the endothelium was removed by gentle rubbing of the intimal surface with a moistened cotton swab.

Tension measurement

Each aortic strip was suspended in an organ bath containing 10 ml of Krebs-Henseleit solution. The composition of the solution was as follows (in mM): NaCl 118, KCl 4.7, $NaHCO_3$ 24.9, $MgSO_4$ 1.18, KH_2PO_4 1.18, $CaCl_2$ 2.5, glucose 11.1, and ascorbic acid 0.057. The solution was saturated with a mixture of 95% O_2 and 5% CO_2 at 37°C (pH 7.4). The developed tension was recorded with an isometric force transducer (7T-15-240, Orientec, Tokyo, Japan). After an equilibration period of 1 hr with a resting tension of 1 g, each strip was contracted with 66.7 mM KCl repeatedly until a reproducible contraction was obtained. A solution containing a high concentration of K^+ solution was made by substituting NaCl with equimolar KCl. Contraction level was expressed as a percentage of the maximal contraction induced by the high potassium solution. Ascorbic acid (0.057 mM) did not affect angiotensin II-induced contraction of aortic strips isolated from WHHL and control rabbits. Relaxation level was expressed as a percentage of the pre-contractile tension induced by phenylephrine (100 nM). Removal of the endothelium was verified by the disappearance of relaxation induced by acetylcholine (1 μM) in strips precontracted with phenylephrine (100 nM).

Measurement of blood lipids

Blood was sampled from a marginal ear artery 18 hr after the last feed. Plasma was separated from the blood samples by centrifugation and stored at −80°C until measurement. Plasma triglyceride and total cholesterol concentrations were measured by enzymatic methods using commercial kits.

Drugs

The drugs used were as follows: angiotensin II, phenylephrine hydrochloride, 5-hydroxytryptamine creatinine sulfate, N^G-nitro-L-arginine, diclofenac and PD123319 (Sigma Chemical, St. Louis, MO, U.S.A.). Angiotensin II, phenylephrine and 5-hydroxytryptamine were dissolved in distilled water to make stock solutions of 0.1 mM, 10 mM and 10 mM, respectively, and diluted with 0.9% NaCl before use. N^G-nitro-L-arginine and diclofenac were dissolved in distilled water to make stock solutions of 10 mM and diluted with 0.9% NaCl before use. PD123319 was dissolved in dimethylsulfoxide to make a stock solution of 10 mM and diluted with 0.9% NaCl before use.

Statistics

Data was expressed as the mean ± S.E.M. Statistical analysis was done using repeated measures ANOVA (analysis of variance) followed by the Scheffé F-test. $P<0.05$ was considered to be statistically significant.

Table 1 Body weight and serum lipid levels of WHHL rabbits and control JW rabbits.

	Body weight (kg)	Serum triglyceride (mg/dl)	Serum total cholesterol (mg/dl)
WHHL rabbits	2.30 ± 0.01*	2035.9 ± 445.4*	1131.5 ± 65.5*
Control rabbits	2.53 ± 0.01	65.0 ± 18.9	53.2 ± 3.6

Data are shown as the mean ± SE. Asterisks denote significant difference from values of the control rabbits (P<0.01). n=17–20.

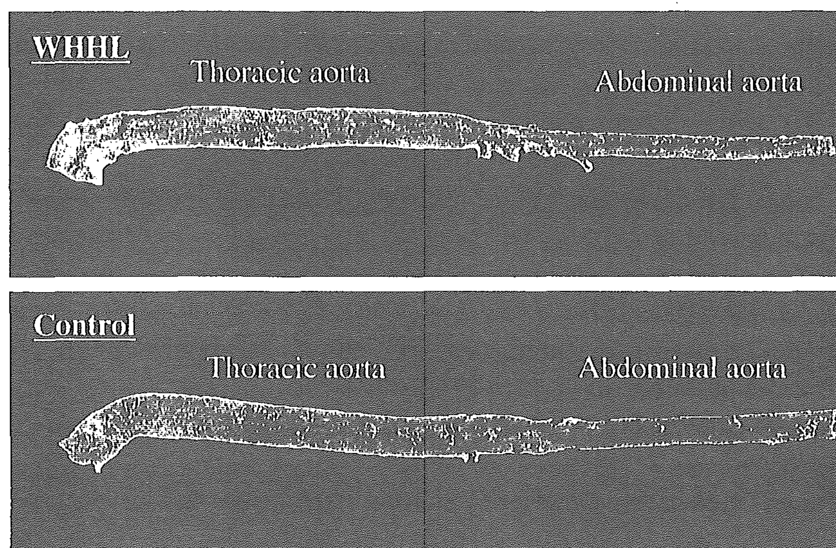

Fig. 1. Representative macroscopic images of aortae isolated from Watanabe hereditary hyperlipidemic (WHHL) and Japanese White (control) rabbits. Note the marked plaques in the thoracic region of the WHHL aorta.

Results

Body weight and blood lipid levels

Table 1 provides a comparison of the body weight and blood lipid levels of the WHHL and control rabbits. Body weight was slightly but significantly lower in the WHHL rabbits than in the control rabbits. Serum triglyceride and total cholesterol levels were markedly higher in the WHHL rabbits than in the control group.

Macroscopic observation of the aortae

Figure 1 compares representative macroscopic images of a descending aorta isolated from both a WHHL and a control rabbit. In the control rabbit aorta, no atherosclerotic plaques were observed. In the WHHL rabbit aorta, the thoracic portion of the descending aorta displayed large plaques, while plaques were not apparent in the abdominal portion of the aorta.

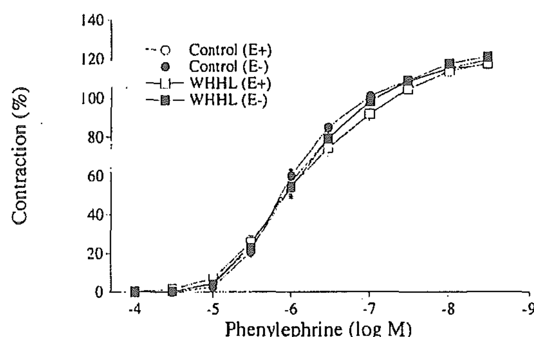

Fig. 2. Phenylephrine-induced contraction of abdominal aortic strips prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. E+, endothelium-intact; E–, endothelium-denuded. n=6–10.

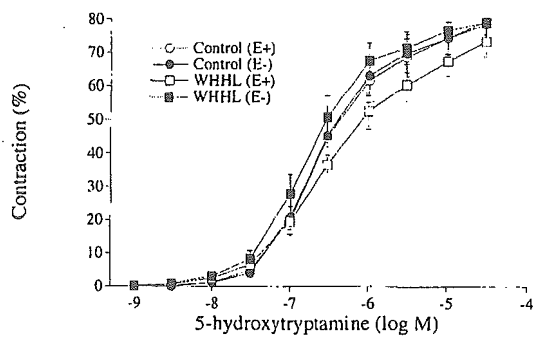

Fig. 3. 5-Hydroxytryptamine-induced contraction of abdominal aortic strips prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. E+, endothelium-intact; E–, endothelium-denuded. n=5–7.

Contractile responses to KCl, phenylephrine and 5-hydroxytryptamine

The maximum level of KCl-induced contraction of aortic strips was not significantly different between WHHL rabbits and control rabbits [8.39 ± 0.42 mN/mg tissue (WHHL) *v.s.* 8.53 ± 0.35 mN/mg tissue (control)]. Thus, we used this as the standard level of contraction for each strip. Both phenylephrine- and 5-hydroxytryptamine-induced contractile responses of aortic strips occurred to the same extent in both the WHHL and control groups (Figs. 2 and 3).

Angiotensin II-induced contractile responses in the presence or absence of endothelium

Figure 4 displays the concentration-force relationships of angiotensin II contracture of the aorta isolated from both WHHL and control rabbits in the presence or absence of endothelium. In each case, the angiotensin II-induced contraction was higher in strips without endothelium than in those with the endothelium. However, the increment in the angiotensin II-induced contraction which occurred after removal of the endothelium was less in the WHHL aorta than in the control aorta. Regardless of the presence of the endothelium, angiotensin II-induced contraction in the WHHL group was significantly lower than in the control group.

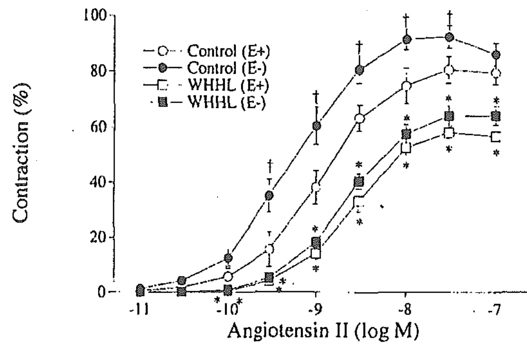

Fig. 4. Angiotensin II-induced contraction of abdominal aortic strips prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. E+, endothelium-intact; E–, endothelium-denuded. *, significantly different ($P<0.05$) from the values of strips isolated from the control rabbits; †, significantly different ($P<0.05$) from the values of strips with endothelium. n=5–12.

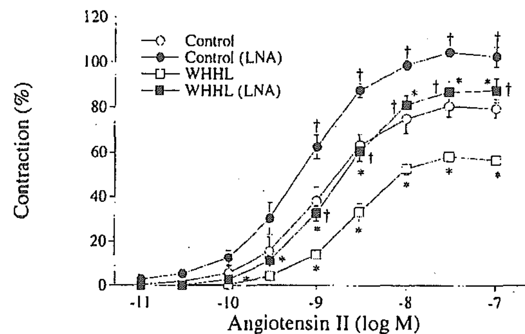

Fig. 5. Angiotensin II-induced contraction of abdominal aortic strips (endothelium-intact) prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. LNA: The aortic strips were pretreated with N^G-nitro-L-arginine (LNA, 100 μM) for 20 min before stimulation with angiotensin II. *, significantly different ($P<0.05$) from the values of strips isolated from the control rabbits; †, significantly different ($P<0.05$) from the values of strips incubated with N^G-nitro-L-arginine. n=5–12.

Angiotensin II-induced contraction in the presence of nitro-L-arginine

In both the WHHL and control groups, pretreatment with nitro-L-arginine significantly enhanced the contractile response to angiotensin II in aortic strips with intact endothelium (Fig. 5). Angiotensin II-induced contraction in the presence of nitro-L-arginine was significantly lower in the WHHL group than in the control group.

Angiotensin II-induced contraction in the presence of diclofenac

In both the WHHL and control groups, the contractile response to angiotensin II in the aorta with the intact endothelium was not significantly affected by diclofenac (Fig. 6). The angiotensin II-induced contraction in the presence of diclofenac was significantly lower in the WHHL group than in the control group.

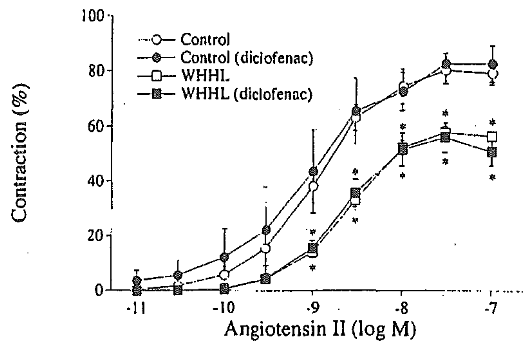

Fig. 6. Angiotensin II-induced contraction of abdominal aortic strips (endothelium-intact) prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. diclofenac: The aortic strips were pretreated with diclofenac (10 μM) for 20 min before stimulation with angiotensin II. *, significantly different ($P<0.05$) from the values of strips isolated from the control rabbits; †, significantly different ($P<0.05$) from the values of strips incubated with diclofenac. n=5–12.

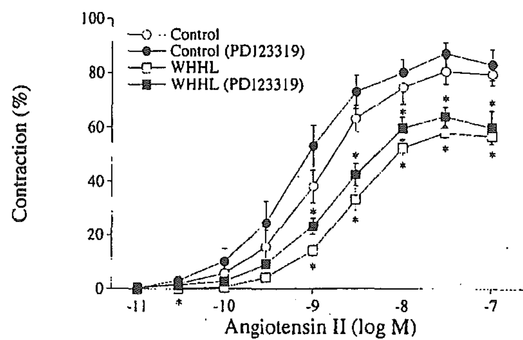

Fig. 7. Angiotensin II-induced contraction of abdominal aortic strips (endothelium-intact) prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. PD123319: The aortic strips were pretreated with PD123319 (1 μM) for 20 min before stimulation with angiotensin II. *, significantly different ($P<0.05$) from the values of strips isolated from the control rabbits. n=5–12.

Angiotensin II-induced contraction in the presence of PD123319

In both the WHHL and control groups, PD123319 only slightly but not significantly increased the angiotensin II-induced contraction of aortic strips with an intact endothelium (Fig. 7). The angiotensin II-induced contraction in the presence of PD123319 was significantly lower in the WHHL group than in the control group.

Acetylcholine-induced relaxing response

Acethylcholine-induced relaxation of aortic strips occurred to the same extent in both the WHHL and control groups (Fig. 8).

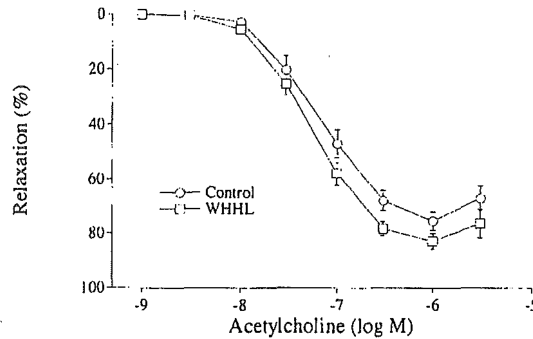

Fig. 8. Acetylcholine-induced relaxation of abdominal aortic strips (endothelium-intact) prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. n=6–10.

Discussion

There have been conflicting results reported about the contractile responses of atherosclerotic arteries to angiotensin II. Dam *et al.* (1997) reported that contractile responses to angiotensin II and methoxamine were decreased in the thoracic aorta but not in the iliac artery of rabbits fed with a cholesterol diet (0.3%) for 12 weeks. On the contrary, Yang *et al.* (1998) found that angiotensin II contraction, as well as its type 1 receptor expression, was increased in the thoracic aorta isolated from rabbits fed with a diet of cholesterol (1%) and coconut oil (4%) for 10 weeks. Moreover, Merkel *et al.* (1990) observed no change in angiotensin II-induced contraction of the abdominal aorta isolated from rabbits fed with a cholesterol-free, casein-rich diet for 10 weeks. These discrepant results might be due to the stage and severity of the atherosclerosis in the vessels used for the experiments. On the other hand, little is known about changes in angiotensin II contraction in arteries of WHHL rabbits. Our recent study has shown that the thoracic aorta isolated from WHHL rabbits displayed a decreased contractile response to angiotensin II, while its type 1 (AT1) receptor expression was increased in the aorta of young WHHL rabbits at 3–4 months of age (Shishido *et al.*, 2004). The present study is the first to demonstrate that angiotensin II-induced contraction was also specifically attenuated in the abdominal aorta of WHHL rabbits, while their 5-hydroxytryptamine-induced contraction was not significantly different from the aorta of control rabbits. However, both the abdominal aorta and the thoracic aorta of diet-induced atherosclerotic rabbits showed augmented contractile responses to 5-hydroxytryptamine (Henry and Yokoyama, 1980; Merkel *et al.*, 1990; Chin *et al.*, 1990). The WHHL rabbit aorta has also been reported to show increased contractility in response to 5-hydroxytryptamine (Yokoyama *et al.*, 1983). The discrepant results of the 5-hydroxytryptamine contraction in the atherosclerotic aorta in these previous studies and those of the present study may also be due to the stage and severity of atherosclerosis, because atherosclerotic plaques were not macroscopically observed in the descending aortae used in the present study. Moreover, the aorta from 1-month-old animals was reportedly more sensitive to 5-hydroxytryptamine than that from 6-month-old

animals (Wines *et al.*, 1989), suggesting that age could also affect the contractile response to 5-hydroxytryptamine.

In the present study, we examined whether endogenous vasodilatory substances were involved in attenuation of the contractile response to angiotensin II in the WHHL rabbit aorta. The decrease in contractile force produced by angiotensin II in the WHHL aorta was also observed in the absence of the endothelium, indicating that the decrease in angiotensin II contraction was not due to vasoactive substances released from the endothelium. NO and prostacyclin are major vasodilatory substances that regulate vascular tone and that are produced in both the vascular intima and media (Moncada *et al.*, 1991 Vane and Botting, 1993; Mitchell and Evans, 1998). Pretreatment of nitro-L-arginine, a NO synthase inhibitor, significantly augmented the contractile response to angiotensin II in the aorta with an intact endothelium in both WHHL and control rabbits. Thus, NO negatively regulates the contractile response to angiotensin II. However, angiotensin II-induced contraction in the presence of nitro-L-arginine was also lower in the WHHL group than in the control group, suggesting that NO is not involved in the diminution of angiotensin II contraction in the WHHL rabbit abdominal aorta. Moreover, acetylcholine-induced endothelium-dependent aortic relaxation, which is mainly due to NO release from the endothelium, was not significantly different between the WHHL and control groups. In contrast, several studies have shown a decrease in endothelium-dependent relaxation in the aorta of WHHL rabbits (Ragazzi *et al.*, 1989; Kolodgie *et al.*, 1990) as well as in the aorta of high-cholesterol-diet-induced atherosclerotic rabbits (Sreeharan *et al.*, 1986; Jayakody *et al.*, 1988). The lack of impairment of endothelium-dependent vasodilation in the WHHL rabbit abdominal aorta in the present study may also be due to a lesser degree of atherosclerosis. Moreover, our recent study demonstrated that attenuation of endothelium-dependent relaxation of the WHHL rabbit thoracic aorta, which displayed markedly large atherosclerotic plaques, was observed only in those animals with hypertriglyceridemia (Shishido *et al.*, 2004), suggesting that hypertriglyceridemia aggravates hypercholesterolemia-induced functional impairment of endothelial cells. The contractile force of the aorta in response to angiotensin II was not affected by pretreatment with diclofenac, a cyclooxygenase inhibitor, while that in the presence of diclofenac was also lower in the WHHL group than in the control group. This finding agrees with the finding of a study by Forstermann *et al.* (1984) that prostaglandin release was enhanced by angiotensin II in both the rabbit coeliac artery and pulmonary artery but not in either the aorta or femoral artery. Therefore, cyclooxygenase products such as prostacyclin do not influence the vascular tone of the angiotensin II response and are not involved in attenuation of the angiotensin II contraction of the WHHL rabbit aorta. Angiotensin II contraction is mediated mainly via stimulation of AT1 receptors in vascular smooth muscle cells. In addition, angiotensin II has recently been reported to induce vasodilation directly via stimulation of its type 2 (AT2) receptor (Widdop *et al.*, 2003). However, hypocontractility to angiotensin II of the WHHL rabbit aorta was also observed in the presence of PD123319, an AT2 receptor antagonist, indicating that AT2 receptor-mediated vasodilation is not involved in the hypocontractility of the WHHL rabbit aorta. The above findings suggest that the decrease in the angiotensin II contraction in the WHHL rabbit aorta is mainly due to an abnormality in smooth muscle contractility, and is not due to changes in the release of vasodilatory substances from the vessel wall. AT1 receptor

expression has rather been shown to be up-regulated in the thoracic aorta of WHHL rabbits (Shishido *et al.*, 2004). Thus, further study including AT1 receptor coupling with downstream signals in vascular smooth muscle cells is needed to clarify the mechanism involved in the attenuated contractile response to angiotensin II in the WHHL aorta. Furthermore, it is also of interest to determine whether other angiotensin II-mediated biological actions in vascular smooth muscle, such as protein synthesis, mitogenesis and hypertrophy, are altered in arteries of WHHL rabbits.

In conclusion, the WHHL rabbit abdominal aorta displays attenuated angiotensin II-induced contraction, which is suggested to be mainly due to an abnormality in the AT1-receptor-mediated contractile pathway of medial smooth muscle.

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PROGRESSION OF SEVERE ATHEROSCLEROSIS AND INCREASED ARTERIAL PULSE PRESSURE IN THE NEWLY DEVELOPED HERITABLE MIXED HYPERLIPIDAEMIC RABBITS

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SUMMARY

1. We have recently segregated a new line of rabbit, named TGH, with severely high levels of plasma triglyceride and cholesterol. The aim of the present study was to investigate the progression of atherosclerosis and haemodynamic parameters in TGH rabbits.

2. Japanese white (JW) and TGH rabbits (24–27 months old) were anaesthetized with ketamine and xylazine. Plasma concentrations of triglyceride were 63.1 ± 8.0 and 446.0 ± 35.2 mg/dL in JW and TGH rabbits, respectively. Blood pressure was measured by a catheter implanted in the femoral artery. Histological examinations were performed using haematoxylin–eosin and elastica–Masson trichrome staining to detect atherosclerotic lesions.

3. The JW rabbits had no atherosclerotic lesions. In TGH rabbits, severe atherosclerotic lesions were observed throughout the aorta, especially in the aortic arch. Basal femoral arterial pressure was not significantly different between JW and TGH rabbits. However, the basal pulse pressure in TGH rabbits (48.3 ± 4.5 mmHg) was significantly greater than that of JW rabbits (28.0 ± 5.6 mmHg). Intravenous infusion of N^G-nitro-L-arginine methyl ester (L-NAME; 26.9 mg/kg) increased the blood pressure of TGH and JW rabbits. There was no significant difference in the response to L-NAME between the two rabbit strains.

4. The present study shows that severe atherosclerotic changes develop in TGH rabbits and suggests that the hyperlipidaemia combined with hypercholesterolaemia and hypertriglyceridaemia is an important factor for promoting atherosclerosis in TGH rabbits. The greater pulse pressure in TGH rabbits may be due to the increased vascular stiffness with atherosclerosis.

5. This newly developed TGH rabbit line of heritable hypertriglyceridaemia with hypercholesterolaemia will become a useful animal model for studies on the role of hyperlipidaemia in the progression of atherosclerosis and in many atherosclerosis-related diseases.

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Key words: arterial blood pressure, atherosclerosis, cholesterol, combined hyperlipidaemia, hypertriglyceridaemia, nitric oxide, pulse pressure, rabbit, triglyceride.

INTRODUCTION

Atherosclerosis is one of the major triggers for ischaemic heart disease, cerebrovascular disease and obstructive arteriosclerosis. Various factors are involved in the progress of atherosclerosis. Of them, hyperlipidaemia, such as hypercholesterolaemia, is regarded as the most widely demonstrated risk factor for these diseases through the progression of atherosclerotic plaques based on epidemiological studies,[1] as well as experimental studies using Watanabe heritable hyperlipidaemic (WHHL) rabbits, which have a defect in their low-density lipoprotein (LDL) receptors. The WHHL rabbits have been used to demonstrate that hypercholesterolaemia promotes fatty streak formation and atherosclerosis at the vascular wall and reduces endothelium-dependent relaxation.[2,3]

Hypercholesterolaemia may cause a progression of the formation of fatty streaks, fibrous plaques and atherosclerosis via uptake of oxidized LDL into foam cells.[4,5] Because cholesterol is metabolised and finally transported into blood as LDL, cholesterol is regarded as the most important factor for the promotion of early stage atherosclerosis. In addition to pathohistological changes, hyperlipidaemia can induce dysfunction of blood vessels. This may be due to endothelial injury caused by lipoproteins, such as oxidized LDL and β very low-density lipoprotein (VLDL).[6] The production of nitric oxide (NO) by activation of endothelial NO synthase (eNOS) is diminished and the endothelium-dependent vasorelaxant responses are decreased in atherosclerosis and hyperlipidaemic conditions.[6,7]

In contrast with the important role of cholesterol, it has not been evaluated, in detail, whether hypertriglyceridaemia is a risk factor for the progression of atherosclerosis. Recent epidemiological studies have demonstrated that hypertriglyceridaemia is a risk factor for coronary heart diseases.[8–10] The remnant lipoproteins that contain triglyceride are hydrolysed by lipoprotein lipase and are able to infiltrate into vessel walls to develop atherosclerotic lesions.[11] Macrophages can take up remnant lipoproteins via surface receptors (LDL receptor, VLDL receptor and apoB48 receptor) to become foam cells. As a result, endothelial function may be impaired, smooth muscle cell proliferation is enhanced and the vascular contractile response to various agonists is enhanced.[5] However, in physiological studies on vascular function, the contribution of hypertriglyceridaemia

to endothelial and medial smooth muscle function has not been determined clearly.[12–14]

We have recently segregated a new rabbit line, namely TGH rabbits, of mixed hyperlipidaemia with markedly high concentrations of plasma triglycerides (TG; > 500 mg/dL in young) and cholesterol[15,16] to investigate the role of mixed hyperlipidaemia in the progression of atherosclerosis. Until now, the haemodynamic parameters of TGH rabbits have not been determined. The aim of the present study was to examine changes in the haemodynamic parameters in TGH rabbits and to evaluate the role of mixed hyperlipidaemia in the progression of atherosclerosis.

METHODS

Animals

Experiments were performed in accordance with the *Guide for Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996; http://www.nap.edu/readingroom/books/labrats/index.html) and under the regulations of the Animal Care Committee of Yamagata University School of Medicine.

Watanabe heritable hyperlipidaemic rabbits were bred since 1991 in the Laboratory Animal Center, Yamagata University School of Medicine. There was wide interindividual variability in plasma TG levels in WHHL rabbits. The definition of hypertriglyceridaemia in the present study was TG > 500 mg/dL in plasma. Based on the level of plasma TG (> 500 mg/dL) of WHHL rabbits, selected inbreedings were repeated up to the seventh generation. The result was that hypertriglyceridaemia had a high penetrance with 93% and finally 100% of phenotype expression at the fourth and fifth generations, respectively. Therefore, these rabbits were named 'TGH rabbits'. We used the seventh generation of TGH rabbits in the present study.

Male 24–27-month-old Japanese white rabbits (weighing 3.7 ± 0.1 kg; $n = 5$) and TGH rabbits (weighing 2.50 ± 0.02 kg; $n = 6$) were used to measuring haemodynamic parameters and for histological examination. In addition, three male young (3 months) and aged (30 months) TGH rabbits were used for histological examination to investigate the progression of atherosclerosis. 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, Tokyo, Japan). Rabbits were anaesthetized with ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg) via the marginal ear vein. Then, anaesthesia was maintained by continuous intravenous infusion of ketamine (0.11 mg/kg per min) and xylazine (0.02 mg/kg per min). Maintenance of body temperature was achieved with the aid of a heating pad.

Measurements of arterial blood pressure and electrocardiogram

Catheters were placed in the right femoral artery for continuous recording of arterial blood pressure and in the left femoral vein for the infusion of drugs. Arterial pressure was measured by a pressure transducer and recorded on a thermal array recorder (RTA 1200M; Nihon Kohden, Tokyo, Japan). Electrodes were attached to the shaved area on each limb for recording the surface electrocardiogram (ECG). The ECG was recorded continuously using limb lead II. In some experiments, the NOS inhibitor N^G-nitro-L-arginine methylester (L-NAME) was infused through the left femoral vein. We prepared a 100 mmol/L stock solution of L-NAME (26.9 mg/mL) and used 26.9 mg/kg L-NAME to inhibit NOS sufficiently and specifically.[17–19]

Histological analysis of aorta

Rabbits were killed with an intravenous overdose of pentobarbitone sodium (300 mg) at the end of each experiment. Thoracic aortas obtained from JW and TGH rabbits were divided into four portions (I, aortic arch; II, proximal thoracic aorta; III, middle thoracic aorta; and IV, distal thoracic aorta) and fixed overnight in 10% formaldehyde at 4°C. Tissues were embedded in paraffin and cut into 4 μm cross-sections. Microscopic examination of haematoxylin–eosin- and elastica–Masson's trichrome-stained sections was performed to assess atherosclerotic changes in the aortas. In the stained cross-sections, the luminal surface and atherosclerotic plaque areas were compared among aortic portions.

Plasma lipid analysis

Blood samples were taken from a marginal ear artery 18 h after the last feeding. All blood samples were centrifuged at 1 000 g for 15 min at 4°C and the plasma was stored at −80°C until assay. Plasma concentrations of total cholesterol and TG were measured by enzymatic methods using SPOTCHEM-EZ (Arkray, Kyoto, Japan).

Statistical analysis

All data are expressed as the mean±SEM. Statistical analysis was performed with unpaired t-tests or Welch's t-tests. To compare changes in blood pressure, non-parametric analysis (Mann–Whitney U-test) was used. $P < 0.05$ was considered statistically significant.

Drugs

Ketamine hydrochloride (Sankyo Pharmaceutical, Tokyo, Japan), xylazine (Bayer, Tokyo, Japan) and L-NAME (Sigma Chemical, St Louis, MO, USA) were used.

RESULTS

Bodyweight and plasma cholesterol and TG levels

The bodyweight of TGH rabbits was significantly lower than that of JW rabbits. Total cholesterol levels in TGH rabbits were significantly higher than those in JW rabbits. In addition, plasma TG levels of TGH rabbits were markedly higher compared with those of JW rabbits. Plasma cholesterol concentrations were 58.2 ± 5.1 and 442.7 ± 27.7 mg/dL in JW and TGH rabbits, respectively. Concentrations of TG were 63.1 ± 8.0 and 446.0 ± 35.2 mg/dL in JW and TGH rabbits, respectively (Table 1).

Histological study

Figure 1 shows the histological changes in each portion of the aortas of JW and TGH rabbits. In JW rabbits, no detectable atheromatous lesions were observed in any portion of the aorta. Examination revealed normal intima containing an intact monolayer of endothelium and a major portion of the media. In contrast, remarkable atherosclerotic

Table 1 Plasma lipid concentrations in adult Japanese white and TGH rabbits

	Bodyweight (kg)	Plasma lipid (mg/dL)		Age (months)	n
		Total cholesterol	Triglyceride		
JW	3.73 ± 0.10	58.2 ± 5.1	63.1 ± 8.0	24.8 ± 0.4	5
TGH	2.47 ± 0.02	442.7 ± 27.7	446.0 ± 35.2	26.7 ± 0.3	6

JW, Japanese white rabbits; TGH, high-triglyceride rabbits.

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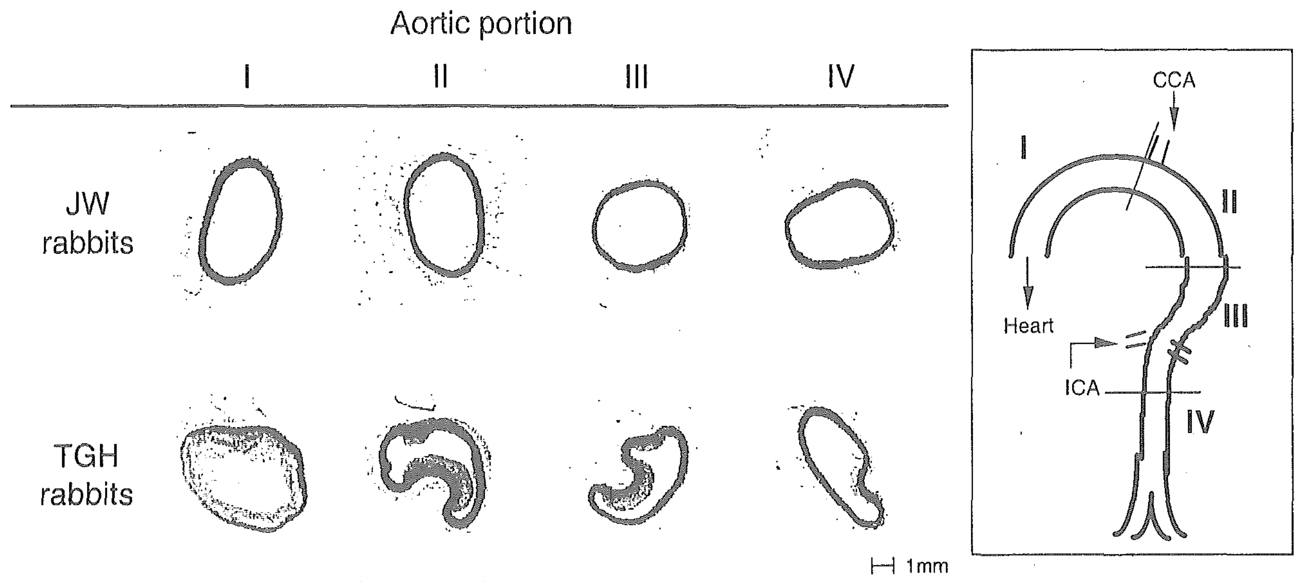

Fig. 1 Representative staining of aortic sections in Japanese white (JW) and TGH (high-triglyceride) rabbits. Portion I, aortic arch; II, proximal thoracic aorta; III, middle thoracic aorta; IV, distal thoracic aorta; CCA, common carotid artery; ICA, intercostal artery.

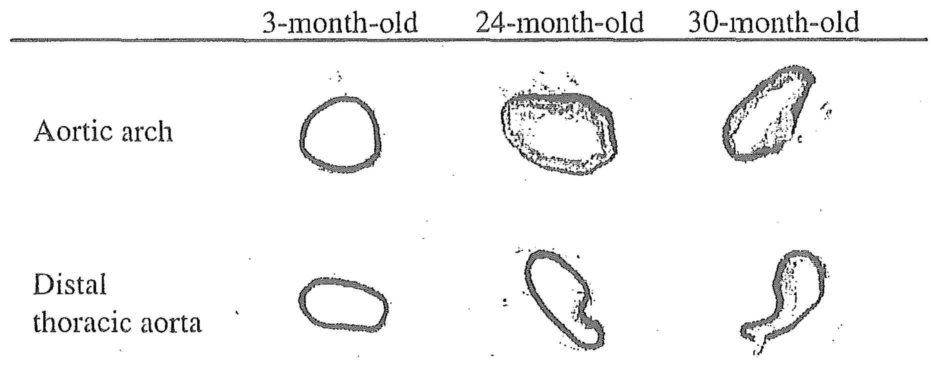

Fig. 2 Age-dependent changes in atherosclerotic lesions in 3-, 24- and 30-month-old TGH rabbits. Representative staining of the aortic arch and distal thoracic aorta.

Table 2 Basal arterial pressure in Japanese white and TGH rabbits

	JW ($n = 5$)	TGH ($n = 6$)
SBP (mmHg)	104.2 ± 8.5	120.8 ± 6.4
DBP (mmHg)	76.2 ± 5.9	72.5 ± 4.5
MAP (mmHg)	85.5 ± 6.3	88.6 ± 4.8
PP (mmHg)	28.0 ± 5.6	48.3 ± 4.5*

Data are the mean±SEM. *$P < 0.01$ compared with Japanese white (JW) rabbits.

TGH, high-triglyceride rabbits; SBP, systolic blood pressure; DBP, *diastolic pulse pressure; MAP, mean arterial pressure; PP, pulse pressure.*

changes were observed throughout the aortas of TGH rabbits, from the proximal to distal portion. Lesions were most extensive in the aortic arch, whereas atherosclerotic changes seemed to be less in the distal thoracic aorta (Fig. 1). The aortic lesions were characterized by cell proliferation, foam cell accumulation and calcification in the intima. In contrast with the atherosclerosis observed in 24-month-old (young adults for normal rabbits, whose life span is 6–7 years) rabbits, young TGH rabbits (3 months old) did not exhibit atherosclerotic changes in the aorta. In 30-month-old TGH rabbits, the atherosclerotic lesion was considerably extended to the distal thoracic aorta (Fig. 2).

Femoral arterial pressure

Basal arterial pressure

Basal femoral arterial pressure in JW rabbits was 104.2 ± 8.5 mmHg in systole and 76.2 ± 5.9 mmHg in diastole, whereas that in TGH rabbits was 120.8 ± 6.4 mmHg in systole and 72.5 ± 4.5 mmHg in diastole. The systolic pressure in TGH rabbits tended to be higher than that in JW rabbits, but there was no significant difference. However, the pulse pressure was significantly greater in TGH than in JW rabbits (48.3 ± 4.5 *vs* 28.0 ± 5.6 mmHg, respectively; Table 2). The heart rate in JW and TGH rabbits was 195 ± 7 and 185 ± 11 b.p.m., respectively.

Effect of L-NAME

To elucidate whether NO was involved in regulating blood pressure, the effect of L-NAME on femoral arterial blood pressure was examined.

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Table 3 Mean arterial pressure and pulse pressure in Japanese white and TGH rabbits after infusion of N^G-nitro-L-arginine methyl ester

	JW (n = 5)	TGH (n = 6)
SBP (mmHg)	122.8 ± 6.2	141.3 ± 9.8
DBP (mmHg)	91.0 ± 3.7	95.2 ± 5.9
MAP (mmHg)	101.6 ± 2.4	110.6 ± 6.9
% Basal value	121.5 ± 8.4†	125.1 ± 3.5†
PP (mmHg)	31.8 ± 7.9	50.8 ± 3.6*
% Basal value	107.0 ± 10.0	98.0 ± 5.5

Data are the mean±SEM. *$P < 0.01$ compared with Japanese white (JW) rabbits; $^†P < 0.05$ compared with basal values.

TGH, high-triglyceride rabbits; SBP, systolic blood pressure; DBP, diastolic pulse pressure; MAP, mean arterial pressure; PP, pulse pressure.

Before infusion of L-NAME, the mean arterial pressure (MAP) in JW and TGH rabbits was 85.5 ± 6.3 and 88.6 ± 4.8 mmHg, respectively. After 10 min, the MAP was 101.6 ± 2.4 and 110.6 ± 6.9 mmHg in JW and TGH rabbits, respectively (Table 3). However, there was no significant difference in the percentage increase in pressure between JW and TGH rabbits (121.5 ± 8.4 and 125.1 ± 3.5%, respectively).

DISCUSSION

In the present study, we investigated haemodynamic parameters in a newly developed rabbit line of heritable hypertriglyceridaemia with hypercholesterolaemia (TGH rabbits) and evaluated the role of mixed hyperlipidaemia in the progression of aortic atherosclerosis histologically.

It is well known that hyperlipidaemia is a major risk factor for atherosclerosis. Epidemiological studies have suggested a relationship between serum TG and the risk of coronary heart disease.[8-10] Recently, it has been demonstrated that hypertriglyceridaemia induces an endothelial dysfunction through increased oxidative stress. The generated superoxide anion can react with NO and produce cytotoxic peroxynitrite.[20] This mechanism may be one possibility for the accelerated atherosclerosis observed in TGH rabbits. In humans, the TG level is an important and independent risk factor for cardiovascular diseases, such as myocardial infarction.[21-25] In addition, an interaction between total cholesterol and TG concentration on the risk of myocardial infarction has been reported.[26,27] Therefore, the new TGH rabbit line may become a useful model of these human diseases associated with hypertriglyceridaemia.

Histopathological changes in the aorta

Of the young rabbits (3 months old), TGH rabbits did not exhibit atherosclerotic changes in the aorta. At 24 months of age (the young-adult age), TGH rabbits showed atherosclerosis and the atherosclerotic lesion was markedly extended to the distal thoracic aorta by 30 months of age.

In JW rabbits, an intact monolayer of endothelial cells covered the aortic lumen and foam cells were not observed in any portion of the aorta examined. In TGH rabbits, endothelial cells were partly detached and fibrous intimal thickening, infiltration of medial smooth muscle cells into the subintima and foam cells were observed. These results suggest that, in TGH rabbits, endothelial cells were damaged and the process of atherosclerosis was proceeding. The production of various cytokines and growth factors by macrophages may further accelerate the migration and growth of medial smooth muscle cells to form atherosclerotic lesions.

The present data show that the degree of atherosclerosis in 24-month-old TGH rabbits is as severe as that in 20–24-month-old WHHL rabbits.[28,29] However, in TGH rabbits, atherosclerotic changes were distributed widely from the aortic arch to the distal thoracic aorta and the pathological changes were most severe in the aortic arch. In contrast, severe histopathological changes tend to occur in the aortic arch and at the bifurcation of arteries in WHHL rabbits.[28] These differences in susceptibility to atherosclerosis may be due to differences in plasma lipids between TGH and WHHL rabbits.

Regulation of basal arterial pressure

Under basal conditions, the systolic and diastolic blood pressures, as well as MAP, of TGH rabbits with severe atherosclerosis were not significantly different those in JW rabbits. These results are consistent with a previous report that the basal blood pressure of WHHL rabbits with atherosclerosis was not higher than that of JW rabbits.[30] Although the blood pressure of TGH rabbits was not significantly different from that of JW rabbits, the pulse pressure of TGH rabbits was significantly greater compared with that of JW rabbits. There is one previous study indicating that the pulse pressure in WHHL rabbits increases gradually with ageing.[31] In addition, 24-month-old Kurosawa and Kusanagi-Hypercholesterolaemic (KHC) rabbits showed greater pulse pressure than 10-month-old KHC rabbits.[32] It should be noted that the increase in pulse pressure observed in these reports may be caused by ageing-related processes rather than hypercholesterolaemia, because elevated plasma cholesterol *per se* may not contribute to the reduced aortic compliance.[33,34] Therefore, the elevated pulse pressure in TGH rabbits compared with that in age-matched JW rabbits in the present study suggests that the elevated pulse pressure may be due to hypertriglyceridaemia or hypertriglyceridaemia with hypercholesterolaemia characterized in TGH rabbits.

It is possible that decreased compliance in atherosclerotic vessels caused the increase in pulse pressure in TGH rabbits, because it has been reported previously that the progression of atherosclerosis reduced compliance in large vessels to increase pulse pressure.[35,36] Pulse pressure arises from the interaction of cardiac stroke volume and the properties of the arterial circulation. In general, increased aortic stiffness of the aorta and large arteries leads to an increase in pulse pressure through a reduction in arterial compliance and effects on wave reflection.[35] Very recently, it has been demonstrated that arterial stiffness is associated with central pulse wave velocity (PWV) and that the PWV correlates well to brachial and central pulse pressure.[37] Therefore, it can be said that central arterial stiffness is closely related to central pulse pressure. Based on these facts, although we did not measure PWV or wall compliance for arterial stiffness directly, the increased pulse pressure in TGH rabbits could be due to arterial stiffness caused by atherosclerosis.

It is quite likely that the elevation of pulse pressure may induce vascular damage and endothelial dysfunction,[36] which can become a possible precursor of atherosclerosis. In fact, it has been found that acetylcholine-induced endothelium-dependent relaxation was decreased in aortas isolated from TGH rabbits.[16] The progression of vascular atherosclerosis and remodelling leads, in turn, to an increase in arterial wall stiffness, thus further amplifying pulse

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pressure. Such a vicious cycle probably promotes the progression of atherosclerosis in TGH rabbits to cause many diseases under hyperlipidaemic conditions. Many clinical studies have reported that baseline pulse pressure is a good predictor of subsequent cardiovascular events.[38–40]

Role of NO in the regulation of arterial pressure

Endothelial cells contribute to the maintenance of homeostasis in vascular function and the regulation of systemic circulation via production of NO and endothelial dysfunction is closely related to the progression of atherosclerosis.[6,7] In hyperlipidaemia, the endothelial cell-dependent relaxation of the aorta is impaired, even in early stages without overt histopathological atherosclerotic changes,[3] because lipoproteins, such as oxidized LDL, could injure the endothelial cells to decrease NO production prior to forming atheromatous plaques. In fact, in WHHL rabbits, it has been reported that the plasma concentration of NO is lower than that in JW rabbits[41] and endothelium-dependent vascular relaxation is decreased.[41] It is possible that the decreased basal NO production as a result of endothelial damage may increase the blood pressure through an increase in the tension of vascular walls. Therefore, in the present study, to evaluate the change in basal production of NO in TGH rabbits, we examined the effect of the NOS inhibitor L-NAME on blood pressure. It has been reported previously that N^G-nitro-L-arginine and its methylesterified L-NAME, at 20 mg/kg, increase arterial pressure with decreasing total peripheral conductance in the rabbits.[17] It has been demonstrated that this dose produces maximum blockade of NOS.[17,18] In addition, these NOS blockers were shown to inhibit the hypotensive actions of acetylcholine and the effects were reversed by L-arginine, but not D-arginine, indomethacin or prazosin, demonstrating that the effects were specific to NOS inhibition.[19]

After the addition of L-NAME, systolic blood pressure increased 16 mmHg in JW rabbits and 22 mmHg in TGH rabbits, with no significant difference in responses between JW and TGH rabbits. Therefore, TGH rabbits may produce sufficient NO, as do JW rabbits, although we did not measure the concentration of plasma NO directly in the present study. There are three isoforms of NOS, namely neuronal (n) NOS, inducible (i) NOS (NOS2) and eNOS (NOS3). Of these, nNOS is distributed throughout central and peripheral nerve cells, as well as pancreatic β cells, and is regulated by intracellular calcium, whereas iNOS, which is induced by lipopolysaccarides in hepatic cells, macrophages, intestinal epithelial cells, vascular smooth muscle cells and glial cells, is regulated independently of calcium. Endothelial NOS exists in vascular endothelial cells and its activity is regulated by calcium and the phosphorylation of enzymes.[42] In TGH rabbits, aortic expression of eNOS mRNA and its protein was equivalent to that of JW rabbits, whereas endothelium-dependent vascular relaxation to acetylcholine was reduced in TGH rabbits compared with JW rabbits.[16] These results suggest that the level of eNOS expression is not directly related to NO production in the aorta from TGH rabbits. It could be speculated that iNOS was induced and activated in vascular smooth muscle cells, as well as in macrophages, within atherosclerotic lesions of TGH rabbits to increase the local production of NO. Therefore, it may be important to examine the role of macrophages or vascular smooth muscle cells as a possible source of NO in TGH rabbits. This problem remains to be elucidated.

Genetic background of TGH rabbits

In order to reveal the mode of inheritance of hypertriglyceridaemia, three rabbits with hypertriglyceridaemia were crossed with two wild-type JW rabbits. The F_1 rabbits did not show either hypertriglyceridaemia or hypercholesterolaemia. By intercrossing five pairs of F_1 rabbits, 33 F_2 animals were obtained. Of these, only seven of the 33 rabbits exhibited both hypertriglyceridaemia and hypercholesterolaemia. These results suggest that, in the TGH rabbits, the phenotype of hypertriglyceridaemia follows a single Mendel's law, exhibiting an autosomal recessive inheritance, which is similar to hypercholesterolaemia shown in WHHL rabbits. At present, the main genetic defects in TGH rabbits have not been clarified. Further studies are needed to determine the cause of the elevated triglyceridaemia in this strain.

In summary, the present study has shown that severe atherosclerotic changes developed in TGH rabbits and suggests that hyperlipidaemia with hypercholesterolaemia and hypertriglyceridaemia is an important risk factor for atherosclerosis in TGH rabbits. In addition, the pulse pressure of TGH rabbits was significantly elevated compared with that of normal rabbits. This newly developed TGH rabbit line with heritable hypertriglyceridaemia will become a useful animal model for studies on the role of hyperlipidaemia in the progression of atherosclerosis and in many atherosclerosis-related diseases.

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Fenofibric Acid, an Active Form of Fenofibrate, Increases Apolipoprotein A-I–Mediated High-Density Lipoprotein Biogenesis by Enhancing Transcription of ATP-Binding Cassette Transporter A1 Gene in a Liver X Receptor–Dependent Manner

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Objective—Fibrates are widely used drugs to reduce plasma triglyceride and increase high-density lipoprotein. Their active forms, fibric acids, are peroxisome proliferator-activated receptor-α activators, but no direct evidence has been demonstrated for their activation of ATP-binding cassette transporter A1 (ABCA1) in relation to clinically used fibrates. We investigated the reaction of fenofibric acid in this regard.

Methods and Results—Fenofibric acid was examined for the effect of increase of ABCA1 activity. It enhanced ABCA1 gene transcription and its protein level in macrophage cell line cells and fibroblasts and increased apolipoprotein A-I–mediated cellular lipid release, all in a dose-dependent manner. Enhancement of the gene transcription was examined by using a reporter assay system for liver X receptor responsive element (LXRE) and its inactive mutant. The results demonstrated that the effect of fenofibric acid is dependent on active LXRE.

Conclusions—Fenofibric acid increased transcription of ABCA1 gene in a liver X receptor–dependent manner. ***(Arterioscler Thromb Vasc Biol.* 2005;25:1193-1197.)**

Key Words: fenofibrate ■ fibrates ■ PPARα ■ ABCA1 ■ HDL ■ cholesterol ■ atherosclerosis

High-density lipoprotein (HDL) is a negative risk factor in coronary atherogenesis,[1] and raising HDL is expected to protect us against atherosclerosis. Such an effect was demonstrated in experimental animals by specific gene expression[2] or inhibition of cholesteryl ester transfer protein (CETP).[3] Although no specific drug is available in clinical use for this purpose, a bile acid–sequestering resin and statins were shown to raise HDL by an unknown mechanism, besides lowering low-density lipoprotein, and subanalysis of these results indicated its independent effect of reducing the atherosclerosis risk.[4–6] Fibric acids, active forms of fibrate drugs and activators of peroxisome proliferator-activated receptor-α (PPARα),[7,8] are also known for an HDL-raising effect. This group of drugs has been widely used for a long time for the treatment of hyperlipoproteinemia, especially types IIb, III, and VI. Fibric acids enhance fatty acid catabolism and accordingly reduce plasma lipid level, predominantly triglyceride (TG). Increase of TG-rich lipoprotein results in increase of TG transfer to HDL in exchange with its cholesteryl ester by CETP, and therefore leads to production of small cholesterol-poor HDL as TG is hydrolyzed.[9,10] Consequently, reduction of TG-rich lipoprotein by fibrates leads to the increase of HDL cholesterol by reversing this mechanism.[11,12] Fibric acids were also shown to enhance transcription of the gene of apolipoprotein A-I (apoA-I) in the liver.[13–15] A PPARα activator, Wy14643, was shown to upregulate the gene of ATP-binding cassette transporter A1 (ABCA1)[16] that mediates and rate-limits biogenesis of HDL by the interaction of helical apolipoprotein and cells.[17–20] ABCA1 expression is enhanced by loading cholesterol to cells via the liver X receptor (LXR),[21,22] presumably because of the increase of oxysterol. The effect of Wy14643 was interpreted by the activation of the LXR pathway as it increased LXR.[16] However, there has been no direct demonstration of the ABCA1 upregulation by fibric acids derived from fibrate drugs clinically used. In mouse atherosclerosis models, PPARα agonists did not appear to enhance ABCA1 expression in atherosclerotic lesion despite their effect of the regression.[23,24] Here we report in vitro observation that fenofibric acid increases the expression of ABCA1 and apoA-I–mediated HDL production. The effect on ABCA1 expression was through the enhancement of the transcription of the ABCA1 gene being dependent on LXR.

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Materials and Methods

Cell Culture

RAW264 cells were maintained in Dulbecco modified Eagle medium (DMEM)/F-12 (1:1) medium (IWAKI Glass) containing 2% TCM serum replacement (purchased from KN) at 37°C in 5% CO_2.[25] Cells in 6-well plates at the concentration of 1.5×10^6 cells per well were incubated 24 hours before the experiments.[25] THP-1 cells (4.0×10^6 cells per well) were differentiated with 3.2×10^{-7} M phorbol 12-myristate 13-acetate (PMA; Wako) in 10% FBS (PAA Laboratories)-RPMI 1640 medium (IWAKI Glass) for 72 hours.[26] BALB/3T3 clone A31[27] (obtained from RIKEN Cell Bank) was incubated in Eagle's minimum essential medium (MEM) with 10% FCS. All cell lines were incubated at 37°C in a humidified atmosphere of 5% CO_2. PPARα activators, fenofibric acid, or Wy14643 (Calbiochem-Novabiochem) were dissolved in dimethyl sulfoxide and added to the culture medium containing 0.2% BSA (Sigma).

Cellular Lipid Release

RAW264 cells were washed with PBS and cultured an additional 48 hours in the presence of fenofibric acid or Wy14643 in DMEM/F-12 (1:1) medium containing 2% TCM and 0.2% BSA. During the last 24 hours of the drug treatment, 300 μmol/L of dibutyryl cAMP (dbcAMP; Wako) and apoA-I (10 μg/mL) were added to the medium.[25] THP-1 cells and BALB/3T3 cells were also treated with the PPARα activators and apoA-I in 0.2% BSA-RPMI 1640 medium and 0.1% BSA-MEM, respectively. Cholesterol and choline-phospholipid released into the medium by apoA-I were determined enzymatically.[25] Adherent cells were dissolved in 0.1 N NaOH for protein determination by bicinchoninic acid protein assay system (Pierce).

Reporter Gene Assay

The constructs of luciferase reporter genes were prepared as described previously.[28] The 5′-flanking region of mouse ABCA1 gene (−1238/+57) was inserted into pGL3 vector (Promega) to generate ABCA1 promotor-luciferase reporter construct (pABCA1-Luc). The reporter plasmid with mutated and inactivated LXR-responsive element (LXRE) (mutant LXRE) was generated by using QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mutations introduced were identical to those reported previously.[21] Cells cultured in 24-well plates (3.0×10^3 cells per well) were washed once with PBS or pABCA1-Luc vector or pABCA1-mutant LXRE-Luc vector were cotransfected with phRL-tk vector (Promega) by Superfect transfection reagent (Qiagen). Three hours after the transfection, cells were washed with PBS and cultured in the presence of fenofibric acid or Wy14643 for 24 hours. Cellular luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega). Results were standardized by the Renilla luciferase activity derived from phRL-tk vector.

Immunoblotting of ABCA1

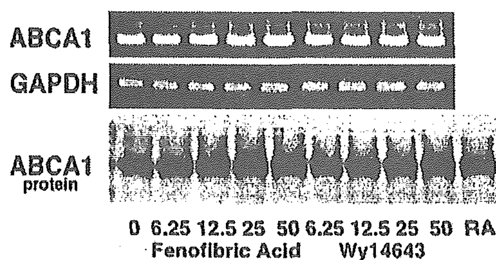
Cells incubated with fenofibrate or Wy14643 for 48 hours were harvested in cold PBS and pelleted by centrifugation. The cell pellet was suspended in 5 mmol/L Tris-HCl, pH 8.5, containing 1% protease inhibitor cocktails (Sigma) and placed on ice for 30 minutes. The cell suspension was centrifuged at 650g for 5 minutes, and the supernatant was centrifuged at 105 000g for 30 minutes to prepare the membrane fraction as a pellet. Immunoblotting of ABCA1 was performed according to the previous method.[26]

Results

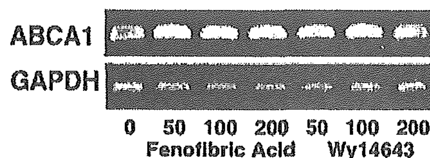
Expression of ABCA1

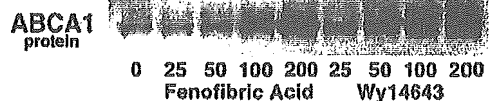
The effects of fenofibric acid and Wy14643 on expression of ABCA1 are shown in Figures 1 and 2. The message of ABCA1 increased by fenofibric acid and Wy14643 in all types of cells examined: RAW264 cells treated with dbcAMP, PMA-differentiated THP-1 cells, and BALB/3T3 cells. ABCA1 protein also increased by the PPARα agonists being demonstrated by its immunoblotting analysis in all these cells (Figures 1 and 2).

RAW 264



THP-1





Balb/3T3

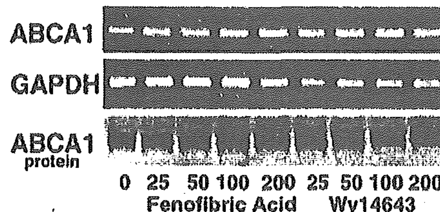


Figure 1. Effects of PPARα agonists fenofibric acid and Wy14643 on expression of ABCA1 in RAW264 cells pretreated with dbcAMP and THP-1 cells differentiated with PMA and BALB/3T3 fibroblasts. Messages of ABCA1 and GAPDH were detected by RT-PCR, and protein level of ABCA1 was determined by immunoblotting, as described in the text in each type of cell in the presence of the agonists (μmol/L) and 9-*cis*-retinoic acid (RA).

ApoA-I–Mediated Cellular Lipid Release

ApoA-I induced release of cellular cholesterol and phospholipids into the medium from the cells examined. PPARα agonists fenofibric acid and Wy14643 increased the apoA-I–mediated release of cholesterol and phospholipids in a dose-dependent manner (Figure 3). The increment of lipids released by the drugs was more prominent in cholesterol than phospholipid in RAW264 cells pretreated by dbcAMP. The maximum effect (102% increase in cholesterol release) was observed when the cells were treated with 25 μmol/L of fenofibric acid.

Reporter Gene Assay

Transcription of the ABCA1 gene was examined by using the reporter genes (pABCA1-Luc) in the dbcAMP-treated RAW264 cells (Figure 4). Fenofibric acid and Wy14642

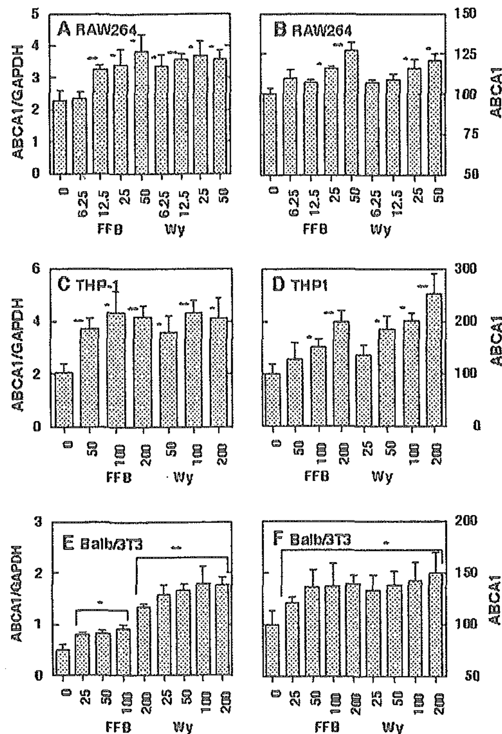


Figure 2. Effects of PPARα agonists fenofibric acid (FFB; μmol/L) and Wy14643 (Wy; μmol/L) on expression of ABCA1. The results of RT-PCR and Western blotting from the same experiments shown in Figure 1 were semiquantified by digital scanning in an Epson GT9500. Message of ABCA1 was standardized for that of GAPDH. Data points represent mean±SE of 3 independent experiments. Significance of the increase from the controls was examined by Student's t test and indicated as $*P<0.05$ and $**P<0.01$.

enhanced transcription of the ABCA1 reporter gene in a dose-dependent manner (Figure 4A and 4B). These effects were cancelled by substitute transfection of the mutant LXRE-containing reporter vector (pABCA1-mutant LXRE-

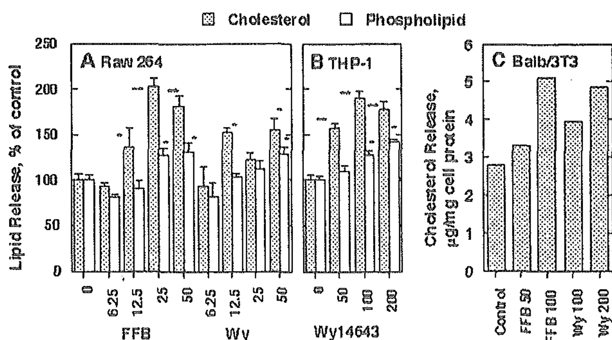


Figure 3. Lipid release by apoA-I from the cells examined in the presence of PPARα agonists fenofibric acid (FFB; μmol/L) and Wy14643 (Wy; μmol/L). Releases of cholesterol and phospholipid are expressed as percentage of the control for RAW264 cells (cholesterol 2.39 μg/mg cell protein and phospholipid 5.89 μg/mg cell protein), and for THP-1 cells (cholesterol 2.45 μg/mg cell protein and phospholipid 4.26 μg/mg cell protein). Data points in A and B represent mean±SE of triplicate measurement, and those in C represent the average of duplicate measurement. Significance of the increase from the controls was examined using Student's t test and indicated as $*P<0.05$ and $**P<0.01$.

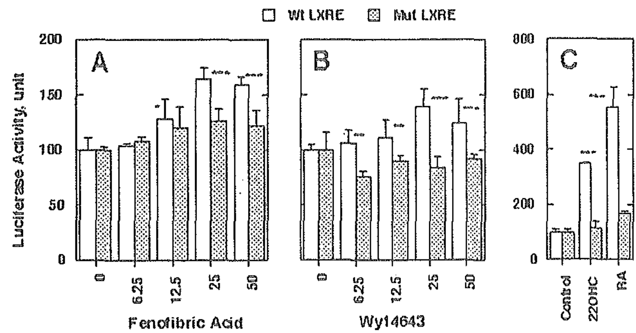


Figure 4. Luciferase reporter gene assay of ABCA1. The reporter genes for the ABCA1 promoter were constructed as described in the text. Wt-LXRE and Mut-LXRE indicate the genes without and with introduction of mutation in LXRE to inactivate the responsive element. The effects of fenofibric acid (FFB; μmol/L) and Wy14643 (Wy; μmol/L) were examined, as well as those of 9-*cis*-retionic acid (RA) and 22-oxysterol (22OHC). Data points represent mean±SE of triplicate measurement. Significance of the increase was examined using Student's t test and indicated as $*P<0.05$ from the blanks (no compound), $**P<0.05$ from the controls (mutant LXRE), and $***P<0.05$ from both.

Luc) to inactivate LXRE (Figure 4A and 4B), whereas 9-*cis*-retionic acid, a ligand for retinoid X receptor (RXR), and 22-oxysterol, a ligand for LXR, failed to increase the transcription of the mutant ABCA1 gene (Figure 4C).

Discussion

PPARs belong to the nuclear receptor superfamily group and act as ligand-activated transcription factors regulating the expression of certain target genes.[29] The PPAR family contains 3 different subtypes, designated PPARα, PPARβ/δ, and PPARγ. PPAR subtypes display distinct expression patterns, different ligand specificities, and distinct biological functions.[30–32] PPARs are activated by fatty acids and its metabolites and accordingly exert various effects in lipid homeostasis.[33] Several subtype-specific synthetic compounds have been developed for clinical use, including fibric acids (PPARα agonist) and glitazones (PPARγ agonist).[34] Fibrates are widely used drugs for hyperlipidemic patients because they significantly improve plasma lipid profiles by reducing TG-rich lipoprotein and raising HDL.[35,36] The primary effects of fibric acids, active forms of fibrates, on plasma lipids have been attributed to their PPARα-mediated expression of the genes of various enzymes that regulate lipid metabolism.[7,8] For HDL metabolism, the effects are partly explained by reduction of plasma TG itself and CETP reaction[9,10] and by increased expression of the apoA-I gene.[13–15] In addition, Wy14643, a nonclinical PPARα activator, was shown to enhance ABCA1 gene expression.[16] Because LXR was also activated in the condition used in that work,[16] and ABCA1 is known to be regulated by the LXR/RXR pathway, it was hypothesized that Wy14643 increases the transcription of ABCA1 gene via the LXR pathway.

We demonstrated the increase of ABCA1 by fenofibric acid, an active form of clinically used fibrate drug fenofibrate, in macrophage cell line cells and in mouse fibroblasts. These effects were also reproduced by a positive control Wy14643. To examine the mechanism, the reporter gene

assay was used via a promoter of the ABCA1 gene by introducing a mutation in LXR response element. Inactivation of this element was verified by abolishment of its response to 9-*cis* retinoic acid and 22-oxysterol, and fenofibrate failed to enhance transcription of the mutant reporter gene. Therefore, PPARα in fact activates the ABCA1 gene by the LXR-dependent pathway. The results were inconsistent with the finding that PPARs form a heterodimeric complex with the RXR (not LXR) and bind to specific PPAR-response elements in the promoter region of target gene.[37,38] However, a direct ligand of RXR, 9-*cis* retinoic acid, failed to activate the mutant gene, consistent with the established finding that dimerization of RXR with LXR is essential for enhancing ABCA1 gene transcription.[21]

Fenofibrate and Wy14643 reportedly have different affinity and distinct specificity to murine and human PPARs. However, both compounds showed equivalent capability in transactivation of the ABCA1 gene. Wy14643 activates not only PPARα but also PPARγ and PPARδ in cell-based transactivation assays[39] at the concentration >30 μmol/L. Activation of PPARδ was suggested to affect the ABCA1-mediated HDL biogenesis on the basis that an agonist of PPARδ induced HDL synthesis in culture cells and in monkeys.[40] Therefore, the effects of Wy14643 may include combined activation of various PPARs. In contrast, fenofibric acid is highly specific for activation of PPARα, at least up to 100 μmol/L.[39] Because C_{max} of fenofibrae is 30 μmol/L when it is orally administrated to human, it is most likely that the effect of this drug on the HDL biogenesis is based on the enhancement of ABCA1 expression by the mechanism shown in this article.

Fenofibrate has been shown to retard progression of coronary atherosclerosis,[41] consistent with the findings of reducing a risk of coronary heart disease by other fibrate drugs.[42,43] The clinical effects of these drugs are attributed to improvement of plasma lipoprotein profile by reducing TG and raising HDL. Although decrease of TG and increase of HDL are linked in human by the action of CETP,[10] the increase of ABCA1 activity may more directly contribute to raising HDL and prevention of lipid accumulation in vascular cells.

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