Cholesterol is required for secretion of very-low-density lipoprotein by rat liver

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To study potential effects of hepatic cholesterol concentration on secretion of very-low-density lipoprotein (VLDL) by the liver, male rats were fed on unsupplemented chow, chow with lovastatin (0.1%), or chow with lovastatin (0.1%) and cholesterol (0.1%) for 1 week. Livers were isolated from these animals and perfused *in vitro*, with a medium containing [2-¹⁴C]acetate, bovine serum albumin and glucose in Krebs–Henseleit buffer, and with an oleate–albumin complex. With lovastatin feeding, the hepatic concentrations of cholesteryl esters and triacylglycerols before perfusion were decreased, although free cholesterol was unchanged. However, hepatic secretion of all the VLDL lipids was decreased dramatically by treatment with lovastatin. Although total secretion of VLDL triacylglycerol, phospholipid, cholesterol and cholesteryl esters was decreased, the decrease in triacylglycerol was greater than that in free cholesterol or cholesteryl esters, resulting in secretion of a VLDL particle enriched in sterols relative to triacylglycerol. In separate studies, the uptake of VLDL by livers from control animals or animals treated with lovastatin was measured. Uptake of VLDL was estimated by disappearance of VLDL labelled with [1-¹⁴C]oleate in the triacylglycerol moiety, and was observed to be similar in both groups. During perfusion, triacylglycerol accumulated to a greater extent in livers from lovastatin-fed rats than in control animals. The depressed output of VLDL triacylglycerols and the increase in triacylglycerol in the livers from lovastatin-treated animals was indicative of a limitation in the rate of VLDL secretion. Addition of cholesterol (either free cholesterol or human low-density lipoprotein) to the medium perfusing livers from lovastatin-fed rats, or addition of cholesterol to the diet of lovastatin-fed rats, increased the hepatic concentration of cholesteryl esters and the output of VLDL lipids. The concentration of cholesteryl esters in the liver was correlated with the secretion of VLDL by the liver. These data suggest that cholesterol is an obligate component of the VLDL required for its secretion. It is additionally suggested that cholesteryl esters are in rapid equilibrium with a small pool of free cholesterol which comprises a putative metabolic pool available and necessary for the formation and secretion of the VLDL. Furthermore, the specific radioactivity (d.p.m./μmol) of the secreted VLDL free cholesterol was much greater than that of hepatic free cholesterol, suggesting that the putative hepatic metabolic pool is only a minor fraction of total hepatic free cholesterol.

INTRODUCTION

The primary function of very-low-density lipoprotein (VLDL), which is synthesized and secreted by the liver, is to transport triacylglycerol (TG) of endogenous origin from the liver to other tissues. In the normal fed animal, the primary substrate for the stimulation of VLDL formation is fatty acid, which results in increased synthesis and secretion of TG, along with the other lipid and protein components of the VLDL [1–5]. Non-esterified fatty acids (NEFA) also stimulate hepatic cholesterol synthesis via the activation of microsomal 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (the rate-limiting enzyme in cholesterol biosynthesis), and cytosolic acetoacetyl-CoA thiolase, acetoacetyl-CoA synthase and HMG-CoA synthase [6–8]. A stimulus for the synthesis of total cholesterol by the liver is the availability of NEFA and the need to form and secrete a VLDL for the transport of TG [5].

We had postulated previously that any of the lipid or

apolipoprotein components of VLDL may, under specific conditions, become rate-limiting, and decrease the hepatic synthesis and secretion of the VLDL [5]. A limitation of the NEFA available for TG synthesis will obviously diminish secretion of the VLDL, but decreased availability or synthesis of cholesterol, phospholipid or protein may also be individually limiting, and under certain circumstances may result in the production of hepatic steatosis. Clearly, it is a tenable hypothesis that cholesterol and phospholipid, the surface lipid components of the VLDL, are essential for the transport of TG. Previous studies from our laboratory have indicated that the hepatic output of cholesterol and phospholipid in VLDL increases with the secretion of TG in the VLDL. Under certain conditions, a direct proportionality has been demonstrated in other laboratories as well as ours [9,10]. A logical extension of this hypothesis is that, in the presence of an inadequate supply or availability of cholesterol, the liver is unable to form adequate amounts of VLDL for the transport of TG. With a diminished

Abbreviations used: VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; NEFA, non-esterified fatty acid; TG, triacylglycerol; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

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metabolic pool of cholesterol, the secretion of VLDL should be decreased. Conversely, replenishing the pool of cholesterol should restore or enhance transport of TG in the VLDL.

To evaluate the role of cholesterol in VLDL formation, studies were carried out with perfused livers isolated from control or lovastatin (mevinolin)-fed rats. Lovastatin is a hypolipidaemic drug, which is a competitive inhibitor of HMG-CoA reductase [11]. To lower the concentration of any putative hepatic metabolic pool of cholesterol required for the secretion of the VLDL, lovastatin was administered to rats in the diet. In addition, livers from normal rats were perfused with lovastatin to observe whether the drug might affect hepatic VLDL secretion when added directly to the medium. To replete the hepatic pool of cholesterol in the lovastatin-fed rats, either free cholesterol or human plasma low-density lipoprotein (LDL) was added *in vitro* to the medium perfusing the livers, or cholesterol was added to the diet of lovastatin-fed rats. The results obtained support the concept that cholesterol is essential in the formation and secretion of the VLDL for the transport of TG. Preliminary reports of this work have appeared [12]; it has also been presented at the Aspen Lipoprotein Conference, Aspen, CO, August 1987.

METHODS

Animals and liver perfusion

Normal male Sprague-Dawley rats obtained from Harlan (Indianapolis, IN, U.S.A.) were maintained on a Purina chow diet, a diet supplemented with 0.1% lovastatin (mevinolin), or a diet supplemented with 0.1% lovastatin and 0.1% cholesterol for 1 week [13]. The light/dark periods were cycled, with lights on from 07:00 to 19:00 h. For diets supplemented with lovastatin, the drug was mixed thoroughly with ground rat chow. For diets additionally supplemented with 0.1% cholesterol, ground chow mixed with lovastatin was used. To attain proper mixing of cholesterol with the chow, the cholesterol was dissolved in a minimal amount of diethyl ether, which was then poured and mixed with the chow. The chow was placed in a drying hood overnight to allow evaporation of the ether. Rats received chow and water *ad libitum*; food intake and body weight were recorded. On the day of the experiments, the rats (250–375 g body wt.) were anaesthetized with an intraperitoneal injection of pentobarbital (60 mg/kg). The livers were isolated surgically and perfused *in vitro* by using the recycling-perfusion apparatus described previously [14,15]. Livers were generally isolated for perfusion between 08:00 and 10:00 h. The livers were perfused with a basal medium containing 6 g of bovine serum albumin/dl, 4 μ Ci of [2-¹⁴C]acetate [sp. radioactivity (1.0–1.2) $\times 10^7$ μ Ci/ μ mol] and 100 mg of glucose/dl in Krebs-Henseleit buffer [14] (pH 7.4). The rate of flow of perfusate was maintained at 35–50 ml/min at 20 cm hydrostatic pressure. An oleate-albumin complex (6 g of purified bovine serum albumin/dl) was infused at a constant rate (11.7 ml/h; 166 μ mol of oleate/h and 4 μ Ci of [2-¹⁴C]acetate/h) [16]. Oleate was added to the medium in all perfusion experiments. Perfusions were maintained for 4 h. Erythrocytes were omitted from the perfusate in these experiments to avoid rapid equilibration of newly synthesized cholesterol with the cholesterol derived from erythrocytes, which would confuse the interpretation of the data [5].

For those perfusions of livers from lovastatin-fed rats in which cholesterol was supplied to the liver, the albumin-oleate complex was prepared with unesterified (free) cholesterol [6] to give a final concentration of 55 mg of cholesterol/dl. In this preparation, the final albumin concentration was 12%, and the starting albumin concentration was 3% in the perfusion medium. No significant differences were observed in hepatic uptake of oleate if the complex with fatty acid was prepared with 6% or 12% albumin. For those experiments in which the effects of human LDL were studied, the lipoprotein was added to the albumin-oleate complex to give a final concentration of 42–47 mg of LDL cholesterol/dl. The LDL was isolated from the plasma of healthy male laboratory personnel. After removal of VLDL and chylomicrons by ultracentrifugation (39000 rev./min for 18 h in a Beckman 50.2 rotor), the LDL was centrifugally isolated at $d < 1.063$ under the same conditions and dialysed against 0.9% NaCl at 4 °C. The LDL was used in the perfusion experiments 3 days after the plasma was obtained.

For those studies in which lovastatin was added to the medium *in vitro*, the drug, dissolved in ethanol, was added to the perfusion medium and infusate to give an ethanol concentration of 0.5%. Under the conditions of these studies, ethanol was also included in control experiments, and the results obtained were not different from those controls without ethanol, and were therefore considered as a single experimental group.

Samples of perfusate were removed for analysis at the end of 2 and 4 h of perfusion. At the termination of the experiment, the livers were perfused with 50 ml of ice-cold 0.9% NaCl, cleansed of adherent extrahepatic tissue, blotted, and weighed. Then 1 g samples of liver were removed for analysis. A 60 ml sample of the final perfusate was collected at the end of the experiment. The nascent VLDL (the VLDL secreted by the perfused liver) was isolated from the perfusate by ultracentrifugation, as described previously [16].

Uptake of VLDL by the liver

VLDL, labelled in the TG moiety with [1-¹⁴C]oleic acid, was prepared *in vitro* by perfusion of livers from normal fed rats, as described previously [16]. The radioactive VLDL was used as the substrate for determination of the rate of hepatic uptake by livers from control and lovastatin-fed animals. In these experiments, [2-¹⁴C]-acetate and oleic acid were omitted from the perfusion medium. Conditions were otherwise identical with the above perfusions. A pulse dose of radioactive nascent VLDL was added to the medium (4.3 μ mol of TG) at zero time after an equilibration period (20 min). The specific radioactivity of the preparation was 2.6×10^5 d.p.m./ μ mol of TG at the start of the experiment. Perfusions were carried out for 4 h. Samples of perfusate were removed at 30 min intervals. Lipids were extracted and separated from samples of perfusate, and radioactivity and mass of TG were measured, as described below. Disappearance of radioactivity and specific radioactivity of TG, as well as uptake of TG mass, were calculated as described previously [16]. Specific radioactivity and uptake of TG were calculated at 2 h of perfusion to decrease errors resulting from re-secretion of VLDL at the later time intervals, and the non-linearity of TG uptake during the experimental period.

Sample analysis

The liver was homogenized, and samples of the liver, VLDL and perfusate were extracted with chloroform/methanol (2:1, v/v) [16,17]. Samples of the washed chloroform extracts were separated into individual lipid classes (TG, free cholesterol, NEFA, cholesteryl ester and phospholipid) by t.l.c. on silica-gel G plates with a solvent mixture of light petroleum (b.p. 60–70 °C)/diethyl ether/acetic acid (84:15:1, by vol.) [18]. Further separation of hepatic free cholesterol from mono- and di-acylglycerol was performed with a solvent mixture of benzene/diethyl ether/acetic acid (50:50:1, by vol.) [9]. The radioactivity in the lipid fractions was measured by liquid-scintillation spectrometry with Bio-Count as a scintillation cocktail. Mass determinations were made on lipid fractions for TG [19], phospholipid [20] and free and esterified cholesterol [21].

Materials

Lovastatin was kindly provided by Mr. A. W. Alberts of Merck, Sharp and Dohme Research Laboratories. Oleic acid was obtained from Nu-Chek-Prep (Elysian, MN, U.S.A.). [2-¹⁴C]Acetate and [1-¹⁴C]oleic acid were purchased from New England Nuclear Corp. Bovine serum albumin (fraction V) was obtained from Miles Laboratories and was delipidated and purified before use as described previously [16]. Thin-layer plates of silica gel G were purchased from Analtech. Bio-Count was purchased from Research Products International. Free cholesterol used was of commercial grade, with 98% purity. All other chemicals used in this study were of analytical grade.

Statistics

Values are reported as means ± standard error of measurement (S.E.M.). Statistical significance of the data was calculated by using a two-tailed Student's *t* test at *P* < 0.05.

RESULTS

There were no significant differences between the weekly food intake of the chow-fed (control), lovastatin-fed, or lovastatin + free cholesterol-fed rats (171 ± 16, 159 ± 10 and 164 ± 23 g/rat respectively). Uptake of oleic acid from the medium by the liver was statistically similar in all of the treatment groups (Table 1). Body and liver weights for each experimental group are also presented in Table 1.

The secretion of the VLDL lipids by the liver is shown in Fig. 1. Treatment of the rats with lovastatin for 1 week decreased the output of all lipid components of the VLDL, compared with the output of VLDL by livers from control rats. The secretion of VLDL TG by the control group was 0.455 ± 0.041 µmol/h per g of liver. Treatment of the rats with dietary lovastatin for 1 week decreased output of VLDL TG to 0.161 ± 0.018 µmol/h per g of liver. Although previous data had suggested an effect [12], the addition of lovastatin *in vitro* produced a slight, but insignificant, decrease of secretion of VLDL TG from control (0.333 ± 0.055 µmol/h per g of liver). The change in the secretion of VLDL phospholipid paralleled that of VLDL TG. However, the decrease in output of free cholesterol and cholesteryl esters of the

Table 1. Effects of treatments on body weight, liver weight, and uptake of NEFA by the liver

Rats were fed for 1 week on a chow diet, a diet supplemented with lovastatin (0.1%), or a diet supplemented with both lovastatin (0.1%) and cholesterol (0.1%). The following experiment groups were identified: CON, chow diet; L(V), chow diet plus lovastatin perfused *in vitro*; L(D), lovastatin in the diet; L(D)+C(D), lovastatin and cholesterol in the diet; L(D)+LDL(V), lovastatin in the diet and LDL perfused *in vitro*; L(D)+C(V), lovastatin in the diet, and cholesterol perfused *in vitro*. Livers were perfused for 4 h in the presence of oleate. The mass of oleate taken up by the liver was measured by disappearance from the medium. The number of perfusions in each group is indicated in parentheses. Values are means ± S.E.M.; ND, not determined. No significant differences in NEFA uptake between treatment groups were observed.

Group	Body wt. (g)	Liver wt. (g)	NEFA uptake (µmol/h per g of liver)
CON (13)	286 ± 13	12.3 ± 0.7	10.3 ± 0.8
L(V) (3)	281 ± 30	13.2 ± 1.7	10.0 ± 0.9
L(D) (14)	271 ± 13	11.3 ± 0.4	11.2 ± 0.5
L(D)+C(D) (4)	332 ± 37	12.6 ± 0.8	ND
L(D)+LDL(V) (4)	296 ± 18	10.9 ± 0.5	12.4 ± 0.5
L(D)+C(V) (4)	278 ± 9	11.2 ± 0.6	12.5 ± 1.0

VLDL was relatively less than that of VLDL TG and VLDL phospholipid. Although dietary lovastatin decreased the TG and phospholipid components of the VLDL by nearly 75% from control, the decreases in the cholesterol and cholesteryl ester components were less than 50% in this group. VLDL cholesterol decreased from 0.050 ± 0.009 for controls to 0.028 ± 0.003 µmol/h per g of liver for the group receiving dietary lovastatin. Similarly, VLDL cholesteryl ester decreased from 0.036 ± 0.004 µmol/h per g of liver for control to 0.019 ± 0.002 µmol/h per g of liver for the group given lovastatin. The addition of either free cholesterol or human LDL to the medium perfusing livers from lovastatin-fed rats increased the secretion of all VLDL lipids by livers from the lovastatin-fed group compared with the group not infused with either free cholesterol or human LDL. The output of VLDL TG for the group given lovastatin and perfused with free cholesterol *in vitro* was 0.237 ± 0.026 µmol/h per g of liver, an increase of 50% from the lovastatin-fed group. Similarly, the VLDL TG for the group given lovastatin and perfused with LDL *in vitro* was 0.377 ± 0.040 µmol/h per g of liver, an increase of 150% over the lovastatin-fed group. With both lovastatin and cholesterol feeding, the output of VLDL TG by the liver was 0.304 ± 0.026 µmol/h per g of liver, an increase of nearly 100% over the lovastatin-fed group. The changes in the output of VLDL TG by the livers were corroborated by the total perfusate TG. When TG synthesis and transport are stimulated by NEFA, we have observed routinely that 85–95% of the total perfusate TG is recoverable in the VLDL [7,22].

Treatment of the rats with lovastatin altered the relative molar composition of the secreted VLDL lipids (Fig. 2). The molar ratio phospholipid: TG was similar in all treatment groups. The ratio of cholesterol: TG, how-

Fig. 1. Secretion of VLDL lipids by the rat liver

Livers were perfused for 4 h. Perfusate was collected, and VLDL ($d < 1.006$) was isolated. Data presented are means $\pm$ S.E.M. Numbers of observations and identification of the treatment groups are as indicated in Table 1. Key: ■, TG; ▨, phospholipid; ▩, cholesterol; ▤, cholesteryl ester. *Value differs ($P < 0.05$) from controls (CON); †Value differs ($P < 0.05$) from lovastatin (diet) [L(D)].

Fig. 2. Molar ratios of lipids relative to TG in the VLDL

Livers were perfused for 4 h, after which the VLDL ($d < 1.006$) was isolated from the perfusate. Lipids were analysed, and the molar ratios were calculated. Values are means $\pm$ S.E.M. Number of observations and identification of the treatment groups are as indicated in Table 1. Key: ■, phospholipid; ▨, cholesterol; ▩, cholesteryl ester. *Value differs ($P < 0.05$) from controls (CON).

Table 2. Concentration of hepatic lipids before and after perfusion

Data in (a) indicate the concentrations of hepatic lipids in the non-perfused livers. These livers were isolated surgically, as if for perfusion, but were not perfused. The lipids of the liver were analysed chemically. Part (b) indicates the concentrations of hepatic lipids after 4 h of perfusion. Part (c) shows the calculated changes in the concentrations of the hepatic lipids. Data presented are means $\pm$ S.E.M.; ND, not determined. Abbreviations are the same as for Table 1. Numbers of observations for the non-perfused livers are given in parentheses. Those for the perfused livers are as indicated in Table 1. *Value differs ($P < 0.05$) from controls (CON); †value differs ($P < 0.05$) from lovastatin diet [L(D)].

		Concn. ($\mu\text{mol/g}$ of liver)		
	Group	TG	Cholesterol	Cholesteryl ester
(a) Non-perfused	CON (4)	2.63 ± 0.21	3.21 ± 0.30	0.84 ± 0.10
	L(D) (4)	$1.94 \pm 0.08^*$	3.56 ± 0.28	$0.44 \pm 0.04^*$
	L(D) + C(D) (5)	$3.82 \pm 0.19^{*\dagger}$	3.55 ± 0.20	$1.16 \pm 0.06^{*\dagger}$
(b) Perfused	CON	4.32 ± 0.28	3.25 ± 0.16	1.07 ± 0.11
	L(V)	3.98 ± 0.23	2.93 ± 0.54	ND
	L(D)	4.84 ± 0.33	3.14 ± 0.12	$0.47 \pm 0.04^*$
	L(D) + C(D)	$8.54 \pm 0.83^{*\dagger}$	3.53 ± 0.12	$1.54 \pm 0.09^{*\dagger}$
	L(D) + LDL(V)	$7.15 \pm 0.29^{*\dagger}$	3.05 ± 0.06	$1.11 \pm 0.06^\dagger$
	L(D) + C(V)	$6.78 \pm 1.31^{*\dagger}$	3.52 ± 0.43	$1.32 \pm 0.16^\dagger$
(c) Change during perfusion	CON	1.69 ± 0.24	0.04 ± 0.01	0.20 ± 0.14
	L(V)	1.35 ± 0.34	-0.21 ± 0.15	ND
	L(D)	$2.86 \pm 0.21^*$	-0.20 ± 0.19	0.07 ± 0.05
	L(D) + C(D)	$4.91 \pm 0.43^{*\dagger}$	0.02 ± 0.02	0.23 ± 0.13
	L(D) + LDL(V)	$5.37 \pm 0.46^{*\dagger}$	-0.11 ± 0.08	$0.79 \pm 0.16^{*\dagger}$
	L(D) + C(V)	$4.81 \pm 1.31^{*\dagger}$	-0.14 ± 0.08	$0.84 \pm 0.16^{*\dagger}$

Table 3. Incorporation of [^{14}C]acetate into cholesterol of the liver and the VLDL

The free cholesterol of the liver and the VLDL was analysed, the radioactivity of these fractions was measured, and the specific radioactivity was calculated. Data presented are means $\pm$ S.E.M. The specific radioactivity of cholesterol was determined as the incorporation of radioactive acetate into cholesterol per mass of cholesterol (average of the two parameters was taken). Numbers of observations are as indicated in Table 1. *Value differs ($P < 0.05$) from controls (CON); †value differs ($P < 0.05$) from lovastatin diet [L(D)].

	Group	Incorporation (d.p.m./h per g of liver)	Sp. radioactivity (d.p.m./ μmol)
Liver	CON	1140 ± 430	1560 ± 600
	L(V)	$20 \pm 5^*$	$28 \pm 12^*$
	L(D)	$12820 \pm 3510^*$	$19040 \pm 4400^*$
	L(D) + C(D)	$2300 \pm 500^{*\dagger}$	$2560 \pm 880^\dagger$
	L(D) + LDL(V)	$14760 \pm 4320^*$	$17760 \pm 3120^*$
	L(D) + C(V)	$18230 \pm 6110^*$	$20040 \pm 4280^*$
VLDL	CON	4690 ± 490	96500 ± 23860
	L(V)	$7 \pm 3^*$	170 ± 70
	L(D)	$10570 \pm 3460^*$	$324670 \pm 98720^*$
	L(D) + C(D)	$380 \pm 140^{*\dagger}$	$5780 \pm 1890^{*\dagger}$
	L(D) + LDL(V)	$3490 \pm 1300^\dagger$	$54340 \pm 21350^\dagger$
	L(D) + C(V)	$4500 \pm 1450^\dagger$	$66030 \pm 19340^\dagger$

ever, increased from 12.6 ± 1.3 for control to 19.0 ± 1.8 for the rats given lovastatin. The ratio of cholesteryl ester: TG increased in a similar manner. Thus, as output of VLDL decreased because of treatment with lovastatin, the secreted particle was enriched in cholesterol and cholesteryl ester relative to TG. It must be reiterated from the data of Fig. 1, however, that the total secretion of cholesterol and cholesteryl esters in the VLDL was decreased by prior treatment of the animal with lovasta-

tin. When free cholesterol or human LDL was added to the medium perfusing livers from lovastatin-fed animals, the cholesterol: TG ratio was also higher than control values (27.1 ± 4.3 for the lovastatin-fed rats perfused with free cholesterol *in vitro* and 20.9 ± 2.9 for the lovastatin-fed group perfused with human LDL). With lovastatin and cholesterol feeding, the cholesteryl: TG and cholesteryl ester: TG ratios were increased over controls (the former ratio was 18.8 ± 1.4 , and the latter ratio was

17.7 ± 1.0). Thus, even though cholesterol supplementation increased the output of VLDL TG, the secreted particle was relatively enriched in cholesterol and cholesteryl ester.

The hepatic concentration of free cholesterol was not affected by treatment with lovastatin (Table 2). The free cholesterol concentration in non-perfused livers of the lovastatin-fed group was similar to that in the control

group. In contrast, the concentration of cholesteryl ester in non-perfused livers of lovastatin-treated rats was approx. 55% of control values. The concentration of hepatic cholesteryl ester in rats given both lovastatin and free cholesterol was above control values. After perfusion with oleate, the hepatic free cholesterol concentration was not changed in any of the groups, including those in which cholesterol or human LDL was infused, or with dietary supplementation of free cholesterol in the diet. During perfusion with oleate, the concentration of hepatic cholesteryl ester did not change in the lovastatin-fed group, but remained low. However, hepatic cholesterol ester concentrations increased to control values when either free cholesterol or human LDL was infused. The concentration of hepatic TG increased in all treatment groups after perfusion with oleate. The accumulation of hepatic TG in the lovastatin-fed group was greater than that of the control group. Moreover, the accumulation of TG in the liver was even greater in the lovastatin-fed rats perfused with either free cholesterol or human LDL *in vitro*, compared with the control ($P < 0.05$). The group of rats given lovastatin and cholesterol also accumulated more TG during the perfusion than did the group given lovastatin only.

The incorporation of [2- ^{14} C]acetate into free cholesterol (d.p.m./h per g of liver) of the liver and the VLDL was also measured as an independent estimate of the effects of lovastatin (Table 3); these incorporations increased approx. 12-fold over controls with livers from the lovastatin-fed group. The effect was expected, resulting from decreased hepatic synthesis of total cholesterol after treatment with lovastatin *in vivo*, and subsequent induction of HMG-CoA reductase [23]. The synthesis of cholesterol *in vivo* remains depressed in the presence of lovastatin, despite the induction of HMG-CoA reductase. However, the drug is rapidly metabolized by the liver, and, when the liver is isolated from lovastatin-fed rats and perfused *in vitro*, stimulation of synthesis of cholesterol is observed. Furthermore, as reported previously, lovastatin added *in vitro* to isolated hepatocytes from normal fed rats decreased the synthesis of cholesterol [12]. In our studies with the perfused livers from normal or lovastatin-fed rats, incorporation of [2- ^{14} C]acetate into free cholesterol of the liver and the VLDL was decreased by over 90% by addition of lovastatin *in vitro*. Also, our laboratory performed a study on a group of livers from rats fed with lovastatin and perfused with lovastatin *in vitro*. The results obtained indicated a substantial decrease in [^{14} C]acetate incorporation into the free cholesterol of the liver and the VLDL from both the control and the lovastatin-fed groups (1040 ± 210 d.p.m./h per g of liver and 620 ± 100 d.p.m./

Fig. 3. Uptake of VLDL TG by the perfused liver

Panel (a) presents the percentage of radioactivity (d.p.m.) remaining in perfusate TG during perfusion. VLDL was labelled primarily in the TG moiety with [1- ^{14} C]oleate, as described in the text, and used as substrate. Panel (b) presents changes in specific radioactivity of the perfusate TG during perfusion of the liver with the VLDL. Data presented are means $\pm$ S.E.M. ($n = 3$ for each point); $\square$, CON; $\blacklozenge$, L(D).

Table 4. Uptake of labelled VLDL TG and net output of TG by the perfused rat liver

Livers were perfused without oleic acid, as stated in the Methods section. The TG of the perfusate was analysed chemically, and the total uptake was calculated, as described in [16]. Data presented ($\mu\text{mol}/2\text{ h}$ per g of liver) are means $\pm$ S.E.M. for three observations. *Value differs ($P < 0.05$) from CON.

	TG uptake (total)	TG re-uptake	TG net output (cumulative)	TG secreted (total)
CON	0.601 ± 0.085	0.312 ± 0.097	0.288 ± 0.102	0.600 ± 0.117
L(D)	0.455 ± 0.051	0.237 ± 0.041	$0.092 \pm 0.015^*$	$0.329 \pm 0.036^*$

h per g of liver for the VLDL). The infusion of free cholesterol or human LDL into livers from lovastatin-fed rats had no apparent effect on the incorporation of [2-¹⁴C]acetate into hepatic cholesterol, but there was a significant decrease in incorporation into the free cholesterol of the VLDL. Combined feeding of lovastatin plus free cholesterol decreased acetate incorporation into the free cholesterol of the liver by over 80 %, and of the VLDL by over 95 %, compared with treatment with lovastatin only. For the liver, this value remained significantly above the control group; however, the incorporation of [¹⁴C]acetate into the VLDL for this group was substantially below the control.

The changes in specific radioactivity (d.p.m./ μ mol) of free cholesterol of the hepatic and VLDL cholesterol mirrored those changes in total incorporation. However, a particularly interesting observation was the considerably higher specific radioactivities of the free cholesterol of the VLDL compared with that of the liver (Table 3). These differences were observed in all experimental groups, although the magnitude of the differences was less in the lovastatin-treated groups. These data suggest that the free cholesterol of the VLDL is derived from a small metabolically active pool which is distinct from the total free cholesterol of the liver, and which is not in rapid equilibrium with the total pool of hepatic free cholesterol. Similar conclusions were derived previously for the relationship between TGs of the VLDL and those of the liver [1].

To rule out the possibility that the apparent decrease in secretion of the VLDL by livers from lovastatin-treated rats was not due to increased uptake of the VLDL by the livers, additional experiments were carried out with VLDL labelled in the TG moiety with [1-¹⁴C]oleic acid. The radioactive VLDL was prepared by perfusion of livers from normal fed rats. Over a 2 h experimental period, no differences were observed in disappearance of perfusate TG radioactivity or mass (Fig. 3a), or calculated uptake of VLDL TG (Table 4). The changes in relative TG specific radioactivity were supportive of decreased secretion of VLDL (Fig. 3b).

DISCUSSION

Treatment of the animals with lovastatin for 1 week depressed severely the secretion of VLDL lipids by the liver. The decreased outward transport of TG from the perfused liver was accompanied by accumulation of TG in the liver, as expected, if the liver was unable to assemble and secrete the VLDL adequately. It must be emphasized that the design of these experiments was not intended to determine the mechanism of action of this drug in patients treated for hypercholesterolaemia, but, rather, lovastatin was used to deplete a putative hepatic metabolic pool of cholesterol, and to study the effects on secretion of the VLDL. Under these experimental conditions, the output of TG, the major lipid component of the VLDL, was particularly decreased, but very significant decreases in phospholipid, free cholesterol and cholesteryl esters were also observed. It was of interest that the depressed secretion of VLDL cholesterol and VLDL cholesteryl ester was less than the decline in output of VLDL TG. As a result, a VLDL particle that was relatively enriched in free and esterified cholesterol was formed. This is expected and reflects the increase in surface components (cholesterol and phospholipid) rela-

tive to core components (primarily TG and some cholesteryl ester) in the secreted VLDL particle. Fewer VLDL particles, with altered composition, are secreted. Such increases in cholesterol relative to TG in the secreted VLDL are seen in various conditions associated with decreased TG secretion, and also vary with the structure of the fatty acid perfusing the liver [24].

In the experiments reported here, human LDL and free cholesterol were used in our perfusions to replete the decreased size of the putative metabolic pool of cholesterol in the liver. When either free cholesterol or LDL was infused into the medium perfusing livers from lovastatin-fed rats, a significant increase in the secretion of VLDL lipids, particularly TG, was observed; the amounts of free and esterified cholesterol secreted in the VLDL approached control values. The quantities of VLDL TG and VLDL phospholipid secreted equalled control values when cholesterol, as LDL, was added to the medium. In the group of rats given cholesterol and lovastatin, an increase in the secretion of VLDL lipids was also observed, although the secretion of VLDL TG and VLDL phospholipid remained below control values.

In these studies in which the recirculating perfusion system was used, it is important to realize that net VLDL was measured, which is the result of both hepatic secretion and uptake of VLDL. Therefore it was necessary to measure uptake of VLDL by the liver separately to determine whether feeding of lovastatin may have affected removal mechanisms of VLDL. Our studies with VLDL labelled with [1-¹⁴C]oleate, primarily in the TG moiety, indicated that uptake of VLDL TG was identical in perfused livers from control and lovastatin-fed rats, and that net accumulation of VLDL in the perfusate was lower in the lovastatin-fed group. It can therefore be concluded that the secretion of VLDL TG is diminished with lovastatin feeding, rather than increased uptake of VLDL by the liver.

In all treatment groups, the hepatic concentration of TG increased after perfusion with oleic acid. This is in agreement with previous studies from our laboratory [1-4]. It was of interest that, after perfusion, hepatic contents of TG were greater in the lovastatin (diet) group than in the controls, whereas the secretion of VLDL was decreased. The decreased ability of the liver to assemble and secrete VLDL apparently induced an accumulation of hepatic TG, as observed in many previous examples of inhibited secretion of the VLDL [9,25-28]. The accumulation of TG, after perfusion with oleate, in livers from lovastatin-fed rats, suggests that the capability for synthesis of TG was not diminished by this drug.

Our data indicate that the concentration of hepatic cholesteryl esters was decreased with the feeding of lovastatin to the animals, and returned to control values with the infusion of free cholesterol, or with free cholesterol supplementation in the diet. Simultaneously, the quantity of VLDL secreted by the liver decreased with lovastatin treatment and increased with cholesterol supplementation, either in the diet or *in vitro*. In agreement with our findings, Stone *et al.* [28] reported recently that addition of cholesterol-rich lipoproteins to the perfused rat liver in the absence of added fatty acid enhanced VLDL output. Our data indicate that a decrease in the hepatic concentration of cholesteryl esters is associated with a decrease in the secretion of VLDL, and when the hepatic cholesteryl esters are restored to control values

Fig. 4. Correlation between secretion of VLDL TG and concentration of cholesteryl esters in the liver

The secretion of VLDL TG was plotted as a function of cholesteryl ester concentration in the liver. Simple regression analysis was performed and the coefficient of correlation was 0.51 ± 0.10 ($P < 0.05$). The equation of the least-squares line was calculated to be $y = 0.07 + 0.29x$.

the secretion of the VLDL is increased. Fig. 4 depicts the calculated correlation between the hepatic concentration of cholesteryl esters and the output of VLDL TG by the liver. When lovastatin was administered to the rats for 1 week, the decrease in total hepatic cholesterol was detected only in the esterified cholesterol fraction. No change in the total free cholesterol amount was detected. It may be that most of the hepatic free cholesterol is primarily associated with the various cellular membranes and is not in rapid equilibrium with the cholesteryl ester pool, and therefore is not readily available as a source of cholesterol for the secretion of the VLDL. We suggest that the cholesteryl esters in the liver are in rapid equilibrium with a small putative metabolic pool of free cholesterol, and this free cholesterol is required for the synthesis of the VLDL, primarily as a surface component, which, in part, determines the amount of VLDL that is secreted by the liver. The existence of this putative metabolic pool of cholesterol available for secretion of plasma lipoproteins (VLDL) is further enhanced by the much higher specific radioactivity (d.p.m./ μ mol) of cholesterol (synthesized from $[2-^{14}\text{C}]\text{acetate}$) of the VLDL compared with that of the liver. This concept would require the action of a cholesterol ester hydrolase in the metabolic processes resulting in the secretion of a VLDL, and the action of acyl-CoA:cholesterol acyltransferase in the storage of cholesteryl esters as precursors of the putative metabolic pool of free cholesterol.

It is of interest that under our experimental conditions, as discussed above, the specific radioactivity and incorporation of radioactive acetate into free cholesterol of the liver and the VLDL by the lovastatin (diet) group were increased dramatically over those of controls in the absence of lovastatin in the perfusate. Although the livers from lovastatin-fed rats may synthesize and secrete VLDL particles containing cholesterol arising from synthesis *de novo* during perfusion, the amount of newly synthesized cholesterol is evidently insufficient to main-

tain normal rates of secretion of the VLDL when stores of cholesteryl esters are depleted. To evaluate hepatic synthesis of cholesterol *in vivo* in animals treated with lovastatin, 5 mCi of $^3\text{H}_2\text{O}$ was injected intraperitoneally into control and lovastatin-fed rats [29]. Then 1 h later, livers were removed, and hepatic free cholesterol was isolated (see the Methods section). These studies were carried out in animals treated identically with those used for the perfusion experiments. Incorporation of $^3\text{H}_2\text{O}$ into free cholesterol was increased by treatment with lovastatin (690 ± 70 and 4890 ± 380 d.p.m./g of liver for control and lovastatin-fed groups respectively; specific radioactivity increased similarly, 200 ± 20 and 1310 ± 110 d.p.m./ μ mol of cholesterol for control and lovastatin-fed groups respectively). These data obtained *in vivo* are similar to those obtained by using $[2-^{14}\text{C}]\text{acetate}$ in the perfused liver. Despite increased synthesis of cholesterol *de novo* induced by lovastatin under these conditions *in vivo*, hepatic concentration of cholesteryl esters was decreased, similar to the data presented in Table 2. In experiments using compactin, an HMG-CoA reductase inhibitor, Fears *et al.* [29] reported that, when the inhibitor was removed by dilution *in vitro*, or decreased below the inhibitory concentration *in vivo*, the activity of induced enzyme was expressed, and cholesterologenesis was increased to rates above control values. It must be assumed that inhibition of hepatic cholesterol synthesis *in vivo*, by administration of lovastatin to the intact rat, or inhibition in the perfused liver *in vitro*, depends on the relative effects of hepatic drug concentration and induction of enzymes of cholesterol synthesis. These opposing effects *in vitro* may vary with time after feeding of drug in the diet, or by parenteral administration. Data supportive of this concept have been obtained by A. W. Alberts (personal communication). These workers measured incorporation of $[^{14}\text{C}]\text{acetate}$ into cholesterol, and HMG-CoA reductase activity in rats treated orally with lovastatin. They obtained data suggestive of increased rates of synthesis of cholesterol under these conditions; nevertheless, calculations (area under the curve) of cholesterol synthesized indicate a decrease in mass with the drug, in agreement with our observations. Clearly, therapy with lovastatin lowered the concentration of cholesteryl ester in the liver and diminished secretion of the VLDL, both of which are reversed by provision of a source of cholesterol to the liver. These are facts independent of the specific mechanism(s) of action of lovastatin. Conceivably, lovastatin may act by mechanisms other than as an inhibitor of cholesterol synthesis to decrease the hepatic pool of cholesteryl ester.

Over two decades ago [30], our laboratory proposed that if the TG, phospholipid and cholesterol 'of the $d < 1.020$ lipoprotein are secreted by the liver in fixed proportions to provide the necessary physical properties for the dispersion of these lipids in an aqueous environment, and if this lipoprotein fraction functions as a vehicle for triglyceride transport, then it is not unreasonable to suggest that interference with the synthesis or availability of any component of the lipoprotein unit, either lipid or protein moiety, could result in decreased capability of the liver to release triglyceride'. Subsequently, this statement was modified by work from this laboratory to recognize the capability of the liver to form larger VLDL particles, with altered ratios of phospholipid: TG and cholesterol: TG under conditions of

Fig. 5. Postulated relationship between the composition of the secreted VLDL and its LDL metabolic product

This Figure suggests a mechanism by which fatty acids may affect the composition of the VLDL, and subsequently its metabolic products. The VLDL secreted by the liver in response to saturated fatty acids is a smaller, denser, particle and contains a higher molar ratio of cholesterol: TG than does the VLDL secreted in response to monounsaturated or polyunsaturated fatty acids [18]. As the VLDL is metabolized by removal of TG and apoproteins to give rise to the LDL, it may be expected that the LDL will contain relatively more cholesterol when derived from saturated fatty acids. Key: ■, TG; ▨, phospholipid (PL); □, cholesterol (C). Abbreviation: IDL, intermediate-density lipoprotein.

increased stimulation of TG synthesis by NEFA, presumably because one or more of the surface components may become limiting [31]. Nevertheless, the process of assembly and secretion becomes saturated before that of TG synthesis, and TG accumulates in the liver. These statements apply to the interpretation of the research reported here. The data reported here suggest to us that the limited availability of cholesterol resulted in decreased formation of VLDL for transport and accumulation of TG in the liver. It is clear from various studies that choline deficiency [32,33] or a decrease in phospholipid synthesis by various procedures [34] decreased the hepatic secretion of TG. It had also been observed previously that chronic administration of a hypocholesterolaemic drug, triparanol, produced a fatty liver [35,36], presumably resulting from a diminished ability to secrete TG. It is known that dietary protein restriction [37] or an imbalance in amino acids [37,38] can give rise to a TG-rich fatty liver, again presumably owing to decreased TG secretion. It is known that addition of puromycin [39] or cycloheximide [40] to the medium will decrease TG secretion, i.e. VLDL output, by the perfused liver.

The work reported here demonstrates that the availability of cholesterol can also regulate the transport of TG in the VLDL, and therefore cholesterol (presumably, free cholesterol) is an obligate requirement for VLDL formation. Our interest has focused on cholesterol because of the known association of hypercholesterolaemia with the development of atherosclerotic cardiovascular disease. Since (1) cholesterol appears to be an obligate component of the VLDL, particularly free cholesterol as a surface

component, (2) the secretion and synthesis of cholesterol are stimulated by fatty acids, which are the primary substrates for the formation of TG and secretion of the VLDL, (3) several enzymes in the pathway of cholesterol biosynthesis are also activated by fatty acids [5–7], and (4) the VLDL cholesterol is the precursor of the cholesterol of plasma intermediate-density lipoprotein and LDL [41], it is reasonable to propose that plasma cholesterol is derived to a significant degree from the secretion of the VLDL, and that this reflects the availability of fatty acid to the liver [42]. Furthermore, the amount of cholesterol secreted in the VLDL particle is also dependent on the structure of the fatty acid, from which the transported TG is derived. The VLDL particles formed and secreted by the livers perfused with saturated fatty acids require a higher molar ratio of free and esterified cholesterol and phospholipid per mol of TG transported than does VLDL secreted by livers perfused with monounsaturated or polyunsaturated fatty acids [18]. The subsequent degradation of the VLDL particles by lipoprotein lipase can theoretically result in LDL particles that are relatively enriched in cholesterol, when derived from VLDL synthesized primarily in response to saturated fatty acids. This may be an important mechanism by which dietary saturated fatty acids increase plasma cholesterol relative to that of equivalent amounts of mono- or poly-unsaturated fatty acids. Fig. 5 illustrates this concept. Clearly, the secretion of cholesterol by the liver reflects the metabolism of fatty acids by the liver and subsequent secretion of the VLDL particle. Furthermore, our present study suggests that the hepatic pool of

cholesteryl esters may be the putative metabolic pool of cholesterol, or, more likely, is in rapid equilibrium with a smaller pool of free cholesterol which is available and necessary for the secretion of VLDL (that is, TG) by the liver. Without an adequate source of free cholesterol, presumably related to the amount of hepatic cholesteryl esters, or derived from exogenous sources (e.g. LDL) the output of VLDL may be diminished, and less endogenous cholesterol derived from the VLDL would contribute to the plasma pool of cholesterol.

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