Absorption of Val–Tyr with *in Vitro* Angiotensin I-Converting Enzyme Inhibitory Activity into the Circulating Blood System of Mild Hypertensive Subjects

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The change in plasma level of dipeptide, Val–Tyr (VY), with *in vitro* angiotensin I-converting enzyme inhibitory activity was investigated after a single oral administration of a VY-drink at doses of 0, 6 or 12 mg given to mild hypertensive subjects. During this protocol for up to 24 h after the intake, patient/subject blood pressure (BP) was measured for a 15 min period at designated times (0, 1, 2, 4, 8, 24 h) with the individual supine. Based on the VY determination, the maximal increment of plasma VY level was observed over the second hour postprandially (12 mg-dose; 2041±148 fmol/ml-plasma). In addition, the plasma VY level increased with the VY dosage. However, no marked BP change was observed with the increase of plasma VY level, suggesting that VY did not exert an acute hypotensive effect. The area under the curve at 12 mg-dose was estimated to be 8644±420 fmol·h/ml-plasma, comparable to that in normotensive subjects. This finding suggests that absorption of VY would not be influenced by a complaint of hypertension.

Key words peptide absorption; hypertension; circulating renin–angiotensin system; column-switching

Recent studies revealed that some specific peptides have diverse physiological functions such as promotion of Ca^{2+} absorption,[1] antioxidant action against unsaturated fatty acids,[2] and in particular, lowering of blood pressure (BP).[3—5] Evidence of the mild antihypertensive effect of a Val–Tyr (VY) drink in hypertensive subjects for a 4 wk-protocol[6] strongly supports the hypothesis that the small VY peptide plays a physiological role in lowering BP. There has been no evidential study, however, on absorption of small bioactive peptides in the human circulating blood system due to lack of a sufficient assaying method.

Studies on peptide transport through membranes have demonstrated that an intestinal absorption of peptide was faster than that of its individual amino acids by jejunal perfusion tests in rat[7,8] and in human.[9,10] According to the reports on the peptide transport system at the intestinal membrane,[11,12] some peptide transporters were identified or cloned as human PepT1 and T2,[11] and a more preferable structure of absorbed peptides or peptidic drugs was found to be di-/tripeptides possessing a free terminal carboxyl group and at least one peptide bond.[12] In parallel with passive permeation the absorption of peptides may be accelerated for those peptides having a high affinity to the proton-dependent system. These findings led us to investigate how much amount of VY can be absorbed in a single oral administration by mild hypertensive subjects. In our previous paper,[13] intact VY absorption with pmol level per ml-plasma was identified in normotensive human subjects. It remains unclear, however, whether VY as a natural angiotensin I-converting enzyme (ACE) inhibitor with the IC_{50} value of 26 μM[14] can be absorbed and can exert an acute hypotensive power as can a therapeutic drug in hypertensive subjects.

Thus, we examined here the pharmacokinetics of VY in mild hypertensive subjects using our proposed column-switching HPLC method with fluorogenic naphthalene 2,3-dialdehyde (NDA) reagent.[15]

MATERIALS AND METHODS

Materials NDA was obtained from Fluka (Tokyo, Japan). All other chemicals were of analytical-reagent grade and used without further purification.

Subjects Mild hypertensive male volunteers of Senmi Ekisu Co. aged 39 to 59 years (n=12, 145.9±3.5 mmHg/93.6±2.1 mmHg, 48.6±2.8 years) participated in the study, and were divided into three groups by dosage (0, 6 or 12 mg-VY). The baseline characteristics are summarized in Table 1. All subjects gave their informed written consent for participation, and the Human Investigation Review Committee of the Institute of Health Science, Kyushu University, approved the study.

Protocol The drink for the study containing 0, 6 or 12 mg of VY in 100 ml was supplied by Senmi Ekisu Co. The subjects were instructed not to consume any beverage nor to smoke during the 12 h prior to the start of the study. After taking urine and blood, the subjects were administered one of the three VY-drinks in each group. Urine was also collected after 24 h to evaluate 24 h-urinary Na^+, K^+ and creatinine excretions.

Blood specimens (7 ml) were taken into a chilled vacutainer tube containing EDTA-2Na (TERUMO, Ltd., Tokyo) at fixed intervals of 1, 2, 4, 8, and 24 h after administration for measurements of plasma angiotensin (Ang) I, Ang II, and VY levels as well as plasma renin activity (PRA). Systolic BP (SBP), diastolic BP (DBP) and heart rate (HR) were measured 3 times in succession with the subjects in the 15 min-supine position before each blood sample was taken. Each sample was then centrifuged at 1500×g for 15 min at 4 °C, followed by immediate ultrafiltration with a Molucut L (<M.W. 5000; Nihon Millipore, Ltd., Yonezawa, Japan) at

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Table 1. Baseline Characteristics of Mild Hypertensive Volunteers before and after Oral Administration of Val–Tyr (VY)

	0 mg-VY dose		6 mg-VY dose		12 mg-VY dose	
Time after intake (h)	0	24	0	24	0	24
Age (years)	48±2.7	—	50±3.9	—	47±4.4	—
Men/women	4/0	—	4/0	—	4/0	—
Blood pressure (mmHg)	144±5.4/99±3.8	—	146±4.7/93±3.5	—	145±3.7/91±4.5	—
Body height (cm)	170.7±3.2	—	170.0±1.3	—	171.8±1.2	—
Body weight (kg)	62.5±1.3	—	67±2.8	—	71±3.8	—
Body mass index (kg/m^2)	21.5±0.7	—	23.1±0.7	—	24.6±1.3	—
Urinary electrolytes (g/l)						
Creatinine	0.8±0.03	0.8±0.03	0.9±0.03	1.0±0.04	1.1±0.03	1.0±0.04
Sodium	4.4±0.9	4.2±1.2	3.2±0.4	3.2±0.7	3.5±0.4	3.2±0.3
Potassium	2.1±1.0	1.7±0.3	1.9±0.7	1.3±0.3	1.9±1.4	1.7±0.5

Values are expressed as mean±S.E. ($n=4$).

4 °C. The ultrafiltered plasma was stored at −30 °C until use.

Measurements of Plasma Angs and VY The plasma sample was subjected to VY determination by our proposed column-switching HPLC method.[15] Briefly, prior to the column-switching HPLC assay, 250 μl of the ultrafiltered plasma was applied to a reversed-phase HPLC (Shimadzu LC-9A instrument, Kyoto, Japan). The HPLC conditions were as follows: column: Cosmosil 5C18-ARII (4.6×250 mm, Nacalai Tesque, Ltd., Kyoto, Japan); elution: 10%—25% CH_3CN in 0.1% trifluoroacetic acid (TFA) (linear gradient of 150 min); flow rate: 0.5 ml/min; monitoring absorbance: 220 nm. The fractions corresponding to the VY elution time of 40 min were collected and dried. The fraction dissolved in 50 μl of 20 mM borate buffer (pH 9.5) was derivatized by adding 50 μl of 0.1 mM NDA solution in methanol and 10 μl of 10 mM sodium cyanide solution in borate buffer for 60 min at ambient temperature. The NDA derivatized sample (50 μl) was then applied to a clean-up Cosmosil 5Ph column (4.6×250 mm) with the linear gradient mode of 40 to 60% CH_3CN (60 min). The zone of retention of NDA-VY (65.5 to 68.5 min) was then heart-cut, and separated on an analytical column (Cosmosil 5C18-ARII). The mobile phase was the 60% CH_3CN in 0.1% TFA containing 5 mM sodium octyl sulfonate as an ion-paired reagent, and the flow rate was 0.4 ml/min. The fluorescence detection (excitation and emission wavelengths: 420 nm and 490 nm, respectively) was done with a fluorescence detector (FP-920S, Nippon Bunko, Tokyo).

Statistical Analysis Values are expressed as the mean±S.E. Changes within each group over time were assessed by the repeated measures two-way analysis of variance (ANOVA) followed by Dunnett's t-test for *post hoc* analysis. $p<0.05$ was considered statistically significant. These analyses were performed with Stat View J5.0 (SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS AND DISCUSSION

Table 1 summarizes the subject features before and after the VY-protocol at VY dosages of 0, 6 and 12 mg. Throughout the 24 h-VY study in mild hypertensive subjects, no significant changes in urinary chemistries were observed compared with the data before administration. Nor were adverse effects such as cough found by administering the VY-drink as previously reported.[6)]

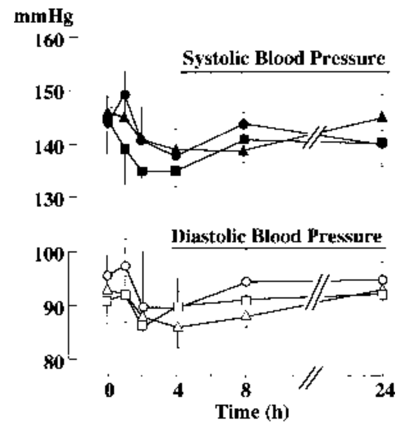

Fig. 1. Mean Systolic (Closed Symbols) and Diastolic Blood Pressure (Open Symbols) in Mild Hypertensive Subjects ($n=4$ in Each Group) at the Val–Tyr (VY) Doses of 0 mg (○, ●), 6 mg (△, ▲), and 12 mg (□, ■)

One hundred milliliters of VY-drink containing each dose was administered orally, and the blood pressure at the 15 min-supine position was measured at fixed intervals of 1, 2, 4, 8, and 24 h. Values are expressed as the mean±S.E. No significant difference of BP within control and VY-groups over time was observed by analyzing the repeated measures of two-way ANOVA.

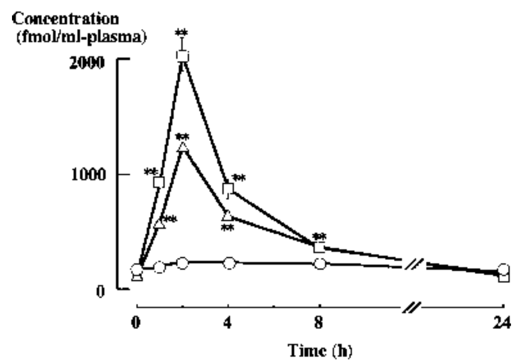

Fig. 2. Time-Course of Plasma Val–Tyr Level after a Single Oral Administration at a Dose of 0 mg (○, Control), 6 mg (△), or 12 mg (□) to Mild Hypertensive Subjects

Plasma Val–Tyr level was measured by the fluorimetric column-switching HPLC method. Values are expressed as the mean±S.E. ($n=4$). ** $p<0.01$ *vs.* control group.

Figure 1 depicts the BP change of the volunteers during the run-in experiment after VY intake. No significant or marked BP reduction after the administration was observed, suggesting that no acute BP lowering effect would be expected by oral intake of a single VY-drink.

Figure 2 shows the change in plasma VY level in mild hy-

Table 2. Pharmacokinetic Parameters of Val–Tyr (VY) in Mild Hypertensive Subjects

Parameter	VY dose (mg)		
	0	6	12
C_{max} (fmol/ml-plasma)	242±26.9	1245±24.5	2041±148
$t_{1/2}$ (h)	—	3.1	3.1
AUC (fmol·h/ml-plasma)	8472±167[a]	6622±512	8644±420

a) The *AUC* for 0 mg VY-dose indicates the *AUC* for endogenous VY during the 24 h-protocol. Data are expressed as mean±S.E., $n=4$. *AUC* was determined from 0 to 24 h for net VY absorption. C_{max}, maximal plasma VY concentration; $t_{1/2}$, elimination half-time; *AUC*, area under concentration.

pertensive subjects after a single oral administration of 6 mg and 12 mg VY dosages. A remarkable and maximal increment of plasma VY level was achieved 2 h (T_{max}) after dosing in both VY-groups, and then declined with an elimination of half time ($t_{1/2}$) of 3.1 h. A significant ($p<0.05$ *vs.* 0 h) accumulation of plasma VY level continued to 8 h. Figure 2 also depicts that VY occurred endogenously in plasma of the mild hypertensive subjects, but the endogenous plasma VY level in the control group did not change significantly during the 24 h-protocol (163 to 243 fmol/ml-plasma). As summarized in Table 2, a dose-dependent increase of maximal VY concentration (C_{max}) in plasma was observed at 2 h; C_{max} at a dosage of 6 mg and 12 mg VY was 5.7 and 9.3 times higher than that of 0 mg dose, respectively. Net *AUC* (area under the curve over 24 h) for a 12 mg VY-dose calculated from overall *AUC* minus *AUC* for endogenous VY showed a 1.3 times higher absorption than that of a 6 mg VY-dose.

Interestingly, all of the pharmacokinetics of VY in the mild hypertensive subjects were in good agreement with those in normotensive subjects (12 mg of VY dosage: C_{max}; 1934±145 fmol/ml-plasma, T_{max}; 2 h, *AUC*; 9185±688 fmol·h/ml-plasma[13]). To date, a human peptide transporter, PepT1, expressed primarily in the small intestine has been cloned as a proton-dependent transporter peptide.[11] Provided that VY was absorbed through the PepT1 transporter specific for di-/tri-peptides,[12] no significant difference in the VY absorption between normotensives[15] and mild hypertensives (Table 2) suggested that the VY transport system *via* PepT1 across the brush border membrane would not be influenced by a complaint of hypertension. Of course, VY absorption by passive permeation through the tight-junction between cells should be considered in this speculation, and an alternative transport experiment will be needed for further elucidation.

An acute pharmacokinetic study of captopril to hypertensive subjects revealed that the maximal plasma level (C_{max} at 100 mg-dose; 1.3 nmol/ml-plasma) was seen 53 min after administration (T_{max}; 53 min, $t_{1/2}$; 100 min).[16] Oral administration study of prodrug-type ACE inhibitor, imidapril, to healthy human,[17] on the other hand, demonstrated that the plasma concentration reached a maximal level (C_{max} at 10 mg-dose) of 70 pmol/ml-plasma after 2 h (T_{max}; 2 h). While the absorption profile of imidapril with T_{max} of 2 h was in good agreement with that in our present VY-study (Fig. 2), the C_{max} of imidapril was 35-fold greater than that of 12 mg-VY dose (Table 2). However, local accumulations of VY at diverse organs should also be considered in investigating the lower absorption of VY in plasma, because it was reported that the ACE inhibitory peptide, Ile–Pro–Pro, was accumulated in the rat abdominal aorta (2 to 5 μg/aorta) after gastric intubation of 141 μg/rat.[18] The report by Okunishi *et al.*[19] supported this possibility, in which spirapril which is a long-acting ACE inhibitor greatly retarded the action of local ACE in lung, kidney, and aorta rather than plasma ACE in spontaneously hypertensive rat.

In summary, this was the first finding in mild hypertensive subjects that small dipeptide, VY, can be absorbed intact into the circulating blood system with the C_{max} of 2 pmol/ml-plasma at 2 h given at a dosage of 12 mg.

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