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Antihypertensive Effect of Angiotensin-Converting Enzyme Inhibitory Peptide RVPSL on Spontaneously Hypertensive Rats by Regulating Gene Expression of the Renin−Angiotensin System

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ABSTRACT: Hen eggs are well-known for their biological functions beyond basic nutrition. In this study, the antihypertensive effect of peptide RVPSL from egg protein has been evaluated by an in vivo model. In addition, the mRNA levels of renin, AT1 receptor, angiotensin-converting enzyme (ACE), and AT2 receptor in the rat kidney were investigated through real-time polymerase chain reaction. The Ang I, Ang II, renin, and aldosterone concentrations of serum were also measured. Our results indicated that the blood pressure of the spontaneously hypertensive rats administered RVPSL for 4 weeks decreased significantly compared to that of the negative group. The mRNA levels of renin, ACE, and AT1 receptor in kidney also decreased significantly. The serum Ang II, renin, and aldosterone concentrations of the treatment group were reduced in comparison to those of the negative group. It is hoped this study will help our understanding and potential use of RVPSL in the treatment or prevention of hypertension.

KEYWORDS: *antihypertensive activity, angiotensin-converting enzyme, blood pressure, peptide, spontaneously hypertensive rats*

INTRODUCTION

Hypertension, which threatens human health worldwide as a major cardiovascular disease (CVD), can lead to stroke, heart failure, and shortened life expectancy.[1] Angiotensin-converting enzyme (ACE), which is an essential part of the renin−angiotensin system (RAS), is a widely accepted enzyme that is considered as the first line of therapy to treat hypertension.[2,3] ACE catalyzes the conversion of angiotensin I (Ang I) to angiotensin II (Ang II), which stimulates aldosterone synthesis and also participates in the regulation of systemic blood pressure.[4] Since the first ACE inhibitory peptide was isolated from snake venom,[5] more researches have focused on the ACE inhibitors.[6] Most antihypertensive drugs were designed on the basis of the snake venom peptide scaffold, such as captopril, which has been used as a potent antihypertensive drug acting essentially as an ACE inhibitor. In addition, the inactivation of ACE also results in an increase of bradykinin, which is a vasodilator. Because of these dual vascular and endothelial protective mechanisms of ACE inhibition, the production of nitric oxide is stimulated, vascular smooth muscle is relaxed, and fibrinolysis is increased.[2]

However, the current antihypertensive drugs bring about undesirable side effects,[7] which has prompted the search for bioactive peptides derived from food proteins due to their beneficial health effects[8−10] and which could be used to prevent and treat hypertension by dietary intervention. For example, some food-derived peptides were reported to possess antihypertensive effects through a classical approach that demonstrated the existence of an in vitro ACE inhibitory activity that inferred the in vivo effects due to this enzyme blockade.[11] However, in some cases, the potency of the ACE blockade does not correlate with the antihypertensive activity in vivo.[12,13]

The purposes of the current work were to evaluate the short- and long-term antihypertensive effect of egg white protein-derived peptide upon spontaneously hypertensive rats by oral administration and to explore the regulation of the expression of major components of the RAS in the kidney of spontaneously hypertensive rats (SHRs), i.e., renin, ACE, and angiotensin type 1 (AT1) receptor and angiotensin type 2 (AT2) receptor by real-time polymerase chain reaction (RT-PCR). In addition, the concentrations of Ang I, Ang II, and ACE in serum were measured with a radioimmune assay (RIA) counter.

MATERIALS AND METHODS

Materials and Reagents. ACE from rabbit lung, hippuryl-L-histidyl-L-leucine (HHL), hippuric acid (HA), HPLC grade acetonitrile, captopril, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich Co. The SV total RNA isolation system was obtained from Promega (Beijing, China), and the Transcriptor First Strand cDNA synthesis kit for RT-PCR was from Roche Diagnostics (Shanghai, China). Premix Ex Taq kits (code DRR390A, Probe qPCR) were purchased from Takara Bio (Dalian, China). The angiotensin II enzyme immunoassay kit and angiotensin I enzyme immunoassay kit were from North Biological Laboratories (Beijing, China). All other reagents were of analytical grade.

Preparation of Peptide from Egg White Protein. The peptide sequence RVPSL,[7] which was isolated and identified from egg white

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Table 1. Primers and Probes Used in Real-Time PCR

gene name		sequence	length (mer)	product length (bp)
renin	-F	5′-GTCCTGTGGGTGTGTATA-3′	18	175
	-R	5′-GAGCAAGATTCGTCCAAA-3′	18	
	-P	5′-(FAM) TCTGGGCTACACGGCTCTCA (eclipse)-3′	20	
Agtr2	-F	5′- CAACGTGTTACTTTGGAA-3′	18	70
	-R	5′-CGGGTAATTCTGTTCTTC-3′	18	
	-P	5′-(FAM) AACATCTGCTGAAGACCAATAGCT (eclipse)-3′	24	
Ace	-F	5′- CCAACGAGTTAGAAGAGTA-3′	19	124
	-R	5′-GCCATTATATTTGTCAGATCA-3′	21	
	-P	5′-(FAM) TCCAGTGACAGACAAGTGCCA (eclipse)-3′	21	
Agtr1a	-F	5′-CAGTCAGAGGTGAATACA-3′	18	132
	-R	5′-AGAGGTAAACATACATTGC-3′	19	
	-P	5′-(FAM) ATTCCATACAGTCTGCCTTGCTCT (eclipse)-3′	24	
β-actin	-F	5′-TATGAGGGTTACGCGCTCCC-3′	20	146
	-R	5′-TCTTTAATGTCACGCACGATTTCC-3′	24	
	-P	5′-(FAM) CCTGCGTCTGGACCTGGCTGGC (eclipse)-3′	22	
GAPDH	-F	5′- TGGTCTACATGTTCCAGTATGACT-3′	24	134
	-R	5′- CCATTTGATGTTAGCGGGATCTC-3′	23	
	-P	5′-(FAM) CCACGGCAAGTTCAACGGCACAGT (eclipse)-3′	24	

protein, was provided by Shanghai Science Peptide Biological Technology Corp. The purity and molecular mass of the synthesized peptide were verified by HPLC and mass spectrometry, respectively. All the products used were dissolved in 0.9% saline to be administered to the rats.

In Vitro ACE Inhibitory Activity Assay. The assay was conducted using a previously reported HPLC method.[7]

Animal Ware. This study was carried out in strict accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Jilin University. All surgery was performed under ether, and all efforts were made to minimize suffering. The protocol conformed to guidelines for the humane care of animals.[14] A total of 30 male SHRs (purchased from Vital River Laboratories), aged 9−10 weeks and weighing 220−270 g, were used in the course of this study. The SHRs were housed in temperature-controlled rooms (22 ± 2 °C) with 12 h light/dark cycles, where spontaneously hypertensive rats could consume tap water and standard diets ad libitum. All SHRs were acclimatized for 3 days with diet before the experiment. The 30 SHRs were assigned to 5 groups: 0.9% saline for the negative group, 10 mg/kg of body mass of captopril for the positive group, and three treatment groups, i.e., 2 mg/kg of body mass of RVPSL for low dose, 10 mg/kg of body mass of RVPSL for medium dose, and 50 mg/kg of body mass of RVPSL for high dose. The rats from the negative group, positive group, and treatment groups were orally administered 1 mL of the corresponding solution from 7:30 a.m. to 8:00 a.m. every day for 4 weeks.

Blood Pressure Measurement. The blood pressure of the SHRs was measured by the tail-cuff method.[15] Before measurement, the rats were kept at 39 °C for 2−3 min to make the pulsation of the tail artery detectable using a blood pressure monitor (model BP-2010A, Softron Co., Tokyo, Japan). The tail cuff was connected to a cylinder of compressed air through an arrangement of inlet and outlet valves that permitted inflation and deflation of the cuff at a constant rate. The systolic blood pressure (SBP) and the diastolic blood pressure (DBP) of the rats were measured by the tail-cuff method before administration and also 5, 10, 15, and 20 h postadministration. Moreover, to guarantee the reliability of the measurements, we established a training period of 4 days before the actual trial time, and during this period the rats were accustomed to the procedure. All measurements were performed by the same person in a quiet environment.

Isolation of RNA from the Rats' Kidneys. Kidney tissues of the spontaneously hypertensive rats that were stored in an ultra-low-temperature freezer at −80 °C (Haier, Beijing, China) were homogenized with a tissue homogenizer under liquid nitrogen conditions. The total RNA of the SHRs' kidneys was isolated using the SV total RNA isolation system Z3105 according to the manufacturer's product instructions (Promega Corp., Beijing, China). The RNA purity and concentration were determined by the A260/A280 ratio (UV2550, Shimadzu, Japan).

Single-Strand cDNA Synthesis. Transcriptor first-strand cDNA was synthesized from 1 μg of RNA using the First Strand cDNA synthesis kit for RT-PCR. A 1 μg portion of total RNA was added to a thin-wall PCR tube on ice, and then 2 μL of 60 μM random hexamer primers was added. RNA-free water was added to a total volume of 13 μL. A 40 μL aliquot of transcriptor reverse transcriptase reaction buffer, 0.5 μL of protector ribonuclease (RNase) inhibitor, 2 μL of deoxynucleotide mix, and 0.5 μL of transcriptor reverse transcriptase were added subsequently to the thin-wall tube, and the contents were incubated at 25 °C for 10 min followed by 55 °C for another 30 min. The reaction was stopped by heating at 85 °C for 5 min. The reaction tube was placed on ice and then stored at −20 °C for an RT-PCR study.

RT-PCR. Real-time PCR was performed for renin, Ang, ACE, AT1 receptor, AT2 receptor, and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the Premix Ex Taq kits on an ABI 7500 real-time PCR system. The primers were ordered from Takara China (Dalian, Liaoning, China). The results are shown in Table 1. PCR reactions were carried out in a 20 μL solution consisting of 2 μL of cDNA template (50 ng), 10 μL of Premix Ex Taq (Probe qPCR), 0.4 μL of forward primer (10 μM), 0.4 μL of reverse primer (10 μM), 0.8 μL of probe solution, 0.4 μL of ROX reference dye II, and 6.0 μL of PCR water. Thermal cycling was initiated with 30 s of denaturation at 95 °C and then by 40 repeats of 5 s at 95 °C and 30 s at 60 °C for melting curve analysis and cooling. The entire process was performed according to the guidelines of the ABI 7500 instrument (Life Technologies Corp., Grand Island, NY). The quantification of a target gene was expressed as the relative expression ratio of the target gene (i.e., ACE, renin, Ang, AT1 receptor, and AT2 receptor) in a sample versus that of a control in contrast to the housekeeping gene GAPDH.

Assay of Serum Ang and Aldosterone Concentrations. The serum Ang I, Ang II, and aldosterone concentrations were determined with the iodine angiotensin I radioimmunoassay kit, iodine angiotensin II radioimmunoassay kit, and iodine aldosterone radioimmunoassay kit, respectively. The above-mentioned samples were measured with an SN-6105 γ RIA counter (Beijing Tayasaf Science & Technology Co., Ltd., Beijing, China) within 20 min after the reaction was stopped. Test samples and standards were measured in duplicate. All of the

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processes were implemented according to the aforementioned guidelines of the manufacturers.

Statistical Analysis. Values are expressed as the means ± SD. Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Student's *t* test. *p* values of <0.05 were considered significant.

RESULTS AND DISCUSSION

Effects of RVPSL on the SBP and DBP of the SHRs in 4 Weeks. There were no mortalities and no significant differences in the final body mass among the experimental SHRs during the 4 weeks. The results demonstrated that the oral administration of saline to SHRs did not lower the values of the SBP and DBP (Figure 1). On the contrary, the SBPs of

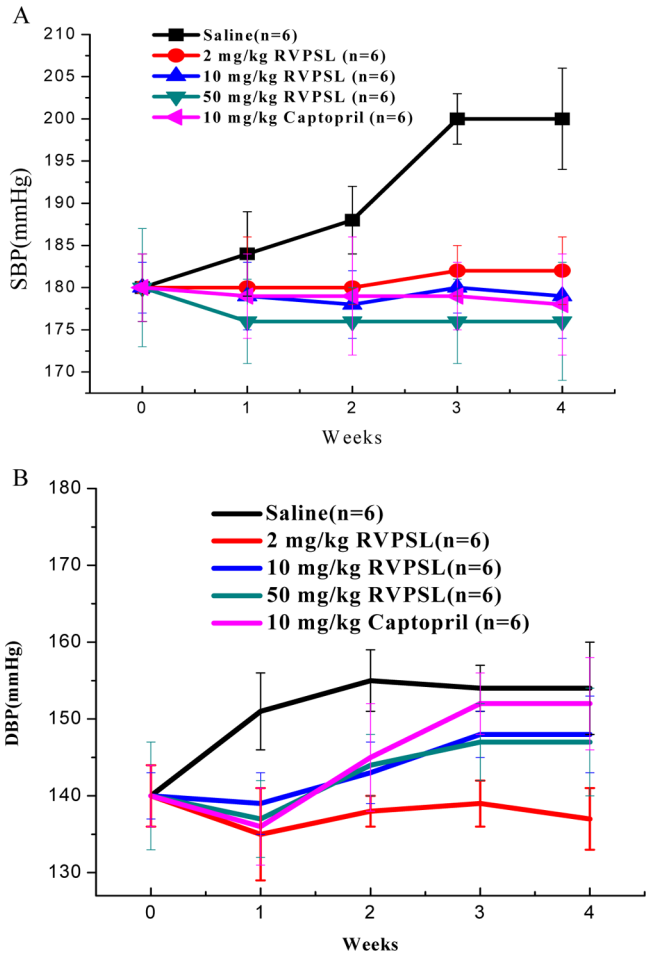

Figure 1. Antihypertensive effects of RVPSL in SHRs: (A) change in the SBP after administration for 4 weeks and (B) change in the DBP after administration for 4 weeks. Each value is expressed as the mean ± SD. A *p* value of <0.05 (*) is considered statistically significant by one-way ANOVA.

SHRs administered saline as the negative group increased from 180 ± 7 to 200 ± 9 mmHg. However, the SBPs of SHRs administered different concentrations of RVPSL as the treatment groups decreased. RVPSL caused a significant dose-dependent decrease in SBP in these animals from the first week until the fourth week. Moreover, the greatest decreases of SBP were obtained when the SHRs were administered 50 mg/kg RVPSL. From the third week after the oral administration with peptide RVPSL, the SBP of the positive control group and that of the treatment groups were significantly lower than that of the negative group.

However, the reduction of DBP in the treatment groups was not significantly higher than that of the negative group from the first week to the fourth week. Some researchers suggested that orally administered bioactive peptide decreased the blood pressure of the SHRs[16] and also demonstrated that the antihypertensive activity of bioactive peptide was blocked by a CCK1 antagonist. In this context, we performed further studies to evaluate the mechanism of antihypertensive peptide derived from egg protein.

Effects of RVPSL on Renin mRNA Expression of the Kidney. Relative renin mRNA levels of the kidney were detected by real-time PCR and quantified as shown in Figure 2A in contrast to GAPDH, which remained stable in comparison to *β*-actin in our study. The expression was analyzed on the fourth week, when a decrease in the treatment group with oral administration of RVPSL (50 mg/kg) and in the positive control group (captopril) was found in contrast to the negative control group (saline). Renin can cleave the substrate angiotensinogen to form Ang I, which is later converted to Ang II. Ang II raises blood pressure by a number of actions: the most important ones being vasoconstriction, sympathetic nervous stimulation, increased aldosterone biosynthesis, and renal actions. Angiotensin II-converting enzyme inhibitors presumably stimulate renin secretion by interrupting angiotensin II feedback inhibition. To some extent, the decrease of renin may contribute to the low production of Ang I and Ang II.

Effects of RVPSL on ACE mRNA Expression of the Kidney. Relative ACE mRNA levels of the kidney were detected by real-time PCR and quantified as shown in Figure 2B in comparison to GAPDH. The expression was also analyzed on the fourth week. A significant decrease in the treatment group with oral administration of RVPSL (50 mg/kg) and in the positive group was found compared to the negative group, but there was no significant difference between the treatment group and the positive group. The results demonstrated that captopril and egg white peptide RVPSL exhibited high activity against ACE in vivo. The antihypertensive effects of captopril and RVPSL were thought to be correlated with their inhibition of ACE activity, which was reflected in our study by the decrease in the mRNA expression of ACE in the kidney for the positive group and RVPSL-treated groups. Consequently, the production of Ang II converted by ACE from Ang I was decreased by the effects of the captopril and/or RVPSL administration. The captopril and the peptide RVPSL are potent inhibitors of angiotensin I-converting enzyme, which is responsible for the conversion of angiotensin I to angiotensin II.

Effects of RVPSL on AT1 and AT2 Receptor mRNA Expression of the Kidney. Relative AT1 receptor and AT2 receptor mRNA levels of the kidney were measured by real-time PCR as shown in parts C and D, respectively, of Figure 2 and compared to the housekeeping gene GAPDH. On the basis of the expression analysis after oral administration for 4 weeks, the results revealed that a significant decrease of AT1 in the treatment group and the positive control group occurred in comparison with the negative control group. However, the AT2 receptor was highly expressed in the treatment group and positive group in contrast to the negative control group. The AT1 receptor seemed to have functions opposite those of the AT2 receptor. Some researchers also demonstrated that the

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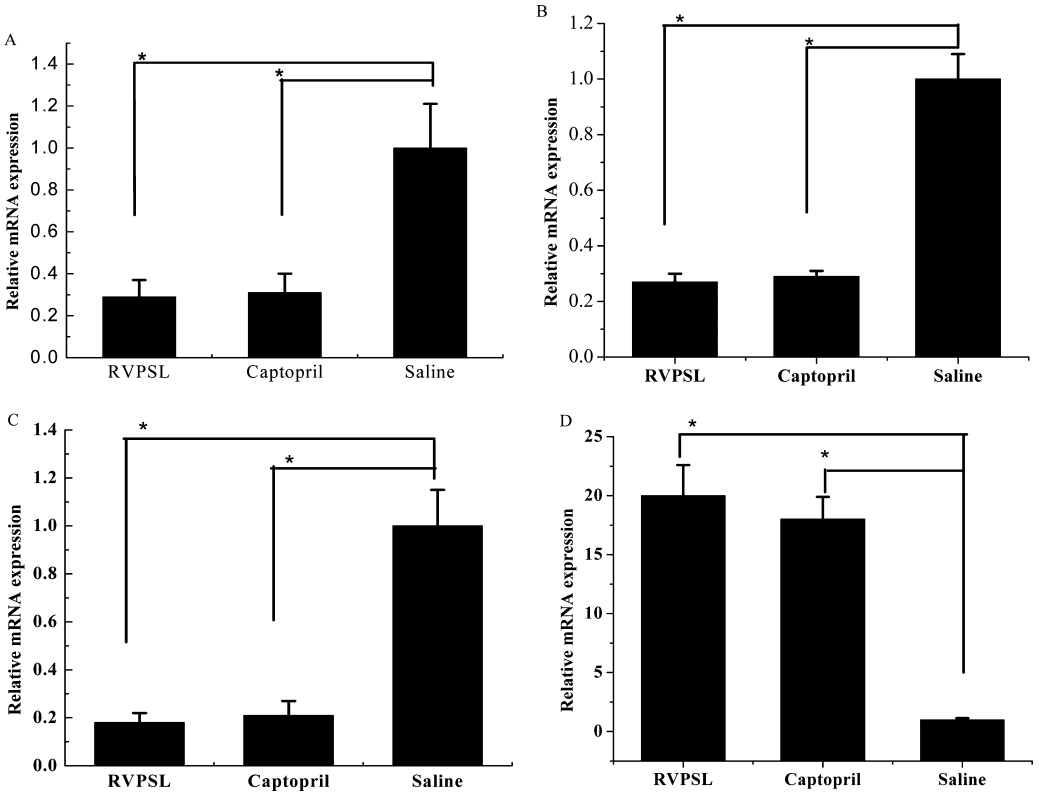

Figure 2. Effect of RVPSL on major RAS components in the SHR kidney: (A) change in relative renin mRNA levels, (B) change in relative ACE mRNA levels, (C) change in relative mRNA levels of the AT1 receptor, and (D) change in the relative mRNA levels of the AT2 receptor . Each value is expressed as the mean ± SD. A p value of <0.05 (*) is considered statistically significant by one-way ANOVA.

AT2 receptors appeared to antagonize the actions of the AT1 receptor.[17]

Effects of RVPSL on the Concentrations of RAS Components of the Serum. The concentrations of serum Ang I, renin, Ang II, and aldosterone were investigated and quantified with an RIA counter and are shown in Figure 3. Compared to the negative group, the serum renin levels of the positive group and high-dose treatment group (50 mg/kg) were significantly lower by 37% and 44% (Figure 3A), respectively. Ang II levels and aldosterone levels of the positive group and high-dose treatment group also declined significantly (Figure 3B,C) in comparison with the negative group. Thus, the peptide RVPSL reduced the blood pressure in combination with the ACE inhibitor or/and renin inhibitor. Although the expression levels of the serum renin of the positive and high-dose treatment groups decreased, the Ang I levels of the positive group and treatment group were not reduced significantly (Figure 3D). Our study on the SHRs gave results similar to those described in another report.[1] In addition, there was no significant difference between the positive group and the high-dose treatment group in the serum Ang I, renin, aldosterone, and Ang II concentrations. In our earlier work, the peptide from egg white protein was shown to have an in vitro ACE inhibition activity with an IC_{50} value of 20 μM,[7] which prompted us to further investigate the in vivo antihypertensive effect of the peptide and explore the corresponding expression of major RAS components in the SHRs. We found that the peptide RVPSL (50 mg/kg) from egg white protein significantly decreased the SBPs of SHRs by 24 mmHg in comparison to the negative group. Miguel[15,18] also evaluated the effects of the hydrolysate of egg white on the short- and long-term arterial blood pressure and suggested that the hydrolysis of egg white with pepsin may be a practical procedure to produce functional bioactive peptides with antihypertensive activity. Ovokinin, a peptide that was hydrolyzed by chymotrypsin from OA 359-364, was found to be able to significantly lower the systolic blood pressure in the SHRs in a dose-dependent manner, when administered orally.[11] Besides, three novel peptides, IQW, IRW, and LKP purified from the ovotransferrin, also exhibited potent activity against ACE based on the quantitative structure−activity relationship of tri- and dipeptides with angiotensin-converting enzyme inhibitory activity.[19] Moreover, IQW was stable against the digestive enzymes.[20] Since the process and pathological characteristics of the SHRs' hypertension are very similar to those of human hypertension,[1] SHRs have generally been used to conduct initial studies on in vivo antihypertensive effects of bioactive peptides. While much is known about diet and physical activity linked to high blood pressure, less is known about the effects of the gene expression level on individual components of RAS in tissues that are responsible for predisposition to cardiovascular disease.[21−24] In this context, we evaluated the mRNA levels of renin, ACE, and AT1 receptor in the rat kidney and found they were significantly reduced. On the contrary, the mRNA level of AT2 receptor in the kidney was upregulated in the treatment group (50 mg/kg) and positive control group compared to the negative group. For the AT1 receptor, the mRNA levels of the positive group and high-dose treatment group (50 mg/kg RVPSL) were decreased significantly. In comparison to that of the negative group, mRNA levels of the AT2 receptor were significantly increased by 20- and 16-fold for the positive group and treatment group (50 mg/kg), respectively. In addition, the

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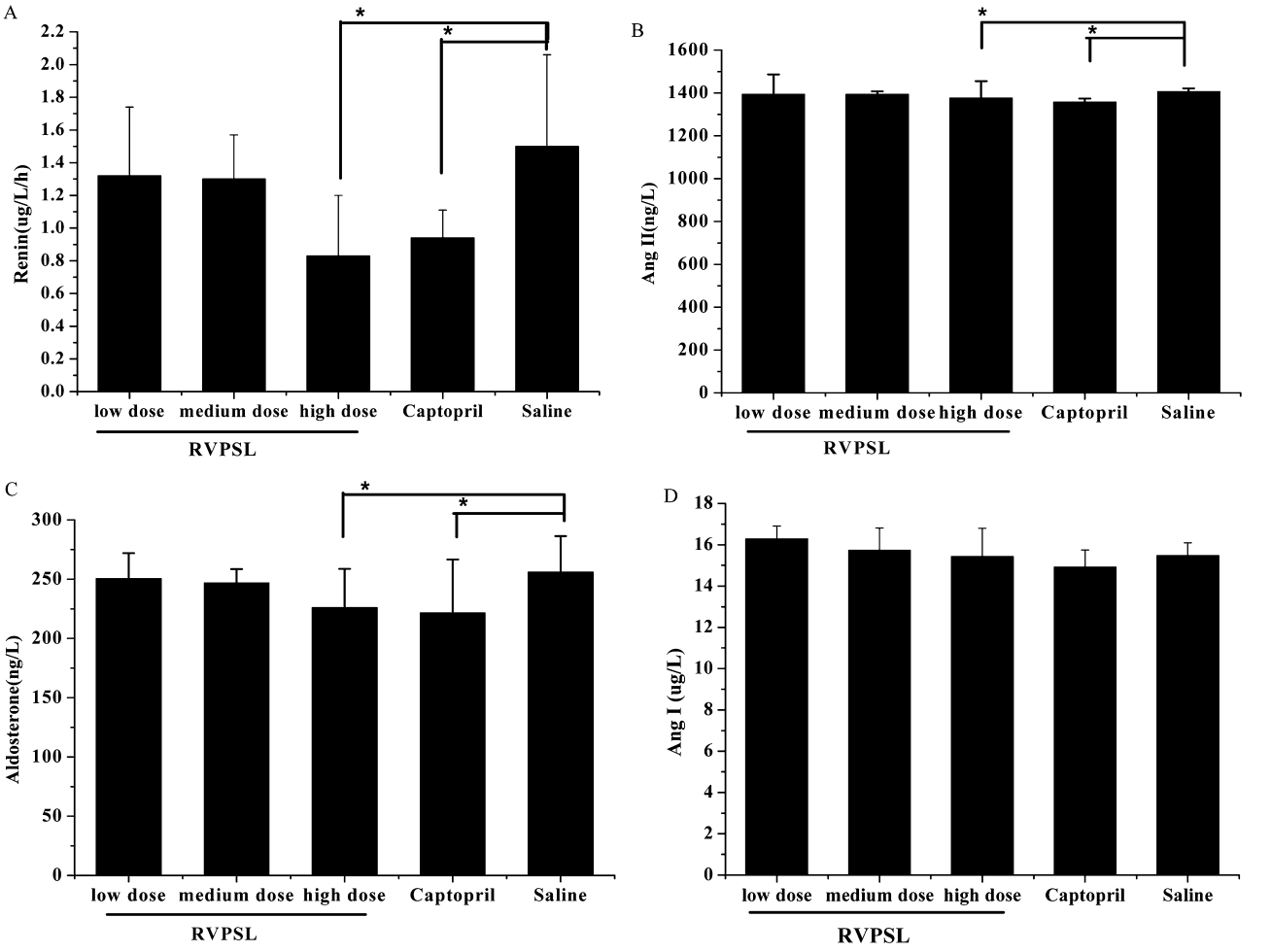

Figure 3. Effects of RVPSL on major RAS components in the serum of SHRs: (A) change in serum renin concentrations, (B) change in serum Ang I concentrations, (C) change in serum Ang II concentrations, and (D) change in serum aldosterone concentrations. Each value is expressed as the mean ± SD. A p value of <0.05 (*) is considered statistically significant by one-way ANOVA.

concentrations of renin, Ang II, and aldosterone in the serum increased significantly in comparison to those of the negative group.

In summary, the current work demonstrated clearly, for the first time, that the peptide RVPSL derived from the egg white significantly decreased the SBP of SHRs in 4 weeks. Also, our results indicated that the peptide RVPSL affected the expression of major RAS components by downregulating the renin, ACE, Ang II, and AT1 receptor while upregulating the AT2 receptor in the kidney or serum of SHRs.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ACE, angiotensin-converting enzyme; Ang I, angiotensin I; Ang II, angiotensin II; AT1, angiotensin type 1; AT2, angiotensin type 2; CCK, cholecystokinin; CVD, cardiovascular disease; DBP, diastolic blood pressure; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HHL, hippuryl-L-histidyl-L-leucine; HA, hippuric acid; TFA, trifluoroacetic acid; RNase, ribonuclease; RIA, radioimmune assay; RAS, renin−angiotensin system; SHRs, spontaneously hypertensive rats; SBP, systolic blood pressure

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