Hepatic and intestinal formation of polar sterols *in vivo* in animals fed on a cholesterol-supplemented diet

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In rats fed on a diet containing 1% cholesterol for 24 h, the decrease in hepatic non-saponifiable lipid synthesis, cholesterologenesis and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity was accompanied by an increase in the proportion of newly synthesized polar sterols *in vivo*. In these animals there was also a strong inverse correlation between the proportion of polar sterols in the non-saponifiable lipid and hepatic HMG-CoA reductase activity. A similar correlation was not observed in animals fed on a normal diet. Cholesterologenesis in the intestine was not as sensitive to inhibition by dietary cholesterol as was that in the liver, and there was no increase in the polar-sterol content of the newly synthesized non-saponifiable-lipid fraction.

INTRODUCTION

During the last several years, much evidence has emerged which supports the hypothesis that enzymically formed oxygenated cholesterol derivatives are directly involved in the normal regulation of cholesterol synthesis (e.g. [1–7]). However, evidence for a relationship between changes in the activity of HMG-CoA reductase, the major regulatory enzyme of cholesterologenesis, and the appearance of biogenetically derived oxysterols was lacking. More recently, it has been shown that, under certain conditions, inhibitors of cytochrome *P*-450 [8] enhance the accumulation of such oxysterols, while HMG-CoA reductase activity is suppressed [9–12]. Under more natural circumstances, cellular HMG-CoA reductase activity becomes suppressed in response to an increase in the availability of exogenous lipoprotein cholesterol. However, it is not known whether enzymically derived oxysterols are involved in the complex chain of events by which these changes are linked. The present results provide evidence that, during the suppression of hepatic cholesterol synthesis and HMG-CoA reductase activity by dietary cholesterol, there is a relative increase in the amounts of labelled oxysterols formed *de novo* in the presence of $^3\text{H}_2\text{O}$. Furthermore, the greater the extent of inhibition, the greater is the relative accumulation of these labelled substances. We believe that this is the first evidence for the existence of such a relationship *in vivo*, under conditions in which the liver responds to an excess of dietary cholesterol.

MATERIALS AND METHODS

Animals and diets

Male Wistar rats were fed and housed as described previously [13]. A cholesterol-containing diet was prepared by grinding pellets of chow to a fine powder and thoroughly mixing with cholesterol (1 g/100 g), which had been re-crystallized three times from a solution of

chloroform and methanol and stored at -20°C . Water containing a solution of vitamin E (20 mg) in ethanol (1 ml) was added to give a stiff paste, and the pellets were reconstituted and dried overnight in a stream of air at room temperature. The control diet was prepared similarly except that cholesterol was omitted. Animals were fed on the diet containing 1% cholesterol or the same diet without cholesterol (controls) for 24 h before the experiment. During this period, animals fed on the high-cholesterol diet consumed 35.3 ± 3.3 g of food/rat, and those on the normal diet consumed 31.5 ± 3.3 g/rat. At the start of the experiment, the animals fed on the cholesterol-containing and the normal diets weighed 271 ± 15.4 g and 269 ± 12.9 g respectively.

Measurement of $^3\text{H}_2\text{O}$ incorporation into non-saponifiable lipids *in vivo*: isolation of labelled sterols and squalene

Rats were injected intraperitoneally with $^3\text{H}_2\text{O}$ (4 mCi, 0.2 ml) between 10:00 h and 11:00 h (i.e. at the midpoint of the dark phase of the light/dark cycle). The rats were then returned to their respective cages; 1 h later the animals were anaesthetized and parts of the liver and intestine (consisting of combined jejunum and ileum [12]) were removed. The incorporation of ^3H into the non-saponifiable-lipid fraction was determined as described previously [12]. Labelled cholesterol, lanosterol and squalene were obtained from the non-saponifiable lipid fraction by t.l.c. [12]. Labelled polar sterols were obtained by eluting the region of the plate between the origin and the cholesterol band with diethyl ether. These substances have the same chromatographic mobility as certain oxygenated precursors of cholesterol [14].

Isolation of liver microsomal fractions and measurement of HMG-CoA reductase activity

The liver microsomal fraction was prepared as described previously [12]. The activities of the 'expressed' and 'total' forms of HMG-CoA reductase were deter-

Abbreviation used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

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Table 1. Effect of cholesterol feeding on hepatic HMG-CoA reductase activity and the accumulation of sterols labelled from $^3\text{H}_2\text{O}$ *in vivo*

Animals were fed with either a normal diet or a similar diet supplemented with 1% cholesterol for 24 h. $^3\text{H}_2\text{O}$ (0.2 ml; 4 mCi) was injected intraperitoneally, and 1 h later the rats were anaesthetized with sodium pentobarbital. A portion of each liver was removed for measurement of HMG-CoA reductase activity. Another portion of liver was frozen in liquid N_2 and used for measurement of incorporation of $^3\text{H}_2\text{O}$ into the non-saponifiable lipid, cholesterol, lanosterol, squalene and polar-sterol fractions. Values marked *, ** and *** are significantly different from the normal fed controls at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively. All values represent means $\pm$ S.E.M. for the numbers of animals shown in parentheses.

	Normal fed	Cholesterol fed
HMG-CoA reductase (expressed activity) (pmol/min per mg of protein)	56.9 $\pm$ 8.3 (10)	19.4 $\pm$ 2.4 (9)***
HMG-CoA reductase (total activity) (pmol/min per mg of protein)	166.8 $\pm$ 23.5 (10)	53.8 $\pm$ 7.2 (9)***
Non-saponifiable lipid synthesis (nmol of $^3\text{H}_2\text{O}$ /min per g of liver)	51.3 $\pm$ 8.5 (12)	21.3 $\pm$ 4.7 (12)***
Cholesterol synthesis (nmol of $^3\text{H}_2\text{O}$ /min per g of liver)	42.6 $\pm$ 7.6 (12)	12.7 $\pm$ 3.4 (12)**
[^3H]Cholesterol synthesized (% of non-saponifiable lipid)	81.8 $\pm$ 1.6 (12)	55.6 $\pm$ 3.6 (12)***
^3H -labelled polar sterols (% of non-saponifiable lipid)	5.4 $\pm$ 1.1 (12)	17.0 $\pm$ 4.0 (12)**
[^3H]Squalene (% of non-saponifiable lipid)	8.7 $\pm$ 1.2 (12)	15.7 $\pm$ 3.2 (12)
[^3H]Lanosterol (including other 4,4-dimethyl sterols) (% of non-saponifiable lipid)	4.5 $\pm$ 0.3 (12)	5.0 $\pm$ 0.8 (12)

mined by preincubation of samples of the microsomal fraction in the presence or absence of alkaline phosphatase. The preincubated microsomal fractions were then assayed for HMG-CoA reductase by the method of Brown *et al.* [15] as described previously [12]. Activity observed after incubation in the absence of phosphatase represents the 'expressed' activity that is present in the livers *in vivo* (i.e. the dephosphorylated form of the enzyme). Activity observed after incubation with phosphatase represents 'total' enzyme activity (i.e. the sum of the activities which were originally present as the phosphorylated and non-phosphorylated forms of the enzyme).

Other analytical methods

Protein was determined by the method of Gieger & Bessman [16]. For determination of the specific radioactivity of the plasma water, a sample of blood (1.0 ml) was taken from the descending vena cava, and the plasma was obtained by centrifugation.

RESULTS

Cholesterol synthesis in the liver

In rats fed on cholesterol for 24 h, the activity of hepatic microsomal HMG-CoA reductase declined as expected. This was true for both the 'expressed' and 'total' forms of the enzyme (Table 1). Carbon flux through the enzyme *in vivo* (as measured by the incorporation of $^3\text{H}_2\text{O}$ into the non-saponifiable-lipid fraction) also decreased considerably after feeding cholesterol for 24 h. There was a decreased contribution of labelled cholesterol to the non-saponifiable lipid (55.6 $\pm$ 3.6%) in the cholesterol-fed animals compared with that observed in the control group (81.8 $\pm$ 1.6%).

This discrepancy was due, in part, to a 3-fold increase in the proportion of labelled polar sterols (i.e. sterols of polarity greater than cholesterol) in the hepatic non-saponifiable lipid derived from the cholesterol-fed animals. There was no change in the proportion of the non-saponifiable lipid contributed by lanosterol (and companion $\text{C}_{30} + \text{C}_{29}$ sterols), but cholesterol feeding produced a small increase in the proportion of labelled squalene (Table 1).

In animals consuming the high-cholesterol diet, there was a strong inverse correlation between the relative accumulation of labelled polar sterols in the hepatic non-saponifiable lipid *in vivo* and the activities of both the 'expressed' and 'total' forms of HMG-CoA reductase (Fig. 1). In other words, the greater the proportion of labelled polar sterols in the non-saponifiable lipid, the greater the extent to which cholesterol feeding suppressed hepatic HMG-CoA reductase. Significant inverse correlations were also observed between labelled polar-sterol accumulation and the rate of formation of non-saponifiable lipid and cholesterol *in vivo* in the cholesterol-fed animals (Fig. 1). Other labelled components of the non-saponifiable lipid, such as squalene, showed no similar correlation with HMG-CoA reductase activity ($r = 0.385$; $P = 0.307$), and the same was true of lanosterol ($r = 0.498$; $P = 0.172$). In contrast with the cholesterol-fed animals, there was no correlation between labelled polar-sterol accumulation and the 'expressed' form of HMG-CoA reductase ($r = 0.361$; $P = 0.305$) in the livers of animals fed on the normal diet.

Cholesterol synthesis in the intestine

In animals fed on the normal diet, the rates of intestinal non-saponifiable lipid and cholesterol synthesis *in vivo* were lower than those observed in the liver (Table 2). In this organ, the inhibitory effect of the high-

Fig. 1. Relationship between hepatic polar sterol formation *in vivo*, cholesterogenesis and HMG-CoA reductase activity in cholesterol-fed rats

Rats were fed on a diet containing 1% cholesterol for 24 h. ³H₂O (4 mCi) was then injected intraperitoneally between 10:00 h and 11:00 h, and the rats were killed 1 h later. A portion of the liver was used to prepare microsomal fractions for assay of the 'expressed' and 'total' activities of HMG-CoA reductase. Another portion of the liver was used for determination of ³H incorporation into the non-saponifiable fraction, cholesterol and polar sterols (i.e. sterols with a chromatographic *R_F* value less than that of cholesterol).

Table 2. Effect of cholesterol feeding on intestinal sterol synthesis *in vivo*

Portions of the ileal and jejunal regions of the intestine were removed from the same animals described in the legend to Table 1. From each animal, these segments were combined and placed in liquid N₂. The incorporation of ³H₂O into the non-saponifiable lipids, and into the fractions containing cholesterol, lanosterol, squalene and polar sterols was determined. All values below represent means ± S.E.M.; the numbers in parentheses represent the numbers of animals used for each measurement. The asterisks represent the levels of significance as defined in the legend to Table 1.

	Normal fed	Cholesterol fed
Non-saponifiable lipid synthesis (nmol of ³ H ₂ O/min per g of intestine)	37.7 ± 6.0 (8)	20.9 ± 2.7 (8)*
Cholesterol synthesis (nmol of ³ H ₂ O/min per g of intestine)	29.5 ± 5.1 (8)	14.5 ± 2.4 (8)*
[³ H]Cholesterol synthesized (% of non-saponifiable lipid)	77.0 ± 3.0 (8)	68.3 ± 4.3 (8)
³ H-labelled polar sterols (% of non-saponifiable lipid)	9.3 ± 2.2 (8)	11.6 ± 2.1 (3)
[³ H]Squalene (% of non-saponifiable lipid)	7.4 ± 1.8 (6)	10.1 ± 2.8 (6)
[³ H]Lanosterol (including other 4,4-dimethyl sterols) (% of non-saponifiable lipid)	8.4 ± 1.0 (6)	6.9 ± 0.9 (6)

cholesterol diet was less pronounced than in the liver. Furthermore, there was no differential effect of cholesterol feeding on the proportion of labelled cholesterol in the non-saponifiable-lipid fraction. Neither was there any difference in the extent to which labelled polar sterols contributed to this fraction. Once again, in contrast with the liver, there was no significant inverse correlation between the rate of labelled non-saponifiable-lipid formation *in vivo* and the relative accumulation of labelled polar sterols in the intestine of animals fed on a high-cholesterol diet ($r = 0.022$; $P = 0.958$).

DISCUSSION

The mechanism by which external cholesterol-containing lipoproteins enter the cell is understood in intimate detail (see, e.g., [17]). However, little is known of the sequence of events linking the appearance of lipoprotein-derived non-esterified cholesterol in the lysosome with the suppression of HMG-CoA reductase activity in the endoplasmic reticulum. In intestinal epithelial cells *in vitro*, this sequence appears to be interrupted by ketoconazole, an inhibitor of cytochrome *P*-450 [9]. It is therefore possible that, when low-density-lipoprotein cholesterol is introduced into the cell, it normally undergoes a cytochrome *P*-450-dependent oxidation, resulting in a sterol which is directly responsible for the suppression of HMG-CoA reductase. Another possibility is that low-density lipoprotein interferes in the cholesterologenic pathway, resulting in the accumulation of an oxysterol precursor of cholesterol, as proposed previously [9,18]. The accumulation of such an oxysterol would be prevented by ketoconazole [9].

In the present work, lipoprotein cholesterol of dietary origin was introduced into the liver *in vivo* in the form of chylomicron remnants [19,20]. The results show that, when cholesterol was present at a concentration of 1% in the diet for 24 h, the suppression of hepatic cholesterologenesis *in vivo* and HMG-CoA reductase activity was accompanied by a relative increase in the accumulation of labelled material with the chromatographic properties of oxysterols. In these animals, the extent of inhibition was inversely proportional to the relative oxysterol content of the non-saponifiable fraction of the cell. In animals fed on the normal diet, the activity of HMG-CoA reductase spanned a similar relative range (3–4-fold variation between highest and lowest) to that in the cholesterol-fed group. However, in the former case, this variability showed no correlation with the labelled polar-sterol content of the non-saponifiable-lipid fraction.

In the cholesterol-fed group, the co-occurrence of low HMG-CoA reductase activities and relatively high amounts of newly formed polar sterols does not prove a causal relationship. Nevertheless, it does indicate that lipoprotein cholesterol, reaching the liver as chylomicron remnants [19,20], results in a redistribution of label among cholesterol and its precursors in such a way as to enhance the relative accumulation of a polar-sterol fraction. The high incorporation of label into this fraction (in some cases this was almost equal to that found in cholesterol itself) suggests that it contained a cholesterol precursor(s) rather than an enzymically derived oxygenated product of exogenous lipoprotein (i.e. dietary) cholesterol. This suggests that, *in vivo*, dietary cholesterol may interfere in the cholesterologenic pathway, resulting in

the accumulation of an oxygenated cholesterol precursor(s). In this respect, a recent report has provided evidence for the accumulation of the cholesterol precursors 32-hydroxycholesterol and 32-oxocholesterol during the suppression of HMG-CoA reductase activity by mevalonate in Chinese-hamster lung cells [21]. Although in the present work we selected a single time point (24 h), it is possible that the proportion of polar sterols to cholesterol and non-saponifiable lipids varies throughout the period over which cholesterol was fed and that this may affect HMG-CoA reductase to different extents.

In the intestine, the inhibitory effect of dietary cholesterol on cholesterologenesis and non-saponifiable lipid formation *in vivo* was less pronounced than in the liver (Table 2). This relative insensitivity of intestinal cholesterologenesis to dietary cholesterol has been noted previously, and it is possible that this may be related to an inability of dietary cholesterol to provoke an increase in the newly synthesized polar-sterol content of the intestinal non-saponifiable lipid (Table 2).

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