Protective Effects of Benidipine Hydrochloride (KW-3049), a Calcium Antagonist, against Experimental Arterial Calcification and Endothelial Dysfunction in Rats

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Protective effects of benidipine hydrochloride (KW-3049) against arterial calcification and its possible mechanisms of action have been investigated. Arterial calcification was induced in rats by combined administration of vitamin D$_2$ (1050000 IU/kg, s.c.) and nicotine (12.5 mg/kg, p.o., b.i.d.) for 6 successive days. Calcium antagonists, benidipine or nifedipine, were given orally twice a day during the same period. The aortic calcium content in vitamin D$_2$ and nicotine-treated (control) rats increased to about 25 times that in normal rats, accompanying an increase of serum calcium level. Benidipine (10 mg/kg, p.o., b.i.d.) reduced the aortic calcium content to about 18% of control rats without reducing the serum calcium level. Although the presence of aortic endothelial cells was observed under light microscopy in control rats, their surfaces were degenerated under scanning electron microscopy. Benidipine exerted a protective effect against these degenerative changes. Acetylcholine-induced endothelial dependent relaxation was attenuated in control rats, compared with that in normal rats. Benidipine significantly improved this attenuation of the relaxation. These results suggest that the anticalcinitic effect of benidipine is accompanied by its protective effect on endothelial cells.

**Keywords** — benidipine; calcium antagonist; arterial calcification; endothelial cell; endothelium-dependent relaxation

**Introduction**

It has been well known that calcium deposition occurs in the medial part of arteries at early phase of atherosclerosis.\(^1\) Furthermore, it has been demonstrated that the calcium content in various kinds of arteries increases according to age.\(^1\) The calcium deposition in arteries may deprive arteries of their elasticity. Previous investigators\(^2\) have demonstrated that several calcium antagonists show protective effects against vitamin D$_2$-induced experimental arterial calcification and have speculated that calcification is caused by the intracellular calcium overload. Calcium antagonists, in general, seem to have anticalcinitic effects in vitamin D-induced calcification models.

Benidipine is a newly-developed 1,4-dihydropyridine calcium antagonist, which is characterized by long-lasting antihypertensive and cardioprotective activities.\(^5\) In the present study, possible anticalcinitic effect of benidipine against vitamin D$_2$ (VD$_2$) and nicotine-induced arterial calcium deposition was examined with special reference to the endothelial function and morphology.

**Materials and Methods**

Male Wistar rats (SLC, Shizuoka Laboratory animal Center, Inc.) weighing 220—320 g were used in this study. Food and water were available ad libitum.

**Administration of VD$_2$, Nicotine and Drugs** — In a preliminary experiment, rats received either VD$_2$ or nicotine, and the effect of either VD$_2$ or nicotine alone on serum and aortic calcium content was determined.

The rats were divided into 8 groups; group 1, normal rats, group 2, control rats, and group 3—8, treated rats. Rats in group 2—8 were given VD$_2$ (1050000 IU/kg, s.c.) in olive oil (2.5 ml/kg) and nicotine (12.5 mg/kg, p.o.) in deionized water (2.5 ml/kg) at about 10:00 a.m. Twelve hours later, 12.5 mg/kg of nicotine was again given orally. Rats in group 1 were given the same volume of olive oil (2.5 ml/kg) s.c. and deionized water (2.5 ml/kg) p.o. as the vehicle. Rats in group 3—8 were given a test drug, benidipine (1, 5, 10 mg/kg) or nifedipine (5, 10,
30 mg/kg), in 0.3% sodium carboxymethyl cellulose (CMC) suspension (2.5 ml/kg) orally twice a day, immediately after the administration of nicotine. The doses of benidine were determined in preliminary studies so that the maximum dose could reduce the aortic calcium content. The doses of nifedipine were determined according to the previous report, which showed that 10 mg/kg of it significantly suppressed the elevation of aortic calcium content. Control rats were given the same volume of the vehicle (CMC) instead of the drug. About 20% of the rats except in the normal group died before the day 7 presumably because of the acute intoxication with nicotine, and they were excluded from the study. These treatments were continued for 6 d, and on the day 7, the rats were killed and used for the analysis. In another series of experiments, nicotine (12.5 mg/kg, p.o., b.i.d.), vitamin D₂ (1050000 IU/kg, s.c., q.d.) or benidine (10 mg/kg, p.o., b.i.d.) alone was given for the reference study.

**Determination of Aortic and Blood Calcium Content** — The thoracic aorta from aortic arch to diaphragm was removed from the rats, cleaned of the surrounding tissues in ice-cold saline and, thereafter, dried at 100 °C for 12 h. The dried tissue was immersed in a mixture of acids (60% HNO₃–60% HClO₄, 1:1), evaporated to dryness and the residue was suspended in 0.06 M HCl containing 5 mM La³⁺. Aortic calcium was determined with an atomic absorption spectrometer (Model Z-6100, Hitachi Co., Ltd., Japan). Blood calcium content was measured with an auto analyzer (Model AU510, Olympus Co., Ltd., Japan).

**Histological Studies** — For light microscopy, the isolated aorta was washed with saline and fixed in 10% formalin. The fixed preparation was cut into transverse sections of 4 µm thick in paraffin and stained with hematoxylin-eosin and silver nitrate (Kossa staining).

For scanning electron microscopy, the isolated aorta was washed with saline and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The specimens were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 h at 4 °C, dehydrated in a graded alcohol series, passed through 100% isoamyl acetate and dried by a critical point method. Mounted samples were coated with gold and examined by scanning electron microscopy (Model JSM-820, JEOL, Japan).

**Endothelium-Dependent Relaxation** — The thoracic aortas were removed and excess connective tissues were excised. The tissues were cut into rings about 5 mm wide and the rings were suspended in 20 ml organ baths filled with oxygenated (95% O₂–5% CO₂) Krebs-Henseleit solution of the following composition (mm): NaCl 117.6, KCl 5.4, MgSO₄ 0.56, CaCl₂ 2.5, NaH₂PO₄ 1.2, NaHCO₃ 25.0 and glucose 11.1. The artery was equilibrated for 1 h in Krebs-Henseleit solution under the resting tension of 1.2 g. The tissue was pre-contracted by an application of 10⁻⁶ M norepinephrine. The concentration of norepinephrine was determined according to the preliminary experiment, in which 10⁻⁶ M of norepinephrine induced submaximum contraction. Acetylcholine (ACH) was applied cumulatively after the contraction reached a plateau. The change in tension was isometrically determined by a force-displacement transducer (SB-1T, Nihon Kohden, Japan) and recorded on a pen-recorder (Type 3066, Yokogawa, Japan).

**Measurement of Blood Pressure** — Systolic blood pressure was measured by plethysmographic tail method (VSM-105K, Ueda Seisakusho Co., Ltd., Japan), at 6 h after the first administration of VD₂ and nicotine each day.

**Drugs Used** — Benidine was synthesized in our laboratory. The sources of other drugs were as follows: nifedipine (Sigma Chemical, St. Louis, MO, U.S.A.), VD₂, nicotine (Wako Pure Chemical, Osaka, Japan), norepinephrine (Sankyo, Tokyo, Japan), acetylcholine (Daiichi Seiyaku, Tokyo, Japan).

**Statistical Analysis** — Results are expressed as the mean ± S.E. Statistical significance was tested by the multiple comparison test. Kruskal Wallis test followed by Scheffe test was used. p values of less than 0.05 were considered to be statistically significant.
**Serum Calcium Level**

In control rats, serum calcium levels increased up to about 16 mg/dl ($p<0.01$ vs normal) (Fig. 1 A). A similar extent of increase was also induced by administration of VD$_2$ alone (15.5 ± 0.6 mg/dl) ($p<0.01$ vs normal) but administration of nicotine alone did not elicit the increase of serum calcium level (10.9 ± 0.2 mg/kg, $n=7$) ($p>0.05$ vs normal). Neither benidipine nor nifedipine exerted any effect on the increase of serum calcium levels.

**Aortic Calcium Content**

The aortic calcium content increased up to $1554 \pm 291 \mu g/g$ dry weight ($p<0.01$ vs normal) following combined administration of VD$_2$ and nicotine, and the content was about 25 times higher than that in normal rats ($60.5 \pm 5.2 \mu g/g$ dry weight) (Fig. 1 B). Administration of VD$_2$ alone increased the aortic calcium content only by 7 times ($465.6 \pm 164 \mu g/g$ dry weight, $n=7$) ($p<0.05$ vs control) although the serum calcium level was raised to the same level as that in control rats. Administration of nicotine alone failed to increase the aortic calcium content ($86.9 \pm 9.2 \mu g/g$ dry weight, $n=7$) ($p>0.05$ vs normal). Benidipine at doses of 1, 5, 10 mg/kg, p.o., b.i.d. reduced the aortic calcium content to $1117 \pm 264$ ($p>0.05$ vs control), $535 \pm 156$ ($p>0.05$), and $333 \pm 54 \mu g/g$ dry weight ($p<0.01$), respectively. Nifedipine at doses of 5, 10, 30 mg/kg, p.o., b.i.d. reduced the aortic calcium content to $1263 \pm 279$ ($p>0.05$ vs control), $1077 \pm 233$ ($p>0.05$), and $507 \pm 204 \mu g/g$ dry weight ($p>0.05$), respectively. Benidipine was more potent than nifedipine. This may be due to the fact that the duration of action of benidipine is longer than that of nifedipine.

**Histological Studies by Microscopy**

Figure 2 shows the light microscopic photograph of the aorta. Kossa staining clearly revealed the calcium deposition in the media of the aorta (Fig. 2A). Calcium deposition was decreased by the administration of benidipine (10 mg/kg) (Fig. 2B).

In the scanning electron micrograph of the aortic luminal surface of normal rats, there were...
A few flat endothelial cells, the surfaces of which were smooth (Fig. 3A). In control rats, the surfaces of cells became rough and fluffy, and most of the cells grew flat in shape (Fig. 3B). Benidipine (10 mg/kg) ameliorated these degenerative changes (Fig. 3C). Nicotine alone did not induce any detectable endothelial injury (photo not shown).

**Acetylcholine (ACh)-Induced Relaxation**

The maximum contractions induced by $10^{-6}$ M norepinephrine in normal, control and benidipine (10 mg/kg)-treated group were $1.10 \pm 0.17$ g, $0.84 \pm 0.10$ g and $1.09 \pm 0.13$ g, respectively. These values were not significantly different from each other. Relaxation of pre-contracted aortic rings was impaired in control rats when determined by the administration of endothelium-dependent vasodilator ACh (Fig. 4). Although almost complete relaxation (98.2 ± 0.9%) was produced by ACh in normal vessels, the maximum relaxation produced in control rats was only 61.5 ± 2.1% ($p<0.01$ vs normal). In benidipine (10 mg/kg)-treated rats, this attenuated relaxation was significantly recovered. In rats treated with nicotine alone, the maximum relaxation by $10^{-5}$ M ACh was 78.6 ± 4.4% ($p<0.01$ vs normal, $n = 10$) of the initial contraction, showing that endothelium-dependent relaxation was also attenuated even by the administration of nicotine alone. ACh ($10^{-5}$ M) elicited full relaxation in vessels from rats treated with vitamin D$_2$ alone or benidipine (10 mg/kg) alone (97.7 ± 2.3% ($n = 3$), 98.2 ± 0.9% ($n = 7$), respectively), these relaxation being not different from that of normal group ($p>0.05$).

**Systolic Blood Pressure**

Figure 5 shows the time course of changes in
Fig. 3. Scanning Electron Micrographs of the Surface of the Endothelium
systolic blood pressure during the experimental period. Throughout 6 d of the experiment, normal rats exhibited constant systolic blood pressure of about 128 mmHg. In control rats, the systolic blood pressure remained almost constant during the first 4 d, and then began to decline, finally reaching to as low as 80 mmHg. In bendipidine-treated rats, the systolic blood pressure declined almost in the same way as in control rats.

**Discussion**

Vitamin D (VD) plays important roles in calcium homeostasis. Administration of overdoses of VD induces hypercalcemia due to the increase in intestinal calcium absorption. In fact, administration of VD$_2$, VD$_3$ or 1-α(OH)D$_3$ causes hypercalcemia and severe calcinosis of soft tissues in rats. On the other hand, administration of nicotine is known to induce the severe degenerative changes in the aortic endothelial cells. This was also confirmed by the present study, which showed that, following 6 d of repeated administration of nicotine alone, the endothelial dependent relaxation was attenuated compared with that of normal rats. The morphological changes of endothelial cells, however, were not noticed presumably due to the short period of nicotine administration. In the present study, administration of nicotine alone did not increase the aortic calcium content. However, combined administration of VD$_2$ and nicotine increased the calcium content more markedly than the single administration of VD$_2$. These results suggest that nicotine can elicit the endothelial injury, resulting in the potentiation of aortic calcinosis occurring in the presence of high blood calcium. Thus, nicotine, in addition to VD$_2$, seems to play a crucial role in the present model of arterial calcinosis.

The present results showed that bendipidine significantly prevents the arterial calcinosis induced by VD$_2$ and nicotine. There are two possible mechanisms of action whereby bendipidine exerted the anticalcinoic effect. First, hypotensive effects may lead to anticalcinoic effect. The aortic calcium content of spontaneously hypertensive rats increases much more than that of normotensive rats according to age, and the long-term prophylactic use of calcium antagonists totally prevents both the increase of blood pressure and the elevation of aortic calcium content. Thus, lowering blood pressure may lead to reduction of aortic calcium content. However, in the present study, bendipidine did not induce...
hypotension during the first 4 d of the experiment in VD$_2$ and nicotine-treated rats. Therefore, it is not likely that the anticalcinoic effect of benidipine is primarily based on the hypertensive effect of this drug in the present study of arterial calcinosis.

The other possible mechanism is based on direct action of the calcium antagonist benidipine. Benidipine can exert effects on smooth muscle cells and/or endothelial cells directly. Mutoh et al. suggested that the protective effect of calcium antagonists on arterial calcinosis is ascribed to their inhibition of slow channels of arterial smooth muscles. This may also be the case with benidipine. In addition, benidipine could act on endothelial cells as well. Endothelial cells protect smooth muscle cells not only physically, but physiologically by releasing several vasoactive and cytoprotective substances such as endothelium-dependent relaxing factor (EDRF) and prostaglandin I$_2$. In the present study, the impairment of ACh-induced relaxation was observed in control rats. This attenuation of the relaxation may be due to the calcium overload into endothelial cells, which results in the decreased release of EDRF. Benidipine (10 mg/kg) significantly improved the attenuated relaxation and it markedly ameliorated the degenerative changes in endothelial cells as examined by scanning electron microscopy. Therefore, benidipine possibly acts on endothelial cells as well as on smooth muscle cells. Although it is not clear whether the injury of endothelial cells precedes that of smooth muscle cells or not, the protection by benidipine of endothelial cells is likely to play some roles in the present model of arterial calcinosis.

It is controversial whether or not there exist voltage-operated calcium channels on the endothelial cell membrane. There are some reports that suggest the existence of such channels. The calcium antagonist nifedipine suppressed the increase in intracellular calcium concentration induced by reactive oxygen in cultured pig endothelial cells. Thus, the protective effect of benidipine on endothelial cells may be exerted by the inhibition of slow channels of endothelial cells. In fact, benidipine markedly improved the EDRF-dependent vasodilation suppressed by splanchnic artery occlusion and reperfusion. However, whether the effect of benidipine on endothelial cells is mediated by slow channel inhibition or not requires further investigation.

In clinical situations, arterial calcinosis gradually proceeds during the life span of healthy humans. Furthermore, atherosclerosis is often accompanied by severe arterial calcinosis. As the therapy for hypertension requires long-term administration of calcium antagonists, benidipine may be expected to inhibit or retard arterial calcinosis when used on a long-term basis as an antihypertensive agent.

In conclusion, the new calcium antagonist benidipine exhibited a protective effect against experimental arterial calcinosis. Moreover, benidipine protected endothelial cells from functional and degenerative changes. The present study suggests that anticalcinoic effect of benidipine involves the protective effect on endothelial cells.

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