Apolipoprotein B overproduction by the perfused liver of the St. Thomas' mixed hyperlipidemic (SMHL) rabbit

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Abstract The St. Thomas' mixed hyperlipidemic (SMHL) rabbit (previously St. Thomas' Hospital rabbit) is a putative model of familial combined hyperlipidemia (FCH). When fed a low (0.08%) cholesterol diet, it exhibits elevations in both plasma cholesterol and triglyceride compared to New Zealand White (NZW) controls. To determine the mechanism for this hyperlipidemia we studied the secretion of apolipoprotein B (apoB)-containing lipoproteins from perfused livers of both young and mature rabbits. During a 3-h perfusion we measured the total cholesterol and triglyceride content of the medium and the cholesterol, triglyceride, and apoB content of very low density lipoprotein ($VLDL)_1$ (S_f 60–400), $VLDL_2$ (S_f 20–60), intermediate (S_f 12–20), and low (S_f 0–12) density lipoproteins (IDL, LDL). Lipoprotein concentrations increased linearly throughout the perfusion period. The rate of cholesterol output was 3-fold higher (459 vs. 137 ng/g liver/min, $P = 0.003$) in SMHL versus NZW rabbits whilst that of triglyceride was similar (841 vs. 662 ng/g liver/min, NS). $VLDL_1$ cholesterol output was elevated 2-fold (232 vs. 123 ng/g liver/min, $P < 0.05$) and $VLDL_2$ + IDL + LDL cholesterol output, 4.5-fold (106 vs. 23 ng/g liver/min, $P < 0.005$) in SMHL versus NZW rabbits. ApoB output in VLDL1 was 38 ng/g liver per min in SMHL and 14 ng/g liver per min in NZW (NS). In SMHL $VLDL_2$ + IDL + LDL apoB was increased 9-fold at 53 versus 6 ng/g liver per min in NZW ($P < 0.001$). We conclude that the SMHL rabbit overproduces apoB-containing lipoproteins particularly in the $VLDL_2$ + IDL + LDL fraction, a characteristic consistent with its use as a model of FCH.—Ardern, H. A., G. M. Benson, K. E. Suckling, M. J. Caslake, J. Shepherd, and C. J. Packard. **Apolipoprotein B overproduction by the perfused liver of the St. Thomas' mixed hyperlipidemic (SMHL) rabbit.** ***J. Lipid Res.*** **1999.** 40: **2234–2243.**

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Familial combined hyperlipidemia (FCH) was first described in 1973 (1–3) as a monogenic disorder distinct from familial hypercholesterolemia and familial hypertriglyceridemia. Affected families show a range of phenotypes, elevated cholesterol, triglyceride or both, with an underlying increase in apoB concentration. The disorder is estimated to be associated with 1 in 10 premature myocardial infarctions and to affect 1–2% of the general population. Currently, diagnosis depends on a knowledge of family histories. However, if a mechanism for the disorder could be elucidated, this may facilitate a clearer description and improved detection of the condition.

Animal studies may help to identify the pathogenesis of the disease. The St. Thomas' Hospital rabbit was first described in 1987 as exhibiting increased though variable plasma lipid levels (4). Elevated very low density lipoprotein (VLDL, S_f 20–400), intermediate density lipoprotein (IDL, S_f 12–20), and low density lipoprotein (LDL, S_f 0–12) cholesterol and LDL triglyceride levels were seen when compared to New Zealand White (NZW) controls. Both the LDL particles and LDL receptors of these rabbits were shown to be normal and on this basis it was proposed that these rabbits have a disorder similar to FCH. Little has appeared in the literature in recent years on this animal model except for a report from Johns Hopkins University on a strain of rabbit derived from St. Thomas' Hospital rabbits that, on investigation, provided evidence for two distinct genetic mechanisms, one acting on triglyceride, and the other on apoB (5). In 1993 the remaining rabbits were used to establish a new colony, whose members have now been renamed the St. Thomas' mixed hyperlipidemic (SMHL) rabbit. When fed a low cholesterol diet the rabbits from the colony developed mixed hyperlipidemia. In preliminary studies performed on a group of moderately hyperlipidemic rabbits, we found that the lipid elevation was due to an increased mass of $VLDL_1$ (S_f 60–400), $VLDL_2$ (S_f 20–60), IDL, and LDL (6).

Having observed a reproducible phenotype in the SMHL

Abbreviations: apoB, apolipoprotein B; VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; FCH, familial combined hyperlipidemia; SMHL, St. Thomas' mixed hyperlipidemic rabbit; NZW, New Zealand white rabbit.

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animals, we undertook a series of liver perfusion experiments to measure hepatic production of apoB-containing lipoproteins in these animals compared to NZW controls. Current concepts of the etiology of FCH in humans suggests that the underlying defect is an overproduction of apoB-containing lipoproteins by the liver (7, 8), possibly as a result of supranormal amounts of fatty acids being delivered to the organ from adipose tissue (9, 10). We therefore tested the hypothesis that SMHL rabbits have a metabolic abnormality of apoB overproduction by the liver.

METHODS

Animals

The SMHL rabbits were maintained as an inbred colony at Froxfield Farms (Froxfield, Nr Petersfield, Hants., UK). Nine SMHL (5 female and 4 male) and 9 age- and sex-matched NZW controls were fed a chow diet (0.005% cholesterol wt/wt) (Stanrab diet, Special Diet Service, Witham, UK) for a minimum of 4 weeks and 10 SMHL and 10 age- and sex-matched NZW rabbits were fed a diet supplemented with a low (0.075% wt/wt) level of cholesterol (0.08% total) for 12 weeks in order to determine the effects of a diet with a slightly elevated cholesterol content on lipoprotein composition and mass.

Plasma cholesterol and triglyceride levels were measured in the inbred colony on 60 young SMHL, 12 young NZW, 16 mature SMHL, and 18 mature NZW male rabbits fed the 0.08% cholesterol diet. From these animals, 17 SMHL rabbits and 19 age-matched NZW controls were selected for liver perfusion experiments on the basis of their lipid levels. The selected rabbits had moderate mixed hyperlipidemia; plasma cholesterol was 4.0 ± 0.8 mmol/l in SMHL versus 1.3 ± 0.2 mmol/l in NZW, $P < 0.005$ and plasma triglyceride was 2.6 ± 0.4 mmol/l in SMHL versus 1.0 ± 0.1 mmol/l in NZW, $P < 0.001$. Six SMHL and 7 NZW rabbits were studied between 11 and 16 weeks of age (young), and the remainder were studied when more than 5 months old (mature). The animals were housed individually in cages under standard conditions and were fed ad libitum the diet containing 0.08% cholesterol. Lipoprotein lipase and hepatic lipase activity were measured in intact animals after administration of 70 IU/kg heparin according to previously published procedures (11).

The procedures involving animals in these studies were subject to UK Home Office regulations.

Surgical procedure

On the day of the perfusion experiment, the animal was terminally anesthetized by the injection of 1 ml Euthatal (Rhône Mérieux Ltd., Harlow, UK) per 1.4 kg body weight plus 1 ml heparin (1000 IU/ml) (Leo Laboratories Ltd., Princes Risborough, UK) into the marginal ear vein. A thoracotomy/laparotomy was performed along the line of the linea alba and the thoracic aorta was ligated using 2-0 silk (David and Geck, Cyanamid of Great Britain Ltd., Gosport, UK). The intestines were displaced to the left and the inferior vena cava (IVC) was cannulated superior to the diaphragm using plastic tubing (internal diameter 2 mm, external diameter 3.2 mm). The hepatic portal vein (HPV) was cannulated (14G IV catheter; Vygon UK Ltd., Cirencester, UK), the IVC was ligated between the renal and hepatic veins (polyamide 66 suture, Ethicon Ltd., Edinburgh, UK), and the common bile duct was cannulated (translucent vinyl tubing, internal diameter 0.63 mm, external diameter 1.4 mm, Portex Ltd., Hythe, UK).

Perfusion

Livers were flushed with 750 ml oxygenated Krebs Henseleit buffer (12) (pH 7.4, 37°C) at 75 cm hydrostatic pressure prior to being connected to the perfusion apparatus. Perfusions were performed at 37°C in a thermostatically controlled room. The apparatus consisted of two peristaltic pumps (Watson Marlow MHRE and 503U), pumping buffer at 100 ml/min with inflow via the HPV and outflow through the IVC. Oxygenation was by a 'Hamilton lung' (13) containing 5 meters of silastic tubing. The pH was constantly monitored (Jenway 3050 portable pH meter, Jenway, UK, Dunmow, UK) and maintained at 7.4 by the addition of 1 M $NaHCO_3$ when necessary. A 3.2 mm internal diameter, 6.4 mm external diameter tubing (AlteSil high strength tubing, Altec, Alton, UK) was used throughout the system. The total volume of recirculating perfusate was 120 ml. It was not necessary to have red blood cells in the perfusate as preliminary experiments showed no difference in liver function, viability, bile output, or apoB production when erythrocytes were present or absent. Their presence caused an artificial increase in the cholesterol concentration in the perfusate, presumably as a consequence of erythrocyte degradation.

A 15-ml sample of perfusate was withdrawn at 0, 60, 120, and 180 min and 5-ml samples were removed at 30, 90, and 150 min. Sample volume was replaced with an equal volume of oxygenated, warmed Krebs Henseleit buffer. The liver and intestines were kept moist by covering with gauze soaked in buffer. The volume of bile produced was recorded. At the end of the experiment the livers were examined and found to be well perfused with no necrotic or hypoxic areas. On three occasions, heparin (about 10 IU) was added to the circulating medium at the end of the 3-h experiment, after all samples had been taken for lipid and lipoprotein analysis. Perfusion was continued for 10 min to release any lipase present in the liver. A heparinized sample was then tested for lipoprotein and hepatic lipase activity (11). Pieces of liver were taken and stored at −70°C for analysis of their lipid content.

Analysis of perfusate

Samples of perfusate were spun (1780 *g*, 10 min) to pellet any cellular material. Cholesterol, cholesteryl ester, triglyceride, phospholipid (Boehringer Mannheim GmbH, Lewes, UK), and glycerol (Randox Laboratories Ltd., Crumlin, UK) were measured with kits. Urea, albumin, AST, and GGT were determined on an Olympus A300 multichannel analyser using manufacturers reagents.

Lipoproteins in the perfusate were concentrated 5-fold by centrifugation at a density of 1.065 g/ml (83,150 *g*, 18 h). The total apoB-containing lipoprotein fraction so obtained was separated into $VLDL_1$, $VLDL_2$, IDL, and LDL by centrifugation in a density gradient constructed of layers of d 1.0988, 1.0860, 1.0790, 1.0722, 1.0641, and 1.0588 g/ml (14, 15). Cholesterol, triglyceride, total protein, and apoB were measured in each fraction. Concentrations were corrected back to that present in the original perfusate sample. Protein was measured by a modification (16) of the method of Lowry et al. (17). Lipoprotein subfractions were treated by the addition of an equal volume of isopropanol to precipitate apoB. The apoB content was calculated by subtracting isopropanol soluble protein from total protein (18).

Extraction of lipid from liver samples

Immediately on thawing, samples of liver were taken and the wet weight was recorded (0.25–0.5 g). Following a modification (19) of the method of Folch et al. (20), samples were homogenized (Janke & Kunkel KG homogenizer, Staufen I Breisgau, Germany) in small volumes of methanol (BDH) and the volume was adjusted to 50 ml with methanol. After standing for 30 min with

occasional mixing, 100 ml chloroform (BDH) was added. After an overnight extraction at 15°C, the samples were filtered (Whatmans No. 1 filter paper, Whatman International Ltd., Maidstone, UK) and protein was precipitated by the addition of 50 ml 0.05% calcium chloride solution. Samples were again maintained overnight at 15°C and the chloroform (bottom) layer was removed and dried on a centrifugal evaporator (Howe). The pellet was redissolved in isopropanol (2 ml) and total cholesterol, cholesteryl ester, triglyceride, and phospholipid contents were measured as above.

Measurement of $VLDL_1$ to LDL conversion during perfusion

Total VLDL was isolated from 50 ml rabbit plasma at d 1.006 g/ml (62,918 *g*, 18 h). From this $VLDL_1$ was prepared by density gradient centrifugation as described above. $VLDL_1$ was then labeled with Na[^{125}I] (Amersham International plc, Amersham, UK) as described previously (21).

Livers from two NZW rabbits (fed standard rabbit chow) and from two SMHL rabbits (fed 0.08% cholesterol-supplemented diet) were prepared as above. After taking a pre-treatment sample at time 0, 5 μCi of ^{125}I-labeled $VLDL_1$ was added to the circulating buffer. During the perfusion $VLDL_1$, $VLDL_2$, IDL, and LDL were isolated as described above; apoB was isolated, resolubilized in 0.1 M NaOH and its specific activity was measured.

[^{3}H]leucine experiments

To demonstrate that the accumulating lipoproteins in the recirculating perfusate represented newly synthesized products, the incorporation of radioactive leucine into apoB was measured during perfusion of livers from two mature NZW and two mature SMHL rabbits. [^{3}H]leucine (100 μCi) (Amersham International plc) (165 Ci/mmol) was added to the perfusate at time 0 and further doses of 100 μCi [^{3}H]leucine were added at 30-min intervals, just after each sample was removed for lipoprotein isolation. The total radioactivity and specific activity of apoB isolated from $VLDL_1$, $VLDL_2$, IDL, and LDL were then determined by radioactivity measurement in a β scintillation counter and assay of apoB protein as described above.

Statistical analyses

Variables were tested for normal distribution. Plasma cholesterol and triglyceride concentrations were transformed to a normal distribution by taking the logarithm of their values. Two sample *t*-tests were performed to determine significance. Where the distribution of a variable or series of variables could not consistently be converted to normal, as was the case with all perfusate sample measurements, a Mann-Whitney *U* test was performed. All statistical analyses were carried out using Minitab version 10. Data are expressed as mean ± standard error of the mean (SEM) unless otherwise stated.

RESULTS

Effect of 0.08% cholesterol diet on plasma lipids and lipoproteins

To uncover the hyperlipidemic phenotype in SMHL rabbits, it was necessary to add a small amount of cholesterol to the diet (**Table 1**). Cholesterol supplementation had no significant effect on plasma lipid levels in NZW rabbits; however, plasma cholesterol was significantly elevated in SMHL rabbits on the 0.08% cholesterol diet compared to SMHL on normal chow and NZW fed either normal or supplemented diet. There were no significant differences in plasma triglyceride concentrations among any of the groups. It was noted that despite having normal total lipid levels, chow-fed SMHL rabbits were dyslipidemic. $VLDL_2$ and IDL mass levels were elevated compared to NZW rabbits fed the same diet ($P = 0.08$, $P = 0.01$, respectively). When fed the cholesterol-supplemented diet, all four apoB-containing lipoproteins were increased in SMHL compared to NZW rabbits fed either normal or supplemented diet.

TABLE 1. Effect of 0.08% cholesterol diet on plasma lipid and lipoprotein levels in SMHL and NZW rabbits

	Cholesterol Diet		Chow Diet	
	NZW (n = 10)	SMHL (n = 10)	NZW (n = 9)	SMHL (n = 9)
Cholesterol (mmol/l)	1.6 ± 0.3	3.8 ± 0.7[a,b]	0.8 ± 0.1	1.1 ± 0.2
Triglyceride (mmol/l)	0.49 ± 0.04	1.3 ± 0.46	0.8 ± 0.2	1.2 ± 0.3
$VLDL_1$ mass (mg/dl)	16 ± 4	97 ± 35[a]	38 ± 11	43 ± 16
$VLDL_2$ mass (mg/dl)	21 ± 5	99 ± 22[a,b]	10 ± 4	20 ± 4
IDL mass (mg/dl)	31 ± 10	108 ± 24[a,b]	7 ± 1	18 ± 3
LDL mass (mg/dl)	15 ± 4	36 ± 6[a,b]	4 ± 1	7 ± 2

Plasma lipid and lipoprotein measurements in NZW and SMHL rabbits fed either normal (0.005% cholesterol) or 0.08% cholesterol diet (mean ± SEM).

Significant differences $P < 0.05$ were determined using the Mann Whitney *U* test.

[a] SMHL vs. NZW on 0.08% cholesterol diet; [b] SMHL on 0.08% cholesterol diet vs. SMHL on normal diet.

Population lipids, lipoproteins, and lipases

In the male rabbits in the colony screened for plasma lipid levels prior to selection of animals for perfusion experiments, plasma cholesterol levels of both young and mature SMHL rabbits were significantly elevated (though variable) compared to NZW rabbits (5.97 ± 0.37 vs. 1.79 ± 0.14 mmol/l, $P < 0.001$ and 3.11 ± 0.95 vs. 1.32 ± 0.23 mmol/l, $P = 0.04$, in young and mature rabbits, respectively (**Fig. 1A**)). Plasma triglyceride levels were significantly elevated in young SMHL rabbits compared to NZW controls (3.31 ± 0.25 vs. 1.14 ± 0.14 mmol/l, $P < 0.0001$ (Fig. 1B)) but not in mature SMHL versus NZW animals. SMHL rabbits showed a decrease with age in plasma lipid concentrations, $P = 0.006$ for young versus mature cholesterol levels and $P < 0.001$ for young versus mature triglyceride levels (Fig. 1) but NZW rabbits did not. Assay of lipoprotein lipase activity in heparinized mature animals (n = 7, in each group) gave values in μmol FFA released/ml plasma per h of 16.0 ± 2.4 in NZW versus 12.4 ± 7.8 in SMHL (NS). Hepatic lipase was 1.7 ± 0.5 μmol FFA released/ml plasma per h in NZW versus 2.1 ± 0.5 μmol/ml per h in SMHL (NS).

Perfusion experiments

Liver perfusion experiments were performed on young and mature SMHL and NZW rabbits that had been fed the 0.08% cholesterol diet from weaning. Liver function tests were normal throughout the perfusions. The livers remained patent and produced bile at 27, 17,

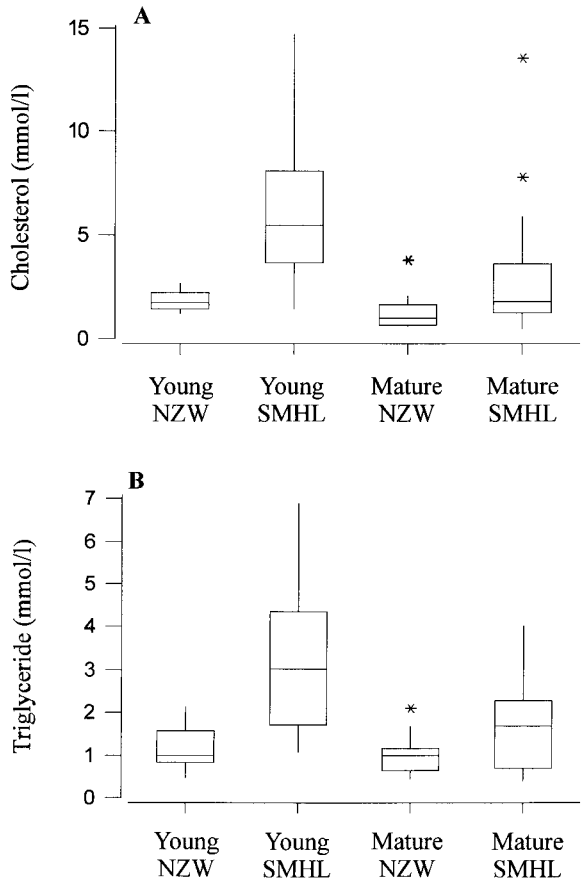

Fig. 1. A: Distribution of plasma cholesterol concentrations in male NZW and SMHL rabbits. Levels were determined in young SMHL rabbits (n = 60), mature SMHL rabbits (n = 16), young NZW rabbits (n = 12), and mature NZW rabbits (n = 18). B: Distribution of plasma triglyceride concentrations in NZW and SMHL rabbits. The horizontal line represents the median; the upper and lower limits of the box are the 75 and 25 percentiles; the vertical lines show the range and the asterisks represent outliers.

29, and 15 μl/liver per min in young NZW, young SMHL, mature NZW, and mature SMHL, respectively (none of these was significantly different). Examination of the livers at the end of the experiment revealed no necrotic or hypoxic areas. Mean age at killing for young NZW versus SMHL rabbits was 3.0 versus 2.8 months, mean body weight was 2.5 versus 2.0 kg, and mean liver weight was 71 versus 74 g. Mean age at killing for the mature NZW versus SMHL rabbits was 9.8 versus 10.2 months, mean body weight was 4.0 versus 3.6 kg, and mean liver weight was 103 versus 92 g.

In those rabbits selected for perfusion studies, the mean plasma cholesterol and triglyceride levels were elevated in SMHL compared to NZW controls and similar to the mean values seen for the whole colony. Thus, in young animals used for perfusion studies, plasma cholesterol was 3.7 ± 0.4 mmol/l in SMHL and 1.0 ± 0.2 mmol/l in NZW (P = 0.001), while plasma triglyceride was 3.6 ± 0.9 mmol/l in SMHL and 0.7 ± 0.07 mmol/l in NZW (P = 0.005). In mature rabbits, plasma cholesterol was 4.1 ± 1.3 versus 1.4 ± 0.3 mmol/l ($P < 0.05$) and plasma triglyceride was 2.0 ± 0.4 versus 1.2 ± 0.1 mmol/l (NS) (SMHL vs. NZW, respectively).

Lipid levels during perfusion

Mean perfusate cholesterol and triglyceride levels rose linearly in all groups of rabbits over the 180 min of the experiment (**Fig. 2**). Only data up to 150 min were available from the perfusate experiments with the young SMHL rabbits. In young rabbits, cholesterol content in the perfusate was barely measurable in the NZW group, rising to 2.0 ± 0.5 μg/g liver after 180 min; however, in the perfusate from the SMHL rabbits, the cholesterol content rose from 5 ± 5 to 58 ± 31 μg/g liver at 150 min and was significantly higher when compared to that from NZW rabbits at all time points from 60 min until the end of the experiment. In young rabbits, the calculated cholesterol output was 380 ± 170 versus 10 ± 6 ng/g liver per min (SMHL vs. NZW, $P < 0.01$). Perfusate cholesterol levels in the mature rabbits rose from 7 ± 5 to 41 ± 11 μg/g liver in NZW rabbits during the experiment and from 6 ± 4 to 96 ± 21 μg/g liver in SMHL rabbits. The cholesterol content of the perfusate was significantly increased in SMHL rabbits in samples taken at 60, 90, and 150 min and the

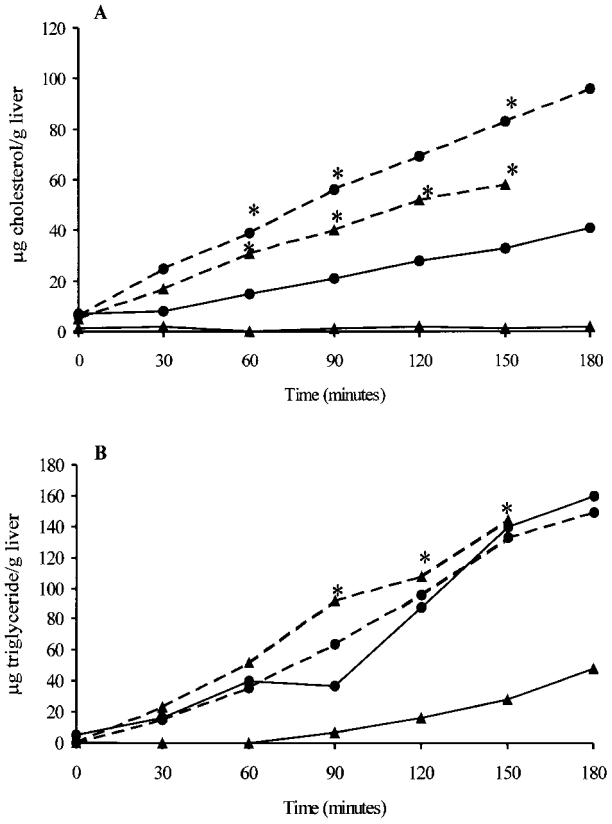

Fig. 2. A: Mean cholesterol output from the liver of NZW and SMHL rabbits during the 3 h of the perfusion study. The point missing at 180 min for young SMHL rabbits is due to insufficient data at that time. Young NZW (—▲—), mature NZW (—●—), young SMHL (---▲---), mature SMHL (---●---). Significant differences (young or mature NZW vs. SMHL) * $P < 0.05$ by the Mann Whitney U test. B: Mean output of triglyceride from the livers of NZW and SMHL rabbits during the 3 h of the perfusion study.

calculated cholesterol output was 510 ± 101 versus 220 ± 67 ng/g liver per min in SMHL versus NZW rabbits (NS). Mature NZW rabbits had a significantly greater cholesterol output compared to young NZW of 220 ± 67 versus 10 ± 6 ng/g liver per min ($P < 0.005$). SMHL rabbits on the other hand did not show a significant effect of age.

The mean triglyceride output was higher in young SMHL rabbits compared to young NZW but this did not reach significance (810 ± 290 vs. 270 ± 100 ng/g liver/min, $P = 0.12$). Perfusate triglyceride rose from 0 to 48 ± 24 μg/g liver in NZW rabbits and from 0 to 144 ± 63 μg/g liver in SMHL rabbits, reaching a significantly higher level when compared to NZW rabbits from 90–150 min. Mature rabbits in both groups showed a similar triglyceride output of 860 ± 130 versus 890 ± 246 ng/g liver per min (SMHL vs. NZW, respectively, NS). Triglyceride perfusate levels rose from 5 ± 4 to 160 ± 50 and 0 to 149 ± 29 μg/g liver in the NZW and SMHL, respectively. Neither NZW or SMHL rabbits showed any significant age related differences in triglyceride output.

Lipoprotein production during perfusion

The lipoproteins in the perfusate were separated and their cholesterol and triglyceride contents were analyzed. Data are shown in **Table 2**. In young rabbits, $VLDL_1$, $VLDL_2$, and IDL cholesterol output were significantly increased in SMHL compared to NZW rabbits but no difference was seen in LDL cholesterol output. $VLDL_2$ triglyceride output was significantly increased in SMHL rabbits. In mature SMHL rabbits, mean IDL triglyceride was increased compared with NZW rabbits ($P < 0.05$, Table 2) but no other significant differences were observed in individual fractions. We then compared the output of triglyceride-rich and cholesterol-rich lipoproteins from the liver. The cholesterol and triglyceride contents of $VLDL_2$, IDL, and LDL (i.e., non $VLDL_1$) were combined as these represent cholesterol-rich lipoproteins and the values obtained were compared with those for the triglyceride-rich $VLDL_1$ (**Fig. 3**). In young rabbits, perfusate levels of $VLDL_1$ cholesterol rose from 0 to 12.5 ± 4 μg/g liver in NZW rabbits and from 0.8 ± 0.3 to 37.8 ± 12.2 μg/g liver in SMHL rabbits (Fig. 3A), being significantly higher in SMHL rabbits at all time points from 30–150 min. $VLDL_1$ cholesterol output was significantly increased in young SMHL rabbits compared to NZW rabbits (237 vs. 60 ng/g liver/min, respectively, Table 2). The sum of $VLDL_1$ + $VLDL_2$ + IDL + LDL cholesterol output in Table 2 for young NZW rabbits appears higher than the corresponding data for perfusion medium total cholesterol content in Fig. 2. The values in Table 2 are more accurate as these fractions were concentrated by centrifugation prior to cholesterol measurement giving a more reliable estimate than the perfusion medium, which in these animals was often at or below the limit of detection of the assay. Non-$VLDL_1$ perfusate cholesterol levels rose from 0.6 ± 0.4 to 4.7 ± 1.7 and from 0.7 ± 0.7 to 18.8 ± 11 μg/g liver in NZW and SMHL rabbits, respectively, and were significantly higher in SMHL rabbits between 90 and 150 min. Non-$VLDL_1$ cholesterol output rate was elevated in SMHL rabbits compared to NZW rabbits (118 ± 56 vs. 21 ± 8 ng/g liver/min, respectively, $P = 0.01$).

In mature rabbits there was no significant difference in mean $VLDL_1$ cholesterol output between the two strains of rabbit (160 vs. 229 ng/g liver/min, NZW vs. SMHL, Table 2). In NZW rabbits, $VLDL_1$ perfusate cholesterol levels rose from 0 to 25 ± 8 μg/g liver (Fig. 3B), and in SMHL rabbits from 0 to 46 ± 11 μg/g liver over the 3 h of the experiment. Although there was a 5-fold increase in SMHL rabbit non-$VLDL_1$ cholesterol output when compared to NZW rabbits, this was not significant (98 ± 28 vs. 23 ± 4 ng/g liver/min). Non-$VLDL_1$ cholesterol levels increased from 0 to 21 ± 7 and 1 ± 1 to 5 ± 2 μg/g liver in SMHL and NZW rabbits, respectively, during the 3-h perfusion, reaching significance at 30, 90, and 150 min.

There were no significant differences in $VLDL_1$ triglyceride output rates in young NZW versus SMHL rabbits (658 vs. 374 mg/g liver/min) largely because of the large inter-animal variability (Table 2). However, $VLDL_1$ triglycer-

TABLE 2. $VLDL_1$, $VLDL_2$, IDL, and LDL cholesterol, triglyceride, and apoB output in young and mature NZW and SMHL rabbits fed a 0.08% cholesterol diet from weaning

	Cholesterol		Triglyceride		ApoB	
Group	NZW	SMHL	NZW	SMHL	NZW	SMHL
Young						
$VLDL_1$	60 ± 19	237 ± 68[a]	374 ± 93	658 ± 177	22 ± 4	79 ± 29[a]
$VLDL_2$	9 ± 5	73 ± 26[c]	17 ± 3	87 ± 44[a]	3 ± 1	36 ± 13[c]
IDL	3 ± 2	40 ± 26[a]	5 ± 2	55 ± 40	1 ± 1	37 ± 23[b]
LDL	2 ± 2	10 ± 7	5 ± 2	9 ± 7	2 ± 1	7 ± 5
Mature						
$VLDL_1$	160 ± 45	229 ± 52	408 ± 97	476 ± 84	10 ± 3	15 ± 4
$VLDL_2$	18 ± 6	65 ± 20	13 ± 3	56 ± 30	2 ± 1	14 ± 5[a]
IDL	8 ± 2	26 ± 10	5 ± 2	19 ± 6[a]	3 ± 1	16 ± 6[a]
LDL	2 ± 1	7 ± 3	4 ± 2	8 ± 3	0	6 ± 2

$VLDL_1$, $VLDL_2$, IDL, and LDL cholesterol, triglyceride, and apoB output (ng/g liver/min) in young and mature NZW and SMHL rabbits (mean ± SEM).

Significant differences [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.005$ between NZW and SMHL rabbits were determined using the Mann Whitney U test.

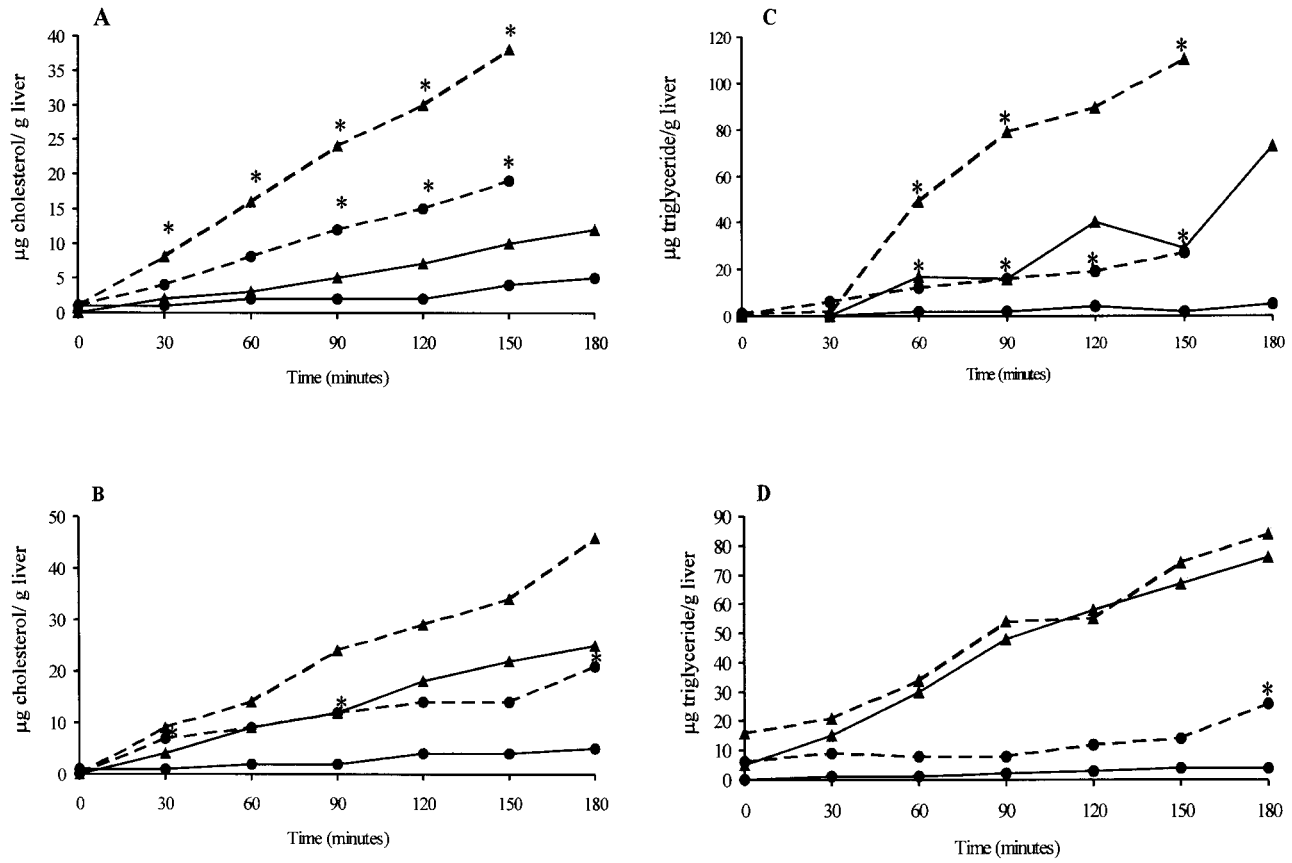

Fig. 3. A: Mean $VLDL_1$ and non-$VLDL_1$ cholesterol output in young NZW and SMHL rabbits. NZW $VLDL_1$ (—▲—), NZW non-$VLDL_1$ (—●—), SMHL $VLDL_1$ (---▲---), SMHL non-$VLDL_1$ (---●---). Significant differences (NZW vs. SMHL) * $P < 0.05$ by the Mann Whitney *U* test. B: Mean $VLDL_1$ and non-$VLDL_1$ cholesterol output in mature NZW and SMHL rabbits. C: Mean $VLDL_1$ and non-$VLDL_1$ triglyceride output in young NZW and SMHL rabbits. D: Mean $VLDL_1$ and non-$VLDL_1$ triglyceride output in mature NZW and SMHL rabbits.

ide concentration was higher in the perfusate from young SMHL versus NZW rabbit at 60, 90, and 150 min (Fig. 3C). Non-$VLDL_1$ triglyceride output was higher in the perfused livers from young SMHL rabbits than from young NZW rabbits (153 ± 93 vs. 20 ± 6 ng/g liver/min, respectively, $P < 0.05$), and non-$VLDL_1$ triglyceride concentrations were significantly higher at all time points from 60 min to 150 min. In mature SMHL perfused livers, $VLDL_1$ triglyceride output was similar to that seen in NZW rabbits (Table 2, Fig. 3D) but non-$VLDL_1$ triglyceride output rates tended to be higher in SMHL compared to NZW rabbits although the difference was not significant (78 ± 38 vs. 30 ± 11 ng/g liver/min, respectively). There was, however, a significant increase in the non-$VLDL_1$ triglyceride concentration in the perfusate from the mature SMHL rabbits after 180 min.

ApoB output from perfused livers

Apolipoprotein B was measured in the individual lipoproteins secreted into the hepatic perfusate. The results are shown in Table 2 and **Fig. 4**. ApoB as a percentage of total lipoprotein was similar in SMHL and NZW rabbits. In $VLDL_1$ it comprised 41% of the total protein in SMHL versus 38% in NZW rabbits, in $VLDL_2$ 60% versus 64%, in IDL 79% versus 82%, and in LDL 75% versus 76% (note that significant amounts of other apoproteins, especially apoE, are commonly found in rabbit IDL and LDL (22, 23) in contrast to the situation for human LDL where apoB is the sole protein). Individual $VLDL_1$, $VLDL_2$, and IDL apoB outputs were significantly elevated in young SMHL rabbits compared to NZW controls (Table 2). The $VLDL_1$ apoB output was 79 mg/g liver per min in SMHL compared to 22 ng/g liver per min ($P < 0.05$). Non-$VLDL_1$ apoB output in young SMHL rabbits was 80 ± 41 ng/g liver per min versus 6 ± 3 ng/g liver per min in NZW rabbits ($P < 0.005$) (Fig. 4A). Likewise, total apoB output was significantly higher in livers from young SMHL rabbits compared to NZW (159 ± 56 vs. 28 ± 7 ng/g liver/min, $P < 0.01$). In contrast to the situation in young animals, livers from mature NZW and SMHL rabbits released $VLDL_1$ apoB at similar rates (Table 2, Fig. 4B). There were no significant differences in $VLDL_1$ apoB concentrations during the perfusion in SMHL and NZW rabbits. However, non-$VLDL_1$ apoB concentrations were significantly increased in SMHL rabbits at all time points from 30 min until the end of the experiment (Fig. 4B). $VLDL_2$ apoB and IDL apoB outputs were increased in mature SMHL versus NZW rabbits (Table 2) and the summated non-$VLDL_1$ apoB output was increased 6-fold in SMHL rabbits relative to NZW (36 ± 12 vs. 6 ± 3 ng/g liver/min, respectively, $P = 0.01$). In mature animals non-$VLDL_1$ apoB

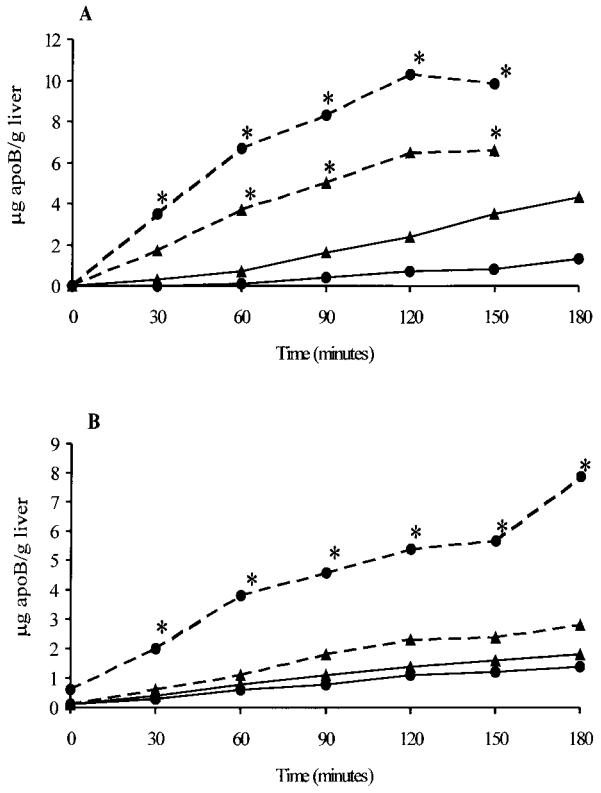

Fig. 4. A: Mean $VLDL_1$ and non-$VLDL_1$ apoB output from young NZW and SMHL rabbits. NZW $VLDL_1$ (—▲—), NZW non-$VLDL_1$ (—●—), SMHL $VLDL_1$ (---▲---), SMHL non-$VLDL_1$ (---●---). Significant differences (NZW vs. SMHL) * $P < 0.05$ by the Mann Whitney *U* test. B: Mean $VLDL_1$ and non-$VLDL_1$ apoB output from mature NZW and SMHL rabbits.

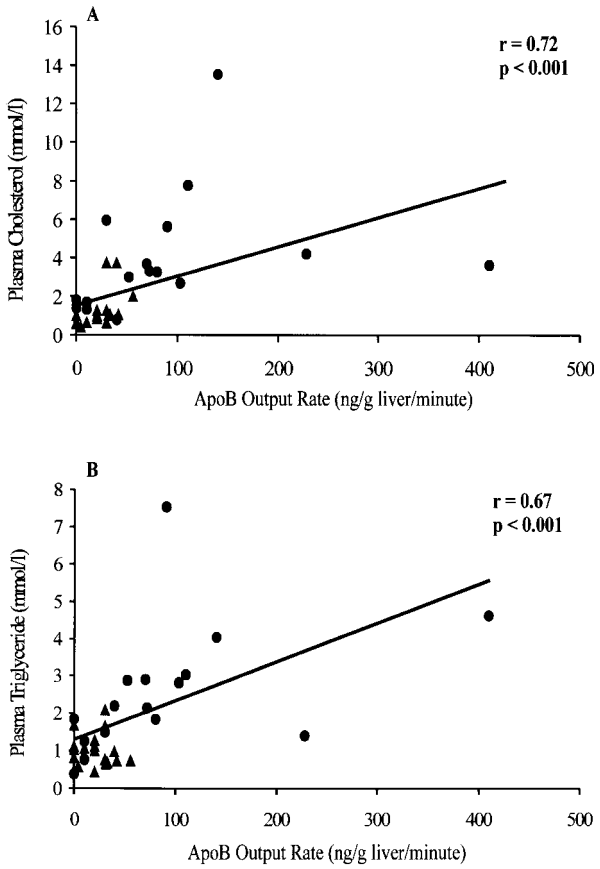

Fig. 5. Correlation of apoB output rates with plasma cholesterol (A) and triglyceride (B) concentrations in NZW rabbits (▲) and SMHL rabbits (●) at the time of killing.

output accounted for 75% of total apoB output. The total apoB output in these mature rabbits was increased in the SMHL rabbit group but this did not achieve significance (48 ± 14 vs. 16 ± 4 ng/g liver/min in SMHL and NZW, $P = 0.07$).

$VLDL_1$ apoB output was significantly increased in the young NZW compared to the mature NZW (22 vs. 10 ng/g liver/min, respectively, $P = 0.04$, Table 2) and in young SMHL versus mature SMHL rabbits (79 vs. 15 ng/g liver/min, respectively, $P < 0.01$, Table 2). No differences were found between the young and mature rabbits of either strain in non-$VLDL_1$ apoB output.

When both young and mature NZW and SMHL rabbits were grouped together, there was a highly significant correlation between the rate of apoB output by the perfused livers and plasma cholesterol levels at time of killing ($r = 0.72$, $P < 0.001$) (**Fig. 5A**). Plasma triglyceride was also significantly correlated with total apoB output rate ($r = 0.67$, $P < 0.001$) (Fig. 5B). Exclusion of the SMHL animal with the highest apoB output (410 ng/g liver/min) as an outlier did not materially alter these relationships (apoB output vs. plasma cholesterol, $r = 0.71$, $P < 0.001$; apoB output vs. plasma triglyceride, $r = 0.68$, $P < 0.001$). Plasma cholesterol correlated strongly with hepatic total cholesterol output ($r = 0.74$, $P < 0.001$) as did plasma triglyceride with hepatic total triglyceride output ($r = 0.49$, $P = 0.003$).

To examine the extent to which $VLDL_1$ was converted to denser lipoproteins during the 3-h perfusion, ^{125}I-labeled $VLDL_1$ was added to the perfusate of two NZW rabbits 10 min prior to the beginning of the experiment. ApoB was isolated to follow the fate of the particles. At time 0, 93% of apoB radioactivity was found in the $VLDL_1$ fraction, with 6% in $VLDL_2$ and 1% in IDL. After 3 h, 77% of the counts remained in $VLDL_1$, 7.5% were found in $VLDL_2$, 2.5% in IDL, and 1.5% in LDL. Likewise in two SMHL perfusions, less than 6% of added ^{125}I-labeled $VLDL_1$ apoB appeared in $VLDL_2$, IDL, or LDL over 3 h. The lack of increase in $VLDL_2$, IDL, and LDL radioactivity indicated that delipidation was limited in this perfusion system. Heparin was used in the surgical preparation for the perfusion, hence at the beginning of the experiments any endothelium-bound lipase present was flushed from the organ and discarded. Addition of heparin at the end of the 3-h perfusion to release any newly formed lipase revealed no lipoprotein lipase or hepatic lipase activity in two of the perfusions where this was carried out and no lipoprotein lipase and barely detectable levels of hepatic lipase in the third.

To show that newly synthesized apoB appeared in both

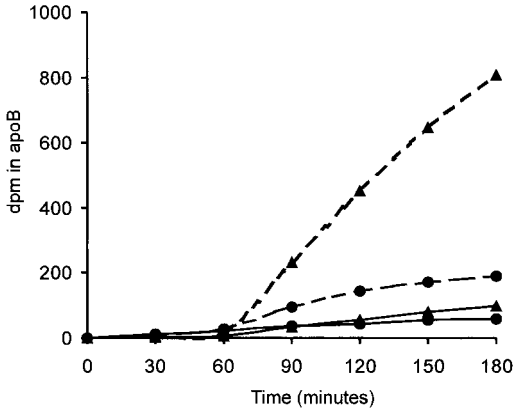

Fig. 6. Appearance of [^{3}H]leucine in apoB of $VLDL_1$ and non-$VLDL_1$ fractions. Two NZW and two SMHL mature rabbits were perfused. To the perfusate was added [^{3}H]leucine at 30-min intervals and apoB was isolated and its radioactivity content was determined. Data shown are the mean radioactivity contents for the two animals in each group. NZW $VLDL_1$ (—▲—), NZW non-$VLDL_1$ (—●—), SMHL $VLDL_1$ (---▲---), SMHL non-$VLDL_1$ (---●---).

$VLDL_1$ and non-$VLDL_1$ fractions, [^{3}H]leucine was added to the perfusate of two mature NZW and two mature SMHL rabbits. There was an initial delay while new apoB was synthesized. By 3 h, leucine radioactivity in $VLDL_1$ apoB rose by an average of 5-fold above the zero time value in NZW and 15-fold in SMHL rabbit livers; in $VLDL_2$ it rose 2- and 6-fold, in IDL, 2- and 2-fold and in LDL, 2- and 3-fold, respectively, showing that the liver was capable of synthesizing lipoproteins across the density spectrum (**Fig. 6**). The specific activity of apoB (dpm per mg apoB) in each lipoprotein fraction was relatively constant throughout the perfusion period, indicating that radioactivity and protein appeared together in a fixed ratio. Further, the specific activity of apoB in $VLDL_2$, IDL, and LDL was similar to that in $VLDL_1$ (data not shown). If substantial amounts of 'cold' lipoprotein from hepatic lymph had contributed to the $VLDL_2$, IDL, or LDL present, then the apoB specific activity in these fractions should have been substantially lower than that in $VLDL_1$.

Liver lipids

Hepatic lipid concentrations were measured after the perfusion experiments. Young and mature NZW and SMHL rabbits were found to have very similar concentrations of cholesterol, free cholesterol, triglyceride, and phospholipids in their livers. Mature NZW rabbits stored more cholesterol (total and free) in their livers than did young NZW rabbits (total cholesterol 15.4 ± 1.9 vs. 6.0 ± 1.1 mg/g wet weight liver, respectively, $P = 0.015$ and free cholesterol 7.4 ± 1.3 vs. 3.3 ± 0.8 mg/g wet weight liver, respectively, $P = 0.017$). The lipids in the livers of SMHL rabbits did not differ with age and were not significantly different from those in NZW (total cholesterol 7.9 ± 2.1 vs. 12.2 ± 2.3 mg/g wet weight liver and free cholesterol 5.0 ± 1.3 vs. 8.2 ± 1.5 mg/g wet weight liver in young vs. mature SMHL rabbits, respectively). Triglyceride concentrations did not vary among the mature rabbits of either strain but young NZW and SMHL rabbits showed a difference (13.0 ± 0.97 vs. 9.7 ± 0.96 mg/g wet weight of liver NZW vs. SMHL, $P = 0.05$). Phospholipid concentrations were significantly higher in mature SMHL rabbits than in mature NZW rabbits (17.3 ± 0.5 vs. 15.4 ± 0.6 mg/g wet weight liver, respectively, $P = 0.046$). When young and mature rabbits were grouped together, the only difference was a significant increase in triglyceride concentration in the NZW rabbits (13.4 ± 0.78 vs. 10.6 ± 1.2 mg/g wet weight of liver NZW vs. SMHL, $P < 0.05$).

DISCUSSION

Appearance of the hyperlipidemic phenotype of SMHL rabbits is dependent on supplementing their diet with a small amount of cholesterol. NZW animals fed the same diet showed no change in plasma lipid levels. However, even on the chow diet, plasma $VLDL_2$ and IDL lipoprotein concentrations were elevated in SMHL rabbits, indicating the presence of a lipoprotein disturbance. In the large series of animals studied from the SMHL rabbit colony, the plasma lipid pattern was variable with increases in both cholesterol and triglyceride being the most common phenotype, but some animals exhibited raised cholesterol only or raised triglyceride only. This phenotypic variability is consistent with the original description of plasma lipid patterns in familial combined hyperlipidemia (1). More detailed examination showed that all four apoB-containing lipoprotein species were elevated in SMHL rabbits fed the cholesterol-supplemented diet. Therefore these animals, derived from the original St. Thomas Hospital stock, express a consistent mixed hyperlipidemia compatible with their potential role as an animal model for FCH. The colony is inbred and as yet no attempt has been made to investigate the genetics of the disorder. In examining the animals over a period of time, it was clear that the hyperlipidemia in male SMHL rabbits decreased with age (Fig. 1) and we have recently reported (24) that plasma cholesterol and triglyceride levels in male SMHL animals decline steadily from 8 weeks of age reaching a plateau at 14–16 weeks. We therefore made the decision to study groups of both young and mature rabbits in the perfusion experiments.

Rabbits used in the perfusion studies had plasma lipid levels close to the mean values of the groups from which they were selected. When all rabbits studied (young and mature) were grouped together, triglyceride, cholesterol, and apoB accumulated in the recirculating medium in an approximately linear manner over the 3-h perfusion period. Output rates for triglyceride were similar to those previously published for perfused rabbit livers (25). Comparing hepatic triglyceride output in the four apoB-containing lipoproteins in SMHL and NZW animals revealed no difference in $VLDL_1$ despite the fact that this was the most abundant triglyceride-carrying particle released from the liver. $VLDL_2$ and IDL triglyceride output rates were significantly increased in SMHL rabbits when both young and mature rabbits were combined, but there was no differ-

ence in LDL triglyceride output. Cholesterol output, however, was substantially higher in $VLDL_1$, $VLDL_2$ and IDL fractions from SMHL versus NZW livers (Table 2, Fig. 3). The 5-fold higher output of cholesterol in $VLDL_2$ + IDL + LDL from SMHL livers was accompanied by a 9-fold increase in apoB released in these denser lipoprotein particles. Given that 55% of apoB released from the livers of SMHL animals appeared in the non-$VLDL_1$ density range and the finding that only a trivial amount of denser lipoproteins is generated by delipidation of $VLDL_1$ during the perfusion (lipase is removed from the liver during the heparin flush at time of surgical preparation of the organ and little activity reappeared during perfusion), we surmised that these rabbits overproduced these denser lipoproteins. Thus the elevation in plasma levels of $VLDL_1$, $VLDL_2$, IDL, and LDL seen in Table 1 is likely to be due to increased hepatic production of these species rather than decreased catabolism. In the original metabolic studies by La Ville et al. (4) on St. Thomas Hospital rabbits, no difference was seen in receptor-mediated catabolism of LDL, and in kinetic experiments with VLDL and LDL tracers in intact animals, an increase in production was suggested as the basis of the hyperlipidemia. A lower fractional catabolic rate was seen for LDL but this was attributed to saturation of receptors by the expanded LDL pool (4). The present perfusion experiments provide direct evidence for apoB overproduction as the underlying cause of the hyperlipidemia in this animal mode. The correlation of apoB output with plasma cholesterol and triglyceride levels provides strong support that apoB output is a major determinant of circulating cholesterol and triglyceride levels (Fig. 5). Despite the differences in lipoprotein production between SMHL and NZW rabbits there was no gross difference in the liver content of cholesterol or triglyceride and it may be that lipid availability was not the basis of the variation in lipoprotein production rates, although critical lipid pools more closely associated with lipoprotein assembly were not measured.

These studies demonstrate again that apoB-containing lipoproteins can be generated by the liver across a wide spectrum of composition and size. Previous in vivo tracer studies from this (26, 27) and other laboratories (28) showed that not all LDL derives from VLDL delipidation. Rather it is necessary to postulate that small VLDL, IDL, and LDL can also be produced directly by the liver to explain satisfactorily the findings of kinetic studies. In earlier rabbit perfusion studies (25) in control and Watanabe Heritable Hyperlipidemic rabbits, virtually all apoB was released as VLDL, leading to the suggestion that 'direct' LDL synthesis was really due to very rapid lipolysis. In young NZW animals we confirm these findings, almost all output of triglyceride and apoB is in the form of VLDL, particularly $VLDL_1$ (Table 2). However, we observed substantial apoB output in mature NZW and SMHL rabbits in the $VLDL_2$ and IDL density intervals. The lack of evidence of extensive lipolysis in the perfusion system and the demonstration that newly synthesized apoB does appear in the non-$VLDL_1$ fraction (i.e., denser lipoproteins did not derive from wash-out of the space of Disse as has been suggested previously (25)) leads us to conclude that direct production of these species does occur.

SMHL rabbits, therefore, have a combined hyperlipidemic phenotype compatible with their use as a potential animal model of FCH. The demonstration that apoB overproduction is the underlying metabolic defect increases the analogy with the human situation. Our finding that the lipoproteins released in this inherited hyperlipidemia can range in size across the entire apoB-containing lipoprotein spectrum highlights a possible mechanism to explain the inter- and intra-individual variability of the lipid phenotype in FCH. An underlying overproduction of apoB-containing lipoproteins could reveal itself as raised VLDL or raised LDL levels.

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