Originals

Serum lipoproteins and cholesterol metabolism in two hypercholesterolaemic rabbit models

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Summary. Serum lipoproteins and key hepatic and intestinal enzymes regulating cholesterol synthesis, esterification and catabolism, namely 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase, acyl coenzyme A: cholesterol-o-acyltransferase (ACAT) and cholesterol 7 α -hydroxylase respectively, were compared in two hypercholesterolaemic rabbit models – the cholesterol-fed animal and the hypercholesterolaemic diabetic animal. Hypercholesterolaemia in the cholesterol-fed animals was reflected in the VLDL and LDL fractions, whereas VLDL and HDL₂ cholesterol levels were elevated in the diabetic animals. The lipoproteins of the cholesterol-fed animals were enriched with cholesterol but the lipoprotein fractions in the diabetic animals were enriched with triacylglycerol. While hepatic HMGCoA reductase activity was significantly reduced in both groups, the activities

of hepatic ACAT and cholesterol 7 α -hydroxylase were significantly increased in the cholesterol-fed animals and significantly reduced in the diabetic animals compared with controls. In the intestine, the activity of HMGCoA reductase was increased and ACAT reduced in the diabetic animals. By contrast, in the cholesterol-fed group, HMGCoA reductase activity was lower and ACAT activity was higher in comparison with the control group. These differences in lipoproteins and cellular cholesterol metabolism between the hypercholesterolaemic rabbit models may explain the differences in susceptibility to atherosclerosis, previously reported in these two animal models.

Key words: Rabbits, diabetes, hypercholesterolaemia, lipoproteins, cholesterol metabolism.

The importance of cholesterol in the pathogenesis of atherosclerosis has been documented by epidemiological [1], biochemical [2] and pathological [3] evidence. The liver and intestine have important roles in the regulation of cholesterol metabolism through their involvement in cholesterol synthesis and esterification, while the liver has an additional role in the catabolism of cholesterol to bile acids. Key enzymes in these processes are 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoA reductase, EC 1.1.1.34), the rate-limiting enzyme of cholesterol biosynthesis [4]; acyl coenzyme A: cholesterol-o-acyltransferase (ACAT, EC 2.3.1.26), the main intracellular enzyme for cholesterol esterification [5] and cholesterol 7 α -hydroxylase (EC 1.14.13.7) which catalyses the committed step in bile acid formation [6].

The susceptibility of the rabbit to the development of hypercholesterolaemia and atherosclerosis [7] makes this a suitable species for the study of the role of cholesterol in the pathogenesis of this disease. Increased serum cholesterol levels are found in association with experimentally – induced diabetes mellitus and

also following ingestion of cholesterol-supplemented diets.

This study compares serum lipoprotein cholesterol levels and the specific activities of HMGCoA reductase, ACAT and cholesterol 7 α -hydroxylase in both the cholesterol-fed and diabetic hypercholesterolaemic rabbit models.

Materials and methods

Animals

Male New Zealand White rabbits weighing 3–4 kg were used in this study and were maintained in a reverse 12 h light cycle (03.00–15.00 hours dark, 15.00–03.00 hours light) and fed Purina rabbit chow and water ad libitum for two weeks prior to commencement of the study. Diabetic animals ($n = 6$) and control animals ($n = 6$) were fed normally throughout the experiments, whereas the cholesterol-fed animals ($n = 6$) received Purina rabbit chow supplemented with 2.5% (weight/volume, w/v) coconut oil and 1% (w/v) cholesterol for 4 days prior to killing.

Chemicals

3-hydroxy-3-methyl (3-¹⁴C)-glutaryl CoA (56.6 mCi/mmol), DL-(2-³H)-mevalonic acid lactone (1.28 Ci/mmol), (1-¹⁴C)-oleic acid (57.0 mCi/mmol), (1 α , 2 α (n)³H)-cholesterol (40 Ci/mmol) and (3-¹⁴C)-cholesterol (58.4 mCi/mmol) were obtained from the Radiochemical Centre (Amersham, UK). β -Nicotinamide adenine dinucleotide phosphate (NADP), β -nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), glucose 6-phosphate, glucose 6-phosphate dehydrogenase, dithiothreitol (DTT), glutathione, human serum albumin (fatty acid free), oleoyl chloride, nicotinamide, mevalonic acid lactone, coenzyme A, cholesterol and alloxan monohydrate were all obtained from Sigma-London Chemical Co. Ltd. (London, UK). Kieselgel 60 was obtained from Merck (Darmstadt, FRG). Insulin zinc suspension (Ultratard MC) was purchased from Novo Laboratories (Copenhagen, Denmark). Visidex and Multistix reagent strips were obtained from Ames Division, (Miles Laboratories Ltd., Slough, UK).

Induction of diabetes

Diabetes was induced by intravenous administration of alloxan monohydrate (140 mg/kg). The alloxan preparation was dissolved in 0–9% (w/v) sodium chloride to constitute a 10% solution immediately prior to injection. The presence of diabetes was confirmed by a Visidex blood sugar reading >22 mmol/l, 48 h after injection. Throughout the study period, the animals were housed in individual metabolic cages and their diabetic control was monitored by daily urinalysis using Multistix reagent strips. The diabetic animals had significant glycosuria throughout the study but ketonuria was prevented by administering insulin zinc suspension (Ultratard MC) once daily.

Blood was taken for lipoprotein cholesterol estimation and elemental analysis from all three groups of animals following a 16 h overnight fast. The animals were killed in the middle of the dark period and their livers and intestines were removed and placed on ice.

Preparation of rabbit microsomal pellet

The livers were minced and then homogenised in 4 volume ice-cold 0.1 mol/l potassium phosphate buffer, pH 7.4, containing 1 mmol/l EDTA and 30 mmol/l nicotinamide. The homogenate was centrifuged at 800 g for 10 min and the resulting supernatant was further centrifuged at 15,000 g for 20 min. This supernatant was then centrifuged at 104,000 g for 60 min to obtain the microsomal pellet. The microsomes were suspended in 0.25 volume 5 mmol/l imidazole/HCl buffer, pH 7.4 containing 0.15 mmol/l NaCl and 5 mmol/l DTT.

Preparation of rabbit intestinal cells

Mucosal cell fractions were obtained from rabbit intestine by differential scraping of the mucosa as described by Dietschy and Siperstein [8]. Cells were homogenised in 0.1 mol/l potassium phosphate buffer, pH 7.4, containing 1 mmol/l EDTA and 30 mmol/l nicotinamide. Due to the relative difficulty in subfractionating intestinal cells compared to liver cells, the activities of HMGCoA reductase and ACAT were measured in the whole homogenate of intestinal cells.

Enzyme assays

HMGCoA reductase was determined as described by Mitropoulos and Balasubramaniam [9], ACAT by the method of Balasubramaniam et al. [10] and cholesterol 7 α -hydroxylase by the method of

Shefer et al. [11]. Prior experimentation with both liver microsomal preparations and intestinal cell whole homogenates from rabbits established optimum assay conditions for the enzymes from both sources with respect to protein concentration and time of incubation [12]. The protein content of the liver and intestinal cell preparations was determined by the method of Lowry et al. [13].

Lipoprotein fractionation

Plasma lipoproteins were separated by a one step density gradient ultracentrifugation technique as follows [14]. Two ml of serum was brought to a density of 1.225 g/ml in the bottom of a modified Beckman ultraclear centrifuge tube (Beckman Instruments Inc., Palo Alto, Calif., USA) by the addition of 770 mg KBr and 50 mg sucrose. This was then layered with salt solutions having densities of 1.225 g/ml, 1.100 g/ml and 1.000 g/ml. Ultracentrifugation was performed using a Beckman L5-50 ultracentrifuge with an SW 41 Ti swinging-bucket rotor head at 20°C at 40,000 rev/min for 22 h ($26.39 \times 10^7 g_{avg} \text{ min}$). The lipoprotein fractions very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein subfractions (HDL₂ and HDL₃) corresponded to the density intervals <1.006 g/ml, 1.006–1.063 g/ml, 1.063–1.100 g/ml and 1.100–1.210 g/ml, respectively. The density profile in the gradient was checked after ultracentrifugation using a four-digit density meter (Anton Paar, Graz, Austria).

Serum cholesterol levels were assayed using a Boehringer Mannheim GmbH diagnostic kit [15] and serum triacylglycerol and phospholipid levels were estimated with Biomerieux (Charbonnières les Bains, France) kits [16]. The protein content of the lipoprotein fractions was measured by a modification [17] of the procedure of Lowry et al. [13].

Statistical analysis

Statistical analysis of changes in the activities of HMGCoA reductase, ACAT and cholesterol 7 α -hydroxylase, between the various groups of animals was performed using the Student's unpaired *t*-test (two-tailed). As the serum lipid levels in the cholesterol-fed and diabetic rabbits were not normally distributed, the Wilcoxon test

Fig. 1. Serum cholesterol levels and distribution in hypercholesterolaemic rabbits. The individual lipoprotein fractions were isolated ultracentrifugally and the cholesterol content of each was measured enzymatically. Control animals ($n = 6$, black bars); diabetic animals ($n = 6$, striped bars); cholesterol-fed animals ($n = 6$, stippled bars). Results are expressed as mean $\pm$ SEM. * $p < 0.05$ different from control animals; ** $p < 0.01$ different from control animals

Table 1. Effect of cholesterol feeding and diabetes on the elemental composition of the lipoprotein subfractions in the rabbit

	Control animals (<i>n</i> = 6)	Diabetic animals (<i>n</i> = 6)	Cholesterol-fed animals (<i>n</i> = 6)
VLDL %			
Triacylglycerol	40.7 ± 4.3	48.6 ± 2.8	13.0 ± 4.7 ^c
Cholesterol	16.5 ± 3.3	10.5 ± 1.4	45.2 ± 5.1 ^c
Phospholipid	18.4 ± 1.7	15.1 ± 1.7	19.8 ± 1.3
Protein	24.3 ± 1.4	25.8 ± 2.8	22.0 ± 3.5
LDL %			
Triacylglycerol	12.1 ± 1.7	23.7 ± 3.2 ^b	2.2 ± 0.4 ^c
Cholesterol	22.4 ± 3.4	14.6 ± 2.7	38.1 ± 1.8 ^c
Phospholipid	27.0 ± 1.2	23.9 ± 2.0	23.6 ± 0.8
Protein	38.5 ± 3.3	37.9 ± 3.3	36.1 ± 1.0
HDL₂ %			
Triacylglycerol	6.9 ± 0.9	15.5 ± 2.3 ^b	2.4 ± 0.6 ^a
Cholesterol	15.4 ± 1.5	12.0 ± 2.0	17.5 ± 0.8
Phospholipid	35.4 ± 2.8	35.8 ± 2.0	26.2 ± 2.1
Protein	42.3 ± 2.0	36.7 ± 2.8	53.9 ± 2.4 ^a
HDL₃ %			
Triacylglycerol	9.2 ± 1.1	11.2 ± 1.6	2.6 ± 0.5 ^c
Cholesterol	9.0 ± 0.8	9.0 ± 1.0	8.9 ± 1.7
Phospholipid	18.9 ± 3.9	20.9 ± 0.3	16.6 ± 1.7
Protein	62.9 ± 3.5	60.9 ± 0.9	71.9 ± 1.3 ^a

The percentage composition was calculated by measuring the triglyceride, cholesterol, phospholipid and protein concentration in each lipoprotein fraction from which the total mass of each fraction was calculated. Each component was then expressed as a percentage of the total particle mass. Results expressed as means ± SEM.

^a *p* < 0.05 different from control animals;

^b *p* < 0.01 different from control animals;

^c *p* < 0.001 different from control animals

(two-tailed) was used in the analysis of this data. The results are expressed as mean ± SEM.

Results

Figure 1 outlines the serum cholesterol concentrations in all three groups. The degree of hypercholesterolaemia in the cholesterol-fed group and the diabetic group was comparable (2.35 ± 0.44 vs 2.45 ± 0.41 mmol/l). These levels were significantly higher (*p* < 0.01) than the cholesterol levels in the control rabbits (1.02 ± 0.20 mmol/l). While the hypercholesterolaemia in the cholesterol-fed animals

was reflected mainly in the VLDL (*p* < 0.01) and LDL (*p* < 0.05) fractions, the elevated serum cholesterol levels in the diabetic group were reflected principally in the VLDL (*p* < 0.01) and HDL₂ (*p* < 0.05) fractions. Total triacylglycerol levels in serum were reduced (*p* < 0.01) in the cholesterol-fed animals (0.222 ± 0.028 mmol/l) and increased (*p* < 0.01) in the diabetic animals (4.05 ± 1.30 mmol/l) compared to the control animals (0.59 ± 0.05 mmol/l). Serum phospholipid levels were significantly higher (*p* < 0.01) in the diabetic animals (2.69 ± 0.37 mmol/l) in comparison with the control group (1.16 ± 0.19 mmol/l). The increase in serum phospholipid levels in the cholesterol-fed animals (1.28 ± 0.11 mmol/l) did not attain statistical significance.

Table 1 demonstrates the elemental composition of the lipoprotein fractions of the three animal groups. In comparison with the control animals, the VLDL and LDL particles in the cholesterol-fed animals were enriched with cholesterol while the proportion of triacylglycerol in these particles was reduced. However, in the diabetic animals, all lipoprotein particles were enriched with triacylglycerol. This was associated with a generalised reduction in the proportion of cholesterol in each fraction and a reduction in the proportion of protein in the HDL₂ and HDL₃ fractions.

The specific activities of HMGCoA reductase, ACAT and cholesterol 7 α -hydroxylase are outlined in Figure 2. HMGCoA reductase activity was significantly reduced in both the cholesterol-fed (*p* < 0.001) and diabetic (*p* < 0.025) rabbits. The specific activity of hepatic ACAT was higher (*p* < 0.001) in the cholesterol-fed animals and lower (*p* < 0.001) in the diabetic animals in comparison with the controls. Similarly, the specific activity of cholesterol 7 α -hydroxylase was increased (*p* < 0.001) in the cholesterol-fed group and reduced (*p* < 0.01) in the diabetic group.

Figure 3 demonstrates the specific activities of HMGCoA reductase and ACAT in the intestines of the three animal groups. HMGCoA reductase activity was higher (*p* < 0.005) in the diabetic animals and lower (*p* < 0.01) in the cholesterol-fed animals compared with controls. By contrast, the specific activity of ACAT was reduced (*p* < 0.001) in the diabetic group and increased (*p* < 0.001) in the cholesterol-fed group in comparison with the normocholesterolaemic control animals.

Fig. 2 a–c. Effect of diabetes and cholesterol feeding on the specific activity of hepatic cholesterol metabolising enzymes. Each enzyme activity was measured in liver microsomal pellets. **a** HMGCoA reductase, **b** (cholesterol-*o*-acyltransferase) ACAT, **c** cholesterol 7 α -hydroxylase. Control animals (*n* = 6, black bars); diabetic animals

(*n* = 6, striped bars); cholesterol-fed animals (*n* = 6, stippled bars). Results are expressed as mean ± SEM. * *p* < 0.01 different from control animals; ** *p* < 0.025 different from control animals; *** *p* < 0.001 different from control animals

Fig. 3 a, b. Effect of diabetes and cholesterol feeding on the specific activity of intestinal cholesterol metabolising enzymes. Both activities were measured in mucosal cell whole homogenates. **a** HMGCoA reductase, **b** (cholesterol-o-acyltransferase) ACAT. Control animals ($n = 6$, black bars); diabetic animals ($n = 6$, striped bars); cholesterol-fed animals ($n = 6$, stippled bars). Results are expressed as mean $\pm$ SEM. * $p < 0.01$ different from control animals; ** $p < 0.005$ different from control animals; *** $p < 0.001$ different from control animals

Discussion

The cholesterol-fed rabbits responded to the exogenously induced hypercholesterolaemia by suppressing endogenous hepatic and intestinal cholesterol synthesis, increasing cholesterol esterification in the liver and the intestine and by increasing cholesterol excretion in bile. The effects of cholesterol feeding on cholesterol metabolising enzymes in the rabbit are supportive of earlier findings in this laboratory [18] and elsewhere in rabbits [19] guinea pigs [20] and hamsters [21]. This reduced synthesis and increased utilisation of cholesterol in the cholesterol-fed animal is therefore an adaptive response to the increased availability of cholesterol [22].

The hypercholesterolaemia in the diabetic rabbits was, by contrast, endogenously induced. While the aetiology of diabetic hypercholesterolaemia has not been clearly defined, studies in many insulinopenic animal models suggest that increased intestinal cholesterologenesis is a contributory factor [23–25]. The elevated intestinal HMGCoA reductase activity in the alloxan-treated rabbits in this study is therefore consistent with this theory and the diabetic animal, like the cholesterol-fed animal, responded to the resultant increased availability of cholesterol by suppressing hepatic cholesterol synthesis. However, in contrast to the cholesterol-fed animals, the activity of ACAT and cholesterol 7 α -hydroxylase was reduced in the diabetic animals. This suggests that decreased hepatic and intestinal cholesterol esterification and a reduction in the rate of catabolism of cholesterol to bile acids, might be contributing factors in the pathogenesis of hypercholesterolaemia in the diabetic rabbit. Such a situation could arise if insulin regulated the activity of these enzymes in the rabbit *in vivo*. However, previous *in vitro* studies in the rat have not demonstrated such an effect [26, 27].

In both diabetic and cholesterol-fed groups, the increased cholesterol appears to have been utilised for lipoprotein formation. While the VLDL particles in the cholesterol-fed animals were rich in cholesterol, the VLDL fractions in the diabetic animals were enriched with tri-

acylglycerol. Thus, the high VLDL cholesterol levels in the diabetic animals reflected the presence of a greater number of VLDL particles, although the quantity of cholesterol in each particle was reduced. By contrast, the elevated VLDL cholesterol levels in the cholesterol-fed animals reflected primarily the enrichment of each VLDL particle with cholesterol. While the ratio of free cholesterol to cholesteryl ester in the VLDL fractions was not quantified, previous studies have shown that these particles are rich in cholesteryl-ester in cholesterol-fed animals [28, 29]. These findings would be consistent with the increased ACAT activity observed in the cholesterol-fed rabbits in this study.

In the cholesterol-fed animals, the elevated VLDL cholesterol levels were accompanied by similar increases in LDL cholesterol. While some VLDL is converted to LDL in the normal rabbit [30, 31], previous kinetic experiments have shown that VLDL catabolism is reduced in the cholesterol-fed rabbit [32]. Since it has been demonstrated that cholesterol feeding in the rabbit causes down regulation of LDL receptors [33], it is likely that the elevated LDL cholesterol levels in the cholesterol-fed animals resulted from impaired LDL catabolism rather than increased VLDL turnover.

In the diabetic animals, the increased VLDL cholesterol levels were accompanied principally by changes in HDL₂ cholesterol while LDL cholesterol levels were only slightly raised. The small increase in LDL cholesterol occurred in a situation where the proportion of cholesterol in each particle was reduced. This suggests that LDL particle number is increased in the diabetic animals. As these LDL particles are enriched with triacylglycerol, it is likely that the increased number of LDL particles resulted from increased VLDL catabolism. Increased hydrolysis of VLDL in the diabetic rabbit could also account for the increased HDL₂ cholesterol levels observed, since during VLDL catabolism in the rabbit, apoprotein transfer from VLDL to HDL has been observed [34].

In conclusion, this study highlights the contrasting changes in cellular and lipoprotein cholesterol metabolism in two different hypercholesterolaemic models. Some investigations of cholesterol metabolism in diabetes have been performed in animals consuming diets rich in cholesterol [35, 36]. The data reported here emphasise the hazards of such an approach since many of the experimental findings may be dietary related and cannot therefore be attributed specifically to diabetes. It has previously been shown that the incidence of atherosclerosis in the diabetic hypercholesterolaemic rabbit model is reduced in contrast to its cholesterol-fed counterpart [37–39]. The differences in hepatic and intestinal cholesterol metabolism in serum lipoproteins between these two models may in part explain this observation.

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