Original Article

Renoprotective Effects of Soy Protein Hydrolysates in N^{ω}-Nitro-L-Arginine Methyl Ester Hydrochloride–Induced Hypertensive Rats

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Pepsin-digested soy protein hydrolysate (SPH) 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 SPH with angiotensin-converting enzyme (ACE) inhibitory potential on blood pressure and renal injuries 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 SPH (at 1%, 3%, or 5%) for 6 weeks. We found that ingestion of SPH ameliorated the development of hypertension during the 6-week experimental period. SPH was also found to ameliorate renal function by decreasing urinary protein excretion and elevating the creatinine clearance rate. The levels of kidney ACE activity, malonaldehyde, tumor necrosis factor-α and plasminogen activator inhibitor-1, and the expression of CYP4A decreased in the 5% SPH group. Consumption of 5% SPH also ameliorated renal damage according to the histopathological analysis. These findings suggest that SPH might ameliorate the elevation of blood pressure and show renoprotective effects in nitric oxide (NO)–deficient rats, and one possible mechanism might be mediation *via* its ACE inhibitory activity. (*Hypertens Res* 2008; 31: 1477–1483)

***Key Words*: soy protein, angiotensin-converting enzyme, kidney**

Introduction

The prevalence of hypertension and related organ damage is increasing worldwide (*1*, *2*). Studies have reported that nitric oxide (NO) and angiotensin II are key factors in regulating blood pressure and cardiorenal remodeling (*3*). Blockade of NO stimulates activation of the renin-angiotensin system (RAS) and leads to elevation of blood pressure and inflammatory changes in vascular tissues (*4*). Studies have shown that administration of NO synthase (NOS) inhibitors such as N^{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME) in animal models causes cardiorenal damage (*4*, *5*). In the RAS, the angiotensin-converting enzyme (ACE) plays an important role in the production of angiotensin II, and ACE inhibitors (ACEIs) have been reported to reduce blood pressure and also retard the progression of cardiorenal remodeling in rats with L-NAME–induced hypertension (*2*, *6*).

Soy-based foods are an important source of dietary protein in Oriental diets. 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 have the potential to improve hypertension *in vivo* (*7*, *8*). Our previous studies found that soy protein may lower blood pressure and retard the loss of renal function in rats with chronic renal failure (*9*). In a spontaneously hypertensive rat model, we found that the hydrophilic portion of soy protein hydrolysate (SPH) retarded the development of hypertension by its ACE-inhibitory activity (*10*). Soy protein was also reported to have beneficial effects on the inflammatory and nutritional statuses in patients with end-stage renal failure (*11*). Thus, we prepared

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Table 1. Systolic and Diastolic Blood Pressure of Rats at the Beginning and 6 Weeks after Ingestion of the Different Experimental Diets

	C	E1	E2	E3
Systolic blood pressure (mmHg)				
0 weeks	113.0±6.3	122.0±3.4	123.8±4.4	122.2±3.4
6 weeks	221.0±16.1[a]	193.5±7.5[a,b]	188.0±6.2[b]	173.5±5.6[b]
Diastolic blood pressure (mmHg)				
0 weeks	83.9±4.6	91.7±4.0	90.7±3.3	94.8±2.6
6 weeks	146.0±12.1[a]	126.8±8.0[a,b]	115.5±6.6[b]	110.5±7.8[b]

Values are presented as the mean±SEM. Different superscript letters in a row indicated a significant difference ($p<0.05$). C, control; E1, 1% SPH; E2, 3% SPH; E3, 5% SPH. SPH, soy protein hydrolysate.

SPH by pepsin-digestion to investigate whether it can preserve renal function and lower blood pressure in a chronic renal-failure model by inhibiting circulating and renal ACE activities. We used an NOS inhibitor to induce hypertension in this study in order to investigate whether SPH can inhibit ACE activity and improve the NO and RAS balance, and thereby reduce oxidative stress and further ameliorate kidney injuries caused by L-NAME.

Methods

Preparation of SPH

Soy protein isolate (Fuji Oil Co., Osaka, Japan) was dissolved in 0.05 mol/L phosphate buffer (pH 7.6) and adjusted to pH 2.0 with 1 Eq/L HCl. The protein solution was pepsin-hydrolyzed 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 grounded into powder as the SPH sample.

Animals and Diets

Thirty-two male Wistar rats (16 weeks old) were purchased from the Laboratory Animal Center of National Taiwan University College of Medicine. Rats were housed in individual cages under a 12-h light-dark cycle at 22±1°C and a relative humidity of 55±5%. The rats were divided into four groups and fed an AIN-93M–based diet containing different amounts of SPH substituted for the casein (Table 1) for 6 weeks: the C (control) group was fed 0% SPH, the E1 group 1% SPH, the E2 group 3% SPH, and the E3 group 5% SPH. All rats were orally administrated 50 mg/kg/d L-NAME (Fluka, Buchs, Switzerland) during the experimental period. Food and water were provided with free access. Body weight and food intake were recorded every week. The animal experiment was approved by the University Committee for Animal Care and Use and followed the guidelines of the National Animal Research Center (Taipei, Taiwan).

Blood, Urine, and Tissue Sampling

After 6 weeks, rats were anesthetized with sodium pentobarbital and sacrificed. Blood samples were collected from the inferior vena cava into tubes containing anticoagulant. Blood samples were immediately centrifuged, and the plasma was stored at −80°C until being analyzed. Plasma albumin, creatinine, urea nitrogen, sodium, potassium, chloride, phosphorous and calcium concentrations were determined using a Hitachi 7170 autoanalyzer (Tokyo, Japan). Twenty-four-hour urine samples were collected using metabolic cages before sacrificing the animals. Urinary protein and urea nitrogen excretion were also analyzed with the autoanalyzer. The creatinine clearance rate (CCr) was calculated as follows:

$$\text{CCr} = \text{urine creatinine (mg/dL)} \times \text{24-h urine volume (mL)/plasma creatinine (mg/dL)} \times 1{,}440 \text{ (min)}.$$

The kidneys were collected, weighed, and divided into several parts for various analyses.

Measurement of Blood Pressure

Blood pressure was measured at 3-week intervals during the experimental period by the tail-cuff method with an electrosphygmomanometer (Model 179; Blood Pressure Analyzer IITC, Woodland Hills, 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.

ACE Activity and Nitrite plus Nitrate (NO_x)

Kidney samples were homogenized in 400 mmol/L phosphate buffer (pH 7.2) containing 340 mmol/L sucrose, 900 mmol/L NaCl, and protease inhibitors (*12*). After centrifugation, the supernatants were stored at −80°C. ACE activities in plasma and kidney homogenates were measured with a spectrophotometric method (*13*). Briefly, we used Hip-His-Leu as the substrate and incubated our samples at 37°C for 80 min. The reaction was stopped by the addition of 1 Eq/L HCl. The hip-

Table 2. Plasma Albumin, Urea Nitrogen, Creatinine, Sodium, Potassium, Chloride, Phosphorus and Calcium Concentrations of Rats at the End of the Study

	C	E1	E2	E3
Albumin (g/dL)	3.92±0.09	4.12±0.10	4.23±0.09	4.12±0.11
Urea nitrogen (mg/dL)	36.5±6.8[a]	21.8 ±3.0[a,b]	22.1±2.2[a,b]	14.7±1.7[b]
Creatinine (mg/dL)	1.15±0.05	1.20±0.22	0.78±0.12	0.95±0.10
Sodium (mEq/L)	138.3±2.7	142.8±3.2	143.0±1.5	136.3±2.8
Potassium (mEq/L)	5.92±0.47	6.53±1.26	5.00±0.19	4.94±0.27
Chloride (mEq/L)	81.7±4.7	90.2±4.9	95.5±2.1	81.2±3.8
Phosphorus (mg/dL)	6.83±0.41	6.87±1.12	6.17±0.31	7.00±0.58
Calcium (mg/dL)	10.17±0.12	10.81±0.48	10.48±0.22	11.03±0.30

Values are presented as the mean±SEM. Different superscript letters in a row indicate a significant difference ($p<0.05$). The various groups are defined in the footnotes of Table 1.

puric acid was extracted with ethyl acetate and determined at 228 nm. ACE activity was expressed as mU/mg protein, and the protein was quantified by the Bradford method (*14*).

Due to the short half-life and low concentration of NO *in vivo*, we evaluated kidney NO levels by measuring its stable metabolites, nitrite (NO_2^-) and nitrate (NO_3^-), by the modified Griess reaction method (*15*).

Antioxidative Enzyme Activities, Malonaldehyde Levels, Tumor Necrosis Factor-α and Plasminogen Activator Inhibitor-1 Concentrations

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, Belfast, UK). Catalase activities were analyzed by the method of Aebi (*16*). Malonaldehyde (MDA) in the plasma and kidneys was measured by the thiobarbituric acid–reactive substance (TBARS) method (*17*). Rat kidneys were homogenized with buffer (pH 7.2) containing protease inhibitors. Tumor necrosis factor-α (TNF-α) and plasminogen activator inhibitor-1 (PAI-1) concentrations in kidney homogenates were measured with an enzyme-linked immunosorbent assay (ELISA) kit (rat TNF-α/TNFSF1A: R&D, Minneapolis, USA; Zymutest rat-PAI-1 RK001A: Hyphen BioMed, Neuville-Sur-Oise, France).

Kidney Cytochrome P450 4A Expression

Renal microsomes were prepared by a previously described method (*18*). Kidneys were homogenized in 10 mmol/L potassium buffer with 250 mmol/L sucrose, 1 mmol/L EDTA, and 0.1 mmol/L phenylmethylsulfonyl fluoride. After centrifugation, microsomal pellets were resuspended in 100 mmol/L potassium buffer (pH 7.3). Protein concentrations of the samples were measured with the Bradford method (*14*). Twenty micrograms of microsomal protein was separated by electrophoresis on 10% SDS-polyacrylamide gels for 2 h at 100 V. The proteins were transferred to a nitrocellulose membrane, and the membrane was blocked overnight in TBST-20 buffer containing 5% nonfat dry milk at 4°C. The membrane was incubated for 2 h with a polyclonal antibody against rat CYP4A (ABR, Golden, USA) at a 1:2,000 dilution in TBS-T buffer containing 1% bovine serum albumin (BSA). The membrane was then washed with TBS-T buffer three times and incubated with goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (Jackson, West Grove, USA) at a 1:10,000 dilution in TBS-T buffer containing 1% BSA. Blots were then washed several times with TBS-T and developed using an enhanced chemiluminescence kit (ECL; Amersham Biosciences, Buckinghamshire, UK).

Histological Analysis

The dissected kidneys of the rats were fixed in 10% formaldehyde. Samples were stained with hematoxylin and eosin (H & E stain). Biopsies were examined on a blinded basis by a pathologist, using a 0-to-2 injury scale, with 0 indicating normal, 1 indicating moderate damage, and 2 indicating severe damage.

Statistical Analysis

Data were analyzed by one-way ANOVA and Fisher's least significant difference test using a Statistical Analysis System (SAS Institute, Cary, USA). Results are expressed as the mean and SEM. Values of $p<0.05$ were considered to indicate statistical significance.

Results

At the end of the study, we found no differences in body weight or food intake among the groups (data not shown). Adding 1% to 5% of SPH as a substitute for casein had no effect on the food intake or the plasma albumin concentration (Table 2, $p>0.05$) in rats.

The systolic blood pressure (SBP) and diastolic blood pressure (DBP) did not differ between the groups at the baseline.

Table 3. Twenty-Four-Hour Urinary Protein, Urea Nitrogen, and Creatinine Clearance Rate (CCr) of Rats at the End of the Study

	C	E1	E2	E3
Protein (mg/d)	25.7±2.5[a]	17.2±3.4[a,b]	20.5±3.2[a,b]	13.8±2.0[b]
Urea nitrogen (mg/d)	84.8±9.1	60.3±5.0	64.9±13.9	51.4±5.5
CCr (mL/min)	0.88±0.11[b]	0.98±0.15[a,b]	1.18±0.13[a,b]	1.40±0.07[a]

Values were presented in mean±SEM. Different superscript letters in a row indicate a significant difference ($p<0.05$). The various groups are defined in the footnotes of Table 1.

Table 4. Kidney Superoxide Dismutase (SOD), Catalase Activity, Malonaldehyde (MDA), Angiotensin-Converting Enzyme (ACE), Tumor Necrosis Factor-α (TNF-α), Plasminogen Activator Inhibitor-1 (PAI-1), and the Nitrite/Nitrate (NO_x) Concentration of Rats at the End of the Study

	C	E1	E2	E3
SOD (U/mg protein)	36.15±38.42	31.12±4.31	41.62±5.61	36.39±5.45
Catalase (U/mg protein)	342.3±38.4	418.1±50.0	445.1±69.2	315.0±28.8
MDA (μmol/mg protein)	1.50±0.05[a]	1.53±0.11[a]	1.16±0.03[a,b]	1.33±0.08[b]
ACE (mU/mg protein)	28.30±0.56[a]	28.32±1.00[a]	24.84±0.78[b]	24.90±0.68[b]
TNF-α (pg/mg protein)	338.6±34.5[a]	284.5±32.2[a,b]	315.3±38.5[a,b]	212.9±38.1[b]
PAI-1 (ng/mg protein)	63.88±4.13[a]	39.87±1.46[b]	38.43±3.54[b]	27.57±6.16[b]
NO_x (μmol/mg protein)	9.39±1.00[b]	10.90±2.10[b]	10.59±1.06[b]	14.43±1.76[a]

Values are presented as mean±SEM. Different superscript letters in a row indicate a significant difference ($p<0.05$). The various groups are defined in the footnotes of Table 1.

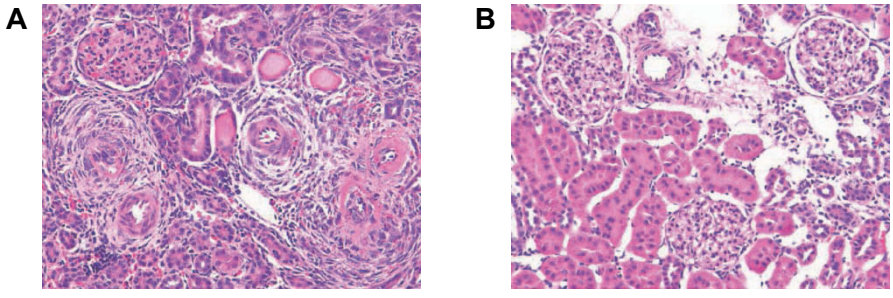

Fig. 1. *Effects of L-NAME and soy protein hydrolysate on small arteries and glomerulus in rat kidney. A: The C group (50 mg/kg/d L-NAME). B: The E3 group (50 mg/kg/d L-NAME with 5% SPH in the diet) (H & E stain ×400).*

After 6 weeks of treatment with L-NAME, both SBP and DBP were significantly elevated, and the diets containing 3% and 5% SPH (the E2 and E3 group) ameliorated the elevation in blood pressure caused by L-NAME (Table 1).

In the analyses of plasma sodium, potassium, chloride, phosphorous, and calcium concentrations, no differences were found among all groups. Blood urea nitrogen of the E3 group was significantly lower than that of the control group ($p<0.05$). There were also lower average values of plasma creatinine concentrations in SPH-fed rats, but no significant difference was found (Table 2). In the urine analysis, we found that urinary protein excretion was significantly lower in the E3 group compared with the C group, and there was also a trend of lower urinary urea nitrogen excretion by the E3 group than the C group ($p=0.058$). We also found that the E3 group fed the diet containing 5% SPH had a higher creatinine clearance rate than the C group (Table 3).

Although no differences were found in SOD or catalase activities between the E3 and the C groups, the MDA concentration of the E3 group was significantly lower than that of the C group (Table 4). Antioxidative enzyme activities and MDA levels in the E1 and E2 group did not differ from those of the C group. Renal ACE activities of the E2 and E3 groups were significantly lower than that of the C group, but there was no difference between the E1 and C groups. The TNF-α concentration in the kidneys was lower in the E3 group than in the C group ($p<0.05$), and lower PAI-1 values were also found in the E1, E2, and E3 groups compared to the control group. The

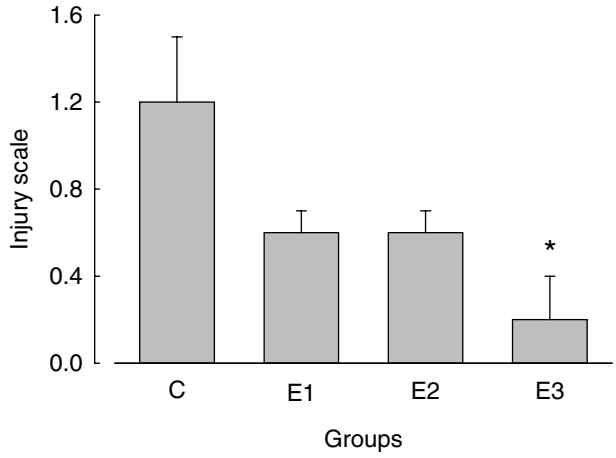

Fig. 2. *Effects of different experimental diets on small artery thickening in rat kidneys. The injury scale was from 0 to 2. Values are presented as the mean±SEM. *Significant difference ($p < 0.05$). C, control; E1, 1% SPH; E2, 3% SPH; E3, 5% SPH.*

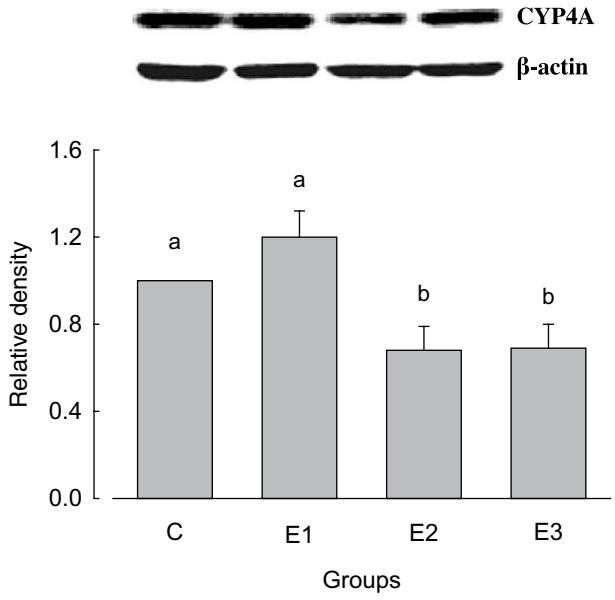

Fig. 3. *Effects of different experimental diets on the expression of CYP4A protein in microsomes from the rat renal cortex. Lanes from left to right were loaded with microsomes prepared from the rat kidney of C (control, 50 mg/kg/d L-NAME), E1 (50 mg/kg/d L-NAME+1% SPH), E2 (50 mg/kg/d L-NAME+3% SPH) and E3 (50 mg/kg/d L-NAME+5% SPH) group. Values are presented as mean±SEM. Different superscript letters indicate a significant difference ($p < 0.05$).*

nitrite plus nitrate concentration in the kidneys of rats in the E3 group was higher than that of the C group, and higher average values were found in the E1 and E2 groups, but without statistical significance.

In the evaluation of histopathological changes in the kidneys, we found that treatment with L-NAME led to reductions in Bowman's space, glomerular congestion, and interstitial inflammation. Hyperplastic changes were also found in the small arteries. Feeding rats the diet containing 5% SPH ameliorated the injuries caused by L-NAME treatment (Fig. 1). The injury scale of small artery thickening in SPH-fed rats decreased, and it was significantly lower in the E3 group compared to the C group (Fig 2).

Figure 3 shows the Western blot of CYP4A and β-actin in kidney homogenates isolated from rats treated with L-NAME and SPH. The semiquantitative results showed that the expressions of CYP4A by the E2 and E3 groups were significantly lower than that of the C group, and no difference was found between the E1 and C groups.

Discussion

In the present study, treatment with an NOS inhibitor elevated blood pressure in rats during the experimental period and caused loss of renal function, including increases in the plasma creatinine level, urea nitrogen level, and proteinuria, and a decrease in the creatinine clearance rate, which results are consistent with previous studies (*4*). We found that consuming a diet containing SPH ameliorated the development of hypertension and ameliorated proteinuria and the reduced creatinine clearance rate. In many clinical studies, ACE inhibitors have been demonstrated to be highly effective against proteinuria and in slowing the loss of the glomerular filtration rate (*2*). In NO-deficient models, captopril, an ACE inhibitor, was also found to normalize SBP, the creatinine clearance rate and proteinuria (*6*). Our previous studies showed that pepsin-digested SPH exhibited ACE inhibitory activities *in vivo* and *in vitro* (*10*, *19*). These results suggest that SPH might ameliorate hypertension and loss of renal function caused by an NO deficiency *via* its ACE inhibition.

In our previous study, we found that SPH was as effective as soy protein in attenuating hypertension and slowing the progression of renal disease in chronic renal failure rats (*20*). SPH derived from soy protein pretreated with digestive enzymes was proven to have ACE inhibitory activity *in vitro* (*21*), and separation of the hydrolysate from peptic-digested soy protein produced 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 (*19*). In addition, hydrolysate derived from pre-digested soy protein was resistant to digestion by proteases of the gastrointestinal tract and showed antihypertensive properties (*7*). Therefore, we used SPH to investigate its renoprotective effects in an L-NAME–induced hypertensive model, and we found a reduction in renal ACE activity and proteinuria in rats fed SPH, but no significant reduction in creatinine was found. Although

captopril was shown to bring high creatinine levels back to normal (*22*), another ACE inhibitor, ramipril, decreased the blood pressure and prevented proteinuria without affecting plasma creatinine in rats with chronic renal failure (*23*). These inconsistent results suggest that different substances with ACE-inhibitory activities may exhibit different effects, and SPH in the present study may have been sufficient to partially correct renal alterations caused by an NO deficiency, but not to completely overcome all of them. In addition, intrarenal NO regulates the angiotensin II response in afferent arterioles and affects renal function (*24*). We also found that the NO_x level in the kidneys was increased with the consumption of SPH; this was consistent with a previous report which showed that soy protein has renal protective effects mediated by restoration of NO production (*25*).

Angiotensin II causes inflammation, production of reactive oxygen species, and blood pressure elevation, and may also be involved in hypertension-induced tissue damage. In our study, we found that the renal MDA concentration in the 5% SPH group was lower than that of the control group, even though no difference was found in antioxidative enzyme activities. Studies have reported that chronic inhibition of NO production increases superoxide production *via* the action of angiotensin II (*26*), and ACE inhibitor therapy prevents this increase of oxidative stress (*27*). One *in vivo* study showed that a soy-rich diet increased the expressions of endothelial NOS and antioxidative enzymes in the vasculature and tissues and led to a reduction of oxidative stress and an increase in NO bioavailability (*28*). However, in rats with chronic nephropathy, consumption of a 20% soy protein diet reduced renal damage without modulating antioxidant enzyme activities (*29*). Thus, SPH with its ACE-inhibitory activity may improve the balance between NO and superoxide to ameliorate renal injuries *via* mechanisms other than modulation of antioxidative enzyme activities.

Studies have demonstrated that blocking the formation of angiotensin II by ACE inhibitors may result in beneficial organ-protective effects in addition to its actions in controlling blood pressure, and this may be explained by the blockade of the angiotensin II–induced pro-inflammatory responses and production of ROS (*30*). In the analysis of TNF-α and PAI-1 levels, our results showed that rats consuming a diet with SPH showed an increased TNF-α and a decreased PAI-1 level in the kidney. Previous animal experiments have shown that angiotensin II infusion led to elevations of TNF-α synthesis and concentration, and these effects were mainly found in the renal tissues (*30*). Imidapril attenuates neointimal formation and TNF-α expression in the vascular wall by blockade of angiotensin II formation and also by activation of the bradykinin-NO system (*31*). In addition, ACE inhibitors may decrease PAI-1 expression *via* bradykinin and promote matrix degradation by activating degradative enzymes to reduce interstitial matrix deposition (*32*). In our histopathological analysis, we also found that SPH ameliorated L-NAME–induced inflammation and vascular and glomerular structural damage in the kidney.

In addition, we found that kidney CYP4A protein expression was down-regulated in SPH-fed rats. Inhibition of NOS led to an increase in CYP4A expression and renal efflux of 20-hydroxyeicosatetraenoic acid (20-HETE) (*33*). 20-HETE is an arachidonic acid metabolite catalyzed by CYP4A, is a potent vasoconstrictor of renal arteries, and is important in the regulation of renal vascular tone. Over-expression of CYP4A caused an increase in vascular 20-HETE production and led to elevations in blood pressure and endothelial dysfunction, including a decrease in NO bioavailability and an increase in oxidative stress (*34*). Studies have also shown that an elevation in the angiotensin II level increases the formation of 20-HETE in the kidneys and peripheral vasculature (*35*) and is related to angiotensin II–induced neointimal growth in injured rat arteries (*36*). Our results indicated that SPH might also affect renal function *via* CYP4A, but further investigation is needed to clarify the underlying mechanisms.

In conclusion, consumption of soy protein hydrolysate can ameliorate the development of hypertension and renal injury in rats with L-NAME–induced hypertension. Our findings that soy protein hydrolysate ameliorates renal damage through its ACE inhibitory activity during NO deficiency reveals one possible mechanism for the beneficial effects of soy protein ingestion, and this might be important in modifying diets to prevent organ damage in the early stage of hypertension.

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