Cholesterol Feeding Alters the Metabolism of Thoracic-Duct Lymph Lipoprotein Cholesterol in Rabbits but not in Rats

By T. G. REDGRAVE

Department of Physiology, University of Melbourne, Parkville, Vic. 3052, Australia

(Received 3 April 1973)

1. Labelled thoracic-duct lymph was collected from rats and rabbits after test meals containing [^{14}C]cholesterol and [$2\text{-}^3\text{H}$]glyceryl trioleate. 2. The metabolism of labelled cholesterol and triglyceride was studied in normally fed and cholesterol-fed rats and rabbits injected with radioactive lymph from the same species. 3. In normally fed animals of both species, 10 min after intravenous administration, about 80% of lymph cholesteryl ester but only about 10% of triglyceride was recovered in the liver after clearance from the plasma. This distribution is consistent with participation of 'remnant' particles in the metabolism of dietary lymph particles. 4. The metabolism of cleared lymph lipoprotein constituents was unchanged in cholesterol-fed rats, but the recovery of cholesteryl ester in the livers of the cholesterol-fed rabbits was decreased to 30% of the cleared dose. 5. The low recovery in cholesterol-fed rabbits was accounted for mainly by increased hydrolysis of cholesteryl ester. 6. It is proposed that differences between rats and rabbits in metabolism of dietary cholesterol might be partly due to the observed enhancement of hydrolysis of lymph lipoprotein cholesteryl ester in rabbits.

For many years the rabbit has served as a useful model of atherogenesis because plasma cholesterol concentration in this species is quickly increased when cholesterol is added to the diet. In contrast the rat is resistant to dietary cholesterol, and significant hypercholesterolaemia does not usually develop unless bile acids and anti-thyroid drugs are also given (Fillios *et al.*, 1956).

In spite of these differences in suitability for studies of experimental atheroma much more is known about the metabolism of cholesterol in rats than in rabbits. In particular knowledge of absorption of dietary cholesterol and its subsequent metabolism is very meagre in rabbits. Thoracic-duct cannulation in rabbits has been technically possible for many years (Sanders *et al.*, 1940), but has been exploited only recently to gain information about cholesterol metabolism (Zilversmit *et al.*, 1967; Fraser & Courtice, 1969; Rudel *et al.*, 1972).

One of the factors that has been suggested to account for the different responses of rats and rabbits to dietary cholesterol is the presence in rabbit thoracic-duct lymph of relatively large amounts of VLD lipoprotein* of intestinal origin (Zilversmit *et al.*, 1967; Rudel *et al.*, 1972). Similarly VLD lipoprotein is found in rat lymph (Windmueller *et al.*, 1970; Ockner *et al.*, 1969) and when the dietary fat load is small or when saturated fats are being absorbed the VLD lipoproteins carry a major portion of

absorbed dietary cholesterol. It has nevertheless been suggested that intestinal VLD lipoproteins might be responsible for the observed differences between rats and rabbits in their responses to cholesterol feeding. Whole lymph was used in the present experiments to obtain an integrated assessment of the metabolism of dietary cholesterol and triglyceride in both species, and also to diminish the possibility of artifactual alterations in lipoproteins that might be produced by isolation techniques.

In these experiments the metabolism of thoracic-duct lipoprotein constituents has been compared in normally fed and cholesterol-fed rabbits and rats. In normally fed animals the metabolism of dietary lipoprotein has been found to be similar in both species. However, after cholesterol feeding the metabolism of dietary triglyceride is unchanged but a clear-cut difference between the two species has been found in the apparent hepatic hydrolysis of lymph lipoprotein cholesteryl ester. This finding suggests that the different responses of rats and rabbits to cholesterol feeding might be related to the activity of hepatic cholesteryl ester hydrolase, and an hypothesis has been advanced that this enzyme is of importance in the regulation of mammalian cholesterol metabolism.

Methods

Collection of lymph lipoproteins

In rats standard techniques (Bollman *et al.*, 1948) were used for cannulation of the thoracic ducts of

* Abbreviation: VLD lipoprotein, very-low-density lipoprotein.

female albino animals. Radioactive lymph was collected after test meals which contained $10\mu\text{Ci}$ of $[4\text{-}^{14}\text{C}]\text{cholesterol}$ and $5\mu\text{Ci}$ of $[2\text{-}^3\text{H}]\text{glyceryl trioleate}$ (The Radiochemical Centre, Amersham, Bucks., U.K.) in 1 ml of a solution of 1% (w/v) cholesterol in corn oil. Lymph was collected in ice without an anticoagulant, stored at 2°C and used for metabolic studies within 24 h of collection. To minimize possible artifacts whole lymph was used rather than ultracentrifugally isolated chylomicrons; any clot was removed with a wooden swab-stick. Details of management of fistulated animals have been given (Redgrave & Zilversmit, 1969).

Lymph collection in rabbits was achieved by cannulation of the cisterna chyli. Female New Zealand rabbits weighing 2.5–4 kg were given intravenously 0.12 mg of atropine and 50–100 mg of sodium thiopentone, and then an endotracheal tube was inserted under direct vision. Despite reports that rabbits have an atropine esterase (Godeaux & Tønnesen, 1949), atropine was found to dry secretions and to decrease post-operative respiratory complications. Anaesthesia was maintained by using halothane and methoxyflurane with O_2 in a closed circuit (Komesaroff & Williamson, 1968). The cisterna was cannulated about 2 cm above the level of the adrenal gland with a vinyl tube (0.8 mm internal diam. and 1.2 mm outside diam.) The tube was secured with an alkyl-2-cyanoacrylate adhesive (Eastman 910; Tennessee Eastman Co., Kingsport, Tenn., U.S.A.). It was not found necessary to remove the left kidney as reported by Rudel *et al.* (1972). A similar tube was placed into the duodenum about 5 cm beyond the pylorus and secured with a purse-string suture.

After the operation rabbits were placed in restraining cages (Bollman, 1948) that had been enlarged and modified. Glucose (5%, w/v, in tap water) was available at all times and 0.15 M-NaCl was infused steadily at 10 ml/h via the intraduodenal tube. Lymph flow varied from 4 to 12 ml/h and could be maintained for 5 or 6 days.

On the first or second post-operative day a test meal was given by the intraduodenal tube. An emulsion was prepared to contain $50\mu\text{Ci}$ of $[4\text{-}^{14}\text{C}]\text{cholesterol}$, $25\mu\text{Ci}$ of $[2\text{-}^3\text{H}]\text{glyceryl trioleate}$, 1 ml of a solution of 1% cholesterol in corn oil and 200 mg of Pluronic F-68 (a copolymer of propylene oxide and ethylene oxide; BASF Wyandotte Corp., Wyandotte, Mich., U.S.A.) in 8 ml of 5% (w/v) glucose, and this was infused via the intraduodenal tube over about 30 min. Lymph was collected from the time it became milky for 6–18 h and the lymph was handled as described above for rat lymph. Under these circumstances $64.4 \pm 5.31\%$ (S.E.M.) of lymph cholesterol radioactivity (six observations) was in particles of $S_r > 400$ when these particles were isolated by using a discontinuous density gradient (Minari & Zilversmit, 1963).

Clearance of lymph lipoproteins

Recipient rats were females weighing 200–250 g and fed *ad libitum* on either commercial rat pellets or the same pellets containing 1% cholesterol and 3% peanut oil for one month before the experiment. Recipient rabbits were also females weighing 3.2–4.3 kg and were fed either commercial rabbit pellets or similar pellets containing 1% cholesterol and 3% peanut oil. The livers of cholesterol-fed rabbits contained 2540–4770 mg of cholesterol, compared with 309–438 mg for the normally fed animals.

In both cases unstarved animals were injected with labelled lymph without anaesthesia. Rats received 1 ml of rat lymph via a tail vein and rabbits received either 10 ml of rabbit lymph given over 2.5 min or 20 ml of lymph over 5 min via an ear vein. Exactly 10 min after completion of injections rats were anaesthetized with ether and rabbits with intravenous pentobarbital, a blood sample was taken, the animals were bled and then either the whole liver (rats) or a 10 g portion (rabbits; with portions from all lobes) was taken for lipid extraction. Plasma volumes were assumed to be 4 ml/100 g body weight for rats, and were calculated for rabbits from the haematocrit and the formula of Bocci & Viti (1966) for blood volume.

Lipids were extracted from liver, plasma and lymph by the method of Folch *et al.* (1957), washed and then samples were applied to 1 g silicic acid columns for separation of cholesteryl esters from free cholesterol and triglyceride as described by Zilversmit (1965), except that because rapid flow was obtained without kieselguhr, silicic acid (SilicAR CC-7, Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.), was used alone. All separations were checked by t.l.c. and radioactivity in portions of the eluates was measured by using previously described methods (Redgrave, 1970).

Results

In rats after 10 min about 80% of injected lymph cholesteryl ester is found in the liver, compared with only about 12% of lymph triglyceride (Table 1). This is entirely consistent with the interpretation of previously reported experiments (Redgrave, 1970; Bergman *et al.*, 1971) indicating the role of cholesteryl ester-enriched 'remnant' particles in the metabolism of chylomicrons. Rather smaller recoveries of both labels in these experiments are perhaps explained by the use of whole lymph instead of isolated chylomicrons or by the use of tail vein injections instead of injections via a jugular cannula. Lymph triglyceride was labelled in the glycerol moiety instead of in the fatty acid moiety as in previous experiments (Redgrave, 1970). Hence in these experiments labelled glycerol arising from the action of lipoprotein lipase in extrahepatic tissues could have been incorporated in some measure into hepatic

glycerides. However, the rather smaller recoveries found indicate that such incorporation was probably insignificant at the time-interval studied.

The feeding of cholesterol to rats produces no alteration in the recoveries of injected radioactivity, as shown in Table 1.

Clearance of lymph lipoprotein constituents in normally fed rabbits is qualitatively very similar to clearance in rats (Table 2). Again a major portion of cleared cholesterol and only a minor portion of triglyceride is recovered in the liver. However, plasma clearance in the rabbit is much less efficient than in the rat and after 10 min $48.1 \pm 6.95\%$ of the injected dose remains in the plasma, compared with only about 20% in rats. Presumably because of their cholesterolemia plasma clearance in cholesterol-fed rabbits was significantly less efficient than in normal rabbits and $75.3 \pm 2.03\%$ of the injected dose was recovered in the plasma after 10 min ($P < 0.05$).

Importantly, in rabbits there is a greater proportion of lymph total cholesterol label than of esterified cholesterol label recovered in the liver, a phenomenon that has not been observed in rats in these or in previous experiments (Redgrave, 1970). Although

apparent in normal rabbits, this finding is much more marked in cholesterol-fed rabbits, as shown in Table 3, and so contributes to the low recovery of esterified cholesterol in cholesterol-fed rabbits (Table 2). Whereas in cholesterol-fed rabbits the recovery of cleared lymph total cholesterol in the liver is only slightly less than in the normal rabbits, the recovery of cleared cholesteryl ester is significantly decreased.

The explanation for this observation in rabbits is clear from Table 3, which shows evidence of rapid hydrolysis of lymph cholesteryl ester. Compared with the injected lymph the proportion of cholesterol in the liver that is unesterified has increased by a factor of 1.27 in normal rabbits, and by 2.65 in cholesterol-fed rabbits. (Unesterified radioactive cholesterol in the two lymph samples used in this study was 19.4 and 25.5% of the total cholesterol radioactivity.) This observation could not be accounted for by preferential hepatic uptake of free cholesterol, because in cholesterol-fed rabbits there is also an enhancement of the proportion of unesterified cholesterol in the plasma (Table 3), leading to a greater combined recovery of free cholesterol than was present in the injected lymph. In normally fed rabbits

Table 1. *Metabolism of lymph lipoprotein cholesterol and triglyceride in rats*

Rats were given 1 ml of fatty lymph labelled with [^{14}C]cholesterol and [$2\text{-}^3\text{H}$]glyceryl trioleate, and the amount of label recovered in the liver 10 min after injection is represented as the percentage of the injected dose cleared from the plasma. Results are means $\pm$ S.E.M.; the numbers of animals are shown in parentheses.

	Recovery in liver (% of injected dose cleared from plasma)		
	Cholesteryl ester	Total cholesterol	Triglyceride
Normally fed rats (9)	81.3 ± 6.32	71.0 ± 5.57	11.7 ± 1.44
Cholesterol-fed rats (12)	78.4 ± 4.50	71.7 ± 3.27	12.3 ± 0.42

Table 2. *Metabolism of lymph lipoprotein cholesterol and triglyceride in rabbits*

Rabbits were given 10 or 20 ml of fatty rabbit lymph labelled with [^{14}C]cholesterol and [$2\text{-}^3\text{H}$]glyceryl trioleate, and the amount of label recovered in the liver 10 min after injection is represented as the percentage of the injected dose cleared from the plasma. Results are means $\pm$ S.E.M.; the numbers of animals are shown in parentheses. Statistical comparison was made by Student's *t* test.

	Recovery in liver (% of injected dose cleared from plasma)		
	Cholesteryl ester	Total cholesterol	Triglyceride
Normally fed rabbits (3)	75.8 ± 5.31	81.5 ± 5.95	7.3 ± 0.80
Cholesterol-fed rabbits (3)	30.1 ± 3.26	61.5 ± 8.00	10.2 ± 2.05
	$P < 0.01$	$P > 0.05$	$P > 0.05$

Table 3. *Hydrolysis of injected lymph lipoprotein cholesteryl ester*

Results are expressed as the fraction of total labelled cholesterol that is unesterified, compared with the fraction of total labelled cholesterol unesterified in injected lymph = 1.00. Hence values greater than 1.00 represent net hydrolysis of cholesteryl ester, and values less than 1.00 represent either net esterification or, more likely, loss of unesterified cholesterol by exchange reactions. Results are means $\pm$ s.e.m.; statistical comparison was made by Student's *t* test; the number in each group is the same as in Tables 1 and 2.

	Free cholesterol in liver		Free cholesterol in plasma	
	Rats	Rabbits	Rats	Rabbits
Normally fed	0.94 $\pm$ 0.037	1.27 $\pm$ 0.034	0.87 $\pm$ 0.052	0.83 $\pm$ 0.107
Cholesterol-fed	1.00 $\pm$ 0.038	2.65 $\pm$ 0.421	0.85 $\pm$ 0.050	1.16 $\pm$ 0.025
	<i>P</i> > 0.05	<i>P</i> < 0.05	<i>P</i> > 0.05	<i>P</i> < 0.05

the proportion of unesterified cholesterol in plasma is similar to that in rats but unesterified cholesterol in the liver is again increased. Although it is impossible to discount the occurrence of some hydrolysis in the plasma, the finding of more extensive hydrolysis in the liver at such short time-intervals makes this unlikely.

Discussion

These experiments show that the metabolism of lymph lipoprotein cholesterol and triglyceride in normal rabbits is qualitatively similar to that in rats. In both species the observed distribution of lymph constituents 10 min after injection is consistent with the initial removal of most of the triglyceride by extrahepatic tissues (Felts & Mayes, 1965) and then rapid uptake of the 'remnant' particles by the liver, so accounting for recovery of dietary cholesterol, most of which is esterified, in this organ (Redgrave, 1970). Although these experiments indicate that plasma clearance of lymph lipoprotein in rabbits might be less efficient than in rats, kinetic studies will be necessary to assess the significance of this observation. Now that similar patterns of lymph lipoprotein metabolism have been shown in rats, rabbits, sheep (Bergman *et al.*, 1971) and dogs (Nestel *et al.*, 1963) it must be concluded that initial removal of most of the chylomicron triglyceride in peripheral tissues by the action of lipoprotein lipase and subsequent hepatic uptake of chylomicron cholesterol in the form of remnant particles is a general phenomenon.

Cholesterol feeding in rats did not alter the pattern of clearance of lymph lipoprotein constituents but in rabbits it was observed that hydrolysis of cholesteryl ester was greatly enhanced. Even in normally fed rabbits significant hydrolysis occurred, apparently in the liver, and cholesterol feeding accelerated the process markedly. Goodman (1962) has shown that hydrolysis of chylomicron cholesteryl ester in rat liver takes several hours, and the lack of hydrolysis

in rats in these experiments is in keeping with his findings. By way of contrast, hydrolysis of cholesteryl ester in rabbits has been shown in these experiments to proceed rapidly and to be enhanced by cholesterol feeding, which is consistent with the observations of Felt & Beneš (1970), who showed that hepatic cholesteryl ester hydrolase activity in rabbits was increased by cholesterol feeding.

Net absorption of cholesterol is greater in rabbits than in rats (Cook & Thompson, 1951; Friedman & Byers, 1954), but even so absorption in the rat is quite efficient. However, rats fed on cholesterol fail to develop hypercholesterolaemia, possibly because they possess the ability to convert absorbed cholesterol into bile acids, and the output of bile acids is considerably increased (Wilson, 1964). On the other hand cholesterol-fed rabbits cannot increase their output of bile acids (Hellström, 1965), so hypercholesterolaemia develops.

The experiments reported here suggest a possible mechanism for these different responses of rats and rabbits to dietary cholesterol. Esterified cholesterol might be the substrate for the initial step in conversion of cholesterol into bile acids (Boyd, 1962). Hence rats, with a low activity of cholesteryl ester hydrolase, convert cholesteryl ester into bile acids efficiently and thereby prevent an accumulation of cholesterol in liver and plasma. In contrast rabbits, with a high activity of cholesteryl ester hydrolase, are unable to convert absorbed cholesterol into bile acids and so unesterified cholesterol will be freely available for lipoprotein synthesis and exchange in the liver-plasma compartment. The unavailability for bile acid synthesis of cholesteryl ester substrate in rabbits will of course be compounded by the observed enhancement of hydrolase activity when cholesterol is fed.

Although this hypothesis accounts satisfactorily for aspects of species differences it is clear that several key points must be resolved before cholesteryl ester hydrolase could be accepted as a regulator of cholesterol metabolism. In particular, the quantitative

importance of the entero-hepatic circulation of cholesterol, the efficiency of intestinal cholesterol esterification and the precise role of cholesteryl esters in bile acid synthesis would all have significant implications for a regulating role of hepatic cholesteryl ester hydrolase, even if the above hypothesis were supported by future experiments. However, if this single enzyme does prove to be a major regulator of mammalian cholesterol metabolism, it should be possible to show within a single species correlations between hydrolase activity and individual susceptibility to hypercholesterolaemia.

In any event the present experiments show clearly that absorbed dietary cholesterol and triglyceride are metabolized in similar fashions in normally fed rats and rabbits. Differences in the transport form of absorbed lipid would therefore appear to be at least not a major determinant of differences in the responses of the two species to dietary cholesterol. Although direct evidence for a role of cholesteryl ester in bile acid synthesis is not available, the interpretation offered to explain the finding in these experiments of enhanced hydrolysis of cholesteryl ester in cholesterol-fed rabbits might lead to an explanation of the inability of rabbits to increase their production of bile acids during cholesterol feeding (Hellström, 1965).

This work was made possible by a grant from the National Heart Foundation of Australia. The skilful technical assistance of Mr. Dennis Vickery and Miss Janice Wilson is gratefully acknowledged.

References

- Bergman, E. N., Havel, R. J., Wolfe, B. M. & Bøhmer, T. (1971) *J. Clin. Invest.* **50**, 1831–1839
- Bocci, V. & Viti, A. (1966) *Quart. J. Exp. Physiol.* **51**, 27–32
- Bollman, J. L. (1948) *J. Lab. Clin. Med.* **33**, 1348
- Bollman, J. L., Cain, J. C. & Grindlay, J. H. (1948) *J. Lab. Clin. Med.* **33**, 1349–1352
- Boyd, G. S. (1962) *Fed. Proc. Fed. Amer. Soc. Exp. Biol. Suppl.* **11**, 86–92
- Cook, R. P. & Thomson, R. O. (1951) *Quart. J. Exp. Physiol.* **36**, 61–74
- Felt, V. & Beneš, P. (1970) *Enzymol. Biol. Clin.* **11**, 511–520
- Felts, J. M. & Mayes, P. A. (1965) *Nature (London)* **206**, 195–196
- Fillios, L. C., Andrews, S. B., Mann, G. V. & Stone, F. J. (1956) *J. Exp. Med.* **104**, 539–554
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
- Fraser, R. & Courtice, F. C. (1969) *Aust. J. Exp. Biol. Med. Sci.* **47**, 723–732
- Friedman, M. & Byers, S. O. (1954) *Amer. J. Physiol.* **179**, 201–215
- Godeaux, J. & Tønnesen, M. (1949) *Acta Pharmacol. Toxicol.* **5**, 95–109
- Goodman, D. S. (1962) *J. Clin. Invest.* **41**, 1886–1896
- Hellström, K. (1965) *Acta Physiol. Scand.* **63**, 21–35
- Komesaroff, D. & Williamson, W. (1968) *Med. J. Aust.* **2**, 391–394
- Minari, O. & Zilversmit, D. B. (1963) *J. Lipid Res.* **4**, 424–436
- Nestel, P. J., Havel, R. J. & Bezman, A. (1963) *J. Clin. Invest.* **42**, 1313–1321
- Ockner, R. K., Hughes, F. B. & Isselbacher, K. J. (1969) *J. Clin. Invest.* **48**, 2367–2373
- Redgrave, T. G. (1970) *J. Clin. Invest.* **49**, 465–471
- Redgrave, T. G. & Zilversmit, D. B. (1969) *Amer. J. Physiol.* **217**, 336–340
- Rudel, L. L., Morris, M. D. & Felts, J. M. (1972) *J. Clin. Invest.* **51**, 2686–2692
- Sanders, A. G., Florey, H. W. & Barnes, J. M. (1940) *Brit. J. Exp. Pathol.* **21**, 254–263
- Wilson, J. D. (1964) *J. Lipid Res.* **5**, 409–417
- Windmueller, H. G., Lindgren, F. T., Lossow, W. J. & Levy, R. I. (1970) *Biochim. Biophys. Acta* **202**, 507–516
- Zilversmit, D. B. (1965) *J. Clin. Invest.* **44**, 1610–1622
- Zilversmit, D. B., Courtice, F. C. & Fraser, R. (1967) *J. Atheroscler. Res.* **7**, 319–329