Dipeptidyl Peptidase III and Hypertension

Novel Therapeutic Role for Dipeptidyl Peptidase III in the Treatment of Hypertension

Xiaoling Pang, Akio Shimizu, Souichi Kurita, Dimitar P. Zankov, Keisuke Takeuchi, Mako Yasuda-Yamahara, Shinji Kume, Tetsuo Ishida, Hisakazu Ogita

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Abstract—Dipeptidyl peptidase III (DPP III) cleaves dipeptide residues from the N terminus of polypeptides ranging from 3 to 10 amino acids in length and is implicated in pathophysiological processes through the breakdown of certain oligopeptides or their fragments. In this study, we newly identified the biochemical properties of DPP III for angiotensin II (Ang II), which consists of 8 amino acids. DPP III quickly and effectively digested Ang II with $K_m = 3.7 \times 10^{-6}$ mol/L. In the in vivo experiments, DPP III remarkably reduced blood pressure in Ang II–infused hypertensive mice without alteration of heart rate. DPP III did not affect hemodynamics in noradrenalin-induced hypertensive mice or normotensive mice, suggesting specificity for Ang II. When DPP III was intravenously injected every other day for 4 weeks after Ang II osmotic minipump implantation in mice, Ang II–induced cardiac fibrosis and hypertrophy were significantly attenuated. This DPP III effect was at least similar to that caused by an angiotensin receptor blocker candesartan. Furthermore, administration of DPP III dramatically reduced the increase in urine albumin excretion and kidney injury and inflammation markers caused by Ang II infusion. Both DPP III and candesartan administration showed slight additive inhibition in the albumin excretion. These results reveal a novel potential use of DPP III in the treatment of hypertension and its protective effects on hypertension-sensitive organs, such as the heart and kidneys. (Hypertension. 2016;68:630-641. DOI: 10.1161/HYPERTENSIONAHA.116.07357.)

Key Words: acute kidney injury ■ angiotensin II ■ blood pressure ■ cardiomegaly ■ dipeptidyl peptidase III ■ kinetics

As an essential part of the classical endocrine system, the renin–angiotensin system (RAS) plays a crucial role in maintaining homeostasis of intravascular blood volume, and thereby contributes to the restoration of arterial blood pressure.1,2 To date, several RAS family members that function independently in different organs have been identified.3-5

The main RAS component is angiotensin II (Ang II), an active octapeptide cleaved from angiotensinogen, catalytically produced by renin and angiotensin-converting enzyme (ACE). Ang II has many cardiovascular functions including the control of blood pressure, cardiac remodeling, and angiogenesis.6-8 Although the action of Ang II in hypertension is not completely elucidated, Ang II is reported to lead to blood pressure elevation by directly causing vasoconstriction, sympathetic nerve stimulation, and aldosterone release.9,10 Furthermore, brain Ang II regulates blood pressure.11 Ang II locally generated in the brain can increase blood pressure by stimulating the Ang II type 1 receptor (AT1R) in the rostral ventrolateral medulla to induce tonic sympathoexcitatory effects. Circulating Ang II has access to circumventricular organs, such as subfornical organ and organum vasculosum of the lamina terminalis, because of lack of the blood–brain barrier, and inhibits baroreflex activity that normally facilitates vasodilation, resulting in the enhancement of vascular tone. Ang II also provokes a wide range of tissue responses, most of which are mediated via AT1R, by promoting apoptosis, inflammation, and fibrosis.12-14 Therefore, ACE inhibitors and Ang II receptor blockers (ARBs), targeting Ang II directly or indirectly, have been widely used to treat hypertension, ventricular remodeling, or coronary heart disease15,16 and are currently the first-line treatment of hypertension, especially when complicated with diabetes mellitus or renal failure.17

Dipeptidyl peptidase III (DPP III, EC 3.4.14.4) is a zinc-dependent aminopeptidase harboring the characteristic HELGH sequence motif18,19 and was first isolated from the bovine pituitary gland.20 DPP III has a total of 6 cysteines:
3 in the lower (Cys19, Cys147, and Cys176) and 3 in the upper catalytic domain (Cys509, Cys519, and Cys654). This enzyme is monomeric in form,21 and its crystal structure shows 2 lobe-like domains separated by a wide cleft.22 As a metalloaminopeptidase, DPP III preferentially cleaves dipeptide residues (eg, Arg–Arg, Ala–Arg, Leu–Arg, or Asp–Arg) from the N terminus of oligopeptides ranging from 3 to 10 amino acid residues or proteins at physiological pH 6–8.20,23,24 Endogenous DPP III has been detected in placenta, erythrocytes, and seminal plasma.25–27 DPP III is implicated in various physiological and pathological processes through the breakdown of certain oligopeptides or their fragments, such as the angiotensins (Ang II, Ang III, and Ang IV) and opioid peptides (enkephalins and endorphins).25,26 However, the details of its enzymatic characteristics and in vivo functions have not been well elucidated to date.

In this study, we analyzed the catalytic activities of DPP III using a synthetic peptide and Ang II and explored the therapeutic potential of the enzyme by investigating its antihypertensive effects in Ang II–infused mice. Furthermore, mutated recombinant DPP III enzymes were generated and studied in comparison with wild-type DPP III. The results showed that wild-type DPP III and a specific DPP III mutant could efficiently digest Ang II. Finally, the administration of DPP III into Ang II–infused hypertensive mice remarkably decreased blood pressure, revealing a novel function and potential therapeutic use of DPP III in the treatment of hypertension.

Methods

Purification of DPP III and Its Mutants
cDNAs encoding full-length or various fragments of rat DPP III were amplified by polymerase chain reaction and subcloned into pGEX-6P1 vector (GE Healthcare, Piscataway, NJ). Glutathione S-transferase–fused DPP III and its mutants were expressed in Escherichia coli transformed with corresponding plasmids at 20°C for 24 to 48 hours. Cells were sonicated in sonication buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, and 1 mmol/L dithiothreitol) and clarified by centrifugation at 20000g for 15 minutes. The resulting cell extract was added to glutathione–Sepharose 4B (GE Healthcare) and incubated for 1 hour. After washing the beads 3x with a buffer containing 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 1 mmol/L dithiothreitol, bound proteins were eluted with an elution buffer (10 mmol/L glutathione, 50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, and 1 mmol/L dithiothreitol). Alternatively, to obtain glutathione S-transferase–cleaved DPP III for administration to mice, protein-bound beads were incubated with PreScission protease (GE Healthcare) in a cleavage buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 1 mmol/L dithiothreitol) at 4°C overnight. Beads were removed with an empty spin column, and the resulting eluate was dialyzed against PBS for 24 hours. Protein concentration was determined by Quick Start Bradford 1x dye reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. Purity of protein was checked by Coomassie brilliant blue staining after SDS-PAGE. All proteins were stored at −80°C until use. Other experimental details are described in the online-only Data Supplement.

Statistical Analysis
Data are expressed as mean±SD. Statistical significance was analyzed using GraphPad Prism software and determined by 1-way ANOVA, 1-way repeated-measure ANOVA, or 2-way repeated-measure ANOVA, as appropriate. If ANOVA was significant, individual difference was evaluated using the Bonferroni post test. A value of P<0.05 was considered to be statistically significant.

Results

Enzymatic Activities and Properties of DPP III Against Ang II
To reveal the enzymatic activity of DPP III against Ang II, we first incubated 1 μmol/L Ang II with 6 nmol/L purified DPP III for various time periods between 0 and 30 minutes. Degraded peptides derived from Ang II were separated by reversed-phase liquid chromatography (Figure 1A) and confirmed by mass spectrometry. Val5-Ang II (1 μmol/L) was used as an internal standard. For incubation periods of 1.5 minutes or longer, the peak of a peptide VYIHPF (Ang IV) resulting from Ang II cleavage was observed. At 5 minutes, peaks of Ang IV and a further cleaved peptide (HIHF) from Ang IV were detected, indicating that Ang II was degraded in a 2-step process by DPP III. The first cleavage produced Ang IV from Ang II and the second cleavage produced IHPF from Ang IV. At 30 minutes, the peaks of Ang II and Ang IV were lost and only the peak of IHFP was observed, indicating that Ang II was completely degraded by DPP III within 30 minutes in this condition.

We selected a reaction time of 2 minutes to determine the reaction velocity (V) of the first cleavage of Ang II (DRVYIHPF→VYIHPF) by DPP III (Figure 1B and 1C), because at this time point, the second cleavage (VYIHPF→HIHF) was considered negligible as the resultant peptide, IHFP, was not detected. The identified kinetic parameters of DPP III required to cleave Ang II are shown in the Table. We performed a similar analysis of the second cleavage using Ang IV as a substrate (Figure 1D; Table), the results of which indicated that the cleavage of Ang IV by DPP III is much more effective than that of Ang II.

Enzymatic Activities of DPP III Mutants
We next constructed a series of truncated mutants of DPP III (Figure 2A and 2B) and examined their ability to degrade a synthetic substrate Arg-Arg-MCA, toward which DPP III exhibited a preferential activity,30 to determine the core region necessary for DPP III activity. Among the mutants tested, DPP III-WT and DPP III-AC1 effectively hydrolyzed the substrate (Figure 2C). DPP III-CE, in which Cys176 was substituted by Glu, showed only slight activity, similar to a previous report.30 In addition, ΔC2, ΔN1, and other mutants showed almost no activity. We then assessed the Ang II-degrading activities of some of the DPP III mutants. DPP III-AC1 (25 nmol/L) completely catalyzed Ang II within 30 minutes, similar to DPP III-WT (Figure 2D and 2E). However, neither DPP III-ΔC2 nor DPP III-ΔN1 cleaved Ang II within 30 minutes, even at concentrations of up to 100 nmol/L (Figure 2F and 2G). These results are consistent with those obtained from experiments using Arg-Arg-MCA as a substrate and indicate that DPP III-WT and DPP III-AC1 possess almost identical enzymatic activities, at least in vitro, for Arg-Arg-MCA and Ang II.

Blood Pressure–Lowering Effect of DPP III in Ang II–Infused Hypertensive Mice
The identification of catalytic properties of DPP III for Ang II prompted us to investigate its potential as an antihypertensive drug, and we therefore examined the in vivo effects of DPP III
Figure 1. Kinetic analysis of dipeptidyl peptidase III (DPP III)–mediated angiotensin II (Ang II) cleavage. A, Ang II was incubated with purified DPP III for the indicated times, and resulting peptides were subsequently analyzed and identified using liquid chromatography–mass spectrometry. X-axis represents elution time on liquid chromatography. Arrowheads indicate DPP III cleavage sites. B, Increasing concentrations of Ang II were incubated with DPP III for 2 min, and resulting peptides were analyzed as in A. Inset shows the lower concentrations of Ang II (0.125, 0.25, and 0.5 μmol/L). C and D, V vs S plot of DPP III–mediated cleavage of Ang II (C) or Ang IV (D). Initial velocity of the reaction (V) was calculated from the data shown in B for Ang II cleavage (C), and analysis similar to B was also performed using Ang IV as a substrate (D). Fitting curve was generated according to the Michaelis–Menten equation. IS indicates internal standard; P, product; S, substrate; and [S], substrate concentrations.
in Ang II–infused hypertensive mice. Before implantation of an Ang II pump (Pre), blood pressure and heart rate were as follows: systolic blood pressure 111±6 mm Hg, diastolic blood pressure 77±8 mm Hg, mean blood pressure 88±6 mm Hg, and heart rate 67±43 bpm (Figure 3A). At 2 to 4 weeks after implantation of the pump, mice were randomly divided into 2 groups: a wild-type DPP III injection group (n=8) or a PBS control injection group (n=12). In both groups, blood pressure was similar and significantly elevated compared with preimplantation levels (systolic blood pressure: DPP III group, 152±19 mm Hg versus PBS group, 119±15 mm Hg; diastolic blood pressure: DPP III group, 106±16 mm Hg versus PBS group, 101±14 mm Hg; and mean blood pressure: DPP III group, 121±15 mm Hg versus PBS group, 119±15 mm Hg) although heart rate was unchanged before and after Ang II infusion (DPP III group, 689±42 bpm versus PBS group, 651±77 bpm). When DPP III (8 μg/g body weight) was injected through the tail vein, blood pressure was dramatically reduced at 1 hour (systolic blood pressure, 97±10 mm Hg; diastolic blood pressure, 63±9 mm Hg; and mean blood pressure, 74±8 mm Hg) and 3 hours (systolic blood pressure, 109±15 mm Hg; diastolic blood pressure, 73±16 mm Hg; mean blood pressure, 85±15 mm Hg) post injection and was restored at 1 and 4 days post injection. Heart rate did not significantly change during the experiments. On the contrary, PBS injection did not affect blood pressure and heart rate to a significant extent. The dose of DPP III (8 μg/g body weight) was determined in preliminary experiments in which different doses of DPP III (1–8 μg/g body weight) were administered to Ang II–induced hypertensive mice, where 8 μg/g body weight of DPP III was shown to be most effective in reducing blood pressure (Figure S1 in the online-only Data Supplement).

To explore how DPP III is metabolized in vivo, blood serum (n=3 at each time point) was obtained pre injection and at 3 hours, 1 day, and 4 days post injection of DPP III, and Western blotting was performed on the serum samples. Administered DPP III was found to be present at 3 hours after injection, was remarkably reduced in 1 day, and almost disappeared by 4 days (Figure 3B). We also evaluated plasma Ang II concentration during the experiment. As expected, Ang II concentration was increased after implantation of the Ang II minipump (Figure 3C). When DPP III was injected and blood samples were collected at 3 hours post injection, Ang II concentration decreased significantly to a lower level than that at preimplantation of the minipump, suggesting that DPP III might cleave endogenous Ang II in addition to ectopically infused Ang II from the minipump.

As the DPP III-ΔC1 mutant had similar enzymatic activity for Ang II as DPP III-WT (as shown in Figure 2), the effect of DPP III-ΔC1 in Ang II–induced hypertensive mice was investigated. We found that DPP III-ΔC1 significantly reduced blood pressure at 3 hours post injection and that blood pressure was restored at 1 day (Figure 4A). However, another DPP III mutant DPP III-ΔN1, which had no enzymatic activity for Ang II (Figure 2), did not alter blood pressure (Figure 4B). To confirm that DPP III specifically affects Ang II–induced hypertension, we used another hypertensive mouse model in which noradrenalin (4 μg/kg per minute) was infused by minipump. Infusion of noradrenalin for 2 to 4 weeks increased systolic blood pressure (143±7 mm Hg), and DPP III injection did not reduce this elevated blood pressure (Figure 4C). Moreover, we found that blood pressure of normotensive mice was not affected by DPP III (Figure 4D). In all the above experimental models, heart rate was not significantly changed. Collectively, these results suggest that the blood pressure–lowering effects of DPP III are specifically mediated by digestion of Ang II in Ang II–infused hypertensive mice.

### Cardioprotective and Renoprotective Effects of DPP III

It is well known that excessive Ang II can damage organs, such as the heart and kidney, and that ACE inhibitors and ARBs have protective effects on these organs by ameliorating Ang II–induced hypertension or by other mechanisms. Therefore, we compared the effects of DPP III and candesartan, an ARB, on the heart and kidney. Candesartan injection (1 μg/g body weight) in Ang II–induced hypertensive mice significantly reduced blood pressure without changing heart rate (Figure 5A), a result similar to that produced by DPP III. Next, mice were regularly (3× per week) injected for 4 weeks with DPP III or candesartan from 2 days after the implantation of the Ang II minipump. Blood pressure, which was measured at 2 and 4 weeks after the implantation, was elevated in Ang II–infused mice (Figure 5B), but was significantly suppressed by the administration of DPP III or candesartan to similar levels. In the heart, Ang II infusion induced cardiac fibrosis, which was markedly reduced by the administration of DPP III or candesartan (Figure 5C). The effects of DPP III were relatively better than those of candesartan although statistical significance was not detected between these 2 treatments. Furthermore, cardiac function was evaluated by echocardiography, and Ang II was shown to increase diastolic left ventricular (LV) posterior wall thickness and LV mass, resulting in the development of cardiac hypertrophy (Figure 5D and 5E). Injection of DPP III for 4 weeks significantly blocked the Ang II–induced increase in left ventricular posterior wall thickness and LV mass, whereas candesartan injection only inhibited the LV mass increase. These results suggest that DPP III has cardioprotective effects against Ang II–induced cardiac damage and hypertrophy, at least similar to those of candesartan.

Finally, we examined the renal protective effects of DPP III. Infusion of Ang II in mice for 4 weeks remarkably increased urine albumin excretion compared with that of the control (Figure 6B). Treatment with DPP III or candesartan inhibited albumin excretion, and treatment with both DPP III and candesartan showed slight additive inhibition although statistical significance was not detected among these 3 treatments. Blood pressure reduction by administration of both DPP III and candesartan in Ang II–infused mice was similar to that of DPP III or

### Table. Kinetic Characteristics of Dipeptidyl Peptidase III for Ang II or Ang IV

<table>
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<th>Substrate</th>
<th>Ang II</th>
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</tr>
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<tbody>
<tr>
<td>( K_v ), mol/L</td>
<td>3.7×10^{-4}</td>
<td>1.7×10^{-6}</td>
<td></td>
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<tr>
<td>( V_{max}, \text{mol/L per s} )</td>
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<td>( k_{cat}, \text{per s} )</td>
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<td>1.5×10^0</td>
<td>2.7×10^6</td>
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Ang II indicates angiotensin II.
Figure 2. Proteolytic activities of dipeptidyl peptidase III (DPP III) and its mutants against Arg-Arg-MCA or angiotensin II (Ang II). A, Schematic representation of purified glutathione S-transferase (GST)-DPP III and its mutants. B, Purified GST-DPP III and its mutants were resolved on SDS-PAGE, followed by Coomassie brilliant blue staining. C, Proteolytic activities of GST-DPP III and its mutants were examined using (Continued)
candesartan alone (Figure 6A, see also Figure 5B). Despite the remarkable reduction of albumin excretion caused by the administration of DPP III, candesartan or both, Periodic Acid Schiff staining did not reveal any change in the kidney tissue, including the glomeruli, after Ang II infusion or additional administration of the agents (Figure 6C). Consistently, the ultrastructure.
Figure 4. Effect of dipeptidyl peptidase III (DPP III) and its mutants on angiotensin II (Ang II)- or noradrenalin (NA)–induced hypertensive mice or normotensive mice. A–D, Blood pressure and heart rate were measured at the indicated times after injection of DPP III-ΔC1 (A), DPP III-ΔN1 (B) in Ang II–infused mice, DPP III in NA-infused mice (C), and DPP III or PBS in normotensive mice into which no agents were infused (D). Data are the mean±SD. ††P<0.01 vs 0 h.
Figure 5. Cardioprotective effect of dipeptidyl peptidase III (DPP III) in angiotensin II (Ang II)–induced hypertensive mice. A, Comparison of blood pressure–lowering effects of DPP III (8 μg/g body weight) and candesartan (1 μg/g body weight) in Ang II–infused mice. The data for DPP III treatment are same as shown in Figure 3A. ††P<0.01 vs 0 h. B, Blood pressure and heart rate measurement during (Continued)
of the kidney, such as the podocytes, was not affected by Ang II infusion (Figure 6D). However, Ang II significantly increased the expression of some kidney injury or inflammation markers (Figure 6E). Treatment with DPP III or both DPP III and candesartan suppressed the increase in these markers, whereas treatment with candesartan attenuated the increase in Ngal and PAI-1 expressions but failed to do so in MCP-1 expression.

Discussion

Hypertension is one of the most common risk factors for cardiovascular diseases and deaths. Lowering blood pressure with antihypertensive drugs reduces organ damages and prevents cardiovascular disease. Although many drug treatments are available, a substantial number of patients with hypertension have uncontrolled blood pressure with conventional drug treatments. A new approach for lowering blood pressure is, therefore, important for the treatment of such patients and the reduction of comorbidities, such as heart failure and chronic kidney diseases. Over the years, many strategies and drug therapies have been used to reduce the precocious effects of hypertension. To date, several types of antihypertensive drugs related to the control of RAS have been developed and approved; renin inhibitors prevent Ang I formation by binding directly to renin to competitively block the digestion of angiotensinogen, ACE inhibitors prevent Ang II production, and ARBs act on AT1R to inhibit Ang II binding to the receptor. However, no treatments are available that directly degrade Ang II itself. Although DPP III was found to be a cytosolic protein in the brain tissue and to cleave dipeptides from the N termini of substrates, such as Ang II and enkephalins, the kinetic details of its hydrolytic activity against Ang II and, to date, its in vivo effects in hypertensive animals have not been well elucidated.

In in vitro analyses, we demonstrated that DPP III cleaved Ang II to Ang IV with $K_m = 3.7 \times 10^{-4}$ mol/L and $V_{\text{max}} = 3.3 \times 10^{-9}$ mol/L per second (26 mmol/h per microgram). A kinetic analysis of Ang II cleavage by DPP III in a previous study determined $K_m = 3.5 \times 10^{-7}$ mol/L and $V_{\text{max}} = 2.3$ nmol/h per microgram, values lower than those seen in our study. As the previous study used DPP III partially purified from the rat brain, the reaction of the second substrate (Ang IV) may affect the kinetics of the first digestion, in accordance with chemical reaction equilibrium rules. Next, we found that a series of DPP III–truncated mutants had almost no enzymatic activity, except for the shortly C-terminal-deleted mutant DPP IIIΔC1. The putative peptidase unit of DPP III is located at amino acid 425 to 663, which is inside the zinc-binding lobe (amino acid 422−668) as determined by X-ray crystallography. In addition to the zinc-binding lobe that includes the peptidase unit, DPP III consists of several characteristic regions, such as the lower lobe and the 5-stranded β-core, and the N terminus of this enzyme is critical for substrate recognition. All of these structural complexities might be necessary to retain full enzymatic activity of DPP III.

In the in vivo experiments, we showed that the administration of DPP III dramatically lowered blood pressure in Ang II–infused hypertensive mice, suggesting the potential of DPP III as an antihypertensive drug. These results propose a novel insight that inhibition of RAS in a mechanism different from that by any conventional antihypertensive drugs indeed decreases blood pressure. It is well known that Ang II binding to its receptor, AT1R, activates reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to generate reactive oxygen species in several tissues. This reactive oxygen species generation contributes to the development of hypertension. In addition, to the digestion of Ang II by DPP III, this enzyme can attenuate Ang II–induced hypertension by reducing reactive oxygen species produced by the Ang II–AT1R signaling pathway. The effective duration of administered DPP III is <1 day, as the decrease in blood pressure caused by DPP III was restored by 1 day and administered DPP III remained scarcely detectable at 1 day (Figure 3). However, regular injection of DPP III successfully suppressed the elevation of blood pressure by Ang II infusion. This effect might be because of the gradual accumulation of subtle amounts of DPP III, which persisted even at 2 days after administration.

We also showed that DPP III had cardioprotective effects by preventing cardiac hypertrophy and fibrosis in Ang II–infused mice, at least similar to candesartan. Several clinical meta-analyses have demonstrated that ARBs have the superiority for reduction of LV mass as an index of cardioprotective effects compared with other types of antihypertensive drugs. On the contrary, in a recent report, long-term ARB therapy was found to significantly reduce mean systolic and diastolic blood pressure in patients with hypertension compared with placebo, but did not produce significant reductions in the risk of heart failure, hospitalization, or mortality. A review article also pointed out that meta-analyses did not show a blood pressure–dependent risk reduction in cardiovascular mortality by treatment with ARB. These lines of evidence, along with our data, suggest that treatment with ARB alone can effectively reduce blood pressure and LV mass, but may not be enough to achieve complete cardioprotection. In fact,
Figure 6. Renal protective effects of dipeptidyl peptidase III (DPP III) in angiotensin II (Ang II)–induced hypertensive mice. A, Systolic blood pressure and heart rate during treatment with or without DPP III plus candesartan in Ang II–infused mice. The data without treatment (Ang II) are same as shown in Figure 5B. **P<0.01 vs Ang II; ††P<0.01 vs Pre. B, Urine samples from each mouse were collected for (Continued)
a recent meta-regression analysis failed to demonstrate a significant relationship between LV hypertrophy and incidence of the composite outcome including all-cause death, myocardial infarction, stroke, or new onset heart failure. Additional therapies are, therefore, required to effectively prevent hypertension-induced cardiac damage and mortality.

Although urinary albumin excretion was markedly increased in Ang II-infused hypertensive mice, the kidney tissue architecture including the glomerulus and podocyte morphology was not damaged in this model. Physical stress on afferent arterioles (hyperfiltration induced by increased renal blood pressure and flow) and an inflammatory microenvironment (increase in inflammatory molecules) caused by Ang II may contribute to the abundant leakage of albumin through the glomeruli. Although the administration of both candesartan and DPP III alone showed slight additive reduction of urinary albumin excretion compared with that of candesartan or DPP III alone, complete inhibition of the RAS might promote protection from Ang II-initiated renal damage and cardiac damage. In clinical trials, renoprotective effect of strong inhibition of Ang II cleavage, its use in conjunction with other RAS inhibitors might improve prognosis of hypertensive subjects.

**Perspectives**

The in vitro enzymatic activity against Ang II and in vivo blood pressure-lowering effects of DPP III were precisely characterized in this study. DPP III showed tissue protection in the heart and kidney at least to a similar extent as candesartan, an ARB, in Ang II–infused hypertensive mice. Because DPP III is endogenously produced in humans, its administration is considered to be relatively safe. Taken together with the results described in this study, this may suggest a new therapy for hypertension, focusing on DPP III.

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**Disclosures**

None.

**References**

DPP III had cardioprotective and renoprotective effects in Ang II–infused pressure was demonstrated using hypertensive mice. The mode of action of DPP III in reduction of Ang II–induced blood pressure was studied in detail.

Novelty and Significance

What Is New?
- Proteolytic activity of DPP III against Ang II and Ang IV was characterized in detail.
- Enzymatic activity of several DPP III mutants was identified.
- The mode of action of DPP III in reduction of Ang II–induced blood pressure was demonstrated using hypertensive mice.
- DPP III had cardioprotective and renoprotective effects in Ang II–infused hypertensive mice.

What Is Relevant?
- DPP III has potential for the development of novel antihypertensive drugs.
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A Novel Therapeutic Role for Dipeptidyl Peptidase III in the Treatment of Hypertension


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Methods

Kinetic Analysis of DPP III Digestion of Ang II
A reaction mixture (100 µL) containing 0–16 µmol/L human Ang II (DRVYIHPF, m/z 524 [M+2H]^{2+}) and 50 mmol/L Tris-HCl buffer, pH 7.2 was prepared, and the reaction started by the addition of 0.5 µL of DPP III enzyme stock solution. The mixture was incubated at 37°C for 0–30 min. At the indicated time, a 20 µL aliquot of reaction mixture was removed and mixed with 2.0 µL of 30% trichloroacetic acid to stop the reaction. A 10 µL aliquot of 10 µmol/L Val5-Ang II (DRVYVHPF, m/z 517 [M+2H]^{2+}) was then added to the mixture as an internal standard. The resultant mixture (10 µL) was injected into an Inertsil peptides C_{18} column (0.3 mm × 150 mm, 4 µm particle size; GL-Science, Tokyo, Japan). Elution was performed at a flow rate of 3.5 µL/min using the following gradient profile: t = 0 min, 20% B; t = 5 min, 30% B; t = 15 min, 36% B; t = 16 min, 60% B; t = 21 min, 60% B. Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in Milli-Q water, and solvent B was 0.1% TFA in acetonitrile-water (90:10, v/v). The gradient flow (90 µL/min) was first constructed using a HPLC system (Shimadzu, Kyoto, Japan) consisting of a SCL-10Avp system controller, a DGU-20A3 degasser, and two LC-20AD pumps, and the flow was then split into column elution and waste using an AC-100-VAR flow splitter (GL-Science, Tokyo, Japan). The elution of peptides was spectrophotometrically monitored at 225 nm using a MU-701 UV-vis detector equipped with an 18-nL flow cell (GL-Science), and chromatograms were analyzed using a Smart Chrom processor (KYA TECH Corporation, Tokyo, Japan). When identification of eluted peptides was required, the flow from the column was directed to an LCQ-Fleet ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA USA) through a nano-electrospray ionization source (AMR Inc., Tokyo, Japan).

The DPP III enzyme cleaved two amino acids of N-terminal human Ang II to produce Ang IV (VYIHPF, m/z 388 [M+2H]^{2+}), which was further cleaved by the enzyme to produce the second products VY (m/z 281 [M+H]^{+}) and IHPF (m/z 513 [M+H]^{+}), as confirmed by mass spectrometry. In this study, we examined both first and second cleavages in detail. For examination of the first cleavage, we selected reaction conditions under which the second cleavage was prevented. The kinetic data were analyzed according to the Michaelis–Menten equation using GraphPad Prism software.

Proteolytic Activity Assay
Proteolytic activity assay using the artificial substrate Arg-Arg-MCA was performed as described previously with some modifications. GST-DPP III proteins in 50 µL of assay buffer (50 mmol/L Tris-HCl, pH 7.5, and 150 mmol/L NaCl) were mixed with 50 µL of assay buffer containing 0.2 mM Arg-Arg-MCA in a 96-well plate. Immediately after the addition of substrate solutions, the increase of fluorescence intensity was kinetically monitored at 440 nm with excitation at 380 nm using an Infinite 200 PRO plate reader (Tecan, Männedorf, Switzerland). Endpoint data were taken from a time point in the linear range of reactions (15 min).

To examine Ang II cleavage by DPP III and its mutants, we employed a
chromatographic technique. Ang II in 200 µL of 50 mmol/L sodium phosphate buffer (pH 8.0) was added with DPP III or its mutant proteins and incubated at 37°C. The reaction was stopped by adding 20 µL of 30% trichloroacetic acid. After centrifugation at 20,000 \( \times \) g for 10 min, supernatants containing peptides were analyzed by reversed-phase chromatographic separation on a COSMOSIL 5C\(_{18}\)-AR-300 column (Nacalai Tesque, Kyoto, Japan) using an acetonitrile/water gradient (5–35% acetonitrile) in the presence of 0.1% trifluoroacetic acid. Chromatograms were processed using Microsoft Excel software.

**Generation of the Hypertensive Mouse Model**

C57BL/6J mice, 8–10 weeks of age, were used as a hypertensive mouse model by subcutaneously implanting a micro-osmotic pump (Alzet model 1004; Direc Corporation, Cupertino, CA, USA) containing Ang II (400 ng/kg/min; Wako Pure Chemical Industries, Osaka, Japan) or noradrenalin (4 µg/kg/min; Tokyo Chemical Industry, Tokyo, Japan) for 2–4 weeks as previously described.\(^2,3\) Ang II- or noradrenalin-infused hypertensive mice were randomly divided into each experimental group. In control mice, a pump with saline was implanted subcutaneously. Mice were housed in the Shiga University of Medical Science Animal Facility and had free access to food and water. The mouse care and experimental procedures performed in this study were reviewed and approved by the Shiga University of Medical Science Animal Care and Use Committee.

**Administration of DPP III or Its Mutants, and Blood Pressure Measurement**

Blood pressure was measured in conscious acclimatized mice using a tail cuff method (BP-98A-L System; Softron, Tokyo, Japan). Animals were maintained at 37–38°C on a heating pad during measurement. DPP III or its mutants dissolved in saline, or PBS was injected via the tail vein. Prior to injection, blood pressure elevation by Ang II- or noradrenalin-infusion (systolic blood pressure \( \geq \) 130 mmHg) was confirmed. At each time point, over 7 measurements were recorded and averaged.

**Measurement of Ang II Concentration by ELISA**

Following intravenous injection of 160 U heparin into each mouse, blood samples were collected in tubes containing 10 mmol/L of sodium-ethylenediaminetetraacetic acid, 4 mM of PMSF and 10 U of heparin followed by centrifugation for 15 min at 1,000 \( \times \) g and 4°C to separate the plasma (~400 µL). After addition of an equal volume of 1% TFA to the plasma, supernatant isolated by 20,000 \( \times \) g centrifugation was applied to a C\(_{18}\) column (Waters Corporation, Milford, MA, USA) to enrich Ang II. The solution eluted with 60% of acetonitrile and 1% TFA was lyophilized and reconstituted with the assay buffer included in the Angiotensin II ELISA kit (ENZO Life Science, Farmingdale, NY, USA). Plasma Ang II level was measured according to the manufacturer's protocol.

**Histological Analyses**

Mice hearts and kidneys were fixed with 4% paraformaldehyde and subsequently embedded in paraffin blocks. Samples were cut in 4 µm thickness and stained by Masson's trichrome or Periodic Acid Schiff (PAS) using standard techniques. Images for
histological analysis were captured by color CCD camera (MicroPublisher 5.0 RTV; Qimaging, Surrey, BC Canada) using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA) mounted on a Nikon FXA light microscope. Extent of fibrosis stained in blue by Masson's trichrome in cardiac sections was quantified as ratio of [sum of blue (collagen-positive) areas / whole area of visible myocardium x 100 (%)] using ImageJ software.

**Transmission Electron Microscopy**

For transmission electron microscopy, we conducted a similar procedure to that described previously. Briefly, the kidneys were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in PBS on ice for 2 h. The samples were washed with PBS, treated with 2% OsO₄ in PBS on ice for 2 h, dehydrated in graded ethanol, and embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate for 2 h and lead nitrate for 3 min, and observed with a Hitachi H-7500 electron microscope.

**Quantitative PCR**

Total RNA was isolated from the kidneys of mice using TRIzol RNA isolation reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan). To compare mRNA expression levels, SYBG-based real-time PCR was conducted using a Roche LightCycler 480 Instrument I (Roche Diagnostics, Mannheim, Germany). Data were quantified by the standard curve method and were adjusted using β-actin mRNA expression level as an internal control, as described previously. Primer sequences for quantitative PCR experiments were as follows: Ngal forward 5'-CCCTGTATGGGAAGACCAAGGA-3' and reverse 5'-CGGTGGGGACAGA GAAGATG-3'; MCP-1 forward 5'-GCCCCACTCACCTGCTGCTACT-3' and reverse 5'-CCTGCTGCTGGTGATCCTCTTGT-3'; PAI-1 forward 5'-GGACACCCTCAGCATGTC A-3' and reverse 5'-CTCTGATGATTCAGCATGTTTC-3'; and β-actin forward 5'-CG TCGTGACATCAAAGAGAA-3' and reverse 5'-TGGATGCCACAGGATTCCAT-3'.

**Reference**

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Figure S1. Administration of different doses of DPP III in Ang II-induced hypertensive mice. After administration of DPP III (1–8 μg/g body weight), systolic blood pressure was measured at the indicated time points.