# Altered Vasoconstrictor Responsiveness in Vitamin D-Induced Arteriosclerotic Rat Aortas

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ABSTRACT—This investigation was undertaken to characterize the vasoconstrictor responsiveness in aortas isolated from a rat model of arteriosclerosis induced by vitamin $D_2$ (VD) administration followed by feeding with a high-cholesterol diet. Cumulative contractile responses to KCl, noradrenaline and serotonin in thoracic aortic strips isolated from arteriosclerotic rats were slightly augmented in concentrations lower than the $EC_{50}$ value of each agent and rather attenuated in their higher concentrations as compared with those from normal rats. Maximum contractions to the agonists were markedly attenuated in arteriosclerotic aortas; the degree of attenuation was greater in rats treated with a combination of VD and cholesterol than in those treated with VD alone. There was a significant negative correlation between the maximum contraction to KCl, noradrenaline or serotonin and the content of calcium or cholesterol ester in aortas. Removal of endothelium markedly enhanced sensitivity and contractility to the agonists in aortic strips from normal rats, whereas the same procedure only slightly enhanced them in aortic strips from arteriosclerotic rats. These results indicate that in arteriosclerotic rat aortas, contractile responsiveness to agonists of vascular smooth muscle cells is impaired with deposition of calcium and cholesterol, and they suggest that augmentation of contractile responses to the agonists in lower concentrations is due to impairment of endothelial function.

*Keywords*: Arteriosclerosis, Vitamin D, High-cholesterol diet, Calcification, Contractile response

Atherosclerosis accompanied with hypercholesterolemia results in altered responsiveness of blood vessels to vasoactive substances in parallel with smooth muscle cell proliferation and lipid deposition. A number of studies have shown augmented responses to serotonin, histamine or ergonovine (1-6), and impaired endothelium-dependent relaxation to a variety of vasodilator agents (7-12) in various regions of arteries with experimental or human atherosclerosis. These changes may predispose atherosclerotic arteries to vasospasm. On the other hand, only a limited number of studies have been carried out on the vascular reactivity of arteriosclerotic vessels without deposition of lipids (13, 14). Henrion et al. (14) have reported that in the aorta from a rat model of vascular calcium overload produced by treatment with vitamin $D_3$ and nicotine, not only endothelium-dependent relaxation to carbachol but also the vasoconstrictive response to noradrenaline is attenuated, while the relaxing responses to sodium nitroprusside is unchanged. Studies in our laboratory have also shown attenuation of vasorelaxation to acetylcholine or the calcium ionophore A23187 in aortas from rats with arteriosclerosis produced by four consecutive doses of vitamin $D_2$ (VD) (15). We also demonstrated that such impairment of endothelium-dependent relaxations to acetylcholine and A23187 was proportional to the degree of calcium deposition and facilitated by cholesterol feeding (15).

The present investigation was designed to characterize the vasoconstrictive responsiveness of the aorta from rats of the arteriosclerosis model made in this laboratory. The relationship between calcium and cholesterol deposition in the arterial wall and contractility of the aorta was also examined.

## MATERIALS AND METHODS

### *Animals and diets*

Male Sprague-Dawley rats (7-week-old, Japan SLC,

Hamamatsu) were housed in an air-conditioned room (23±1℃ and 60±10% humidity) under an artificial 12-hr light/dark cycle (7:00 AM−7:00 PM). Animals received a purified basal diet or a high-cholesterol diet containing 1.5% cholesterol and 0.5% cholic acid. The composition of these diets was as described in a previous paper (15).

*Vitamin $D_2$-induced arteriosclerosis*

Arteriosclerosis in the aorta was produced by the previously described protocol (15). Rats 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 $D_2$ (VD, $3\times10^5$ 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 a 6-week period.

*Aortic preparations*

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. Then the thoracic aortas were excised, immediately placed in the Krebs-Henseleit solution (118.4 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 10.0 mM glucose) and excess connective tissues removed. Helical strips (approx. 2 mm × 15 mm) were then prepared. Each strip was fixed vertically under a resting tension of 1.5 g in a 10-ml organ-bath filled with the solution (37℃, pH 7.4) described above, which was continuously aerated with a gas mixture of 95% $O_2$ and 5% $CO_2$. Each strip was allowed to equilibrate for 90 min before the start of the experiments, with the bathing medium changed every 10 min during this period. Isometric tension change was measured by a force-displacement transducer (Model T-7, NEC San-Ei, Tokyo) coupled to an ink-writing oscillograph (Model 8K21, NEC San-Ei). In some preparations, the endothelium was disrupted mechanically by gentle ablation of the luminal surface with a small steel spatula. Denudation of the endothelium was confirmed pharmacologically by the disappearance of the $10^{-6}$ M ACh-induced relaxation together with the preservation of the $10^{-7}$ M nitroprusside sodium-induced relaxation or ascertained morphologically as described in our previous report (16).

*Contractile responses*

Before the addition of the agonist to be tested, in each preparation, contractions were repeatedly induced by KCl (60 mM) and then by noradrenaline ($10^{-7}$ M) until a constant response was obtained. After the tissue was allowed to return to a resting tension by washing with the buffer solution, KCl (5−60 mM), noradrenaline ($10^{-9}-10^{-5}$ M) or serotonin ($10^{-9}-10^{-4}$ M) was cumulatively added to the bath medium. Contractile responses were expressed as a percentage of the mean of the maximum contractions developed by each agent in the endothelium-intact aortic preparation from the control animals.

*Cholesterol and calcium contents in the aortic preparations*

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℃ 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℃. Hydrolyzates were evaporated under vacuum and used for the determination of calcium. Cholesterol in the serum and aorta was measured by the fluoroenzymatical method as described previously (17). Aortic calcium was measured with an atomic absorption spectrophotometer (Model 180-60, Hitachi, Tokyo).

*Drugs*

Drugs used in the present experiments were as follows: vitamin $D_2$ (VD), potassium chloride (KCl), papaverine hydrochloride, and nitroprusside sodium (Nacalai Tesque, Inc., Kyoto); noradrenaline (Sankyo Co., Ltd., Tokyo); serotonin hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.); acetylcholine chloride (ACh, Daiichi Pharmaceutical Co., Ltd., Tokyo).

*Statistical analyses*

The data are expressed as the mean±S.E.M. In each protocol, the number of strips studied was also the number of rats used. Statistical analysis of the data was performed by Student's *t*-test for unpaired observations. Linear correlations were performed according to the least squares method.

## RESULTS

*Baseline data*

Body weight did not significantly change in all four groups of rats at the end of the study. Biochemical findings in the aorta showed a marked accumulation of calcium in the VD and VD+CHOL groups and a significant accumulation of cholesterol, particularly cholesterol ester, in the VD+CHOL group. Arteriosclerotic lesions were histologically evident in the aorta from rats of the VD and VD+CHOL groups. The arteriosclerotic aorta

was characterized by medial calcification and intimal cell proliferation. In addition, a mild lipid deposition was observed in some preparations from the VD+CHOL group. These are similar to ones reported already (15), so data are not shown here.

*Contractile responses in endothelium-intact strips*

The helical strips of thoracic aorta did not show spontaneous or rhythmic contraction. Cumulative addition of KCl (5 to 60 mM), noradrenaline ($10^{-9}$ to $3\times10^{-6}$ M) or serotonin ($10^{-9}$ to $3\times10^{-5}$ M) induced concentration-dependent contractions in the endothelium-intact strips isolated from the control group. The maximum tension was attained with KCl at 60 mM, noradrenaline at $3\times10^{-6}$ M and serotonin at $3\times10^{-5}$ M, respectively (Figs. 1, 2 and 3). The $pD_2$ values and maximum responses to the agonists in the control, CHOL, VD and VD+CHOL groups are shown in Table 1.

In the arteriosclerotic aortic strips from the VD and VD+CHOL groups, the contractile responses to KCl tended to be augmented at lower concentrations (5 to 20 mM), as compared with those in the normal aortic strips from the control group (Fig. 1). The aortas from the VD group slightly increased in sensitivity to KCl as described by the $pD_2$ values ($-\log EC_{50}$) for KCl (Table 1), but the increase was not significant. In contrast, at higher concentrations (40 to 60 mM) of KCl, the contractile response was significantly attenuated in the VD+CHOL group, compared to that in the control group (Fig. 1). The maximum contractions elicited by KCl in the VD and VD+CHOL groups were 78% (not significantly different) and 72% ($P<0.01$) of the control, respectively. On the other hand, in the aortic strips from the CHOL group, the concentration-contractile response curve to KCl was comparable to the control (Fig. 1).

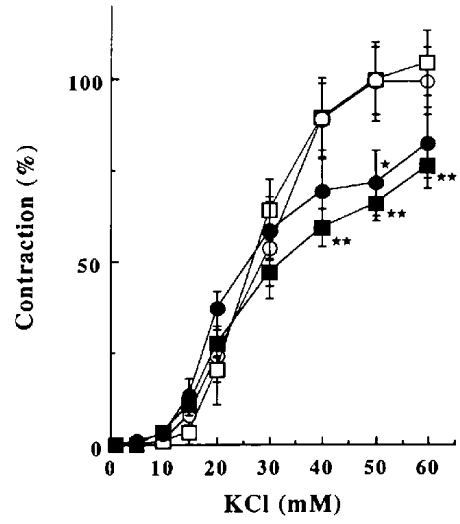


**Fig. 1.** Concentration-contractile response curves to potassium chloride (KCl) in the endothelium-intact aortic strips isolated from rats in the control (○, n=6), CHOL (□, n=6), VD (●, n=8), and VD+CHOL (■, n=8) groups. Contractile responses are expressed as a percentage of the mean of the maximum contractions at 60 mM KCl in the preparation from rats in the control group. Each point represents the mean±S.E.M. *$P<0.05$, **$P<0.01$, as compared with the control group.

The similar pattern was observed for the concentration-contractile response curves to noradrenaline in the endothelium-intact aortic strips from four groups (Fig. 2). The contractile responses to noradrenaline in lower concentrations ($10^{-9}$ to $10^{-7}$ M) were slightly augmented in the VD and VD+CHOL groups; at a concentration of $10^{-8}$ M, there was a significant difference between the control and VD groups. The $pD_2$ value for noradrenaline in the VD group was significantly ($P<0.05$) higher than that in the

**Table 1.** Evaluation of contractile responses on the thoracic aortas isolated from rats in the control, CHOL, VD and VD+CHOL groups

| | Group | | | |
|---|---|---|---|---|
| | Control | CHOL | VD | VD+CHOL |
| n | 6 | 6 | 8 | 8 |
| $pD_2$ value | | | | |
| Potassium chloride | 1.54±0.05 | 1.55±0.02 | 1.65±0.02 | 1.57±0.02 |
| Noradrenaline | 7.11±0.17 | 7.44±0.09 | 7.61±0.10* | 7.47±0.09 |
| Serotonin | 6.09±0.05 | 6.23±0.10 | 6.51±0.12** | 6.42±0.08** |
| Maximum response (g) | | | | |
| Potassium chloride | 1.27±0.09 | 1.32±0.11 | 0.99±0.08 | 0.91±0.06** |
| Noradrenaline | 1.16±0.10 | 1.07±0.12 | 0.98±0.09 | 0.85±0.10* |
| Serotonin | 1.25±0.15 | 1.17±0.08 | 1.00±0.14 | 0.80±0.14* |

Each value represents the mean±S.E.M. *$P<0.05$, **$P<0.01$, as compared with the control group.

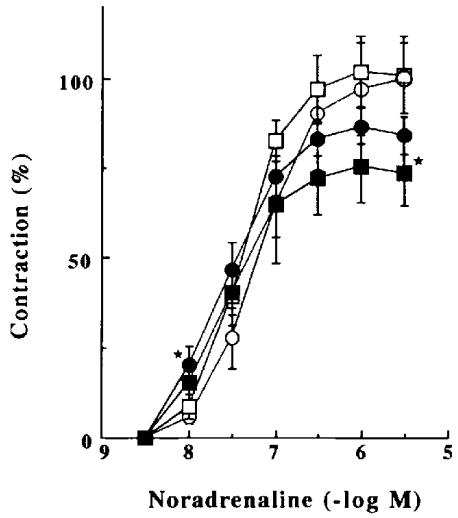


**Fig. 2.** Concentration-contractile response curves to noradrenaline in the endothelium-intact aortic strips isolated from rats in the control (○, n=6), CHOL (□, n=6), VD (●, n=8), and VD+CHOL (■, n=8) groups. Contractile responses are expressed as a percentage of the mean of the maximum contractions at $3\times10^{-6}$ M noradrenaline in the preparation from rats in the control group. Each point represents the mean±S.E.M. *P<0.05, as compared with the control group.

control group, although the difference did not reach statistical significance in the VD+CHOL group (Table 1). The maximum contractions elicited by noradrenaline in the VD and VD+CHOL groups were 84% (not significantly different) and 73% (P<0.05) of the control, respectively. The contractile response to noradrenaline in the aortic strips from the CHOL group was slightly larger than that in the control (Fig. 2).

Serotonin also caused patterns similar to the concentration-contractile responses to KCl and noradrenaline (Fig. 3). The contraction caused by serotonin at a concentration of $3\times10^{-7}$ M in aortic preparations from the VD group was significantly (P<0.01) larger than the corresponding contraction of those from the control group. The $pD_2$ values for serotonin were significantly (P<0.01) higher in the VD and VD+CHOL groups than that in the control group (Table 1). At concentrations higher than $10^{-6}$ M, serotonin caused significantly smaller contractions in the VD+CHOL group than those in the control group. The maximum contractions elicited by serotonin in the VD and VD+CHOL groups were 80% (not significantly different) and 64% (P<0.05) of the control, respectively. The contractile response to serotonin in the aortic strips from the CHOL group was comparable to the control (Fig. 3).

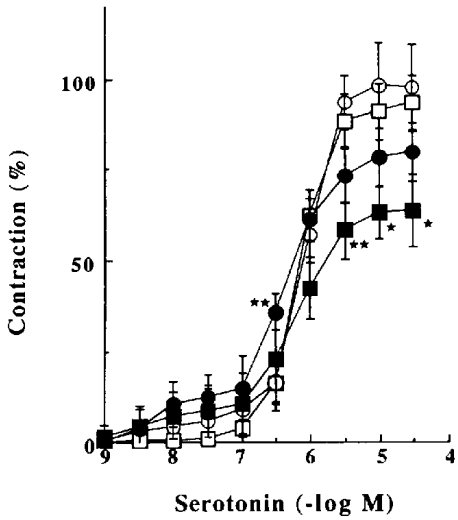


**Fig. 3.** Concentration-contractile response curves to serotonin in the endothelium-intact aortic strips isolated from rats in the control (○, n=6), CHOL (□, n=6), VD (●, n=8) and VD+CHOL (■, n=8) groups. Contractile responses are expressed as a percentage of the mean of the maximum contractions at $3\times10^{-5}$ M serotonin in the preparation from rats in the control group. Each point represents the mean±S.E.M. *P<0.05, **P<0.01, as compared with the control group.

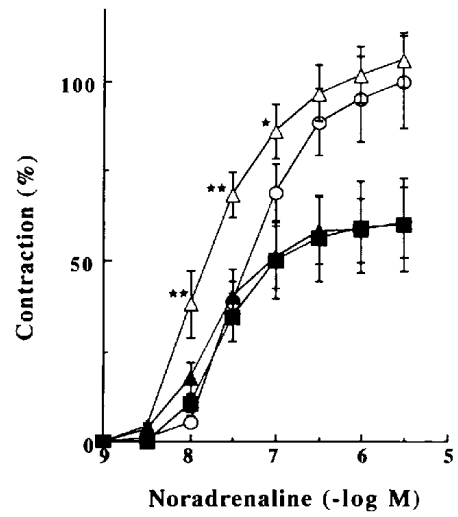


**Fig. 4.** Effects of endothelial denudation on the contractile responses to noradrenaline in aortic strips isolated from rats in the control (○, endothelium-intact; △, endothelium-denuded) and VD+CHOL (■, endothelium-intact; ▲, endothelium-denuded) groups. Contractile responses are expressed as a percentage of the mean of the maximum contractions at $10^{-6}$ M noradrenaline in the aortic preparation with endothelium from rats in the control group. Each point represents the mean±S.E.M. *P<0.05, **P<0.01, as compared with the respective endothelium-intact preparation.

*Contractile responses in the endothelium-denuded strips*

Contractile responses to noradrenaline in the endothelium-denuded aortic strips were compared with those in the endothelium-intact aortic strips, from the control and VD+CHOL groups. In the strips from the control group, mechanical removal of the endothelium resulted in an enhancement of sensitivity to noradrenaline ($pD_2$ value: $7.78\pm0.08$ in the denuded strips vs. $7.30\pm0.12$ in the endothelium-intact strips, $P<0.01$) and an enhanced contractility to noradrenaline; there was a significant difference in contractions produced in the concentrations from $10^{-8}$ to $10^{-7}$ M, although maximum tension in the denuded strips was 106% (not significantly different) of the intact strips (Fig. 4). In contrast, such augmentative effects of endothelium removal observed in the control group was nearly lost in the arteriosclerotic strips from the VD+CHOL groups. Furthermore, in the VD+CHOL group, a slightly increased contraction observed at lower concentrations of noradrenaline in the endothelium-intact strips disappeared in the endothelium-denuded strips (Fig. 4). Similar results were obtained in the case of serotonin-induced contractions (data not shown).

*Calcium and cholesterol*

A significant negative correlation was found between the maximum contractile response to KCl and the calcium content ($r=-0.549$, $n=16$, $P<0.05$) in the endothelium-intact strips from the VD and VD+CHOL groups and also between the maximum contractile response to KCl and the cholesterol ester content ($r=-0.722$, $n=14$, $P<0.01$) in the endothelium-intact strips from the CHOL and VD+CHOL groups (Fig. 5). A similar significant correlation was noted between the maximum contractile response to noradrenaline and the calcium content ($r=-0.636$, $n=16$, $P<0.01$), and the cholesterol ester content ($r=-0.577$, $n=14$, $P<0.01$), and also between the maximum contractile response to serotonin and the calcium content ($r=-0.502$, $n=16$, $P<0.05$), and the cholesterol ester content ($r=-0.689$, $n=14$, $P<0.01$).

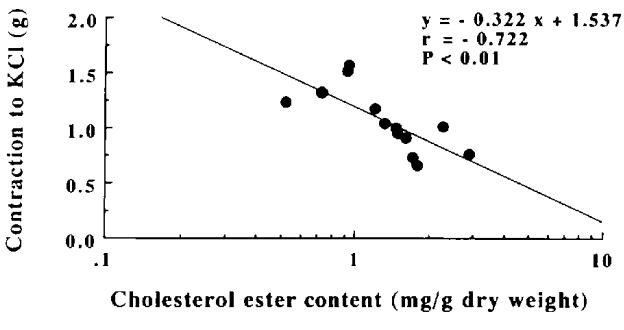


**Fig. 5.** Linear correlation between the maximum contraction at 60 mM potassium chloride (KCl) and cholesterol ester content in thoracic strips from rats in the CHOL and VD+CHOL groups.

## DISCUSSION

Excess VD-induced arteriosclerosis in rat aortas is considered to be a model of Monkeberg's sclerosis (18), which is characterized by calcification and degenerative changes in the media (19, 20). Cholesterol feeding in addition to excess VD treatment aggravates the morphological changes with an appreciable accumulation of cholesterol ester in the aorta (15).

In the aorta isolated from rats with such experimental arteriosclerosis, we demonstrated that the contractile responses to KCl, noradrenaline or serotonin were slightly augmented in lower concentrations and attenuated at higher concentrations. The maximum contractions to all the contractile agonists used were markedly attenuated in the aortic strips with arteriosclerosis, particularly that induced by the combination of VD and the high-cholesterol diet. Baraka and Bekemeier (13) have reported that the increase in perfusion pressure in response to noradrenaline or ATP is reduced in the isolated perfused hind legs of rats with arteriosclerosis induced by toxic doses of VD. Furthermore, Henrion et al. (14) have reported that the vasoconstrictor response to noradrenaline is diminished in aortic rings from rats treated with vitamin $D_3$ plus nicotine. As regards to the experimental atherosclerosis induced by a high-cholesterol diet, to our knowledge, there are only a few reports showing reduced adrenergic vasoconstriction (11, 21), and other studies have demonstrated potentiation of the contractility to KCl and serotonin (1, 2, 4) or no change of that to noradrenaline (5, 9, 22).

Although the exact mechanism involved in the decreased contractility in the VD-induced arteriosclerotic aortas is not yet known, it is reasonable to assume that it occurs through structural damage in the media, as shown by morphological changes involving extracellular calcification and degeneration together with cholesterol deposition. In the aorta from our arteriosclerotic rats, there was a negative correlation between the maximum contraction to KCl, noradrenaline or serotonin and the content of calcium as well as cholesterol, particularly cholesterol ester. Therefore, it is conceivable that both calcification and cholesterol deposition exert a great influence on the contractile machinery of vascular smooth muscle cells. It has been reported that injection of 1,25-dihydroxy-VD, the biologically most active metabolite of VD, to rats enhances the contractile responses to noradrenaline and serotonin (23). Thus, it is probable that the vasoconstrictor responses to the agonists had been affected by an elevated plasma 1,25-dihydroxy-VD level or hypercalce-

mia following VD administration. However, this possibility is eliminated by the fact that an excess dose of VD in rats does not result in elevation of plasma 1,25-dihydroxy-VD, probably due to homeostatic mechanisms (24), and also by the fact that the VD-induced hypercalcemia is restored to normal about one week later (25). At any rate, hypercholesterolemia per se was not crucial in attenuating the contractile response to agonists but only facilitated this attenuating effect in our arteriosclerotic rat aortas, although in animals other than rats, hypercholesterolemia exerted a variety of effects on the vascular contractile responses to KCl, noradrenaline and serotonin (1, 3, 4, 26, 27). The discrepancy between the diminished contractility and the unaffected relaxing capacity reported previously (14, 15) for the vascular smooth muscle from arteriosclerotic animals remains to be clarified.

Slight augmentation of the contractile response to noradrenaline as well as serotonin in lower concentrations observed in the strips of arteriosclerotic aorta disappeared by removal of the endothelium; the contraction to noradrenaline in the denuded strips was markedly reduced in the arteriosclerotic aorta as compared with the normal aorta. In addition, the endothelial removal markedly increased the maximum response and sensitivity to noradrenaline in the normal aortas, whereas the same procedure scarcely increased them in the arteriosclerotic aortas. We previously demonstrated that endothelium-dependent relaxations to acetylcholine and A23187 were impaired in the rat aortas with arteriosclerosis induced by VD (15). These findings suggest that the slightly augmented vasoconstriction to agonists in lower concentrations in arteriosclerotic aortas with intact endothelium is due to an impairment of the endothelium-dependent relaxation. Suppression or removal of the endothelium could enhance vasoconstriction elicited by different contractile agents (28). Release of endothelium-derived relaxing factor may interfere with the development of the contraction (29). On the other hand, Du and Woodman (30) have reported that there is no apparent relationship between loss of endothelium-dependent relaxation and the observed changes in adrenergic vasoconstriction in experimental hypercholesterolemia and atherosclerosis. Additional work is needed to clarify this important role of the endothelium in the modulation of contractile responses in the arteriosclerotic and atherosclerotic vessels.

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