmol, NEN Life Science Products, MA, USA) into cholesterol and triglyceride. Cells were incubated with or without a test compound for indicated time periods. [1-^{14}C]Acetic acid was added to the cells 2 h prior to harvest. Cells were washed two times with phosphate-buffered saline, and lipids were extracted by incubating the cells with 750 μl of hexane/2-propanol (3:2) 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 to plastic-backed thin layer chromatography sheets, and developed two times, first with toluene/isopropyl ether (1:1) for 10 min and then with heptane for 15 min. The radioactivities in the cholesterol and triglyceride fractions were analyzed by a BAS 2000 imaging plate system (Fuji Film, Tokyo, Japan).

2.7. Statistical analysis

Statistical analysis was conducted using the software package, SAS 6.12 (SAS Institute Japan, Tokyo, Japan). In the time-course study in WHHL rabbits, the percent change from baseline during administration (day 1 and day 4) was analyzed by repeated measures ANOVA, followed by Student's *t*-test for comparison with controls. In other experiments, statistical evaluation was basically performed

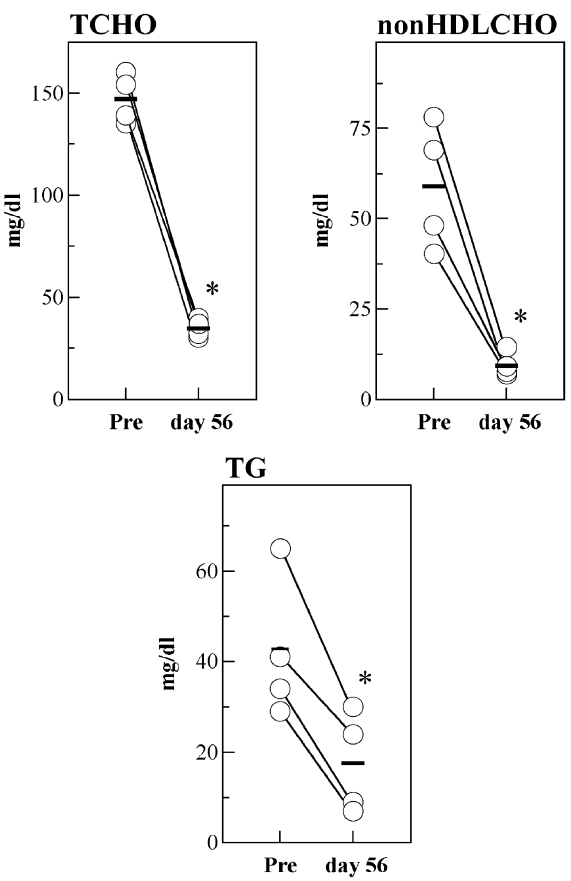

Fig. 2. Effect of ER-27856 on plasma lipids in rhesus monkeys. Rhesus monkeys were treated orally with 10 mg/kg of ER-27856 for 8 weeks. Plasma total cholesterol (TCHO), non-HDL cholesterol (non-HDLCHO), and plasma triglyceride (TG) concentrations of four monkeys at the end of treatment (day 56) were compared with pre-treatment values (Pre). Bars represent the means of plasma lipid concentration ($n = 4$). An asterisk represents a significant difference from the pre-treatment value, with $p < 0.01$ by paired *t*-test.

by means of a one-way ANOVA, followed by Dunnett's *t*-test for comparison with controls.

3. Results

3.1. Hypolipidemic effect of ER-27856 in rhesus monkeys

To study the effect of squalene synthase inhibition on plasma cholesterol and triglyceride, ER-27856 was orally administered to rhesus monkeys for 8 weeks (Fig. 2). ER-27856 reduced plasma cholesterol by 76%. Analysis of lipid components indicated that non-HDL cholesterol was decreased 84%, suggesting that the decrease of non-HDL cholesterol mainly contributed to the reduction of plasma cholesterol. Importantly, ER-27856 also reduced plasma triglyceride by 59%. These data suggest that ER-27856 has a potent activity to decrease both plasma cholesterol and triglyceride in rhesus monkeys.

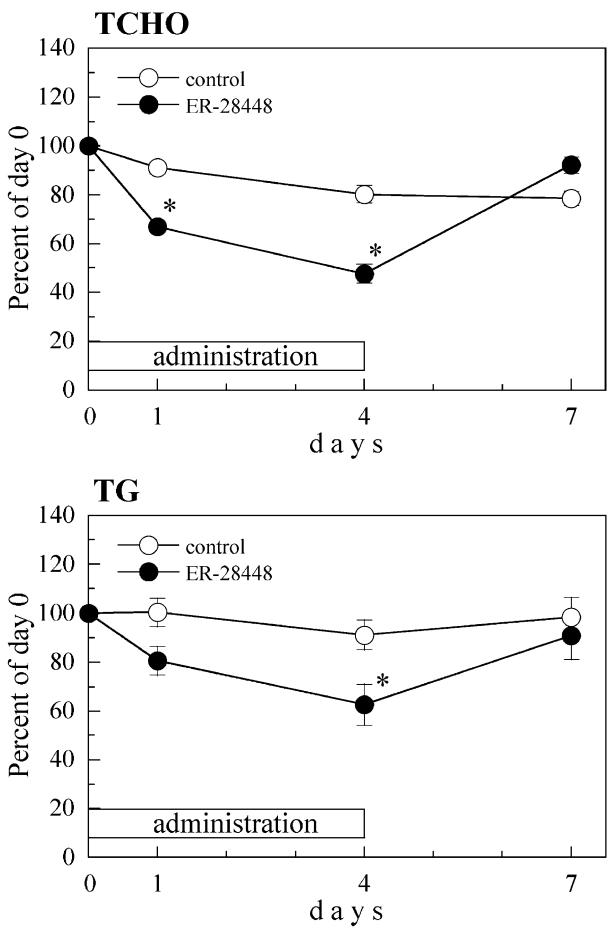

Fig. 3. Effect of ER-28848 on plasma lipids in heterozygous WHHL rabbits. Heterozygous WHHL rabbits were intravenously treated either with vehicle (open circles, $n = 4$) or with 2 mg/kg of ER-28448 (closed circles, $n = 5$) for 4 days. Blood samples were taken before feeding and prior to administration on days 0, 1, 4, and 7. Plasma samples were analyzed for plasma total cholesterol (TCHO) and plasma triglyceride (TG). Each point represents the mean S.E.M. of percent values from day 0. Data during administration were analyzed by repeated measures ANOVA, followed by *t*-test for comparisons with controls. An asterisk represents a significant difference from the control with $p < 0.05$ by post hoc Student's *t*-test.

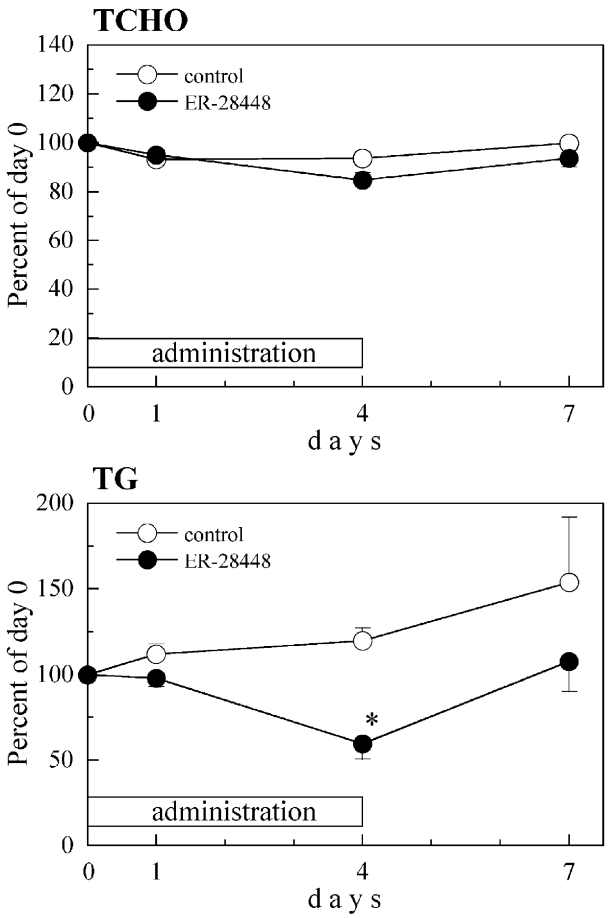

Fig. 4. Effect of ER-28448 on plasma lipids in homozygous WHHL rabbits. Homozygous WHHL rabbits were intravenously treated with either vehicle (open circles, $n = 3$) or 2 mg/kg of ER-28448 (closed circles, $n = 3$) for 4 days. Blood samples were taken before feeding and prior to administration on days 0, 1, 4, and 7. Plasma samples were analyzed for plasma total cholesterol (TCHO) and plasma triglyceride (TG). Each point represents the mean ± S.E.M. of percent values from day 0. Data during administration were analyzed by repeated measures ANOVA, followed by *t*-test for comparison with controls. Variance of data in plasma cholesterol was not significant. An asterisk represents a significant difference from the control, with $p < 0.05$ by post hoc Student's *t*-test.

3.2. Hypolipidemic effect of ER-28448 in heterozygous and homozygous WHHL rabbits

To determine the role of LDL receptor in the triglyceride-lowering effect of squalene synthase inhibitor, we intravenously administered ER-28448 to WHHL rabbits for 4 days. In heterozygotes, ER-28448 significantly reduced plasma cholesterol and triglyceride (Fig. 3). The effects were statistically significant versus the control at day 1 and day 4 for cholesterol, and at day 4 for plasma

Table 1
Effect of ER-27856 on plasma lipids in homozygous WHHL rabbits: comparison with atorvastatin and bezafibrate

	Treatment	Dose	*N*	TCHO	TG
Experiment 1	Control		4	−5.2±4.3	13.9±18.2
	ER-27856	10	4	−11.4±3.2	−40.9±14.2†
	ER-27856	20	4	−9.2±4.7	−45.2±4.0†
Experiment 2	Control		6	−2.8±1.1	−5.5±2.0
	Atorvastatin	10	4	−7.6±2.0	−12.8±5.1
	Atorvastatin	30	4	−18.0±9.4	−7.9±11.0
	Bezafibrate	10	4	−5.5±0.4	−3.5±7.3
	Bezafibrate	30	4	−6.6±0.9	28.9±22.1
Experiment 3	Control		5	−8.2±2.2	31.3±8.4
	ER-27856	30	5	−15.9±4.4	−54.0±3.4†
	Atorvastatin	30	5	−13.7±0.7	16.0±13.7
	Atorvastatin	100	5	−15.5±2.8	8.8±17.0

Homozygous WHHL rabbits were treated orally with ER-27856, atorvastatin, or bezafibrate for 4 days. Blood samples were taken and plasma total cholesterol (TCHO) and triglyceride (TG) were determined. Doses were expressed in mg/kg/day. Each value represents the mean ± S.E.M. percent change from baseline.

†A significant difference from the control with $p < 0.05$ by Dunnett's *t*-test.

triglyceride. At day 4, ER-28448 decreased plasma cholesterol by 52% and plasma triglyceride by 37% from the pre-treatment values. At 3 days after the end of treatment, both plasma cholesterol and triglyceride had recovered to the pre-treatment levels.

In homozygous WHHL rabbits, the cholesterol-lowering effect of ER-28448 was not statistically significant at any time examined (Fig. 4). On the other hand, ER-28448 significantly reduced plasma triglyceride, and the decrease at day 4 was 40% from the pre-treatment value. These data indicate that ER-28448 reduced plasma cholesterol through an LDL receptor dependent mechanism, but reduced plasma triglyceride through an LDL receptor-independent mechanism.

3.3. Hypolipidemic effects of ER-27856, atorvastatin, and bezafibrate in homozygous WHHL rabbits

In order to study the effect of squalene synthase inhibitor in plasma triglyceride in comparison with HMG-CoA reductase inhibitors and PPARα agonists, we orally administered ER-27856, atorvastatin, and bezafibrate to homozygous WHHL rabbit for 4 days (Table 1). Neither ER-27856, atorvastatin nor bezafibrate decreased plasma cholesterol. LDL receptor may play a key role in the reduction of plasma cholesterol by these agents. In contrast, ER-27856 potently reduced plasma triglyceride by 41% at 10 mg/kg, 45% at 20 mg/kg, and 54% at 30 mg/kg. However, atorvastatin and bezafibrate failed to lower plasma triglyceride at dose levels up to 100 and 30 mg/kg, respectively. These results indicate that the mechanism through which ER-27856 reduced plasma triglyceride is distinct from that of atorvastatin or bezafibrate, and that LDL receptor is not important for the reduction of plasma triglyceride by ER-27856.

3.4. Effects of ER-27856, RPR-107393, and atorvastatin on lipid biosynthesis in primary hepatocytes isolated from homozygous WHHL rabbits

In order to elucidate the mechanism through which plasma triglyceride is decreased by squalene synthase inhibitor, we examined the inhibitory effects of ER-27856, RPR-107393, another squalene synthase inhibitor, and atorvastatin on lipid biosynthesis in primary cultured hepatocytes isolated from homozygous WHHL rabbits (Fig. 5). In 2 h incubation, ER-27856 (1 μM), RPR-107393 (10 μM), and atorvastatin (1 μM) completely inhibited cholesterol biosynthesis without significant effect on triglyceride biosynthesis. In 24 h incubation, however, ER-27856 and RPR-107393 potently reduced triglyceride biosynthesis by

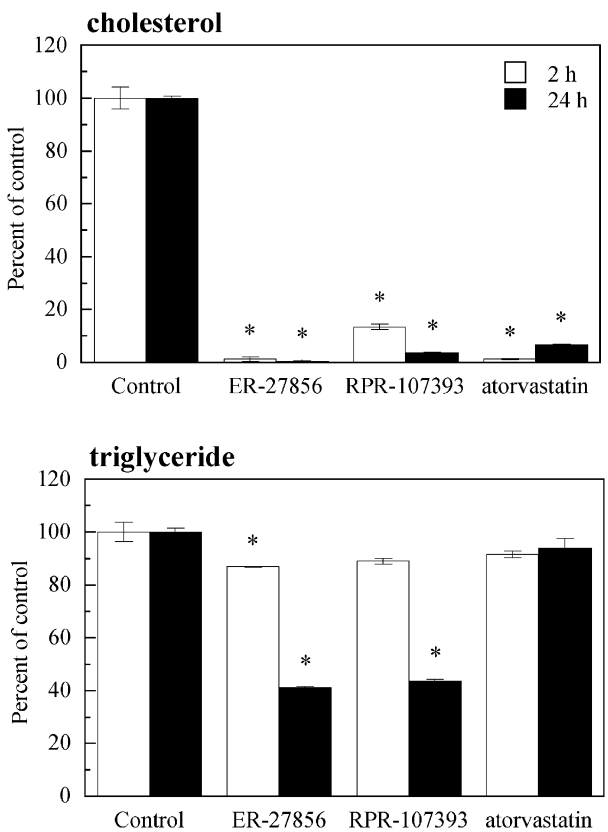

Fig. 5. Effect of ER-27856, RPR-107393, and atorvastatin on cholesterol and triglyceride biosynthesis in hepatocytes isolated from homozygous WHHL rabbits. In 24-h experiments (closed column), hepatocytes were incubated with compounds for 18 h. At that point, the media were changed, compounds were re-added to corresponding wells, and cells were incubated for a further 4 h before [1-^{14}C]acetic acid was added. In 2-h experiments (open column), compounds and [1-^{14}C]acetic acid were added simultaneously. Cells were incubated for 2 h in the presence of [1-^{14}C]acetic acid. The radioactivities in cellular cholesterol and triglyceride fractions were expressed as the mean S.E.M. in percent of control ($n = 3$). An asterisk represents a significant difference from the corresponding control with $p < 0.01$ by Dunnett's *t*-test.

59% and 56%, respectively, in addition to continued inhibition of cholesterol biosynthesis. Even though atorvastatin inhibited cholesterol biosynthesis, it did not reduce triglyceride biosynthesis. These results suggest that the complete inhibition of squalene synthase leads to the reduction of triglyceride biosynthesis, and that squalene synthase inhibitors lower plasma triglyceride by reducing hepatic triglyceride biosynthesis in WHHL rabbits.

4. Discussion

Squalene synthase inhibitors have been reported as potent lipid-lowering drugs. RPR-107393 reduced plasma triglyceride in marmosets (Amin et al., 1997), in which pravastatin, an HMG-CoA reductase inhibitor, was reported not to affect plasma triglyceride levels (Miyazaki and Koga, 1998). In a recent report, a squalene synthase inhibitor, YM-53601, but not pravastatin reduced plasma triglyceride in hamsters fed with normal or high-fat diet (Ugawa et al., 2000). We have recently reported that ER-28448 and its prodrug, ER-27856, are potent and selective inhibitors of squalene synthase in rats and rhesus monkeys (Hiyoshi et al., 2000). In this study, ER-27856 potently reduced plasma triglyceride in rhesus monkeys. These results suggest that squalene synthase inhibitors may involve an additional effect on plasma triglyceride.

ER-28448 potently lowered plasma cholesterol by 52% in WHHL rabbit heterozygotes. On the other hand, the cholesterol-lowering effect of ER-28448 was not significant and ER-27856 and atorvastatin failed to lower plasma cholesterol in homozygotes. These results demonstrated that LDL receptor is a common mediator of the reduction of plasma cholesterol by cholesterol biosynthesis inhibitors in WHHL rabbits. Unlike plasma cholesterol lowering, the reduction of plasma triglyceride by ER-28448 was independent of hepatic LDL receptor activity. ER-28448 reduced plasma triglyceride not only in heterozygous, but also in homozygous WHHL rabbits. These data suggest that squalene synthase inhibitors may affect triglyceride metabolism through an unidentified mechanism.

The mechanism through which squalene synthase inhibitor decreases plasma triglyceride seems to be different from that of HMG-CoA reductase inhibitor. The lipid-lowering effect of HMG-CoA reductase inhibitors is mediated by the induction of hepatic LDL receptor (Bilheimer et al., 1983; Reihner et al., 1990) and by the decrease of very low-density lipoprotein (VLDL) secretion from the liver as secondary responses to the inhibition of cholesterol biosynthesis (Ginsberg et al., 1987; Arad et al., 1990; Bakker-Arkema et al., 1996). Naka et al. reported that both the stimulation of hepatic LDL receptor-mediated clearance of VLDL particles and the suppression of hepatic VLDL secretion were involved in the triglyceride-lowering effect of fluvastatin in heterozygous WHHL rabbits (Naka et al., 1998). However, HMG-CoA reductase inhibitors did not alter plasma triglyceride levels in homozygous WHHL rabbits (Watanabe et al., 1981; Khachadurian et al., 1991; Shiomi and Ito, 1994, 1999; Shiomi et al., 1999). Therefore, the increase of LDL receptor-mediated VLDL clearance, rather than the reduction of hepatic VLDL secretion, may contribute to the triglyceride-lowering effect of HMG-CoA reductase inhibitors in heterozygous WHHL rabbits. In our study, 10 mg/kg of ER-27856 but not 100 mg/kg of atorvastatin reduced plasma triglyceride in homozygous WHHL rabbits, in agreement with previous findings on other HMG-CoA reductase inhibitors. Because the dose of atorvastatin used in this study was high enough to inhibit hepatic cholesterol biosynthesis, it is likely that the triglyceride-lowering effect of squalene synthase inhibitors was independent of the inhibition of cholesterol biosynthesis per se.

One possibility is that plasma triglyceride reduction by squalene synthase inhibitor is mediated by PPARα. Agonist-bound PPARα binds to retinoic X receptor to form a functional heterodimeric complex that activates genes that contain a peroxisome proliferator responsive element in their promoters. PPARα agonists such as bezafibrate reduce plasma triglyceride levels by multivalent mechanisms, including the induction of triglyceride hydrolysis and hepatic fatty acid uptake, and the reduction of hepatic triglyceride production. Recently, farnesol was reported to stimulate epidermal keratinocyte differentiation via PPARα activation (Hanley et al., 2000). Because the inhibition of squalene synthase increased farnesol and its derivatives in cultured cells (Bergstrom et al., 1993) and in liver (Keller, 1996; Amin et al., 1997; Vaidya et al., 1998), it is possible that squalene synthase inhibitors affect triglyceride metabolism by increasing farnesol and its derivatives, which act as native ligands for PPARα. In this study, however, bezafibrate was not effective in homozygous WHHL rabbits, indicating that reduction of plasma triglyceride by squalene synthase inhibitors was not mediated by PPARα activation.

Mechanisms through which squalene synthase inhibitors reduced plasma triglyceride presumably involve either decreased secretion and/or increased clearance of plasma triglyceride. In primary cultured hepatocytes isolated from homozygous WHHL rabbits, 24-h treatment with ER-27856 resulted in a decrease of triglyceride biosynthesis. These data indicate that ER-27856 lowered plasma triglyceride by reducing the rate of hepatic triglyceride biosynthesis. The reduction of triglyceride biosynthesis was also observed in hepatocytes treated with RPR-107393. Therefore, it is likely that the reduction of triglyceride biosynthesis which decrease the triglyceride content in nascent VLDL resulted in the squalene synthase inhibitor-specific reduction of plasma triglyceride in animal models. This hypothesis was supported by an observation in cholestyramine-fed rats. ER-27856 and RPR-107393 showed significant plasma triglyceride reduction, which was attributed to the decrease of hepatic triglyceride secretion (data not shown). On the

other hand, squalene synthase inhibitors did not affect plasma triglyceride clearance either in homozygous WHHL rabbits or in cholestyramine-fed rats.

In conclusion, we demonstrated that ER-28448 and ER-27856 but not atorvastatin reduced plasma triglyceride in LDL receptor-deficient rabbits. The triglyceride-lowering effect was not attributed to the inhibition of cholesterol biosynthesis, but to the reduction of triglyceride biosynthesis. Studies with squalene synthase inhibitors may provide a new approach to treat patients with mixed dyslipidemia by reducing both plasma cholesterol and triglyceride.

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