Impairment of Endothelium-Dependent Relaxation in Aorta from Rats with Arteriosclerosis Induced by Excess Vitamin D and a High-Cholesterol Diet

Satomi Kitagawa, Yu Yamaguchi, Masaru Kunitomo, Noriko Imaizumi and Motohatsu Fujiwara

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Mukogawa Women's University,
11-68 Koshien Kyuban-cho, Nishinomiya 663, Japan

ABSTRACT — The present investigation was undertaken to characterize the relaxing responsiveness in aortic strips from rats with arteriosclerosis, which was produced by excess vitamin D\(_2\) (VD) administration followed by treatment with or without a high-cholesterol diet for 6 weeks (VD + CHOL and VD group, respectively). This arteriosclerotic aorta was characterized by medial calcification and intimal cell proliferation. Helical strips of thoracic aorta were suspended in oxygenated Krebs-Henseleit solution. Under precontraction with noradrenaline, endothelium-dependent relaxations to acetylcholine were significantly attenuated in the VD and VD + CHOL as compared with the control. Relaxation to calcium ionophore A23187 was also significantly attenuated in the VD + CHOL. However, the relaxing responses to acetylcholine and A23187 in aortas from rats fed a high-cholesterol diet alone remained unaffected. Nitroglycerine caused an equal degree of relaxation in aortas from control and arteriosclerotic rats. There was a significant negative correlation between the relaxing response to acetylcholine and the calcium content in the aorta. These results indicate that in arteriosclerotic rat aortas, the endothelium-dependent relaxation to acetylcholine is impaired in proportion to the degree of calcification, and such impairment is facilitated by cholesterol feeding but can not be attributed to hypercholesterolemia or vascular cholesterol deposition.

Keywords: Endothelium-dependent relaxation, Arteriosclerosis, Vitamin D, High-cholesterol diet, Calcification

Atherosclerosis is a disease of the arterial intima characterized by the accumulation of lipids and proliferation of smooth muscle cells, and endothelial injury is considered to be an initiating event in this disorder (1). A series of recent studies has reported that endothelium-dependent relaxations induced by a variety of vasodilator agents including acetylcholine (ACh), substance P and thrombin are markedly impaired in the isolated atherosclerotic arteries of rabbits (2-6), pigs (7, 8), monkeys (9, 10) and humans (11-13), whereas endothelium-independent relaxations induced by nitroglycerin (NG) and sodium nitrite are preserved (5, 9-11), enhanced (6) or reduced (3, 13). Decreases in production or release of endothelium-derived relaxing factor (EDRF) are considered to be the principal mechanism for abnormal endothelium-dependent relaxations in atherosclerosis (14-17).

However, there have been no report investigating the arterial reactivities in atherosclerotic vascular preparations from rats, because this animal species is resistant to the development of experimental atherosclerosis. Although degenerative arterial lesions could be developed in rats by the administration of excess vitamin D, the lesions are sclerotic rather than atheromatous in morphological features (18, 19). Such arteriosclerosis is accompanied by calcification in the arterial media, which resembles Mönckeberg's arteriosclerosis (20). Very recently, vascular endothelial dysfunction has been shown in the aortic rings isolated from rats treated with vitamin D\(_3\) plus nicotine (21).

The purpose of the present study is to characterize the endothelium-dependent and-independent relaxations in the aorta from arteriosclerotic rats treated with excess vitamin D followed by feeding with or without a high-cholesterol diet.
MATERIALS AND METHODS

Male Sprague-Dawley rats (7 weeks old, Japan SLC, Hamamatsu) were housed in an air-conditioned room (23 ± 1°C and 60 ± 10% humidity) under an artificial 12 hr light/dark cycle (7:00 a.m.–7:00 p.m.). Diets used in this study were a purified basal diet and a high-cholesterol diet. The basal diet contained 20% casein, 63.2% sucrose, 10% corn oil, 2% agar, 0.8% vitamin mixture and 4% salt mixture. The high-cholesterol diet consisted of the basal diet with 1.5% cholesterol and 0.5% cholic acid.

Animals were divided into 4 groups. For 4 consecutive days, groups I and II were orally given olive oil (2.0 ml/kg of body weight, once daily) and groups III and IV were orally given vitamin D2 (VD, 3 × 105 IU/2.0 ml olive oil/kg of body weight, once daily). Thereafter, group I (control group, n = 6) and group III (VD group, n = 8) were maintained on the basal diet and group II (CHOL group, n = 6) and group IV (VD + CHOL group, n = 8) were maintained on the high-cholesterol diet for the 6-week period. After the 6-week period, the rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) and killed by bleeding from a cannula inserted into the abdominal aorta, and the thoracic aorta was excised for measurement of relaxing responses to ACh, calcium ionophore A23187 and NG, contents of cholesterol and calcium, and for histological studies. The blood was centrifuged at 3,000 rpm for 10 min and the serum preserved at −40°C for lipid analysis. The thoracic aortas were immediately placed in the Krebs-Henseleit solution (118.4 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, 25.0 mM NaHCO3, 5.6 mM glucose) and excess connective tissues removed. Helical strips (approx. 2 mm × 15 mm) were then prepared. In some preparations, the endothelium was disrupted mechanically by gentle ablation of the luminal surface with a small steel spatula. Each strip was fixed vertically under a resting tension of 1.5 g in a 10-ml organ-bath filled with the solution (37°C, pH 7.4) described above, which was continuously aerated with a gas mixture of 95% O2 and 5% CO2, and then it was allowed to equilibrate for 90 min before the start of the experiments. The bathing medium was changed every 10 min during this period. Isometric tension change was measured by a force-displacement transducer (Model T-7, NEC San-Ei, Tokyo, Japan) coupled to an ink-writing oscillograph (Model 8K21, NEC San-Ei).

Relaxants, ACh (10−10–10−5 M), calcium ionophore A23187 (10−9–10−6 M) and NG (10−9–10−5 M), were added to the bath medium when the 10−7 M noradrenaline-induced contraction reached a plateau, which was approx. 80% of the maximal contraction. The relaxing response was expressed in terms of percent decrease of the maximal relaxation developed by papaverine (10−4 M). Denudation of the endothelium was confirmed pharmacologically by the disappearance of the 10−6 M ACh-induced relaxing response.

At the end of each experiment, the strips used were subjected to the determination of aortic cholesterol and calcium contents. The strips were freeze-dried to a constant weight and the lipids were subsequently extracted at 50°C for 20 min with chloroform-methanol (2:1 v/v), and the extracts were used for the determination of cholesterol. The delipidated strips were hydrolyzed in sealed hydrolysis tubes with 6 N HCl for 24 hr at 105°C. Hydrolyzates were evaporated under vacuum and used for the determination of calcium. Cholesterol in serum and aorta was measured by the fluoroenzymatic method as described previously (22). Aortic calcium was measured with an atomic absorption spectrophotometer (Model 180-60, Hitachi, Japan).

For histopathological examinations, the remaining thoracic aortas proximal to the excised portion were fixed in 10% buffered formalin. Frozen sections were prepared, cut at 8-micron thickness and stained with oil red O and hematoxylin for visualization of the presence of neutral lipid and with Von Kossa stain for visualization of calcium deposits.

Drugs used in the present experiments were as follows: VD (Nacalai Tesque Inc., Kyoto, Japan), ACh chloride (Daichi Pharmaceutical Co., Ltd., Tokyo, Japan), calcium ionophore A23187 and NG (Millisrol, Nihon Kayaku Co., Ltd., Tokyo, Japan), noradrenaline (Sankyo Co., Ltd., Tokyo, Japan), and papaverine hydrochloride (Nacalai Tesque).

In each protocol, the number of strips studied was also the number of rats used. The data are expressed as mean ± S.E.M. Statistical analyses were done with Student’s t-test for unpaired comparison.

RESULTS

Growth rate

Initial body weights were 246 ± 3 g (n = 18) and 247 ± 3 g (n = 12) in VD-treated animals (VD and VD + CHOL groups) and VD-non-treated animals (control and CHOL groups), respectively. The animals which were given VD for 4 consecutive days showed rapid loss of body weight with an extremely lessened intake of food after the last VD administration. The maximum loss (approx. 50 g) of weight was on day 4 after the last VD administration. Thereafter, the body weight rapidly recovered as food consumption increased. During the
course of this experiment, animals were fed the basal or high-cholesterol diet by pair-feeding. At the end of the experiment (the 6th week after stopping VD administration), body weights were 416 ± 9 g (n = 6), 403 ± 12 g (n = 6), 408 ± 16 g (n = 8), 390 ± 12 g (n = 8) in the control group, CHOL group, VD group and VD + CHOL group, respectively. There was no significant difference among them. The mortality rate was 11% in either the VD or VD + CHOL group.

**Cholesterol in the serum and aorta and calcium in the aorta**

Table 1 shows the serum cholesterol concentrations and the aortic cholesterol and calcium contents in four groups of rats at the 6th week. A significant increase in the serum cholesterol concentration occurred in the CHOL and VD + CHOL groups as compared to the control group fed the basal diet. There was no significant difference between the control and the VD group. A highly significant increase in the content of aortic cholesterol, particularly cholesterol ester, was observed in the VD + CHOL group. A small but significant increase in aortic cholesterol content was also observed in the CHOL group.

A marked increase in the aortic calcium content occurred in both the VD and VD + CHOL groups; the value in the VD + CHOL group was higher than that in the VD group.

**Histological findings**

In rats fed the basal diet (control group) or the high-cholesterol diet (CHOL group), the aorta was normal in microscopic appearance. The aortas in rats treated with VD (VD and VD + CHOL groups) displayed a variety of pathologic changes involving medial degeneration and calcification (Fig. 1) and showed lysis and fragmentation of the elastic lamina. Some preparations exhibited mesenchymal cell proliferation and lipid deposition in the intima, but not atheroma formation. The histological findings in the VD + CHOL group were comparable to those seen in the VD group, but the occurrence of lipid materials stained by oil red O was apparently more frequent in the VD + CHOL group than in the VD group although no quantitative comparisons were made.

**ACh-, A23187- and NG-induced relaxation**

Figure 2 shows representative recordings of changes in isometric tension of the helical thoracic aorta strips from the control and VD + CHOL groups. In preparations from the control group, ACh (10⁻¹⁰ - 10⁻⁷ M) produced a concentration-related relaxation in the endothelium-intact strips precontracted by 10⁻⁷ M noradrenaline, but such relaxation disappeared in the endothelium-denuded strips. The endothelium-dependent relaxation by ACh showed a marked decrease in preparations from arteriosclerotic rats in the VD + CHOL group as compared to that in the control. The contractile response to 10⁻⁷ M noradrenaline in aortas from the VD + CHOL group was almost the same as that in the control. The ACh- and A23187-induced endothelium-dependent relaxations were almost completely inhibited by treatment of the strips with 10⁻⁴ M N⁶-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase (data not shown).

Figure 3 shows the concentration-relaxing response curves to ACh in the endothelium-intact thoracic aortas precontracted by 10⁻⁷ M noradrenaline. Relaxations by ACh markedly decreased in the strips from arteriosclerotic rats (the VD and VD + CHOL groups) as compared to those of the control. The maximum relaxations by ACh in preparations from the VD and VD +

### Table 1. Serum cholesterol concentrations and aortic cholesterol and calcium contents in rats treated with excess vitamin D and/or the high-cholesterol diet

| Group      | n | Total cholesterol (mg/100 ml) | Total cholesterol (mg/g dry weight) | Cholesterol ester (mg/g dry weight) | Calcium (mg/g dry weight) |
|------------|---|-------------------------------|------------------------------------|-----------------------------------|--------------------------|
| Control    | 6 | 98 ± 3                         | 5.62 ± 0.16                        | 0.639 ± 0.115                    | 0.28 ± 0.02              |
| CHOL       | 6 | 189 ± 17**                    | 6.05 ± 0.12*                      | 0.926 ± 0.143*                   | 0.24 ± 0.03              |
| VD         | 8 | 107 ± 9                       | 5.52 ± 0.29                       | 0.673 ± 0.083                    | 3.40 ± 0.94**            |
| VD + CHOL  | 8 | 197 ± 14**                    | 7.04 ± 0.35**                     | 1.782 ± 0.183**                  | 5.40 ± 0.89**            |

Animals were orally given vitamin D₃ (VD, 3 × 10³ IU/kg, once daily) for 4 consecutive days and then maintained on the basal diet (VD group) or the high-cholesterol diet (VD + CHOL group) for subsequent 6 weeks. VD-untreated animals were also maintained on the basal diet (control group) or the high-cholesterol diet (CHOL group) alone. n: Number of animals. Each value represents the mean ± S.E.M. *P < 0.05, **P < 0.01, as compared with the control group.
Fig. 1. Photographs showing damage and heavy calcification in the internal and medial elastic membranes (upper panel: Von Kossa stain, ×150) of the aorta in the VD group and fibrous proliferation and lipid staining materials in the intima (lower panel: oil red O stain, ×150) of the aorta in the VD + CHOL group.
CHOL groups were 81% (P < 0.05) and 65% (P < 0.01) of the control, respectively. On the other hand, relaxation to ACh in the aortas from hypercholesteremic, non-arteriosclerotic animals (the CHOL group) remained unaffected or was enhanced at 10^{-8} M.

A23187 also produced a concentration-related relaxation, and the relaxation was abolished by endothelial removal. The relaxing responses to A23187 were attenuated significantly in the aortas from the VD + CHOL group, but not in those from the VD group; the maximum relaxation in the VD + CHOL group was 79% (P < 0.01) of the control. A23187-induced relaxation in preparations from the CHOL group was preserved (Fig. 4).

**Fig. 3.** Concentration-response curves to acetylcholine (ACh) in aortic strips with intact endothelium isolated from rats in the control ( ), CHOL ( ), VD ( ), and VD + CHOL ( ) groups. Aortic strips are precontracted with 10^{-7} M noradrenaline. Relaxation is expressed as a percentage of that induced by 10^{-4} M papaverine. The response of endothelium-denuded strips ( , n = 6). Each point represents the mean ± S.E.M. *P < 0.05, **P < 0.01, as compared with the control group.
The extent of NG-induced relaxations was almost the same in both endothelium-intact and -denuded aorta strips (data not shown). The concentration-relaxing response curves to NG in the endothelium-intact aorta strips showed similar patterns in all four groups, and no significant difference was detected in the means of the corresponding values on each curve (Fig. 5).

A linear negative correlation was found between the calcium content and the relaxing response to 10^{-5} and 10^{-6} M ACh (r = -0.590, n = 16, P < 0.05 and r = -0.560, n = 16, P < 0.05, respectively) in the thoracic aorta strips from the VD-treated animals (the VD and VD + CHOL groups) (Fig. 6). However, there was no significant correlation between the cholesterol ester content and the relaxing response to 10^{-5} and 10^{-6} M ACh (r = -0.498, n = 14, n.s. and r = -0.444, n = 14, n.s., respectively) in the aorta strips from the cholesterol-fed animals (the CHOL and VD + CHOL groups). Similarly, no correlation was observed between the calcium content in the aorta and response to \(3 \times 10^{-7} M\) A23187 (r = -0.298, n = 16, n.s.).

**DISCUSSION**

It has been reported that the relaxing response to ACh in rat aorta is also mediated by EDRF (23), which plays an important role in the modulation of vascular tone (24, 25). The endothelium-dependent relaxation to ACh was markedly impaired in the thoracic aortas isolated from rats with excess VD-induced arteriosclerosis. The sclerotic aortas were characterized by calcification and degenerative changes in the media with cell proliferation in the intima, as has been previously shown (18, 19, 26). Cholesterol feeding in addition to VD treatment facilitated the impairment of ACh-induced relaxation, along with aggravation of the morphological changes and the accumulation of aortic calcium and cholesterol. In contrast, the endothelium-independent relaxation to NG was hardly affected, indicating that the impaired relaxing response to ACh in arteriosclerosis is not due to a disturbance of smooth muscle function. Very recently, Henrion et al. (21) have reported that in the isolated aorta of young rats treated with vitamin D₃ plus nicotine, the endothelium-dependent vasodilation by carbachol is attenuated together with vascular calcium overload, but the effects of sodium nitroprusside are unchanged. These changes in vaso-reactivity closely resemble our observations with ACh, A23187 and NG in isolated thoracic aorta strips. However, nicotine does not appear to be essential for the production of arteriosclerosis with vascular dysfunction.
We also observed that cholesterol feeding alone caused long-term hypercholesterolemia, but the relaxing response to ACh was not impaired, but rather enhanced. In such preparations, no vascular injury was observed microscopically. Similar findings have been reported in the circumflex coronary arteries of long-term hypercholesterolemic dogs (9). This animal species is considered to be resistant to the development of hypercholesterolemia and atherosclerosis as in the case of rats. On the other hand, even in an acute or mild degree of hypercholesterolemia at an early stage of atherosclerosis, the endothelium-dependent relaxation is impaired in rabbits (4) and pigs (8). The present results, together with such previous reports, indicate that the impairment of the endothelium-dependent relaxation in rat aorta cannot be attributed to hypercholesterolemia and that the arteriosclerotic or atherosclerotic endothelial injury is prerequisite to the impaired relaxation.

ACh-induced endothelium-dependent relaxation is known to be mediated by stimulation of muscarinic receptors of endothelial cells, while calcium ionophore A23187-induced endothelium-dependent relaxation is due to an increase in the calcium influx into the endothelial cells without activation of a specific membrane receptor (27). In atherosclerotic arteries, one might argue that the mechanism of impairment of endothelium-dependent relaxation to ACh differs from that to A23187. Bossaller et al. (11) reported that endothelium-dependent relaxation to ACh was abolished, while preserving the relaxation to A23187, in atherosclerotic human coronary artery and rabbit aorta. Other investigators showed that there was impairment of both receptor-mediated and nonreceptor-mediated endothelium-dependent relaxations in atherosclerotic arteries (7, 12, 15, 16). The present results showed that the extent of decrease in endothelium-dependent relaxation to ACh was greater than that in case of A23187. Thus, in VD-induced arteriosclerosis, functional damage to receptor-mediated relaxation appears to occur prior to that to the nonreceptor-mediated one.

A negative correlation between the impairment of relaxation to ACh and the cholesterol content has been shown in the aorta from atherosclerotic rabbits (5). In the aorta from our arteriosclerotic rats, there was a negative correlation between the degree of relaxation to ACh and the calcium content, but not the cholesterol (cholesterol ester) content. Therefore, vascular calcification induced by VD seems to be essential for the impairment of endothelium-dependent relaxation. However, we do not know yet why calcification in the aorta exerts such a great influence on endothelium-dependent relaxation without affecting NG-induced relaxation despite the noticeably degenerative appearance in the vascular smooth muscle cells. The intimal thickening was seen along with vascular calcification. The intimal dysfunction in the relaxing response might have been unveiled prior to the medial dysfunction by an unknown mechanism.

The impairment of endothelium-dependent relaxation has been demonstrated in spontaneously hypertensive rats (28) and stroke-prone spontaneously hypertensive rats (29). Furthermore, calcium antagonists protected the rabbit aorta from the loss of endothelium-dependent relaxation and the accumulation of cholesterol (30,
31), and they delayed the progression of atherosclerosis (32, 33). The calcium contents in the aortic wall increase with age, hypertension, arteriosclerosis and atherosclerosis (34, 35). Thus, it is possible that vascular calcium overload is associated with either the impairment in endothelial cell function to produce and release EDRF or the disturbance of its diffusion or binding to smooth muscle cells. Further studies are required to clarify the causal relationship between impairment of endothelium-dependent relaxation and calcium deposition in the sclerotic aorta.

Acknowledgments
This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (No. 03268102) from the Ministry of Education, Science and Culture, Japan and a grant from the Smoking Research Foundation, Japan.

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