Table 4
Blood Pressure Levels and Carotid Atherosclerosis in Groups Distributed by C(−1652)T Genotypes

Male	CC	CT	TT	P
n (%)	519(47.1%)	438(39.7%)	146(13.2%)	
SBP (mmHg)	129.3 ± 0.7	128.8 ± 0.8	129.3 ± 1.4	0.850
DBP (mmHg)	81.4 ± 0.5	80.6 ± 0.5	80.8 ± 0.9	0.355
%Hypertension	41.4	39.0	35.6	0.419
IMT (mm)	0.898 ± 0.005	0.903 ± 0.005	0.892 ± 0.009	0.896
Max-IMT (mm)	1.729 ± 0.035	1.745 ± 0.038	1.650 ± 0.067	0.492
Plaque Score	4.6 ± 0.2	4.9 ± 0.2	4.5 ± 0.3	0.981
Female	CC	CT	TT	P
n (%)	579(48.0%)	481(39.9%)	146(12.1%)	
SBP (mmHg)	129.1 ± 0.7	128.8 ± 0.8	127.5 ± 1.5	0.405
DBP (mmHg)	79.0 ± 0.4	79.1 ± 0.5	78.5 ± 0.8	0.727
% Hypertension	35.6	36.0	34.9	0.973
IMT (mm)	0.846 ± 0.004	0.852 ± 0.004	0.848 ± 0.008	0.601
Max-IMT (mm)	1.332 ± 0.021	1.368 ± 0.023	1.371 ± 0.042	0.257
Plaque Score	2.4 ± 0.1	2.6 ± 0.1	2.7 ± 0.2	0.143

SBP: systolic blood pressure, DBP: diastolic blood pressure, IMT: intima-media thickness. Values are mean ± SE.

3.2. Study population

Table 1 shows the clinical characteristics of the present subjects by sex. Most variables (i.e., age, body mass index, diastolic blood pressure, percentage of current alcohol drinking, smoking, diabetes, and hypertension) were significantly higher in men than in women, but percentage of dyslipidemia, serum total cholesterol and HDL cholesterol levels were significantly higher in women than in men. There were no significant differences in systolic blood pressure.

3.3. Association of three polymorphisms with blood pressure and carotid arterioscleorsis

We investigated the possible association of three SNPs in the human *HGF* gene with blood pressure and carotid atherosclerosis in a population-based sample (the Suita Study) that consisted of 2412 participants. The frequencies of each genotype are described in Tables 4-6. The genotype frequencies of all analyzed polymorphisms were consistent with Hardy-Weinberg equilibrium. There were no significant differences in the genotype frequencies of polymorphisms for either sex. Tables also show systolic and diastolic blood pressure levels, and carotid IMT and Plaque Scores in each genotype of the three polymorphisms.

After full adjustment of all confounding factors (age, body mass index, current smoking status, alcohol consumption, presence of diabetes mellitus and dyslipidemia), there was no significant association between the three genotypes and blood pressure levels or the prevalence of hypertension in all subjects and in each sex.

Table 5
Blood Pressure Levels and Carotid Atherosclerosis in Groups Distributed by A43839T Genotypes

Male	AA	AT	TT	P
n(%)	555(59.8%)	304(32.8%)	69(7.4%)	
SBP (mmHg)	129.2 ± 0.7	128.4 ± 0.9	130.6 ± 2.0	0.981
DBP (mmHg)	81.3 ± 0.4	80.2 ± 0.6	81.7 ± 1.2	0.550
%Hypertension	38.0	41.1	34.8	0.522
IMT(mm)	0.897 ± 0.005	0.896 ± 0.006	0.888 ± 0.013	0.643
Max-IMT (mm)	1.733 ± 0.034	1.691 ± 0.046	1.743 ± 0.098	0.703
Plaque Score	4.8 ± 0.2	4.4 ± 0.2	4.5 ± 0.5	0.200
Female	AA	AT	TT	P
n(%)	636(61.5%)	340(32.9%)	59(5.7%)	
SBP (mmHg)	129.3 ± 0.7	127.4 ± 1.0	131.2 ± 2.3	0.578
DBP (mmHg)	79.0 ± 0.4	78.7 ± 0.5	78.9 ± 1.3	0.790
%Hypertension	36.6	32.1	44.1	0.135
IMT (mm)	0.854 ± 0.004	0.842 ± 0.005	0.837 ± 0.012	0.039
Max-IMT (mm)	1.390 ± 0.021	1.310 ± 0.028	1.232 ± 0.065	0.003
Plaque Score	2.7 ± 0.1	2.3 ± 0.2	1.8 ± 0.4	0.002

SBP: systolic blood pressure, DBP: diastolic blood pressure, IMT: intima-media thickness. Values are mean ± SE.

Table 6
Blood Pressure Levels and Carotid Atherosclerosis in Groups Distributed by C644222T Genotypes

Male	CC	CT	TT	*P*
n (%)	7(0.7%)	194(19.1%)	817(80.3%)	
SBP (mmHg)	125.7 ± 6.2	128.7 ± 1.2	129.3 ± 0.6	0.547
DBP (mmHg)	80.0 ± 3.9	81.2 ± 0.7	80.9 ± 0.4	0.780
%Hypertension	14.2	38.1	40.3	0.334
IMT (mm)	0.895 ± 0.039	0.885 ± 0.008	0.900 ± 0.004	0.096
Max-IMT (mm)	2.066 ± 0.287	1.690 ± 0.057	1.732 ± 0.028	0.856
Plaque Score	2.4 ± 0.9	2.4 ± 0.2	2.5 ± 0.1	0.414
Female	CC	CT	TT	*P*
n (%)	11(1.0%)	207(18.6%)	897(80.5%)	
SBP (mmHg)	130.2 ± 5.3	128.2 ± 1.2	129.1 ± 0.6	0.616
DBP (mmHg)	80.0 ± 3.0	78.2 ± 0.7	79.0 ± 0.3	0.439
%Hypertension	36.4	31.9	37.0	0.383
IMT (mm)	0.825 ± 0.030	0.841 ± 0.007	0.850 ± 0.003	0.161
Max-IMT (mm)	1.317 ± 0.162	1.362 ± 0.036	1.349 ± 0.017	0.844
Plaque Score	6.2 ± 1.5	4.4 ± 0.3	4.8 ± 0.1	0.414

SBP: systolic blood pressure, DBP: diastolic blood pressure, IMT: intima-media thickness. Values are mean ± SE.

Although no association was found between carotid IMT, Plaque Scores and *A43839T* genotype in male subjects, women with the A allele showed significantly thicker IMT and greater Plaque Scores than those with the T allele (Table 5). There was no association between carotid IMT, Plaque Scores and genotypes of the two SNPs, *C(-1652)T* and *C44222T*, in both male and female subjects (Tables 4 and 6).

4. Discussion

Although a number of reports have suggested a strong association between the severity of hypertension and serum HGF levels, there have been few reports that investigated the association between cardiovascular disease and *HGF* gene polymorphisms by direct sequencing. Of those identified single nucleotide polymorphisms, 11 were not deposited in the public database. We have performed a large genetic epidemiological study of the Japanese general population regarding three candidate SNPs in the promoter and intron of the *HGF* gene. There was no significant association between the three *HGF* SNPs and blood pressure or the prevalence of hypertension. Interestingly, female subjects with the A allele of *A43839T* in intron 8 had more severe carotid atherosclerosis than those with the T allele.

4.1. HGF and hypertension, atherosclerosis

Clinical studies have demonstrated a positive correlation between serum HGF concentrations and blood pressure. These studies have gone on to show that serum HGF concentrations in hypertensive patients were significantly higher than those seen in normotensive control subjects [5,6]. In an experimental setting, serum HGF concentrations were significantly increased in spontaneous hypertensive rats compared to Wistar-Kyoto rats at any age, and there was a positive association between serum HGF concentration and blood pressure level [17].

A number of reports have also suggested that serum HGF concentrations are increased in proportion to the development of hypertensive target organ damage. The circulating level of HGF was elevated in patients with myocardial infarction [18] and peripheral arterial disease [9,19,20]. Furthermore, serum HGF concentrations were significantly correlated with the hyperemic response of forearm blood flow and pulse wave velocity [21]. Alternatively, it is apparent that endothelial cell dysfunction may promote abnormal vascular growth, and this vascular remodeling clearly plays an important role in the pathophysiology of atherosclerosis. In normotensive subjects, serum HGF was suggested to maintain the vascular structure and stimulate tissue regeneration in an autocrine-paracrine manner [19,22]. In patients with hypertension or diabetes mellitus, this local HGF system was disturbed by transforming growth factor-β or angiotensin II with the resultant development of abnormal vascular smooth muscle cell growth [17,23].

4.2. HGF SNPs, hypertension, and atheroscleorosis

Polymorphisms of several growth factor genes have been investigated because they may potentially play a key role in the maturation of atheromatous lesions. Among these growth factors, HGF was of particular interest because it could have cardiovascular protective effects in several disorders including hypertension, diabetes mellitus, and cardiovascular diseases.

In the present study, we identified 21 SNPs in the *HGF* gene, and determined the genotype of three of these polymorphisms in more than 2000 individuals. Our results showed that there were no significant associations between *HGF* genotypes and blood pressure levels or the prevalence of hypertension. However, the A allele of *A43839T* in intron 8 was significantly associated with increased severity

of carotid atherosclerosis in females. The reason for this sex-specific effect in our study subjects is unclear. The interaction of HGF with angiotensin II and/or transforming growth factor β could contribute to some of the difference seen in our patients population. Although still controversial [24], renin-angiotensin system related genetic variation tends to appear in male individuals in Japanese [13] and Caucasians [25,26]. Additionally, a T → C transition at nucleotide 869 of the *transforming growth factor β* gene has been reported to be one of the candidate susceptibility loci for hypertension only in the female Japanese population [27]. We assume that there might exist some link or interaction between HGF and the genetic variation of these two growth factors.

Our results suggest that the HGF gene located at chromosome 7q11.2-q21 is a candidate susceptibility locus for atherosclerosis in Japanese women. The polymorphism conferring increased susceptibility for atherosclerosis was located in the intron region without amino acid substitution. Thus, it is possible that the *A43839T* polymorphism in intron 8 of the *HGF* gene is in linkage disequilibrium with some other polymorphisms which are actually responsible for the development of atherosclerosis. To elucidate the exact mechanisms and clinical implications of the association, further functional and linkage disequilibrium analyses are required.

In conclusion, we identified 21 SNPs in the *HGF* gene including 11 SNPs that have never been reported. The present study provides the first evidence that *HGF* may be a candidate susceptibility loci that affects the progression of atherosclerosis in Japanese subjects.

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Chronic hypertriglyceridemia in young watanabe heritable hyperlipidemic rabbits impairs endothelial and medial smooth muscle function

Tetsuro Shishido[a,*], Katsunari Tasaki[b], Yasuchika Takeishi[a], Satoshi Takasaki[a], Takuya Miyamoto[a], Makoto Itoh[a], Hiroki Takahashi[a], Isao Kubota[a], Tsunekata Ito[c], Yumi Katano[d], Ichiro Wakabayashi[b], Hitonobu Tomoike[e]

[a] *First Department of Internal Medicine, Yamagata University School of Medicine, 2-2-2 Iida-Nishi, Yamagata, 990-9585, Japan*
[b] *Department of Hygiene & Preventive Medicine, Yamagata University School of Medicine, Yamagata, Japan*
[c] *Laboratory Animal Center, Yamagata University School of Medicine, Yamagata, Japan*
[d] *Division of Clinical Nursing and Pathophysiology, Yamagata University School of Medicine, Yamagata, Japan*
[e] *National Cardiovascular Center, Suita, Japan*

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Abstract

Several studies have suggested that hypertriglyceridemia is a common risk factor for coronary heart disease. Although increasing serum levels of triglyceride correlate with hypercoagulability, little is known about the contribution of hypertriglyceridemia to vascular function. We successfully segregated two lines of rabbits with genetically-determined severely high (TGH; 2764 ± 413 mg/dl) and moderately high (TGL; 191 ± 12 mg/dl) levels of triglyceride, but with comparable levels of total cholesterol, from Watanabe heritable hyperlipidemic rabbits. To determine whether hypertriglyceridemia was involved in alterations of vascular function, we conducted isometric tension studies and analyzed protein expression on thoracic aortic rings isolated from young (3–4 month) TGH, TGL and Japanese White rabbit (JW). No difference in percentage of plaque area in the thoracic aorta was found between TGH and TGL. Relaxing responses, evoked by sodium nitroprusside were similar in JW, TGL and TGH, but endothelium-dependent relaxation to acetylcholine was impaired in TGH compared with JW or TGL (maximal relaxation in JW; 83.5 ± 2.7%, TGL; 79.9 ± 5.3%, TGH; 59.1 ± 5.7%, $p<0.05$). Relaxation to A23187 was also attenuated in TGH compared with JW, but not significantly different between TGL and JW. Endothelium-independent relaxation elicited by isoproterenol in TGH was significantly decreased compared with JW or TGL (maximal relaxation in JW; 95.2 ± 2.6% TGL; 91.0 ± 4.9%, TGH; 75.1 ± 5.2%, $p<0.05$). Protein expression of angiotensin II type-1 receptor was increased in TGH and that of nitric

* Corresponding author. Tel.: +81-23-628-5302; fax: +81-23-628-5305.
E-mail address: tshishid@med.id.yamagata-u.ac.jp (T. Shishido).

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oxide synthases-3 was attenuated in TGH compared with TGL. This is the first study showing that endothelium-dependent and -independent vascular relaxation under the condition of combined hyperlipidemia was severely impaired as compared to that under only hypercholesterolemia. These results suggest that hypertriglyceridemia aggravates functional impairment induced by hypercholesterolemia in endothelial and smooth muscle cells.

Keywords: Hypertriglyceridemial; Combined hyperlipidemia; EDRF; Isoproterenol; Vascular relaxation; Endothelial dysfunction; WHHL; NOS3; Angiotensin II

Introduction

Hyperlipidemia has been implicated as a common risk factor for cardiovascular disease (Gordon et al., 1977; Hulley et al., 1980). Although elevated TG levels are predictive of subsequent occurrence of coronary heart disease and mortality in healthy persons, adjustment for HDL cholesterol and fasting serum glucose eliminates the independent relationship between serum TG levels and cardiovascular events (Castelli et al., 1977). However, recent epidemiological studies have demonstrated that hypertriglyceridemia is identified as one of the potential risk factors for coronary heart disease (Egger et al., 1999; Rosenson et al., 1999). Lines of evidence have shown that hypercholesterolemia induces endothelial dysfunction, smooth muscle cell proliferation, enhanced vascular reactivity to some contractile agonists, infiltration of monocytes into vessel walls, and production of reactive oxygen species (Ross, 1993). Studies using Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal model with deficient low-density lipoprotein (LDL) receptors, have demonstrated that hypercholesterolemia causes progression of atherosclerosis and fatty streak formation in the vascular wall, which correlates with reduced endothelium-dependent relaxation (Tagawa et al., 1991, 1993). However the contribution of chronic hypertriglyceridemia to endothelial and medial smooth muscle function is largely undefined (de Man et al., 2000; Inoue et al., 1998). Several studies have demonstrated that flow-mediated vasodilatation is impaired in patients with hypertriglyceridemia (Lundman et al., 2001a; Lupattelli et al., 2000). It has been reported that aortic rings pre-incubated with triglyceride-rich fat emulsion attenuates endothelial-dependent vasodilation (Lundman et al., 2001b). However, little is known about the potential mechanisms for vascular dysfunction induced by chronic hypertriglyceridemia.

In order to elucidate the different roles of cholesterol and triglyceride in vascular dysfunction, we successfully segregated two lines of heritable hypercholesterolemic rabbit models with high (TGH) and low (TGL) levels of triglyceride (Takasaki et al., 1999). The aim of the present study was to use these animal models to clarify the role of hypertriglyceridemia in the alteration of vascular function. We examined differences in vasoconstrictor and relaxing function of thoracic aortic rings isolated from TGH and TGL rabbits.

Methods

Materials

The pharmacological agents used in the present study were acetylcholine chloride, calcium ionophore A23187, isoproterenol, sodium nitroprusside, angiotensin II, and phenylephrine hydrochloride. Krebs-

Henseleit solution contained (mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_2$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2, glucose 11.1, and ascorbic acid 0.057. All chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Animals

The experimental procedures conform to the guiding principles of the animal core and use in Yamagata University School of Medicine. Male WHHL rabbits, which were segregated into two lines of severely high triglyceride (TGH, n = 12) and moderately high TG level (TGL, n = 11), and Japanese White rabbits (JW, n = 19) aged 3–4 months were used in the present study. All animals were housed individually in a controlled environment with unlimited access to water and were fed standard rabbit chow (120 g/day, Labo R Grower, Nihon Nosan Kogyo, Ltd., Tokyo, Japan). The animals were anesthetized with an intravenous administration of 30 mg/kg sodium pentobarbital, and segments of the descending thoracic aorta were carefully removed and immediately immersed in ice-cold Krebs-Henseleit solution for isometric tension studies.

Isometric tension recording

After the connective tissue was carefully removed, the rings from the lower thoracic aorta (3 mm width) were mounted on two stainless steel triangular clips and placed into organ baths filled with 10 ml oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution maintained at 37 °C. The endothelium was gently removed in some rings by rubbing with a small wooden stick in order that the endothelium-independent relaxation and contractile responses in denuded rings could be studied. Isometric tension was monitored continuously using transducers (T7-15-240, Orientec Co, Tokyo, Japan) connected to a data acquisition system (RJ1000, San-ei, Tokyo, Japan). The rings were equilibrated under a resting tension of 2 g for 60–90 minutes, and the buffer was changed every 15 minutes (Tagawa et al., 1991, 1993). The rings were pre-contracted with phenylephrine (1 μM) and relaxed with acetylcholine (1 μM) to test endothelial integrity (Dam et al., 1997). After 60 minutes of equilibration, high potassium solution (66.7 mM) was added with intervals during which the resting tension was re-adjusted to 2 g (Hirata et al., 1992). High potassium solution was prepared by replacing NaCl with equimolar KCl. The cumulative contractile responses of the aortic ring to angiotensin II and phenylephrine were studied in the absence and presence of endothelium. Contraction was expressed as percentage of the maximal contraction by high potassium solution. To assess relaxation, cumulative concentration response curves were obtained with endothelium-dependent vasodilators, acetylcholine and calcium ionophore A23187, and endothelium-independent vasodilators, sodium nitroprusside and isoproterenol. Endothelium denuded rings were used to study endothelium-independent relaxation. Relaxation was expressed as a percentage of the pre-contractile tension induced by phenylephrine at levels of 30–50% of maximal contraction by high potassium solution (Kolodgie et al., 1990; Tagawa et al., 1991). EC_{50} was defined as the concentration of agonist at which 50% of the maximal response was obtained.

After completion of each experiment, some rings were washed with Krebs-Henseleit solution and removed from the apparatus, cut open, and were stained with Sudan III. Finally the stained strips were

photographed, and the total surface and atherosclerotic plaque areas were assessed quantitatively using NIH Image (version 1.55). Results were expressed as the percentage of the arterial surface covered by atherosclerotic lesions (Tagawa et al., 1993).

Microscopic analysis of atherosclerotic lesion

Descending thoracic aortas obtained from TGL and TGH (n = 5 in each) were fixed in 10% formaldehyde, and embedded in paraffin. Quantitative analysis of lesion composition was performed as previously described (Shiomi et al., 1994). Elastica-Goldner staining was performed to determine intimal thickening. Cell components of the plaque that were quantitatively evaluated included macrophages, smooth muscle cells, and collagen. Immunohistochemical staining was performed using RAM-11 (Dako A/S, Glastrup, Denmark) and monoclonal antibody against a-smooth muscle actin (Dako A/S, Glastrup, Denmark). Immunostaining was carried out with a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) according to the manufacturer's instructions. The area of macrophages and smooth muscle were expressed as the percentage of the total intimal lesion area in each section reacting positively with diaminobenzidine (Shiomi et al., 1994). Collagen was defined as fibers staining cobalt blue with Azan-Mallory stain. The medial thickening, total cell number, and percentage of fractional fibrosis in the aortic media of vessel sections without atheroma were also determined (Fujii et al., 1999).

Lipid analysis

Blood samples were taken from a marginal ear artery 18 hr after the last feeding. All blood samples were centrifuged at 4 °C, and the plasma was stored at − 80 °C until assay. Plasma concentrations of total cholesterol, triglyceride and glucose were measured by enzymatic methods using a SPOTCHEM-EZ (ARKRAY Co, Kyoto, Japan).

Protein extraction and assay

Aortic samples were homogenized in a lysis buffer containing 25 mM Tris-HCl, 50 mM NaCl, 2% NP-40, 0.5% Na-deoxycholate, 0.2% SDS, 200 μM $NaVO_3$, 100 mM NaF and protease inhibitor mixture, and then centrifuged at 14,000 g for 15 min at 4 °C. The supernatant was collected, and the protein concentration of each sample was measured using BCA protein assay reagents (Pierce Chemical Co., Rockford, IL, USA). For SDS–PAGE, equal amounts of protein were added on 8–12% polyacrylamide gel, electrophoretically separated, and transferred to PVDF membrane. After blocking with a buffer containing 5% non-fat milk and 0.2% Tween-20 for 1 hr at room temperature, the membrane was incubated with either anti-nitric oxide synthase (NOS)-3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or anti-angiotensin II type-1 receptor (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibody. After washing three times with PBS, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Promega Co., Madison, WI, USA) for 1 hr at room temperature. The membrane was washed again and the signals were visualized by enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ, USA).

Statistical analysis

The data are expressed as mean ± SEM for the isometric tension studies. Statistical analysis was performed with one factor ANOVA for vascular relaxation, lipid profile, body weight and protein expression, and other parameters were analyzed by a two-way ANOVA followed by an appropriate post-hoc test. A value of $P < 0.05$ was considered significant.

Results

Body weight and plasma levels of cholesterol, triglyceride and glucose

Body weights of TGL and TGH were significantly lower than those of JW, but there was no difference between TGH and TGL (Table 1). Total cholesterol levels in TGH and TGL were remarkably higher than in JW, however there was no significant difference in total cholesterol level between TGL and TGH (Table 1). Plasma triglyceride levels of TGH were markedly higher compared with JW and TGL (Table 1). The plasma levels of fasting glucose were not different among the three groups.

Macroscopic evaluation of fatty streak formation

No plaques were observed in the rings from JW rabbits. In the descending thoracic aortic rings from WHHL, fatty streaks were formed, but there was no significant difference in the percentages of the plaque areas between TGH (32.5 ± 1.4%, n = 71 rings) and TGL (28.8 ± 1.5%, n = 61 rings).

Microscopic evaluation of fatty streak formation

We made microscopic comparisons of smooth muscle cell proliferation, macrophage infiltration, and collagen deposition in atheroma of TGL and TGH. The percent area in atheroma of smooth muscle

Table 1
Body weight and plasma levels of total cholesterol, triglyceride and glucose in JW, TGL and TGH

	JW	TGL	TGH
Body weight (kg)	2.53 ± 0.58 (n=19)	2.29 ± 0.07* (n=11)	2.08 ± 0.07** (n=12)
Total cholesterol (mg/dl)	59 ± 5 (n=10)	1081 ± 59$^{\#}$ (n=11)	1465 ± 167$^{\#}$ (n=12)
Triglyceride (mg/dl)	63 ± 8 (n=10)	191 ± 12 (n=11)	2764 ± 413$^{\#,\dagger}$ (n=12)
Fasting glucose (mg/dl)	160 ± 16 (n=10)	149 ± 4 (n=11)	149 ± 8 (n=12)

Values are mean ± SEM.
*$P < 0.05$.
**$P < 0.01$.
$^{\#}P < 0.0001$ vs. JW.
$^{\dagger}P < 0.0001$ vs. TGL.

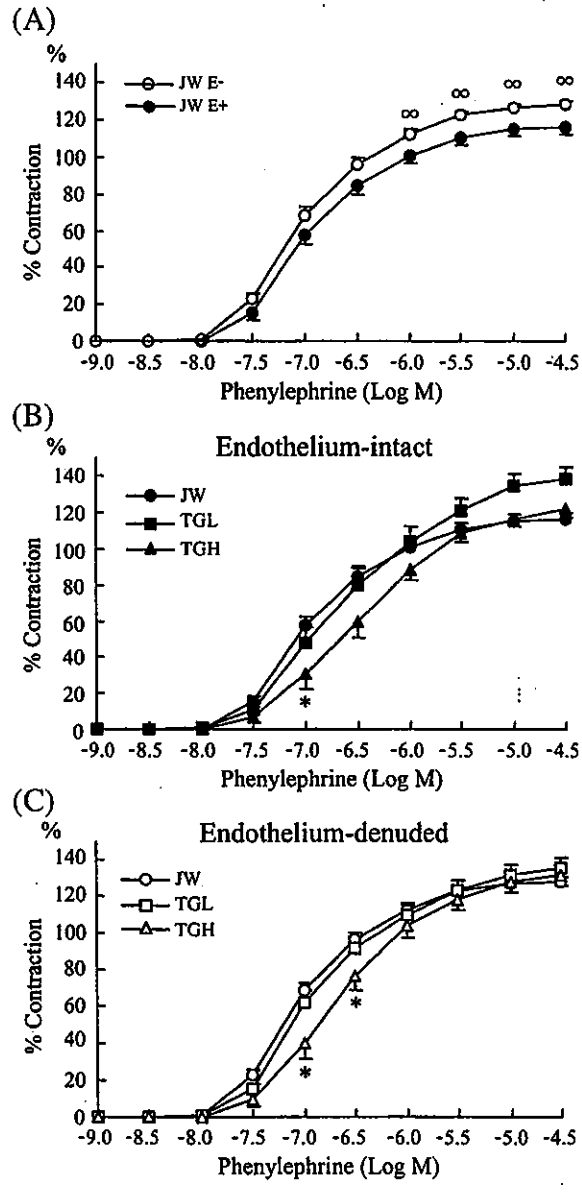

Fig. 1. Phenylephrine-induced contraction of thoracic aortic rings from JW (circles), TGL (squares) and TGH (triangles). Filled symbols and open symbols indicates aortic rings with intact and denuded endothelium, respectively. Contraction was expressed as percentage of the maximal contraction produced by high potassium. Each concentration-response curve was plotted by taking the respective mean ± SEM values from at least 8 separate experiments for each group. E, endothelium. ∞, significant difference compared with endothelium-intact rings ($P<0.05$). *, significant difference compared with aortic rings from JW ($P<0.05$).

cells, macrophage and collagen were similar in TGL and TGH (smooth muscle cell; 21.9 ± 2.1% vs. 17.3 ± 1.7%; macrophage, 20.0 ± 3.4% vs. 15.1 ± 2.1%; collagen, 31.9 ± 2.0% vs. 27.8 ± 1.6% in TGL and TGH, respectively). Total cell numbers of the media were similar in TGL and TGH (2580 ± 120 /mm^2 and 2580 ± 60 /mm^2, respectively). There was no significant difference in medial thickness between TGL and TGH (251 ± 12 μm and 252 ± 10 μm, respectively). Furthermore, total fractional fibrosis in the media was not different in TGL and TGH (34.2 ± 0.5% and 28.4 ± 0.3%, respectively).

Comparison of contractile response to phenylephrine

No differences in contraction induced by high potassium solution were detected among JW, TGH and TGL. In JW, the contractile response to phenylephrine was significantly augmented in denuded rings compared with intact rings (Fig. 1A and Table 2). However in TGH and TGL, there was no difference in phenylephrine-induced contraction between endothelium-denuded and -intact rings. The cumulative concentration-response curve to phenylephrine in TGH was shifted to the right compared with JW in both endothelium-intact and -denuded rings, respectively ($P<0.05$) (Fig. 1B and C). In endothelium-denuded rings, the EC_{50} value of phenylephrine-induced contraction was also lower in TGH than JW (Table 2). However, there was no significant difference in the EC_{50} values between JW and TGL.

Table 2
Contractile and relaxing responses in JW, TGL and TGH

Stimulations		E	JW	TGL	TGH
Phenylephrine Hydrochloride	*max contraction (%)*	+	116.3 ± 3.7[a]	130.5 ± 3.8	122.3 ± 4.8
	EC 50 (10^{-7} M)	+	1.2 ± 0.2	3.2 ± 1.0	3.2 ± 1.1*
	max contraction (%)	–	128.6 ± 2.2	135.9 ± 4.9	132.2 ± 5.6
	EC 50 (10^{-7} M)	–	1.0 ± 0.2	1.5 ± 0.3	2.4 ± 0.5*
Angiotensin II	*max contraction (%)*	+	73.2 ± 6.7	37.0 ± 7.1**	24.4 ± 5.8**
	EC 50 (10^{-9} M)	+	2.1 ± 1.0	3.3 ± 1.4	3.7 ± 0.6
	max contraction (%)	–	85.5 ± 2.3	48.2 ± 6.4**	35.1 ± 6.5**
	EC 50 (10^{-9} M)	–	0.9 ± 0.3	3.0 ± 0.9	3.9 ± 1.0*
Acetylcholine	*max relaxation (%)*	+	83.5 ± 2.7	79.9 ± 5.3	59.1 ± 5.7**,†
	EC 50 (10^{-8} M)	+	3.8 ± 0.6	3.9 ± 0.9	6.3 ± 1.6
A23187	*max relaxation (%)*	+	96.0 ± 2.0	91.4 ± 4.7	74.5 ± 6.5*
	EC 50 (10^{-9} M)	+	2.7 ± 1.6	7.8 ± 3.7	2.6 ± 0.6
Sodium Nitroprusside	*max relaxation (%)*	–	106.8 ± 2.5	105.0 ± 2.1	104.0 ± 1.7
	EC 50 (10^{-9} M)	–	13.5 ± 5.5	4.6 ± 1.3	4.9 ± 1.2
Isoproterenol	*max relaxation (%)*	–	95.2 ± 2.6	91.0 ± 4.9	75.1 ± 4.1**,†
	EC 50 (10^{-8} M)	–	1.5 ± 0.3	2.1 ± 0.4	4.2 ± 1.5

Values are reported as mean ± SEM. E, endothelium.
[a] $P<0.05$ vs. endothelium-denuded rings.
*$P<0.05$.
**$P<0.01$ vs. JW.
† $P<0.05$ vs. TGL.

Comparison of contractile responses to angiotensin II

In JW, TGL and TGH, contraction elicited by angiotensin II were similar in endothelium-denuded and -intact rings (Fig. 2 and Table 2). Contractile responses to angiotensin II were significantly impaired in TGH and TGL compared to JW, while there was no significant difference in angiotensin II-induced contraction between TGH and TGL (Fig. 2 and Table 2).

Comparison of endothelium-dependent relaxation to acetylcholine and calcium ionophore A23187

Endothelium-dependent relaxation to acetylcholine was attenuated in aortas from TGH compared with those from JW and TGL, but there was no difference between JW and TGL (Fig. 3A). In the aortic segments obtained from TGH, maximal relaxation to acetylcholine was markedly

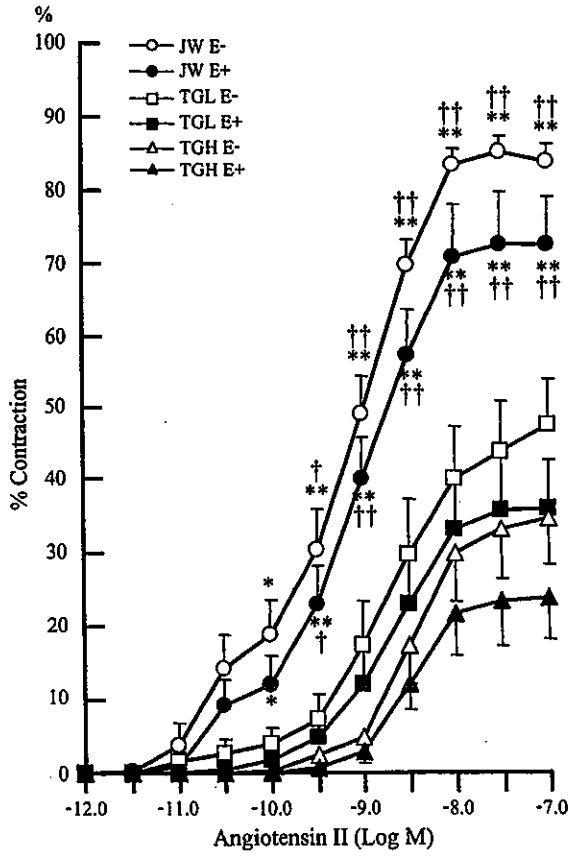

Fig. 2. Angiotensin II-induced contraction of thoracic aortic rings from JW, TGL and TGH. Contraction was expressed as percentage of the maximal contraction produced by high potassium. The results are mean ± SEM values from at least 8 separate experiments in each group. Abbreviations and symbols are the same as in Fig. 2. † and ††, significant difference compared with aortic rings from TGL ($P<0.05$ and $P<0.01$, respectively). * and **, significant difference compared with aortic rings from TGH ($P<0.05$ and $P<0.01$, respectively).

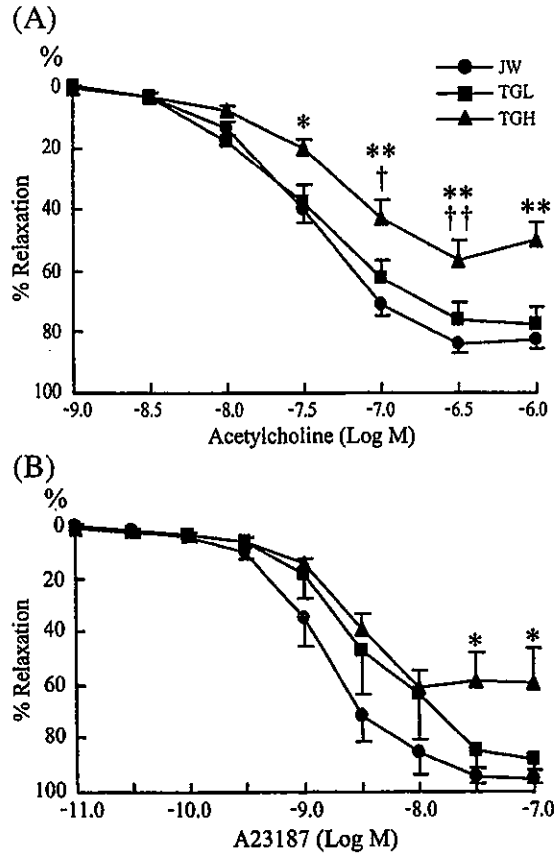

Fig. 3. Acetylcholine (A) and calcium ionophore A23187 (B)-induced relaxing responses in thoracic aortic rings with intact endothelium from JW, TGL, and TGH. Data are mean ± SEM values from at least 8 separate experiments. * and **, significant difference compared with aortic rings from JW ($P<0.05$ and $P<0.01$, respectively). † and ††, significant difference compared with aortic rings from TGL ($P<0.05$ and $P<0.01$, respectively).

attenuated compared with those from TGL and JW (Table 2). Responses to another endothelium-dependent vasodilator, calcium ionophore A23187, were also reduced in TGH compared to those in JW (Fig. 3B). The level of maximum relaxation by calcium ionophore A23187 in TGH was significantly lower than that in JW, but there was no significant difference between TGL and JW (Table 2).

Comparison of endothelium-independent relaxation to sodium nitroprusside

To determine whether decreased response to NO was involved in diminished endothelium-dependent relaxation in TGH, we compared concentration-response curves evoked by the NO donor, sodium nitroprusside. Relaxing responses elicited by sodium nitroprusside were similar in JW, TGL and TGH (Fig. 4A and Table 2).

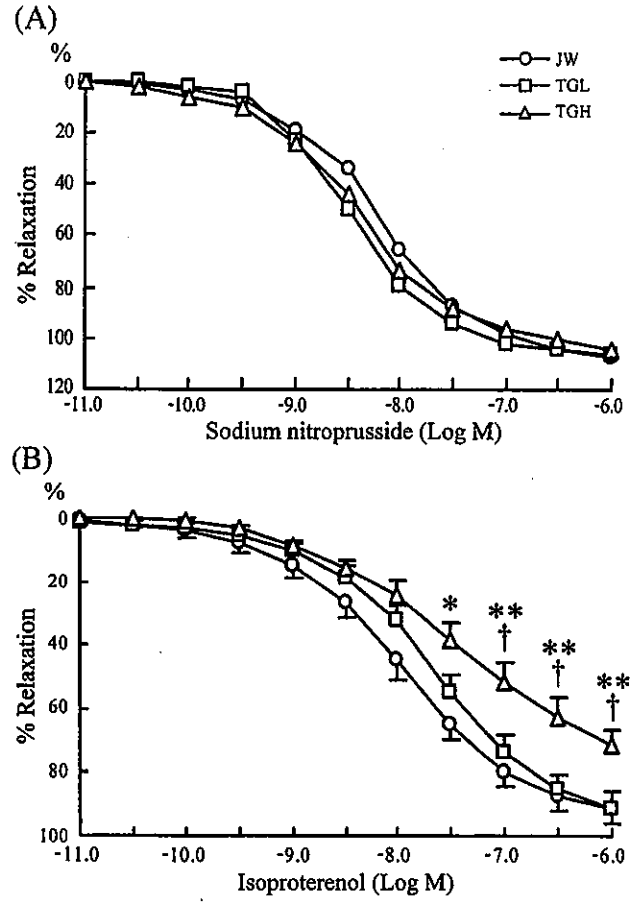

Fig. 4. Sodium nitroprusside (A)- and isoproterenol (B)-induced relaxing responses in thoracic aortic rings without endothelium from JW, TGL and TGH. Abbreviations and symbols are the same as in Fig. 4.

Comparison of endothelium-independent relaxation in response to isoproterenol

The nonselective β agonist, isoproterenol, produced concentration-dependent relaxation of intact rings. The relaxing response induced by isoproterenol was significantly attenuated in TGH compared with JW and TGH (Fig. 4B). Maximal relaxation induced by isoproterenol in TGH was significantly diminished compared to those in JW and TGL, while there was no significant difference between JW and TGL (Table 2).

Protein expressions of angiotensin II type-1 receptor and NOS3

Protein expressions of angiotensin II type-1 receptor and NOS3 were examined. The expression of angiotensin II type-1 receptor was increased 1.5- and 2.6- fold in TGL and TGH, respectively, compared with JW. The expression of angiotensin II type-1 receptor was significantly increased only in TGH,

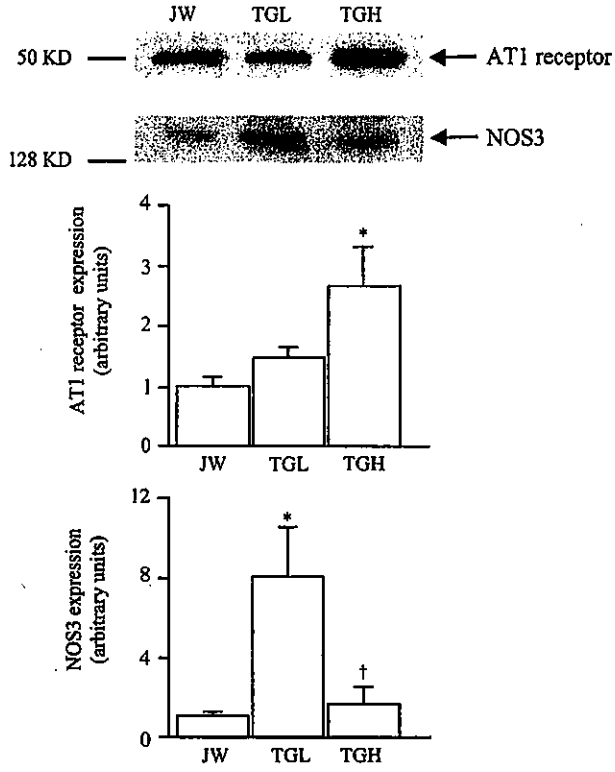

Fig. 5. Representative Western blots and densitometric data of angiotensin II type-1 (AT1) receptor and NOS3 in JW, TGL and TGH. *, significant difference compared with JW ($P<0.05$). †, significant difference compared with TGL ($P<0.05$).

when compared with JW (Fig. 5). NOS3 expression in aorta was significantly increased in TGL compared with JW, while in TGH it was significantly attenuated compared with TGL (Fig. 5).

Discussion

In this study, isometric tension studies using isolated aortic rings with comparable areas of atherosclerotic plaque demonstrated that hypertriglyceridemia attenuated endothelium-dependent relaxation which was associated with decreased EDRF release, and impaired medial smooth muscle cell function accompanied by decreased NOS3 and increased angiotensin II type-1 receptor expression.

Many lines of evidence suggest that hypertriglyceridemia is a marker of increased risk for coronary heart disease (Egger et al., 1999; Rosenson, 1999). Many studies have shown that very low-density liopoprotein (VLDL) produces remnant lipoprotein or small dense LDL, and triglyceride-rich lipoproteins accumulate in endothelial cells (Rosenson, 1999). Hypertriglyceridemic VLDL causes down-regulation of tissue plasminogen activator and surface localized-plasmin generation, and increases plasminogen activator inhibitor-I (Stiko-Rahm et al., 1990). Thus, hypertriglyceridemia causes a prothorombotic state, which can contribute to induction of the atherogenic state in vascular wall (Dichtl et al., 1999; Nagornev and Rabinovich, 1998). Growing evidence has demonstrated that flow-mediated

vasodilatation is impaired in patients with hypertriglyceridemia (de Man et al., 2000; Egger et al., 1999; Inoue et al., 1998; Lupattelli et al., 2000). However, the potential mechanisms for vascular dysfunction induced by chronic hypertriglyceridemia have yet to be defined.

We successfully segregated two lines of WHHL with severely high (TGH) and moderately high (TGL) levels of serum triglyceride. Plasma levels of total cholesterol were not significantly different between TGH and TGL, and plasma levels of glucose were similar in JW, TGL and TGH. In this study, plaque qualities such as smooth muscle cell infiltration, macrophage infiltration, and collagen deposition were similar between TGL and TGH. Moreover in our pilot study, in the young state, there was no significant difference between TGH and TGL in coronary stenosis. This study is the first to report that more prominent vascular dysfunction occurs in the presence of combined hyperlipidemia as compared with hypercholesterolemia alone, as was shown in combined hyperlipidemic rabbit.

In this study using aged 3 to 4 month old rabbits, it was found that endothelium-dependent relaxation to acetylcholine was attenuated in TGH compared with JW and TGL. Moreover, relaxation to the more potent endothelium-dependent vasodilator, calcium ionophore A23187 (Kolodgie et al., 1990), was also attenuated in TGH compared to JW. Several studies have demonstrated that hypercholesterolemia induces endothelial dysfunction (Habib et al., 1986; Verbeuren et al., 1986); however, in this study, endothelium-dependent relaxation was similar in JW and TGL. One possible explanation for this difference is that WHHL are hyperlipidemic from birth and may have adapted to high cholesterol environment (Kolodgie et al., 1990). It has been reported that NOS3 expression in both mRNA and protein levels in WHHL aortas are increased (Kanazawa et al., 1996). Thus, increased expression of NOS3 was contributed to preservation of endothelium-dependent relaxation in TGL. In our present study, endothelium-mediated vasodilatation was decreased in TGH, whereas relaxation evoked by sodium nitroprusside, an endothelium-independent vasodilator, was similar in JW, TGL and TGH. These results indicated that the response to NO was similar in JW, TGL and TGH, but production of EDRF was attenuated in TGH. Moreover, at an early stage of atherosclerosis, hypertriglyceridemia in addition to hypercholesterolemia is necessary to induce vascular endothelial dysfunction that is involved in vasorelaxation (Hirono et al., 1996; Lundman et al., 2001a; Ross, 1993; Tagawa et al., 1991).

Both decreased (Dam et al., 1997) and enhanced (Yang et al., 1998) contractions elicited by angiotensin II in hypercholesterolemic rabbits have been reported. One possible explanation for these contrasting results is that vascular size and/or percentage of plaque area vary among the various studies. In the present study, responses to angiotensin II were similarly attenuated in TGH and TGL, suggesting that in TGH and TGL medial smooth muscle cells were impaired by hypercholesterolemia. However, the expression of angiotensin II type-1 receptor in aorta was increased in TGH compared with JW, and contraction to phenylephrine was attenuated only in TGH, but not TGL, compared to JW. It has been reported that expression of angiotensin II type-1 receptor is increased in the aorta of hyperlipidemic animals (Yang et al., 1998), and angiotensin II type-1 receptor mediated-signaling pathways contributed to vascular dysfunction. These results indicated that hypertriglyceridemia also evokes smooth muscle cell dysfunction more prominently. The angiotensin II type-2 receptors are thought to have an effect opposite to that of angiotensin II type-1 receptors and play a pivotal role in anti-neointimal formation (Akishita et al., 2000) and vascular relaxation (Israel et al., 2000) through, in part, NO production. The angiotensin II type-2 receptors are expressed in fetal condition, and are re-expressed in certain pathological conditions such as inflammation and vascular injury (Akishita et al., 2000). Therefore, changes in angiotensin II type-2 receptor expression may be one explanation for the impairment of contraction evoked by angiotensin II in TGL and TGH.

We found that relaxation elicited by isoproterenol was reduced in rings from TGH compared with JW and TGL, but there was no significant difference between JW and TGL. Recently, cAMP-dependent NO release pathways have been reported in the endothelium (Zhang and Hintze, 2001). Since in the present study we used denuded rings to investigate relaxation evoked by isoproterenol, the reduced relaxation to isoproterenol in TGH may represent a medial smooth muscle dysfunction. Several studies demonstrated that the cAMP-mediated vasodilator mechanism was not altered in WHHL (Hirata et al., 1992). However in aortic smooth muscle cells obtained from hypercholesterolemic rabbits, basal and NaF-stimulated cAMP generation were decreased, and induction of oxidized LDL in rabbits inhibited cAMP-mediated vasodilatation (Galle et al., 1992; Schmidt et al., 1993). These data suggest that hypertriglyceridemia also impairs smooth muscle cell function. Similar to endothelial dysfunction, hypercholesterolemia alone did not induce α1- and β-adrenergic dysfunction, and combined dyslipidemia was necessary to induce them in aortas from young rabbits.

In conclusion, our studies using aortas from TGH demonstrated that both endothelium-dependent and -independent relaxation responses were impaired, and smooth muscle contractile responses were also attenuated. These vascular dysfunctions were the first to appear in the atherogenic state. These observations support the concept that hypertriglyceridemia accelerates functional impairment of endothelial and smooth muscle cells induced by hypercholesterolemia.

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遺伝性高中性脂肪血症家兎における動脈硬化病変の進展および大腿動脈圧

下田智子，石幡　明*，伊藤恒賢**，大和田一雄**，
會田智美，利　美賀子，片野由美

山形大学医学部看護学科臨床看護学講座
*山形大学医学部器官機能統御学講座腫瘍分子医科学分野
**山形大学医学部動物実験施設

（平成16年10月13日受理）

要　　旨

高コレステロール血症は、粥状動脈硬化の形成に密接に関わっている[1),2)]。その一方で、高コレステロール血症に合併した高中性脂肪血症が、動脈硬化の進展にどのような役割を果たしているのかについては、まだ一定の見解が得られていない。山形大学医学部動物実験施設では、Watanabe heritable hyperlipidemic (WHHL) rabbits を中性脂肪値にしたがって選抜交配することにより、中性脂肪が高値の群（high-triglyceride rabbit: TGH）と低値の群（low-triglyceride rabbit: TGL）の２系統の遺伝疾患モデルを樹立した。そこで、本研究では TGH の動脈硬化病変の分布と循環動態について検討した。

実験には、24ヶ月齢の日本白色家兎（Japanese White rabbit: JW）と TGH を用いた。摘出した大動脈の動脈硬化病変を、Elastica-Masson trichrome 染色により検討した。また、ケタミンとキシラジンの静脈内投与による持続麻酔下において、大腿動脈圧を測定した。大腿静脈に挿入したカニューレより、NO 合成酵素阻害薬である N^G-nitro-L-arginine methyl ester（L-NAME）を投与し、血圧の経時的変化を測定した。

JW では、検討した大動脈のいずれの部位でも、全く動脈硬化病変を認めなかった。一方、TGH では大動脈弓部に顕著な動脈硬化病変を認めた。定常時血圧は、JW と TGH の間に有意な差はなかった。しかし、TGH の脈圧は、JW よりも有意に大きかった。また、NO 合成酵素阻害薬である L-NAME の投与によって、JW、TGH いずれにおいても血圧が有意に上昇した。しかし、L-NAME 投与後の血圧上昇率には、JW と TGH の間に明らかな差は認められなかった。以上の結果から、TGH の大動脈では動脈硬化が進展していること、それにより血管壁の弾性低下をきたし脈圧を増大させることが示唆された。

キーワード：高脂血症、中性脂肪、動脈硬化、血圧、NO

別刷請求先：片野由美（山形大学医学部看護学科臨床看護学講座）〒990-9585　山形市飯田西２－２－２