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Decreased Arterial Responses in WHHL Rabbits, an Animal Model of Spontaneous Hypercholesterolemia and Atherosclerosis

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Abstract: *We examined changes in blood pressure and blood flow of the arteries of WHHL and Japanese white rabbits after intravenous bolus injections of acetylcholine (3.0 μg/kg), bradykinin (0.5 μg/kg), and sodium nitroprusside (3.0 μg/kg) under a condition of anesthesia. These vasodilators lowered the blood pressure and increased the blood flow in WHHL and Japanese white rabbits. The changes in the hemodynamic parameters of WHHL rabbits after injection of sodium nitroprusside were similar to those of Japanese white rabbits. This suggests that the relaxation response of the tunica media was not diminished in WHHL rabbits. In contrast, the changes in the hemodynamic parameters of WHHL rabbits after injection of acetylcholine or bradykinin were significantly lower than those in Japanese white rabbits. In the histopathological and immunohistological examination, atherosclerotic lesions were observed in the ascending aortas of WHHL rabbits. In the surface of the atheromatous plaques, CD31-positive endothelial cells disappeared partly and the accumulation of RAM-11-positive macrophages was observed in these regions. In addition, plasma NO_2^- and NO_3^- levels of WHHL rabbits were significantly lower than those of Japanese white rabbits. These findings suggest that relaxation responses derived from arterial endothelial cells were probably depressed in WHHL rabbits due to dysfunction or denudation of the arterial endothelial cells.*

Key words: *atherosclerosis, blood flow, blood pressure, endothelial cell function, vasodilator, WHHL rabbit*

Introduction

It has been reported that the development of hypercholesterolemia and atherosclerosis induced lowering of the arterial endothelial cell function [2, 3, 8, 18]. Arterial endothelial cells play an important role in the relaxation of arteries [5, 8], in addition to infiltration of blood cells into arterial intima, thrombogenesis, pro-

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gression and destabilization of atherosclerotic plaques, and acute coronary syndromes [17]. This suggests strongly that the relaxation response is diminished in arteries with atherosclerotic lesions.

Several *in vitro* studies have demonstrated that vasorelaxation responses were reduced in arteries with atherosclerotic lesions compared with normal arteries [2, 3, 9]. Although *in vitro* studies have demonstrated that relaxation responses of arteries without atherosclerotic lesions were normal in hypercholesterolemic WHHL rabbits [2, 3, 9], the vasorelaxation response stimulated by acetylcholine was reduced even in normal arteries of hypercholesterolemic state [19]. To examine the discrepancies between the *in vitro* arterial vasorelaxation response and observations of dysfunction of arterial endothelial cells in hypercholesterolemic states, we examined the *in vivo* arterial vasorelaxation responses of the carotid arteries of WHHL and Japanese white rabbits. Since WHHL rabbits suffer from severe atherosclerotic lesions constantly after maturation due to spontaneous hypercholesterolemia [13], we examined the changes in the hemodynamic parameters of the arteries of WHHL rabbits as indices of the arterial relaxation response.

Materials and Methods

Animals

We used seven male WHHL rabbits bred at Kobe University [13] and seven male Japanese white rabbits (Kitayama Labes Co. Ltd., Ina, Japan) aged 12 months old. All aspects of animal experimentation and care were conducted according to the Guidelines of Animal Experimentation of Kobe University.

Measurement of Blood flow and blood pressure

Rabbits were anesthetized by intravenous injection of Ketaral (ketamine, 10 mg/kg) and xylazine (3 mg/kg), and anesthesia was maintained by an intravenous drip infusion of 0.1% ketamine in 500 ml of a physiological electrolytic solution through the marginal ear vein at a rate of 2.0 μg of ketamine/min. The rabbits were then placed in a supine position. A 20G indwelling catheter was inserted into the femoral artery and was connected to a blood pressure transducer (SPB-108, Viggo-Spectramed Japan, Tokyo, Japan) for monitoring blood pressure with a DC Strain Amplifier AS2102 (NEC San-ei Instruments Ltd., Osaka, Japan). Blood flow was monitored in the carotid artery with an Electromagnetic Blood Flowmeter MFV-3200 (Nihon Kohden Co., Tokyo, Japan) using a blood flow probe (FB-030T, Nihon Kohden Co., Tokyo, Japan). The blood pressure and blood flow were recorded with PowerLab/8SP (AD Instruments Japan Inc., Nagoya, Japan).

Injection of vasodilators

To examine the relaxation responses of arteries derived from arterial endothelial cells, we injected bradykinin and acetylcholine at concentrations to obtain a sufficient decrease in blood flow as reported previously [4]. Bradykinin (Biosciences Inc., La Jolla, CA, USA) and acetylcholine (ICN Biomedicals Inc., Aurora, Ohio, USA) were resolved by saline at a concentration of 0.5 μg/ml and 3.0 μg/ml, respectively. To examine the relaxation response of arteries derived from arterial media, we injected sodium nitroprusside dihydrate (SNP, ICN Biomedicals Inc., Aurora, Ohio, USA) at a concentration to obtain a sufficient decrease in blood flow as reported previously [4]. SNP was dissolved in saline at a concentration of 3.0 μg/ml. These vasodilators (1.0 ml/kg) were injected intravenously into the marginal ear vein for about 15 s, 30 min after an intravenous bolus administration of the anesthetic agents. A 10 min injection interval between the two different vasodilators was sufficient to restore hemodynamic parameters to the basal values.

Measurement of plasma NO_2^-, NO_3^- and lipid levels

Plasma NO_2^- and NO_3^- (NOx) levels were measured with high-performance liquid chromatography (ENO-10, Eicom Co., Kyoto, Japan) equipped with a stainless-steel column (NO-PAK, Eicom Co., Kyoto, Japan) [1]. Plasma samples were deproteinized by methanol and the supernatants were filtered through a 0.22 μm filter (Millipore Co., Bedford, MA, USA) before measurement. Plasma cholesterol and triglyceride levels were measured enzymatically using plasma obtained after 12 h fasting.

Preparation of histopathological sections

After monitoring the blood pressure and blood flow, anesthetized rabbits were sacrificed by exsanguination from the femoral artery and perfused with saline. The aorta and carotid arteries were excised and immersed in

Table 1. Basal plasma lipid and NOx levels, and hemodynamic parameters of WHHL and Japanese white rabbits

	N	Body weight (kg)	Plasma lipid level (mmol/l)		Plasma NOx (μmol/l)		Blood pressure (mm/Hg)		Blood flow (ml/min)
			Total cholesterol	Triglyceride	NO_2^-	NO_3^-	Systolic	Diastolic	
Japanese white rabbits	7	3.44 ± 0.06	0.3 ± 0.1	0.9 ± 0.04	0.53 ± 0.06	48.2 ± 12.1	118 ± 11	83.0 ± 7.3	22.7 ± 4.5
WHHL rabbits	7	3.21 ± 0.07	15.4 ± 1.0	2.2 ± 0.3	0.36 ± 0.04	7.3 ± 0.7	119 ± 7.6	66.7 ± 4.9	27.1 ± 2.4
		P=0.046	*P<0.001*	*P<0.001*	*P=0.038*	*P=0.020*	*P=0.965*	*P=0.101*	*P=0.405*

Values are presented as means ± the standard error of the means. Statistical analyses were carried out with Student's t-test or Aspin-Welch's t-test.

periodate-lysine-paraformaldehyde fixative. After immersion-fixation, the segments prepared from the ascending aortas and carotid arteries were embedded in paraffin and were sectioned serially at 4 μm thickness. Each section was stained with elastic van Gieson staining, or immunohistochemical staining with monoclonal antibodies specific for endothelial cells (CD31; Dako A/S, Glastrup, Denmark) [12] or rabbit macrophages (RAM-11; Dako A/S, Glastrup, Denmark) [16]. Immunohistochemical staining was carried out with Dako EnVision + Polymer reagent (Dako A/S, Glastrup, Denmark) or Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) [14].

Statistical analysis

Values are presented as mean ± the S.E.M. Statistical analysis was carried out using Student's t-test or Aspin-Welch's t-test for comparison between WHHL and Japanese white rabbits and by Paired t test for comparison between before and after vasodilator injection. Values of $P<0.05$ were regarded as statistically significant.

Results

Basal plasma lipid levels, NOx levels, and hemodynamic parameters

The basal hemodynamic parameters of WHHL rabbits were not significantly different from those of Japanese white rabbits (Table 1). However, compared with Japanese white rabbits, the plasma lipid levels of WHHL rabbits were significantly higher and NOx levels were significantly lower.

Atherosclerotic lesions

In WHHL rabbits, severe atherosclerotic lesions were observed in the ascending aorta and carotid sinus but not in the region of the carotid arteries where the blood flow probe was set (Fig. 1). Japanese white rabbits had no arterial lesions. In addition, CD31-positive cells disappeared partly from the surface of atherosclerotic lesions of WHHL rabbits. In the CD31-negative superficial area, macrophage accumulation was observed. Endothelial cells in the lesion-free arteries were positive for CD31-immunohistochemical staining.

Effects of bradykinin on hemodynamic parameters

Bradykinin lowered the systolic and diastolic blood pressure, and increased the blood flow in both WHHL and Japanese white rabbits (Fig. 2). Compared with Japanese white rabbits, the increase in the blood flow of WHHL rabbits was decreased by 57% (P=0.032).

Effects of acetylcholine on hemodynamic parameters

Acetylcholine lowered the systolic and diastolic blood pressure, and increased the blood flow in both WHHL and Japanese white rabbits (Fig. 3). Compared with Japanese white rabbits, the increase in the blood flow of WHHL rabbits was decreased by 64% (P=0.017).

Effects of sodium nitroprusside on hemodynamic parameters

SNP lowered the systolic and diastolic blood pressure, and increased the blood flow in both WHHL and Japanese white rabbits (Fig. 4). However, there were no significant differences in the changes in the hemodynamic parameters between WHHL and Japanese white rabbits. In addition, both the increase in the blood flow and the decrease in the blood pressure induced by SNP were relatively mild compared with the changes induced by bradykinin and acetylcholine.

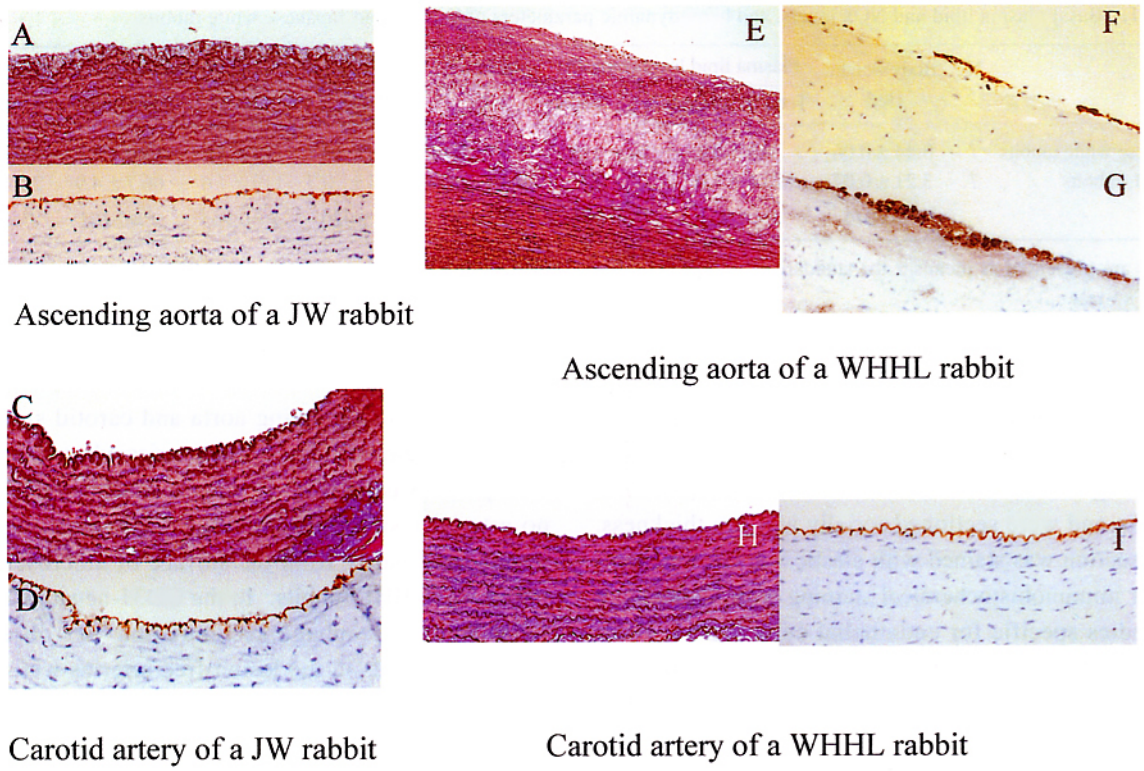

Fig. 1. Microphotographs of the ascending aorta and carotid artery of Japanese white and WHHL rabbits. (A) Ascending aorta of a Japanese white rabbit (elastic van Gieson staining; original magnification: × 66). (B) CD31 immunohistostaining for endothelial cells of a Japanese white rabbit's ascending aorta (original magnification: × 132). (C) Carotid artery of a Japanese white rabbit (elastic van Gieson; original magnification: × 132). (D) CD31 immunohistostaining for endothelial cells of a Japanese white rabbit's carotid artery (original magnification: × 132). (E) Ascending aorta of a WHHL rabbit (elastic van Gieson staining; original magnification: × 66). (F) CD31 immunohistostaining for endothelial cells of a WHHL rabbit's ascending aorta (original magnification: × 132). (G) RAM-11 immunohistostaining for macrophages of a WHHL rabbit's ascending aorta (original magnification: × 132). (H) Carotid artery of a WHHL rabbit (elastic van Gieson staining; original magnification: × 132). (I) CD31 immunohistostaining for endothelial cells of a WHHL rabbit's carotid artery (original magnification: × 132).

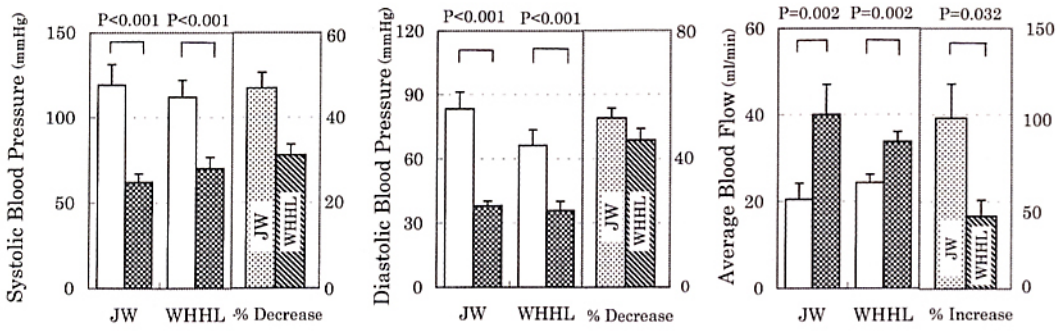

Fig. 2. Effects of bradykinin on hemodynamic parameters of Japanese white and WHHL rabbits. □, Before bradykinin injection; ▩, After bradykinin injection; ▨, % decrease or increase in each hemodynamic parameter of Japanese white rabbits; ▧, % decrease or increase in each hemodynamic parameter of WHHL rabbits. Error bars indicate the standard error of the means. Statistical analyses were carried out with the Paired t-test for comparison between before and after vasodilator injection, and with Student's t-test or Aspin-Welch's t-test for comparison between Japanese white and WHHL rabbits.

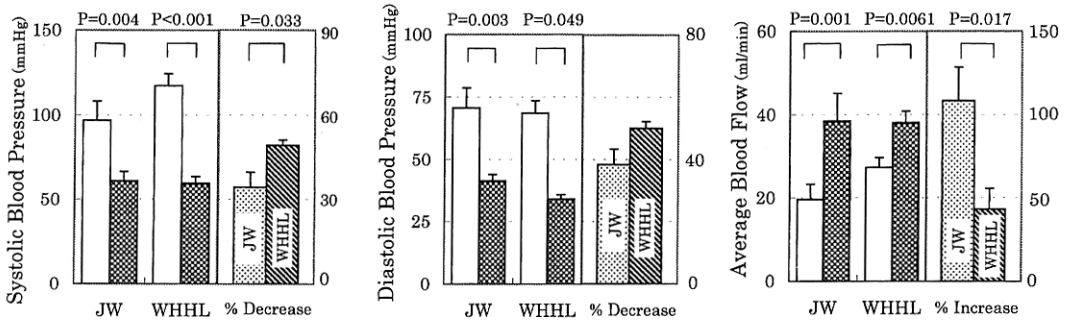

Fig. 3. Effects of acetylcholine on hemodynamic parameters of Japanese white and WHHL rabbits. □, Before acetylcholine injection; ▩, After acetylcholine injection; ▨, % decrease or increase in each hemodynamic parameter of Japanese white rabbits; ▧, % decrease or increase in each hemodynamic parameter of WHHL rabbits. Error bars indicate the standard error of the means. Statistical analyses were carried out with the Paired t-test for comparison between before and after vasodilator injection, and with Student's t-test or Aspin-Welch's t-test for comparison between Japanese white and WHHL rabbits.

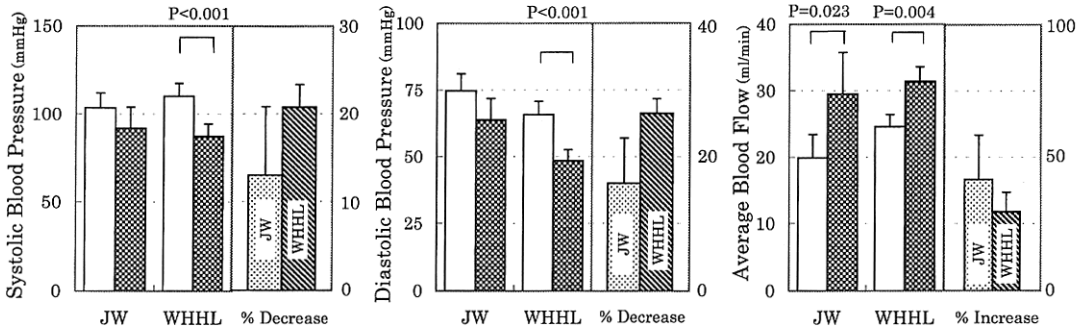

Fig. 4. Effects of sodium nitroprusside on hemodynamic parameters of Japanese white and WHHL rabbits. □, Before sodium nitroprusside injection; ▩, After sodium nitroprusside injection; ▨, % decrease or increase in each hemodynamic parameter of Japanese white rabbits; ▧, % decrease or increase in each hemodynamic parameter of WHHL rabbits. Error bars indicate the standard error of the means. Statistical analyses were carried out with the Paired t-test for comparison between before and after vasodilator injection.

Discussion

In this study, we demonstrated that in carotid arteries with no atherosclerotic lesions, the increase in the blood flow after an intravenous bolus injection of bradykinin or acetylcholine was lower in WHHL rabbits than in normal rabbits. In addition, CD31-positive endothelial cells were partly disappeared in the proximal ascending aortas of WHHL rabbits and the plasma NOx levels were markedly lower compared with the normal rabbits.

Plasma NOx is derived from nitric oxide synthesized mainly by arterial endothelial cells, neuronal cells, and activated macrophages and smooth muscle cells. Nitric oxide is well known as an endothelium-derived relaxing factor [5, 8]. Although macrophages and smooth muscle cells in atherosclerotic lesions can play a role in the increase in plasma NOx levels, synthesis of nitric oxide is probably decreased in the endothelial cells covering atherosclerotic lesions because of denudation or dysfunction. In histopathological or immunohistological staining of the arteries, severe atherosclerotic lesions were observed in the ascending aortas of WHHL rabbits (Fig. 1). In these sections, the CD31-positive endothelial cells disappeared partly in the superficial area of atheromatous plaques where macrophages were accumulated. In addition, the large main arteries of WHHL rabbits are generally covered with atherosclerotic lesions after maturation. Therefore, the low plasma NOx levels of WHHL rabbits may be caused by denu-

dation or dysfunction of arterial endothelial cells at the sites of atherosclerotic lesions.

Although *in vitro* studies have shown that vasorelaxation responses stimulated by acetylcholine or bradykinin were normal in the arteries with no atherosclerotic lesions of WHHL rabbits [2, 3, 9], the present *in vivo* study demonstrated that the increases in the blood flow induced by injection of these vasodilators were markedly lower in normal carotid arteries of WHHL rabbits compared with normal rabbits. In WHHL rabbits aged about 12 months, there were no atherosclerotic lesions in the penetrating arteries and only mild lesions were observed at the bifurcations or junctions of basal cerebral arteries [7]. Therefore, the peripheral cerebral circulation was probably normal in WHHL rabbits aged about 12 months. This suggests that the peripheral cerebral circulation in WHHL rabbits did not affect the carotid blood flow. On the other hand, the ascending aorta suffered from severe atherosclerotic lesions involving denudation of endothelial cells as shown Fig. 1. This suggests that the proximal atherosclerotic lesions, especially the denudation or dysfunction of the endothelial cells, may have affected not only the increase in blood flow but also the relaxation response of the carotid arteries of WHHL rabbits through a decrease in the secretion of endothelium-dependent relaxation factors in the present study. Since several *in vitro* studies have demonstrated that vasodilators induced relaxation of carotid arteries or aortic rings with or without atherosclerotic lesions in WHHL rabbits [2, 3, 9], the increase in the blood flow with a decrease in the blood pressure in the present study suggests that an injection of bradykinin or acetylcholine enlarged the carotid arteries in addition to the peripheral arteries. Consequently, we suggested that the low level increase in the blood flow induced by bradykinin or acetylcholine in the carotid arteries of WHHL rabbits was probably due to depressed arterial endothelial cell function at the proximal carotid sinus and ascending aorta.

The changes in the hemodynamic parameters by SNP were relatively mild and there were no significant differences between WHHL and Japanese white rabbits. It has been reported that SNP induced vasorelaxation due to the direct action to the tunica media [6]. We set the probe of the blood flow meter at a lesion-free site of the carotid arteries. Therefore, the results suggest that the vasorelaxation response of the tunica media is probably normal at lesion-free site of carotid arteries in WHHL rabbits.

In the present study, the effects of bradykinin on the blood pressure of the femoral artery were different from those of acetylcholine. Ayajiki *et al.* [2] reported that the *in vitro* relaxation response of the carotid arteries with or without atheromatous lesions by acetylcholine and substance P, another vasodilator, differed. Regarding this difference, it has been reported that the endothelial cell-derived relaxation response to substance P, histamine, and a calcium ion ionophore were not impaired by atherosclerosis in human coronary and subcutaneous arteries [15]. Therefore, the mechanism of vasorelaxation by bradykinin and acetylcholine may differ. The pathway of endothelium-dependent vasorelaxation by acetylcholine or bradykinin is considered to mainly consist of three pathways: the nitric oxide pathway, the prostaglandin I_2 pathway [11], and the endothelium-derived hyperpolarizing factor pathway [10, 11]. The influence of bradykinin on these pathways may be different from that of acetylcholine.

In conclusion, the present study showed that the vasorelaxation responses derived from arterial endothelial cells were depressed in WHHL rabbits, even though the arteries had no atherosclerotic lesions, and that the relaxation response dependent on the tunica media was normal in lesion-free arteries.

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