Hydroxymethylglutaryl CoA reductase and the modulation of microsomal cholesterol content by the nonspecific lipid transfer protein

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Abstract The influence of membrane cholesterol content on 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase, EC 1.1.1.34) in rat liver microsomes was investigated. Microsomes were enriched in cholesterol by incubation with egg phosphatidylcholine-cholesterol vesicles and the nonspecific lipid transfer protein from rat liver. By this method, the microsomal cholesterol content was 2.5-fold enhanced up to final concentrations of 140 nmol cholesterol per mg microsomal protein. In another experiment, microsomes isolated from rats fed a cholesterol-rich diet were depleted of cholesterol by incubation with egg phosphatidylcholine vesicles and the transfer protein. Both cholesterol enrichment and depletion had virtually no effect on the microsomal HMG-CoA reductase activity. In another set of experiments, normal rat liver microsomes were incubated with human serum, resulting in a rise of microsomal cholesterol content. This was reflected in an increase of acyl-CoA:cholesterol acyltransferase activity but failed to have an effect on HMG-CoA reductase.—**van Heusden, G. P. H., and K. W. A. Wirtz.** Hydroxymethylglutaryl CoA reductase and the modulation of microsomal cholesterol content by the nonspecific lipid transfer protein. *J. Lipid Res.* 1984. **25:** 27–32.

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The microsomal enzyme 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase (EC 1.1.1.34), which converts HMG-CoA into mevalonic acid, controls the rate of cholesterol biosynthesis (1). HMG-CoA reductase activity in liver is inhibited when rats are fed a cholesterol-rich diet (1). The exact molecular mechanism of this feedback regulation is not known. A major factor in this regulation is the synthesis and degradation of HMG-CoA reductase (1–4). In addition, phosphorylation-dephosphorylation is thought to play a role in the short-term regulation of this enzyme (5, 6). A direct effect of microsomal cholesterol levels on HMG-CoA reductase activity has also been reported. This enzyme was shown to be strongly inhibited when rat liver microsomes were enriched with cholesterol by incubation with serum (7, 8). Moreover, Arrhenius plots of HMG-CoA reductase were different when the microsomes that were used were isolated from rats fed

either a normal or a cholesterol-rich diet (7, 9, 10). On the other hand, no direct effect on HMG-CoA reductase activity was observed when cholesterol dissolved in organic solvents (11, 12) or lipoproteins isolated from rat liver (2) and human serum (12) were included in the incubation mixtures.

In this report a possible direct modulation of HMG-CoA reductase by microsomal cholesterol content was studied in more detail. Microsomes were enriched or depleted of cholesterol by use of the nonspecific lipid transfer protein (nsL-TP) isolated from rat liver (13, 14). This protein transfers cholesterol as well as natural diacylphospholipids between membranes and appears identical to sterol carrier protein 2 (14–16). When the acceptor membranes were deficient in cholesterol, this protein was shown to mediate a net transfer of cholesterol to these membranes (16, 17). By transferring cholesterol to rat liver microsomes, nsL-TP stimulated the acyl-CoA:cholesterol acyltransferase activity of these membranes (16, 18, 19). Microsomal cholesterol levels in the present study were also elevated by incubation with human serum as described by Venkatesan and Mitropoulos (7) and Mitropoulos et al. (8).

MATERIALS AND METHODS

Materials

DL-[3-^{14}C]Hydroxymethylglutaryl CoA and R-[5-^{3}H]mevalonic acid were obtained from New England Nuclear, Boston, MA. Unlabeled DL-hydroxymethylglutaryl CoA and mevalonic acid were purchased from Sigma, St. Louis, MO and Fluka, AG, Switzerland, respectively. Glucose-6-phosphate dehydrogenase was a product of Boehringer, Mannheim, G.F.R. Cholesterol was obtained

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; nsL-TP, nonspecific lipid transfer protein.

from Merck, Darmstadt, G.F.R. and was purified before use by recrystallization in sequence from glacial acetic acid, methanol, and acetone (20). Egg yolk phosphatidylcholine was purchased from Sigma. nsL-TP was purified from rat liver as described previously (14) and stored at −20°C in 50% glycerol–50 mM potassium phosphate–5 mM β-mercaptoethanol, pH 6.8.

Preparation of microsomes

Mature male Wistar rats were fed either a normal diet or a diet supplemented with 2% (w/w) cholesterol for 2 days before being killed. Animals were killed at 10 PM and the livers were homogenized in 0.25 M sucrose–0.005 M potassium phosphate–0.001 M EDTA, pH 6.8, (SEP) yielding a 20% (w/v) homogenate. After subsequent centrifugations for 10 min at 1000 g and for 20 min at 20,000 g, the supernatant (a 10-ml aliquot) was applied on a discontinuous sucrose gradient (total volume of 30 ml) consisting of layers of 25, 35, 39, and 50% (w/w) sucrose in 5 mM potassium phosphate (pH 6.8)–5 mM dithioerythritol. After centrifugation for 3 hr at 26,000 rpm in a Beckman SW27 rotor, the microsomes at the interface between 35 and 39% sucrose were collected. The sample was diluted with two volumes of SEP and the microsomes were sedimented by centrifugation for 1 hr at 105,000 g. The pellet was resuspended in SEP, at a concentration of approximately 10 mg/ml. After addition of dithioerythritol (10 mM), the microsomes were stored at −20°C and used within 1 week after preparation.

Preparation of vesicles

Lipids dissolved in chloroform–methanol 1:1 (v/v) were transferred to a glass tube. Upon removal of the organic solvents by a stream of nitrogen, the lipid residue was dispersed in SEP (10 μmol/ml) by sonication for 10 min at 50 MHz with a Branson sonifier at 0°C under a nitrogen atmosphere. Multilamellar structures and titanium particles were sedimented by centrifugation for 30 min at 105,000 g. The supernatant containing the vesicles was used immediately.

Incubation of microsomes with nsL-TP and vesicles

Microsomes (1.25 mg of protein) were mixed with vesicles consisting of either an equimolar mixture of egg phosphatidylcholine and cholesterol or egg phosphatidylcholine alone in a volume of 0.8 ml of SEP. The reaction was initiated by addition of nsL-TP in 0.2 ml of 50% glycerol–50 mM potassium phosphate–5 mM β-mercaptoethanol and continued for 30 min at 37°C. SEP was added and the microsomes were sedimented by centrifugation for 60 min at 100,000 g through a cushion of 2 ml of 21% sucrose (w/w) in a SW41 rotor tube. The pellet was resuspended in 0.3 ml of SEP and aliquots were taken for protein and cholesterol determination. After

addition of dithioerythritol (final concentration of 10 mM), HMG-CoA reductase activity was determined the same day.

Incubation of microsomes with serum

Human serum was heat inactivated and centrifuged as described by Mitropoulos et al. (8). The supernatant was lyophilized and stored at −20°C; before use it was reconstituted by dissolving in water (8). Liver microsomes (6.6 mg of protein) were incubated with 21 mg of reconstituted serum in 2 ml of 0.25 M sucrose–0.003 M imidazole, pH 7.4, for 60 min at 37°C. Subsequently 2 ml of ice-cold buffer was added and the mixture was centrifuged for 50 min at 150,000 g. The microsomal pellet was resuspended in SEP and treated further as described above.

Assay of HMG-CoA reductase

HMG-CoA reductase was measured essentially as described by Philipp and Shapiro (21). Microsomal protein (125 μg) was preincubated for 5 min at 37°C in 50 μl of SEP containing 10 mM dithioerythritol. Then 30 μl of 20 mM EDTA-KOH, pH 6.8, containing 5.2 μmol of glucose-6-phosphate, 0.52 μmol of NADP, and 1 unit of glucose-6-phosphate dehydrogenase was added. After another 5 min, 20 μl of 1 mM DL-[3-^{14}C]HMG-CoA (2200 dpm/nmol) was added and the incubation was continued for another 20 min. The reaction was stopped with 25 μl of 4 N HCl containing 3.8 μmol R-[5-^{3}H]mevalonic acid (5000 dpm/μmol). The mixture was incubated for 15 min at 37°C to convert mevalonic acid into mevalonic acid lactone. Precipitated protein was removed by centrifugation (5 min at 2000 g) and 100 μl of the mixture was directly applied on a silica gel G thin-layer plate (Merck, Darmstadt, G.F.R.). After development with toluene–acetone 1:1 (v/v), the mevalonic acid lactone spot was scraped off, and the ^{3}H and ^{14}C radioactivity were measured in a Packard Prias scintillation counter. [14]C Radioactivity was corrected for the recovery of [^{3}H]mevalonic acid.

Assay of acyl CoA:cholesterol acyltransferase

Acyl CoA:cholesterol acyltransferase was measured by the incorporation of [1-^{14}C]oleoyl-CoA into cholesteryl ester as described previously (19). Before initiation of the reaction, the microsomes (0.25 mg of protein) were routinely preincubated for 10 min at 37°C in 0.2 ml of SEP; incubation was for 10 min at 37°C.

Analytical procedures

Protein was measured according to Lowry et al. (22). Lipids were extracted from the microsomal preparations by the method of Bligh and Dyer (23). Free cholesterol in the lipid extract was measured enzymatically with a Merckotest kit (Merck, Darmstadt, G.F.R.).

RESULTS

HMG-CoA reductase activity in cholesterol-enriched microsomes

Mitropoulos et al. (24) have shown that HMG-CoA reductase activity was particularly high in rat liver microsomes of a density between 1.15 and 1.17 g/ml. Here we have used microsomes of this density to measure effects of cholesterol enrichment on HMG-CoA reductase activity (see Materials and Methods). In order to achieve this enrichment, microsomes (1.25 mg of protein) were incubated with vesicles (1.2 μmol of lipid) consisting of an equimolar amount of phosphatidylcholine and cholesterol in the absence and presence of nsL-TP (**Table 1**). In the absence of nsL-TP, the microsomal cholesterol content increased from 56 nmol to 80 nmol of cholesterol per mg microsomal protein. When nsL-TP was included in the incubation mixture, microsomes were obtained that contained as much as 141 nmol of cholesterol per mg protein. Under these conditions the phospholipid content of the reisolated microsomes was not significantly altered (data not shown). Determination of HMG-CoA reductase activity in these microsomes indicated that increased cholesterol levels had very little effect (Table 1, experiments 1 and 2). Similar results were obtained when the activity was determined directly on the preincubation mixture (Table 1, experiments 3 and 4). Moreover, the K_m for HMG-CoA was not affected by preincubation of microsomes with cholesterol-containing vesicles and nsL-TP (data not shown).

HMG-CoA reductase activity in microsomes isolated after a cholesterol-rich diet

Rats were fed a diet containing 2% cholesterol for 2 days and the liver microsomes were isolated by density gradient centrifugation. In agreement with previous studies (1), HMG-CoA reductase activity was drastically reduced whereas the microsomal cholesterol content was slightly enhanced (cf. Table 1 and **Table 2**). Incubation of the microsomes with phosphatidylcholine vesicles and nsL-TP lowered the cholesterol content from 65 nmol to a maximum of 40 nmol cholesterol per mg protein (Table 2). This depletion of cholesterol, however, had no significant effect on HMG-CoA reductase activity (experiment 1). A similar lack of effect was observed when the HMG-CoA reductase activity was measured on the total preincubation mixture without reisolation of the microsomes (Table 2, experiments 2 and 3). This agrees with the idea that there is no direct relationship between microsomal cholesterol levels and HMG-CoA reductase activity.

HMG-CoA reductase activity in microsomes incubated with serum

In the light of the above results, we thought it of interest to repeat the experiments by Mitropoulos et al. (7, 8). These investigators increased the cholesterol content of microsomes by incubation with serum. Under these conditions they reported that the HMG-CoA reductase activity was nearly completely inhibited, whereas the acyl CoA:cholesterol acyltransferase activity was greatly stimulated. We have confirmed that preincubation of microsomes with serum very significantly enhanced the acyl CoA:cholesterol acyltransferase activity in conjunction with an increased cholesterol level (**Table 3**). In contrast, HMG-CoA reductase activity was hardly affected.

DISCUSSION

In the present study we have clearly demonstrated that the manipulation in vitro of cholesterol levels in rat liver

TABLE 1.   HMG-CoA reductase activity in microsomes enriched in cholesterol

Additions During Preincubation		Microsomal Cholesterol Content		HMG-CoA Reductase Activity[a]			
Cholesterol	nsL-TP	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 3	Expt. 4
nmol	*μg*	*nmol · mg protein^{-1}*		*pmol · min^{-1} · mg protein^{-1}*			
		56	57	454	434	377	274
600		80	75	364	367	325	242
	17	49	43	465	547	372	196
600	17	141	125	384	495	392	272

Rat liver microsomes (1.25 mg of protein) were preincubated with vesicles (1.2 μmol of lipid) containing equimolar amounts of egg phosphatidylcholine and cholesterol, and the indicated amounts of nsL-TP for 30 min at 37°C. The enzyme assays and chemical determinations were performed as described under Methods.
[a] In experiments 1 and 2, HMG-CoA reductase activity was determined in microsomes reisolated after preincubation; in experiments 3 and 4, the reductase activity was determined on the total preincubation mixture without reisolation of the microsomes.

TABLE 2. HMG-CoA reductase activity in microsomes isolated from rats fed a cholesterol-rich diet, after reduction of the microsomal cholesterol content

Additions During Preincubation		Microsomal Cholesterol Content	HMG-CoA Reductase Activity[a]		
Phosphatidylcholine	nsL-TP		Expt. 1	Expt. 2	Expt. 3
nmol	*µg*	*nmol · mg protein⁻¹*	*pmol · min⁻¹ · mg protein⁻¹*		
		65	154	64	50
1200		51	131	60	58
	17	59	138	67	59
1200	17	40	127	64	57

Rat liver microsomes (1.25 mg of protein) were preincubated with egg phosphatidylcholine vesicles (1.2 µmol of lipid) and the indicated amounts of nsL-TP for 30 min at 37°C. The enzyme assays and chemical determinations were performed as described under Methods.

[a] In experiment 1, HMG-CoA reductase activity was determined in microsomes reisolated after preincubation; in experiments 2 and 3, the reductase activity was determined on the total preincubation mixture.

microsomes has no significant effect on HMG-CoA reductase activity. On the other hand, feeding rats a cholesterol-rich diet inhibited this enzymatic activity (Table 2; ref. 1). Furthermore, temperature–activity relationships for HMG-CoA reductase resulted in linear Arrhenius plots for cholesterol-fed animals as compared to broken Arrhenius plots for control animals (7, 9, 10). These observations were interpreted to indicate that microsomal cholesterol levels have a direct effect on the functioning of HMG-CoA reductase. However, our studies in vitro fail to provide any support for this concept. In a recent study (25), a cholesterol-rich diet was fed to rats carrying various Morris hepatomas. As a result, HMG-CoA reductase activity was found to be inhibited in the microsomes from the host liver, but not in the hepatomas. Despite the observed inhibition, the Arrhenius plots showed a break at a temperature (i.e., 24°C) comparable to that of the control. This strongly suggests that microsomal cholesterol levels, HMG-CoA reductase activity, and Arrhenius plot characteristics are not directly related.

Mitropoulos and coworkers (7, 8) demonstrated that HMG-CoA reductase activity was strongly inhibited upon incubation of microsomes with serum. Under identical conditions of incubation we have failed to confirm this observation. On the other hand, we did confirm that acyl CoA:cholesterol acyltransferase activity was enhanced by this treatment, concomitant to an increase of microsomal cholesterol levels. At present we have no explanation for this discrepancy.

Microsomal cholesterol levels were increased by incubation of rat liver microsomes with cholesterol containing vesicles and nsL-TP (Table 1). Previous studies have shown that under comparable conditions microsomal acyl CoA:cholesterol acyltransferase activity was as much as 5-fold enhanced (16, 19). It is very likely that the increase of this enzymatic activity is directly related to the expanded microsomal cholesterol pool. This is in agreement with the idea that microsomal cholesterol pools are suboptimal for this enzyme (26, 27). From the present study it is evident that microsomal cholesterol pools have no direct effect on HMG-CoA reductase activity. In this respect it is also of interest that microsomes from Morris hepatomas have higher HMG-CoA reductase activities than the host liver despite increased levels of microsomal cholesterol (25, 28).

Rat liver contains soluble proteins that modulate HMG-

TABLE 3. Cholesterol content, HMG-CoA reductase activity, and acyl CoA:cholesterol acyltransferase activity in microsomes incubated with serum

Addition During Preincubation	Microsomal Cholesterol Content	HMG-CoA Reductase Activity	Acyl CoA:Cholesterol Acyltransferase Activity
	nmol · mg protein⁻¹	*pmol · min⁻¹ · mg protein⁻¹*	*pmol · min⁻¹ · mg protein⁻¹*
	55	176 ± 38[a]	124
serum	70	234 ± 43	276

Rat liver microsomes (6.6 mg of protein) were preincubated with buffer or serum (21 mg of protein) for 60 min at 37°C. The enzyme assays and chemical determinations were performed as described under Methods.

[a] Means ± SD for four independent experiments.

CoA reductase in vitro. A protein (molecular weight of 10,300) has been purified which, upon addition to microsomes, gave a 3-fold stimulation of this enzyme (29). Recently, evidence has been provided that HMG-CoA reductase can be inhibited by an $FeSO_4$-dependent cytosolic protein (30). HMG-CoA reductase was also inhibited by oleic acid and oleoyl coenzyme A. This inhibition was reversed by addition of crude rat liver cytosol, possibly due to the presence of Z-protein which has a strong affinity for these ligands (31). Previous studies have strongly suggested that nsL-TP is identical to sterol carrier protein 2 which is intimately involved in cholesterol biosynthesis (15, 16). However, it follows from Tables 1 and 2 that in the absence of vesicles nsL-TP itself has no direct effect on HMG-CoA reductase activity, in agreement with the studies of Trzaskos and Gaylor (16). At present it remains to be elucidated whether the ability of nsL-TP to modulate membrane cholesterol levels in vitro has any bearing on the regulation of cholesterol metabolism in vivo.█

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