Hypertriglyceridemia in Watanabe Heritable Hyperlipidemic Rabbits Was Associated with Increased Production and Reduced Catabolism of Very-Low-Density Lipoproteins

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Key Words

Hypertriglyceridemia · Watanabe heritable hyperlipidemic rabbits · Lipoprotein lipase · Hypercholesterolemia

Abstract

Watanabe heritable hyperlipidemic (WHHL) rabbit is an animal model for human familial hypercholesterolemia. Recently, we segregated a new mutant of WHHL rabbits with plasma levels of triglycerides (TG) >500 mg/dl (designated as TGH-WHHL). To investigate the underlying mechanisms for hypertriglyceridemia, we compared TGH-WHHL with WHHL rabbits with lower plasma TG levels (<250 mg/dl, designated as TGL-WHHL). A Triton WR-1339 injection experiment revealed that TGH-WHHL rabbits had increased secretion and decreased clearance of TG-rich lipoproteins. Furthermore, TGH-WHHL rabbits had lower a post-heparin activity of lipoprotein lipase and a higher cholesterol ester transfer protein activity than TGL-WHHL rabbits. Cultured hepatocytes isolated from TGH-WHHL rabbits showed a higher secretion rate of TG and cholesterol than those of TGL-WHHL rabbits. In addition, TGH-WHHL rabbits exhibited marked insulin resistance. These data suggest that hypertriglyceridemia exhibited by WHHL rabbits is caused by both increased production and impaired catabolism of TG-rich lipoproteins and associated with insulin resistance.

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Introduction

There are growing lines of evidence showing that hypertriglyceridemia is a potential risk factor for coronary artery disease [1–4]. It has been demonstrated that a therapeutic lowering of plasma triglycerides (TG) reduces the occurrence of coronary artery disease [5]. Watanabe heritable hyperlipidemic (WHHL) rabbits were established 30 years ago by Dr. Watanabe at Kobe University, Japan, and have been used as an excellent animal model for human familial hypercholesterolemia [6]. WHHL rabbits show spontaneous hypercholesterolemia and atherosclerosis due to a genetic defect of low-density lipoprotein (LDL) receptors [6–8]. In our facility, we bred WHHL rabbits obtained from Kobe University and observed that there was a large range of plasma levels of TG (ranging from 150 to 1,050 mg/dl) among WHHL descendants. After selected inbreeding, we segregated 2 WHHL colonies; one with plasma levels of TG >500 mg/dl (designated as TGH-WHHL) and another with relatively lower plasma levels of TG (<250 mg/dl), designated as TGL-WHHL rabbits [9, 10]. These 2 kinds of WHHL rabbits with different plasma levels of TG allowed us to investigate the possible mechanisms of hypertriglyceridemia and its relationship with glucose metabolism and insulin sensitivity. We found that severe hypertriglyceridemia in

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TGH-WHHL rabbits was essentially caused by both increased production and delayed catabolism of TG-rich lipoproteins. In addition, hypertriglyceridemia was associated with impaired glucose metabolism and insulin resistance in TGH-WHHL rabbits. TGH-WHHL rabbits may become a suitable model for the study of combined hyperlipidemia and metabolic syndrome.

Materials and Methods

In this study, we bred 2 colonies of WHHL rabbits based on the plasma levels of TG, as described previously: TGH-WHHL and TGL-WHHL rabbits [11]. Rabbits used for this study were male and 4, 7 and 12 months old. Age- and sex-matched Japanese white (JW) rabbits from Kumagai (Saitama, Japan) were used as a normal control. The 3 groups of rabbits were maintained individually in a controlled environment with free access to water and a chow diet (Labo-R-Grower, Nihon Nosan Kogyo, Ltd., Tokyo, Japan). All animal experiments were performed with the approval of the Animal Care Committee of the University of Yamagata and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Analysis of Plasma Lipids and Enzyme Activity

Blood was collected from a marginal ear vein after overnight fasting. Blood samples were centrifuged at 2,000 rpm for 10 min, at 4°C. The plasma TG and total cholesterol (TC) levels were measured by Abbott Vision kits (Dinabot, Ltd., Tokyo, Japan) [12].

Post-heparin enzymatic activity of lipoprotein lipase (LPL) and hepatic lipase (HL) was measured using an enzymatic determination of the released free fatty acids [13]. Substrates were prepared for LPL in pH 8.2 and for HL in pH 8.8. Selective assays of LPL and HL were feasible by inactivating HL with sodium dodecyl sulfate and by inactivating LPL with 1 M NaCl, respectively. The concentrations of free fatty acids were measured by an enzymatic kit (NEFA kit-K; Nippon Shoji Kaisha, Ltd., Tokyo, Japan). Plasma cholesterol ester transfer protein (CETP) activity was measured as reported previously [14].

TG Secretion and Catabolic Rates in vivo

Rabbits, 12 month old, were used after overnight fasting. The secretion rate of TG into systemic circulation was measured according to the method reported by others [15]. Briefly, Triton WR-1339 solution (200 mg/ml in 0.85% NaCl; Sigma Chemical, St. Louis, Mo., USA) was injected into a marginal ear vein at a dose of 400 mg/kg of body weight, and blood samples were collected at 30 and 45 min from the opposite ear vein. The rate of a linear increase in TG was adjusted to plasma volume as a measure of the total TG secretion rate (TGSR) per rabbit. The plasma volume was estimated from the linear relationship between body weight and plasma volume [16]. TGSR was calculated by the formula: $TGSR = (TG_1 - TG_0)/T_1/2 \times PV + (TG_2 - TG_0)/T_2/2 \times PV$, where TG_0 is the basal plasma TG concentration (mg/dl), TG_1 the first plasma TG concentration after Triton WR-1339 injection, TG_2 the second plasma TG concentration after Triton WR-1339 injection, T_1 the time of first sampling in minutes after Triton WR-1339 injection, T_2 the time of second sampling in minutes after Triton

WR-1339 injection, and PV the estimated plasma volume based on the linear relationship between body weight and plasma volume using the following formula: $PV = \text{body weight (g)} \times 0.07$. The fractional catabolic rate (FCR) for plasma TG was calculated as being TGSR divided by plasma TG pool size (PTG) which is calculated from $PTG = TG_0 \times PV$.

TG Secretion from Cultured Hepatocytes

Rabbits, 4 months old, were sacrificed by overdose injection of sodium pentobarbital, and hepatocytes were isolated by in situ perfusion of collagenase solution as described by Seglen [17]. The isolated cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and plated at $6 \times 10^6/100$ mm fibronectin-coated dishes. After incubating for 24 h, cells were washed with phosphate-buffered saline to remove cellular debris and serum. The hepatocytes were incubated with serum-free medium. The lipoprotein concentrations in conditioned media were analyzed by a high-performance liquid chromatography [18]. Cellular lipids were extracted with hexane/isopropyl alcohol (3:2). After evaporation of the organic solvent, the lipids were resuspended in ethanol, and cellular TG was measured with an enzymatic kit (Ono Pharmacy and Wako Chemical, Tokyo, Japan).

Measurements of Insulin Sensitivity and Glucose Utilization

An intravenous glucose tolerance test (IVGTT) was performed in rabbits after they were fasted overnight. The glucose solution (0.6 g glucose/kg body weight) was injected into a marginal vein, and a 2-ml blood sample was drawn at 5, 10, 15, 20, 30, 45, 60, 90 and 120 min, respectively. Plasma glucose (PG) levels were determined by the glucose oxidase procedure using a Vision analyzer (Dinabot, Ltd., Japan), and insulin levels were measured by enzyme immunoassay (Sankyo, Tokyo, Japan). ΣIRI (immunoreactive insulin) and ΣPG were also determined based on the data of IVGTT.

Histological Analysis of the Pancreas

Three rabbits from each group were sacrificed for histopathological examinations. The whole pancreas was removed from the duodenal loop and divided into 3 pieces (proximal, middle and distal) and they were fixed in 10% buffered formalin. The sections were cut at a thickness of 4 μm and stained with hematoxylin and eosin. For immunohistochemical staining, the sections were rinsed in distilled water for 5 min at room temperature and were treated with 0.3% H_2O_2 in methanol for 30 min to suppress endogenous peroxidase activity. The sections were washed in Tris buffer solution for 20 min and incubated with monoclonal antibody against insulin (Abcam Inc., Cambridge, Mass., USA) for 30 min. After washing for 10 min, the sections were incubated with 1:1,000 diluted biotinylated secondary antibody for 30 min followed by another washing. Then, the sections were incubated with Vectastain ABC Reagent for 30 min. After washing, the sections were incubated for 2 min in peroxidase substrate solution and then counterstained with hematoxylin. The islets $>150 \mu\text{m}$ in diameter were calculated under a light microscope using an image analyzer (Olympus VIP-21C, Tokyo, Japan).

Statistical Analysis

Comparisons were mainly made between TGH-WHHL and TGL-WHHL rabbits or between TGL-WHHL and JW rabbits us-

Table 1. Body weight and plasma lipids

	4 months (n = 4)	7 months (n = 6)	12 months (n = 4)
Body weight, kg			
TGH	2.3 ± 0.1	2.7 ± 0.2	3.0 ± 0.2
TGL	ND	2.8 ± 0.3	3.1 ± 0.1
JW	2.5 ± 0.2	3.0 ± 0.2	3.4 ± 0.1
Plasma TG, mg/dl			
TGH	1,176 ± 175 ^b	1,497 ± 351 ^{a, b}	756 ± 34 ^{a, b}
TGL	ND	244 ± 28 ^c	197 ± 51 ^c
JW	76 ± 8	88 ± 13	48 ± 5
Plasma TC, mg/dl			
TGH	1,291 ± 68 ^b	1,287 ± 123 ^{a, b}	923 ± 66 ^{a, b}
TGL	ND	919 ± 36	728 ± 35
JW	57 ± 8	76 ± 10	78 ± 12

Data are expressed as the mean ± SEM. ND = Not determined. p values for multiple comparison are as follows: ^a p < 0.01, TGH vs. TGL; ^b p < 0.01, TGH vs. JW; ^c p < 0.01 TGL vs. JW.

ing the unpaired Student's t test. Data are expressed as the mean ± SEM. Correlation coefficients among continuous variables were evaluated by a simple linear regression analysis. A p value <0.05 was considered statistically significant.

Results

Basal Concentrations of Plasma Lipids and Body Weight

As shown in table 1, 2 WHHL rabbits exhibited marked hyperlipidemia (12- to 16-fold increase in TC and 3- to 17-fold increase in TG at 6 months) compared with normal JW rabbits. When a comparison was made between TGH-WHHL and TGL-WHHL rabbits, plasma TG levels of TGH-WHHL rabbits were 6-fold (6 months) and 3.8-fold (12 months) higher than those of TGL-WHHL rabbits. In addition, plasma TC levels were also higher in TGH-WHHL than in TGL-WHHL rabbits. There was no difference in body weight between WHHL and normal JW rabbits.

TG Secretion and Catabolic Rates in vivo

To explore the possible mechanism for higher TG levels found in TGH-WHHL rabbits, the Triton WR-1339 experiment was performed. The TGSR was calculated from the linear fitting of a TG time-concentration relationship. The TGSR of TGH-WHHL rabbits was significantly higher than that of TGL-WHHL and JW groups

Fig. 1. Secretion of TG from rabbits after Triton WR-1339 injection. Basal TG concentrations (upper panel) and TGSR (middle panel) and FCR (lower panel) were compared. Data are expressed as the means ± SEM. n = 4 for TGH-WHHL, n = 5 for TGL-WHHL, and n = 3 for JW rabbits. * p < 0.01.

(fig. 1). However, the TGSR of TGL-WHHL rabbits was similar to that of JW rabbits even though plasma TG levels of TGL-WHHL rabbits were higher than those of JW rabbits, suggesting that different mechanisms may be operative in 2 WHHL rabbits. The FCR was markedly decreased in both TGH-WHHL and TGL-WHHL rabbits compared with JW rabbits.

TG Secretions in Cultured Hepatocytes

TG and TC contents in the conditioned media produced by cultured hepatocytes were increased in a time-dependent manner in all groups (fig. 2). However, hepatocytes of TGH-WHHL rabbits produced a significantly higher amount of both TG and TC than those in TGL-WHHL rabbits. Hepatic production of TG and TC in TGL-WHHL rabbits showed an increased tendency compared with JW rabbits, but this was not statistically significant.

Activity of LPL, HTGL and CETP

Post-heparin plasma LPL activity of TGH-WHHL rabbits was significantly lower than that of TGL-WHHL

Fig. 2. TG and cholesterol contents in the conditioned media by cultured hepatocytes were measured in triplicate. Values are the means $\pm$ SEM. * $p < 0.01$, TGH-WHHL vs. TGL-WHHL. $n = 3$ for each group.

Table 2. Fasting plasma glucose and insulin and sum of IVGTT

	TGH-WHHL (n = 5)	TGL-WHHL (n = 7)	JW (n = 7)
Fasting PG, mg/dl	170 $\pm$ 12 ^{a, b}	175 $\pm$ 8 ^c	139 $\pm$ 6
Fasting IRI, mIU/ml	11.1 $\pm$ 2.3 ^{a, b}	5.8 $\pm$ 0.7	4.7 $\pm$ 0.7
Σ PG, mg/dl	3,221 $\pm$ 193 ^a	3,263 $\pm$ 114 ^c	2,745 $\pm$ 123
Σ IRI, mIU/ml	152 $\pm$ 17.6 ^a	100 $\pm$ 13.4	78 $\pm$ 8.9

Σ PG and Σ IRI are the sum of PG and IRI concentrations at each time point during the IVGTT (fig. 3).

Data are expressed as the mean $\pm$ SEM. ^a $p < 0.01$, TGH vs. TGL; ^b $p < 0.01$, TGH vs. JW; ^c $p < 0.01$, TGL vs. JW.

rabbits (7.7 ± 1.6 mmol/ml/h in TGH WHHL vs. 14.3 ± 1.0 mmol/ml/h in TGL-WHHL; $p < 0.01$; $n = 6$ for each group), while there was no significant difference in HTGL activity between the 2 WHHL rabbits (data not shown). The levels of plasma CETP activity were higher in TGH-WHHL rabbits than in the other 2 groups (7.8 ± 1.2 nmol/ml/min in TGH-WHHL vs. 4.2 ± 1.3 nmol/ml/min in TGL-WHHL; $p < 0.01$). There was a linear relationship between CETP activities and plasma levels of TG but not TC in TGH-WHHL rabbits (data not shown).

Intravenous Glucose Tolerance Test

Both TGH-WHHL and TGL-WHHL rabbits at fasting state exhibited hyperglyceridemia, but TGH-WHHL rabbits also showed hyperinsulinemia compared with TGL-WHHL and JW rabbits (table 2). The response of PG and insulin to an intravenous glucose load was exam-

ined by IVGTT. As shown in figure 3 and table 2, both TGH-WHHL and TGL-WHHL rabbits showed impaired glucose clearance after 120 min, but TGH-WHHL rabbits were more prominent than TGL-WHHL rabbits. The levels of 120 min PG and IRI were apparently higher in TGH-WHHL and TGL-WHHL than in JW rabbits. After analyzing the levels of plasma TG and insulin, we found that correlation coefficients were significant between Σ IRI and TG (fig. 4), whereas there was no significant correlation between Σ PG and TG (data not shown).

Histological Examinations of Islets

We also examined the islet histological changes, and representative micrographs are shown in figure 5. Quantitative analysis of the islet size distribution showed TGH-WHHL rabbits had more hypertrophied islets ($>300 \mu\text{m}$) than the other 2 groups.

Discussion

In the present study, using 2 kinds of WHHL rabbits with different levels of plasma TG, along with JW wild-type rabbits, we investigated the possible mechanisms responsible for hypertriglyceridemia in TGH-WHHL rabbits. We found that hypertriglyceridemia in TGH-WHHL rabbits was essentially caused by enhanced hepatic production and reduced catabolism of TG-rich lipoproteins. This notion is supported by both the Triton WR-1339 injection study and measurements of TG secretion from isolated hepatocytes: TGH-WHHL rabbits had a higher capability of TG production compared with TGL-WHHL or JW groups. In combined hyperlipidemia, increased

Fig. 3. IVGTT was performed as described in Materials and Methods. Values are expressed as the means $\pm$ SEM. $n = 5$ for TGH-WHHL, $n = 7$ for TGL-WHHL, and $n = 7$ for JW rabbits.

production of apoB-100 is a common underlying metabolic characteristic [19]. The microsomal TG transfer protein (MTP) plays an obligatory role in the production of apoB-containing lipoproteins [20]. In the present study, there were significant increases in both TG and TC levels in TGH-WHHL rabbits compared with TGL-WHHL or JW rabbits; however, the MTP mRNA expression in both the liver and small intestine was not significantly increased by Northern blotting (data not shown), suggesting that there is an alternative pathway independent of MTP in the pathogenesis of hypertriglyceridemia of TGH-WHHL rabbits.

In the present study, TGH-WHHL rabbits also show a higher activity of CETP compared with TGL-WHHL and JW rabbits. However, it is not known whether high CETP directly causes hypertriglyceridemia in TGH-WHHL rabbits.

TG catabolism is mediated by several factors, including LPL-mediated hydrolysis of TG in the peripheral tissue, hepatic uptake at the binding site which recognizes apoE on the surface of parenchymal cells, LDL receptor-mediated catabolism of very low density lipoprotein, and

Fig. 4. Correlation between fasting levels of TG and Σ IRI.

Fig. 5. Histological analysis of the pancreas. Hematoxylin-eosin (HE) and immunohistochemical staining for insulin (IHC) of islets. $\times 200$.

Table 3. The distribution of pancreas islets in various sizes

	<200 μm	250 μm	300 μm	350 μm	>400 μm
TGH (n = 3)	147 $\pm$ 31	112 $\pm$ 19 ^b	54 $\pm$ 8 ^{a, b}	27 $\pm$ 4 ^{a, b}	18 $\pm$ 5 ^{a, b}
TGL (n = 3)	89 $\pm$ 19	54 $\pm$ 20	19 $\pm$ 6	6 $\pm$ 2	3 $\pm$ 2
JW (n = 3)	71 $\pm$ 14	36 $\pm$ 6	10 $\pm$ 3	3 $\pm$ 0.6	0

^a $p < 0.01$, TGH vs. TGL; ^b $p < 0.01$, TGH vs. JW.

extrahepatic cellular uptake by very-low-density lipoprotein receptors. In the current study, TGH-WHHL rabbits had lower LPL activities than TGL-WHHL rabbits, which is consistent with the suppressed level of the fractional catabolic rate of plasma TG. Therefore, the combined effects of decreased LPL activity and enhanced CETP activities in TGH-WHHL rabbits favor the pathogenesis of hypertriglyceridemia.

Hypertriglyceridemia is a hallmark for metabolic syndrome in humans and is frequently associated with

insulin resistance [4]. In the current study, we also demonstrated that TGH-WHHL rabbits had impaired glucose metabolism and insulin resistance. TGH-WHHL rabbits showed an increased number of hypertrophic islets in the pancreas accompanied by hyperinsulinemia. Although it is not completely clear how hypertriglyceridemia affects insulin resistance, the current study indicates that TGH-WHHL rabbits may serve as a model for the study of combined familial hyperlipidemia and metabolic syndrome.

In summary, our results have shown that causes of hypertriglyceridemia in TGH-WHHL rabbits are multifactorial: increased synthesis and decreased catabolism of TG-rich lipoproteins along with low LPL and high CETP activities. Our study also suggests that hypertriglyceridemia is closely associated with insulin resistance.

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