A role for smooth endoplasmic reticulum membrane cholesterol ester in determining the intracellular location and regulation of sterol-regulatory-element-binding protein-2

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Cellular cholesterol homeostasis is regulated through proteolysis of the membrane-bound precursor sterol-regulatory-element-binding protein (SREBP) that releases the mature transcription factor form, which regulates gene expression. Our aim was to identify the nature and intracellular site of the putative sterol-regulatory pool which regulates SREBP proteolysis in hamster liver. Cholesterol metabolism was modulated by feeding hamsters control chow, or a cholesterol-enriched diet, or by treatment with simvastatin or with the oral acyl-CoA:cholesterol acyltransferase inhibitor C1-1011 plus cholesterol. The effects of the different treatments on SREBP activation were confirmed by determination of the mRNAs for the low-density lipoprotein receptor and hydroxymethylglutaryl-CoA (HMG-CoA) reductase and by measurement of HMG-CoA reductase activity. The endoplasmic reticulum was isolated from livers and

separated into subfractions by centrifugation in self-generating iodixanol gradients. Immunodetectable SREBP-2 accumulated in the smooth endoplasmic reticulum of cholesterol-fed animals. Cholesterol ester levels of the smooth endoplasmic reticulum membrane (but not the cholesterol levels) increased after cholesterol feeding and fell after treatment with simvastatin or C1-1011. The results suggest that an increased cellular cholesterol load causes accumulation of SREBP-2 in the smooth endoplasmic reticulum and, therefore, that membrane cholesterol ester may be one signal allowing exit of the SREBP-2/SREBP-cleavage-regulating protein complex to the Golgi.

Key words: acyl-CoA:cholesterol acyltransferase, cholesterol feeding, HMG-CoA reductase, LDL receptor, simvastatin.

INTRODUCTION

Cellular cholesterol levels are tightly regulated by the membrane-bound transcription factors, sterol-regulatory-element-binding proteins (SREBPs) [1–3]. Three forms of SREBP exist: SREBP-2, which is considered to be most active in regulation of genes involved in cholesterol homeostasis; SREBP-1a, involved in both cholesterol and fatty acid metabolism; and SREBP-1c, which is mainly involved in regulation of genes involved in fatty acid biosynthesis [2,4–8]. SREBP-1c is predominant in liver, while the SREBP-1a is predominant in cultured cell lines [2]. The membrane-bound precursor form of SREBP consists of approx. 1150 amino acid residues; the N-terminal segment (approx. 500 amino acids) is the mature transcription factor and the C-terminal segment serves to anchor the protein in the membrane through a hairpin loop made up of two transmembrane domains, and also provides a C-terminal cytosolic domain involved in protein–protein interactions [2,3,9]. When cellular cholesterol levels fall, the N-terminal segment is released through proteolysis and moves to the nucleus, where it activates transcription of genes involved in cholesterol synthesis and uptake by the cell. Proteolysis of membrane-bound SREBP-2 is dependent on association with SREBP-cleavage-activating protein (SCAP)

[10,11]. Formation of the SREBP-2–SCAP complex facilitates two proteolytic steps [12–17]. The first step is catalysed by site 1 protease (S1P) and cleaves the luminal loop of SREBP-2 [12–14]. This allows a second proteolytic step, catalysed by membrane-bound zinc-metalloprotease site 2 protease (S2P), which releases the N-terminal mature form of SREBP [15–17].

For many years the existence of a ‘putative cholesterol-regulatory pool’ involved in determining the activity of key enzymes in cholesterol homeostasis has been postulated [18]. However, despite the considerable recent progress in understanding the role of SREBP, the intracellular site of the putative regulatory pool remains unidentified. Nor is it clear whether cholesterol, a different sterol, or another molecule is involved [18]. In the present study, we set out to identify and locate the intracellular sterol-regulatory pool. Most research on the mechanism of activation of SREBP has been carried out using tissue culture cell lines, frequently after genetic manipulation. However, in animals, cholesterol homeostasis through SREBP activation is regulated by physiological factors, including a major role played by nutrition. Ultimately, it is necessary to extend studies of SREBP activation from tissue culture cell lines to whole animals. In the present study we have chosen to investigate the cholesterol-regulatory pool in the hamster, which is an established

Abbreviations used: SREBP, sterol-regulatory-element-binding protein; SCAP, SREBP-cleavage-activating protein; S1P, site 1 protease; S2P, site 2 protease; HMG-CoA, hydroxymethylglutaryl-CoA; LDL, low-density lipoprotein; ER, endoplasmic reticulum; SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum; ACAT, acyl-CoA:cholesterol acyltransferase; TAG, triacylglycerol; HPTLC, high performance thin layer chromatography; CHO, Chinese-hamster ovary; VLDL, very-low-density lipoprotein; LDLr, low-density-lipoprotein receptor.

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model for studies of lipoprotein metabolism [19–22] and has been shown to regulate cholesterol metabolism through activation of SREBP-2 [23]. To modulate the release of the mature form of SREBP-2, and hence the size of the putative sterol regulatory pool, hamsters were fed a diet enriched in cholesterol or were fed a statin (simvastatin). The relative effects of cholesterol ester and cholesterol were also investigated by treating hamsters with an orally administered acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor (C1-1011). Our rationale and experimental design is similar to that used by others investigating SREBP in hamster liver [23]. Modification of the hepatic cellular cholesterol load, through dietary or drug manipulation, results in new steady states of SREBP-activated gene expression. However, as the mature form of SREBP is rapidly degraded in the nucleus, the signalling mechanism that modulates proteolysis of intracellular SREBP also reaches a new steady state. Thus the 'sterol-regulatory pool' remains depressed under conditions of cholesterol loading and elevated under conditions of cholesterol depletion.

We made an initial assumption, based on existing literature, that the endoplasmic reticulum (ER) is the most probable site of the sterol regulatory pool. To investigate the distribution of SREBP-2 and the ER lipids, we used a centrifugation method recently developed in this laboratory, in which the total ER is separated into rough ER (RER) and smooth ER (SER) in self-generating gradients of iodixanol [24]. Moreover, each of these major fractions is separated into subfractions allowing fine resolution of the continuous ER compartment. By analysis of the ER subfractions, we have made the novel observations that, under conditions of cholesterol excess, the SREBP-2 precursor is predominantly in the SER and, under conditions of cholesterol depletion, SREBP-2 is in the RER. Parallel analysis of the membrane lipids of ER subfractions showed that cholesterol ester levels of the SER membranes increased in cholesterol-fed hamster liver and decreased in simvastatin- and ACAT inhibitor-treated hamster liver. Although it is well established that feeding cholesterol activates hepatic ACAT and increases total intracellular cholesterol esters, this is the first study in which the lipid compositions of ER subfraction membranes have been measured and correlated with the intracellular site and activation of SREBP-2. The results suggest that the amount of SER membrane cholesterol ester may signal cellular cholesterol levels and indirectly or directly modulate proteolysis of SREBP-2.

EXPERIMENTAL

Materials

Simvastatin was a gift from Merck Sharpe Dohme (Enfield, Middx, U.K.); the orally administered ACAT inhibitor C1-1011 was a gift from Dr Max Walker (GlaxoWellcome Stevenage, Herts, U.K.). Optiprep (60% iodixanol) and Maxidens were purchased from Lipotek Ltd (Upton, Merseyside U.K.). Hybridoma cells expressing anti-SREBP-2 (7D4), which was raised against amino acids 32–250 of hamster SREBP-2 [25], were purchased from A.T.C.C., cultured and the monoclonal antibody purified by Antibody Technologies Limited (Sheffield University, Sheffield, U.K.).

Animals

Male DSNI Golden Syrian hamsters (120–140 g) used for these studies were bred in the Joint Animal Breeding Unit, University of Nottingham. The animals were maintained on Rodent Maintenance diet 3 powdered form (RM3, Special Diet Services, Witham, Essex, U.K.) and exposed to a 12 h light/dark cycle.

The following experimental diets were fed for 2 weeks: chow; chow supplemented with 0.5% cholesterol; chow mixed with simvastatin (0.013%, w/w); and control chow supplemented with 0.5% cholesterol mixed with the ACAT inhibitor, C1-1011 (0.0033%, w/w) [26]. Hamsters had free access to food and water and were killed at 09:00 h, the end of the dark period.

Subcellular fractionation

Livers were removed from hamsters and homogenized in 0.25 M sucrose. ER-enriched vesicles (microsomes) were prepared and separated into subfractions in self-generated gradients of iodixanol (20% initial concentration), as described previously for rabbit liver [24]. The gradients were unloaded by upward displacement with Maxidens and were collected in 20 fractions. The total microsomes and gradient fractions were characterized by assay of protein, NADPH-cytochrome *c* reductase and RNA as described previously [24] and contained no detectable galactosyltransferase (Golgi marker), succinic dehydrogenase (mitochondrial marker), acid phosphatase (lysosome marker) or 5' nucleotidase (plasma membrane marker). The gradient fractions, which consist of closed membrane vesicles, were separated into membrane and luminal contents by carbonate treatment [24,27]. In previous experiments we have shown that luminal markers (protein disulphide isomerase and newly synthesized albumin) are absent from the membrane fraction but recovered in the content fraction, and that repeated treatment of the membranes with sodium carbonate does not increase the amount of very-low-density-lipoprotein (VLDL), apolipoprotein B or lipid released into the content fraction.

Lipid extraction and analysis

Lipids were extracted from aliquots of the total homogenates, total microsomes and the gradient fractions, and the neutral lipids were separated by high performance thin layer chromatography (HPTLC), stained and quantified using laser densitometry as described previously [24,28].

Immunoblotting analysis

SREBP-2 was detected by immunoblotting after separation of the gradient fraction proteins by SDS/PAGE on 3–20% (w/v) polyacrylamide gradients using 7D4 as primary antibody and anti-mouse IgG coupled to alkaline phosphatase as secondary antibody [29,30]. The protein composition of the fractions in sample buffer was assayed and the same amount of protein (100 µg) was applied to each well. In practice, 30–100 µl of sample made up to 100 µl with sample buffer was applied to wells.

mRNA determination

Liver (100 mg) was removed, immersed and stored in RNA-later (Ambion, Austin, TX, U.S.A.). Prior to homogenization total RNA was extracted [31] and mRNA levels for 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and low-density lipoprotein (LDL) receptor (LDLR) were determined by solution hybridization RNase protection assay as described previously [32,33].

Other assays

ACAT activity was determined as described previously [24] and HMG-CoA reductase was assayed as described in [34].

RESULTS

Effect of diet or drug treatment on the cholesterol, cholesterol ester and triacylglycerol (TAG) content of liver and isolated microsomes

Compared with chow-fed controls, the unesterified cholesterol content of total microsomes was not significantly altered by feeding cholesterol or by simvastatin treatment, although the cholesterol content of the whole liver fell by approx. 40 % in response to simvastatin treatment (Table 1). The major effect of either treatment was on the cholesterol ester content, which increased in response to cholesterol feeding in both microsomes and liver and fell in response to simvastatin, and on the TAG content of whole liver, which fell in response to cholesterol feeding and rose in response to simvastatin treatment (Table 1). The unesterified cholesterol content of the microsomes and, to a lesser extent, of the whole liver was apparently maintained within fairly narrow limits, with the excess cholesterol being esterified. In an attempt to increase microsomal cholesterol levels, we also investigated the effect of feeding an ACAT inhibitor (CI-1011) with 0.5 % cholesterol. Under these conditions, the cholesterol ester content of microsomes and whole liver fell. However, there was no change in the unesterified cholesterol content of the microsomes, but a decrease in the total liver unesterified cholesterol and an increase in TAG content of the liver. Treatment of hamsters with an ACAT inhibitor apparently overrides the effects of cholesterol feeding on liver and microsomal lipids and mimics the effect of simvastatin treatment. It is probable that this is due, at least in part, to inhibition of cholesterol absorption in the gut by the orally administered CI-1011.

TAG, unesterified cholesterol and cholesterol ester are minor lipid components of the microsomal membranes accounting for approx. 5 %, 2 % and 0.3 % respectively of the total lipid, which is predominantly phospholipid. There were no significant differences between the TAG and unesterified cholesterol content of the membranes, prepared by carbonate treatment of the microsomes from livers of hamsters treated in the four different ways (Table 2). However, the cholesterol ester content was increased by 63 % in membranes from livers of cholesterol-fed hamsters compared with control chow ($P < 0.01$) and decreased by 52 % and 41 % in membranes prepared from livers of hamsters treated with simvastatin and ACAT inhibitor + cholesterol respectively ($P < 0.01$).

Table 2 Lipid composition of microsomal membranes prepared from livers of hamsters subjected to diet or drug treatment

Total microsomes were prepared from livers of hamsters. The microsomal vesicles were separated into membrane and luminal content fractions and the lipids were extracted and analysed by HPTLC as described in the Experimental section. Results are the means $\pm$ S.D. for the membrane composition ($\mu\text{g}/\text{mg}$ of original membrane protein) from six experiments. Student's t test was used to compare experimental samples with the chow-fed control value; * $P < 0.01$, ** $P < 0.002$, unmarked values $P > 0.05$.

	Unesterified cholesterol ($\mu\text{g}/\text{mg}$ of protein)	TAG ($\mu\text{g}/\text{mg}$ of protein)	Cholesterol ester ($\mu\text{g}/\text{mg}$ of protein)
Cholesterol-fed	16.79 $\pm$ 1.22	39.25 $\pm$ 6.13	3.12 $\pm$ 0.42**
Chow-fed	14.81 $\pm$ 3.63	31.68 $\pm$ 4.71	1.97 $\pm$ 0.34
Simvastatin-treated	13.23 $\pm$ 2.34	36.11 $\pm$ 7.8	0.91 $\pm$ 0.19**
ACAT inhibitor + cholesterol	15.25 $\pm$ 1.99	29.83 $\pm$ 4.02	1.18 $\pm$ 0.23**

Expression of HMG-CoA reductase and the LDLr in livers of hamsters subjected to diet or drug treatment

Cholesterol feeding reduced mRNA levels for HMG-CoA reductase and the LDLr by 35 % and 15 % respectively compared with the chow-fed controls, whereas treatment with simvastatin or ACAT inhibitor + cholesterol increased mRNA levels for HMG-CoA reductase by 362 % and 212 % respectively and for the LDLr by 188 % and 186 % respectively (Table 3). HMG-CoA activity in liver microsomes showed a relatively large variation between individual hamsters; however, there was a significant decrease in activity by ~ 60 % in the cholesterol-fed hamster liver microsomes and increased activities of 27- and 14-fold respectively in microsomes from hamsters treated with simvastatin or ACAT inhibitor + cholesterol (Table 3).

SREBP-2 distribution in ER gradient fractions prepared from livers of hamsters subjected to different dietary or drug treatments

To determine the distribution of SREBP-2 in different compartments of the ER, the microsomes from livers of hamsters were

Table 1 Effect of diet or drug treatment on the lipid composition of hamster liver homogenate and total microsomes

Hamsters were subjected to diet or drug treatment as described in the Experimental section. The livers were removed, homogenized and total microsomes isolated. Proteins were assayed and the lipids were extracted from aliquots of the homogenates and the microsomes and quantified by HPTLC. Results are the means $\pm$ S.D. from six experiments. Student's t test was used to compare values from experimental samples with the control value (chow-fed); * $P < 0.01$, ** $P < 0.002$, unmarked values $P > 0.05$.

	Protein (mg/g of liver)	Unesterified cholesterol (mg/g of liver)	TAG (mg/g of liver)	Cholesterol ester (mg/g of liver)	Phospholipid (mg/g of liver)
Homogenate					
Cholesterol-fed	230.07 $\pm$ 68.036	2.89 $\pm$ 0.19	2.02 $\pm$ 0.19**	33.84 $\pm$ 0.80**	317.94 $\pm$ 66.11
Chow-fed	210.95 $\pm$ 14.36	2.48 $\pm$ 0.64	4.42 $\pm$ 0.12	2.59 $\pm$ 0.18	253.01 $\pm$ 27.40
Simvastatin-treated	229.56 $\pm$ 15.87	1.52 $\pm$ 0.27**	5.09 $\pm$ 0.36*	0.43 $\pm$ 0.06**	302.20 $\pm$ 67.26
ACAT inhibitor + cholesterol	213.01 $\pm$ 12.76	1.57 $\pm$ 0.06**	9.34 $\pm$ 0.58**	0.31 $\pm$ 0.06**	308.41 $\pm$ 53.10
Microsomes					
Cholesterol-fed	20.30 $\pm$ 2.48	0.55 $\pm$ 0.04	1.11 $\pm$ 0.18	0.271 $\pm$ 0.063*	19.95 $\pm$ 3.32
Chow-fed	24.80 $\pm$ 4.43	0.67 $\pm$ 0.13	1.75 $\pm$ 0.31	0.174 $\pm$ 0.034	20.97 $\pm$ 3.69
Simvastatin-treated	26.65 $\pm$ 2.63	0.56 $\pm$ 0.12	3.23 $\pm$ 0.78*	0.0597 $\pm$ 0.019**	22.76 $\pm$ 2.32
ACAT inhibitor + cholesterol	27.53 $\pm$ 1.12	0.69 $\pm$ 0.02	2.75 $\pm$ 0.98	0.0839 $\pm$ 0.005**	20.02 $\pm$ 1.07

Table 3 Effect of diet or drug treatment on expression of HMG-CoA reductase and the LDLr in hamster liver

mRNA levels for HMG-CoA reductase and the LDLr were determined by the RNase protection assay, as described in the Experimental section. The results are means $\pm$ S.D. (amol of mRNA/ μ g of total liver mRNA) from three separate preparations. The activity of HMG-CoA reductase was determined in microsomes isolated from livers of treated animals as described in the Experimental section. The results are means $\pm$ S.D. from four preparations. Student's *t* test was used to compare values from experimental samples with the chow-fed control value; **P* < 0.01, ***P* < 0.002, unmarked values *P* > 0.05.

	LDLr mRNA (amol/ μ g of liver mRNA)	HMG-CoA reductase mRNA (amol/ μ g of liver mRNA)	HMG-CoA reductase activity (pmol/min per mg of protein)
Cholesterol-fed	4.83 $\pm$ 0.52*	2.09 $\pm$ 0.35*	1.51 $\pm$ 0.81*
Chow-fed	5.67 $\pm$ 0.48	3.25 $\pm$ 0.49	2.68 $\pm$ 0.92
Simvastatin-treated	10.64 $\pm$ 0.97**	11.75 $\pm$ 0.89**	34.50 $\pm$ 10.43**
ACAT inhibitor + cholesterol	10.57 $\pm$ 0.81**	6.89 $\pm$ 1.09**	17.83 $\pm$ 4.72**

Figure 1 Distribution of organelle markers on iodixanol gradients

Total microsomes were prepared from livers of chow-fed hamsters and separated in self-generating gradients of iodixanol as described in the Experimental section. Fractions were collected from the top of the gradient (light-end) first. The protein (●), NADPH-cytochrome *c* reductase activity (◇), phospholipid (◆) and RNA (○) were determined. Recoveries of each of these components from the gradients were 85–90%. In order to compare the distribution of the components, the activities are plotted as the % recovery in each fraction, with RNA plotted on the right-hand axis. Results plotted are means $\pm$ S.D. (*n* = 4).

separated in self-generating gradients of iodixanol. As we have shown previously [21,24], this procedure separates the microsomal vesicles into two major peaks. The heavier peak, fractions 13–20, contains the RER-derived vesicles (identified by RNA and NADPH-cytochrome *c* reductase), and the lighter peak, fractions 1–9, contains SER-derived vesicles (identified by NADPH-cytochrome *c* reductase without RNA) with a peak in fractions 3–7 (Figure 1). The RER peak is heterogeneous such that the fractions towards the denser part of the peak are enriched with bound ribosomes and have lower NADPH-cytochrome *c* reductase activity compared with those towards the less dense end of the peak. There was no significant difference between the protein, phospholipid and marker distributions in gradient fractions from livers of hamsters subjected to diet/drug treatment (results not shown).

Immunodetectable SREBP-2 was at the highest concentration in gradient fractions 15–21 from livers of chow-fed hamsters (Figure 2). These fractions are coincident with the RER peak. A

Figure 2 Distribution of SREBP-2 in gradient fractions

Total microsomes were prepared from livers of hamsters subjected to diet or drug treatment and separated in self-generating gradients of iodixanol as described in the Experimental section. Gradient fractions were dissolved in sample buffer and the protein content determined. The same amount of protein (100 μ g) was applied to each well (30–100 μ l made up to 100 μ l with sample buffer before application). Alternate fractions from the gradient (fraction 1 is the less dense end of the gradient) were separated, using a single gel for each gradient, by SDS/PAGE and SREBP-2 was detected by immunoblotting as described in the Experimental section. The immunoblots illustrated are typical of four separate experiments.

similar distribution of SREBP-2 was observed in gradient fractions from livers of hamsters treated with ACAT inhibitor + cholesterol (Figure 2). After treatment of hamsters with simvastatin, immunodetectable SREBP-2 showed a slight shift to the less dense gradient fractions 11–17 (Figure 2). In fractions prepared from livers of hamsters fed cholesterol, SREBP-2 was at the highest concentration in fractions 3–9, coincident with the SER peak, although SREBP-2 was also detected in the denser fractions 13–17 (Figure 2). It is difficult to quantify immunoblots; however, there was no marked change in the apparent amount of SREBP in the microsomes and the same amount of protein was applied to each well. We have also measured the SREBP-2 protein by ELISA and this does not alter significantly. The effect of the different treatments was thus to cause a shift in the intracellular site of SREBP-2 to the SER from the RER, under conditions of cholesterol loading.

Lipid composition of membranes of ER gradient fractions prepared from livers of hamsters subjected to different dietary or drug treatments

The total unesterified cholesterol content of microsomal membranes (Table 2) was not altered significantly by the different diet and drug treatments. However, as we observed changes in distribution of SREBP-2 in the ER, we looked for differences in the distribution of the ER lipids, which might signal cholesterol loading. If a small pool of unesterified cholesterol is involved in signalling this might change with SREBP-2 distribution and activation, but be masked by the total membrane pool of cholesterol.

The unesterified cholesterol, cholesterol ester and TAG content of the total microsomes and membranes of gradient fractions

Figure 3 Lipid composition of total microsomes and membrane fractions of liver microsomes separated on iodixanol gradients

Livers were removed from chow-fed hamsters and total microsomes were isolated and separated in self-generating gradients of iodixanol as described in the Experimental section. Fractions were collected from the top of the gradient (light-end) first. Lipids were extracted from aliquots of gradient fractions without further treatment (total microsomes). Further aliquots of gradient fractions were pelleted by centrifugation, separated into membrane and luminal content fractions and the lipids were extracted (membranes). The lipids of luminal contents are not plotted; however, membrane and luminal content recoveries were $> 80\%$. Results are the means $\pm$ S.E.M. ($n = 4$). ●, Total fraction; ○, membrane fraction; in the latter case the symbols sometimes overlap the error bars.

increased through the gradient from the RER to the SER (Figure 3). As we have reported previously [24], when the gradient fractions were opened by carbonate treatment, TAG, unesterified cholesterol and cholesterol ester destined for assembly into secreted VLDL was found in the luminal contents, particularly of the SER peak fractions (Figure 3). Fractions from livers of cholesterol-treated hamsters showed similar distributions between membrane and luminal lipids, except that there was increased luminal TAG in fractions from the simvastatin- and ACAT inhibitor + cholesterol-treated hamster livers and decreased luminal TAG in the fractions from the livers of cholesterol-fed hamsters (results not shown).

There was no significant difference between the unesterified cholesterol content of the membranes prepared from gradient fractions from livers of hamsters subjected to the different diet or drug treatments (Figure 4). Apart from a small increase in the membrane TAG in fractions 1–5 from livers of animals treated with simvastatin or fed cholesterol, there was no significant difference in the TAG content in gradient fractions (Figure 4).

Figure 4 Lipid composition of membrane fractions prepared from livers of hamsters subjected to diet or drug treatment

Experiments were performed as described in Figure 3 on hamsters subjected to diet and drug treatments. Only the data for membrane lipid compositions (unesterified cholesterol, TAG and cholesterol ester) are plotted. The cholesterol ester content of the dense fractions (7–20) is plotted with a smaller scale in the inset of the top graph. Results are the means $\pm$ S.E.M. ($n = 4$). In some cases the error bars are concealed by the symbols. ■, Cholesterol fed; □, chow fed; ●, simvastatin treated; ○, ACAT inhibitor treated.

However, after cholesterol feeding, the membrane cholesterol ester of fractions 5–20, which correspond to the lighter part of the SER plus the RER, increased 2-fold compared with chow-fed controls ($P < 0.002$ comparing individual fractions). After simvastatin treatment, the cholesterol ester of all fractions (1–20) was reduced 2–3-fold ($P < 0.002$) compared with chow-fed controls, whereas after treatment with ACAT inhibitor + cholesterol, the cholesterol ester of the SER (fractions 1–8) was reduced ($P < 0.002$) compared with chow-fed controls, but that of the RER peak (fractions 8–20) was not. Both ACAT inhibitor and simvastatin increased expression of the LDLr and HMG-CoA reductase compared with chow feeding. However, cholesterol ester was reduced in the SER fractions and not the RER fractions from ACAT inhibitor + cholesterol-treated hamsters, suggesting that cholesterol ester in the SER is important rather than that in the RER.

One mechanism by which membrane cholesterol ester might be altered by cholesterol feeding or simvastatin treatment is

Figure 5 ACAT activity of gradient fractions

Total microsomes were prepared from livers of hamsters subjected to diet or drug treatment and separated in self-generating gradients of iodixanol as described in the Experimental section. ACAT activity (pmol/min per mg of protein) was determined on aliquots of the total microsomes (lower graph) and gradient fractions (upper graph). The results plotted are the means $\pm$ S.D. ($n = 4$). ■, Chow fed; □, cholesterol fed; ●, simvastatin treated; ○, ACAT inhibitor treated.

through modulation of ACAT activity. There was considerable variation in the specific activity of ACAT in the gradient fractions between individual hamsters resulting in large S.D.s. However, the distribution of ACAT activity was similar in all gradients with the peak activity in the SER (Figure 5). The specific activity of ACAT was increased both in SER fractions 5–8, which also exhibited an increase in membrane cholesterol ester, and in the total microsomes from cholesterol-fed hamsters. However, simvastatin treatment had no significant effect compared with the chow-fed controls (Figure 5). These results suggest that the level of ACAT activity in the ER is not the limiting factor regulating membrane cholesterol ester levels. Unexpectedly, the ACAT activity of the SER fractions from livers of hamsters treated with ACAT inhibitor fell by only approx. 30%, although treatment of hamsters *in vivo* with ACAT inhibitor reduced the cholesterol ester of total microsomes and SER subfractions. However, when the ACAT inhibitor was added directly to the isolated fractions, activity was completely abolished suggesting that the inhibitor was washed out during preparation of subcellular fractions.

Relationship of microsomal HMG-CoA reductase activity and cholesterol ester levels

HMG-CoA reductase is an indicator of gene expression (although this is approximate because HMG-CoA reductase is also regulated by post-translational degradation which is stimulated by increased cellular cholesterol). The amount of cholesterol ester in preparations of microsomes from individual hamsters treated in the four different ways correlated with the microsomal HMG-CoA reductase activity ($r^2 = 0.79$). The relationship suggests that there is a threshold of approx. 5 μ g of cholesterol ester/mg of microsomal protein below which HMG-CoA reductase activity

Figure 6 Relationship of HMG-CoA reductase activity to lipid composition of microsomes

Total liver microsomes were prepared from livers of hamsters subjected to diet or drug treatment. HMG-CoA activity and the lipid composition were determined as described in the Experimental section. The data for individual hamsters are plotted. Cholesterol ester correlated with HMG-CoA reductase activity ($y = 400x^{-2.4}$, $r^2 = 0.79$). There was no correlation between TAG ($y = 0.21x^{1.011}$, $r^2 = 0.31$) or cholesterol ($y = 41.40x^{-2.6}$, $r^2 = 0.003$) with HMG-CoA reductase activity. ■, Cholesterol fed; □, chow fed; ●, ACAT inhibitor + cholesterol treated; ○, simvastatin treated.

is increased and above which activity is reduced (Figure 6). Although there appeared to be a tendency for HMG-CoA reductase activity to increase with increased TAG and cholesterol the correlation was poor ($r^2 = 0.31$ for TAG and 0.003 for cholesterol).

DISCUSSION

The liver plays a central role in whole body cholesterol homeostasis. It is the main site of endogenous cholesterol synthesis, removes plasma lipoproteins from the circulation, secretes cholesterol as VLDL, and excretes cholesterol in bile. Co-ordination of cholesterol metabolism is orchestrated through modulation of proteolysis of the precursor form of SREBP. The signal which links cellular cholesterol loading or depletion with proteolysis of SREBP has not been identified. The rationale of the present investigation was that modulation of cholesterol homeostasis, coupled with subcellular fractionation, may reveal the sterol-regulatory pool and its intracellular site. Because the nuclear form of SREBP-2 is rapidly degraded by proteolysis, the altered sterol-regulatory pool will persist during dietary or drug treatment.

Most of the investigations of the molecular mechanisms involved in SREBP proteolysis have been carried out using genetically manipulated cultured cells including Chinese-hamster ovary (CHO) and HEK-293 cells. It is difficult to directly compare cultured cells with hepatocytes, since the ER/secretory compartment is usually far less developed in cultured cells and there is little SER. In CHO cells, approx. 20–40% of the SREBP-2 forms a complex with all of the SCAP, which is located in the ER [10]. Complex formation is necessary for the first proteolytic cleavage step of the luminal loop of SREBP by S1P [10,13,35]. However, in cholesterol-loaded or cholesterol-depleted CHO cells, the proportion of SREBP which co-precipitates with SCAP is similar, suggesting that this association is not sterol regulated [10]. Susceptibility of SCAP oligosaccharides to endoglycosidase H suggests that cholesterol depletion causes SCAP to move to the Golgi before returning to the ER, while under conditions of cholesterol loading SCAP remains in the ER [36,37]. Active forms of S1P are located in the ER and the Golgi [37–39]. The model/mechanism that reconciles all of these observations is that SCAP binds SREBP and, when a decrease in cellular cholesterol levels is signalled, the complex moves from the ER to the Golgi or pre-Golgi (e.g. the *cis*-Golgi network) compartment by a process requiring membrane budding [40]. Proteolysis of SREBP takes place and SCAP recycles to the ER. In experiments in which S1P is relocated to the ER from the Golgi, SREBP hydrolysis is not dependent on SCAP [39]. Thus, when SCAP senses a decrease in the cellular cholesterol content it escorts SREBP to the active S1P-containing compartment.

In the context of the model described above, an explanation for our observations is that newly synthesized SREBP-2 is incorporated into the RER membrane, and part of the SREBP forms a complex with SCAP and moves through the continuous membrane to the SER. From here it moves to the Golgi and the mature SREBP-2 is released by proteolysis. However, under conditions of cholesterol loading, the SREBP-2 remains in the SER. SREBP-2 is not detected in fraction 1 at the top of the gradient, although cholesterol ester does increase in the membranes of this fraction. This is consistent with retention of SREBP-2 in the SER as it moves from its site of synthesis, the RER, to the SER and encounters increased membrane cholesterol ester. Under conditions of cholesterol depletion and in untreated hamsters, SREBP-2 is only detected in the RER. This may be because SREBP-2 reaching the SER under these conditions is rapidly transferred to the Golgi and more SREBP-2 is synthesized in the RER.

Cholesterol ester synthesis has been implicated as a regulator of VLDL production by the liver, although not all studies have reached this conclusion (reviewed in [41,42]). Spady et al. [43] recently showed that overexpression of human ACAT-1 in mice results in increased production of VLDL and increased liver cholesterol ester levels, but no change in liver cholesterol levels. Overexpression of human ACAT-1 in hamsters did not alter expression of the LDLr. At first sight, this differs from our findings that an increase in SER cholesterol ester was associated with decreased expression of the LDLr. However, changes in bulk liver cholesterol ester [43] may not necessarily reflect changes in the very small membrane pool. Moreover, there are two forms of ACAT: ACAT-1, which is ubiquitous, and ACAT-2, which is found in liver and intestine [44–48]. It has been suggested that ACAT-1 (which was used by Spady et al. [43]) may provide cholesterol esters for storage, whereas ACAT-2 (which is in liver and intestine) produces cholesterol esters for secretion in VLDL.

Total cellular cholesterol levels are signalled to the SREBP/SCAP/S1P. The signal involved must be produced in proportion to the cholesterol load and be readily inactivated or removed, so

that signalling is stopped, when cholesterol levels fall. When cellular cholesterol levels increase, cholesterol is transferred to the ER and esterified; thus ER cholesterol ester is an indicator of cholesterol loading. Moreover, the membrane cholesterol ester pool is extremely small compared with membrane cholesterol and is removed from the membrane (and thus inactivated) to cytosolic stores or for secretion as a component of VLDL. Thus ER cholesterol ester better fits the role of a signalling molecule than unesterified cholesterol and alters in parallel with changes in SREBP-2 localization and modification of gene expression.

We cannot exclude the possibility that a small part of the membrane unesterified cholesterol is regulatory and that the cholesterol ester levels reflect inactivation of this pool. However, the method used for lipid analysis is very sensitive and revealed differences in membrane unesterified cholesterol ester levels, which are far smaller than the membrane cholesterol pool. It is also possible that changes in ER membrane unesterified cholesterol levels are masked by contamination with plasma membrane vesicles, which are highly enriched in cholesterol. However, plasma membrane vesicles and caveolae would move to the top of the iodixanol gradient used [49] and, although they may contribute to the total microsome unesterified cholesterol, they would not contaminate the ER gradient fractions.

The mechanism by which SER membrane cholesterol ester might modulate transfer of SCAP/SREBP to the S1P-containing compartment is unknown. The ER is a continuous membrane; however, transfer from the SER to the *cis*-Golgi network requires vesicle formation and membrane budding has been implicated in SREBP/SCAP transfer [40]. A role for cholesterol ester in vesicle formation, through changes in the physical structure of the bilayer and in recruitment of SCAP (which has a sterol-binding domain) into vesicles budding from the SER, is feasible but requires further investigation.

This work was supported by project grants from the Biotechnology and Biological Sciences Research Council (BBSRC) to J.A.H., A.J.B. and A.M.S. and by a BBSRC studentship to C.R.I.

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Received 16 March 2001/3 May 2001; accepted 20 June 2001