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Original Article

Effects of the combination of Herba Epimedii and Semen Plentaginis on the aortic ACE2/Angiotensin-(1-7)/Mas receptor axis and blood pressure in spontaneously hypertensive rats

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Abstract: This study evaluated the effects of Herba Epimedii-Semen Plentaginis (HESP) on blood pressure and aortic intima media thickness (IMT) in spontaneously hypertensive rats (SHR) and explored if these effects correlated with the aortic angiotensin-converting enzyme 2-angiotensin-(1-7)-Mas receptor (ACE2/Ang-(1-7)/Mas receptor) axis. Twenty-four SHR were randomly assigned to the following three groups: SHR-control, low-dose HESP (12.5 g/kg), and high-dose HESP (25 g/kg). Systolic blood pressure and diastolic blood pressure (SBP/DBP) were measured once per week in conscious rats using a tail-cuff plethysmograph. After 4 weeks of treatment, thoracic aortas and plasma were harvested to measure IMT, nitric oxide (NO), endothelial nitric oxide synthase (eNOS), and Ang-(1-7) levels, as well as angiotensin-converting enzyme 2 (ACE2), Mas receptor, and neuronal nitric oxide synthase (nNOS) protein expression. Four weeks of HESP treatment significantly decreased SBP, DBP, and aortic IMT compared to the SHR-control group ($P < 0.05$ or $P < 0.01$). Moreover, HESP significantly increased plasma Ang-(1-7) levels, aortic Ang-(1-7), NO, and eNOS levels. Moreover, HESP treatment significantly increased ACE2, Mas receptor, and nNOS protein expression. These beneficial effects were particularly pronounced in the high-dose HESP group. In conclusion, HESP attenuates SBP, DBP, and aortic IMT by upregulating the ACE2/Ang-(1-7)/Mas receptor axis and nNOS/eNOS signaling pathways in SHR.

Keywords: Compatibility of Herba Epimedii and Semen Plentaginis, ACE2/Ang-(1-7)/Mas receptor axis, neuronal nitric oxide synthase, vascular remodeling, spontaneously hypertensive rats

Introduction

Growing evidence suggests that the angiotensin-converting enzyme 2/angiotensin-(1-7)/Mas receptor axis (ACE2/Ang-(1-7)/Mas receptor axis) is involved in the pathogenesis of essential hypertension [1-3]. ACE2 can catalyze angiotensin II (Ang II) into Ang-(1-7), which has vasodilatory, anti-proliferative, and anti-oxidative effects mediated via Mas receptor activation [4]. Thus, the ACE2/Ang-(1-7)/Mas axis represents an endogenous counter regulatory pathway within the renin angiotensin system (RAS) that can antagonize the deleterious effects of Ras activation [5].

Damage to the ACE2/Ang-(1-7)/Mas axis can result in excessive vasoconstriction, endothelial dysfunction, and vascular smooth muscle cell proliferation and migration [6, 7], which can all subsequently contribute to development of hypertension. Therefore, pharmacological agents that activate the ACE2/Ang-(1-7)/Mas receptor axis may represent a promising therapeutic target to treat hypertension [8, 9].

Nitric oxide (NO) plays an essential role in various physiological processes. Ang-(1-7) produces its effects through a NO-dependent mechanism [10]. As a compensatory mechanism to attenuate hypertension, Ang-(1-7) upregulates neuronal nitric oxide synthase (nNOS) expression in response to hypertensive conditions [11]. Apart from the nervous system, nNOS is also expressed in human blood vessels [12]. NO produced by activated nNOS plays a key role in relaxation of peripheral blood vessels [13, 14]. In addition, Ang-(1-7) mediates endothelial nitric oxide synthase (eNOS) activation through the Mas receptor [5].

Several traditional Chinese medicinal patent agents have been used to effectively manage essential hypertension [15]. HESP is composed of Herba Epimedii and Semen Plantaginis in a 1:1 ratio. Herba Epimedii, known as Yinyanghuo in Chinese, is traditionally used to treat skeletal diseases [16] and impotence [17] in China. Herba Epimedii can tonify kidney Yang and dispel wind-damp and is traditionally used to relieve stress, fatigue, and dizziness [18]. Clinical research has shown that Herba Epimedii significantly improves hypertension associated with fatigue, dizziness, and other symptoms [19, 20] and reduces blood pressure in hypertensive patients and animals [21]. When Herba Epimedii extract tablets were used to treat 115 cases of hypertension, blood pressure was decreased by 10.7 kPa/5.33 kPa (80/40 mmHg) and the overall response rate was 78% [22]. Semen Plentaginis has dump cleaning and diuresis promoting effects in traditional Chinese medicine. Modern pharmacology has demonstrated that the ingredients of HESP possess antihypertensive properties [23-25]. The antihypertensive effect of Semen Plentaginis is mainly mediated through diuresis and sodium and potassium excretion. Its effective component can excite parasympathetic ner ves and inhibit sympathetic nerves, resulting in dilation of peripheral blood vessels and decreased blood pressure [26, 27].

Our previous study showed that the combination of Herba Epimedii and Semen Plentaginis (HESP) decreased the plasma level of Ang-(1-7) in spontaneously hypertensive rats (SHR) [28] and produced a significant antihypertensive effect in patients with mild to moderate hypertension [29]. Furthermore, HESP can prevent target organ damage [30], regulate plasma lipids [30], and improve insulin resistance [31]. However, the underlying mechanisms of HESP's protection are not fully characterized. Therefore, we conducted this study to investigate the effect of HESP on systolic blood pressures (SBP) and diastolic blood pressures (DBP), as well as on aortic intima media thickening (IMT) in SHR, focusing on the role of the aortic ACE2/Ang-(1-7)/Mas receptor axis.

Materials and methods

Drugs and reagents

HESP tablets were provided by the First Affiliated Hospital of Guangzhou University of Chinese Medicine. The tablets were composed of Herba Epimedii and Semen Plantaginis (Table S1) in 1:1 ratio (each tablet was equivalent to 5 g of crude drug and the dosage for an adult was 75 g per day). Anti-ACE2 and anti-nNOS were purchased from Abcam, anti-Mas receptor was from Bioss, and anti-GAPDH was from Shanghai KangChen Bio-tech Inc. Horseradish peroxidase (HRP)-labeled secondary antibody was purchased from Southern Biotech Co. The Immunoprecipitation Assay (RIPA) Lysis kit was purchased from Beyotime Biotechnology and the Bicinchoninic acid protein assay kit was purchased from Nanjing KeyGen Biotech. Co. Ltd. The Ang-(1-7) enzyme-linked immunosorbent assay (ELISA) kit was bought from Kamiya Biomedical (Seattle, WA, USA). The NO reagent kit was from Nanjing Jiancheng Bioengineering Institute. The eNOS ELISA kit was bought from Shanghai Yuanmu Bio Technology Co., Ltd.

Animals and experimental protocol

Twenty-four male SHR (10-11 weeks old, 160-180 g body weight) and eight age-matched Wistar-Kyoto (WKY) rats were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All rats were housed in a temperature (20-22°C) and humidity (50-60%) controlled room with a 12-h dark-light cycle. Animals were fed a standard rodent diet and had free access to tap water. After 10 days of adaptation, SHR were randomly assigned to the following three groups: SHR-control, SHR + low-dose HESP (12.5 g/kg), and SHR + high-dose HESP (25 g/kg) (n = 8 per group). Another eight WKY rats were selected as normal controls. SHR-control and WKY rats received normal saline. The rats were intragastrically administered saline and HESP treatments for 4 successive weeks. The current study was approved by the Ethics Committee of Animal Research at Guangzhou University of Chinese Medicine and all experimental procedures followed the international guidelines for the use and care of experimental animals.

Arterial blood pressure measurements

SBP/DBP were determined by indirect tail-cuff plethysmography (ALC-NIBP Noninvasive blood pressure measurement system) in conscious rats. Rats were warmed to a temperature of 32°C for 10 min before blood pressure measurements were recorded. At least 3 measurements (less than 5 mmHg difference in value)

were performed on each rat and we obtained the average of the recorded measurements as the final blood pressure. SBP/DBP were determined once a week at the same time each week.

Sample collection and preparation

Following the last blood pressure measurement, rats were anesthetized with 10 mL/kg chloral hydrate through intraperitoneal injection. Arterial blood (2 mL) was taken from the thoracic aorta. Uncoagulated blood was centrifuged at 3,000 r/min for 10 min to collect the plasma. Thoracic aortas, 4-5 mm in length, were harvested under sterile conditions and then frozen in liquid nitrogen for further analysis.

Determination of Ang-(1-7), NO, and eNOS concentrations

Ang-(1-7) levels in the thoracic aorta and plasma were determined by ELISA according to the manufacturer's instructions. The value of each specimen was determined by an enzyme-linked immunosorbent analyzer. Aortic and plasma Ang-(1-7) levels were calculated using a standard curve. Aortic NO levels were detected by measuring nitrite (NO_2) according to the manufacturer's instructions. Aortic eNOS levels were determined by ELISA according to the manufacturer's protocol. Aortic levels of eNOS were obtained from a standard curve.

Determination of Mas receptor and ACE2 expression

Mas receptor and ACE2 expression were detected by immunohistochemistry according to the manufacturer's instructions. Briefly, 2-μm thick aortic sections were deparaffined with xylene, then rehydrated in a descending series of alcohol concentrations. The sections were treated with boiling citrate buffer in a microwave oven for antigen retrieval. Endogenous peroxides were inactivated by incubation with 3% hydrogen peroxide for 10 min. The sections were incubated with an anti-ACE2 antibody (1:5,000 dilution) or an anti-Mas receptor antibody (1:1,000 dilution) overnight at 4°C. After washing with phosphate buffered saline (PBS) three times, the sections were incubated with the biotin-conjugated secondary antibody (1:2,000 dilution) for 30 min at room temperature. The sections were incubated with 0.01% diaminobenzidine and counterstained with hematoxylin, then photographed with a light microscope at 400× magnification. As a negative control, the primary antibody was removed and the sections were incubated with normal serum.

Morphological analysis of thoracic aortas

Morphometric analysis was made in transversal tissue sections under a light microscope (Olympus CX41, Japan). Aortic IMT was quantified using ImagePro plus 5.0 software (Media Cybernetics, Rockville, MD, USA). The average of three measurements was recorded for each animal.

Determination of Mas receptor, ACE2, and nNOS protein levels

Western blot analysis was used to analyze aortic Mas receptor, ACE2, and nNOS protein expression. In brief, total protein was extracted by homogenizing thoracic aortas in ice-cold RIPA lysis buffer. Protein concentrations were measured using the Bicinchoninic acid protein assay kit according to the manufacturer's instructions. Protein samples (30 μg) were loaded on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred electrophoretically onto polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies: anti-ACE2 (1:5,000 dilution), anti-Mas receptor (1:1,000 dilution), anti-nNOS (1:1,000 dilution), or anti-GAPDH (1:10,000 dilution) overnight at 4°C after blocking with 5% skim milk solution. GAPDH was selected as an internal control. Subsequently, the membranes were incubated with the secondary antibody (1:1,000 dilution) at 37°C for 60 min and then exposed to a fluorescent luminescent substrate. Protein band intensity was determined using ImagePro plus 5.0 software (Media Cybernetics, Rockville, MD, USA). Relative protein levels of Mas receptor, ACE2, and nNOS were calculated relative to GAPDH levels.

Data analysis

All values were expressed as mean ± standard deviation (SD). All experimental data were summarized from at least three independent experiments. Differences in mean values of various groups were analyzed by ANOVA. Differences

Table 1. Systolic blood pressures changes (mmHg)

Group	n	Week 1	Week 2	Week 3	Week 4
WKY	8	134 ± 10	138 ± 9	134 ± 3	132 ± 6
SHR-control	8	222 ± 17	215 ± 4	210 ± 7	213 ± 16$^{\#}$
Low-dose HESP	8	216 ± 18	201 ± 2	189 ± 6	179 ± 3$^{*,\triangle}$
High dose HESP	8	217 ± 16	193 ± 3	175 ± 7	158 ± 5$^{*,\triangle,\blacktriangle}$

Values are mean ± SD. *P < 0.05, versus SHR-control group; $^{\#}$P < 0.05, versus WKY group; $^{\triangle}$P < 0.05, versus low-dose HESP group, $^{\blacktriangle}$P < 0.01, versus week 1. HESP: Combination of Herba Epimedii and Semen Plentaginis.

Table 2. Diastolic blood pressures changes (mmHg)

Group	n	Week 1	Week 2	Week 3	Week 4
WKY	8	101 ± 10	106 ± 9	100 ± 6	99 ± 8
SHR-control	8	167 ± 18	161 ± 7	160 ± 6	157 ± 16$^{\#}$
Low-dose HESP	8	169 ± 16	150 ± 9	139 ± 6	137 ± 6*
High dose HESP	8	164 ± 7	143 ± 8	132 ± 9	122 ± 8$^{*,\#,\triangle}$

Values are mean ± SD. *P < 0.05, versus SHR-control group; $^{\#}$P < 0.05, versus WKY group; $^{\triangle}$P < 0.05, versus low-dose HESP group. HESP: Combination of Herba Epimedii and Semen Plentaginis.

before and after medication in the same group were compared with the paired t-test. Statistical analyses were conducted using the Prism software package (GraphPad v5, San Diego, CA, USA). A *p*-value < 0.05 was considered statistically significant.

Results

Effects of HESP on arterial blood pressure

Tables 1 and **2** show SBP and DBP changes, respectively, throughout the 4-week HESP treatment period. At the end of the experiment, SBP and DBP were markedly increased in the SHR-control group compared to the normal WKY group (all P < 0.05). SBP and DBP were markedly decreased in the HESP group compared to the control group after 4 weeks of treatment (all P < 0.05). High-dose HESP treatment had a more pronounced antihypertensive effect compared to low-dose HESP (P < 0.05).

Effect of HESP on aortic IMT

Figure 1A shows representative immunohistochemical staining images of aortic IMT. Aortic IMT was thicker in the SHR-control group compared to the normal WKY group. Treatment with HESP decreased aortic IMT relative to the SHR-control group. As shown in **Figure 1B**, aortic IMT in the SHR-control group was markedly increased compared to the normal WKY group (175.9 ± 29.5 μm *vs.* 120.7 ± 13.8 μm; P < 0.05). Treatment with HESP significantly decreased aortic IMT to 149.5 ± 25.1 μm at the low-dose and to 134.9 ± 14.1 μm at the high-dose compared to the SHR-control group (all P < 0.05). The high-dose HESP treatment attenuated aortic IMT to a greater extent compared to the low-dose HESP (P < 0.05).

Effects of HESP on aortic and plasma Ang-(1-7) concentrations

Figure 2 shows aortic (A) and plasma (B) Ang-(1-7) concentrations. After 4 weeks of HESP treatment, the aortic Ang-(1-7) concentration in the SHR-control group was significantly reduced compared to the normal WKY group (0.11 ± 0.01 ng/mg *vs.* 0.79 ± 0.04 ng/mg; P < 0.01). Treatment with HESP significantly increased the aortic Ang-(1-7) concentration to 0.23 ± 0.06 ng/mg at the low-dose and to 0.50 ± 0.04 ng/mg at the high-dose compared to the SHR-control group (all P < 0.01). Similarly, the plasma Ang-(1-7) concentration in the SHR-control group was markedly decreased compared to the normal WKY group (0.68 ± 0.08 ng/mg *vs.* 1.54 ± 0.03 ng/mg; P < 0.01). HESP treatment significantly increased the plasma Ang-(1-7) concentration to 1.13 ± 0.02 ng/mg at the low-dose and to 1.23 ± 0.01 ng/mg at the high-dose compared to the SHR-control group (all P < 0.01). High-dose HESP treatment was associated with higher aortic and plasma Ang-(1-7) concentrations compared to the low-dose (all P < 0.01). Moreover, linear correlation analysis (**Figure 3**) suggests that there is an inverse relationship between aortic Ang-(1-7) concentration and SBP (y = -109.37x + 214.89; R^2 = 0.8273) or DBP (y = -77.149x + 161.44; R^2 = 0.7816).

Effects of HESP on aortic NO and eNOS concentrations

As shown in **Figure 4A**, the aortic NO concentration was significantly reduced in the SHR-control group compared to the normal WKY group (68.0 ± 0.6 μmol/ml *vs.* 127.76 ± 14.21 μmol/ml; P < 0.01). HESP markedly increased the aortic NO concentration to 81.124 ± 3.93

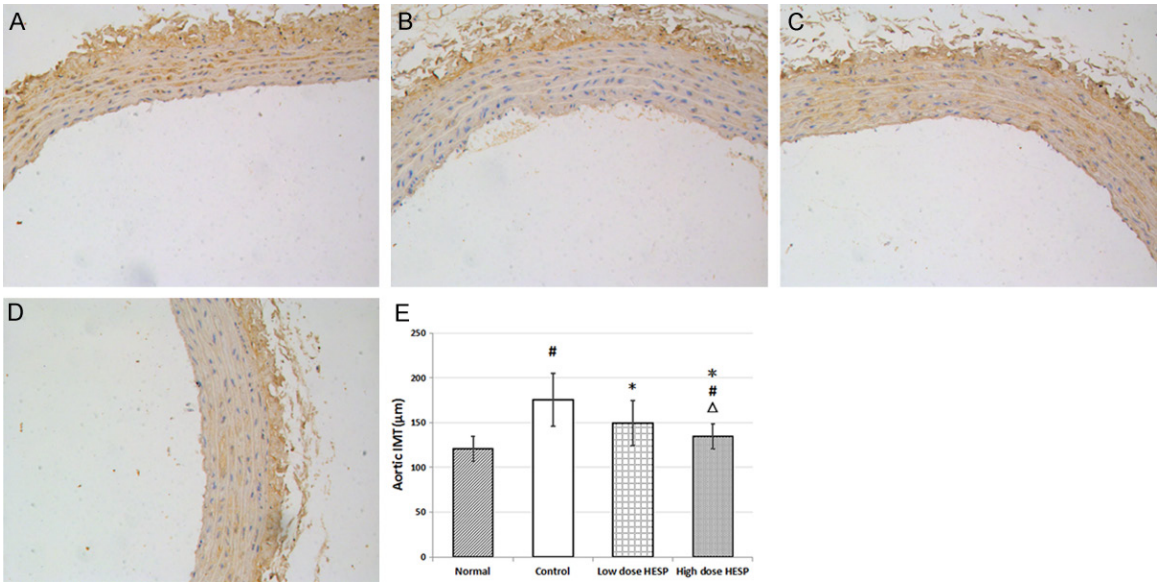

Figure 1. Effect of HESP on aortic intima media thickness (IMT). Normal WKY group (A), SHR-control group (B), Low dose HESP group (C), High dose HESP group (D). Morphometric analysis (E) showed that HESP treatment significantly decreased aortic IMT at 4 weeks compared to the SHR-control group. Values are mean ± SD (n = 5). $^{*}P < 0.05$, versus SHR-control group; $^{\#}P < 0.05$, versus WKY group; $^{\Delta}P < 0.05$, versus low-dose HESP group.

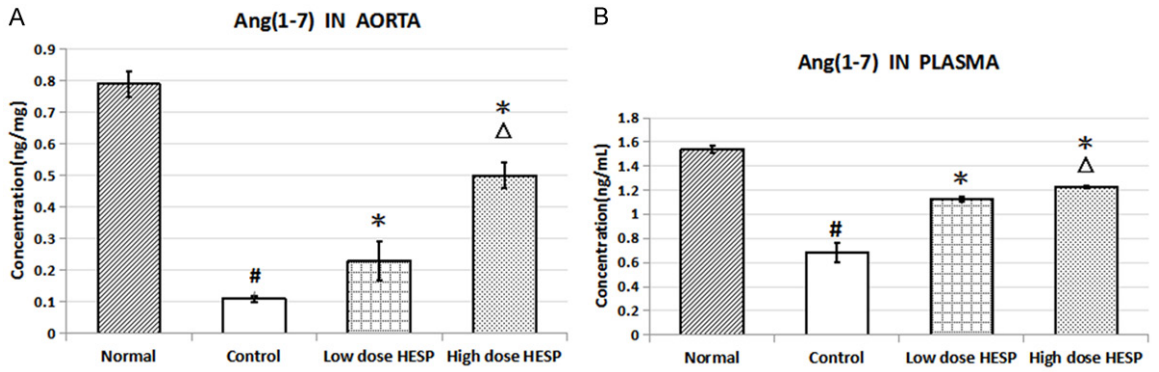

Figure 2. Effects of HESP on aortic (A) and plasma (B) Ang-(1-7) concentrations. Ang-(1-7) concentration was measured by ELISA. HESP treatment increased aortic and plasma Ang-(1-7) concentrations compared to the SHR-control group. Values are mean ± SD (n = 7). $^{*}P < 0.01$, versus SHR-control group; $^{\#}P < 0.01$, versus WKY group; $^{\Delta}P < 0.01$, versus low-dose HESP group.

μmol/ml at the low-dose and to 103.84 ± 7.01 μmol/ml at the high-dose compared to the SHR-control group (all P < 0.01). High-dose HESP treatment significantly increased the aortic NO concentration compared to low-dose (P < 0.01). As shown in **Figure 4B**, the aortic eNOS concentration was reduced in the SHR-control group compared to the normal WKY group (0.82 ± 0.13 μM/g *vs.* 8.84 ± 3.55 μM/g; P < 0.01). HESP treatment increased aortic eNOS concentration to 2.57 ± 0.98 μM/g at the low-dose and to 4.63 ± 1.05 μM/g at the high-dose compared to the SHR-control group (all P < 0.01). The aortic eNOS concentration was significantly higher in the high-dose HESP group compared to the low-dose HESP group (P < 0.01).

Effects of HESP on Mas receptor and ACE2 expression

Figure 5 shows representative images of Mas receptor and ACE2 expression as detected by immunohistochemical staiing. Brown staining represents positive immunostaining of the Mas receptor and ACE2. Blue staining indicates cell

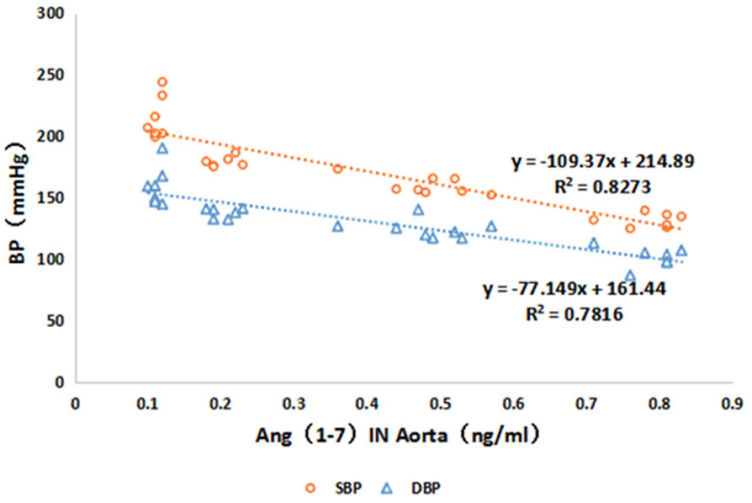

Figure 3. Effects of HESP on aortic NO (A) and eNOS (B) concentrations. NO and eNOS were measured using the nitric reductase method. HESP treatment increased aortic NO and eNOS concentrations compared to the SHR-control group. Values are mean ± SD (n = 7). *P < 0.01, versus SHR-control group; #P < 0.01, versus WKY group; ΔP < 0.01, versus low-dose HESP group.

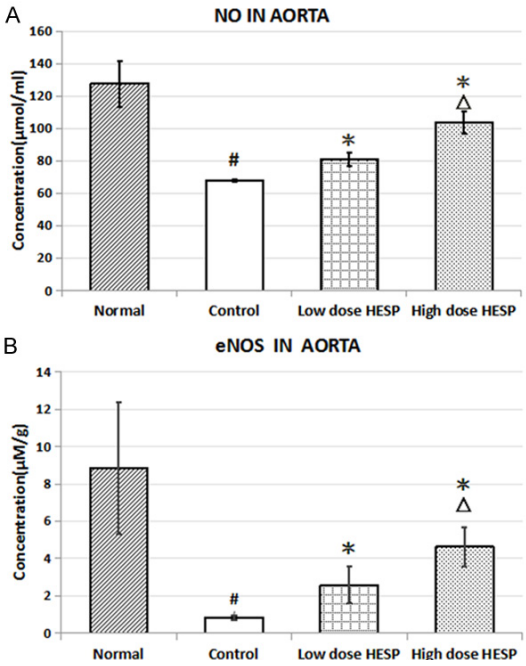

Figure 4. Correlation analysis between aortic Ang-(1-7) concentration and SBP/DBP. There was an inverse relationship between aortic Ang-(1-7) and both SBP or DBP, respectively.

nuclei. Positive Mas receptor and ACE2 expression was mainly located in the vascular smooth muscle cells in the media and endothelial cells in the intima. Mas receptor and ACE2 expression in the SHR-control group were decreased compared to the normal WKY group. By contrast, HESP treatment significantly increased Mas receptor and ACE2 expression.

Effects of HESP on Mas receptor, ACE2, and nNOS protein expression.

Figure 6A shows representative images of aortic Mas receptor, ACE2, and nNOS protein expression. Protein bands corresponding to Mas receptor, ACE2, and nNOS expression in the SHR-control group were weaker in intensity compared to the normal WKY group and the HESP-treated group. Mas receptor, ACE2, and nNOS expression were markedly downregulated in the SHR-control group compared to the normal WKY group (P < 0.05). HESP treatment upregulated Mas receptor, ACE2, and nNOS expression relative to the SHR-control group (all P < 0.01). High-dose HESP treatment increased aortic Mas receptor, ACE2, and nNOS protein expression compared to the low-dose (all P < 0.05).

Discussion

The principal findings of this study include: 1) HESP treatment for 4 weeks decreased SBP and DBP and reduced aortic IMT; 2) HESP increased aortic and plasma Ang-(1-7) concentrations; and 3) HESP increased Mas receptor and ACE2 protein expression in the thoracic aorta. Furthermore, HESP increased aortic NO and eNOS concentrations, as well as upregulated nNOS protein expression in the thoracic aorta. Collectively, these results indicate that HESP is an antihypertensive agent that inhibits vascular remodeling. Furthermore, our data suggested that these effects were mediated by upregulating the Mas receptor, ACE2, and nNOS protein expression and increasing eNOS concentration.

Chronic hypoxia can activate ACE2, enrich Ang-(1-7), and increase expression of the Mas receptor in hypertensive mice [32]. At the same

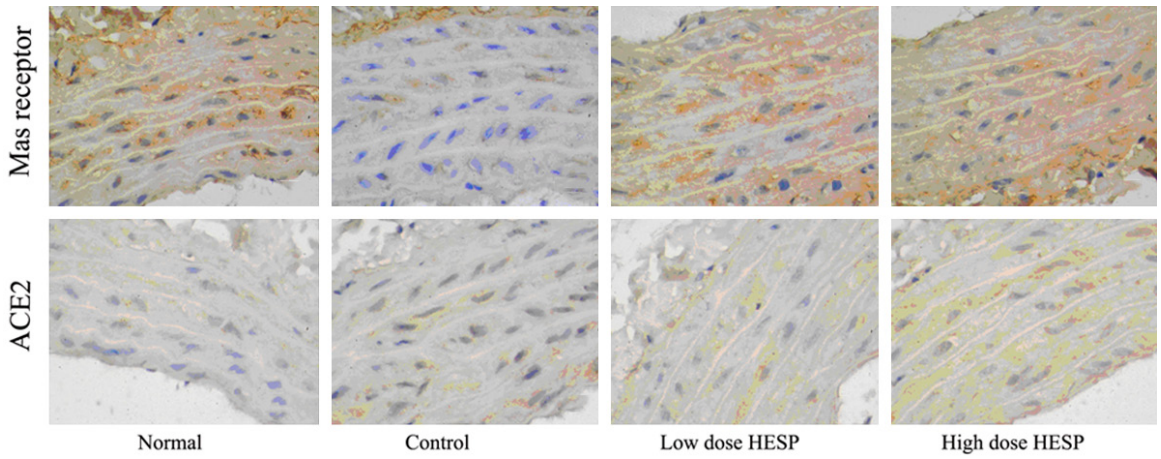

Figure 5. Aortic Mas receptor and ACE2 immunohistochemical staining. Representative images of the Mas receptor and ACE2 in different groups (400× magnifications).

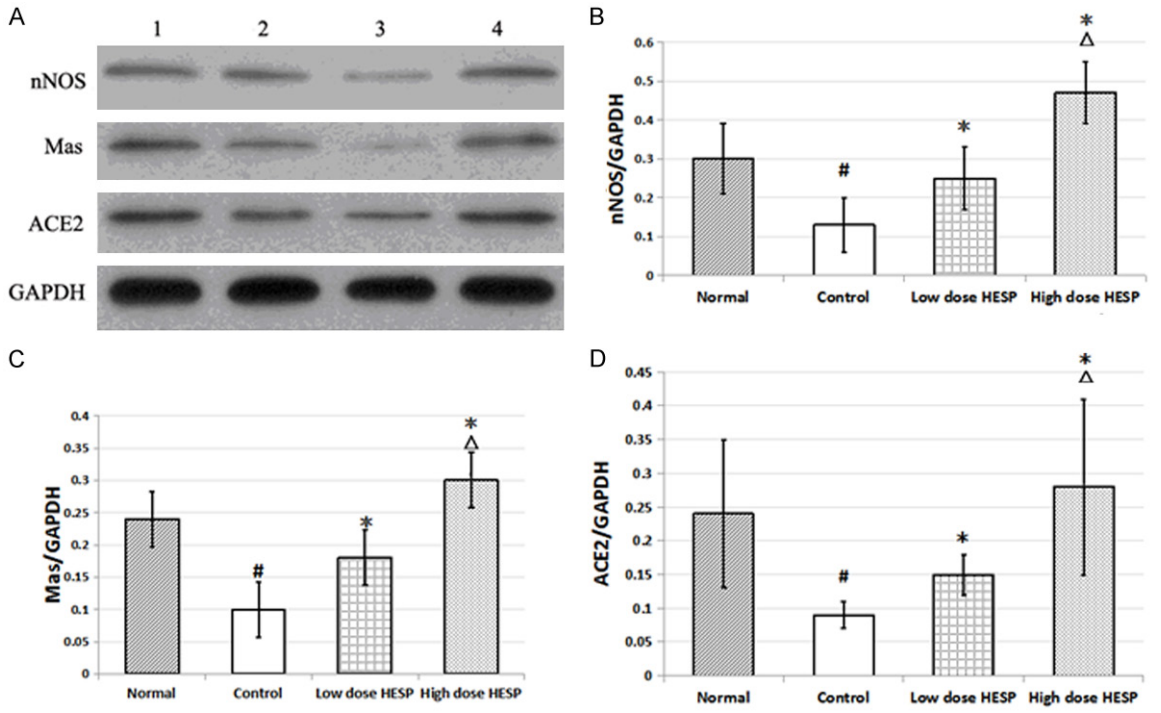

Figure 6. Effects of HESP on aortic Mas receptor, ACE2, and nNOS expression. Aortic Mas receptor, ACE2, and nNOS protein expression were analyzed using Western blot. (A) Representative protein bands of aortic Mas receptor, ACE2, and nNOS: Band 1-High-dose HESP group; Band 2-Low-dose HESP group; Band 3-SHR-control group; and Band 4-WKY group. HESP treatment increased nNOS (B), Mas receptor (C), and ACE2 (D) protein expression compared to the SHR-control group. Values are mean ± SD (n = 5). $^{*}P < 0.05$, versus SHR-control group; $^{\#}P < 0.05$, versus WKY group; $^{\Delta}P < 0.05$, versus low-dose HESP group.

time, blood pressure in the hypertensive mice decreased to normal levels, suggesting that the ACE2/Ang-(1-7)/Mas axis plays a key role in lowering blood pressure.

SHR are the most studied animal model of essential hypertension [33]. Elevated blood pressure gradually develops in SHR from 5 to 6 weeks of age to full hypertension by adults.

ACE2 mRNA levels and protein expression decreased significantly in the SHR group, and the degree of the reduction negatively correlated with blood pressure [2], which demonstrates that the ACE2/Ang-1-7/Mas axis is involved in the pathological process of hypertension in SHR.

In the present study, SHR displayed elevated SBP and DBP compared to age-matched WKY rats and the ACE2/Ang-(1-7)/Mas axis signaling pathway in the aorta downregulated significantly. HESP treatment reduced blood pressure after 4 weeks in SHR and upregulated the ACE2/Ang-(1-7)/Mas axis signaling pathway in the aorta. The antihypertensive effect and elevated ACE2/Ang-(1-7)/Mas axis were more pronounced in the high-dose HESP group.

Vascular remodeling is a crucial pathological alteration in the progression of hypertension [34]. Framingham's study reported that abnormal vascular wall thickening of the small arteries has been found in prehypertension. This thickening is a predisposing factor for progression of prehypertension to clinical hypertension [35]. Manios' study found that carotid artery IMT in prehypertensive patients was higher compared to IMT in normotensive subjects [36]. Our study showed that aortic IMT was increased in the untreated SHR, indicative of significant aortic remodeling. In addition to the blood pressure lowering effect, treatment with HESP for 4 weeks also significantly reduced aortic IMT compared to the untreated SHR. This finding suggests that HESP at the tested doses reverses aortic remodeling via its antihypertensive effects.

The renin angiotensin system (RAS) is a principal target for regulating blood pressure. Ang II is a key regulatory peptide of the RAS. In addition to Ang II, Ang-(1-7) is a pivotal regulatory peptide that exerts vasodilatory effects. ACE2 is an important enzyme in Ang-(1-7) formation. Activation of ACE2 could lower arterial blood pressure and attenuate end-organ damage caused by hypertension [37, 38]. Ang-(1-7) is an endogenous peptide of the Mas receptor, which is a G protein-coupled receptor [39]. ACE2 can increase the degradation of Ang II into Ang-(1-7) through the Mas receptor [40, 41]. The ACE2/Ang (1-7)/Mas receptor axis represents a counter regulatory RAS pathway, which opposes the vasoconstrictive action of the ACE/Ang II/AT_1 receptor system. An imbalance between the ACE2/Ang (1-7)/Mas receptor axis and the ACE/Ang II/AT1 receptor arm may contribute to the progression of hypertension. Additionally, the ACE2/Ang-(1-7)/Mas pathway has been shown to regulate vascular remodeling [42]. In this study, we showed that ACE2 and the Mas receptor localizes in the thoracic aorta. Furthermore, Western blot analysis demonstrated that ACE2 and Mas receptor protein expression were approximately 40% lower in the thoracic aorta of untreated SHR compared to age-matched WKY controls. Moreover, aortic and plasma Ang-(1-7) concentrations were lower in the untreated SHR compared to the age-matched WKY rats. Importantly, there was an inverse association of aortic Ang-(1-7) concentration with arterial blood pressure. When compared to the untreated SHR, HESP treatment increased aortic ACE2 and Mas receptor protein expression, as well as increased the aortic Ang-(1-7) concentration. These findings suggest that HESP activates the ACE2/Ang (1-7)/Mas receptor axis.

Endothelial dysfunction is considered an early marker of essential hypertension [43]. NO is synthesized by a family of NOSs, which includes inducible NOS (iNOS), eNOS, and nNOS. NO is an important modulator of vascular tone and blood pressure [44]. A decrease in NO production, as a result of endothelial dysfunction, can result in elevated blood pressure [45, 46]. A previous study demonstrated a relationship between Ang-(1-7)-induced reduction in arterial blood pressure with an increase in cardiac eNOS and nNOS expression in SHR [10]. Acute NOS inhibition increased blood pressure [47]. Our study showed that aortic nNOS expression was approximately 50% lower in the SHR-control group compared to the age-matched normal group. Accordingly, the untreated SHR showed significantly lower aortic NO and eNOS concentrations compared to the normotensive rats. HESP treatment was associated with a significant increase in aortic NO and eNOS concentrations, suggesting that HESP-mediated effects on blood pressure and aortic remodeling may correlate with increased production or release of NO. Also, treatment with HESP significantly increased aortic nNOS protein expression and increased eNOS levels. This finding indicates that HESP, at least in part, promotes NO production or release through modulating aortic nNOS and eNOS production. The antihy-

pertensive effect of traditional medicine is relatively weak, but it has few side effects and markedly improves symptoms associated with hypertension. Our study found that HESP can correct the imbalance of aortic RAS through activating the ACE2/Ang-(1-7)/Mas axis, reducing blood pressure, and inhibiting vascular remodeling. This has clinical value for treating complications of vascular remodeling in hypertension and erectile dysfunction.

There are some potential limitations in this study. First, the effects of HESP on the ACE/Ang II/AT1 receptor axis were not evaluated. Second, iNOS-induced oxidative stress correlates with hypertension [48]; however, we did not evaluate changes in iNOS and failed to explain whether HESP could affect aortic and plasma iNOS levels. Our future studies will focus on these issues.

Conclusions

The present study demonstrates that treatment with HESP for 4 weeks significantly reduces SBP and DBP and attenuates aortic IMT in SHR. These beneficial effects of HESP are at least partly correlated with activation of the ACE2/Ang (1-7)/Mas receptor axis and the nNOS/eNOS signaling pathways.

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Disclosure of conflict of interest

None.

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Table S1. Ingredient of Herba Epimedii-Semen Plentaginis Herb-pair

Chinese name	English herb name	Latin herb name	Family	Species	TCM classification
Chē qián zǐ	Plantago asiatica	Plantaginis semen	Plantaginaceae	Plantago	Clearing damp and promoting diuresis
Yín yáng huò	Epimedium	Epimedium brevicornu Maxim	Berberidacea	Epimedium	Tonifying kidney-yang