JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY ARTICLE

J. Agric. Food Chem. 2011, 59, 559–563 559
DOI:10.1021/jf104126a

One-Week Antihypertensive Effect of Ile-Gln-Pro in Spontaneously Hypertensive Rats

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The antihypertensive effect of an angiotensin I-converting enzyme (ACE) inhibitory peptide Ile-Gln-Pro (IQP), whose sequence was derived from *Spirulina platensis*, was investigated in spontaneously hypertensive rats (SHRs) for 1 week. The weighted systolic blood pressure (SBP) and diastolic blood pressure (DBP) of the peptide IQP-treated group were significantly lower than those of the negative control group from the third and fourth days, respectively. Accompanying the blood pressure reduction, a significant regulation of the expression of major components of the renin−angiotensin system (RAS) was found in the treatment group, including downregulation of the mRNA levels of renin, ACE, and the angiotensin II type 1 (AT1) receptor in the kidney, as well as serum angiotensinogen (Ang), ACE, and angiotensin II (Ang II) concentrations. The treatment group also showed upregulation of mRNA expression of the angiotensin II type 2 (AT2) receptor in the kidney. Our findings suggested that IQP might be of potential use in the treatment of hypertension.

KEYWORDS: ACE inhibitory peptide; Ile-Gln-Pro; renin−angiotensin system; antihypertension

INTRODUCTION

Hypertension is a major disease that threatens human health worldwide. Without effective medication, persistent hypertension can lead to stroke, heart failure, arterial aneurysm, chronic kidney failure, and shortened life expectancy (*1*, *2*). Since Skeggs et al. (*3*) first illustrated the crucial effect of the renin−angiotensin system (RAS) on regulating cardiovascular function, this endocrine system has been the focus of many studies on treating and preventing hypertension (*4*). In the RAS, renin converts angiotensinogen (Ang) to inactive decapeptide angiotensin I (Ang I), which is then cleaved by two amino acid residues from the C terminal to form angiotensin II (Ang II) by angiotensin-converting enzyme (ACE) (*5*). Ang II plays its physiological roles by interacting with specific transmembrane G-protein-coupled receptors. When Ang II binds to its high-affinity angiotensin II type 1 (AT1) receptor, Ang II activates the AT1 receptor and causes vasoconstriction by stimulating the synthesis of aldosterone in the zona glomerulosa of the adrenal gland and promoting remodeling or restructuring of the blood vessel wall (*6*, *7*). Stimulation of the angiotensin II type 2 (AT2) receptor, on the other hand, counterbalances the effects of AT1 receptor stimulation, thereby inhibiting proliferation, vasodilation, and natriuresis (*6*, *8*).

In recent decades, ACE inhibitors have been broadly applied to treat and prevent hypertension. These drugs include captopril, the first synthetic antihypertensive medicine (*9*). Because synthetic inhibitors with non-natural sequences induce various side effects, such as cough, allergy, edema, and kidney damage (*10*), ACE inhibitors derived from natural resources, especially food proteins, have attracted more and more attention in many countries. Researchers have obtained ACE inhibitory peptides from various natural resources (*11*−*13*) and verified their *in vivo* antihypertensive effects in animal models or human trials (*14*−*16*). We recently isolated an ACE inhibitory tripeptide, Ile-Gln-Pro (IQP), from the blue algae *Spirulina platensis* (*17*), which is well-known for its high protein content and comprehensive amino acid composition (*18*). The IC_{50} of IQP was determined as $5.77 \pm 0.09\ \mu mol/L$, and the antihypertensive effect of IQP was proven in spontaneously hypertensive rats (SHRs) by a single dose in 24 h (*17*). However, the antihypertensive effect of repeated administration and the regulatory function on the RAS of IQP has not yet been investigated.

The purposes of the present study were therefore to investigate the 1 week antihypertensive effect of repeated treatment with IQP in SHRs and to explore the regulation of the expression of major components (renin, Ang, Ang II, ACE, and AT1 and AT2 receptors) of the RAS in the kidney and sera of SHRs.

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Published on Web 12/23/2010

MATERIALS AND METHODS

Materials and Reagents. IQP was synthesized by the solid-phase method on a peptide synthesizer (model PSSM-8; Shimadzu, Kyoto, Japan) in our laboratory. The purity was verified to be higher than 95% by analytical high-performance liquid chromatography coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Sodium pentobarbital was purchased from Sigma-Aldrich (St. Louis, MO). SV Total RNA Isolation System was obtained from Promega (Madison, WI), and the First Strand cDNA Synthesis Kit for RT-PCR (AMV) was from Roche Diagnostics (Mannheim, Germany). SYBR Premix Ex *Taq*II Kits (Perfect Real-Time PCR) were purchased from Takara Bio (Shiga, Japan). The Rat Total Angiotensinogen Assay Kit, Angiotensin II Enzyme ImmunoAssay Kit, and ACE ImmunoAssay Kit were from Immuno-Biological Laboratories (Gunma, Japan), Phoenix Pharmaceuticals (Burlingame, CA), and Research and Diagnostics Systems (Minneapolis, MN), respectively. All other reagents, unless otherwise specified, were obtained from Wako Pure Chemical Industries (Osaka, Japan) and of analytical or guaranteed reagent grade.

Animals and Blood Pressure Measurement. A total of 15, male, 8-week-old, specific pathogen-free SHRs, weighing 235 ± 7 g, were purchased from Charles River Laboratories (Kanagawa, Japan) and kept in an air-conditioned room (22 ± 1 °C) with a 12 h light and dark cycle. The rats were acclimatized for 7 days with a diet based on the American Institute of Nutrition (AIN)-93G formula (*19*) and became accustomed to the following procedure for measuring blood pressure: the systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) of the rats were measured by the tail-cuff method every day. In consideration of the blood pressure fluctuation induced by nervousness, the weighted SBP and DBP were defined as follows: weighted SBP or DBP = SBP or DBP/HR × AveHR, where AveHR was the average heart rates of all SHRs in the measurement. Before measurement, the rats were kept at 38 °C for 5−10 min to make the pulsations in the tail artery detectable. Five measurements were taken; the highest and lowest were discarded, and the remaining three were averaged. All measurements were performed by the same person in a quiet environment (*16*).

From the eighth day, the rats were assigned to three groups: a negative control, a positive control, and a treatment group, according to the balanced average blood pressure of each group. Between 10:30 a.m. and 11:30 a.m. each day for 1 week, the rats in different groups were orally administrated 1 mL of the following solution by gastric intubation with KN-349 NIS tubes (Natume Seisakusho, Tokyo, Japan): 0.9% saline for the negative control group, 10 mg/kg of captopril per rat dissolved in 0.9% saline for the positive control group, and 10 mg/kg of IQP per rat dissolved in 0.9% saline for the treatment group, whose peptide dose was chosen on the basis of the results of our preliminary experiments (data not shown). The blood pressure was measured before administration each day. On the last day (the 14th day), the rats were anaesthetized by intraperitoneal injection of pentobarbital sodium at a dose of 50 mg/kg of body weight and killed after their blood pressure was measured. Blood was drawn from the hearts, and kidneys were removed carefully and weighed. The blood samples were centrifuged at 1200 *g* for 20 min, and the serum samples obtained were stored at −20 °C until analyzed. The kidneys were quickly frozen in liquid N_2 and kept at −80 °C.

The care and treatment of the rats were in accordance with The Ethical Guide for the Care and Use of Laboratory Animals, Chiba University. The animal experiments were approved by the Ethics Committee for Animal Experiments of Chiba University.

Isolation of RNA. Kidney tissues stored at −80 °C were homogenized with a tissue minimizer (Tekmar, Cincinnati, OH), and total RNA was isolated using the SV Total RNA Isolation System according to the instructions of the manufacturer. RNA purity and concentration were determined spectrophotometrically (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE) by the ratio of A_{260}/A_{280}. RNA integrity was verified by 0.8% agarose formaldehyde gels and ethidium bromide staining under ultraviolet light.

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). First-strand cDNA was synthesized from 1 μg of RNA using the First Strand cDNA Synthesis Kit for RT-PCR (AMV). Real-time PCR was performed for renin, Ang, ACE, and AT1 and AT2 receptors, as well as for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using the SYBR Premix Ex *Taq*II Kit on a Roche Real-Time PCR Lightcycler System, version 3.5 (Roche Applied Science, Indianapolis, IN). The primers were ordered from Sigma-Aldrich Japan (Tokyo, Japan) and listed as in **Table 1** (*20*, *21*). PCR reactions were carried out in 20 μL volumes consisting of 2 μL of cDNA template (10 ng), 10 μL of SYBR Premix Ex *Taq*II (2×), 0.8 μL of forward primer (10 μM), 0.8 μL of reverse primer (10 μM), and 6.4 μL of PCR water. Thermal cycling was initiated with 10 s denaturation at 95 °C and followed by 45 cycles of 5 s at 95 °C and 20 s at 65 °C and then 1 cycle of 0 s at 95 °C, 15 s at 60 °C, 0 s at 95 °C, and 30 s at 40 °C for melting curve analysis and cooling. Except for 0.1 °C/s for the heating step from 60 to 95 °C in the melting curve analysis stage, the heating or cooling rate was 20 °C/s for all steps. All of the process was implemented according to the guidelines of the manufacturers.

Table 1. Primers Used in Real-Time RT-PCR

primer	sequences
Ang	sense: 5′-GCCCAGGTCGCGATGAT-3′ anti-sense: 5′-TGTACAAGATGCTGAGTGAGGCAA-3′
renin	sense: 5′-AACATTACCAGGGCAACTTTCACT-3′ anti-sense: 5′-ACCCCCTTCATGGTGATCTG-3′
ACE	sense: 5′-CACCGGCAAGGTCTGCTT-3′ anti-sense: 5′-CTTGGCATAGTTTCGTGAGGAA-3′
AT1	sense: 5′-CGGCCTTCGGATAACATGA-3′ anti-sense: 5′-CCTGTCACTCCACCTCAAAACA-3′
AT2	sense: 5′-CAATCTGGCTGTGGCTGACTT-3′ anti-sense: 5′-TGCACATCACAGGTCCAAAGA-3′
GAPDH	sense: 5′-GGGCAAGGTCATCCCTGAGCTGAA-3′ anti-sense: 5′-GAGGTCCACCACCCTGTTGCTGTA-3′

The quantification of a target gene was expressed as the relative expression ratio of the target gene in a sample versus that of a control in comparison to the reference gene (i.e., the housekeeping gene GAPDH). A mathematical model for calculating the relative expression ratio follows (*22*):

$$\text{ratio} = [(E_{\text{target}})^{\Delta CP_{\text{target}}(\text{control}-\text{sample})}]/[(E_{\text{ref}})^{\Delta CP_{\text{ref}}(\text{control}-\text{sample})}]$$

where E_{target} is the real-time PCR efficiency of the target gene transcription, E_{ref} is the real-time PCR efficiency of the reference gene transcription, $\Delta CP_{\text{target}}$ is the crossing point (CP) deviation of the control versus sample of the target gene transcription, and ΔCP_{ref} is the CP deviation of the control versus sample of the reference gene transcription. The real-time PCR efficiency was calculated according to $E = 10^{[-1/\text{slope}]}$ (*23*), where the slope was determined by the corresponding standard curve for the real-time PCR of a transcripted gene.

Enzyme-Linked Immunosorbent Assay (ELISA). Serum Ang and ACE concentrations were measured with IBL Rat Total Angiotensinogen Assay Kit and R&D ACE ImmunoAssay Kit, respectively, by the double-antibody solid-phase sandwich method. The serum Ang II concentration was measured with Phoenix Pharmaceuticals Angiotensin II Enzyme ImmunoAssay Kit by competitive enzyme immunoassay. Samples in 96-well microplates were read by a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA) within 20 min after the reaction was stopped. Test samples and standards were measured in duplicate. Microplate Manager Version 5.2.1 software, which was equipped with the spectrophotometer, automatically calculated sample concentrations according to corresponding standard curves with its four-parameter logistics and log−logit function. All of the processes were implemented according to the guidelines of the manufacturers.

Statistical Analysis. Results are expressed as mean values with their standard errors (SEs). The total variation was estimated by one-way analysis of variance (ANOVA) followed by post-hoc Scheffe's multiple comparison test with Excel Toukei (version 6.0; Esumi, Tokyo, Japan). The level of statistical significance was $p < 0.05$.

RESULTS

Effects of IQP on Growth Parameters of the SHRs. There were no mortalities among the experimental animals, and no significant differences were observed in the final body weight, body weight gain, carcass weight, or kidney ratio among the groups

Table 2. Growth and Diet Parameters of the SHRs during the 1 Week Experimental Course[a]

group	initial body weight (g)	body weight gain (g/7 days)	liquid intake (g/7 days)	diet efficiency[b] (%)	kidney ratio[c] (%)	carcass weight (g)
negative control (saline)	233 ± 6	24 ± 6	260 ± 50	2.6 ± 0.5	0.96 ± 0.07	189 ± 6
positive control (captopril, 10 mg/kg)	237 ± 10	18 ± 8	310 ± 20	1.5 ± 0.6	0.98 ± 0.06	193 ± 6
treatment (IQP, 10 mg/kg)	235 ± 4	20 ± 3	290 ± 20	2.0 ± 0.4	1.02 ± 0.07	195 ± 5

[a] Data are means ± SE; $n = 5$. [b] The diet efficiency = body weight gain per 100 g diet/7 days. [c] The kidney ratio = kidney weight × 100/carcass weight.

over the whole 7 day experimental course (**Table 2**). Liquid intake and diet efficiency, however, showed noticeable (although not significant) differences among the groups. Average liquid intake of the positive control group and that of the treatment group increased 18.0 and 10.4% compared to the negative control group, but diet efficiency decreased 39.6 and 25.4%, respectively.

Effects of IQP on Blood Pressure Changes of the SHRs in 1 Week. The weighted SBP and DBP of the SHRs in the different groups are shown in **Figure 1**. From the third day after treatment started, the weighted SBP of the positive control group and that of the treatment group were significantly lower than that of the negative group. The decrease persisted and accelerated. On the sixth day after treatment started, the weighted SBPs were 163 ± 1 and 165 ± 3 mmHg for the positive control group and the treatment group, respectively; these values were 33 and 32 mmHg lower than the original, respectively. These reductions corresponded to 5.5 and 5.4 mmHg per day, clearly indicating an antihypertensive effect (**Figure 1A**).

Furthermore, as shown in **Figure 1B**, the weighted DBP of the positive control group and that of the treatment group were significantly lower than that of the negative control group from the fourth day after the treatment started, and on the sixth day, the weighted DBPs were 133 ± 3 and 136 ± 5 mmHg, respectively, which were both 24 mmHg lower than the original. The reductions corresponded to 4.0 mmHg per day in both groups.

Effects of IQP on mRNA Levels of Major RAS Components in the Kidney. Kidney mRNA levels of renin, Ang, ACE, and AT1 and AT2 receptors were detected by real-time PCR and quantified as illustrated in **Figure 2** in comparison to the housekeeping gene GAPDH. The Ang gene could not be detected in kidneys of the SHRs in the three groups after 45 PCR cycles (data not shown). In comparison to those of the negative group, the renin mRNA levels were significantly decreased, and the reductions even reached 99 and 97% for the positive and treatment groups, respectively (**Figure 2A**). There was no significant difference in renin mRNA levels between the positive group and the treatment group.

Gene expression levels of ACE and the AT1 receptor kept the same trends as renin among the groups (panels **B** and **C** of **Figure 2**). The ACE mRNA levels of the positive and treatment groups were decreased significantly, by 94 and 89%, respectively, compared to the negative group. For the AT1 receptor, the mRNA levels were decreased significantly, by 97 and 96%, respectively. Similarly, both ACE and the AT1 receptor showed no significant difference between the positive group and the treatment group.

The AT2 receptor, however, presented contrary tendencies compared to renin, ACE, and the AT1 receptor (**Figure 2D**). In comparison to those of the negative group, mRNA levels of the AT2 receptor were increased significantly, by 37 ± 7- and 39 ± 9-fold for the positive and treatment groups, respectively. Moreover, although there was no significant difference between the positive group and the treatment group, mRNA expression of the AT2 receptor for the treatment group was higher than that for the positive control group.

Effects of IQP on Concentrations of Major RAS Components in the Serum. Serum Ang, ACE, and Ang II concentrations were measured and quantified by ELISA, as illustrated in **Figure 3**.

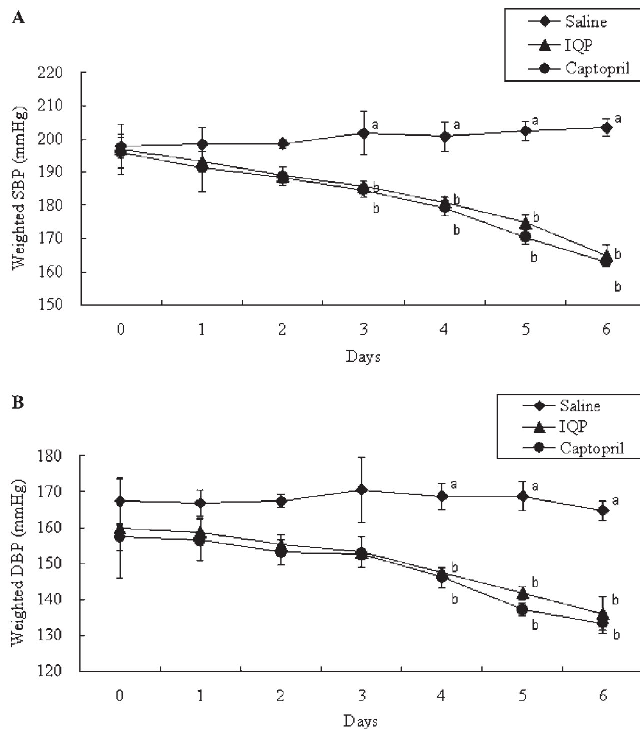

Figure 1. One-week antihypertensive effects of IQP in SHRs: (A) change in the weighted SBP during the 1 week experimental course and (B) change in the weighted DBP during the 1 week experimental course. Each value was expressed as the mean ± SE; $n = 5$. A value of $p < 0.05$ was considered statistically significant by one-way ANOVA followed by post-hoc Scheffe's test. Values at the respective times with dissimilar lowercase letters were significantly different.

In comparison to that of the negative group, serum Ang levels of the positive and treatment groups were decreased significantly, by 16 and 13%, respectively, ACE levels were decreased significantly, by 30 and 28%, respectively, and Ang II levels were also reduced significantly, by 60 and 56%, respectively. There was no significant difference between the positive group and the treatment group for serum Ang, ACE, and Ang II concentrations.

DISCUSSION

The aim of the present study was to explore the effects of IQP, an ACE inhibitor whose sequence was derived from *S. platensis*, on blood pressure and the expression of major RAS components in SHRs over a 1 week experimental course. In the current work, we demonstrated clearly, for the first time, that IQP treatment significantly decreased the weighted SBP and DBP in 1 week and affected the expression of major RAS components by down-regulating renin, Ang, ACE, Ang II, and AT1 while upregulating AT2 in the kidney or sera of SHRs.

Because SHR develops hypertension whose process and pathological characteristics are very similar to those of human hypertension, this strain has generally been used to conduct initial studies on antihypertensive effects of functional food products

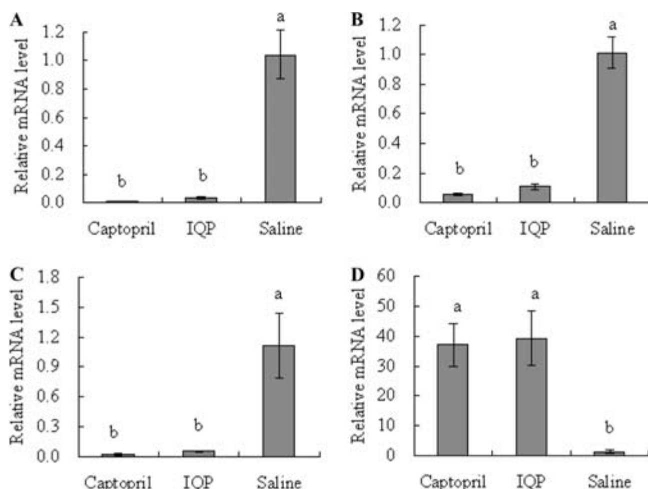

Figure 2. One-week regulatory effect of IQP on major RAS components in the SHR kidney: (A) change in renin mRNA levels, (B) change in ACE mRNA levels, (C) change in mRNA levels of the AT1 receptor, and (D) change in the mRNA levels of the AT2 receptor. Each value was expressed as the mean ± SE; $n = 5$. A value of $p < 0.05$ was considered statistically significant by one-way ANOVA followed by post-hoc Scheffe's test. Values with dissimilar lowercase letters were significantly different.

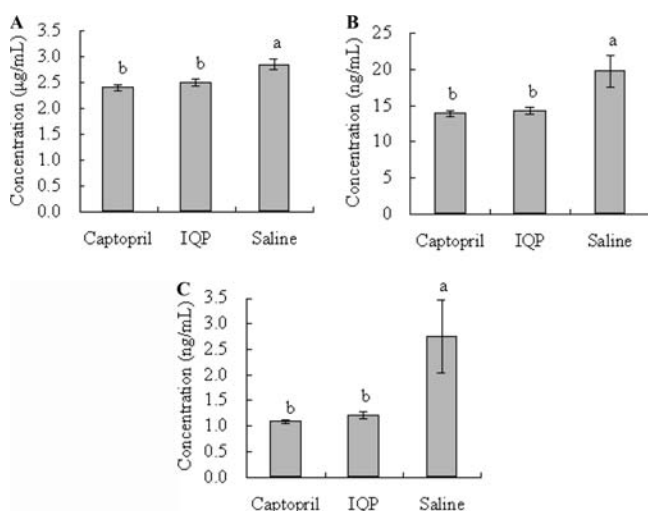

Figure 3. One-week regulatory effects of IQP on major RAS components in the sera of SHRs: (A) change in serum Ang concentrations, (B) change in serum ACE concentrations, and (C) change in serum Ang II concentrations. Each value was expressed as the mean ± SE; $n = 5$. A value of $p < 0.05$ was considered statistically significant by one-way ANOVA followed by post-hoc Scheffe's test. Values with dissimilar lowercase letters were significantly different.

and bioactive peptides derived from food proteins (*24*, *25*). In our study, no significant difference in the growth parameters and no deaths were observed among the animals of the different experimental groups, indicating the safety of IQP during the treatment course. It should be noted that liquid intake and diet efficiency showed noticeable (although not significant) differences in the positive control and treatment groups compared to the negative control group. The reason for this might be the inhibition of ACE to slow body weight gain in this rat strain and to induce thirst and increased water intake in rats (*26*, *27*). Moreover, the 8-week-old SHRs exhibited typical hypertension symptoms, with average SDP higher than 190 mmHg and DBP higher than 160 mmHg, and captopril treatment attenuated SHR hypertension significantly. Although the IC_{50} value of IQP (5.77 ± 0.09 μmol/L) was more than 260-fold higher than that of captopril (ranging from 0.005 to 0.023 μmol/L), oral administration of IQP resulted in a significant decrease in blood pressures, very close to the antihypertensive levels of captopril. Fujita and Yoshikawa (*28*) reported that, in comparison to captopril, ACE inhibitory peptides derived from food proteins might possess higher *in vivo* activity levels than expected by *in vitro* activities and concluded that this could be explained by their higher affinity to target tissues and their slower elimination. Miguel et al. (*16*) postulated that there might be other mechanisms of bioactivities of these peptides for lowering the arterial blood pressure of SHR, such as interactions with receptors expressed in the gastrointestinal tract, effects on the central nervous system, and/or correlations with the radical scavenging and antioxidant activities (*29*, *30*). In our study on the isolation and purification of IQP from *S. platensis*, we found that enzymatic hydrolysates with high ACE inhibitory activity usually possess high levels of 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity at the same time (data not shown).

Over the past 2 decades, bioactive peptides derived from hydrolysates or enzymatic digests of food proteins have been the focus of hypertension therapeutics by virtue of their improved bioactivities and no side-effect profiles (*11*−*13*). Although many studies have demonstrated their antihypertensive properties for hypertensive animals (*12*, *14*, *15*), little was known about their *in vivo* regulatory effects on individual components of RAS in tissues and the blood cycle. In the current study, the mRNA levels of renin, ACE, and the AT1 receptor in the kidney and the concentrations of Ang, ACE, and Ang II in the sera were significantly suppressed but the mRNA level of the AT2 receptor in the kidney was upregulated in the captopril-administered positive control group. IQP treatment achieved similar effects on the SHRs, except that the mRNA level of the AT2 receptor for the IQP-treated group was upregulated significantly more than that for the captopril-administered positive control group, while the downregulation of other RAS components was lower than that for the positive control group. The AT1 and AT2 receptors were reported to be functionally distinct and to employ different signal transduction pathways (*6*, *31*). Stimulation of the AT2 receptor counterbalanced or possibly opposed the effects of AT1 receptor stimulation, thereby inhibiting proliferation, vasodilation, natriuresis, and stimulation of apoptosis (*32*). Our findings suggest that the antihypertensive effect of IQP accompanies its regulatory function on RAS and that, in comparison to captopril, the effects of IQP may occur more through the regulation of the AT2 receptor. This may explain why the effect of IQP is a little weaker and slower than the effect of captopril (*17*), because the AT2 receptor interacts with Ang II through a low-affinity binding site, whereas the AT1 receptor interacts through a high-affinity binding site (*32*), which might cause a difference in the binding time.

The antihypertensive effects of ACE inhibitors were thought to be correlated with their inhibition of ACE activity and, hence, the obstruction of Ang II formation (*6*, *7*), which was reflected in our study by the decrease in mRNA expression of ACE in the kidney as well as the concentrations of ACE and Ang II in the sera for the positive control and IQP-treated groups. However, treatment with captopril or IQP also significantly affected other RAS components, including renin, Ang, and AT1 and AT2 receptors, as mentioned above. We postulated that obstruction or suppression of the physiological effects of Ang II, such as vasoconstriction, pro-inflammation, and disturbance of extracellular fluid balance (*7*, *33*), may have triggered a "positive feedback" effect on the RAS and, hence, resulted in the regulation of its major components (*34*). Ang II expression was reported to affect the synthesis of Ang and renin (*6*, *34*), suggesting that ACE inhibitors

might also play regulatory roles on the expression of other RAS components, such as AT1 and AT2 receptors. Nonetheless, the detailed mechanism underlying *in vivo* antihypertensive effects of ACE inhibitory peptides derived from natural resources, including IQP, should be further investigated.

ABBREVIATIONS USED

ACE, angiotensin I-converting enzyme; Ang, angiotensinogen; Ang I, angiotensin I; Ang II, angiotensin II; AT1, angiotensin II type 1; AT2, angiotensin II type 2; ANOVA, analysis of variance; CP, crossing point; DBP, diastolic blood pressure; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HR, heart rate; IQP, Ile-Gln-Pro; RAS, renin−angiotensin system; SBP, systolic blood pressure; SHR, spontaneously hypertensive rat.

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Received for review October 23, 2010. Revised manuscript received December 7, 2010. Accepted December 7, 2010. This work was supported in part by grants from the Overseas Study Program ([2007]3021) of the China Scholarship Council, the Fundamental Research Funds for the Central Universities of China (BLYX200935 and TD2010-3), the Scientific Research Foundation for Returned Overseas Chinese Scholars, the State Education Ministry of China, and a Japanese Government (MEXT) Scholarship from the Ministry of Education, Culture, Sports, Science, and Technology.