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Nuclear Factor κB-Dependent Anti-inflammatory Effects of *s*-Allyl Cysteine and *s*-Propyl Cysteine in Kidney of Diabetic Mice

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ABSTRACT: Renal protection of *s*-allyl cysteine (SAC) and *s*-propyl cysteine (SPC) in diabetic mice against inflammatory injury was examined. Each agent at 0.5 and 1 g/L was added to the drinking water for 10 weeks. SAC or SPC intake significantly reduced the plasma blood urea nitrogen level and increased creatinine clearance ($P < 0.05$). These treatments significantly lowered the renal level of reactive oxygen species, nitric oxide, interleukin-6, tumor necrosis factor-α, and prostaglandin E_2 in diabetic mice ($P < 0.05$). Renal mRNA expression of inducible nitric oxide synthase, cyclooxygenase-2, protein kinase C (PKC)-α, PKC-β, and PKC-γ was enhanced in diabetic mice ($P < 0.05$); however, SAC or SPC treatments dose dependently declined mRNA expression of these factors ($P < 0.05$). Nuclear factor κB (NF-κB) activity, mRNA expression, and protein production in kidney of diabetic mice were significantly increased ($P < 0.05$). SAC or SPC intake dose dependently suppressed NF-κB activity, NF-κB p65 mRNA expression, and protein level ($P < 0.05$). Diabetes also enhanced renal protein expression of mitogen-activated protein kinase ($P < 0.05$). SAC and SPC, only at a high dose, significantly suppressed protein production of p-p38 and p-ERK1/2 ($P < 0.05$). Renal mRNA expression and protein generation of peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ were significantly down-regulated in diabetic mice ($P < 0.05$), but the intake of SAC or SPC at high dose up-regulated PPAR-α and PPAR-γ ($P < 0.05$). These findings support that SAC and SPC are potent anti-inflammatory agents against diabetic kidney diseases.

KEYWORDS: *s-allyl cysteine, s-propyl cysteine, diabetes, NF-κB, MAPK, PPAR*

INTRODUCTION

Diabetic renal injury, or so-called diabetic nephropathy, is one diabetic complication. It has been documented that inflammation contributes to the development of diabetic nephropathy, which impairs renal functions and exacerbates the severity and mortality of diabetes.[1,2] The overproduced pro-inflammatory cytokines and chemokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α in patients with diabetic nephropathy have been reported.[3,4] These inflammatory factors not only raise diabetes-associated renal inflammatory stress but also disturb systemic immune functions. Nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) are crucial signaling pathways responsible for transcriptional activation or induction of genes that are involved in the initiation of inflammatory reactions. Diabetic pathological stress, like hyperglycemia, stimulates the activation of NF-κB and MAPK pathways, which evoke the excessive production of inflammatory, oxidative, and even fibrotic molecules and promote the pathogenesis of diabetic nephropathy.[5,6] In addition, protein kinase C (PKC) is an upstream regulator for pro-inflammatory cytokines, and enhanced PKC activity contributes to inflammatory deterioration under diabetic conditions.[7] Consequently, renal dialysis, and even renal replacement, is necessary for people with diabetic nephropathy to survive. Thus, any agent(s) with anti-inflammatory activities to lower inflammatory cytokines that suppress NF-κB, MAPK, and PKC may potentially prevent or delay the development of diabetic renal injury. On the other hand, peroxisome proliferator-activated receptors (PPARs), especially PPAR-α, could block NF-κB and MAPK pathways, which in turn declines inflammatory progression.[8,9] Thus, the therapeutic potentials of PPAR agonists have been suggested.

s-Allyl cysteine (SAC), and *s*-propyl cysteine (SPC) are two hydrophilic cysteine-containing compounds naturally formed in *Allium* plants such as garlic and onion.[10,11] Our past study found that the intake of these compounds at 1 g/L exhibited antidiabetic protection including antihyperglycemic, antihyperlipidemic, and antihemostatic effects in diabetic mice.[12] Our other study indicated that these compounds at the dosage of 1 g/L inhibited hepatic release of IL-6 and TNF-α in acetaminophen-induced mice.[13] In addition, these compounds could decrease the brain level of reactive oxygen species (ROS), a stimulator for NF-κB, in mice under Alzheimer's diseaselike conditions.[14] Kim et al.[15] reported that SAC could suppress NF-κB activation and lower nitric oxide (NO) production in interferon-γ-stimulated RAW264.7 cells. Those previous studies implied that SAC and SPC are potent anti-inflammatory agents. However, it remains unknown that they could alleviate diabetic nephropathy via reducing inflammatory stress. Also, less information is available regarding their impact upon upstream inflammation-associated regulators such as NF-κB, PPARs, and MAPK in kidney tissue.

The major purpose of this study was to investigate the renal protection of SAC and SPC in diabetic mice against

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inflammatory injury. The possible action modes from these agents were evaluated.

MATERIALS AND METHODS

Animals and Diets. Male Balb/cA mice, 3−4 weeks old, were obtained from National Laboratory Animal Center (National Science Council, Taipei City, Taiwan). Mice were housed on a 12 h light:dark schedule; water and mouse standard diet were consumed ad libitum. The use of mice was reviewed and approved by China Medical University Animal Care Committee (99-22-N). To induce diabetes, mice with body weights of 24.3 ± 0.7 g were treated with streptozotocin (40 mg/kg body weight in 0.1 mol/L citrate buffer, pH 4.5) ip for 5 consecutive days. The blood glucose level was monitored on day 10 from the tail vein using a one-touch blood glucose meter (Lifescan, Inc., Milpitas, CA). Mice with fasting blood glucose levels ≥14.0 mmol/L were used for this study. After diabetes was induced, mice were divided into several groups (10 mice per group).

Experimental Design. SAC (99%) and SPC (99.5%) were supplied by Wakunaga Pharmaceutical Co. (Hiroshima, Japan). Each agent at 0.5 and 1 g/L was added to the drinking water. All mice had free access to food and water at all times. The consumed water volume, feed intake, and body weight were recorded weekly. Twenty-four hour urine output collected by metabolic cage was measured at week 9. After 10 weeks of supplementation, mice were fasted overnight and sacrificed with carbon dioxide. Blood and kidney were collected. Plasma was separated from erythrocytes immediately. Kidney at 0.1 g was homogenized on ice in 2 mL of phosphate buffer saline (PBS, pH 7.2), and the filtrate was collected. The protein concentrations of plasma or kidney filtrate were determined by the method of Lowry et al.[16] using bovine serum albumin as a standard. In all experiments, the sample was diluted to a final concentration of 1 g protein/L using PBS, pH 7.2.

Blood and Urinary Analyses. The plasma glucose level was measured by a glucose HK kit (Sigma Chemical Co., St. Louis, MO). The plasma insulin level was measured by using a rat insulin radioimmunoassay kit (Linco Research Inc., St. Charles, MO). The plasma blood urea nitrogen (BUN), plasma creatinine (Cr), and urinary Cr concentrations were detected by a Beckman Autoanalyzer (Beckman Coulter, Fullerton, CA). The creatinine clearance rate (CCr) was calculated according to Cockcroft−Gault formula, [urinary Cr (mg/dL) × urinary volume (mL)] ÷ [plasma Cr (mg/dL) × length of urine collection (min)], and expressed as mL/min/100 g body weight.

Determination of ROS, NO, and Prostaglandin E $(PGE)_2$. The method described in Gupta et al.[17] was used to measure the ROS level. Briefly, 10 mg of renal tissue was homogenized in 1 mL of ice cold 40 mM Tris−HCl buffer (pH 7.4) and further diluted to 0.25% with the same buffer. Then, samples were divided into two equal fractions. In one fraction, 40 μL of 1.25 mM 2′,7′-dichlorofluorescin diacetate in methanol was added for ROS estimation. Another fraction, in which 40 μL of methanol was added, served as a control for autofluorescence, which was determined at 488 nm excitation and 525 nm emission using a fluorescence plate reader. The production of NO was determined by measuring the formation of nitrite. Briefly, 100 μL of supernatant was mixed with 100 μL of Griess reagent after centrifugation at 10000*g* for 15 min at 4 °C. The optical absorbance at 540 nm was measured and compared with a sodium nitrite standard curve. The production of PGE_2 was determined using a PGE_2 EIA kit (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's instructions.

Renal Cytokines Determination. Perfused renal tissue was homogenized in 10 mM Tris-HCl buffered solution (pH 7.4) containing 2 M NaCl, 1 mM ethylenediaminetetraacetic acid, 0.01% Tween 80, and 1 mM phenylmethylsulfonyl fluoride and centrifuged at 9000*g* for 30 min at 4 °C. The resultant supernatant was used for cytokine determination. The levels of IL-6 and TNF-α were measured by ELISA using cytoscreen immunoassay kits (BioSource International, Camarillo, CA). Samples were assayed in duplicates according to the manufacturer's instructions.

Activity of Total NOS and Cyclooxygenase-2 (COX-2). The method described in Sutherland et al.[18] was used to measure the total NOS activity. Briefly, it was determined via incubating 30 μL of homogenate with 10 mM β-nicotinamide adenine dinucleotide phosphate, 10 mM L-valine, 3000 U/mL calmodulin, 5 mM tetrahydrobiopterin, 10 mM $CaCl_2$, and a mixture of 100 μM L-arginine containing L-[^{3}H]arginine. The COX-2 activity was assayed by a commercial assay kit (Cayman Chemical Co.) and colorimetrically monitoring the appearance of oxidized *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine at 590 nm.

Measurement of Renal Glomeruli PKC Activity. The method described in Koya et al.[19] was used to measure the glomeruli PKC activity. Briefly, bilateral kidneys were dissected and homogenized in ice-cold RPMI1640 media containing 20 mM HEPES. Glomeruli were isolated by removing the capsules and passed through sieves of various sizes. After they were washed twice with RPMI1640 media containing 20 mM HEPES and once with a mixed salt solution, glomeruli were incubated with a salt solution for 15 min in the presence or absence of 100 μM PKC-specific substrate, RTLRRL, and followed by adding 5 mg/mL digitonin and 1 mM ATP mixed with γ-[^{32}P]ATP (<1500 cpm/pmol). The reaction was stopped by 5% trichloroacetic acid, then spotted onto P81 phosphocellulose paper, and washed four times with 1% phosphoric acid and once with acetone. The amount of incorporated radioactivity into the substrate was determined by scintillation counting. The PKC activity was normalized by the corresponding protein content.

NF-κB p50/65 Assay. NF-κB p50/65 DNA binding activity in nuclear extract of kidney tissue was determined by a commercial kit (Chemicon International Co., Temecula, CA). The binding of activated NF-κB was examined by adding a primary polyclonal anti-NF-κB p50/p65 antibody, and a secondary antibody conjugated with horseradish peroxidase, and the 3,3′,5,5′-tetramethylbenzidine substrate. The absorbance at 450 nm was read. Values are expressed as relative optical density (OD) per mg protein.

Real-Time Polymerase Chain Reaction (PCR) for mRNA Expression. The total RNA was isolated using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA). One microgram of RNA was used to generate cDNA, which was amplified using Taq DNA polymerase. PCR was carried out in 50 μL of reaction mixture containing Taq DNA polymerase buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 200 mM dNTP, 2.5 mM $MgCl_2$, and 0.5 mM concentration of each primer) and 2.5 U Taq DNA polymerase. The specific oligonucleotide primers of targets are shown in Table 1. The cDNA

Table 1. Forward and Reverse Primers for Real-Time PCR Analysis

target	forward	reverse
COX-2	5′-CAG CAA ATC CTT GCT GTT-3′	5′-TGG GCA AAG AAT GCA AAC ATC-3′
iNOS	5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′	5′-TGT CAG AGA GCC TCG TGG CTT TGG-3′
PPAR-α	5′-CTG CAG AGC AAC CAT CCA GAT-3′	5′-GCC GAA GGT CCA CCA TTT T-3′
PPAR-γ	5′-TCC GTG ATG GAA GAC CAC TC-3′	5′-CCC TTG CAT CCT TCA CAA GC-3′
PKC-α	5′-GAA CCA TGG CTG ACG TTT AC-3′	5′-GCA AGA TTG GGT GCA CAA AC-3′
PKC-β	5′-TTC AAG CAG CCC ACC TTC TG-3′	5′-AAG GTG GCT GAA TCT CCT TG-3′
PKC-γ	5′-GAC CCC TGT TTT GCA GAA AG-3′	5′-GTA AAG CCC TGG AAA TCA GC-3′
NF-κB p50	5′-GGA GGC ATG TTC GGT AGT GG-3′	5′-CCC TGC GTT GGA TTT CGT G-3′
NF-κB p65	5′-GCG TAC ACA TTC TGG GGA GT-3′	5′-CCG AAG CAG GAG CTA TCA AC-3′
GAPDH	5′-TGA TGA CAT CAA GAA GGT GGT GAA G-3′	5′-CCT TGG AGG CCA TGT AGG CCA T-3′

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Table 2. Water Intake (WI, mL/Mouse/Day), Feed Intake (FI, g/Mouse/Day), Body Weight (BW, g/Mouse), Kidney Weight (g/Mouse), Kidney Weight-to-Body Weight Ratios (g/100 g BW), and Urine Output (mL/Mouse/Days) of Nondiabetic (non-DM), Diabetic Mice (DM) Consumed Water, or 0.5 or 1 g/L SAC or SPC[a]

			DM + SAC		DM + SPC	
	non-DM	DM	0.5	1	0.5	1
			WI			
week 2	1.8 ± 0.7 a	3.1 ± 1.1 b	3.2 ± 0.8 b	3.4 ± 1.0 b	3.0 ± 0.6 b	3.3 ± 1.2 b
week 10	2.1 ± 0.6 a	7.9 ± 2.0 d	6.4 ± 1.3 c	5.3 ± 1.2 b	6.8 ± 0.9 c	5.0 ± 0.7 b
			FI			
week 2	1.0 ± 0.4 a	2.1 ± 0.8 b	2.0 ± 0.9 b	1.8 ± 1.0 b	2.2 ± 0.7 b	2.0 ± 0.5 b
week 10	2.1 ± 1.0 a	8.4 ± 2.2 d	7.0 ± 1.6 c	5.3 ± 1.4 b	6.8 ± 1.5 c	5.1 ± 0.9 b
			BW			
week 2	23.9 ± 1.2 b	20.8 ± 1.9 a	20.0 ± 1.5 a	20.5 ± 1.3 a	21.1 ± 1.6 a	20.2 ± 1.0 a
week 10	35.1 ± 3.4 d	11.3 ± 1.8 a	14.4 ± 1.2 b	16.8 ± 0.8 c	15.0 ± 1.1 b	17.1 ± 0.7 c
kidney weight, week 10	0.51 ± 0.12 a	0.42 ± 0.10 a	0.43 ± 0.05 a	0.47 ± 0.06 a	0.41 ± 0.05 a	0.45 ± 0.09 a
kidney/BW, week 10	1.45 ± 0.23 a	3.76 ± 0.31 d	3.09 ± 0.15 c	2.73 ± 0.20 b	2.71 ± 0.25 b	2.63 ± 0.19 b
urine volume, week 9	0.63 ± 0.13 a	7.04 ± 1.12 c	6.54 ± 0.78 c	4.85 ± 0.61 b	6.37 ± 1.03 c	4.52 ± 0.59 b

[a]Data are means ± SDs, $n = 10$. For letters a–d, means in a row without a common letter differ, $P < 0.05$.

Table 3. Plasma Level of Glucose (mmol/L), Insulin (nmol/L), BUN (mg/dL), and CCr (mL/min/100 g Body Weight) of Nondiabetic (non-DM), Diabetic Mice (DM) Consumed Water, or 0.5 or 1 g/L SAC or SPC[a]

			DM + SAC		DM + SPC	
	non-DM	DM	0.5	1	0.5	1
glucose	9.2 ± 1.1 a	28.6 ± 3.3 d	23.5 ± 1.9 c	18.2 ± 1.5 b	22.6 ± 2.0 c	17.2 ± 1.3 b
insulin	12.9 ± 1.3 e	4.0 ± 0.7 a	5.1 ± 0.4 b	6.5 ± 0.9 c	6.2 ± 1.1 c	8.1 ± 0.8 d
BUN	5.8 ± 0.5 a	60.2 ± 4.1 d	48.5 ± 3.2 c	29.7 ± 2.6 b	45.3 ± 2.9 c	25.7 ± 3.0 b
CCr	1.64 ± 0.31 e	0.39 ± 0.08 a	0.56 ± 0.07 b	0.83 ± 0.10 c	0.77 ± 0.06 c	1.07 ± 0.11 d

[a]Data are means ± SDs, $n = 10$. For letters a–d, means in a column without a common letter differ, $P < 0.05$.

Table 4. Renal Level of ROS (nmol/mg Protein), NO (μM/mg Protein), IL-6 (pg/mL), TNF-α (pg/mL), and PGE_2 (pg/g Protein) of Nondiabetic (non-DM), Diabetic Mice (DM) Consumed Water, or 0.5 or 1 g/L SAC or SPC[a]

			DM + SAC		DM + SPC	
	non-DM	DM	0.5	1	0.5	1
ROS	0.23 ± 0.04 a	1.38 ± 0.11 d	0.96 ± 0.09 c	0.46 ± 0.06 b	1.05 ± 0.08 c	0.53 ± 0.05 b
NO	5.3 ± 1.0 a	32.6 ± 1.3 d	25.1 ± 0.9 c	19.6 ± 0.7 b	23.5 ± 0.8 c	18.8 ± 1.0 b
IL-6	21 ± 3 a	241 ± 20 d	193 ± 15 c	124 ± 10 b	201 ± 18 c	135 ± 12 b
TNF-α	18 ± 2 a	335 ± 27 d	276 ± 21 c	200 ± 14 b	290 ± 22 c	215 ± 16 b
PGE_2	983 ± 55 a	2138 ± 167 f	1790 ± 89 e	1320 ± 49 c	1594 ± 101 d	1131 ± 70 b

[a]Data are means ± SDs, $n = 10$. For letters a–f, means in a column without a common letter differ, $P < 0.05$.

was amplified under the following reaction conditions: 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Twenty-eight cycles were performed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, the housekeeping gene), and 35 cycles were performed for others. Generated fluorescence from each cycle was quantitatively analyzed by using the Taqman system based on real-time sequence detection system (ABI Prism 7700, Perkin-Elmer Inc., Foster City, CA). In this study, the mRNA level was calculated as a percentage of the control group.

Western Blot Analysis. Kidney tissue was homogenized in buffer containing 0.5% Triton X-100 and protease-inhibitor cocktail (1:1000, Sigma-Aldrich Chemical Co.). This homogenate was further mixed with buffer (60 mM Tris-HCl, 2% SDS, and 2% β-mercaptoethanol, pH 7.2) and boiled for 5 min. The sample at 40 μg of protein was applied to 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, Bedford, MA) for 1 h. After blocking with a solution containing 5% nonfat milk for 1 h to prevent nonspecific binding of antibody, the membrane was incubated with mouse anti-NF-κB p50 (1:1000), anti-NF-κB p65 (1:1000), anti-MAPK (1:2000), anti-PPAR-α (1:1000), or anti-PPAR-γ (1:1000) monoclonal antibody (Boehringer-Mannheim, Indianapolis, IN) at 4 °C overnight, followed by reacting with horseradish peroxidase-conjugated antibody for 3.5 h at room temperature. The detected bands were quantified by Scion Image analysis software (Scion Corp., Frederick, MD), and GAPDH was used as a loading control.

Statistical Analysis. All data were expressed as the mean ± standard deviation (SD). Statistical analysis was done using one-way analysis of variance, and posthoc comparisons were carried out using Dunnet's t test. P values <0.05 were considered as significant.

RESULTS

As shown in Table 2, the intake of SAC or SPC dose dependently decreased water intake and feed intake and increased body weight in diabetic mice ($P < 0.05$). Diabetes caused a significant increase in the kidney weight-to-body weight ratios ($P < 0.05$). The intake of SAC or SPC significantly declined these ratios ($P < 0.05$) but only at high dose lowered urine output ($P < 0.05$). SAC or SPC intake also significantly reduced glucose and BUN levels (Table 3, $P < 0.05$) and increased insulin and CCr levels ($P < 0.05$). As shown in Table 4, SAC or SPC intake dose dependently lowered renal levels of ROS, NO, IL-6, TNF-α, and PGE_2 in

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Table 5. Renal Activity of Total NOS (pmol/min/mg Protein), COX-2 (U/mg Protein), and PKC (pmol/min/mg Protein) in Nondiabetic (non-DM), Diabetic Mice (DM) Consumed Water, or 0.5 or 1 g/L SAC or SPC[a]

			DM + SAC		DM + SPC	
	non-DM	DM	0.5	1	0.5	1
total NOS	8.4 ± 1.0 a	41.3 ± 1.7 d	34.5 ± 1.9 c	21.7 ± 1.3 b	31.8 ± 1.6 c	20.5 ± 1.0 b
COX-2	0.31 ± 0.06 a	2.71 ± 0.13 f	2.21 ± 0.19 e	1.44 ± 0.07 c	1.80 ± 0.10 d	0.95 ± 0.08 b
PKC	6.3 ± 0.5 a	21.4 ± 2.0 d	17.2 ± 1.8 c	12.4 ± 0.9 b	17.1 ± 1.1 c	13.2 ± 0.6 b

[a]Data are means ± SDs, $n = 10$. For letters a–f, means in a column without a common letter differ, $P < 0.05$.

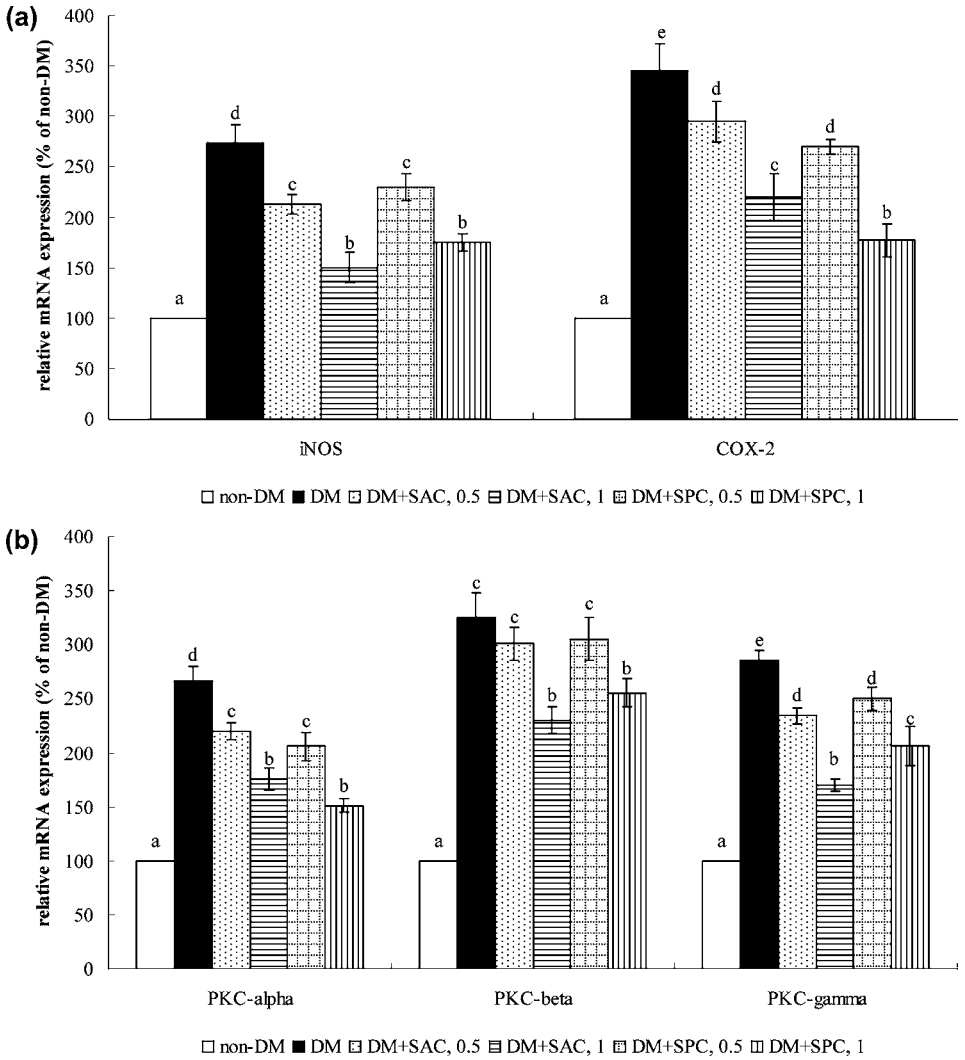

Figure 1. mRNA expression of iNOS, COX-2 (a), PKC-α, PKC-β, and PKC-γ (b) in kidney of nondiabetic (non-DM), diabetic mice (DM) consumed water, or 0.5 or 1 g/L SAC or SPC. Data are means ± SDs, $n = 10$. Means among bars without a common letter differ; $P < 0.05$.

diabetic mice ($P < 0.05$). Renal activities of total NOS, COX-2, and PKC were enhanced in diabetic mice; however, SAC or SPC treatments diminished the activity of these factors (Table 5, $P < 0.05$). Renal mRNA expression of iNOS, COX-2, PKC-α, PKC-β, and PKC-γ was raised in diabetic mice (Figure 1, $P < 0.05$). SAC or SPC treatments dose dependently declined mRNA expression of iNOS, COX-2, PKC-α, and PKC-γ ($P < 0.05$).

NF-κB activity, mRNA expression, and protein production in kidney of diabetic mice were significantly increased (Figure 2, $P < 0.05$). SAC or SPC intake dose dependently suppressed NF-κB activity, NF-κB p65 mRNA expression, and protein level ($P < 0.05$) but only at high dose down-regulated NF-κB p50 mRNA expression and its protein production ($P < 0.05$). Diabetes also enhanced protein production of MAPK (p38, ERK1/2, and JNK) (Figure 3, $P < 0.05$). SAC and SPC, only at high dose, significantly suppressed protein production of p-p38 and p-ERK1/2 ($P < 0.05$). As shown in Figure 4, renal mRNA expression and protein level of PPAR-α and PPAR-γ were significantly down-regulated in diabetic mice ($P < 0.05$). The intake of SAC or SPC at high dose up-regulated PPAR-α and PPAR-γ expression ($P < 0.05$).

DISCUSSION

On the basis of the increased urine output and BUN levels and decreased CCr, these diabetic mice exhibited pathological

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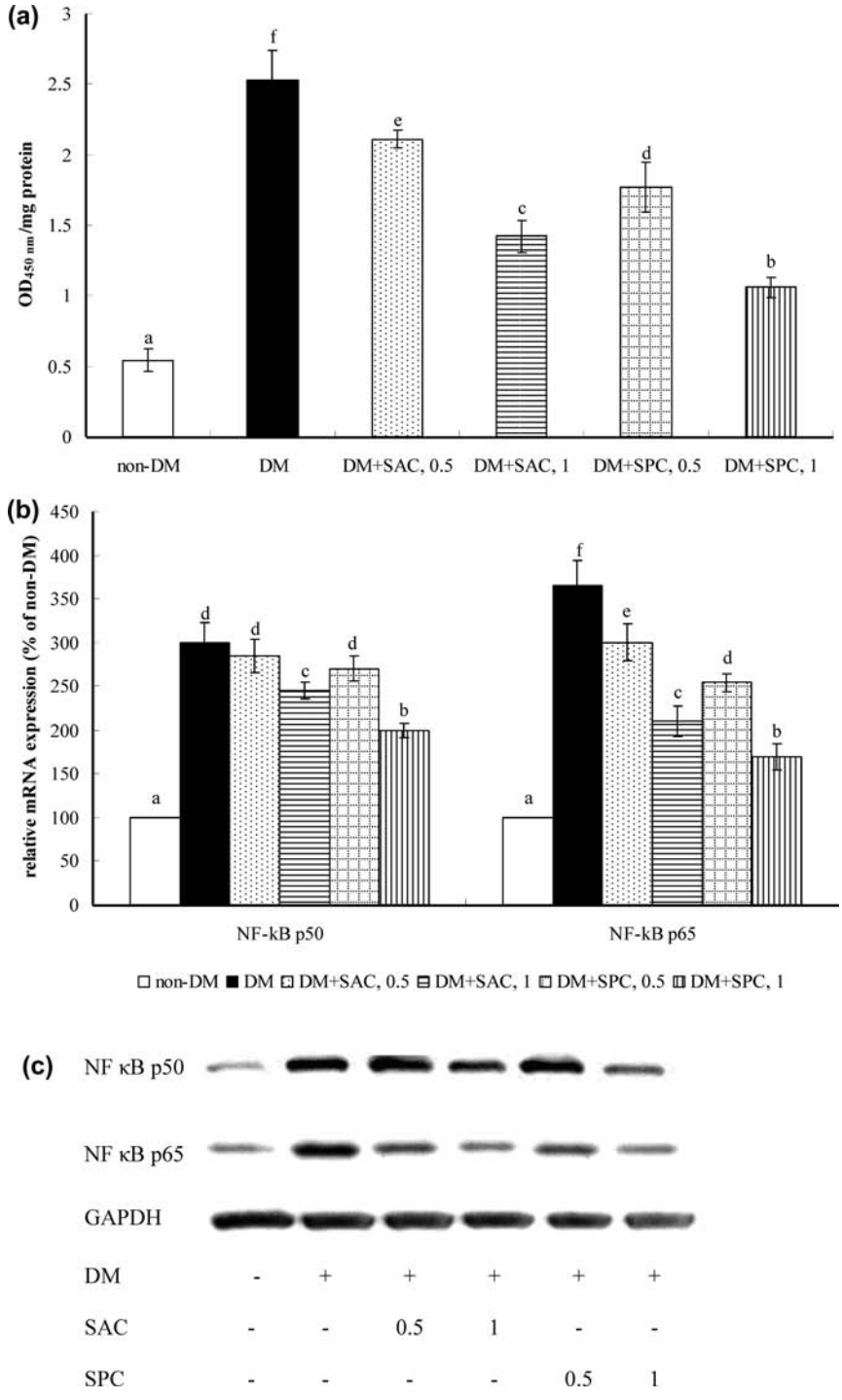

Figure 2. Activity (a), mRNA expression (b), and protein production (c) of NF-κB p50 and NF-κB p65 in kidney of nondiabetic (non-DM), diabetic mice (DM) consumed water, or 0.5 or 1 g/L SAC or SPC. Data are means ± SDs, $n = 10$. Means among bars without a common letter differ, $P < 0.05$.

characteristics of diabetic renal disease. The increased kidney weight-to-body weight ratios, an indicator of renal hypertrophy,[20] in these diabetic mice also suggested the development of diabetic nephropathy. Moreover, the enhanced renal release of IL-6, TNF-α, and PGE_2 revealed that an inflammatory response was involved in the progression of diabetic nephropathy. However, we found that the intake of SAC or SPC effectively lowered renal levels of IL-6, TNF-α, and PGE_2. Both mRNA expression and Western blot data indicated that SAC or SPC intake regulated renal expression of inflammation-associated molecules, NF-κB, MAPK, PKC, COX-2, and PPAR and mitigated inflammatory stress. The decreased urine output, BUN level, kidney weight-to-body weight ratios, and elevated CCr in SAC- or SPC-treated mice also agreed that diabetes-induced renal hypertrophy had be attenuated, and renal functions had been improved. These findings support that these compounds could provide anti-inflammatory protection against diabetic nephropathy through molecular and transcriptional actions.

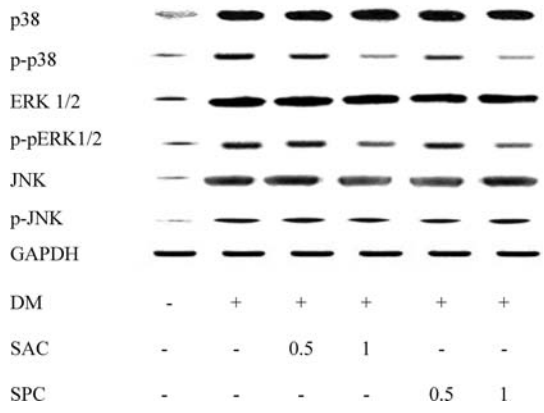

Figure 3. Protein production of MAPK in kidney of nondiabetic (non-DM), diabetic mice (DM) consumed water, or 0.5 or 1 g/L SAC or SPC. Data are means ± SDs, $n = 10$.

Both hyperglycemia and ROS are crucial activators for signal transduction cascades of NF-κB and MAPK.[21] Once activated, NF-κB regulates the gene expression of many mediators involved in inflammatory reactions, such as iNOS, TNF-α, and COX-2.[22,23] Therefore, NF-κB has been considered as a target for novel anti-inflammatory therapy. Our previous and present studies found that SAC and SPC are effective antihyperglycemic and anti-ROS agents.[12−14] Thus, the intake of these compounds could alleviate diabetic progression via decreasing these upstream stimulators. Furthermore, we found that dietary supplementation of SAC or SPC markedly suppressed the activity, mRNA expression, and protein production of NF-κB p50 and p65 in kidney, which in turn reduced renal activity and expression of iNOS and COX-2. These findings not only explained the lower release of pro-inflammatory cytokines, nitric oxide, and PGE_2 but also supported that the anti-inflammatory actions of SAC and SPC were NF-κB-dependent. In addition, the activation of MAPK pathways, especially p38 MAPK pathway, in kidney of diabetic mice induced the production of IL-6, IL-1, and TNF-α and facilitated the progression of diabetic nephropathy.[24,25] We notified that SAC or SPC, at high dose, abated phosphorylation of p38 and pERK1/2 based on the observed lower protein expression of p-p 38 and p-p ERK1/2. Via down-regulating p38 and ERK1/2 MAPK pathways, the downstream inflammatory reactions were diminished, and renal production of IL-6 and TNF-α was decreased. These results indicated that the anti-inflammatory actions of these agents against diabetic nephropathy were partially MAPK-dependent.

Elevated activity of NOS and COX-2 enhanced the generation of nitric oxide and PGE_2, which raised oxidative and inflammatory stress.[26,27] Our present study found that either SAC or SPC effectively declined renal activity and/or mRNA expression of total NOS, iNOS, and COX-2, which subsequently reduced the production of nitric oxide and PGE_2 in diabetic mice. Thus, the anti-inflammatory effect of SAC or SPC was partially due to their direct inhibition on NOS and COX-2. In addition, it is reported that increased activity of PKC, especially PKC-α and PKC-β, promoted inflammatory deterioration of diabetic nephropathy, through altering enzyme

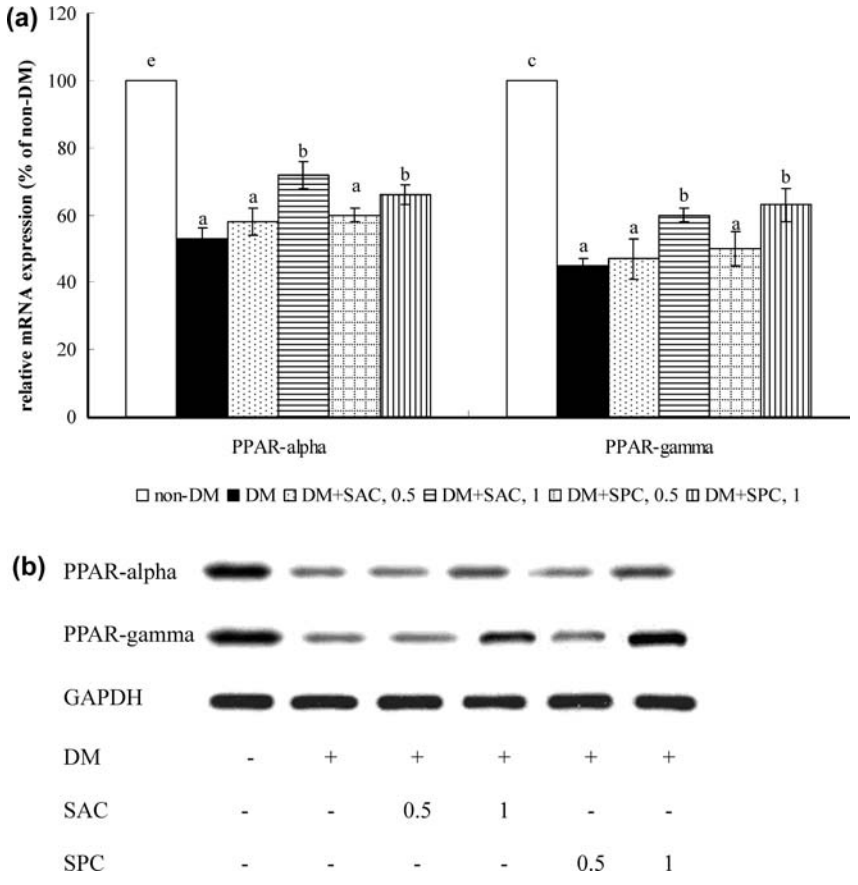

Figure 4. mRNA (a) and protein production (b) of PPAR-α and PPAR-γ in kidney of nondiabetic (non-DM), diabetic mice (DM) consumed water, or 0.5 or 1 g/L SAC or SPC. Data are means ± SDs, $n = 10$. Means among bars without a common letter differ, $P < 0.05$.

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activity of Na^+-K^+-ATPase and MAPK and increasing the formation of pro-inflammatory cytokines.[28] Kumar et al.[29] reported that PKC activation under hyperglycemic condition led to a preferential activation of NF-κB p65, which was further responsible for the up-regulation of other genes associated with diabetic pathogenesis. We notified that enhanced renal activity and mRNA expression of PKC-α, -β, and -γ in diabetic mice were counteracted by SAC or SPC, which contributed to alleviate renal inflammatory stress via diminishing the activation of NF-κB and decreasing renal release of IL-6 and TNF-α. These results implied that SAC and SPC had the ability to directly mediate NOS, COX-2, and PKC. Obviously, these compounds could attenuate diabetic nephropathy via multiple actions.

PPARs are nuclear transcription factors, and activated PPARs could reduce the production of pro-inflammatory cytokines, COX-2, and iNOS by inhibiting the transcriptional activity of NF-κB p65.[30] The renal protective effects of PPAR-α or PPAR-γ agonists against inflammation have been reported.[31,32] Those authors indicated that PPAR-α agonist could diminish renal inflammation via mitigating oxidative stress and decreasing phosphorylation of p38 and JNK in kidney. In our present study, SAC or SPC intake at high dose restored renal mRNA expression and protein production of PPAR-α and PPAR-γ, which further suppressed NF-κB activation and lowered the formation of inflammatory factors. Apparently, these compounds at high doses could ameliorate diabetic nephropathy via up-regulating PPARs. These findings indicated that SAC and SPC could be considered as PPAR activators. Besides regulating inflammatory signaling, PPARs also mediate insulin sensitivity and lipid metabolism.[33] Therefore, the improved glycemic control as observed in those diabetic mice could be partially ascribed to SAC or SPC enhance PPARs expression.

It is interesting to find that antidiabetic effects of SAC and SPC were not identical. SPC possessed greater up-regulating activity upon PPAR-α, and SAC was greater in down-regulating NF-κB. It seems that the allyl group of SAC and propyl group of SPC determined their functions. Although our studies indicated that SAC and SPC could regulate renal inflammatory-associated factors and exhibit anti-inflammatory effects, further study is necessary to examine their efficiency and safety before they are used for human. Furthermore, it should be pointed out that it may not be appropriate to obtain these compounds by supplementing the diet with garlic or other *Allium* plants because the content of these compounds changes from species to species and from season to season.[34]

In summary, the dietary intake of SAC and SPC suppressed protein expression of NF-κB, MAPK, and PKC in kidney of diabetic mice, which in turn declined renal activity and mRNA expression of NOS and COX-2, finally lowered the formation of ROS, nitric oxide, IL-6, TNF-α, and PGE_2, as well as improved renal functions. Therefore, SAC and SPC were potent anti-inflammatory agents against diabetic nephropathy.

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