**Note**

**Accumulation of ACE Inhibitory Tripeptides, Val-Pro-Pro and Ile-Pro-Pro, in Vascular Endothelial Cells**

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Received April 20, 2012; Accepted May 22, 2012; Online Publication, September 7, 2012
[doi:10.1271/bbb.120299]

The antihypertensive peptides, Val-Pro-Pro and Ile-Pro-Pro, were successfully detected in the aorta of spontaneously hypertensive rats after orally administering these peptides by a guanidine-thiocyanate treatment to prevent proteolysis. Cy3-labeled versions of both peptides were localized in the endothelial cells of arterial vessels in the rats. The accumulation of both peptides in the endothelial cells suggested in vivo inhibitory activity of the angiotensin I-converting enzyme.

**Key words:** Val-Pro-Pro; Ile-Pro-Pro; Cy3-VPP; Cy3-IPP; vascular endothelial cells

A variety of bioactive peptides have been developed and used in functional food products based on clinical evidence. Most of the peptides have been discovered in hydrolyzed food proteins, synthetic peptides and fermented food products.1,2 Among these bioactive peptides, two antihypertensive peptides, Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), were the first to be isolated from milk fermented with *Lactobacillus helveticus* and have since been extensively studied for their antihypertensive effects on spontaneously hypertensive rats (SHRs)3,4 and in several clinical trials.5–8 A recent meta-analysis of these clinical trials has revealed a significant reduction in blood pressure after treating with VPP and IPP.9,10

Most clinical trials have reported a gradual drop in blood pressure by repeated uptake of these peptides over two to three weeks;5–7 however, our previous clinical study using a single administration of a test sample containing VPP and IPP revealed no significant reduction in blood pressure. One previous clinical study has reported limited absorption of IPP in circulating blood, although there was no result for VPP absorption.12 Quantification of VPP and IPP within the target organ after the absorption of these bioactive peptides from circulating blood is necessary to confirm the in vivo ACE inhibitory effects of VPP and IPP on the target organs. We quantified the amounts of VPP and IPP in various organs and prevented possible degradation of these peptides by adding denaturing agents in the extraction process; this would enable us to elucidate the mild in vivo effects observed in many clinical studies and to gain a pathophysiological understanding of the in vivo actions of VPP and IPP. Fluorescent Cy3-labeled VPP and IPP were prepared and used in histological staining for a more precise analysis of peptide localization in the target organs.

Adult (30–31-week-old, 385–427 g) male SHRs were obtained for the animal study, from Charles River Japan (Kanagawa, Japan). The rats were fed on a commercially available CE-2 diet (Clea Japan, Tokyo, Japan) and water, and were exposed to conventional conditions by controlling the temperature (24 ± 3 °C) and humidity (55 ± 5%). The rats were acclimatized to those conditions for about a week before the experiment began. All experimental procedures were approved by the Animal Research Committee at Calpis R&D Centre. After their acclimatization, the rats were assigned to two groups: the control group (n = 3) and tripeptide group (n = 6). The rats of the tripeptide group were fasted overnight, before 100 mg of VPP and IPP/kg of body weight (Kurabou, Osaka, Japan) was administered by gavage. To the control group, 1 mL of distilled water/kg of body weight was administered. Each group was killed by taking whole blood from the abdominal aorta 1 h after the administration. The blood, kidneys, liver, lungs and aorta were prepared from the SHR for a tripeptide analysis.

VPP and IPP in the various tissues were quantified after suspending 1 g of each tissue (100 mg of the aorta tissue) in 10 mL of 5.5 M cold guanidine-thiocyanate (Gu-SCN) to prevent proteolytic degradation of the tripeptides in the tissue. The peptides from the plasma were prepared by adding 4 volumes of Gu-SCN to the plasma sample. The suspension was homogenized at 10,000 rpm (Polytron PT-10-35 GT, Kinematica) for 2 min on ice. An equal volume of 10% TCA was added to the extract before centrifuging at 5,000 g for 10 min. The supernatant was collected and passed through a 0.45-μm filter (the peptide solution). Two mL of this peptide solution was loaded into a Sep-pak tC18 (Waters) cartridge column. The cartridge was washed with 2 mL of water, and the peptides containing VPP...
and IPP were eluted by washing with 2 mL of a 30% ethanol solution. The eluted peptides were dried and then dissolved in 0.6 mL of H$_2$O for a peptide analysis. VPP and IPP were measured by using the LC-MS method according to a previous report, but with some modifications, using VPP and IPP isotopes as internal standards. Extracts containing VPP and IPP were mixed with the VPP isotope, Val($^{13}$C$_3$,$^{15}$N)-Pro($^{13}$C$_3$,$^{15}$N)-Pro, and the IPP isotope, Ile-Pro($^{13}$C$_3$,$^{15}$N)-Pro, and applied to LC-MS. The specific signals of VPP and IPP were respectively quantified by using the signal peaks from the isotopes of VPP and IPP as internal standards. The amounts of VPP and IPP in the various tissues are expressed as µg of peptides per g of tissue (weight) and plasma.

A histological microscopic analysis of the arterial vessels was made after preparing Cy3-VPP and Cy3-IPP. A 100-µg amount of VPP or IPP, purchased from Kurabo (Osaka, Japan), was dissolved in 1 mL of 0.1 M NaHCO$_3$ at pH 8.3. A 100-µL amount of the Cy3 monoreactive NHS ester dissolved in dimethyl sulfoxide (DMSO) (10 mg/mL) was then added to 1 mL of the peptide solution. The mixture was stored at room temperature for 3 h in the dark. Each reaction mixture for VPP or IPP was then loaded into sep-pak tC18 cartridge columns. Each cartridge was washed step wise with 3 mL of 0, 10, 20, 30, or 40% ethanol/water. Cy3-VPP and Cy3-IPP were recovered in the 20% ethanol fractions. Ethanol was removed from the eluted fraction by drying, and the peptides were dissolved in 1 mL of distilled water. A 25-week-old male spontaneously hypertensive rat, purchased from Charles River Japan, was then fasted for 8 h before being sacrificed. The arterial vessel was isolated and washed 3 times with phosphate-buffered saline (PBS), before being cut into 1-cm-long sections. Each piece of the arterial vessel was incubated in a pre-warmed supplement-free Humedia-EG2 medium (Kurabo, Japan) at 37°C for 20 min. Each piece was then incubated for 60 min at 37°C with the pre-warmed supplement-free Humedia-EG2 medium containing 5 µg/mL of Cy3-VPP and Cy3-IPP, with or without 1 mg/mL of VPP and IPP. A piece was then washed 3 times with PBS, and thin-sectioned samples of 10 µm in thickness of Cy3-VPP and Cy3-IPP was visualized by using a BX51 fluorescence microscope (Olympus, Tokyo, Japan) with an excitation wavelength of 590 nm and an emission wavelength of 570 nm. Specific staining of the endothelial cells was performed on the same sections which were then kept in 2% hydrogen peroxide in methanol for 5 min after rehydrating to remove the endogenous peroxidase activity, before being washed with PBS. The von Willebrand factor (vWF) was stained in the arterial vessel by using the protein denaturant, guanidine thiocyanate (Gu-SCN) (Fig. 1). The amounts of VPP and IPP in the homogenate of the aorta were at least 10 µg and 14 µg, these being higher than the IC$_{50}$ values for VPP (9 µg) and IPP (5 µg). The present study thus revealed the accumulation and condensation of VPP and IPP into the aorta and lung even at lower peptide levels in the circulating blood system.

In a previous study, the C$_{max}$ and T$_{max}$ values for IPP, which were observed 40 min after administering 20.4 mg to fasted subjects, were 0.897 nmol/L (12). Our previous clinical trial also showed similar results for the C$_{max}$ and T$_{max}$ values of VPP and IPP 30 min after administration, the respective values being 0.58 and 2.31 nmol/L (unpublished data). Both C$_{max}$ values for serum VPP and IPP were too low to expect any ACE inhibitory effect in the circulating blood, since the values were lower than their IC$_{50}$ values (the concentration required for 50% ACE inhibition) of 9 µg for VPP and 5 µg for IPP. However, in the present animal study with SHR, we successfully isolated higher amounts of VPP and IPP in several tissue extracts compared with the plasma, preventing the proteolysis of ACE inhibitory peptides in the tissues by using the protein denaturant, guanidine-thiocyanate (Gu-SCN) (Fig. 1). The amounts of VPP and IPP in the aorta extract (10 and 14 µmol/L, respectively) and lung (5.0 and 5.9 µmol/L, respectively) were high enough to explain the in vivo ACE inhibitory effects of these peptides. Taking into account the results of the present animal study and previous clinical trials, the pharmacokinetic analysis of VPP and IPP suggested their concentration was more important in the target tissues than in the circulating blood system.

Cy3 was introduced into VPP and IPP at the amino terminal to detect the localization of VPP and IPP in vivo ACEI effects of VPP and IPP in a pharmacokinetic study using various tissues, after the oral administration of VPP and IPP to SHR. The inhibitory reagents were investigated to prevent the predicted proteolysis of active peptides in the extraction process. In a recovery study using a lung extract, VPP and IPP spiked into the extract were quickly hydrolyzed to PP, VP and IP, even if 1% sodium dodecyl sulfate (SDS) or a 1% protease inhibitor cocktail was present (data not shown). In contrast, high recovery of the peptides was obtained if 5.5 M cold guanidine-thiocyanate (Gu-SCN) was added during the extraction process (data not shown). High amounts of VPP and IPP were successfully isolated from the aorta of SHR (100 mg of VPP and IPP) when a 5.5 M Gu-SCN solution was used in the tissue extraction process (Fig. 1). The respective amounts of VPP and IPP in the homogenate of the aorta were at least 10 µg and 14 µg, these being higher than the IC$_{50}$ values for VPP (9 µg) and IPP (5 µg). The present study thus revealed the accumulation and condensation of VPP and IPP into the aorta and lung even at lower peptide levels in the circulating blood system.

The amounts of VPP and IPP in the various tissues are shown as peptide µg/g of wet weight of the tissue or g of plasma 1 h after a single oral administration of 100 mg of VPP or IPP/kg of body weight. *p < 0.05 and *p < 0.1 vs. plasma by a Wilcoxon analysis.
in the aorta of SHR. The incorporation of Cy3 did not significantly change the ACE inhibitory activities of the Cy3-labelled peptides when compared with non-labelled peptides (12 and 8 μM, respectively). After incubating the arterial vessel with Cy3-VPP and Cy3-IPP, the vessel was thin-sectioned (10 μm) and observed under a fluorescence microscope (Fig. 2A and D). The fluorescent signal originating from Cy3-VPP and Cy3-IPP (Fig. 2B) was significantly decreased by adding a 200-fold amount of non-labelled VPP and non-labelled IPP to the incubation mixture (Fig. 2E). The Cy3 signal also disappeared upon adding a 200-fold amount of non-labelled VPP or non-labelled IPP (data not shown). Using the same sectioned tissue, we could show von Willenbrand factor (vWF) staining (Fig. 2C and F) in the same position as the Cy3 fluorescence, suggesting that the Cy3-labelled peptides were localized in the endothelial cells of the arterial vessel. The arterial vessel might have been the major target for VPP and IPP incorporation, and the two peptides most likely existed as stable intact forms in the endothelial cells.

Dose-dependent antihypertensive effects of VPP and IPP, ranging from 0.5 to 10 mg/kg of BW, in SHR have been reported. In our first pharmacokinetic study, we applied higher doses of VPP and IPP for the oral administration to SHR (100 mg/kg of BW) to aid the detection of both peptides in various tissues. VPP and IPP accumulated in endothelial cells of the arterial vessels after the oral uptake of the peptides. A high dosage of VPP and IPP (100 mg/kg of BW) orally administered to SHR resulted in levels high enough to reduce the blood pressure due to inhibition of the local ACE activity. The endothelial cells, which had been stained by using the vWF method or Cy3-peptides, accounted for about 5% of the volume in the arterial vessel, as assessed by calculating the corresponding area (Fig. 2). This calculation suggests that the respective VPP and IPP levels in the endothelial cells must have exceeded 200 and 280 μmol/L. If the peptides were localized in the endothelial cells of the arterial vessel, the concentrations of both peptides in the endothelial cells would have been about 100-fold higher than that obtained in the homogenized aorta shown in Fig. 1. Dose-dependent accumulation of both peptides in the aorta could be expected to enable a lower dosage of peptides, about 1 mg of VPP and IPP/kg BW, being enough to show inhibitory activity in the aorta. This expectation is supported by the results of a microarray analysis of the aorta: the gene profile was linked to the ACE inhibitory effect of VPP and IPP in the aorta from SHR after consecutive treatments with both peptides. The importance of the accumulation of both peptides into the endothelial cells of the arterial vessel that was observed in the present study had also been suggested by a study showing that the vasodilatory activity of VPP and IPP in the aortic tube was mainly regulated by the endothelial cells of the aortic vessel throughout the release of nitrogen oxide (NO).

ACE is widely distributed in a large variety of tissues and body fluids. The source of circulating ACE in the plasma is thought to be the pulmonary endothelial cells. Post-translational processing causes the secretion of ACE from the membrane into the blood serum. ACE is mainly expressed in endothelial cells which also express type 1 and type 2 receptors for angiotensin II. The localization of ACE in endothelial cells may therefore play an important role in controlling blood pressure in vivo. The accumulation of VPP and IPP in the endothelial cells may not have been caused by the first action of ACE in the endothelial cells, because there was no condensation of VPP and IPP with ACE low-affinity resin (data not shown). In contrast, VPP and IPP may have shared affinity components in the endothelial cells of the arterial vessel, because the Cy3-VPP plus
Cy3-IPP signals in the endothelial cells disappeared after adding an excess amount of non-labeled VPP or non-labeled IPP.

In conclusion, the results of the present pharmacokinetic study strongly suggest that the antihypertensive peptides, VPP and IPP, might accumulate in the endothelial cells of the arterial vessel and could have ACE inhibitory activity in the endothelial cells after an oral administration. However, the dose-dependent accumulation and changes in VPP and IPP in the target organs should be investigated for a more detailed understanding of the in vivo mechanism for both peptides.

References