Studies on the Mechanism of Antihypertensive Action by Nicotianamine

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Summary Nicotianamine (NA), which is obtained from vegetables, lowers blood pressure through the renin-angiotensin system, and we clarified that NA preferentially inhibits the activity of angiotensin I-converting enzyme (ACE)—a zinc-containing enzyme. In this study, we elucidated the mechanism of antihypertensive action of NA through the Magnus method by using rat aortic blood vessels. Angiotensin I-induced contractions were inhibited by NA in a concentration-dependant manner. Because NA did not inhibit angiotensin II-induced contractions, it was believed that NA inhibited ACE activity in vascular smooth muscles. NA did not affect KCl-induced contractions, but it affected norepinephrine-induced contractions to a small extent. NA exerted similar effects on endothelium-denuded and endothelium-intact blood vessels. Therefore, the antihypertensive action of NA did not play a role in the opening of voltage-dependent calcium channels, but this effect influenced vasoconstriction by the activation of α-adrenergic receptors. These results suggest that after absorption from the intestinal tract, NA may exert antihypertensive effects via 2 mechanisms: direct inhibition of ACE in vascular smooth muscle and activation of α-adrenergic receptors.
Key Words nicotianamine, angiotensin I-converting enzyme (ACE), rat aorta, hypertension

Angiotensin I-converting enzyme (ACE) plays an important role in vascular homeostasis; it is an ectoenzyme of vascular endothelial cells. In addition, ACE is a component of the renin-angiotensin and kallikrein-kinin systems: it activates angiotensin I into angiotensin II, a strong vasopressor, and inactivates bradykinin (BK), a vasodilator. BK induces vasodilation by 2 pathways: it stimulates the production of endothelium-derived relaxing factor and affects the release of prostacyclin (PGI$_2$) (*1, 2*). Therefore, ACE inhibitor not only lowers the blood pressure but also protects organs such as the brain, heart, and kidney (*3*).

Nicotianamine (NA) is ubiquitously present in higher plants (*4*). We previously reported that NA exhibits antihypertensive effects by preferentially inhibiting the activity of circulatory and tissue ACE, which are zinc-containing enzymes (*5*). Moreover, we reported that long-term administration of NA exerts an antihypertensive effect in spontaneously hypertensive rats (SHR) (*6*) and that NA is absorbed from the intestine and released in the blood after administration (*7*). Since NA was detected in blood, we presumed that NA exerts a direct action on blood vessels. We investigated the mechanism of NA action on the aorta. The development of various hypotensive drugs has facilitated the lowering of blood pressure. However, every medicine has certain adverse effects. If it can be proved that NA regulates blood pressure by some mechanism, the prevention or improvement of hypertension without using drugs is possible through daily dietary intake.

In this study, we investigated whether NA directly inhibited the action of the constrictive factor, which acts on vascular smooth muscle, by using aortic blood vessels extracted from a normal rat. Hypertension is one of the well known factors that induces vascular endothelium disorders (*8*). Hence, we investigated the effect of an NO-synthesis inhibitor on endothelium-derived relaxation factor.

MATERIALS AND METHODS

Materials. Human angiotensin I and angiotensin II were obtained from Peptide Institute (Osaka, Japan). Norepinephrine was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Potassium chloride was obtained from Yanagishima Pharmaceutical Co., Ltd. (Tokyo, Japan). Other agents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Aortic ring preparation. All animal handling was performed in accordance with the guidelines of the committee for animal experimentation of our university and notification no. 88, 2006, of the Ministry of the Environment. Seven-week-old male Wistar rats were purchased from Tokyo Laboratory Animal Science Co., Ltd. (Tokyo, Japan), and fed on a standard laboratory diet (CE2; CLEA Japan, Inc., Tokyo) and tap water ad libitum. All animals were housed in cages and maintained in a 12 h light-dark cycle. Temperature and humidity were controlled at $23\pm2°C$ and $55\pm5\%$, respectively. Between the age of 8 and 10 wk, the animals were killed by exsanguination from the abdominal aorta for ring preparation. The thoracic aorta was quickly removed and carefully cleaned of surrounding
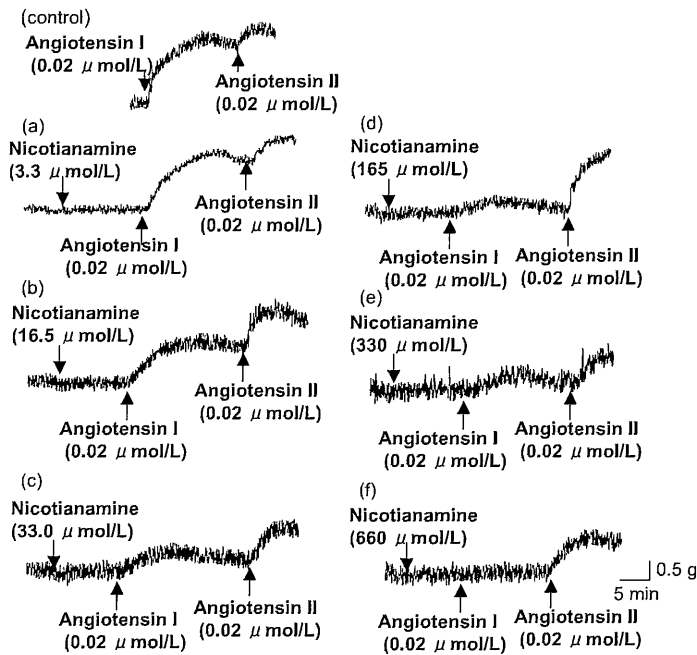
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Fig. 1. Typical traces of vasodilator action after the addition of nicotianamine to angiotensin I- or angiotensin II-induced contractile ring preparations. Constriction was noted in aortic ring preparations pretreated with nicotianamine followed by treatment with 0.02 μmol/L angiotensin I or angiotensin II. Control: Contraction without addition of NA (100%). (a) 3.3 μmol/L NA; (b) 16.5 μmol/L NA; (c) 33.0 μmol/L NA; (d) 165 μmol/L NA; (e) 330 μmol/L NA; and (f) 660 μmol/L NA.

connective tissue, and immediately transferred into PSS buffer bubbled with 95% O_2/5% CO_2 gas at 37°C. The composition of PSS buffer was (mmol/L): NaCl, 145; KCl, 5; Na_2HPO_4, 1; $CaCl_2$, 2.5; $MgSO_4$, 0.5; glucose, 10; and HEPES, 5 (pH 7.4). After 45 min, the aorta was cut into 3–4 mm wide rings, and the ring segments were mounted in organ baths filled with 5 mL PSS buffer. To prepare rings without an endothelium layer, the layer was removed with a scrap of paper. The segments were then stretched using a preload of 2 g and equilibrated for 45 min until stabilized. Contractile response of the aortic ring preparation was measured using a force transducer (Easy Magnus System; Iwashi-ya Kishimoto, Kyoto) connected to a data treatment system (Quad-161 and Data Trax; Physio-Tech, Tokyo).

Experiments on angiotensin I- or angiotensin II-induced contractions. To determine the effect of NA on the contractile inhibition of endothelium-denuded aortic rings, we treated the rings with angiotensin I (0.02 μmol/L) dissolved in the PSS buffer, and determined the maximum contractile tension. After 10 min, the rings were treated with angiotensin II (0.02 μmol/L). To eliminate the decline of reaction caused by desensitization in the estimation of contractions induced by angiotensin I or angiotensin II, we treated each ring separately with the constricting agent once for each reaction. Accordingly, we treated a different ring with NA (3.3–660 μmol/L) and estimated constrictive inhibition at 10 min after treatment with angiotensin I.

Experiments on norepinephrine-induced contractions. Norepinephrine (10 μmol/L) was added to endothelium-denuded or endothelium-intact rings, and the maximum contractile tension was recorded (100%).

The rings were washed with PSS buffer and stabilized for 30 min, and subsequently treated with NA (165–660 μmol/L). Norepinephrine was added to the ring preparations after 10 min, and the inhibition of contractile tension was estimated. Further, the effect of nitric oxide (NO) was examined by exposing the endothelium-intact rings to 1 μmol/L N^G-l-nitro-L-arginine methyl ester (L-NAME) for 10 min before NA addition. L-NAME is an inhibitor of NO synthesis.

Experiments on KCl-induced contractions. Endothelium-denuded and endothelium-intact rings were used to investigate the effect of NA on KCl-induced contractions (KCl, 60 mmol/L). Arterial ring contraction was induced by treatment with KCl in PSS buffer, and the maximum contractile tension was recorded. The rings were then washed using PSS buffer, stabilized for 30 min, and treated with NA (165–660 μmol/L). KCl was added after 10 min, and the inhibition of contractile tension was estimated.

All reagents were cumulatively applied to the organ bath containing 5 mL PSS buffer so that the final concentration would reach a plateau. Results were estimated by decrease to maximal response.

Statistical analysis. The constriction rate of 3 rings was presented as the mean ± SE. Data were analyzed by using the one-way analysis of variance (ANOVA) test. Significant differences of the means were evaluated by Student's t-test at a level of $p < 0.05$.

RESULTS

Effects on angiotensin I- or angiotensin II-induced contractions

The typical trace of aortic ring is indicated in Fig. 1.

Contraction of aortic rings was observed after treatment with angiotensin I (0.02 μmol/L) and angiotensin II (0.02 μmol/L) in PSS buffer, and the maximum contractile tension was recorded (control). Figure 1 (a–f) shows the effect of NA addition in each of the abovementioned solutions. NA (33.0–660 μmol/L) inhibited angiotensin I-induced contractions in a concentration-dependent manner. However, it did not affect contractile tension induced by angiotensin II. The average of contractile tension of 3 angiotensin I-treated rings is indicated in Fig. 2. Addition of NA at ≦16.5 μmol/L did not inhibit angiotensin I-induced contractions, but concentrations ≧33.0 μmol/L significantly inhibited the contractions ($p<0.05$).

Effects on norepinephrine-induced contractions

We examined norepinephrine-induced contractile inhibition after NA (165–660 μmol/L) addition in endothelium-denuded rings, endothelium-intact rings, and endothelium-intact rings after pretreatment with L-NAME. A typical trace after NA addition (660 μmol/L) is indicated in Fig. 3. The average of 3 rings is indicated in Table 1. Addition of 165 μmol/L of NA did not inhibit norepinephrine-induced contraction in either endothelium-denuded or endothelium-intact rings. However, pretreatment with 330 μmol/L or more of NA significantly inhibited norepinephrine-induced contraction ($p<0.05$), and this effect was observed regardless of the presence or absence of the endothelium. Similar results were obtained in the case of endothelium-denuded
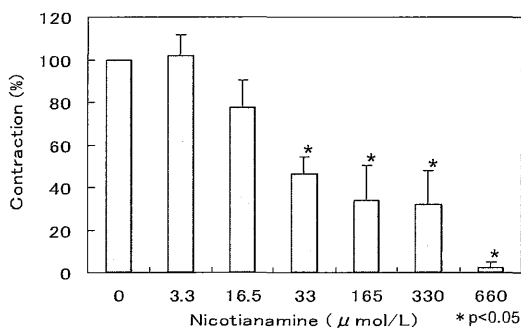
rings and endothelium-intact rings pretreated with L-NAME.

Effects on KCl-induced contractions

Addition of 660 μmol/L of NA, which inhibited angiotensin I- and norepinephrine-induced contractions, did not inhibit KCl-induced contractions (KCl, 60 mmol/L) in either endothelium-denuded or endothelium-intact rings (data not shown).

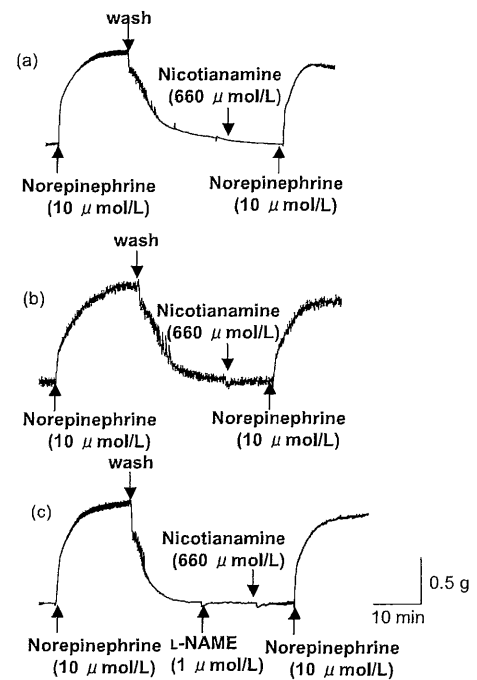## DISCUSSION

Norepinephrine, potassium ions, and angiotensin

Fig. 3. Typical traces of vasodilator action after the addition of 660 μmol/L nicotianamine to norepinephrine-induced contractile ring preparations. Contraction of the aortic rings was observed after the addition of 10 μmol/L norepinephrine, and the maximum contractile tension was recorded. After washing with PSS buffer and subsequent stabilization, inhibition of the contraction after the addition of NA was observed. (a) Typical trace using endothelium-denuded aortic rings. (b) Typical trace using endothelium-intact aortic rings. (c) Endothelium-intact aortic rings were treated with 1 μmol/L of L-NAME.

Fig. 2. Effect of nicotianamine addition on angiotensin I-induced contraction of endothelium-denuded aortic preparations. The values are mean±SE of 3 experiments. *$p<0.05$, significant difference as compared to control (NA, 0 μmol/L).

Table 1. Effect of nicotianamine on norepinephrine-induced contraction of endothelium-denuded and endothelium-intact aortic preparations.

	L-NAME (1 μmol/L)	Nicotianamine (μmol/L)			
		0	165	330	660
Endothelium-denuded	−	100	103.5±3.5	90.0±2.54*	77.1±11.62*
Endothelium-intact	−	100	100±0	90.3±2.25*	82.2±1.99*
	+	100	100±0	92.8±3.2*	84.8±1.45*

The values are mean±SE of 3 experiments.
*$p<0.05$; significant difference as compared to the control (NA, 0 μmol/L).

induce contractions of vascular smooth muscle, and consequently lead to the elevation of blood pressure. The contractile effect of these 3 inducers is mediated by the entry of extracellular calcium ions into vascular smooth muscle cells. However, the mechanism of action varies with different inducers. Norepinephrine induces vascular contractions by the activation of α-adrenergic receptors localized to the cell membrane surface (9). KCl-induced contractions are produced via the opening of voltage-dependent calcium channels (10). Angiotensin I, by itself, does not possess direct vascular contractile activity; angiotensin-induced aortic contractions are generated by angiotensin II, which is formed by the conversion of angiotensin I by ACE localized to the vascular wall (11). In other words, suppression of vasoconstriction after the addition of angiotensin I suggests the inhibition of activity of ACE, which is localized to the aortic vascular wall. In this case, vasoconstriction by the direct addition of angiotensin II was suppressed, which was believed to be due to the blockage of the AT_1 receptor.

We found that KCl-induced contractions were not inhibited after the addition of 660 μmol/L of NA—the maximum concentration used in this experiment. Therefore, we inferred that the antihypertensive action of NA did not involve voltage-dependent calcium channels. However, the addition of NA did not exert a marked concentration-dependent effect on the norepinephrine-induced contractions in the endothelium-denuded aortic preparations or endothelium-intact aortic preparations. Therefore, NA might influence the activation of α-adrenergic receptors localized to the vascular smooth muscle cell membrane. The resulting contraction rates were similar to those obtained for the endothelium-intact rings treated with L-NAME, suggesting that NA did not enhance the activity of the endothelium-dependent relaxation factor that induces vasodilation.

NA suppressed angiotensin I-induced constriction in a concentration-dependent manner. Therefore, it might directly inhibit ACE activity in the aortic vascular smooth muscle. The role of tissue ACE in vivo is not yet clear; only the activity of ACE in the aorta has been correlated to blood pressure (12). After intake, NA is absorbed from the intestine, incorporated in the liver, and released in the blood. In a previous report, NA exerted specific inhibitory effects on rat aortic ACE in vitro (5). In this experiment, we extracted blood vessels from the experimental animals and determined that NA plays a role in the regulation of blood pressure by inhibiting ACE in the aortic vascular wall.

Studies on Tsukuba hypertensive mice (THM) ($n=10$) have detected 9.57 μmol/L of NA in the plasma at 1 h after the administration of 1 mg NA/20 g mouse (7). Further, we found that 30–40 mg of NA can be safely administered to humans daily in the form of food ingredients containing high NA content (unpublished data). If the result on THM is applied to the human, it is possible to calculate where 0.13 μmol/L of NA is detected in blood when the human of 60 kg in weight takes 40 mg

of NA. It is believed that this concentration of NA in the blood corresponds to that at which aorta ACE can be regulated, considering the IC_{50} value of aortic ACE in SHR was 0.13 μmol/L (5). In this study, we determined that the addition of 33.0 μmol/L NA significantly inhibited angiotensin I-induced contraction and 330 μmol/L of NA significantly inhibited norepinephrine-induced contraction. The possibility that a daily NA intake can prevent and improve high blood pressure was suggested.

Various components of food origin have direct influence on vascular smooth muscle contraction, including peptides isolated from sardine muscle (13); dried bonito (14) and seaweed (15); radish leaf extract (16); a component of garlic (17); and tannin extract from cinnamonomi cortex (18). The functions of these substances are not similar to those of NA of non-protein amino acid: NA suppresses the activity of ACE in the aortic vascular wall in a concentration-dependent manner, and it shows influence on the activation of α-adrenergic receptors. On the other hand, captopril, an ACE inhibitor, suppresses endogenous norepinephrine-induced vasoconstriction (14). On a similar line of thought, the possibility that NA may regulate blood pressure by these 2 mechanisms was suggested.

The Dietary Approaches to Stop Hypertension (DASH) clinical trial demonstrated that a diet that emphasizes fruits, vegetables, and low-fat dairy products, includes whole grains, nuts, fish, and poultry, and is reduced in fats, red meats, sweets, and sugar-containing beverages can be highly effective in lowering blood pressure (19). The results of this study indicate that the antihypertensive effect of various vegetables was clearly attributed to NA, which suppresses blood pressure and regulates blood vessel contraction. Adequate vegetables intake is expected to be a very effective method to prevent the development of or to improve mild hypertension. In the future, it is necessary to investigate the efficacy of NA for the treatment of SHR with established hypertension.

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