Age-Associated Decrease in Plasma Cholesterol and Changes in Cholesterol Metabolism in Homozygous Watanabe Heritable Hyperlipidemic Rabbits

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We examined the cholesterol metabolism of homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal model deficient in low-density lipoprotein (LDL) receptors, to clarify the mechanism of the age-associated decrease of plasma total cholesterol, one of the properties of WHHL rabbits. The rabbits were examined at several ages: after weaning at 3 months, at sexual maturation at 6 months, at 12 months, and at 24 months, equivalent to about 35 years of age in humans. Plasma total cholesterol, triglyceride, and phospholipid levels decreased with aging by about 45%. These reductions were mainly dependent on a decrease in the LDL fraction. In the liver microsomal fraction, although there were no age-related changes in the cholesterol concentration and cholesterol 7α-hydroxylase (C7H) activity, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity increased and acyl-coenzyme A:cholesterol acyltransferase (ACAT) activity decreased with aging. The lipolytic activity varied with aging. The secretion rate of very–low-density lipoprotein (VLDL) cholesterol as determined by injection of Triton WR-1339 decreased significantly with aging, while the catabolic rate of VLDL cholesterol was about 2-fold higher in the oldest group versus the young groups. From these results, we conclude that the age-associated decrease in plasma cholesterol in WHHL rabbits is related not only to a decrease in the secretion rate of VLDL cholesterol but also to an increase in the catabolic rate.

ALTHOUGH epidemiological studies show that plasma lipid concentrations are well known to increase with age in Western societies, age-associated changes in plasma lipids vary by animal species and experimental conditions.[1-5] Plasma cholesterol is considered to be regulated by endogenous cholesterol synthesis in the liver, absorption of dietary cholesterol from the intestine, and clearance of plasma lipoproteins. In hepatic cholesterol metabolism, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase plays an important role in cholesterol synthesis, acyl-coenzyme A:cholesterol acyltransferase (ACAT) esterifies free cholesterol to cholesterol ester, and cholesterol 7α-hydroxylase (C7H) converts cholesterol to bile acids. Regarding lipoprotein metabolism, lipolytic activity in the plasma and receptors for lipoproteins regulate the plasma cholesterol level.

In homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal model of low-density lipoprotein (LDL) receptor deficiency which shows spontaneous hypercholesterolemia[6-9] and atherosclerosis,[10,11] plasma cholesterol gradually decreases with aging after maturation, by an unknown mechanism. Such age-associated decreases in plasma lipids affect the evaluation of hypolipidemic effects or antiatherosclerotic effects of medications.[12-15] We have attempted to examine the mechanism of the age-associated decrease in plasma cholesterol, one of the unknown properties of WHHL rabbits. In the present study, we determined the activities of the liver microsomal enzymes that mediate hepatic cholesterol metabolism. We also examined very–low-density lipoprotein (VLDL) metabolism.

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MATERIALS AND METHODS

Animals

The WHHL rabbits in this study were bred at Kobe University School of Medicine.[11] We used 32 rabbits, with 8 animals each in four age groups at 3, 6, 12, and 24 months. In rabbits, 6 months is the age of sexual maturation and 24 months of age is equivalent to about 35 years of age in humans as calculated from the average life spans.[16] Four rabbits in each age group were used for preparation of the liver microsomal fractions. The other 4 rabbits in each group were used to assess the lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) activity, plasma volume, and VLDL secretion rate. Each group consisted of an equal number of females and males. We used both genders in this study, not only because plasma lipid levels were similar in male and female rabbits in our WHHL colony[11] but also because female rabbits do not show natural ovulation and an ovulation cycle. In rabbits, ova are released from the ovaries when stimulated by coupling or similar stimulation.

To equalize the rabbits' nutrition and ensure that the rabbits did not become obese, they were housed individually in metal cages and fed 120 g/d of standard chow for experimental rabbits (CR-3; Clea Japan, Tokyo, Japan). The cholesterol content was less than 4 mg/100 g chow. This limited feeding did not appear to affect the rabbits' growth. The body weight was 2.01 ± 0.36 kg (mean ± SD) at age 3 months, 3.11 ± 0.23 kg at 6 months, 3.30 ± 0.25 kg at 12 months, and 3.32 ± 0.24 kg at 24 months. All animal experiments were performed according to the guidelines for the care and use of experimental animals at Kobe University School of Medicine.

Measurement of Microsomal Enzyme Activity and Cholesterol Concentration

The rabbits were anesthetized with an intravenous injection of sodium pentobarbital (25 mg/kg) and perfused with cold saline solution (4°C). Liver microsomal fractions were prepared according to the method of Kovanen et al.[17] HMG-CoA reductase activity was determined by the method of Kuroda and Endo,[18] in which [^{14}C]mevalonolactone converted from [^{14}C]HMG-CoA was separated by thin-layer chromatography, and then the radioactivity was counted. ACAT activity was determined by the method of Lichtenstein and Brecher,[19] in which [^{14}C]cholesteryl oleate synthesized from [^{14}C]oleoyl-CoA and microsomal cholesterol was separated using thin-layer chromatography and then the radioactivity was counted. C7H activity was determined by high-performance liquid chromatography (HPLC)[20] using a 2-part method: first, 7α-hydroxylation of microsomal cholesterol was per-

Table 1. Lipoprotein Lipid Concentrations (mg/dL) in WHHL Rabbits at Each Age

Variable	Whole Plasma	VLDL	IDL	LDL	HDL
Total cholesterol					
3 mo	916 ± 177	98 ± 48	132 ± 61	680 ± 115	6 ± 2
6 mo	701 ± 97†	73 ± 35	86 ± 52*	536 ± 55†	7 ± 1
12 mo	596 ± 89†	71 ± 34	94 ± 23	425 ± 112†	6 ± 1
24 mo	508 ± 55†	49 ± 19†	57 ± 28†	393 ± 46†	8 ± 4†
Triglyceride					
3 mo	332 ± 123	56 ± 37	54 ± 39	218 ± 58	4 ± 6
6 mo	253 ± 75	42 ± 24	35 ± 25	170 ± 38*	8 ± 7
12 mo	247 ± 115	73 ± 66	46 ± 27	143 ± 34†	3 ± 3
24 mo	189 ± 73†	30 ± 14	25 ± 22*	125 ± 45†	9 ± 8
Phospholipid					
3 mo	565 ± 106	51 ± 26	77 ± 40	405 ± 60	33 ± 5
6 mo	432 ± 54†	36 ± 18	48 ± 29*	317 ± 29†	32 ± 5
12 mo	364 ± 49†	38 ± 22	54 ± 19	244 ± 48†	29 ± 5
24 mo	312 ± 41†	26 ± 9*	32 ± 20†	226 ± 28†	28 ± 7

NOTE. Data are the mean ± SD.
*$P < .05$, †$P < .01$ *v* 3 mo by William's multiple-comparison test.

formed, resulting in the conversion of 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-on. The final product was determined with a HPLC apparatus equipped with a Finepak SIL column (JASCO, Tokyo, Japan). Microsomal cholesterol was extracted with 5 vol isopropyl alcohol per 1 vol microsomal fraction, and cholesterol accumulated in the liver was extracted according to the method of Folch et al.[21] The concentration of extracted cholesterol was measured enzymatically.[12]

Measurement of Plasma LPL and HTGL Activity

Plasma LPL and HTGL activity was measured in heparinized plasma according to the method of Muir,[22] with slight modification. The plasma was incubated with a mixture containing triolein as a substrate, and the free fatty acid (FFA) level in the mixture was measured. HTGL was determined as the lipolytic activity in the plasma after inhibition of LPL by 1.0 mol/L NaCl.[23] LPL activity was calculated by subtracting HTGL activity from the total lipolytic activity of the plasma.

Measurement of VLDL Secretion Rate

The VLDL secretion rate was determined by a method using Triton WR-1339[24] after overnight fasting to eliminate the influence of chylomicrons. Triton WR-1339 blocks the degradation of VLDL,[25,26] and the VLDL secretion rate determined by a Triton injection is well reflective of the secretion rate of VLDL in vivo.[27,28] Triton WR-1339 at 200 mg/mL in 0.15 mol/L NaCl solution was injected into an ear vein at a dose of 400 mg/kg body weight. Blood samples were obtained from a marginal ear vein before and 6 hours after Triton injection, and the VLDL was fractionated by ultracentrifugation. The increase in the secretion rate of VLDL was calculated by dividing the difference between VLDLs obtained before and 6 hours after Triton injection by 6 hours. The VLDL secretion rate was calculated by multiplying the increase of VLDLs by the plasma volume. Plasma volume was determined by a dye dilution method using Evans blue[29] at 3 weeks before Triton injection. The VLDL catabolic rate was calculated by dividing the VLDL secretion rate by VLDL total mass.[30,31] VLDL total mass was calculated by multiplying the plasma volume by the VLDL concentration determined before Triton injection.

Fractionation of Plasma Lipoprotein and Measurement of Lipid Concentrations

Blood samples were obtained for plasma lipid measurements after overnight fasting. Total cholesterol, triglyceride, and phospholipid concentrations were measured enzymatically.[12] Lipoproteins were fractionated by ultracentrifugation to yield the following fractions: VLDL (density < 1.006 g/mL), intermediate-density lipoprotein ([IDL] 1.006 ≤ density < 1.019 g/mL), LDL (1.019 ≤ density < 1.063 g/mL), and high-density lipoprotein ([HDL] density ≥ 1.063 g/mL).

Materials

RS-3-hydroxy-3-methyl [3-^{14}C]glutaryl-coenzyme A (58.6 Ci/mol), [^{14}C]oleoyl-coenzyme A, and [^{3}H]cholesteryl oleate were obtained from New England Nuclear (Boston, MA). Triton WR-1339 was purchased from Nakalai Tesque (Kyoto, Japan).

Statistical Analysis

The data are presented as the mean ± SD. Statistical analysis was performed using William's multiple-comparison test. Williams[32] reported this statistical test to be suitable for populations that consist of dose-dependent groups. A *P* level less than .05 was considered statistically significant.

RESULTS

Plasma Lipid Concentrations

Table 1 shows lipoprotein lipid concentrations for the WHHL rabbits according to age group. Total plasma cholesterol showed an age-dependent decrease of 45% (from 916 ± 177 mg/dL at age 3 months to 508 ± 55 mg/dL at 24 months, respectively, $P < 0.01$). Plasma triglyceride and phospholipid also decreased significantly with age. The decline in plasma lipid concentrations mainly reflected a decrease in LDL (from 680 ± 115 mg/dL at 3 months to 393 ± 46 mg/dL at 24 months in cholesterol, $P < .01$). Although VLDL and IDL lipid levels also tended to decrease with age, HDL lipid levels did not show an age-dependent change.

Microsomal Enzyme Activity

Table 2 summarizes the microsomal enzyme activity and cholesterol content according to age. HMG-CoA reductase activity was similar between ages 3 and 6 months, and then age-dependently increased from 2.87 ± 0.99 pmol/min/mg protein at age 6 months to 7.11 ± 0.42 pmol/min/mg protein at

Table 2. Microsomal Enzyme Activity in WHHL Rabbits at Each Age

Age Group	Microsomal Enzyme Activity (pmol/min/mg protein)			Hepatic Cholesterol Content	
	HMG-CoA Reductase	ACAT	C7H	Microsome (μg/mg protein)	Whole Liver (mg/g wet tissue)
3 mo	2.93 ± 0.90	66.6 ± 20.8	5.91 ± 1.45	12.3 ± 2.5	2.51 ± 0.32
6 mo	2.87 ± 0.99	27.7 ± 16.3†	4.61 ± 0.39*	10.6 ± 2.4	2.60 ± 0.27
12 mo	5.30 ± 2.71*	28.1 ± 11.0*	4.95 ± 0.96	9.7 ± 0.7	2.49 ± 0.17
24 mo	7.11 ± 0.42†	30.1 ± 11.7*	5.00 ± 0.92	10.6 ± 1.9	2.32 ± 0.12

NOTE. Data are the mean ± SD.
*$P < .05$, †$P < .01$ *v* 3 mo by William's multiple-comparison test.

Table 3. LPL and HTGL Activity in Postheparin Plasma of WHHL Rabbits at Each Age

Age Group	Lipolytic Activity (μmol FFA/min/mL)	
	LPL	HTGL
3 mo	0.191 ± 0.038	0.045 ± 0.015
6 mo	0.165 ± 0.019	0.060 ± 0.022
12 mo	0.237 ± 0.102	0.072 ± 0.010*
24 mo	0.190 ± 0.087	0.064 ± 0.013

NOTE. Data are the mean ± SD.
*$P < .05$ *v* 3 mo by William's multiple-comparison test.

24 months ($P < .01$). In contrast, ACAT activity decreased significantly from 66.6 ± 20.8 pmol/min/mg protein at 3 months to 27.7 ± 16.3 pmol/min/mg protein at 6 months ($P < .01$), and then remained essentially constant until 24 months. C7H activity, microsomal cholesterol content, and overall hepatic cholesterol content did not show any age-dependent change.

Plasma LPL and HTGL Activity

LPL activity in heparinized plasma was similar among the age groups. HTGL activity tended to increase with age from 0.045 ± 0.015 μmol FFA/min/mL at 3 months to 0.064 ± 0.013 μmol FFA/min/mL at 24 months (Table 3).

VLDL Secretion Rate

Table 4 shows the VLDL secretion rate by age group. When total cholesterol or phospholipid were used as an index, the VLDL secretion rate decreased significantly with age over the 21-month observation period, from 36.4 ± 8.4 to 17.9 ± 3.0 mg/h ($P < .01$, total cholesterol) and from 39.1 ± 9.6 to 21.2 ± 2.7 mg/h ($P < .01$, phospholipid), respectively. In addition, the secretion rate of VLDL triglyceride tended to decrease.

Table 5 shows the lipid composition of VLDL obtained 6 hours after the Triton injection. Since Triton WR-1339 inhibits VLDL degradation, VLDL obtained after a Triton injection more closely resembles nascent VLDL rather than plasma VLDL.[33] The total cholesterol content of the VLDL fraction was significantly reduced with aging, from 20.0% ± 4.1% at age 3 months to 14.2% ± 3.6% at 24 months ($P < .01$). The phospholipid content also decreased significantly with aging (17.2% ± 1.8% *v* 14.7% ± 2.4%, $P < .05$). In contrast, the triglyceride content significantly increased with aging from 62.8% ± 5.8% to 71.1% ± 5.9% ($P < .01$).

Table 4. VLDL Secretion Rate in WHHL Rabbits at Each Age

Age Group	VLDL Secretion Rate (mg/h)		
	Total Cholesterol	Triglyceride	Phospholipid
3 mo	36.4 ± 8.4	178 ± 66	39.1 ± 9.6
6 mo	27.4 ± 5.3*	191 ± 46	35.0 ± 7.2
12 mo	24.0 ± 4.5†	175 ± 43	30.6 ± 9.1
24 mo	17.9 ± 3.0†	120 ± 38	21.2 ± 2.7†

NOTE. Data are the mean ± SD.
*$P < .05$, †$P < .01$ *v* 3 mo by William's multiple-comparison test.

Table 5. Lipid Composition of Newly Secreted VLDL in WHHL Rabbits at Each Age

Age Group	VLDL Lipid Composition (%)		
	Total Cholesterol	Triglyceride	Phospholipid
3 mo	20.0 ± 4.1	62.8 ± 5.8	17.2 ± 1.8
6 mo	15.0 ± 1.3*	69.8 ± 1.7*	15.2 ± 0.6
12 mo	14.7 ± 1.2*	70.7 ± 1.4†	14.6 ± 0.8*
24 mo	14.2 ± 3.6†	71.1 ± 5.9†	14.7 ± 2.4*

NOTE. Data are the mean ± SD.
*$P < .05$, †$P < .01$ *v* 3 mo by William's multiple-comparison test.

VLDL Catabolic Rate

Table 6 shows the VLDL catabolic rate for each age group. The catabolic rate of VLDL cholesterol increased significantly for 24-month-old rabbits (0.564 ± 0.193 per hour, $P < .05$) compared with 3-month-old rabbits (0.313 ± 0.159 per hour). In addition, the catabolic rate of VLDL triglyceride and VLDL phospholipid also increased about 2-fold, although these differences were not significant.

DISCUSSION

In the present study, we examined the age-associated decrease in plasma cholesterol after maturation in homozygous WHHL rabbits, one of the unknown properties of this rabbit model. The decrease was remarkable after sexual maturation (age 6 months). Significant age-associated changes were observed, including an increase of hepatic microsomal HMG-CoA reductase activity and VLDL catabolism and a decrease of hepatic microsomal ACAT activity, VLDL cholesterol secretion, and the cholesterol content of newly secreted VLDL.

The plasma cholesterol level is regulated by cholesterol absorption from the intestine,[1,3,4,34,35] bile acid synthesis,[35,36] LDL clearance from the plasma,[37-39] VLDL secretion from the liver,[5] and direct clearance of VLDL by several receptors. Since the chow for WHHL rabbits contains less than 4 mg cholesterol per 100 g chow and WHHL rabbits have almost no LDL receptor function,[6-9] cholesterol absorption and LDL clearance supposedly do not contribute to the age-associated plasma cholesterol decrease in WHHL rabbits. Therefore, we consider that a decrease in VLDL secretion and an increase in direct VLDL clearance play an important role in the age-associated plasma cholesterol decrease in WHHL rabbits.

In hepatic cholesterol metabolism, the cholesterol content of microsome fractions and cholesterol accumulation in the liver were not changed from 3 to 24 months of age. The cholesterol pool in the liver is considered to be affected by both cholesterol supply, which consists of cholesterol uptake from the plasma

Table 6. VLDL Catabolic Rate in WHHL Rabbits at Each Age

Age Group	VLDL Catabolic Rate (per hour)		
	Total Cholesterol	Triglyceride	Phospholipid
3 mo	0.31 ± 0.16	2.71 ± 1.90	0.63 ± 0.31
6 mo	0.31 ± 0.07	3.29 ± 0.74	0.71 ± 0.17
12 mo	0.30 ± 0.06	3.61 ± 1.70	0.67 ± 0.17
24 mo	0.56 ± 0.19*	5.79 ± 3.02	1.18 ± 0.52

NOTE. Data are the mean ± SD.
*$P < .05$ *v* 3 mo by William's multiple-comparison test.

and cholesterol biosynthesis, and cholesterol consumption, which consists of bile acid synthesis and VLDL cholesterol secretion. Since LDL receptors have almost no function in WHHL rabbits,[6-9] the cholesterol supply in the liver supposedly consists of cholesterol synthesis mediated by HMG-CoA reductase and LDL receptor–independent LDL uptake from the plasma. Consequently, we consider that LDL uptake through the LDL receptor–independent pathway appears to decrease with aging in WHHL rabbits because HMG-CoA reductase activity increased, VLDL cholesterol secretion decreased, and C7H activity did not change with aging.

Regarding VLDL catabolism, the lipolytic activity did not show age-associated changes, while the catabolic rate of VLDL increased. Although we do not have any evidence to explain these results, we consider that VLDL may be directly degraded through several lipoprotein receptors related to atherosclerosis or accumulation of visceral fat. It is reported that these receptors are expressed in the foam cells or adipocytes. Kita et al[40] and Ishii et al[41] reported that cholesterol-rich WHHL-VLDL is recognized by the receptor for β-VLDL in macrophages and stimulated cholesterol ester synthesis. Recently, Hiltunen et al[42] reported that many foam cells in atherosclerotic lesions express scavenger receptors, VLDL receptors, and LDL receptor–related protein. These receptors can bind VLDL.[43-47]

In contrast to WHHL rabbits, plasma cholesterol levels increase with age in humans in Western societies, but individuals in non-Western societies do not show an age-associated increase in plasma cholesterol.[48,49] Kasim[50] reports that an increase in the LDL concentration with age in humans in Western societies reflects a decrease in LDL receptors due to an increase in the hepatic cholesterol pool. The increase in hepatic cholesterol results from an increase in cholesterol absorption from the intestine and a decrease in the biliary excretion of cholesterol.[50] The differences in cholesterol metabolism with aging between Western humans and WHHL rabbits may result from an almost absent LDL receptor function, a low-cholesterol diet, and an absence of age-dependent changes in bile acid synthesis in WHHL rabbits.

We conclude that a decrease in the cholesterol content of newly secreted VLDL and an increase in VLDL catabolism play an important role in the age-dependent plasma cholesterol decrease in WHHL rabbits.

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