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Tissue Distribution of Antihypertensive Dipeptide, Val-Tyr, after its Single Oral Administration to Spontaneously Hypertensive Rats

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Abstract: The distribution of an antihypertensive dipeptide, Val-Tyr (VY), in the tissues of spontaneously hypertensive rats (SHR) was investigated in this study. A single oral administration of VY (10 mg/kg) to 18-week-old SHR resulted in a prolonged reduction of systolic blood pressure (SBP) up to 9 h (SBP_{0h} 198.0 ± 3.6 mmHg; SBP_{9h} 154.6 ± 3.5 mmHg). As a result of VY determination, a roughly 10-fold higher increment of plasma VY level was observed at 1 h than that at 0 h, whereas thereafter the level declined rapidly. In tissues, VY was widely accumulated in the kidney, lung, heart, mesenteric artery and abdominal aorta with the area under the curve over 9 h of more than 40 pmol h/g tissue; of these a higher VY level was observed in the kidney and lung. In addition, a mean resident time (MRT) for each tissue (>5 h except for liver) revealed that VY preferably accumulated in the tissues rather than in the plasma (MRT 3.8 h). Significant reductions of tissue angiotensin I-converting enzyme activity and angiotensin II level were found in the abdominal aorta as well as in the kidney, suggesting that these organs could be a target site associated with the antihypertensive action of VY.

Keywords: hypertension; angiotensin I-converting enzyme; antihypertensive peptide; renin–angiotensin system; tissue accumulation

INTRODUCTION

Some advanced studies on the antihypertensive effect of peptides have provided clinical evidence that chronic administration of angiotensin I-converting enzyme (ACE, EC 3.4.15.1) inhibitory peptides may improve the borderline hypertensive stage. To date, antihypertensive food products containing active peptides have been accepted by the Ministry of Health, Labor and Welfare in Japan as a FOSHU (foods for specific health uses) product. Our clinical trial using sardine muscle hydrolysate in mild essential hypertensive volunteers also demonstrated a successful antihypertensive effect, in which Val-Tyr (VY) contributed to a significant reduction of blood pressure (BP) during a 4-week experimental period [1]. The finding that VY was a competitive ACE inhibitor with a K_i value of 3.03 μM [2] suggested that even a natural small peptide may induce a positive antihypertensive effect via ACE inhibition as well

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as therapeutic drugs. Contrary to the therapeutic prevalence of ACE inhibitory peptides, no detailed studies on the antihypertensive mechanism of active peptide have been performed so far, due to the difficulty of assaying small peptides in the blood and tissues. Although accumulation of Val-Pro-Pro with ACE inhibitory activity in the abdominal aorta of spontaneously hypertensive rat (SHR) has been reported by Masuda *et al.* [3], where the peptide was detected at a level of 4.3 μg/rat aorta at 6 h after gastric intubation, its bioavailability or pharmacokinetics in SHR still remains unclear.

One possible reason why VY intake induced a significant BP reduction is the suppression of the renin–angiotensin system (RAS), contributing to a vasodilatation and/or natriuresis. However, there is no research yet showing that active peptides, including VY, with *in vitro* ACE inhibitory activity act on the human RAS. According to our previous studies clarifying the antihypertensive effect induced by VY dosing, the effect could be explained partly by the following experimental findings [4–6]:

1. VY can be absorbed intact into the human circulating blood system without any hydrolysis by peptidases during the absorption process [4,5];
2. Enhanced human circulating RAS was suppressed by the absorbed VY [6].

The latter fact that VY acted on human RAS was proved by using transgenic hypertensive mice (Tsukuba Hypertensive Mice, THM) having both the human renin gene and human angiotensinogen gene. However, it was impossible to make clear the distinct BP depressor mechanism of VY, because the RAS occurs in diverse organs, and each RAS plays an independent physiological role in regulating BP [7]. Our previous finding that the long-lasting BP lowering effect of VY did not correlate with a transient inhibition of the circulating RAS [6] strongly suggested that VY peptide would exert a considerable role in tissue BP regulation systems. Therefore, it is important to assess the accumulation of antihypertensive peptide in diverse organs pharmacodynamically, because not only the RAS, but also other systems such as kinin–kallikrein and nerve systems are involved in BP regulation [8]. Hence, in the present study, target sites of VY accumulation were clarified plasma and tissue ACE activities as well as angiotensin (Ang) II level were also checked to clarify their action against diverse RASs.

MATERIALS AND METHODS

Materials

Naphthalene 2,3-dialdehyde (NDA) as a fluorogenic reagent was purchased from Fluka (Tokyo, Japan). Sodium 1-octanesulphonate (SOS) as an ion-pair reagent was purchased from Wako (Osaka, Japan). Hippuryl-His-Leu (Hip-His-Leu) as a synthetic ACE substrate was obtained from the Peptide Institute (Osaka, Japan). Aprotinin and chymostatin were purchased from Sigma (St Louis, MO, USA). Nonidet P-40 (polyoxyethylene octylphenyl ether) was obtained from Wako Chemicals (Tokyo, Japan) and used as a sufficient detergent of ACE extraction as described by Horiuchi *et al.* [9]. All other reagents were purchased from Nacalai Tesque Ltd (Kyoto, Japan).

BP Measurement

SHRs (16-week-old male SHR/NCrj, Charles River Japan, Kanagawa) were fed a laboratory diet (CE-2, Clea Japan, Tokyo) and given water *ad libitum*. All rats were individually housed for 2 weeks at $21^\circ \pm 1\,^\circ$C and $55\% \pm 5\%$ humidity under controlled lighting from 8:30 to 20:30. For BP measurement (started at 10:00), two groups of rats ($n = 5$, each group) were studied. Single oral administration of VY was done in 18-week-old SHR (324.7 ± 4.7 g), in which the dosage of 10 mg/kg dissolved in 1 ml of saline solution was given by gavage. Control rats were administered with the same volume of saline solution. Systolic BP (SBP) and heart rate (HR, bpm) were measured at 0, 1, 3, 6 and 9 h after administration. The BP measurement was performed five times by a tail-pulse pick up method with a Softron BP system (Softron BP-98A, Tokyo, Japan) after warming the rat in a warm holder kept at $39.0^\circ \pm 0.5\,^\circ$C for 10 min. An average of three measurements, except for the highest and lowest values was recorded for each animal. This experiment was carried out under the guidance of the Animal Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No.105, 1973) and Notification (No.6, 1980 of the Prime Minister's Office) of the Japanese Government.

Measurement of Plasma or Tissue ACE Activity

For these experiments, 25 rats (18-week-old SHR) were used and divided into five groups: 0 h group after oral administration of 10 mg/kg of VY, 1 h, 3 h, 6 h, and 9 h groups. At each fixed time in the experiment the rats were killed by taking whole blood from the abdominal aorta (~10 ml) into a 20 ml chilled (4 °C) tube containing 0.5 mg of heparin under ethyl ether anaesthesia. The blood sample was immediately centrifuged at 3500 × **g** for 10 min at 4 °C and 50 µl of the separated plasma was then used to measure plasma ACE activity. The right kidney, lungs, heart, abdominal aorta and mesenteric artery were then excised from the freshly killed rat. After weighing, the chopped tissue (100 mg of each tissue) was placed in 500 µl of 50 mmol/l Tris-HCl buffer (pH 7.8) containing 30 mmol/l KCl, 5 mmol/l magnesium acetate, 0.25 mol/l sucrose and 0.5% Nonidet P-40. Each tissue was then homogenized using a micro-homogenizer (Iuchi S-203, Tokyo, Japan) for 30 s cooled on ice. The homogenate was then centrifuged at 12 000 × **g** for 20 min at 4 °C and the supernatant was used immediately to measure tissue ACE activity.

Plasma or tissue ACE activity was assayed on the day of the experiment according to the method of Horiuchi *et al.* [9]. Namely, the assay was started by adding an aliquot (50 µl) of the plasma sample or the supernatant from each tissue extract to 200 µl of 5 mmol/l Hip-His-Leu solution (100 mmol/l phosphate buffer (pH 8.3) containing 300 mmol/l NaCl) and incubated at 37 °C for 30 min. In the lung preparation, prior to the assay the supernatant was diluted 20 times with the buffer due to the high concentration of ACE. The assay was stopped by adding 750 µl of 3% methaphospholic acid. After centrifuging for 5 min at 10 000 × **g**, a 20 µl-aliquot was injected into a reversed-phase high-performance liquid chromatography (HPLC, Shimadzu LC-10A instrument, Kyoto, Japan) to determine the amount of liberated hippuric acid from Hip-His-Leu by ACE. The HPLC conditions were as follows: the column was a Cosmosil 5C18-ARII (4.6ϕ × 250 mm, Nacalai Tesque, Kyoto, Japan); elution was 35% CH_3CN in 0.1% trifluoroacetic acid (TFA); flow rate was 0.35 ml/min; monitoring absorbance at 228 nm. Plasma or tissue ACE activity (mU/ml plasma or mU/mg protein, respectively) was calculated from the increasing amount (nmol) of hippuric acid per min. The protein concentration of the tissue preparation was measured by a Dc Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin standards.

Measurements of Plasma or Tissue Angiotensin II and VY Levels

Blood or tissue at each fixed time as described above were subjected to an assay for Ang II and VY levels. The blood sample was taken in a chilled tube containing 0.2 mg/ml of aprotinin, 0.3 mg/ml of chymostatin and 0.5 mg/ml of EDTA-2Na, and immediately centrifuged at 3500 × **g** for 10 min at 4 °C. Then, 200 µl of 0.1% TFA solution was added to 200 µl of the separated plasma to prevent unintended hydrolysis by peptidases. The solution was subjected to an ultrafiltration with a Molucut L (<M.W. 5000; Nihon Millipore Ltd, Yonezawa, Japan) at 4 °C, and the filtrate was stored at −30 °C until measurement of metabolites. The chopped tissues (100 mg) including livers prepared as described above were subjected to an extraction with 500 µl of 50 mmol/l Tris-HCl buffer (pH 7.8) containing 30 mmol/l KCl, 5 mmol/l magnesium acetate, 0.25 mol/l sucrose, 0.1 mg of aprotinin, 0.15 mg of chymostatin, 0.5 mg of EDTA-2Na and 0.5% Triton X-100 for measurement of tissue metabolites. Each tissue was then homogenized using the micro-homogenizer for 30 s cooled on ice, and 200 µl of 0.1% TFA solution was added rapidly. The extract was then centrifuged at 12 000 × **g** for 20 min at 4 °C, and the supernatant obtained was used for Ang II and VY determinations.

An aliquot (200 µl) of the supernatant was then applied to a Sep-Pak Plus C18 Cartridge (Waters, Milford, MA, USA). An extract with 3 ml of 35% CH_3CN in 0.1% TFA solution was used for VY determination, and a subsequent extract with 60% CH_3CN in 0.1% TFA solution was used for Ang II determination. The extract for Ang II assay was evaporated to dryness, and subjected to an ELISA (Peninsula Laboratories, Inc., San Carlos, CA, USA) method. For VY determination, the 35% CH_3CN extract was evaporated to dryness, and dissolved in 50 µl of water. According to our proposed column-switching HPLC method [10], the concentration of VY in each tissue or plasma was assayed. Briefly, after a first reversed-phase HPLC run (column, Cosmosil 5C18-ARII (4.6ϕ × 250 mm); elution, 10% –25% CH_3CN in 0.1% TFA (150 min); flow rate, 0.4 ml/min), a fraction corresponding to the VY elution time of 40 min was collected and dried. The VY fraction was resolved in 50 µl of 20 mmol/l borate buffer (pH 9.5). Then, it was derivatized

by adding 50 µl of 0.1 mmol/l NDA solution in methanol and 10 µl of 10 mmol/l 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 (4.6ϕ × 250 mm) with the linear gradient mode of 40% to 60% CH_3CN (60 min). The zone of the 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 60% CH_3CN in 0.1% TFA containing 5 mmol/l SOS, and the flow rate was 0.4 ml/min. Fluorescence detection (excitation and emission wavelengths of 420 nm and 490 nm, respectively) was done with a fluorescence detector (FP-920S, Nippon Bunko, Tokyo, Japan). The validity of these extraction procedures was ascertained by a spiked test: the recoveries of VY and Ang II from spiked (100 fmol/ml) plasma being 95.0 ± 5.6 and 92.4 ± 2.4 fmol/ml, respectively; recoveries of VY and Ang II from spiked (10 pmol VY/g tissue and 500 pmol Ang II/g tissue) lung extract were 9.4 ± 0.5 pmol/g tissue and 527.3 ± 55.0 pmol/g tissue, respectively.

Pharmacokinetic Constant of VY in SHR

The pharmacokinetic constant of VY (10 mg/kg) after a single oral administration to SHR was evaluated during the 9 h protocol. The t_{max} (time to reach the maximum concentration) and $t_{1/2}$ (half-life) values were calculated by plotting the VY level at each fixed time against the logarithmic time (h). The C_{max} (maximum concentration) in this study was defined as the maximum VY level of the levels obtained at each time. The *MRT* (mean resident time) was calculated by dividing the area under the moment curve $(AUMC)_{0-9\ h}$ by $AUC_{0-9\ h}$ (area under the curve over 9 h)).

Statistical Analysis

The values are expressed as the mean ± SEM. The statistical significance within the group over time was assessed using a two-factor analysis of variance (ANOVA) followed by Dunnett's *t*-test for *post hoc* analysis. $P < 0.05$ was considered statistically significant. The analysis was performed with Stat View J5.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Acute BP Lowering Effect of VY in SHR

In the present study, 18-week-old SHR ($n = 5$) were given a single oral dose of 10 mg/kg VY. As shown in Figure 1, a significant of reduction systolic blood pressure of 13.7 mmHg at 1 h after the VY administration was observed (SBP at 0 and 1 h: 198.0 ± 3.6 and 184.3 ± 2.4 mmHg, respectively; $p < 0.05$), and the effect continued to 9 h (SBP 154.6 ± 3.5 mmHg) ranging from a reduction ratio of 6.9% to 21.9%. These long-lasting BP reductions were also significant against the control group. During this protocol up to 9 h, the heart rate did not change significantly (data were not shown) (e.g. HR at 0 and 1 h: 391.7 ± 18.1 and 416.2 ± 12.3 bpm, respectively).

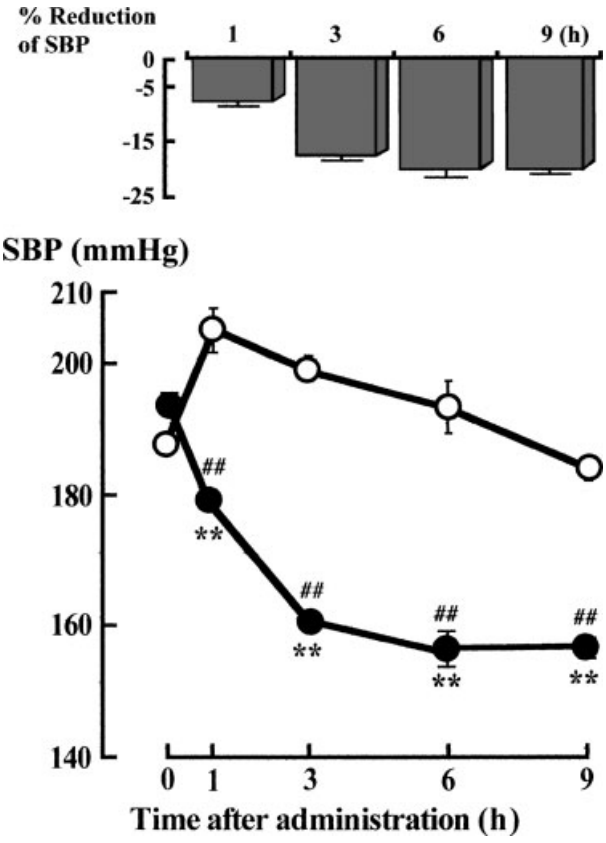

Figure 1 Change in systolic blood pressure of 18-week-old spontaneously hypertensive rats (SHR) after a single oral administration of 10 mg/kg Val-Tyr (●) or control (○), and the reduction ratio against 0 h. Each value is expressed as mean ± SEM ($n = 5$). $^{**}p < 0.01$, compared with 0 h; $^{\#\#}p < 0.01$ compared with the control group.

Plasma and Tissue VY Levels

Figure 2 shows the changes in plasma and tissue concentration of VY after the oral administration of VY (10 mg/kg) to SHR. As shown in Figure 2, a roughly 10-fold higher increment of plasma VY level was observed at 1 h (3.7 ± 0.04 pmol/ml plasma) than that at 0 h (0.3 ± 0.03 pmol/ml plasma), whereas thereafter the level declined rapidly. Namely, the plasma VY level of 0.7 ± 0.05 pmol/ml plasma at 6 h revealed a rapid elimination of VY ($t_{1/2}$ 2.7 h) from the circulating blood system in SHR. The *AUC* of VY was estimated to be 8.2 ± 0.15 pmol h/ml plasma within this protocol (Table 1). Similar to this plasma VY profile, a significant increase of VY level was observed in each tissue as well. The increments of each tissue VY level with a t_{max} of about 1 h, except for the lung (Table 1), suggested that the absorbed VY was rapidly accumulated into the tissues. According to the calculated $t_{1/2}$ values for each tissue, the metabolism of VY at the kidney was as rapid as in the plasma. However, the VY accumulation in the kidney showed a unique behavior that once the level had decreased to the baseline level at 6 h, it began to increase thereafter as shown in Figure 2. In other tissues, a slower metabolism of accumulated VY than that in the plasma was observed. The *MRT* also revealed that VY preferably accumulated in the kidney, lung, heart, mesenteric artery and abdominal aorta rather than in the plasma and liver. In addition, the $AUC_{0-9\ h}$ levels shown in Table 1 indicated that VY was highly and widely accumulated in the tissues rather than in the circulation; among the tissues the $AUC_{0-9\ h}$ for the kidney and lung showed relatively high levels of 104.8 ± 15.6 pmol h/g tissue and 126.6 ± 28.9 pmol h/g tissue, respectively. Based on the calculated $AUC_{0-9\ h}$ values shown in Table 1, a total amount of VY from each tissue and plasma after the 10 mg/kg (or 3 mg/rat) dosing was estimated to be 310.0 ± 46.1 ng/rat during the 9 h protocol period.

Plasma and Tissue ACE Activities

Figure 3 summarizes the changes in plasma and tissue ACE activities after the VY dosing at 1 and 6 h. On commencement of the VY dosing, there were no significant ($p > 0.05$) changes in the plasma ACE activity during the protocol, compared with the baseline level ($ACE_{plasma\text{-}0h}$ 44.9 ± 0.3 mU/ml). In the heart and mesenteric artery, the tissue ACE activities did not show any significant changes, regardless of the significant accumulation of VY in both tissues (Figure 2). On the contrary, the pulmonary ACE activity as well as the renal ACE activity was still suppressed at 6 h after dosing ranging from a reduction ratio of 26.1% to 34.6% ($ACE_{lung\text{-}0h}$ 70.3 ± 5.7 mU/mg protein; $ACE_{lung\text{-}6h}$ 46.0 ± 4.0 mU/mg protein; $p < 0.05$). In addition, the aortic ACE activity was significantly decreased during the VY dosing period; the highest reduction ratio (45.7%) among the tissues tested was obtained for the abdominal

Table 1 Pharmacokinetics of Val-Tyr after a Single Oral Administration of 10 mg/kg Val-Tyr to 18-week-old Spontaneously Hypertensive Rats

Tissue	t_{max} (h)	C_{max} (pmol/ml plasma or g-tissue)	$t_{1/2}$ (h)	*AUC* (pmol h/ml plasma or g-tissue)	*MRT* (h)
Plasma	0.8	3.7 ± 0.04	2.7	8.2 ± 0.15	3.8
Liver	1.0	32.2 ± 6.9	3.4	79.2 ± 11.0	4.1
Kidney	0.9	47.7 ± 5.3	2.7	104.8 ± 15.6	6.1
Heart	0.9	19.6 ± 0.6	4.4	42.2 ± 11.9	7.7
Lung	(6.3)	31.4 ± 6.2	(10.5)	126.6 ± 28.9	6.9
Abdominal aorta	1.0	20.9 ± 2.4	5.0	60.9 ± 6.4	6.2
Mesenteric artery	1.0	26.0 ± 0.9	3.8	64.9 ± 5.9	5.1

Data are the mean ± SEM ($n = 5$).
t_{max}, time to reach maximum concentration; C_{max}, maximum concentration;
$t_{1/2}$, half-life; *AUC*, area under the curve; *MRT*, mean resident time.
t_{max} and $t_{1/2}$ were calculated by plotting VY level at each fixed time up to 9 h against logarithmic time (h), except for kidney and lung. Both values for kidney were obtained from the VY levels at 1, 3 and 6 h, and for lung the data were obtained at 6 and 9 h, so that the value shown in parentheses is taken as 'tentative' data
C_{max} was defined as a maximum VY level among the levels obtained at each fixed time.

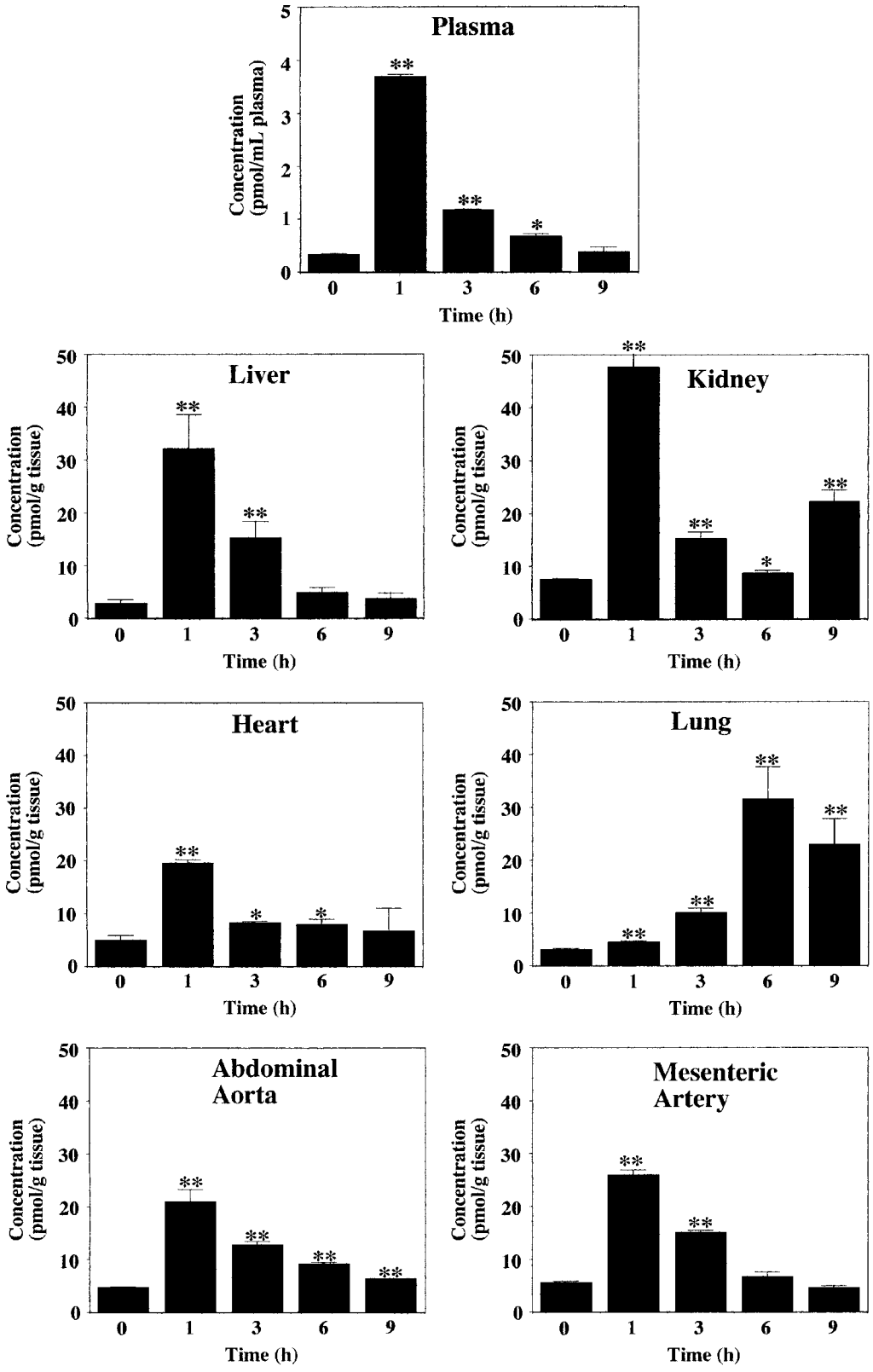

Figure 2 Time-course of plasma and tissue concentrations of Val-Tyr after a single oral administration of 10 mg/kg Val-Tyr to 18-week-old SHR. Each value is expressed as mean ± SEM ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with 0 h.

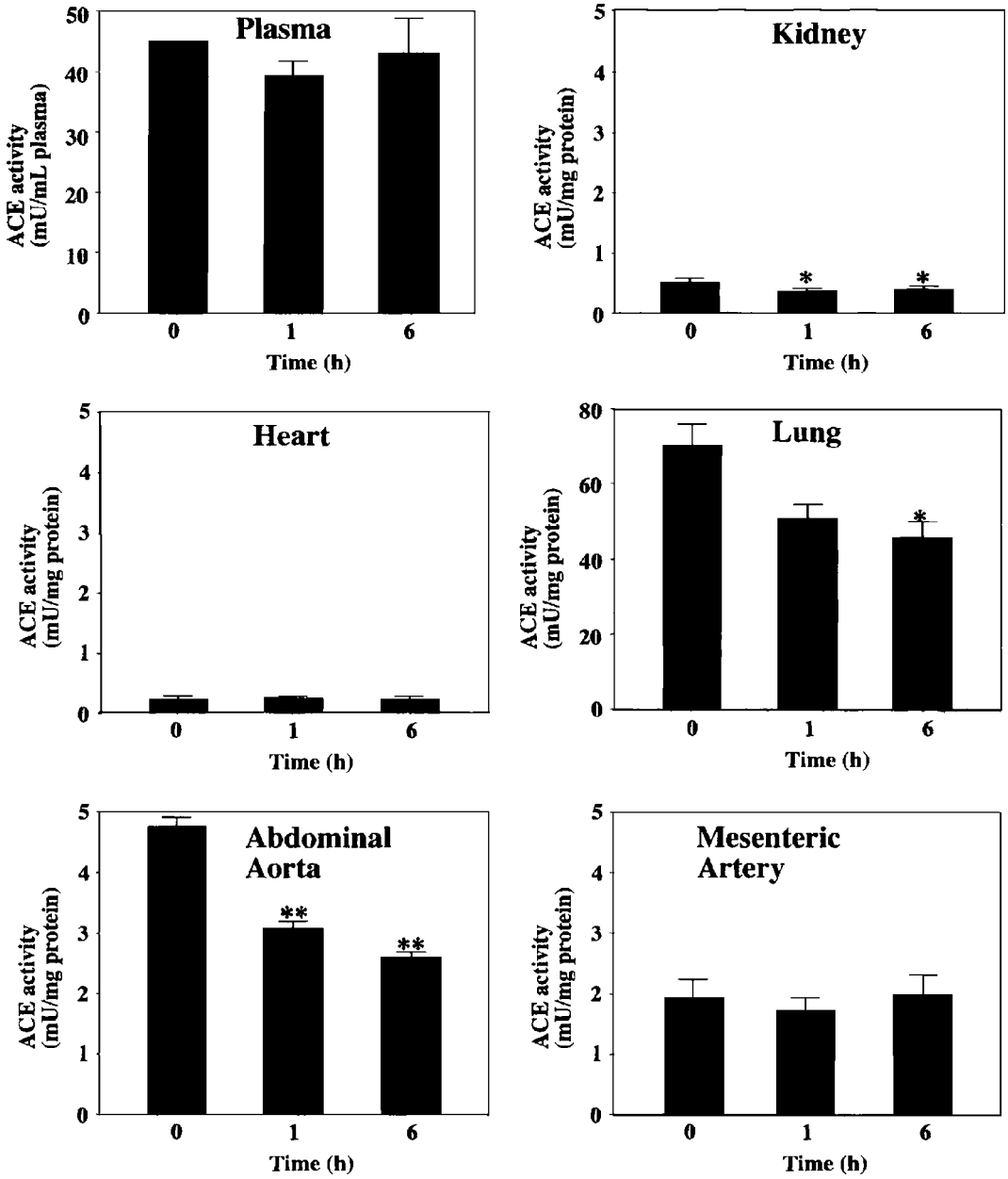

Figure 3 Change in plasma and tissue angiotensin I-converting enzyme (ACE) activities after the Val-Tyr dosing. ACE activity was assayed on the day of experiment by adding an aliquot (50 μl) of plasma sample or the supernatant from each tissue extract to a 200 μl of 5 mmol/l Hip-His-Leu solution (100 mmol/l phosphate buffer (pH 8.3) containing 300 mmol/l NaCl) and incubated at 37 °C for 30 min. The amount of liberated hippuric acid from Hip-His-Leu was determined with reversed-phase HPLC. Each value of change in ACE activity is expressed as mean ± SEM ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with 0 h.

aorta at 6 h ($ACE_{aorta\text{-}0h}$ 4.75 ± 0.16 mU/mg protein; $ACE_{aorta\text{-}6h}$ 2.58 ± 0.11 mU/mg protein; $p < 0.01$).

Plasma and Tissue Ang II Levels

The concentration of Ang II, a prominent vasopressor peptide in the circulating and local RAS metabolisms, was determined. Figure 4 depicts changes in the plasma and tissue Ang II levels after the VY dosing. The plasma Ang II level at 0 h in 18-week-old SHR was 62.2 ± 4.8 fmol/ml plasma. Although the Ang II level tended to decrease at 1 h transiently (44.3 ± 7.5 fmol/ml plasma; $p = 0.136$),

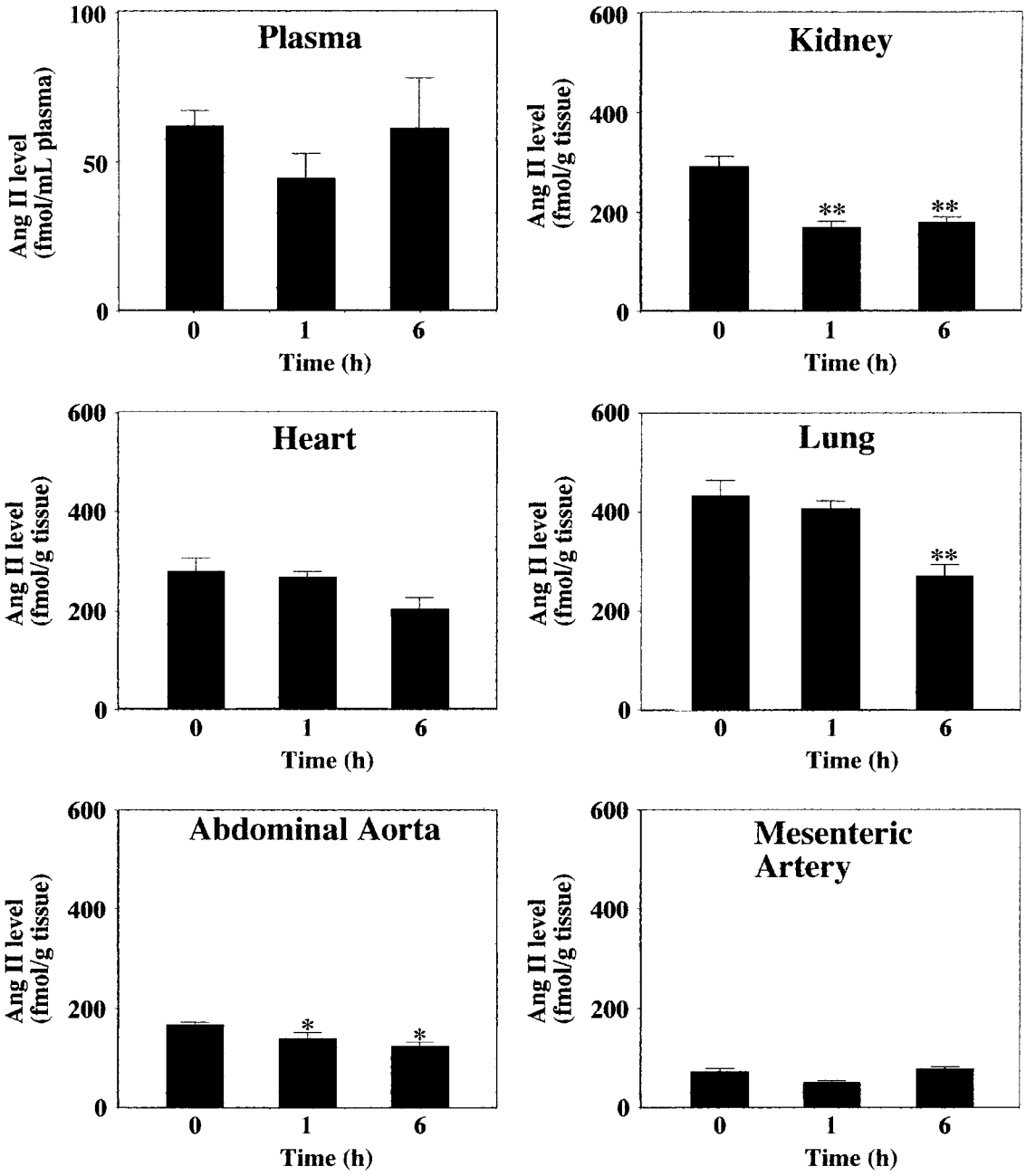

Figure 4 Change in plasma and tissue angiotensin (Ang) II levels after the Val-Tyr dosing. Ang II level in plasma or tissue extract was assayed by an ELISA method. Each value of change in Ang II level is expressed as mean ± SEM ($n = 5$). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with 0 h.

no significant changes in the level in the circulation were observed during the period, being compatible with the change of plasma ACE activity (Figure 3). This suggested that VY was not directly associated with an inhibition of plasma ACE. In the mesenteric artery as well as in the heart, no significant changes in Ang II level were observed. In the lung, a significant reduction of Ang II level was observed at 6 h (Ang $II_{lung\text{-}0h}$ 431.8 ± 32.7 fmol/g tissue; Ang $II_{lung\text{-}6h}$ 269.0 ± 24.3 fmol/g tissue; $p < 0.01$). Contrary to the transient reductions of Ang II levels in the above tissues, the VY dosing provided a prolonged reduction of Ang II in the kidney and abdominal aorta. In the

kidney, a decrease of Ang II was observed at 1 h (Ang $II_{kidney\text{-}0h}$ 292.5 ± 23.0 fmol/g tissue; Ang $II_{kidney\text{-}1h}$ 179.3 ± 13.0 fmol/g tissue; $p < 0.01$) and continued to 6 h (Ang $II_{kidney\text{-}6h}$ 184.6 ± 12.5 fmol/g tissue; $p < 0.01$). However, at 9 h an increase in renal Ang II level (Ang $II_{kidney\text{-}9h}$ 243.3 ± 21.1 fmol/g tissue) as well as in ACE activity ($ACE_{kidney\text{-}9h}$ 0.39 ± 0.03 mU/mg protein) was observed in accordance with the second increase in the renal VY level at 9 h (Figure 2), although data are not shown in Figures 3 and 4. A long and significant decrease in Ang II level was also observed in the abdominal aorta (Ang $II_{aorta\text{-}0h}$ 167.5 ± 6.2 fmol/g tissue; $ACE_{aorta\text{-}6h}$ 127.3 ± 9.2 fmol/g tissue; $p < 0.05$), in good agreement with the change of tissue ACE activity (Figure 3).

DISCUSSION

Our previous report on the antihypertensive effect of VY in THM revealed that the absorbed VY suppressed the circulating RAS [6]. However, a discrepancy between a return of the plasma Ang II level to the baseline level and a prolonged blood pressure reduction at 6 h strongly suggested that an alternative depressor mechanism rather than a retardation of the circulating RAS could be involved in the antihypertensive effect of VY. In the present study using 18-week-old SHR, the absence of change in the plasma ACE activity (Figure 3) also demonstrated that VY in the circulation did not serve in a prolonged BP reduction via ACE inhibition (Figure 1). Our studies concur with the work of Cohen *et al.* [11], who concluded that a 24 h BP reduction in SHR after acute treatment with a therapeutic ACE inhibitor, captopril, (10 mg/kg) was not a result of a short (15 min) inhibition of serum ACE. A rapid removal of VY from the circulation (t_{max} : 0.8 *h*, $t_{1/2}$; 2.7 h, *MRT*; 3.8 h, see Table 1 and Figure 2) as well as captopril so that half of the plasma captopril disappeared within 2 h from human blood [12,13] also supported a lesser involvement of the circulating VY in the prolonged antihypertensive effect.

Hence, in order to clarify the target sites of VY, tissue VY concentrations in the kidney, heart, lung, abdominal aorta, mesenteric artery and liver were determined. The pharmacokinetics of VY after oral administration to 18-week-old SHR were clarified as shown in Table 1. There has been no report on the distribution profile of ACE inhibitory peptides to date. The present study demonstrated for the first time that VY was highly and widely accumulated in the tissues rather than in the circulation, and the major accumulation of VY with an $AUC_{0-9\ h}$ of more than 100 pmol h/g tissue occurred in the kidney and lung. In contrast, Masuda *et al.* [3] reported that 4.3 μg/rat tissue of Val-Pro-Pro was detected in the aorta at 6 h after its oral administration to SHR. Unlike their report, aortic VY showed a low tissue concentration of 3.4 ± 0.4 ng/rat tissue (9.2 ± 0.3 pmol/g tissue) at 6 h, suggesting that the magnitude of the tissue accumulation of peptides may depend greatly on their amino acid composition or sequence. In the liver, 79.2 ± 11.0 pmol h/g tissue of VY was detected in intact form. As Sakakibara *et al.* [14] have already pointed out, Tyr residues in the liver tissue are susceptible to a sulfation reaction by dopa/tyrosine sulfotransferase. However, no observation of sulfated VY was detected when VY was incubated with a liver homogenate taken from SHR (data not shown). This indicated that the dipeptide form of VY would no longer undergo a sulfation reaction at the Tyr residue in the liver, followed by an intact secretion into the circulation without any derivatization.

Based on the result that the RAS exists in diverse tissues such as the lung, heart, aorta, kidney, testicles and brain [7], studies on the regulation of tissue RASs during ACE inhibition by therapeutic drugs have been performed. Okunishi *et al.* [15] have demonstrated that spirapril, which was a long-lasting ACE inhibitor, greatly affected tissue ACEs in the lung, heart, kidney, aorta and mesenteric artery, whereas no suppression was observed in the brain RAS. A concordant result was reported by Chai *et al.* [16], in which the target site of ACE inhibitors (lisinopril and perindopril) were the kidney, lung and blood vessels. They also reported that ACE inhibition in the testis and brain may be restricted due to the lipophilicity or the blood-brain barrier of ACE inhibitors. As in the case of the therapeutic ACE inhibitors, in the present study, the pharmacological effect of VY on tissue RASs was examined to clarify the prolonged BP reduction in SHR (Figure 1). As shown in Figure 3, the time-course and degree of ACE activity by VY varied between the tissues. For the kidney and abdominal aorta a long suppressive effect on ACE activity and the Ang II level (Figure 4) was observed, being correlated with the reduction in blood pressure. Thus, both tissues would be candidates for the target site of VY. In the mesenteric artery, which determines the systemic and peripheral blood pressure, ACE activity as well as the Ang II level, transiently tended to decrease

at 1 h, but no significant differences were observed. This unexpected result may be due in part to a difference in the VY elimination rate from each tissue (mesenteric artery MRT, 5.1 h; abdominal aorta MRT, 6.2 h) or a rapid return of the VY level in the mesenteric artery to the baseline level at 6 h (Figure 2). In addition, as the C_{max} of the VY level at 6 h in the lung (Figure 2) correlated with a delayed suppression of pulmonary ACE activity (Figure 3), the VY accumulation rate into the lung would be slow. A similar delay of ACE suppression in the lung was reported by Jackson *et al.* [17], in which perindopril (8 mg/kg dose) inhibited pulmonary ACE with a t_{max} of 8 h, whereas the aortic and renal ACE was maximally suppressed within 1–2 h. However, further investigation would be needed to clarify these specific actions of VY in the lung.

To date, the aortic RAS has been recognized as a candidate for developing atherosclerosis via Ang II stimulation [18,19]. In addition, Okunishi *et al.* [15] reported that a close relationship between enhancement of the tissue ACE activity and BP promotion with age in SHR was observed only for the abdominal aorta. In the present study, VY dosing in SHR resulted in a long-lasting reduction of the Ang II level and ACE activity in the abdominal aorta. Although there was no report on the time-course of the aortic Ang II level in the acute and chronic protocol of the peptides, the result suggested that the accumulated VY would improve the physiological function of the aortic vessel. Further studies on the effects of aortic VY on the mRNA level of ACE, NO production, hypertropy of vascular smooth muscle cell, AT1 receptor or Ca^{2+} channel and so on [20–22] is needed to clarify the potential functions of aortic VY, and are now in progress.

In conclusion, it was found that VY was highly accumulated in the tissues (kidney, heart, lung, abdominal aorta, mesenteric artery and liver) rather than in the circulation. Neither circulating RAS, nor tissue RASs in the mesenteric artery and heart was altered significantly by the VY administration, whereas VY greatly suppressed the aortic and renal RASs. Taken as a whole, the abdominal aorta and kidney were probably the target sites of the antihypertensive peptide, VY, in this experiment.

REFERENCES

1. Kawasaki T, Seki E, Osajima K, Yoshida M, Asada K, Matsui T, Osajima Y. Antihypertensive effect of valyl-tyrosine, a short chain peptide derived from sardine muscle hydrolyzate, on mild hypertensive subjects. *J Human Hypertens* 2000; **14**: 519–523.
2. Matsui T, Li C-H, Tanaka T, Maki T, Osajima Y, Matsumoto K. Depressor effect of wheat germ hydrolysate and its novel angiotensin I-converting enzyme inhibitory peptide, Ile-Val-Tyr, and the metabolism in rats and human plasma. *Biol Pharm Bull* 2000; **23**: 427–431.
3. Masuda O, Nakamura Y, Takano T. Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides to spontaneously hypertensive rats. *J Nutr* 1996; **126**: 3063–3068.
4. Matsui T, Tamaya K, Seki E, Osajima K, Matsumoto K, Kawasaki T. Val-Tyr as a natural antihypertensive dipeptide can be absorbed into the human circulatory blood system. *Clin Exp Pharm Physiol* 2002; **29**: 204–208.
5. Matsui T, Tamaya K, Seki E, Osajima K, Matsumoto K, Kawasaki T. Absorption of Val-Tyr with *in vitro* angiotensin I-converting enzyme inhibitory activity into the circulating blood system of mild hypertensive subjects. *Biol Pharm Bull* 2002; **25**: 1228–1230.
6. Matsui T, Hayashi A, Tamaya K, Matsumoto K, Kawasaki T, Murakami K, Kimoto K. Depressor effect induced by dipeptide, Val-Tyr in hypertensive transgenic mice is in part due to the suppression of human circulating renin-angiotensin system. *Clin Exp Pharm Physiol* 2003; **30**: 262–265.
7. Johnston CI, Burrell LM, Perich R, Jandeleit K, Jachson B. The tissue renin-angiotensin system and its functional role. *Clin Exp Pharm Phys* 1992; **19**(Suppl. 19): 1–5.
8. Ehlers MRW, Riordan JF. Angiotensin-converting enzyme. In *Hypertension*, Laragh JH, Brenner BM (eds). Raven Press: New York, 1995; 1217–1231.
9. Horiuchi M, Fujimura K, Terashima T, Iso T. Method for determination of angiotensin-converting enzyme activity in blood and tissue by high-performance liquid chromatography. *J Chromatogr* 1982; **233**: 123–130.
10. Matsui T, Tamaya K, Kawasaki T, Osajima Y. Determination of angiotensin metabolites in human plasma by fluorimetric high-performance liquid chromatography using a heart-cut column-switching technique. *J Chromatogr B* 1999; **729**: 89–95.
11. Cohen ML, Kurz KD. Angiotensin converting enzyme inhibition in tissues from spontaneously hypertensive rats after treatment with captopril or MK-421. *J Pharmacol Exp Ther* 1982; **220**: 63–69.
12. Jarrott B, Drummer O, Hooper R, Anderson A, Miach P, Louis W. Pharmacokinetic properties of captopril after acute and chronic administration to hypertensive subjects. *Am J Cardiol* 1982; **49**: 1547–1549.
13. Jankowski A, Skorek A, Krzysko K, Zarzycki PK, Ochocka RJ, Lamparczyk H. Captopril: determination

in blood and pharmacokinetics after single oral dose. *J Pharm Biomed Anal* 1995; **13**: 655–660.
14. Sakakibara Y, Takami Y, Zwieb C, Nakayama T, Suiko M, Nakajima H, Liu MC. Purification, characterization, and molecular cloning of a novel rat liver dopa/tyrosine sulfotransferase. *J Biol Chem* 1995; **270**: 30 470–30 478.
15. Okunishi H, Kawamoto T, Kurobe Y, Oka Y, Ishii K, Tanaka T, Miyazaki M. Pathogenic role of vascular angiotensin converting enzyme in the spontaneously hypertensive rat. *Clin Exp Pharm Physiol* 1991; **18**: 649–659.
16. Chai SY, Perich R, Jackson B, Mendelsohn AO, Johnston CI. Acute and chronic effects of angiotensin-converting enzyme inhibitors on tissue angiotensin-converting enzyme. *Clin Exp Pharm Physiol* 1992; **19**: (suppl. 19): 7–12.
17. Jackson B, Cubela RB, Johnston CI. Inhibition of tissue angiotensin converting enzyme by perindopril: *in vivo* assessment in the rat using radioinhibitor binding displacement. *J Pharmacol Exp Ther* 1988; **245**: 950–955.
18. Ruiz-Ortega M, Lorenzo O, Ruperez M, Esteban V, Suzuki Y, Mezzano S, Plaza JJ, Egido J. Role of the renin-angiotensin system in vascular diseases. *Hypertension* 2001; **38**: 1382–1387.
19. Weiss D, Kools JJ, Taylor R. Angiotensin II-induced hypertension accelerates the development of atherosclerosis in apoE-deficient mice. *Circulation* 2001; **103**: 448–454.
20. Shiota N, Miyazaki M, Okunishi H. Increase of angiotensin converting enzyme gene expression in the hypertensive aorta. *Hypertension* 1992; **20**: 168–174.
21. Nishijo N, Takamine S, Sugiyama F, Kimoto K, Taniguchi K, Horiguchi H, Ogata T, Murakami K, Fukamizu A, Yagami K. Vascular remodeling in hypertensive transgenic mice. *Exp Anim* 1999; **48**: 203–208.
22. Marsden PA, Brenner BM, Ballermann BJ. Mechanism of angiotensin action on vascular smooth muscle, the adrenal and the kidney. In *Hypertension*, Laragh JH, Brenner BM (eds). Raven Press: New York, 1995; 1247–1271.

