

Soy protein hydrolysate ameliorates cardiovascular remodeling in rats with L-NAME-induced hypertension[☆]

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Abstract

Pepsin-digested soy protein hydrolysate has been reported to be responsible for many of the physiological benefits associated with soy protein consumption. In the present study, we investigated the effects of soy protein hydrolysate with angiotensin-converting enzyme (ACE) inhibitory potential on the blood pressure and cardiovascular remodeling in rats with *N*_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME)-induced hypertension. Rats were fed a diet containing L-NAME (50 mg/kg body weight) with or without soy protein hydrolysate (1%, 3% or 5%) for 6 weeks. We found that ingestion of soy protein hydrolysate retarded the development of hypertension during the 6-week experimental period without affecting the amount of food intake. Although there was no difference in plasma ACE activity or tissue nitric oxide levels, ACE activity in the heart of rats consuming soy protein hydrolysate was significantly lower than that of the control group. Moreover, cardiac malonaldehyde and tumor necrosis factor- α concentrations were also lower in the soy protein hydrolysate group. No difference in plasminogen activator inhibitor-1 level was found in plasma or cardiovascular tissue. In the histopathological analysis, we also found that soy protein hydrolysate ameliorated inflammation and left ventricle hypertrophy in the heart. These findings suggest that soy protein hydrolysate might not only improve the balance between circulating nitric oxide and renin–angiotensin system but also show beneficial effects on cardiovascular tissue through its ACE inhibitory activity.

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Keywords: Soy protein; Hypertension; Nitric oxide; Angiotensin-converting enzyme; Tumor necrosis factor; Cardiovascular remodeling

1. Introduction

Hypertension is one of the main factors causing cardiovascular disease. Lifestyle and dietary habits may affect blood pressure and cardiovascular risk factors. Studies have reported that nitric oxide (NO) and angiotensin II are key factors in regulating blood pressure and cardiovascular tissue structures [1]. Inhibiting the production of NO stimulates the activation of the renin–angiotensin system (RAS) and leads to elevation of blood pressure and cardiovascular inflammatory changes [2]. Administration of NO synthase (NOS) inhibitors like *N*_ω-nitro-L-arginine

methyl ester hydrochloride (L-NAME) in animal models causes cardiorenal damage [3,4]. Angiotensin-converting enzyme (ACE) is important in the production of angiotensin II. Treatment with ACE inhibitors in rats with L-NAME-induced hypertension was reported not only to reduce blood pressure but also to retard the progression of cardiorenal remodeling [4,5].

Soy-based foods are an important source of dietary protein in Asian countries. A study with Chinese women found that soy food consumption was associated with a lower risk of coronary disease [6]. In a chronic renal failure rat model, soy protein hydrolysate (SPH) also exhibited blood pressure-lowering effects [7]. Many studies have reported that hydrolysates derived from dietary protein exhibit various physiological effects. Some of the protein hydrolysates were reported to have ACE inhibitory activity and may had the potential to improve hypertension in vivo [8,9]. Our previous

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study used pepsin-digested SPH to determine the effects of soy protein after digestion in vivo, and we found that it leads to a reduction in heart ACE activity in spontaneously hypertensive rats [10]. Therefore, we used a NOS inhibitor to induce hypertension and cardiovascular remodeling in this study to investigate whether SPH can inhibit ACE activity and improve the NO and RAS balance in order to reduce oxidative stress and further ameliorate cardiac and vascular injuries caused by L-NAME.

2. Materials and methods

2.1. Preparation of SPH

Soy protein isolate (Protein Technologies International, St. Louis, MO, USA) was dissolved in buffer and adjusted to pH 2.0 with 1 N HCl. The protein solution was hydrolyzed with pepsin for 24 h at 37°C. The solution was then adjusted to pH 7.0 and centrifuged. The hydrophobic precipitate was discarded, and the hydrophilic supernatant was lyophilized and ground into powder as the SPH sample.

2.2. Animals and diets

Forty male Wistar rats (16 weeks old) were purchased from the Laboratory Animal Center of National Taiwan University (Taipei, Taiwan). Rats were housed in individual cages under a 12-h light–dark cycle at 22±1°C and a relative humidity of 55±5%. Rats were divided into four groups (10 rats for each group) and fed a diet based on AIN-93M for 6 weeks containing different amounts of SPH substituted for the casein (Table 1): a control group (C) with 0% SPH; rats fed 1% SPH (E1); rats fed 3% SPH (E2); and rats fed 5% SPH (E3). All rats were administered orally 50 mg/kg per day of L-NAME (Fluka, Switzerland) during the experiment period. Food and water were provided with free access. Body weight and food intake were recorded every week. The

Table 1
Diet composition (g/kg)

Ingredient ^a	C	E1	E2	E3
Cornstarch	460	460	460	460
Dextrin	150	150	150	150
Casein	140	130	110	90
SPH	0	10	30	50
Sucrose	100	100	100	100
Soy oil	40	40	40	40
Cellulose	50	50	50	50
Mineral mix	37.5	37.5	37.5	37.5
Vitamin mix	10	10	10	10
D,L-Methionine	10	10	10	10
Choline	2.5	2.5	2.5	2.5

C; control group; E1, rats fed 1% SPH; E2 rats fed 3% SPH; E3, rats fed 5% SPH.

^a Cornstarch was from the Samyang Genex (Seoul, Korea). Dextrin, Casein (high-N), cellulose (nonnutritive bulk), mineral mixture (AIN-93M), vitamin mixture (AIN-93M), D,L-methionine and choline were obtained from the ICN Biochemicals (Aurora, OH). Sucrose and soy oil were from the Taiwan Sugar Co. (Taipei, Taiwan).

animal experiment was approved by the University Committee for Animal Care and Use and following the guidelines of the National Animal Research Center (Taipei, Taiwan).

2.3. Measurement of blood pressure

Blood pressure was measured at 3-week intervals during the experimental period by the tail-cuff method with an electrophygmomanometer (Model 179, Blood Pressure Analyzer IITC, Woodland Hills, CA, USA). After being starved for 12 h, rats were put into restrainers and at least five readings were recorded. The maximum and minimum values were discarded, and the blood pressure was calculated as the average of the remaining three values.

2.4. Blood and tissue sampling

After 6 weeks, rats were sacrificed after being anesthetized with sodium pentobarbital. Blood samples were collected from the interior vena cava into tubes containing anticoagulant. Blood samples were immediately centrifuged, and the plasma and red blood cells (RBCs) were stored at –80°C until being analyzed. Plasma lipids, glucose, albumin, uric acid, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a Hitachi 7170 autoanalyzer (Tokyo, Japan). Hearts and aortas were collected, weighed and divided into several parts for various analyses. Heart and aorta samples were homogenized in 400 mM phosphate buffer (pH 7.2) containing 340 mM sucrose, 900 mM NaCl and protease inhibitors [11]. After centrifugation, the supernatants were stored at –80°C.

2.5. Angiotensin-converting enzyme activity

ACE activities in plasma and tissue homogenates were measured with a spectrophotometric method [12]. Briefly, we used Hip-His-Leu as the substrate and incubated our samples at 37°C for 80 min. The reaction was stopped by addition of 1 N HCl. The hippuric acid was extracted with ethyl acetate and determined at 228 nm. ACE activity was expressed as milliunit per milligram of protein, and the protein was quantified by the Lowry method.

2.6. Nitrite plus nitrate (NO_x)

Due to the short half-life and low concentration of NO in vivo, we evaluated plasma and tissue NO levels by measuring its stable metabolites, nitrite (NO₂⁻) and nitrate (NO₃⁻), by the modified Griess reaction method [13]. The principle of the assay was reduction of nitrate by vanadium (III) in combination with the detection by acidic Griess reaction.

2.7. Antioxidative enzyme activities and malonaldehyde levels

RBCs were washed with saline and diluted with phosphate buffer. Superoxide dismutase (SOD) activities in RBCs and tissue homogenates were analyzed by commercial kits (Randox, UK). Catalase activities were analyzed by the

method of Aebi [14]. Malondialdehyde (MDA) in plasma, heart and aorta was measured by the thiobarbituric acid-reactive substance method [15].

2.8. TNF- α and PAI-1 expressions

Plasma, heart and aorta levels of tumor necrosis factor- α (TNF- α) and plasminogen activator inhibitor-1 (PAI-1) were measured with enzyme-linked immunosorbent assay kits [rat TNF- α /TNFSF1A, R&D, USA; Zymutest rat-PAI-1 (antigen/activity), Hyphen BioMed, France].

2.9. Histological analysis

The dissected hearts and aortas of the rats were fixed in formaldehyde. Samples were stained with hematoxylin and eosin. The biopsies were examined by a pathologist on a blinded basis. Intima thickening of the rat aortas was observed at $\times 200$ magnification. To evaluate the inflammation and hypertrophy of the heart, cardiac tissues were observed at $\times 100$ magnification, and we measured the thickness of the thickest part of the left ventricular wall at $\times 2$ magnification with scales.

2.10. Statistical analysis

Data were analyzed by one-way ANOVA and Fisher's least significant difference test using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Results are expressed as the mean and S.E.M. A P value of $<.05$ was taken as the level of statistical significance.

3. Results

3.1. Body weight and nutritional status

During the experimental period, adding 1% (the E1 group), 3% (the E2 group) and 5% (the E3 group) of SPH to the diet did not affect the food intake of the rats. At the end of the study, the average body weights of the C, E1, E2 and E3

Table 2
Plasma lipids, albumin, AST, ALT, glucose and uric acid in rats with L-NAME-induced hypertension

	C	E1	E2	E3
Alb (g/dL)	3.9 \pm 0.1	4.1 \pm 0.1	4.3 \pm 0.1	3.9 \pm 0.2
TC (mmol/L)	1.68 \pm 0.15	1.88 \pm 0.19	1.58 \pm 0.24	2.08 \pm 0.24
TG (mmol/L)	0.28 \pm 0.03	0.39 \pm 0.07	0.35 \pm 0.06	0.33 \pm 0.07
HDL-C (mmol/L)	1.26 \pm 0.16	1.31 \pm 0.15	1.20 \pm 0.21	1.62 \pm 0.21
LDL-C (mmol/L)	0.23 \pm 0.02	0.27 \pm 0.03	0.21 \pm 0.03	0.27 \pm 0.03
AST (IU/L)	89.4 \pm 3.5	114.4 \pm 17.3	116.0 \pm 19.8	102.9 \pm 9.3
ALT (IU/L)	24.9 \pm 2.0	35.6 \pm 5.7	46.6 \pm 14.9	48.5 \pm 8.5
BS (mg/dL)	165.7 \pm 5.6	148.8 \pm 5.8	179.7 \pm 14.1	167.3 \pm 22.7
UA (mg/dL)	1.5 \pm 0.1	1.7 \pm 0.2	1.5 \pm 0.2	1.6 \pm 0.1

Data are expressed as mean \pm S.E.M. ($n=10$). A description of the experimental groups is given in the footnote to Table 1. Alb, albumin; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein; LDL, low-density lipoprotein; BS, blood sugar; UA, uric acid.

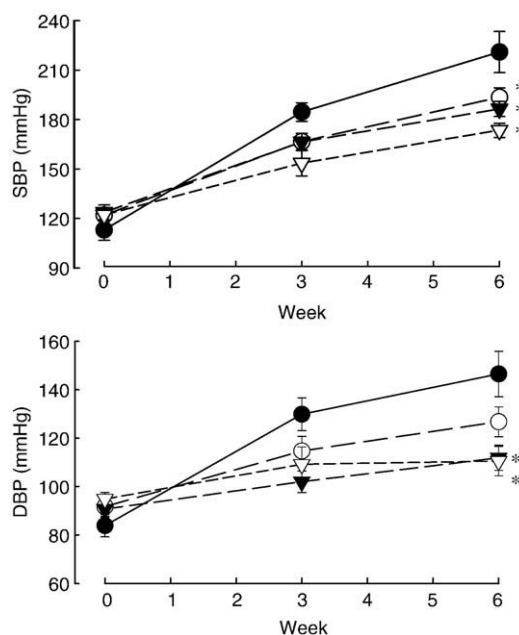


Fig. 1. Changes in blood pressure in rats with L-NAME-induced hypertension. The SBP of the E1 (○), E2 (▼) and E3 (▽) groups was significantly lower than that of the control group (●) in the sixth week. The DBP of the E2 and E3 groups was also lower than the control group in the sixth week. Data are presented as the mean \pm S.E.M. ($n=10$). A description of the experimental groups is given in the footnote to Table 1. * $P<.05$, significantly different.

groups were 337.3 \pm 9.9, 342.5 \pm 31.5, 381.8 \pm 25.4 and 328.1 \pm 21.0 g, respectively. No difference was found among the four groups of animals. In the analysis of plasma lipids, glucose, albumin, AST and ALT, there were also no differences among the four groups, and this indicates that SPH had no effect on the growth or nutritional status compared with the casein group (Table 2).

3.2. Nitrite and nitrate

At the end of the study, the combined plasma nitrite and nitrate level was significantly higher in the E3 group than in the three other groups. Nitrite and nitrate levels in the heart and aorta did not significantly differ among all groups (Table 3).

3.3. Blood pressure and ACE activity

Rats treated with L-NAME for 3 weeks had significantly higher systolic and diastolic blood pressures (SBP,

Table 3
Nitrite and nitrate concentration in rats with L-NAME-induced hypertension

	C	E1	E2	E3
Plasma (μ M/ml)	123.8 \pm 4.5 ^b	114.3 \pm 13.8 ^b	151.3 \pm 20.5 ^b	289.3 \pm 52.5 ^a
Heart (μ M/mg protein)	4.5 \pm 0.4	4.5 \pm 0.5	5.9 \pm 0.7	5.9 \pm 0.5
Aorta (μ M/mg protein)	22.0 \pm 9.2	14.5 \pm 2.6	18.3 \pm 3.0	13.6 \pm 1.2

^{a,b}Significantly different ($P<.05$). Values are expressed as the mean \pm S.E.M. ($n=10$). A description of the experimental groups is given in the footnote to Table 1.

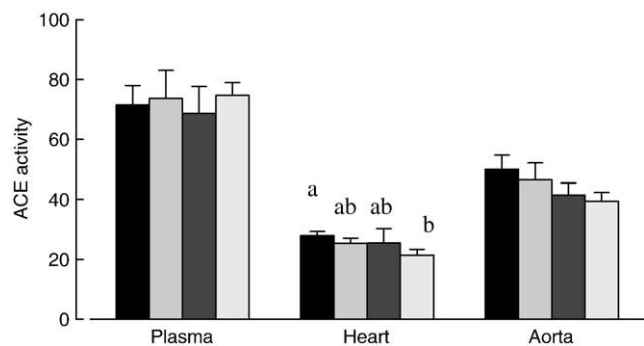


Fig. 2. ACE activity in plasma (mU/ml) and tissues (mU/mg protein) in rats with L-NAME-induced hypertension in the sixth week. Bars presented from the left to the right are the C, E1, E2 and E3 groups. Data are presented as mean±S.E.M. ($n=10$). ^{a,b} $P<.05$, significantly different.

184.6±5.6 mmHg; DBP, 129.8±6.7 mmHg) compared to the baseline (113.0±6.3/83.9±4.6 mmHg), and values continued to rise through the end of the study (221.0±12.5/146.0±9.4 mmHg). At the end of the study, the SBP and DBP of the E2 and E3 groups were significantly lower than those of the C group (Fig. 1). No difference was found in plasma ACE activity among the four groups. However, heart ACE activity of the E3 group was significantly lower than that of the C group. Rats consuming SPH had lower average value of aorta ACE activity, but no significant difference was found (Fig. 2).

3.4. TNF- α and PAI-1

The heart TNF- α level of the E3 group was significantly lower than that of the control group. TNF- α in the aorta of rats consuming SPH was lower than that of the control group, although no significant difference was found (Fig. 3). In the analysis of PAI-1, we found that the different experiment diets had no significant effects on PAI-1 levels in the plasma, heart and aorta.

3.5. MDA and antioxidative enzyme activity

MDA levels, and SOD and catalase activities in the plasma did not differ among all groups at the end of the

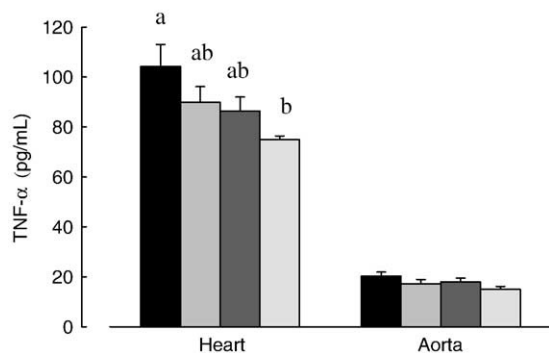


Fig. 3. TNF- α in heart and aorta in rats with L-NAME-induced hypertension in the sixth week. Bars presented from the left to the right are the C, E1, E2, and E3 groups. Data are presented as mean±S.E.M. ($n=10$). ^{a,b} $P<.05$, significantly different.

Table 4

SOD, catalase and malonaldehyde concentration in the plasma, heart and aorta of rats with L-NAME-induced hypertension

	C	E1	E2	E3
SOD (U/mg protein)				
RBCs	1.47±0.13	2.51±0.29	1.68±0.29	1.78±0.15
Heart	9.64±0.50 ^a	7.77±0.64 ^b	7.85±0.53 ^{a,b}	6.55±0.31 ^b
Aorta	11.01±0.98	9.68±1.12	9.81±0.75	9.82±0.84
Catalase (U/mg protein)				
RBCs	96.56±16.44	109.13±14.96	106.07±11.62	130.66±17.36
Heart	13.61±1.63	14.25±3.60	9.62±0.93	10.05±0.90
Aorta	49.00±5.21	46.73±6.22	35.01±2.89	45.90±4.00
MDA (μ M/mg protein)				
Plasma	6.45±1.07	5.41±0.87	7.01±0.51	5.27±0.80
Heart	0.91±0.06 ^a	0.86±0.05 ^{ab}	0.64±0.04 ^{a,b}	0.77±0.06 ^b
Aorta	1.27±0.08 ^a	0.89±0.09 ^b	1.20±0.17 ^{a,b}	0.84±0.06 ^b

^{a,b}Significantly different ($P<.05$). Values are expressed as the mean±S.E.M. ($n=10$). A description of the experimental groups is given in the footnote to Table 1.

study. The E3 group had significantly lower MDA levels in the heart and aorta compared to the C group. The E3 group also had lower SOD activity in the heart than the C group, but no significant difference was found in the analysis of catalase activity (Table 4).

3.6. Histological analysis of the heart and aorta

Rats treated with L-NAME were found to have thickened intima and vacuolated endothelium in the aorta, and a diet containing SPH ameliorated this vascular remodeling (Fig. 4A and B). We also found that SPH improved L-NAME-induced inflammation, fibrosis (Fig. 4C and D) and left ventricle wall thickening in the heart. The wall of the left ventricular in rats consuming 5% SPH was significantly thinner than that of the control group (2.77±0.14 vs. 3.23±0.1 mm).

4. Discussion

In the present study, we found that SPH retarded the development of L-NAME-induced hypertension in rats during the 6-week experimental period. There is an important interaction between NO and angiotensin II, and their balance may be influential in maintaining homeostasis and regulation of vascular tone [16]. Many studies have reported that chronic blockade of NO synthesis by NOS inhibitors like L-NAME causes endothelial dysfunction, a significant increase in blood pressure and further pathological injuries to the cardiovascular system and kidneys, which may lead to aggravation of hypertension [11,17]. Treatment with ACE inhibitors or AT₁-receptor antagonists exhibited amelioration of NO impairment. In the European Trial on Reduction of Cardiac Events with Perindopril in Stable Coronary Artery Disease study, researchers demonstrated that ACE inhibition up-regulated eNOS expression and activity as well as reduced the angiotensin II level [18].

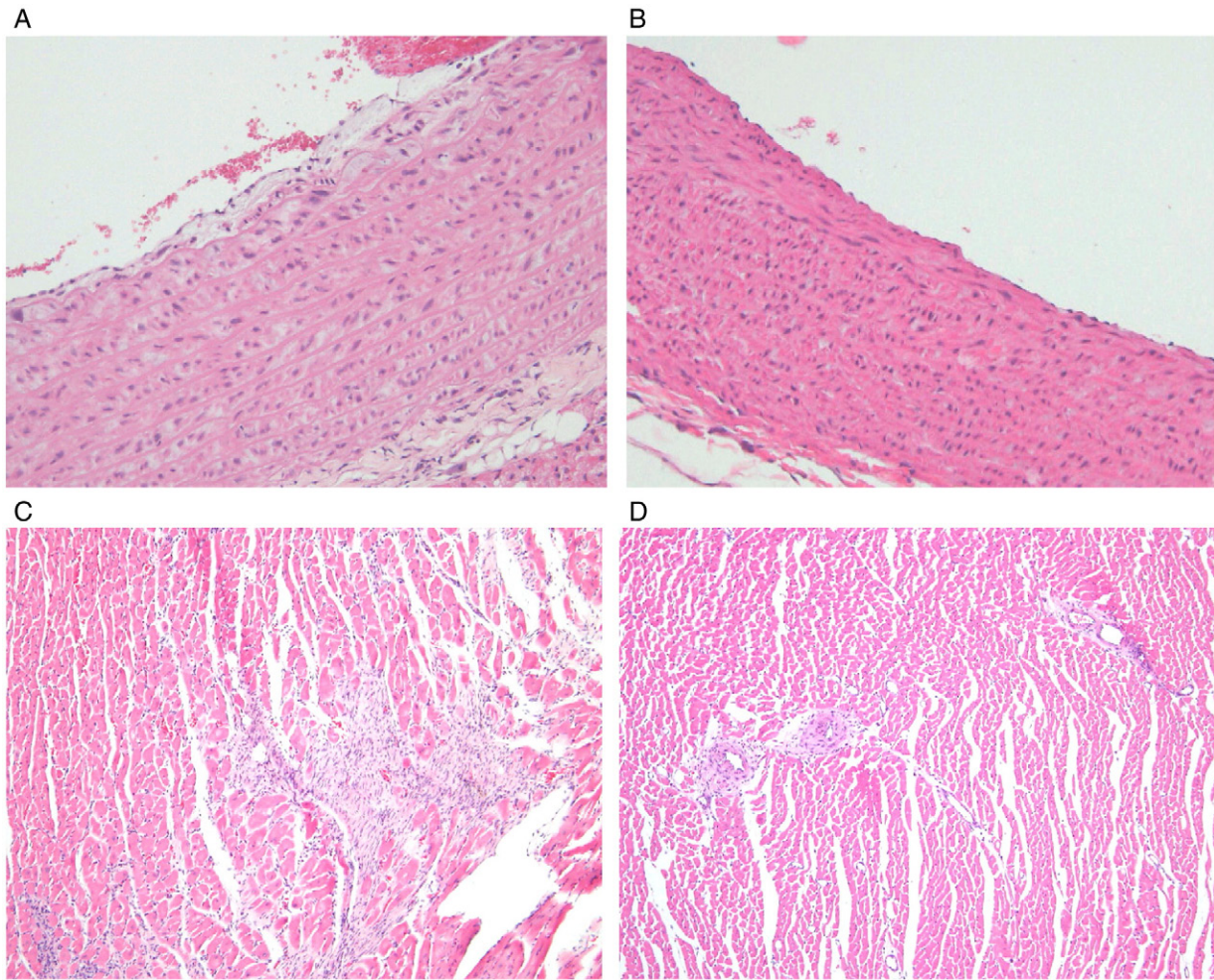


Fig. 4. Effects of L-NAME and SPH on rat aorta and heart. (A) Aorta of the C group (50 mg/kg per day of L-NAME). (B) Aorta of the E3 group (50 mg/kg per day of L-NAME with 5% SPH in diet). (C) Heart of the group. (D) Heart of the E3 group (HE stain, original magnification $\times 400$).

Treating rats with L-NAME-induced hypertension with enalapril and losartan retarded the progression of hypertension and increased eNOS mRNA expression and the plasma nitrite/nitrate concentration [19]. In our study, all L-NAME-treated rats had significantly higher heart ACE activities compared with the baseline, which is consistent with previous studies. Although no significant difference was shown in plasma ACE activity among groups at the end of the study, we found that rats consuming diets containing 3% and 5% SPH had lower SBP and DBP than the control group. In addition, we also found that the plasma NO level was higher in the 5% SPH group. Our results suggest that the intake of SPH may prevent L-NAME-induced elevation of blood pressures, and the effect may be mediated by improvement in the NO and angiotensin II balance in vivo.

In this study, we found that consumption of SPH lowered heart ACE activity, and there was also a reduction in aorta ACE activity, although no statistically significant difference was found. However, there was no significant difference in heart and aorta NO levels, and this result was the same as a previous study in which captopril had no effect on heart and

kidney nitrite/nitrate concentrations in NO-deficient hypertensive rats [20]. Chronic inhibition of NO synthesis was reported to lead to tissue damage through the AT₁ receptor independent of its effects on SBP [21]. In a study infusing asymmetric dimethylarginine, an endogenous NOS (eNOS) inhibitor, in mice, it was demonstrated that long-term effects of an eNOS inhibitor were not entirely mediated by inhibition of NO synthesis, but direct increases in ACE and oxidative stress via the AT₁ receptor were found to be involved [22]. Treatment with ACE inhibitors, but not diuretics, showed antifibrotic effects in L-NAME-induced myocardial structural changes [23,24]. Moreover, many studies have reported that the balance between NO and angiotensin II may be crucial to the cardiovascular remodeling that occurs with hypertension, and the repair of this balance is compulsory to prevent cardiovascular damage that may be independent of the changes in blood pressure [1]. These findings suggest that SPH might suppress the effects of angiotensin II through the AT₁ receptor by its ACE inhibitory activity in addition to reversing the effect of L-NAME on NO levels.

ACE activity in tissues is also thought to be involved in the generation of superoxide anions and the release of TNF- α and PAI-1 that may cause sustained hypertension [25]. Angiotensin II stimulates PAI-1 and ROS and contributes to further inflammation and thrombosis; moreover, the activated inflammatory cells release ACE that increases angiotensin II production through a local paracrine feedback system [26]. In our study, we found that rats fed peptic-digested soy protein had lower MDA levels in the heart and aorta, while no changes in circulating MDA were found. However, soy protein with or without isoflavones showed no direct effect on the antioxidant capacity [27]. This indicates that SPH may improve vascular functions not directly by scavenging free radicals but by reducing the production of free radicals through lowering angiotensin II level. In addition, we found that rats consuming a diet containing SPH had a lower TNF- α concentration in the heart, which was dose-dependent. Although the change in TNF- α in the aorta was not significant, there was a lower average value of aorta TNF- α levels in rats consuming SPH. Recent studies have demonstrated that the interplay between RAS and tumor necrosis factor in the heart is important in the progression of cardiac remodeling [28]. In the present study, we also found that SPH may improve the intima thickening and inflammation in the cardiac tissue caused by administration of L-NAME. Therefore, we suggest that SPH inhibits ACE activity in cardiovascular tissues by decreasing the effects of angiotensin II, which may lead to a reduction in both oxidative damage and further inflammatory reactions. Studies have also shown that the expression of PAI-1 increased in vascular lesions and cardiac injuries [29,30], and endogenous NO and ACE activity is important in regulating PAI-1 levels [31]. An animal study has shown that NO inhibits the release of PAI-I, and L-NAME may induce vascular PAI-I expression [32]. However, we found no differences among groups in terms of the level of the PAI-1 antigen or its activity in the plasma, aorta and heart. Although some studies have reported that ACE inhibitors might abolish the effects of L-NAME on PAI-1 expression, another study showed that long-term administration of the ACE inhibitor, ramipril, or L-arginine alone had no effect on the fibrinolytic balance in vivo; the PAI-1 antigen level and activity decreased only when treated with a combination of ramipril and DNP [31]. Another reason might be that ACE inhibitors with different lipophilic properties and tissue affinities have dissimilar physiological influences in vivo [33]. The inconsistencies of these studies may require further investigation to clarify the underlying mechanisms.

The peptide derivatives of many dietary protein hydrolysates are reported to have physiological effects [8,34], and these enzyme-pretreated protein hydrolysates were shown to be resistant to pepsin and pancreatin [35]. SPH derived from soy protein pretreated with digestive enzymes was proved to have ACE inhibitory activity in vitro [36], and separation of

the hydrolysate from peptic-digested soy protein afforded some ACE inhibitory peptides including Ile-Ala, Tyr-Leu-Ala-Gly-Asn-Gln, Phe-Phe-Leu, Ile-Tyr-Leu-Leu and Val-Met-Asp-Lys-Pro-Gln-Gly [37]. These findings support the consumption of soy protein possibly retarding the development of hypertension and progression of cardiovascular damage by peptides produced after digestion. Further work on isolating and identifying the peptides derived from gastrointestinal-digested soy protein is needed to clarify the mechanisms of the effects of soy protein on blood pressure and the cardiovascular system in vivo.

We concluded that the consumption of soy protein may, mainly through its enzyme-digested hydrolysate, retard the development of hypertension in a chronic NO-deficient rat model and may protect the cardiovascular system against injuries from endothelial dysfunction through its ACE inhibitory activity. Our finding that SPH ameliorates cardiovascular damage by its ACE inhibitory activity during NO deficiency at least revealed one possible mechanism of the beneficial effects of soy protein ingestion during endothelial dysfunction, and this might be important in modifying diet to prevent early organ damage in people with high cardiovascular disease risks.

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