**Note**

**Determination of Endogenous Peptides with in Vitro ACE Inhibitory Activity in Normotensive Human Plasma by the Fluorometric HPLC Method**

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An in vitro degradation test of angiotensin (ANG) II or III in normotensive supine human plasma from 9 healthy male subjects confirmed the production of smaller ANG metabolites with angiotensin I-converting enzyme inhibitory activity. These metabolites were identified as ANG (3-8), ANG (4-8), ANG (5-8), and ANG (3-4), whose respective peptide concentrations were determined by our proposed naphthalene-2,3-dialdehyde (NDA)-HPLC method to be 64 ± 9, 39 ± 5, 176 ± 22, and 197 ± 35 fmol/ml of plasma.

**Key words:** ACE; angiotensin metabolites; HPLC

The renin-angiotensin (R-A) system is well known to deeply affect blood pressure (BP) and to be localized in such diverse tissues as the lung, kidney, blood vessel, and brain cells. In the R-A system, angiotensin (ANG) II has both biological activities as activation of vasoconstriction and retention of Na⁺ in kidneys via the promotion of aldosterone release. An angiotensin I by the action of the angiotensin I-converting enzyme (ACE, EC 3.4.15.1) is mainly formed from ANG I by the action of the angiotensin I-converting enzyme (ACE, EC 3.4.15.1). This, direct blockage or inactivation of ACE by inhibitors is very effective for lowering BP. Many synthetic ACE inhibitors have thus been developed and applied to clinical usage. More than 400 peptides with ACE inhibitory activity have already been isolated and identified from natural resources for the food industry to prepare a physiologically functional food. In our series of studies, we have isolated twenty ACE inhibitory peptides from an enzymatic hydrolyzate of sardine muscle. Among these peptides, one surprising finding was that the sequence of the tripeptide, Arg-Val-Tyr, from the Bacillus licheniformis alkaline protease (2.4L, type FG, Nacalai Tesque, Co., and EC 3.4.15.1) hydrolyzate of sardine muscle is a part (ANG (2-4)) of the sequence of hypertensive ANG II. According to this finding, we have already obtained the interesting results with rats that (i) among the thirteen synthetic ANG II fragments, seven showed potent in vitro ACE inhibitory activity (IC₅₀ < 200 μM), (ii) four ACE inhibitory fragments (ANG (3-8), ANG (4-8), ANG (5-8), and ANG (3-4)) were produced by the in vitro ANG III degradation test in spontaneously hypertensive rat (SHR) plasma, and (iii) intravenous administration of ANG (3-4), one of the four fragments, to SHR, resulted in a diastolic blood pressure (DBP) being significantly (p < 0.01) decreased (22.6%) with a dose of 20 mg/kg. These results led us to speculate that when in vitro ACE inhibitor peptides with the same sequence of ANGs existed in blood, they would have the possibility to show an in vitro antihypertensive effect, although their bioavailability and in vivo activity have not been clarified. In order to clarify the in vivo biological activity of these smaller ANG fragments, it is necessary to demonstrate their existence and bioavailability. However, there has been little systematic research to confirm the occurrence of endogenous smaller ANG metabolites in human plasma because of the absence of an adequate analytical method. In our previous paper, the fluorometric HPLC method with naphthalene-2,3-dialdehyde (NDA) was proposed to determine ANG I, II, and III in human plasma. In this paper, we reported the determination of the endogenous smaller ANG metabolites or corresponding peptides with in vitro ACE inhibitory activity in normotensive human plasma by this method.

Human plasma was obtained from 9 healthy male volunteer students at Kyushu University of 22 to 26 years of age (24 years mean age) who gave their informed consent prior to the study. The subjects were instructed not to consume any beverages and not to smoke from 12h prior to the protocol. Before breakfast, venous blood samples (20 ml) were taken after assuming a 30-min supine posture. Each blood sample was drawn into chilled vacutainer tubes containing sodium heparin and EDTA-2Na (Terumo, Tokyo, Japan). After immediately centrifuging at 1500 × g for 15 min at 4°C, the plasma was obtained. Supine systolic/diastolic BP (SuSBP/SuDBP) was consecutively measured 3 times prior to the blood sampling protocol. Plasma renin activity (PRA) was determined by a PRA assay kit (Ciba Geigy Co., Hyogo, Japan).

The in vitro preparation procedure for the ANG metabolism was the same as described in our previous paper. An aliquot (300 μl) of plasma obtained from the heparin vacutainer tube was added to a test tube containing d-Leu-d-Leu (Sigma Chemical Co., St. Louis, MO, U.S.A.; 0.4 mm final concentration) and ANG II or III (Sigma Chemical Co., St. Louis, MO, U.S.A.; 6.25% w/v final concentration). The plasma sample was incubated for 6 min at 37°C, and the incubation stopped by adding 40 μl of 10% trichloroacetic acid to the test tube (1% final concentration). The plasma mixture was then ultrafiltered through Molucut L (< M.W. 5000, Nihon Millipore, Yonezawa, Japan). The ultrafiltered plasma sample was directly applied to a reversed-phase column (Cosmosil 5C₁₈·AR, 4.6φ x 250 mm; Nacalai Tesque, Kyoto, Japan) for HPLC (Shimadzu LC-9A instrument, Kyoto, Japan) and then eluted with a linear CH₃CN gradient (10–25%, 150 min) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.5 ml/min.

The endogenous ANG metabolites in normotensive human plasma were then determined. An aliquot (10 ml) of the plasma obtained from the EDTA-2Na vacutainer tube was immediately ultrafiltered through Molucut L (< M.W. 5000) to prevent any degradation of endogenous ANG metabolites by proteases, before being concentrated to 0.5 ml by evaporation under reduced pressure. The sample was then directly applied to the Cosmosil 5C₁₈·AR column and eluted with a linear CH₃CN gradient.

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*Abbreviations:* ACE, angiotensin I-converting enzyme; ANG, angiotensin; R-A, renin-angiotensin; NDA, naphthalene-2,3-dialdehyde; HPLC, high-performance liquid chromatography.

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Determination of Endogenous Peptides in Human Plasma

Fig. HPLC Elution Profiles from the in Vitro Degradation of Angiotensin III in Normotensive Human Plasma at 37°C for 6 min.
The initial concentration of ANG III was 6.25% w/v. Column, Cosmosil SCX,AR (4.6μ x 250mm); solvent system, 10% to 25% CH3CN (150 min) in 0.1% TFA; flow rate, 0.5 ml/min; column oven, 30°C; absorbance monitoring, 220 nm.

(10–25%, 150 min) in 0.1% TFA at a flow rate of 0.5 ml/min. The fractions corresponding to the elution of each synthetic ANG metabolite were collected and dried. These fractions were rechromatographed in the same column (ANG (3–4), 5–15% CH3CN in 0.1% TFA, 100 min; ANG (5–8) and (4–8), 15–25%, 100 min; ANG (3–8), 20–25%, 100 min), and collected and dried.

The peptides corresponding to the ANG metabolites in their fractions were determined by our proposed fluorometric HPLC method with NDA. Briefly, to 50 μl of a sample solution prepared by adding a 20 mM borate buffer (pH 9.5) into the final fractionation tube, 10 μl of a 10 mM sodium cyanide solution in the borate buffer and 50 μl of a 0.1 mM NDA (Fluka, Tokyo, Japan) solution in methanol were added. After reacting for 60 min at ambient temperature, each NDA-converted sample (50 μl) was applied to the SCX,AR column and eluted with a linear CH3CN gradient in 0.1% TFA (ANG (3–4) and ANG (3–8), 45–55% CH3CN (100 min); ANG (4–8) and ANG (5–8), 30–45% CH3CN (100 min)). Fluorescence detection (excitation and emission wavelengths of 420 nm and 490 nm, respectively) was done with a Shimadzu RF-10A instrument equipped with a 150W xenon lamp. The NDA-ANG peaks in human plasma were identified by comparing the retention times with those of corresponding NDA-synthetic ANG peaks with a Shimadzu SPD-10AV UV detector attached to the front of the fluorescence detector.

The radioimmunoassay (RIA) of ANG II in 1.0 ml of plasma used an Angiotensin II RIA kit (Nichols Institute Diagnostics B.V., The Netherlands).

We primarily attempted to confirm the in vitro degradative production of the smaller ANGS from vasopressor ANG II or III in normotensive human plasma. The figure shows the HPLC patterns with and without the addition of ANG III to human plasma. After incubating an excess amount (6.25% w/v) of ANG III with plasma for 6 min, a decrease in the amount of ANG III (6.14% w/v) and four new peaks marked with arrows were observed, although after incubating the plasma without adding ANG III and the plasma pre-inactivated by 10% TCA with added ANG III, these peaks were not apparent within the UV detection limit. A Shimadzu LC-6A amino acid analyzer and Shimadzu PSQ-1 protein sequencer were used to identify the corresponding ANG (3–4), Tyr-Ile-His-Pro-Phe (ANG (4–8)), Tyr-Ile-His-Pro-Phe (ANG (4–8)), and Val-Tyr-Ile-His-Pro-Phe (ANG (3–8)). These four peptides were also produced for the ANG II study (data not shown). Consequently, the four ANG metabolites would be likely to exist in human plasma.

To confirm the existence of the endogenous ANG metabolites in normotensive human plasma, the concentrations of ANG I and II and of the four peptides corresponding to the sequence of ANG (3–4), ANG (4–8), ANG (3–8) (Val-Tyr, Ile-His-Pro-Phe, Tyr-Ile-His-Pro-Phe, and Val-Tyr-Ile-His-Pro-Phe, respectively) were determined by our proposed NDA-HPLC method, using 9 healthy male subjects (Table). ANG III could not be determined by the present method with a detection limit of 1.4 fmol/ml of plasma. The validity of the results is supported by the agreement (no significant difference (p > 0.05)) between the ANG II concentration determined by the present method (32 ± 6 fmol/ml of plasma mean) and the RIA method (20 ± 4 fmol/ml of plasma). The average value of SuSBP/SuDBP and PRA for the subjects were 110 ± 2.5/59 ± 3.1 mm of Hg and 1.35 ± 0.57 ng/ml/h, respectively. As shown in the Table, ANG I and II, and the four peptides existed in supine human plasma at concentrations of more than 40 fmol/ml of plasma (mean values: ANG I, 304 ± 43 fmol/ml of plasma; ANG II, 32 ± 6; ANG III, <1.4; Val-Tyr-Ile-His-Pro-Phe, 64 ± 9; Tyr-Ile-His-Pro-Phe, 39 ± 5; Ile-His-Pro-Phe, 176 ± 22; Val-Tyr, 197 ± 35). In particular, Val-Tyr was the predominant peptide in normotensive supine human plasma among these.

The in vitro study, using normotensive human plasma, in which ANG II or ANG III was incubated with human plasma for 6 min at 37°C, revealed the production of ANG (3–8), ANG (4–8), ANG (5–8), and ANG (3–4). In addition, when the concentrations of...
the corresponding peptides in normotensive supine human plasma from 9 healthy male subjects were determined by our proposed NDA-HPLC method, Val-Tyr was found to be the most predominant peptide among them (mean value of 197 ± 35 fmol/ml of plasma). Allard et al.15 have already demonstrated the metabolism of ANG (3–4) from ANG II in cultured mouse spinal cord cells, so that part of Val-Tyr found in the human circulatory R-A system would possibly result from the metabolism of ANGs. Specification of the precursor of these four peptides, especially of Val-Tyr, and of the metabolic pathway for ANGs in huma plasma is necessary to confirm their involvement in the R-A system, and this is now under investigation.

References


Table
Concentrations of Angiotensins and Peptides with in Vitro ACE Inhibitory Activity in Normotensive Human Supine Plasma

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* Determined by the NDA-converted fluorometric HPLC method.
* Determined by the radioimmunoassay method.
* ND, not determined.