

Regulation of squalene epoxidase activity in rat liver

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Abstract Regulation of squalene epoxidase activity was examined in rat hepatic microsomes. The hepatic squalene epoxidase activity was high in the dark period and low in the light period. Three percent cholestyramine feeding increased the hepatic squalene epoxidase activity by 2.5-fold, and the administration of lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, increased its activity by 2.1-fold. Co-administration of cholestyramine and lovastatin had a synergistic effect increasing the activity by 7.0-fold. On the other hand, cholesterol feeding reduced hepatic squalene epoxidase activity to 18%. The addition of sodium fluoride, a phosphatase inhibitor, and the treatment of the microsomes with ATP-Mg²⁺ had no effect on enzyme activity. ■ HMG-CoA reductase activity has been reported to be regulated by cholesterol and unidentified metabolite(s) derived from mevalonate. However, since squalene epoxidase is less responsive than the reductase to the treatment with lovastatin, squalene epoxidase is thought to be regulated only by cholesterol pool. —Satoh, T., Y. Hidaka, and T. Kamei. Regulation of squalene epoxidase activity in rat liver. *J. Lipid Res.* 1990. **31**: 2095–2101.

Supplementary key words cholesterol synthesis • 2,3-oxidosqualene • lovastatin • HMG-CoA reductase

In an accompanying paper (1) we reported that squalene epoxidase activity is regulated by the intracellular level of cholesterol in HepG2 cells (human hepatoma cell line). L-654, 969, an analogue of lovastatin (HMG-CoA reductase inhibitor), increased squalene epoxidase activity in cultured cells, and low density lipoprotein (LDL) decreased it. In contrast to the regulation of HMG-CoA reductase, squalene epoxidase activity was thought to be suppressed only by sterols, not by nonsterol derivative(s) of mevalonate in HepG2 cells.

Eilenberg and Shechter (2) reported that squalene epoxidase is an important site in the cholesterol synthetic pathway in rats and Chinese hamster ovary cells. However, their data were qualitative, but not quantitative, because the method for determination of squalene epoxidase activity was not well established. Therefore, we developed a method to determine squalene epoxidase activity in rat hepatic microsomes, and examined the regulatory mechanism of this enzyme in vivo by dietary and drug treatment of rats.

MATERIALS AND METHODS

Materials

Lovastatin (3) was prepared by Merck Sharp and Dohme Research Laboratories (Rahway, NJ). Cholestyramine was purchased from Bristol-Myers (Tokyo, Japan). All other chemicals used were same as described in the accompanying study (1).

Animals

Female Sprague-Dawley rats aged 6 weeks and weighing 132–168 g (at the time of killing) were used in this study. These animals were purchased from Charles River Japan (Atsugi, Japan). Rats were kept on a reversed light regime (6 AM–6 PM, dark, 6 PM–6 AM, light) for 2 weeks. Animals were treated for 1 week as follows. In the nontreated control group (A), rats were given a normal diet (CE-2, Nihon Clea, Tokyo, Japan). In group B, rats were given 3% cholestyramine in the diet. A diet containing 2% cholesterol and 2% squalene was given to rats in C and D groups, respectively. In group E, rats were given a normal diet and were administered 0.5% carboxymethylcellulose (CMC) suspension as a control vehicle at 10 AM every day. Lovastatin (50 mg/kg, 0.1 ml/kg) suspended in 0.5% CMC was administered using a stomach tube to rats fed a normal diet in group F at 10 AM every day. In group G, rats fed a 3% cholestyramine diet were given 50 mg/kg of lovastatin at 10 AM every day. Five rats were used in each group. Each rat was given tap water ad libitum. Rats were killed at 10 AM, 24 h after the last drug treatment unless otherwise stated.

Preparation of microsomes

The microsomal squalene epoxidase from rat liver was prepared according to Yamamoto and Bloch (4) with some modifications. Rats were bled from the heart under ether anesthesia; livers were perfused with 50 ml of cold 0.1 M

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LDL, low density lipoproteins; CMC, carboxymethylcellulose.

Tris-HCl buffer, pH 7.5, via the portal vein, minced, and homogenized with 2 volumes of the same buffer, pH 7.5. After the homogenate was twice centrifuged at 9,750 g for 10 min, the supernatant was recentrifuged at 105,000 g for 60 min. The microsomes were washed once with 0.1 M Tris-HCl buffer, pH 7.5, and used for the assay of squalene epoxidase. The 105,000 g supernatant was used as the S_{105} fraction. All fractions were kept at -80°C until use.

Assay of squalene epoxidase

Squalene epoxidase activity was assayed as described in the accompanying paper (1) except for the minor changes of reagent concentration and incubation time. The standard assay mixture consisted of a total volume of 0.3 ml containing 0.75 mg/ml microsomes, 67 mM Tris-HCl buffer, pH 7.5, 100 μM FAD, 1 mM NADPH, 1 mM EDTA, 0.1% Triton X-100, 0.3 mM AMO 1618 (an inhibitor of 2,3-oxidosqualene lanosterol cyclase) (5), and 8 μM [^3H]squalene (20,000 dpm) dispersed in 0.075% Tween 80. Incubation was for 30 min at 37°C . Enzyme activity is expressed in pmol of 2,3-oxidosqualene formed per mg of microsomal protein per min.

In the experiment to determine the effect of S_{105} fraction on squalene epoxidase activity, enzyme activity was determined with 3.4 mg/ml protein of S_{105} fraction in the place of Triton X-100.

Assay of HMG-CoA reductase

HMG-CoA reductase activity was determined according to the method described by Shapiro and Rodwell (6) and Edwards, Lemongello, and Fogelman (7).

Determination of serum total cholesterol

Serum total cholesterol was measured by an autoanalyzer (Centrifichem, Encore, Baker Instruments Co., Allenton, PA), using an enzymatic assay kit (Determiner TC 555, Kyowa Medex Co., Tokyo, Japan).

Determination of cholesterol content in the liver

Lipid in the liver was extracted according to Folch, Lees, and Sloane Stanley (8). The lipid extract was evaporated under a stream of nitrogen gas, and redissolved in isopropyl alcohol. Total cholesterol concentration was determined as described above.

Determination of protein

The protein concentrations were determined according to the method of Lowry et al. (9) using bovine serum albumin as a standard.

Determination of squalene

Fifty μl of liver homogenate was mixed with 4 volumes of isopropyl alcohol. After centrifugation, the supernatant fraction was analyzed for squalene by high performance liquid chromatography (HPLC, JASCO, Tokyo, Japan). Ten

μl of sample was injected onto a Capcell pak C18 column (5 μm , I.D. 4.6×150 mm) (Shiseido, Tokyo, Japan) equipped with a pre-column (LiChrospher 100, RP-18e, E. Merck, Darmstadt, West Germany). Acetonitrile-isopropyl alcohol-water 70:28:2 (v/v) was used as a mobile phase. The squalene peak was detected by absorption at 210 nm. A calibration curve was established with known injected amounts of squalene ranging from 0.002 to 0.02 μg .

Data analysis

Statistical analysis of the data was carried out using Wilcoxon's rank sum test. The variations in all mean values in the table and figures are expressed as standard deviation (SD).

RESULTS

Squalene epoxidase assay in rat hepatic microsomal preparation

The method for determination of squalene epoxidase activity in rat liver microsomes was examined. The optimum Triton X-100 concentration depended on the microsomal protein concentration. Squalene epoxidase activity was maximally activated by 0.1% Triton X-100 at protein concentrations between 0.5 and 1.0 mg/ml (data not shown). Therefore, 0.1% Triton X-100 and 0.75 mg/ml protein concentration were selected for the routine assay. As shown in Fig 1a, the enzyme activity in microsomal preparations obtained from cholestyramine-treated rats was almost saturated at substrate concentrations higher than 8 μM . At a substrate concentration of 8 μM , 2,3-oxidosqualene formation was linear with incubation time up to 30 min (Fig. 1b). For microsomal protein concentrations from 0.25 to 0.75 mg/ml, linearity of enzyme activity was observed (Fig. 1c). FAD and NADPH were essential for enzyme reaction (data not shown). AMO 1618 (5) was needed to block 2,3-oxidosqualene lanosterol cyclase activity (data not shown). Under standard assay conditions, the K_m value for squalene was 4.2 μM .

Serum total cholesterol levels

Rats were fed 2% cholesterol to expand the cholesterol pool, 2% squalene to expand the squalene pool, treated with 3% cholestyramine to increase the excretion of cholesterol and/or bile acids, and treated with lovastatin, an inhibitor of HMG-CoA reductase, to inhibit mevalonate synthesis. Serum total cholesterol levels are shown in Fig. 2. None of these treatments affected the serum total cholesterol levels.

Effects of cholesterol-lowering agents, and cholesterol and squalene feeding on the squalene epoxidase activity

Since hepatic HMG-CoA reductase activity shows circadian variation (10), we examined the effects of the time of killing the rats on squalene epoxidase activity. As shown in Fig 3a, the enzyme activity was 2.5-fold higher in the

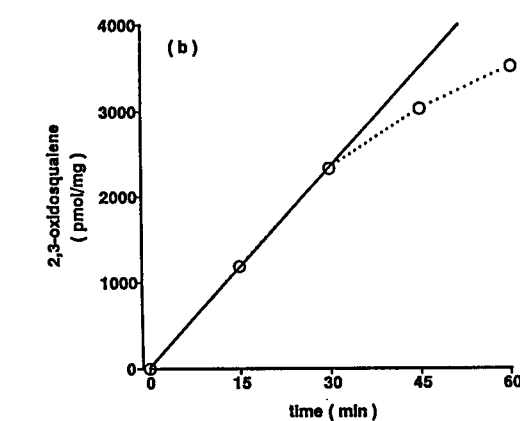
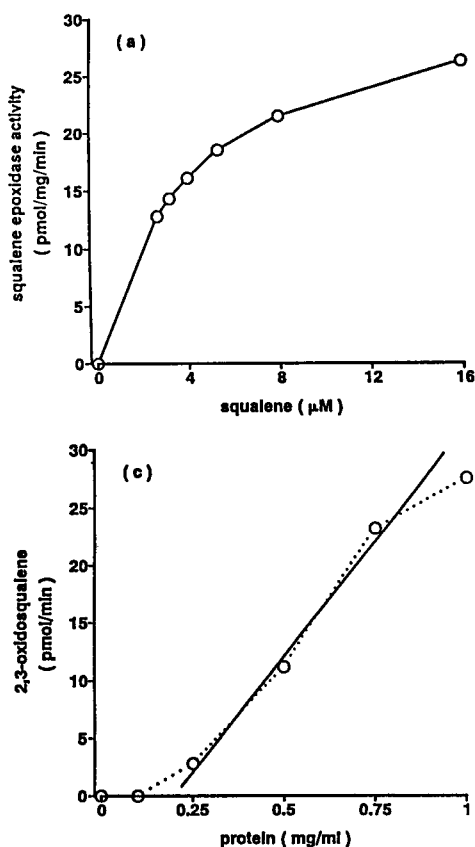


Fig. 1. Assay conditions of squalene epoxidase activity in rat liver. Rat liver microsomes were prepared as described in Materials and Methods. Squalene epoxidase activity in hepatic microsomes was determined under the following conditions. (a) The concentration of squalene in the reaction mixture was varied as indicated at 0.75 mg/ml protein and for 30 min incubation in the presence of 0.1% Triton X-100. (b) Incubation times were varied as indicated at 0.75 mg/ml protein and 8 μM squalene in the presence of 0.1% Triton X-100. (c) Protein concentration was varied as indicated at 8 μM squalene and for 30 min incubation in the presence of 0.1% Triton X-100. Each value represents the mean of duplicate determinations.

dark period than in the light period. HMG-CoA reductase activity in the dark period was also higher than that in the light period (Fig 3b). Therefore, rats were killed in the dark period (4 h after the onset of dark period : D4) and the enzyme activities were determined.

As shown in Fig. 4, squalene epoxidase activity in microsomes from rats treated with cholestyramine was significantly higher (average 2.5-fold) than the values obtained from the control rats. Lovastatin also induced squalene epoxidase activity (average 2.1-fold) under these conditions. Co-administration of cholestyramine and lovastatin increased enzyme activity synergistically (average 7.0-fold). On the other hand, 2% cholesterol feeding reduced squalene epoxidase activity to 18%. Squalene feeding also decreased enzyme activity significantly. However, squalene feeding increased microsomal squalene concentration (data not shown). Therefore, squalene epoxidase activity was determined again in the same substrate concentrations by adding exogenous squalene. In this experiment, squalene feeding also decreased the enzyme activity significantly (data not shown).

In order to examine whether these changes in squalene epoxidase activity were due to the change of the phosphorylation state and/or in the amount of the enzyme protein, the properties of the enzyme induced by cholestyramine feeding were investigated. Squalene epoxidase activity in liver microsomes prepared with or without 50 mM sodium fluoride, a phos-

phatase inhibitor (11), was determined (Table 1). Sodium fluoride had no effect on the increased squalene epoxidase activity in cholestyramine-treated rats or on the enzyme activity in control rats. To determine the effect of ATP-Mg²⁺ on the enzyme activity, microsomes were preincubated in the presence or absence of ATP and/or Mg²⁺ for 20 min at 37°C before assay. The control activities were decreased by this treatment because EDTA was excluded from the

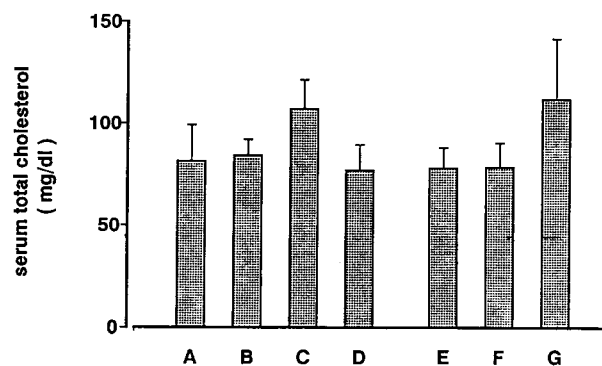


Fig. 2. Effects of various treatments on serum total cholesterol levels in rats. Rats were treated for 1 week as follows: A, normal diet; B, 3% cholestyramine diet; C, 2% cholesterol diet; D, 2% squalene diet; E, treated control (0.5% CMC); F, 50 mg/kg lovastatin; G, 50 mg/kg lovastatin plus 3% cholestyramine. Each value represents the mean ± SD (n = 5).

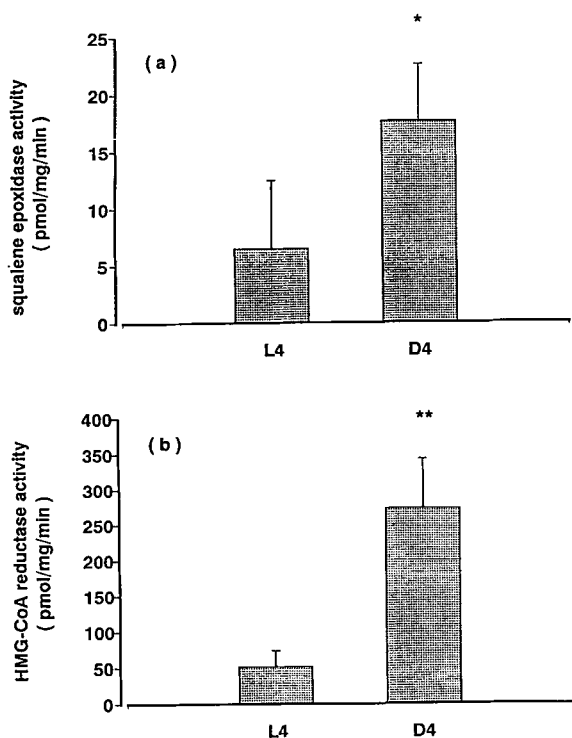


Fig. 3. Effects of time of killing on (a) squalene epoxidase and (b) HMG-CoA reductase activities. Rats were killed in the dark period (D4) and in the light period (L4). Rat liver microsomes were prepared as described in Materials and Methods. Squalene epoxidase and HMG-CoA reductase activities in hepatic microsomes were determined as described in Materials and Methods. Each value represents the mean \pm SD ($n = 5$); * $P < 0.05$; ** $P < 0.01$; light period as the reference.

preincubation mixture. In this assay condition, however, ATP-Mg²⁺ did not affect the enzyme activity (Table 1).

The enzymatic properties of induced squalene epoxidase were examined by the Lineweaver-Burk plot analysis. V_{max} of the enzyme from rats treated with cholestyramine was increased, while K_m value was constant (data not shown). These results indicated that the increase in the enzyme activity by cholestyramine treatment was not due to change in the affinity of the enzyme for squalene.

The effect of the concentration of Triton X-100 on the squalene epoxidase activity was examined. The enzyme activity induced by cholestyramine feeding was stimulated by Triton X-100 in the same manner as with the control enzyme (data not shown). The concentration of 0.1% Triton X-100, used as standard assay conditions, was optimum to give full activation. The increased activity produced by cholestyramine treatment was not due to change in the optimum concentration of detergent for enzyme activation.

Rat squalene epoxidase in microsomes has been reported to be activated by soluble fraction (4, 12). As shown in Fig. 5, soluble fractions prepared from control rats, cholestyramine plus lovastatin-treated rats, and cholesterol-fed rats activated squalene epoxidase activity to the same degree.

Therefore, the soluble fraction is not thought to participate in the regulation of squalene epoxidase activity in rat liver.

HMG-CoA reductase responded to the various treatments as recorded in the literature (13) and the 2% squalene diet depressed the HMG-CoA reductase as did the 2% cholesterol diet (data not shown).

Liver lipid levels

Liver lipid levels are shown in Table 2. Cholesterol and squalene feeding increased the hepatic total cholesterol concentrations. Cholestyramine feeding did not affect the hepatic concentration of cholesterol. Lovastatin treatment increased hepatic total cholesterol levels. Co-administration of cholestyramine and lovastatin did not affect them. Squalene feeding increased hepatic squalene levels, but cholesterol feeding decreased them.

DISCUSSION

In the accompanying paper (1), we reported that L-654,969, a potent competitive inhibitor of HMG-CoA reductase, increased squalene epoxidase activity dose-dependently, and that LDL and 25-hydroxycholesterol decreased the activity in HepG2 cells. In the presence of mevalonate, LDL, or oxysterol, this effect of L-654,969 was abolished. Actinomycin D and cycloheximide treatment suppressed this enzyme induction by L-654,969. The change in squalene epoxidase activity is thought to be caused by the levels of enzyme protein in HepG2 cells.

A nonionic detergent, Triton X-100, has been shown to stimulate the squalene epoxidase activity in rat liver micro-

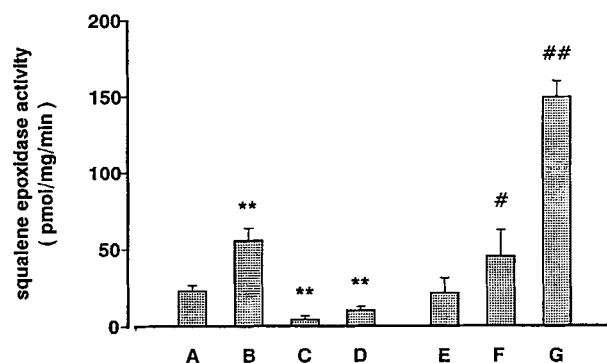


Fig. 4. Effect of various treatments on activity of squalene epoxidase in rat liver. Rats were treated for 1 week as follows: A, normal diet; B, 3% cholestyramine diet; C, 2% cholesterol diet; D, 2% squalene diet; E, treated control (0.5% CMC); F, 50 mg/kg lovastatin; G, 50 mg/kg lovastatin plus 3% cholestyramine. Squalene epoxidase activity in hepatic microsomes was determined as described in Materials and Methods. Each value represents the mean \pm SD ($n = 5$); ** $P < 0.01$, normal diet group (A) as the reference; # $P < 0.05$, ## $P < 0.01$, treated control group (E) as the reference.

TABLE 1. Effect of phosphorylation/dephosphorylation on squalene epoxidase activity

Diet	Addition to Microsome-Preparative Solution	Treatment	Squalene Epoxidase Activity <i>pmol/mg/min</i>
Experiment 1			
Normal diet	none		7.59
Normal diet	NaF		9.14
3% Cholestyramine diet	none		88.9
3% Cholestyramine diet	NaF		88.6
Experiment 2			
Normal diet	none	no addition	2.86
Normal diet	none	Mg ²⁺	2.86
Normal diet	none	ATP	3.07
Normal diet	none	ATP + Mg ²⁺	3.19
3% Cholestyramine diet	none	no addition	43.2
3% Cholestyramine diet	none	Mg ²⁺	46.4
3% Cholestyramine diet	none	ATP	52.5
3% Cholestyramine diet	none	ATP + Mg ²⁺	47.6

Microsomes were prepared with 0.1 M Tris-HCl buffer (pH 7.5) with or without 50 mM NaF and squalene epoxidase activity was measured (experiment 1). In experiment 2, microsomes (7.5 mg/ml) were preincubated in the presence or absence of 2 mM ATP and/or 4 mM MgCl₂ for 20 min at 37°C before assay. After the preincubation, mixture was diluted 10-fold with the same buffer, and squalene epoxidase activity was determined as described in Materials and Methods. Each value represents the mean of duplicate determinations.

somes (14). Therefore, we assayed squalene epoxidase activity in the presence of Triton X-100. In the presence of 0.1% Triton X-100, the reaction was linear with protein concentrations and incubation times under standard assay conditions. Under the experimental conditions, in which linearity was observed between protein concentration and enzyme activity, the regulatory mechanism of squalene epoxidase was investigated in rats.

In the present study, squalene epoxidase was shown to be regulated by the intracellular pool of cholesterol *in vivo*. Feeding 3% cholestyramine increased the hepatic squalene epoxidase activity 2.5-fold, and the administration of lovastatin (HMG-CoA reductase inhibitor) also increased its activity 2.1-fold. Co-administration of cholestyramine and lovastatin increased squalene epoxidase activity synergistically. On the other hand, cholesterol feeding reduced the activity to 18%.

HMG-CoA reductase activity in the liver was also increased by treatment with cholestyramine and decreased by cholesterol feeding, as was squalene epoxidase. However, lovastatin treatment increased HMG-CoA reductase 18-fold (data not shown). HMG-CoA reductase activity has been reported to be regulated by cholesterol and/or unidentified metabolite(s) derived from mevalonate (15). Therefore, lovastatin inhibits the formation of cholesterol and metabolite(s) derived from mevalonate, resulting in the remarkable increase in HMG-CoA reductase activity. On the other hand, squalene epoxidase was thought to be regulated only by cholesterol pool. These results were confirmed *in vitro* studies using HepG2 cells (1).

Cohen et al. (16) reported that 4% cholestyramine feeding increased hepatic squalene synthetase 2.8-fold in rats. Squalene

synthetase has been reported to be a secondary regulation site in the cholesterol synthetic pathway (16, 17). Therefore, squalene epoxidase is also thought to be a secondary regulatory enzyme in cholesterol synthetic pathway. Squalene synthetase has been reported to be regulated only by cholesterol (17). Squalene epoxidase activity is also regulated only by cholesterol pool. Cohen et al. (16) also reported that there was no circadian variation in squalene synthetase activity in rats. However, squalene epoxidase was found

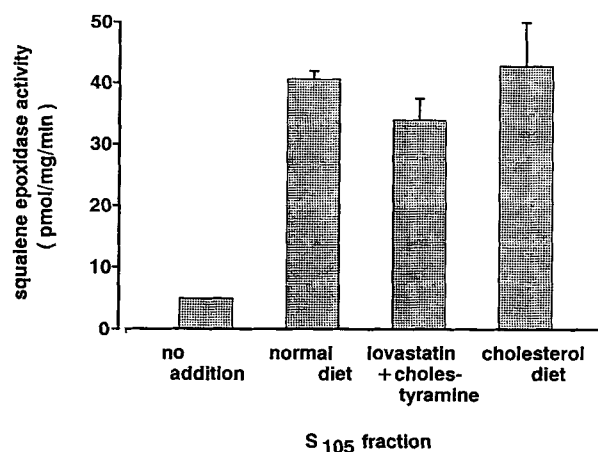


Fig. 5. Effect of cytosolic (S₁₀₅) fractions on microsomal squalene epoxidase activity. Rats were treated for 1 week as follows: normal diet, 50 mg/kg lovastatin plus 3% cholestyramine, and 2% cholesterol diet. S₁₀₅ fractions were prepared from each rat liver as described in Materials and Methods. Squalene epoxidase activity was determined at 0.5 mg/ml of microsomal protein in the presence of each S₁₀₅ fraction (3.4 mg protein/ml) or in their absence (no addition). Each value represents the mean ± SD (n = 5).

TABLE 2. Effect of cholesterol feeding, squalene feeding, cholestyramine feeding, and lovastatin treatment on hepatic cholesterol and squalene content in rats

Treatment	Total Cholesterol	Squalene
	mg/g tissue	μg/g tissue
A) Normal diet	2.84 ± 0.18	320 ± 83
B) 3% Cholestyramine	2.90 ± 0.33	428 ± 93
C) 2% Cholesterol	8.18 ± 1.46 **	105 ± 61*
D) 2% Squalene	3.68 ± 0.22 **	4100 ± 1483**
E) 0.5% CMC	2.82 ± 0.34	236 ± 87
F) 50 mg/kg Lovastatin	3.86 ± 0.61 **	117 ± 42
G) 3% Cholestyramine + 50 mg/kg lovastatin	3.26 ± 0.34	208 ± 127

Rats were treated as indicated for 1 week, and lipids in the liver were extracted and measured as described in Materials and Methods. Each value represents the mean ± SD (n = 5).

*P < 0.05; **P < 0.01; the normal diet group (A) or the 0.5% CMC group (E) served as the reference.

to show circadian variation. Enzyme activity was high in the dark period and low in the light period, similar to HMG-CoA reductase. Circadian variation of HMG-CoA reductase was reported to be regulated by the insulin cycle (10). Squalene epoxidase may also be regulated by insulin.

It has been reported that HMG-CoA reductase activity is controlled by a phosphorylation/dephosphorylation mechanism (18, 19). However, the regulation of squalene epoxidase activity does not seem to involve the phosphorylation/dephosphorylation process.

S₁₀₅ fractions derived from control rats, cholesterol-fed rats, and cholestyramine plus lovastatin-treated rats stimulate enzyme activity to a similar degree. Activator in soluble fractions does not seem to participate in the regulation of squalene epoxidase activity.

None of the treatments showed any effect on serum total cholesterol levels as described by Endo et al. (20) Serum cholesterol levels in rats were strictly regulated by many mechanisms. Therefore, these manipulations are not thought to affect serum total cholesterol levels in rats.

Cholesterol feeding increased hepatic cholesterol levels. Unexpectedly, lovastatin treatment increased hepatic cholesterol levels in rats. The enzyme activities and liver lipids were determined 24 h after the final drug treatment. Therefore, after disappearance of the drug from the liver, induced enzyme activities in cholesterol synthetic pathway might increase the hepatic concentration of cholesterol. The activities of squalene epoxidase and HMG-CoA reductase are thought to be regulated by a specific regulatory pool of cholesterol in the liver. Squalene feeding also decreased squalene epoxidase activity. Exogenous squalene is thought to be converted to cholesterol and to increase the hepatic cholesterol concentration. Cholestyramine treatment slightly increased hepatic squalene levels, but cholesterol treatment decreased squalene levels in the liver. Cholestyramine increased the whole pathway of cholesterol synthesis, but cholesterol feeding decreased it. These regulation mechanisms may affect the squalene concentration in liver.

Eilenberg et al. (2) also demonstrated that co-administration of cholestyramine and lovastatin increased squalene epox-

idase activity, and cholesterol feeding decreased its activity. However, their results were qualitative. This is the first report that squalene epoxidase activity is regulated by hepatic cholesterol, but not by nonsterol derivative(s) from mevalonate. ■

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