Nicotianamine Preferentially Inhibits Angiotensin I-Converting Enzyme

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Summary Nicotianamine (NA) is a nonprotein amino acid that inhibits the angiotensin I-converting enzyme (ACE) in the renin-angiotensin system (RAS). The purpose of this study is to prove that NA contributes to the suppression of hypertension by preferential inhibition of ACE. On comparison with EDTA—a chelator—we found that the inhibition pattern of NA for ACE is that of mixed inhibition and that NA exhibits weak chelation effects for zinc, copper, and cobalt ions. Therefore, we investigated whether NA inhibited zinc-containing enzymes other than ACE *in vitro*. The results revealed that NA does not inhibit leucine aminopeptidase or alkaline phosphatase in rat serum. On the other hand, NA demonstrated specific inhibitory effects for rat serum ACE and aortic ACE. These results suggest that the preferential inhibition of circulatory and tissue ACE by NA can contribute to the suppression of hypertension.

Key Words nicotianamine, angiotensin I-converting enzyme (ACE), renin-angiotensin system (RAS), hypertension

The renin-angiotensin system (RAS) plays an important role in the maintenance of blood pressure and fluid and electrolyte homeostasis. The primary enzymes involved in this process are renin (EC 3.4.23.15) and angiotensin I-converting enzyme (ACE) (EC 3.4.15.1). ACE is a zinc-dependent carboxypeptidase that hydrolyzes di- and tri-peptides from the C-terminus of peptides. Each active site of the enzyme appears to bind one atom of zinc; this is crucial for its catalytic activity. Zinc is considered to participate directly in the catalysis of peptide substrates since its replacement with other divalent cations affects the rate of conversion with little effect on the substrate affinity.

ACE inhibitors are now widely used for the treatment of patients with hypertensive disorders. However, a medicine is accompanied by side effects. Many investigators (1–5) have attempted to prepare and isolate an ACE inhibitor in various food substances. We have isolated and purified nicotianamine (NA) from several kinds of plants (6–8). It has been reported that a long-term administration of NA exerts an antihypertensive effect in spontaneously hypertensive rats (SHR) (7) and that it is absorbed from the intestine and detected in the blood after administration into the stomach in Tsukuba hypertensive mice (THM) (8). Furthermore, we established a quick and simple assay system for identifying the presence of NA in plants using the high performance liquid chromatography (HPLC) method (9). If it can be proved that NA is absorbed from the intestinal tract and that it effectively affects the RAS that regulates the blood pressure, the prevention or improvement of hypertension without depending on a drug is possible

through adequate daily dietary intake.

NA, a chelator of metals, is ubiquitously present in higher plants. It chelates metal ions—such as iron—in plants and mediates their transport; further, it is considered to contribute to their stability within cells (10).

In this study, we examined the chelation properties of NA with respect to its selectivity for ACE. Further, we examined whether its antihypertensive effects were attenuated due to the chelation of other metal ions.

MATERIALS AND METHODS

Materials. Hippuryl-L-histidyl-L-leucine (Hip-His-Leu) as a synthetic ACE substrate was obtained from the Peptide Institute (Osaka, Japan). ACE obtained from rabbit lung acetone powder and *p*-nitrophenyl phosphate liquid substrate system (pNPP) were purchased from Sigma Chemical (St. Louis, MO, USA). L-Leucine-*p*-nitroanilide (LPA) was obtained from Merck Ltd. (Frankfurter, Germany). Ethylenediaminetetraacetic acid disodium salt (EDTA) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

ACE inhibitory activity assay. The ACE inhibitory activity was measured by using a modified method (11) of Cushman and Cheung (12); that is, 500 μ L of 7 mM Hip-His-Leu in 200 mM borate buffer (pH 8.3), 400 μ L of 2 M NaCl, 40 μ L of distilled water, and 30 μ L of the inhibitor were incubated with 30 μ L of ACE (156.6 mU/mL) at 37°C for 30 min. The reaction was stopped by adding 500 μ L of 1 M HCl. The hippuric acid released by the action of ACE was extracted with 3 mL of ethyl acetate. After centrifugation at 3,000 rpm for 10 min, 2 mL of the supernatant was dried at 60°C for 2 h *in vacuo* by a speed back concentrator (VC-960, TAITEC, Aichi, Japan). The residue was dissolved in 1 mL of dis-

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tilled water, and the absorbance was measured at 228 nm. The concentration of the inhibitor that is required for 50% inhibition of the ACE activity was defined as the IC_{50} value.

A kinetic study was performed under identical conditions. The concentration range of Hip-His-Leu was 0.35–4.2 mM. The inhibition patterns for ACE were determined by using Lineweaver-Burk plots. In addition, a similar experiment was performed with EDTA as a representative chelator. The experiment included inhibitor concentrations of 5 $\mu\text{g/mL}$ and 3 $\mu\text{g/mL}$; further, the control mixture did not contain any inhibitor.

Effect on ACE inhibition by addition of metals. The measurement of ACE inhibitory activity was performed after the addition of various metals to the inhibitor; that is, 30 μL of 10 $\mu\text{g/mL}$ NA or EDTA and 3 μL of 0–1,000 μM metal were mixed in 400 μL of 2 M NaCl. The metals used in this experiment were NiCl_2 , FeCl_2 , CaCl_2 , MnCl_2 , ZnCl_2 , CuSO_4 , and CoCl_2 . The reaction was started by the addition of 500 μL of 7 mM Hip-His-Leu in borate buffer (pH 8.3) and 30 μL of ACE (156.6 mU/mL) in a method similar to that employed in the measurement of ACE inhibitory activity.

Leucine aminopeptidase (LAP) inhibitory activity assay. The LAP inhibitory activity was measured by using LPA as a substrate. We added 200 μL of 1–4 mg/mL NA in 10 μL of SHR serum; this mixture was preincubated at 37°C for 10 min. The reaction was started by adding 1 mL of 2 mM LPA in Tris buffer (pH 7.6). After incubation at 37°C for 90 min, the reaction was stopped by adding 300 μL of 30% acetic acid, and the absorbance was measured at 410 nm. In addition, 30 μL of 5–100 mg/mL EDTA in 10 μL of SHR serum was added, and the mixture was processed in a manner similar to that described above. The results were indicated using IC_{50} values for LAP activity in 10 μL of serum.

Alkaline phosphatase (ALP) inhibitory activity assay. The ALP inhibitory activity was measured by using pNPP. We added 200 μL of 1 mg/mL NA to 10 μL of SHR serum; this mixture was preincubated at 37°C for 10 min. The reaction was started by adding 1 mL of pNPP. After incubation at 37°C for 30 min, the reaction was stopped by adding 300 μL of 3 M NaOH, and the absorbance was measured at 405 nm. Further, 30 μL of 0.25–5 mg/mL EDTA was added to 20 μL of SHR serum, and the mixture was processed in a manner similar to that described above. The results were indicated using IC_{50} values for ALP activity in 10 μL of serum.

Inhibitory activity of NA for SHR serum ACE and aortic ACE in comparison with pure commercial ACE. In this experiment, we used 18-wk-old male SHR. Blood samples were collected from the abdominal aorta under anesthesia with ether. The samples were centrifuged at 3,500 rpm for 15 min to obtain serum. The serum were stored at -80°C until further use. Following blood collection, the rats were sacrificed and the thoracic aorta was excised from each rat. After washing with cold saline, the aorta samples were cut and homogenized in 2 mL of 200 mM borate buffer (pH 8.3). The samples

Fig. 1. The inhibition patterns of nicotianamine and EDTA for ACE using Lineweaver-Burk plots. The concentration range of Hip-His-Leu was 0.35–4.2 mM. (A) Nicotianamine; (B) EDTA. ○ inhibitor: 5 $\mu\text{g/mL}$; ● inhibitor: 3 $\mu\text{g/mL}$; × control: no inhibitor. Each graph was plotted using the median value of five measurements.

were centrifuged at 12,000 rpm for 20 min, and the supernatant was collected and stored at -80°C until further use. In a manner identical to that used for measuring the ACE inhibitory activity, NA, EDTA, or Captopril was added to the reaction mixture as the inhibitor in 7 mM Hip-His-Leu and 2.0 M NaCl. The reaction was started by adding SHR-serum ACE, aortic ACE, or pure commercial ACE.

RESULTS

Figure 1 shows the inhibition patterns of NA and EDTA for ACE with Lineweaver-Burk plots. For EDTA, the K_m value did not change, while the V_{max} value exhibited a change. Therefore, the pattern was identified as that of noncompetitive inhibition. For NA, both the K_m and V_{max} values exhibited change; therefore, the pattern was identified as that of mixed inhibition. This implied that the inhibition effect of NA on ACE did not solely depend on the effect of chelation.

We investigated the chelation effect of NA by adding metal ions to the mixture used for the measurement of ACE inhibitory activity. Figure 2 (A–D) shows the metal ions that did not suppress the ACE inhibition by NA. The ACE inhibitory activity of NA did not vary even after adding 1,000 μM of NiCl_2 , FeCl_2 , CaCl_2 , or MnCl_2 . On the other hand, a decrease was observed in the ACE

Fig. 2. Effect on ACE inhibition by the addition of metals. The ACE inhibitory activity after adding 3 μL of various metals (0–1,000 μM) was measured. (A) NiCl_2 ; (B) FeCl_2 ; (C) CaCl_2 ; (D) MnCl_2 ; (E) ZnCl_2 ; (F) CuSO_4 ; (G) CoCl_2 . $\circ$ Nicotianamine; $\bullet$ EDTA. The values are the median of triplicate measurements.

inhibitory activity of EDTA following the addition of 1,000 μM of NiCl_2 or CaCl_2 and of more than 100 μM of FeCl_2 or MnCl_2 . Figure 2 (E–G) shows the metal ions that attenuated the ACE inhibition by NA. A decrease in the ACE inhibitory activity of NA was observed following the addition of 1,000 μM ZnCl_2 and of more than 200 μM CuSO_4 or CoCl_2 . HgCl_2 or MgCl_2 did not affect the ACE inhibitory activity of NA or EDTA even at concentrations of 1,000 μM .

We further investigated the preferential ACE inhibition by NA. We examined the inhibition effects of NA for LAP and ALP, which are zinc-containing enzymes in SHR serum. The results are shown with respect to the 50% inhibition concentrations of NA or EDTA for these zinc-containing enzymes in 10 μL of serum (Table 1). The measurement was performed more than twice and reliable values averaged. The concentration of EDTA

Table 1. IC_{50} values of NA or EDTA on zinc-containing enzyme in SHR serum.

	Nicotianamine (μM)	EDTA (μM)
ACE	0.256	9.624
LAP	1,746	460
ALP	1,350	46.97

required for the inhibition of LAP and ALP in the rat serum was approximately 50 times and 5 times of that required for ACE inhibition, respectively. On the other hand, the concentration of NA required for the inhibition of LAP and ALP was approximately 7,000 times and 5,000 times of that required for ACE inhibition, respectively.

Fig. 3. Inhibitory effects of nicotianamine, EDTA, and captopril for SHR serum ACE. (A) Nicotianamine; (B) EDTA; (C) Captopril. ○ SHR serum ACE; ● pure commercial ACE. The measurement was performed more than three times and shown by the median value. For nicotianamine, the IC_{50} value was $7.78 \mu\text{g/mL}$ for SHR serum ACE and $1.6 \mu\text{g/mL}$ for pure commercial ACE. For EDTA, the IC_{50} value was $281 \mu\text{g/mL}$ for SHR serum ACE and $3.13 \mu\text{g/mL}$ for pure commercial ACE. For captopril, the IC_{50} value was $0.063 \mu\text{g/mL}$ for SHR serum ACE and $0.036 \mu\text{g/mL}$ for pure commercial ACE.

Figure 3 indicates the inhibitory effects of three types of ACE inhibitors on pure commercial ACE and SHR serum ACE. For captopril—a typical ACE inhibitor with competitive inhibition for ACE—the IC_{50} value was $0.036 \mu\text{g/mL}$ for pure commercial ACE and $0.063 \mu\text{g/mL}$ for SHR serum ACE. Thus, specific inhibition for both varieties of ACE was observed. On the other hand, for EDTA—a chelation inhibitor with noncompetitive inhibition for ACE—the IC_{50} value was $3.13 \mu\text{g/mL}$ for pure commercial ACE and $281 \mu\text{g/mL}$ for SHR serum ACE, which was approximately 80 times greater than that for pure commercial ACE. Therefore, we inferred that the specificity of EDTA for serum ACE was low. For NA, the IC_{50} value was $1.6 \mu\text{g/mL}$ for pure commercial ACE and $7.78 \mu\text{g/mL}$ for SHR serum ACE, which was slightly lesser than 5 times the value for pure commercial ACE; this demonstrated the specific inhibitory effect of NA on serum ACE.

Figure 4 shows the inhibitory effects for aortic ACE.

Fig. 4. Inhibitory effects of nicotianamine and EDTA for SHR aortic ACE. (A) Nicotianamine; (B) EDTA. △ SHR aortic ACE; ● pure commercial ACE. The measurement was performed more than three times and shown by the median value. For nicotianamine, the IC_{50} value was $1.33 \mu\text{g/mL}$ for SHR aortic ACE and $1.6 \mu\text{g/mL}$ for pure commercial ACE. For EDTA, the IC_{50} value was $46 \mu\text{g/mL}$ for SHR aortic ACE and $3.13 \mu\text{g/mL}$ for pure commercial ACE.

For NA, the IC_{50} value for aortic ACE was $1.33 \mu\text{g/mL}$, which was nearly equal to that for pure commercial ACE. For EDTA, the IC_{50} value for aortic ACE was $46 \mu\text{g/mL}$, which was approximately 15 times greater than that for pure commercial ACE.

DISCUSSION

In previous studies, a majority of the functional components with antihypertensive effects that were derived from food were peptidergic ACE inhibitors. In addition, nonpeptidic components, such as phytic acid (13), gamma aminobutyric acid (14), anthocyanin (15), etc., have been obtained. However, detailed reports regarding the mechanisms of the antihypertensive effects of these inhibitors are few.

NA is a nonprotein amino acid, which was discovered in tobacco leaves by Noma and Noguchi in 1971 (16). Thereafter, it was isolated from many plants, and its wide distribution in the plant kingdom has been confirmed. NA is a metal chelator that exists in higher plants (10). Furthermore, it has been reported that gramineous plants synthesize mugineic acid, which is a metal chelator derived from NA (17). Kinoshita et al. (18) found that NA isolated from soy sauce demonstrates ACE inhibitory activity in vitro and has antihypertensive effects in SHR.

We have isolated NA from several kinds of plants. The administration of NA to SHR (7) and THM demon-

strated that it was absorbed from the intestinal tract; further, it exhibited antihypertensive effects by inhibiting the activity of circulatory and tissue ACE (9). However, since various metal ions exist in vivo, it is necessary to further investigate the preferential inhibition of ACE by NA with respect to its chelation properties.

We investigated the inhibition patterns of NA for ACE and demonstrated that EDTA—a typical chelator—functioned via noncompetitive inhibition, while NA evidenced mixed inhibition. Thus, it was illustrated that the chelation of zinc was not the only mechanism by which NA inhibited ACE. Kinoshita et al. (19) compared the inhibitory effects of NA and EDTA for the zinc-containing enzymes ACE and carboxypeptidase A and for the copper-containing enzyme aminopeptidase M. Their results indicated that the range of the inhibitory activity of NA was similar to that of EDTA for carboxypeptidase A and aminopeptidase M; however, for ACE, the inhibitory activity of NA was 15 times greater than that of EDTA. Therefore, Kinoshita et al. suggested that the chelation effect of NA was not solely responsible for its ACE inhibitory activity. In our experiment, the ACE inhibition patterns of NA were examined using Lineweaver-Burk plots; our results indicated that in addition to the chelation effects illustrated by Kinoshita et al., NA demonstrated patterns of mixed inhibition.

Next, we investigated the chelation effect of NA on various metal ions. Prior to measuring the ACE inhibitory activity of NA, we examined the reactions between NA and several metal ions. We observed that EDTA had a chelation effect on all of the metal ions that we used in our experiment and that the addition of certain concentrations of these metals caused a decrease in its ACE inhibition. With respect to NA, we observed that it had a weak chelation effect for zinc, copper, and cobalt ions.

Further, we compared the differences between the inhibitory activities of NA and EDTA for SHR serum ACE and for the zinc-containing enzymes LAP and ALP. We observed that NA had a high affinity for ACE with a very low value of IC_{50} , while a 5,000–7,000 times greater concentration of NA was necessary to inhibit LAP and ALP. On the other hand, the IC_{50} value of EDTA for the inhibition of LAP and ALP was 5–50 times greater than that for ACE. Thus, it was demonstrated that NA specifically inhibited ACE and had a low specificity for the other zinc-containing enzymes, i.e., LAP and ALP. These results suggested that since the intestinal absorption of NA was possible, the preferential inhibition of ACE by NA could effectively suppress hypertension.

Apart from the RAS present in circulatory systems, there are independent RASs in vivo in local tissues such as the lung, heart, kidney, adrenal gland, aorta, and brain. Moreover, it has been demonstrated that they are controlled by mechanisms different from those for the circulatory RAS (20). Therefore, we used aortic extracts to examine the specificity of NA for tissue ACE. We examined the specificity of NA for various sources of ACE in comparison to pure commercial ACE in the case of metal enzymes other than ACE being included in the

aorta or serum. In addition, we used captopril and EDTA, which are typical inhibitors showing competitive inhibition for ACE, in order to compare the inhibitory effects of NA. We observed that captopril specifically inhibited both pure commercial ACE and serum ACE in nearly equal concentrations. On the other hand, the concentration of EDTA required for inhibiting serum ACE was greater than 80 times that required for inhibiting pure commercial ACE; this result indicated the low specificity of EDTA for serum ACE. The concentration of NA required for inhibiting serum ACE was slightly less than 5 times that required for inhibiting pure commercial ACE; this result indicated the specific inhibition of serum ACE by NA.

In addition, when we administered 1 mg of NA to THM, NA was detected $2.85 \pm 1.337 \mu\text{g/mL}$ in blood after 1 h (8). In this study, the IC_{50} value of NA on SHR serum ACE was $7.78 \mu\text{g/mL}$; it was less than 3 times that of blood concentration for 1 h after administration. Therefore, it was sufficiently shown that NA suppressed ACE activity in vivo.

Furthermore, unlike EDTA, NA inhibits aortic ACE in concentrations identical to those that inhibit pure commercial ACE.

NA is a chelator that is widely distributed in higher plants (10) and is absorbed in the blood following its intake (9). However, because of NA has chelate effects, it was thought that ACE inhibitory activity has not been exerted by chelating with other metal ions in vivo. This study examined the selectivity of NA for ACE. The results suggested that NA preferentially acted on circulatory ACE without influencing other zinc-containing enzymes and that it contributed to the suppression of hypertension by selectively inhibiting tissue ACE.

In conclusion, it is suggested that NA is useful for the prophylaxis or suppression of hypertension. Since it is found in natural foods and is easy to include in the daily diet, it can be used for the prevention of hypertension without any side effects.

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