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Anti-inflammatory and Antifibrotic Effects of Naringenin in Diabetic Mice

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ABSTRACT: Renal protective effects of naringenin at 0.5, 1, and 2% of the diet in diabetic mice were examined. Naringenin supplemented at 1 and 2% increased its deposit in liver and kidney of diabetic mice. Compared with the diabetic control group, naringenin treatments at 1 and 2% lowered plasma levels of glucose and blood urea nitrogen, as well as increased insulin level and creatinine clearance ($P < 0.05$). Naringenin treatments dose-dependently reduced renal tumor necrosis factor-α level and expression ($P < 0.05$) but only at 1 and 2% significantly decreased production and expression of interleukin (IL)-1β, IL-6, and monocyte chemoattractant protein-1 ($P < 0.05$). Naringenin intake at 2% decreased renal formation and expression of type IV collagen, fibronectin, and transforming growth factor-β1 ($P < 0.05$). This compound at 1 and 2% lowered protein kinase C activity and suppressed nuclear factor κB (NF-κB) p65 activity, mRNA expression, and protein production in kidney. However, this agent only at 2% diminished NF-κB p50 activity, mRNA expression, and protein production ($P < 0.05$). These results indicate that naringenin could attenuate diabetic nephropathy via its anti-inflammatory and antifibrotic activities.

KEYWORDS: *naringenin, diabetes, nuclear factor κB, protein kinase C*

INTRODUCTION

Diabetic renal injury, or so-called diabetic nephropathy, is one diabetic complication. It has been documented that inflammation and fibrosis contribute to the development of diabetic nephropathy, which deteriorates renal functions and exacerbates the severity and mortality of diabetes.[1−3] The overproduction of pro-inflammatory cytokines and chemokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and monocyte chemoattractant protein (MCP)-1 in patients with diabetic nephropathy has been reported.[4,5] These inflammatory factors not only raise diabetes-associated renal inflammatory stress but also disturb systemic immune functions. The up-regulation of renal transforming growth factor (TGF)-β1 is the other characteristic of diabetic nephropathy, because increased TGF-β1 leads to massive production of extracellular matrix (ECM), including fibronectin and type IV collagen.[6,7] These ECMs, also called structural and supporting molecules, elicit glomerular trophy, basement membrane thickening, and mesangial expansion and finally lead to renal fibrosis. Both nuclear factor κB (NF-κB) and protein kinase C (PKC) are upstream regulators for pro-inflammatory cytokines and ECMs. The activated NF-κB and enhanced PKC activity promote inflammatory and fibrotic progression under diabetic conditions.[3,8] Consequently, renal dialysis, and even renal replacement, is necessary for people with diabetic nephropathy to survive. Thus, any agent with anti-inflammatory and antifibrotic effects may potentially prevent or delay the development of diabetic renal injury.

Naringenin is a flavonoid commonly occurring in many plant foods such as grapefruit, lemon, and tomato. It has been documented that this compound possesses antioxidative and antihyperlipidemic activities.[9,10] The antidiabetic effects of naringenin have been investigated. Mulvihill et al.[10] reported that the dietary intake of this compound at 1−3% improved insulin resistance. Li et al.[11] indicated that the antihyperglycemic action of naringenin was partially ascribed to its inhibition of renal glucose uptake and renal glucose reabsorption. So far, less information is available regarding the protective effects of naringenin against diabetic renal injury. In addition, the study of Lin and Lin[12] revealed that naringenin could modulate the release of pro-inflammatory cytokines, IL-6 and TNF-α, and exhibit anti-inflammatory activities in primary mouse splenocytes. Liu et al.[13] reported that naringenin decreased the expression of ECMs in rat hepatic stellate cells and mitigated fibrotic progression. Those previous studies suggested that naringenin is an anti-inflammatory and antifibrotic agent; however, it is unclear that this compound is able to alleviate renal inflammatory and fibrotic stress under diabetic conditions.

The purpose of this study was to investigate the anti-inflammatory and antifibrotic effects of naringenin in the kidney of diabetic mice. The possible action modes of this agent for renal protection were also evaluated. These results are helpful for understanding the potent application of naringenin as an antidiabetic agent.

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MATERIALS AND METHODS

Materials. Naringenin (99%) was purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals used in these measurements were of the highest purity commercially available.

Animals. Male BALB/cA mice, 3−4 weeks old, were obtained from the 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 the China Medical University Animal Care Committee. To induce diabetes, mice with body weights of 23.1 ± 1.2 g were treated with a single intravenous (iv) dose (50 mg/kg) of streptozotocin dissolved in citrate buffer (pH 4.5) into the tail vein under fasting status. 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. Naringenin at 1−3% was used in the animal study of Mulvihill et al.[10] Our present study examined the antidiabetic effects of this compound at lower doses, 0.5−2%. Thus, naringenin at 0.5, 1, or 2 g was mixed with 99.5, 99, or 98 g of powdered diet (PMI Nutrition International LLC, Brentwood, MO) and supplied to diabetic mice. All mice had free access to food and water at all times. Consumed water and feed volumes and body weights were recorded. Plasma levels of glucose and insulin were measured at weeks 1 and 10. Urine output was measured at week 9. After 10 weeks of supplementation, mice were killed with carbon dioxide. Blood was collected, and plasma was separated from erythrocytes immediately. Liver and kidney were collected and weighed. Each organ at 0.1 g was homogenized in 2 mL of ice-cold phosphate buffer saline (PBS, pH 7.2). The homogenate was further passed through a Whatman no. 1 filter paper, and the filtrate was collected. The protein concentration of organ filtrate was determined according to the method of Lowry et al.[14] 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. One group of mice without diabetes (non-DM) and consuming normal diet was used for comparison.

Analysis of Naringenin Content. The content of naringenin in plasma, liver, and kidney was analyzed by a HPLC method described in Cao et al.[15] Briefly, sample was incubated with a mixture containing glucuronidase (89.4 units/mL), sodium acetate buffer (0.78 mM, pH 4.8), and ascorbic acid (0.1 mM). Ethyl acetate was used to partition sample, followed by vortexing for 30 s and centrifuging at 6000*g* for 10 min. After evaporation of the ethyl acetate layer, the residue was reconstituted with methanol and subjected to HPLC analysis. Quercetin was used as an internal standard. The LC-2010A HPLC system (Shimadzu, Kyoto, Japan) equipped with an RP-18 column (LiChrospher 5 μm, 250 mm × 4.6 mm i.d.) and a SPD-M10AV photodiode array detector was used. The mobile phase was acetonitrile−water containing 0.5% acetic acid solution (29:71). The extraction recovery of this method was 95−98%. The coefficients of variation for interday and intraday assays were 4.3 and 6.0%, respectively. The limits of detection and quantification were 0.01 and 0.02 μmol/L, respectively.

Measurement of Plasma Glucose, Insulin, Blood Urea Nitrogen (BUN), and Creatinine Clearance (CCr). The plasma glucose level (mmol/L) was measured by a glucose HK kit (Sigma Chemical Co., St. Louis, MO). Plasma insulin (nmol/L) was measured by a rat insulin RIA kit (Linco Research Inc., St. Charles, MO). BUN, plasma creatinine (Cr), and urinary Cr concentrations were detected by a Beckman Autoanalyzer (Beckman Coulter, Fullerton, CA). CCr was calculated and expressed as mL/min/100 g body weight.

Cytokine Analyses. 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 phenylmethanesulfonyl fluoride and centrifuged at 9000*g* for 30 min at 4 °C. The resultant supernatant was used for cytokine determination. IL-1β, IL-6, TNF-α, and MCP-1 levels were measured by ELISA using cytoscreen immunoassay kits (BioSource International, Camarillo, CA). Samples were assayed in duplicates according to the manufacturer's instructions. The sensitivity of the assay with the detection limit was at 5 pg/mg protein for IL-1β and IL-6 and at 10 pg/mg protein for TNF-α and MCP-1.

NF-κB p50/65 Assay. NF-κB p50/65 DNA binding activity in the 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, a secondary antibody conjugated with horseradish peroxidase, and the 3,3′,5,5′-tetramethylbenzidine substrate. Absorbance at 450 nm was read. Values are expressed as relative optical density (OD) per milligram of protein.

Measurement of Urinary Albumin and Renal Level of TGF-β1, Fibronectin, and Type IV Collagen. Urinary albumin concentration was measured by a commercial ELISA Albuwell M kit (Exocel Inc., Philadelphia, PA). Renal cortex was homogenized with ice-cold PBS containing 0.05% Tween 20. After centrifuging at 9000*g* for 15 min at 4 °C, the supernatants were used for measuring renal TGF-β1 level (ng/mg protein), which was quantified by a commercial ELISA kit (R&D Systems, Minneapolis, MN). Fibronectin (mg/mg protein) was assayed using rabbit anti-rat fibronectin antibody and quantified by solid-phase immunoenzymic ELISA.[16] Type IV collagen concentration was measured by a Collagen IV M kit (Exocell Inc.), which measured both intact and fragments of type IV collagen.

Determination of Renal Glomeruli PKC Activity. The method described in Koya et al.[17] was used to determine glomeruli PKC activity. Briefly, kidney was homogenized in ice-cold RPMI1640 medium containing 20 mM HEPES. Glomeruli were isolated by removing the capsules and passed through sieves of various sizes. After washing twice with RPMI1640 medium containing 20 mM HEPES (pH 7.4) and once with a mixed-salt solution (137 mM NaCl, 5.4 mM KCl, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 5.5 mM glucose, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 5 mM EGTA, 2.5 mM $CaCl_2$, and 20 mM HEPES, pH 7.4), glomeruli were further 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. Sample was then spotted onto P81 phosphocellulose paper and washed four times with 1% phosphoric acid and once with acetone. The amount of radioactivity incorporated into the substrate was determined by scintillation counting. Glomerular PKC activity was normalized by the corresponding protein content.

Real-Time Polymerase Chain Reaction (RT-PCR) for mRNA Expression. Renal tissue was homogenized in guanidine thiocyanate, and 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 $MgCl_2$, 0.5 mM of each primer) and 2.5 U of Taq DNA polymerase. The specific oligonucleotide primers of targets are as follows: IL-1β (forward) 5′-TGT GGC TGT GGA GAA GCT GT-3′, (reverse) 5′-CAG CTC ATA TGG GTC CGA GA-3′; TNF-α (forward) 5′-GCA TGA TCC GCG ACG TGG AA-3′, (reverse) 5′-AGA TCC ATG CCG TTG GCC AG-3′; MCP-1 (forward) 5′-ATG CAG GTC CCT GTC ATG-3′, (reverse) 5′-GCT TGA GGT GGT TGT GGA-3′; TGF-β1 (forward) 5′-CCG CAA CAA CGC CAT CTA TGA-3′, (reverse) 5′-GGG GGT CAG CAG CCG GTT AC-3′; fibronectin (forward) 5′-TGT GAC CAG CAA CAC GGT G-3′, (reverse) 5′-ACA ACA GGA GAG TAG GGC GC-3′; NF-κB p50 (forward) 5′-GGA GGC ATG TTC GGT AGT GG-3′, (reverse) 5′-CCC TGC GTT GGA TTT CGT G-3′; NF-κB p65 (forward) 5′-GCG TAC ACA TTC TGG GGA GT-3′, (reverse) 5′-CCG AAG CAG GAG CTA TCA AC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH, the housekeeping gene) (forward) 5′-TGA TGA CAT CAA GAA GGT GGT GAA G-3′, (reverse) 5′-CCT TGG AGG CCA TGT AGG CCA T-3′. The cDNA was amplified under the

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Table 1. Naringenin Content in Plasma, Liver, and Kidney from Nondiabetic Mice (Non-DM) and Diabetic Mice (DM) Consuming a Normal Diet or 0.5, 1, or 2% Naringenin[a]

	non-DM	DM	DM + naringenin, 0.5%	DM + naringenin, 1%	DM + naringenin, 2%
plasma, μmol/L	–[b]	–	–	–	0.11 ± 0.06 a
liver, μmol/g	–	–	0.14 ± 0.05 a	0.72 ± 0.17 b	1.30 ± 0.22 c
kidney, μmol/g	–	–	–	0.26 ± 0.10 a	0.84 ± 0.13 b

[a]Data are the mean ± SD, $n = 10$. Means in a row without a common letter differ, $P < 0.05$. [b]Means too low to be detected.

Table 2. Water Intake (WI), Feed Intake (FI), Body Weight (BW), Urine Output (UO), and Organ Weight (OW) of Nondiabetic Mice (Non-DM) and Diabetic Mice Consuming a Normal Diet (DM) or 0.5, 1, or 2% Naringenin at Weeks 1 and 10[a]

	non-DM	DM	DM + naringenin, 0.5%	DM + naringenin, 1%	DM + naringenin, 2%
WI, mL/mouse/day					
1	1.7 ± 0.4 a	3.1 ± 0.8 b	3.5 ± 1.0 b	3.7 ± 0.6 b	3.6 ± 0.9 b
10	2.3 ± 0.6 a	6.9 ± 1.1 c	7.0 ± 1.2 c	6.6 ± 1.0 c	5.4 ± 0.7 b
FI, g/mouse/day					
1	2.0 ± 0.7 a	2.7 ± 0.4 a	2.6 ± 0.5 a	2.5 ± 0.8 a	2.9 ± 0.7 a
10	3.1 ± 0.5 a	6.6 ± 1.2 c	6.4 ± 0.9 c	6.1 ± 1.0 c	5.3 ± 0.6 b
BW, g/mouse					
1	21.5 ± 1.4 a	20.6 ± 1.5 a	20.7 ± 0.8 a	20.2 ± 1.0 a	21.0 ± 1.1 a
10	29.3 ± 2.0 c	13.2 ± 1.7a	13.9 ± 1.0 a	14.4 ± 0.9 a	16.7 ± 1.1 b
UO, mL/mouse/day					
10	0.56 ± 0.08 a	6.23 ± 1.10 c	6.06 ± 0.76 c	5.82 ± 0.42 c	4.89 ± 0.57 b
OW, g/mouse					
heart	0.27 ± 0.05 a	0.22 ± 0.06 a	0.24 ± 0.05 a	0.23 ± 0.03 a	0.25 ± 0.04 a
liver	1.47 ± 0.09 a	1.63 ± 0.13 a	1.61 ± 0.07 a	1.59 ± 0.11 a	1.54 ± 0.10 a
kidney	0.45 ± 0.08 a	0.53 ± 0.06 a	0.48 ± 0.10 a	0.50 ± 0.04 a	0.49 ± 0.07 a

[a]Data are the mean ± SD, $n = 10$. Means in a row without a common letter differ, $P < 0.05$.

Table 3. Plasma Levels of Glucose, Insulin, BUN, and CCr of Nondiabetic Mice (Non-DM) or Diabetic Mice (DM) Consuming a Normal Diet or 0.5, 1, or 2% Naringenin at Week 10[a]

	non-DM	DM	DM + naringenin, 0.5%	DM + naringenin, 1%	DM + naringenin, 2%
glucose, mmol/L	9.7 ± 1.1 a	25.8 ± 2.3 d	26.0 ± 2.5 d	22.9 ± 1.8 c	20.3 ± 1.0 b
insulin, nmol/L	13.4 ± 1.0 c	4.1 ± 0.5 a	4.6 ± 1.0 a	5.4 ± 0.8 b	5.7 ± 0.6 b
BUN, mg/dL	6.7 ± 0.4 a	51.6 ± 3.9 d	50.8 ± 4.0 d	44.2 ± 3.4 c	38.7 ± 2.5 b
CCr, mL/min/100 g BW	1.57 ± 0.08 d	0.42 ± 0.03 a	0.49 ± 0.05 a	0.68 ± 0.06 b	0.87 ± 0.07 c

[a]Data are the mean ± SD, $n = 10$. Means in a row without a common letter differ, $P < 0.05$.

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 GAPDH, and 35 cycles were performed for the 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, mRNA level was calculated as a percentage of the non-DM group.

Western Blot Analysis of NF-κB. Kidney tissue was homogenized in buffer containing 0.5% Triton X-100 and protease-inhibitor cocktail (1:1000, Sigma-Aldrich Chemical Co., St. Louis, MO). 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. 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, membrane was incubated with mouse anti-NF-κB p50 or anti-NF-κB p65 monoclonal antibody (Boehringer-Mannheim, Indianapolis, IN) at 4 °C overnight and followed by reaction 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 Dunnett's t test. Statistical significance is defined as $P < 0.05$.

RESULTS

Naringenin supplement at 1 and 2% increased its deposit in the liver and kidney of diabetic mice (Table 1, $P < 0.05$). As shown in Table 2, mice with 2% naringenin treatments had significantly lower water intake, feed intake, and urine output, as well as higher body weight, than the diabetic control group at week 10 ($P < 0.05$). Plasma levels of glucose, insulin, BUN, and CCr are presented in Table 3. Compared with the diabetic control group, naringenin treatments at 1 and 2% decreased glucose and BUN levels, as well as raised insulin and CCr levels ($P < 0.05$).

Renal levels and mRNA expression of inflammatory factors are shown in Table 4 and Figure 1. Naringenin treatments dose-dependently reduced TNF-α production and mRNA expression ($P < 0.05$), but only at 1 and 2% significantly lowered the levels and expression of IL-1β, IL-6, and MCP-1 ($P < 0.05$). Renal levels of fibrotic factors are presented in Table 5. Naringenin intake at 1 and 2% lowered urinary

Table 4. Renal Levels of Inflammatory Cytokines (IL-1β, IL-6, TNF-α, and MCP-1) in Nondiabetic Mice (Non-DM) and Diabetic Mice Consuming a Normal Diet (DM) or 0.5, 1, or 2% Naringenin at Week 10[a]

	non-DM	DM	DM + naringenin 0.5%	DM + naringenin, 1%	DM + naringenin, 2%
IL-1β, pg/mg protein	15 ± 3 a	221 ± 23 d	201 ± 21 d	166 ± 18 c	120 ± 16 b
IL-6, pg/mg protein	19 ± 4 a	237 ± 24 d	212 ± 18 d	170 ± 19 c	129 ± 20 b
TNF-α, pg/mg protein	16 ± 5 a	254 ± 25 e	201 ± 20 d	169 ± 17 c	117 ± 15 b
MCP-1, pg/mg protein	17 ± 3 a	226 ± 15 d	213 ± 17 d	172 ± 13 c	138 ± 14 b

[a]Data are the mean ± SD, n = 10. Means in a row without a common letter differ, $P < 0.05$.

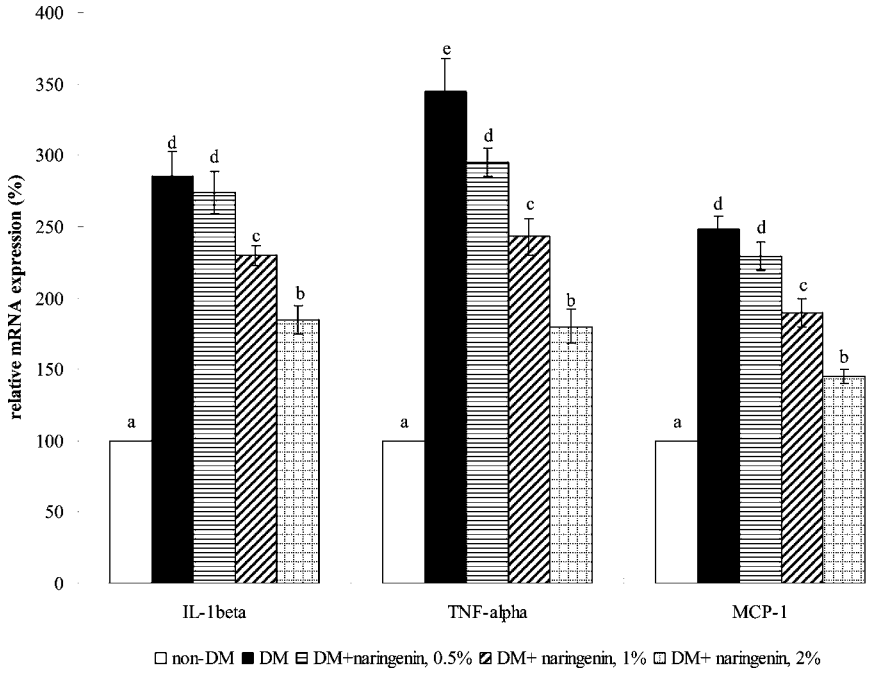

Figure 1. mRNA expression of renal IL-1β, TNF-α, and MCP-1 in nondiabetic mice (non-DM) and diabetic mice (DM) consuming a normal diet or 0.5, 1, or 2% naringenin at week 10. Data are the mean ± SD, n = 10. Means among bars without a common letter differ, $P < 0.05$.

Table 5. Urinary Level of Albumin and Renal Levels of Fibronectin, Type IV Collagen, and TGF-β1 in Nondiabetic Mice (Non-DM) and Diabetic Mice (DM) Consuming a Normal Diet or 0.5, 1, or 2% Naringenin at Week 10[a]

	non-DM	DM	DM + naringenin, 0.5%	DM + naringenin, 1%	DM + naringenin, 2%
urinary					
albumin, μg/day	19 ± 5 a	165 ± 15 d	159 ± 9 d	127 ± 13 c	98 ± 10 b
renal					
fibronectin, mg/mg protein	2.47 ± 0.18 a	5.68 ± 0.37 c	5.43 ± 0.24 c	5.05 ± 0.10 b	4.16 ± 0.15 b
type IV collagen, ng/mg protein	9 ± 3 a	80 ± 11 c	76 ± 9 c	72 ± 6 c	60 ± 8 b
TGF-β1, ng/mg protein	13 ± 4a	74 ± 7 c	71 ± 5 c	69 ± 8 c	57 ± 4 b

[a]Data are the mean ± SD, n = 10. Means in a row without a common letter differ, $P < 0.05$.

albumin, and renal fibronectin levels ($P < 0.05$) and at 2% diminished renal production of type IV collagen and TGF-β1 ($P < 0.05$). As shown in Figure 2, only 2% naringenin significantly suppressed mRNA expression of fibronectin and TGF-β1 ($P < 0.05$).

Naringenin supplement dose-dependently decreased NF-κB p65 activity, but only 2% abated NF-κB p50 activity (Figure 3A, $P < 0.05$). NF-κB p65 mRNA expression and protein level were suppressed by 1 and 2% naringenin treatments, and p50 mRNA expression and protein level were repressed by 2% naringenin treatments (Figure 3B,C, $P < 0.05$). As shown in Figure 4, renal PKC activity was mitigated by 1 and 2% naringenin intake ($P < 0.05$).

DISCUSSION

Naringenin is a natural flavonoid responsible for the bitter taste in several plant foods. The study of Ortiz-Andrade et al.[18] revealed that naringenin exerted its antidiabetic effect by lowering carbohydrate absorption from the intestine, which alleviated postprandial blood glucose increase. Kannappan and Anuradha[19] reported that naringenin enhanced insulin sensitivity in fructose-fed animals. In our present study, the dietary supplement of naringenin at high dose (2%) increased its deposit in kidney and liver, elevated insulin level, and improved glycemic control in diabetic mice. Thus, the antidiabetic effects of this compound might also include stimulating insulin secretion. Furthermore, we found the intake of naringenin decreased renal inflammatory and fibrotic stress

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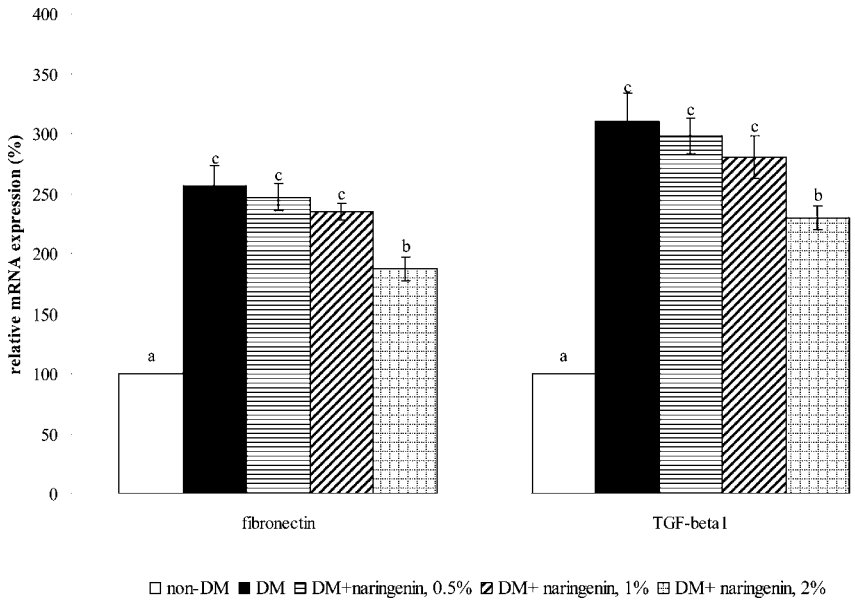

Figure 2. mRNA expression of renal fibronectin and TGF-β1 in nondiabetic mice (non-DM) and diabetic mice (DM) consuming a normal diet or 0.5, 1, or 2% naringenin at week 10. Data are the mean ± SD, n = 10. Means among bars without a common letter differ, $P < 0.05$.

and maintained renal functions in diabetic mice. These findings indicate that naringenin is a potent agent against diabetic nephropathy.

IL-1β, IL-6, and TNF-α are central mediators for the regulation of inflammatory biomarkers, and the overproduction of these mediators facilitates the progression of diabetes-associated inflammation, endothelial dysfunction, and coagulation.[2,20] Furthermore, TNF-α induces neutrophil accumulation and activation, which promotes immune disorders in diabetic individuals.[21] We found that naringenin supplement substantially decreased the production and mRNA expression of IL-1β, IL-6, and TNF-α in kidney. These results indicated that this compound attenuated renal inflammatory injury via down-regulation of these inflammatory mediators. In addition, MCP-1 is a chemotactic factor that activates monocytes and macrophages and recruits monocytes to the sites of injury.[22] The increased renal MCP-1 level as we observed further supported that renal inflammation was marked, and these diabetic mice were at high risk for further renal complications. Meanwhile, our results revealed that the intake of naringenin markedly mitigated renal MCP-1 release and expression, which implied that this compound could protect kidney against inflammation via diminishing the activation of monocytes and macrophages and lowering the recruitment of monocytes. TGF-β1 is a key regulator of ECM genes because it could induce the expression of type IV collagen and fibronectin in mesangial cells[23,24] and further caused renal dysfunction such as albuminuria. Our data indicated that naringenin at 2% suppressed the production and expression of TGF-β1, which subsequently lowered the generation of type IV collagen and fibronectin and, consequently, alleviated albuminuria. These findings indicated that this compound could ameliorate fibrosis occurring in diabetic nephropathy. The decreased BUN and urine output and elevated CCr as we observed in those mice also agreed with naringenin intake benefitting renal functions. It is interesting to note that this compound exhibited anti-inflammatory stress at 1 and 2%, but its antifibrotic effect was remarkable only at 2%. Thus, higher doses and/or longer supplement time of this agent seems necessary to attenuate renal fibrosis for diabetic individuals.

NF-κB is activated by cell stress associated stimuli including hyperglycemia, growth factors, and oxidative stress. Enhanced activation of NF-κB has been demonstrated in human diabetic nephropathy, and the activated NF-κB is subsequently responsible for the regulation of inflammation mediators such as TNF-α and IL-6.[25,26] In our present study, naringenin at 2% markedly suppressed activity, mRNA expression, and protein production of NF-κB p50 and p65, which in turn diminished the formation of inflammatory cytokines and ameliorated renal inflammatory injury. Thus, the anti-inflammatory activities of this compound could be ascribed to its down-regulation on NF-κB, an upstream regulator. We notified that naringenin treatments dose-dependently declined NF-κB p65 activity and expression, but only at 2% did it effectively reduce p50 activity and expression. Obviously, this compound was more effective in modulating NF-κB p65. It is suggested that the nuclear level of NF-κB p65 is positively correlated with renal activation of NF-κB pathway in diabetic mice.[27] Because naringenin at lower dose (0.5%) was able to mediate renal NF-κB p65, the observed lower renal production of TNF-α in mice treated by this dose could be explained. On the other hand, NF-κB is a key transcription regulator for fibrotic factors including TGF-β1 and fibronectin.[28,29] Thus, the suppressive effects from naringenin upon renal NF-κB activation also enhanced the decrease in the expression of TGF-β1 and fibronectin, which consequently alleviated renal fibrotic stress. The inhibition of PKC activity causes a reduction in NF-κB activation, which in turn limited the transcription of its downstream factors.[30,31] Our data revealed naringenin treatments abated renal PKC activity. Therefore, it is possible that the intake of this agent diminished renal PKC activity and mitigated NF-κB expression and activity, which subsequently decreased the production of inflammatory and fibrotic factors and finally improved renal functions.

Although naringenin at 2% exhibited effective anti-inflammatory and antifibrotic activities in diabetic mice, further studies are necessary to examine its safety before it is used for

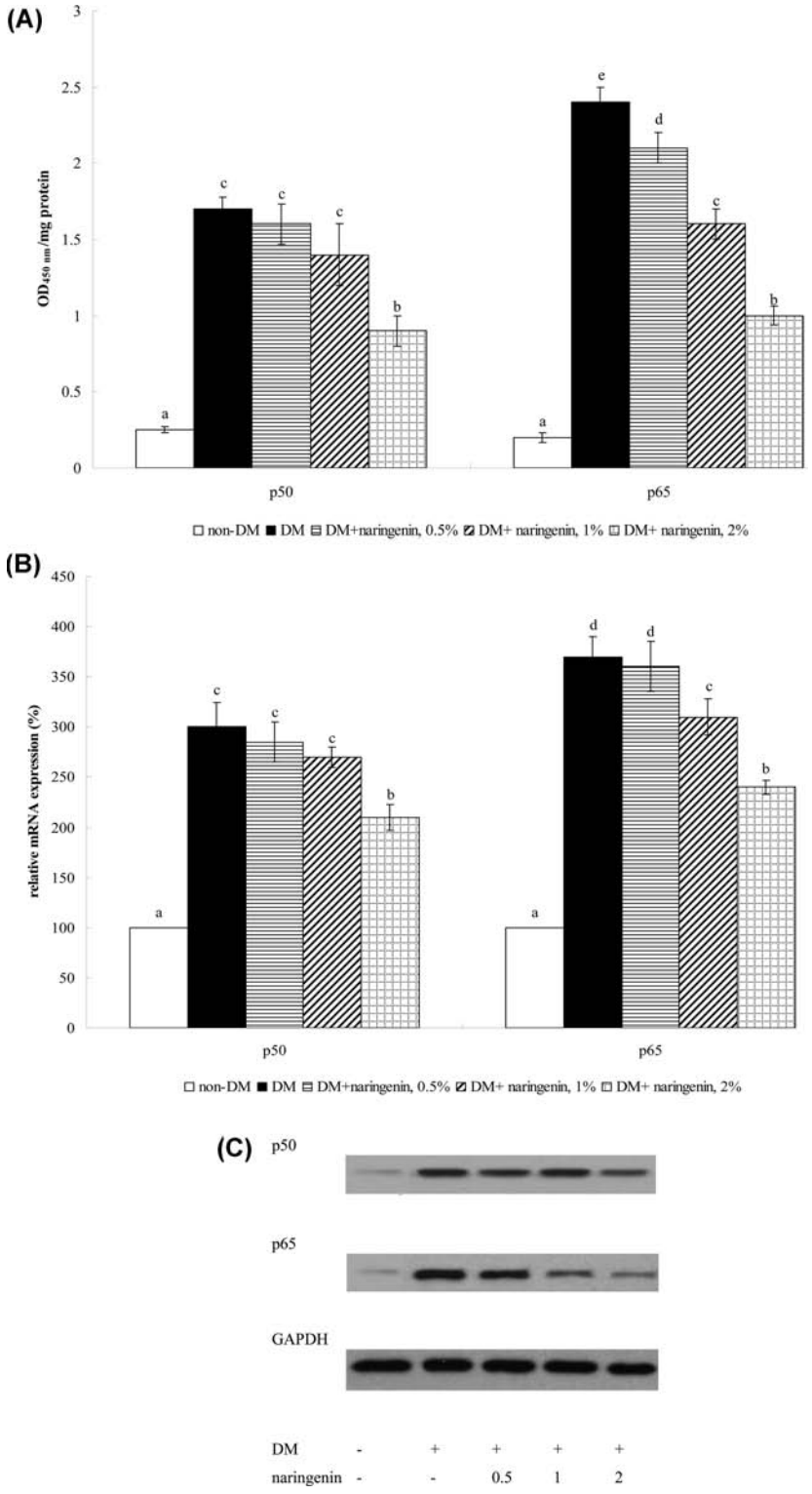

Figure 3. Renal activity (A), mRNA expression (B), and production (C) of NF-κB p50 and p65 in nondiabetic (non-DM) and diabetic mice (DM) consuming a normal diet or 0.5, 1, or 2% naringenin at week 10. Data are the mean ± SD, $n = 10$. Means among bars without a common letter differ, $P < 0.05$.

humans. The study of Felgines et al.[32] indicated that rats had marked bioavailability of naringenin-7-rhamnoglucoside and naringenin-7-glucoside, predominant flavanones in grapefruit. Bredsdorff et al.[33] reported that humans had greater bioavailability for naringenin glycosides. It seems that the bioactivity of other naringenin glycosides warrants attention. Further studies are encouraged to investigate the protective effects of naringenin glycosides against diabetic progression.

In summary, naringenin provided renal anti-inflammatory and antifibrotic protection for diabetic mice. Naringenin intake decreased the expression and production of inflammatory cytokines such as TNF-α and MCP-1 and fibrotic factors such

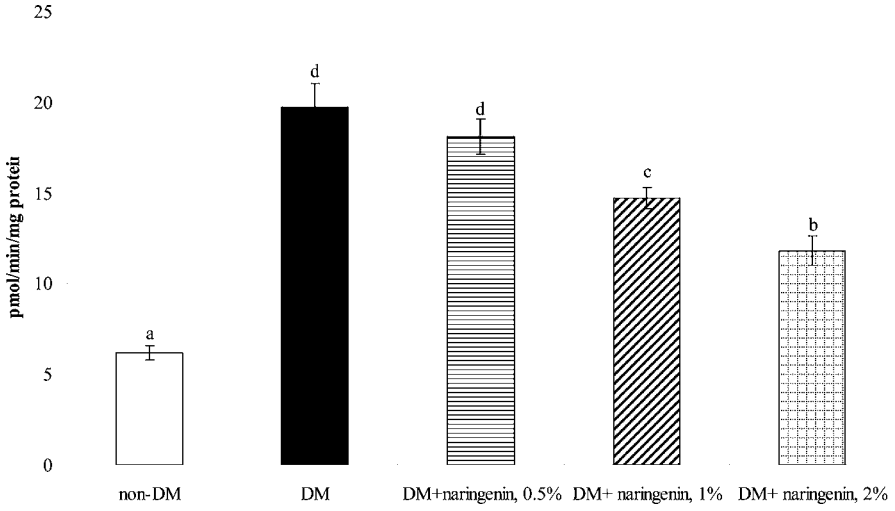

Figure 4. Renal PKC activity (pmol/min/mg protein) in nondiabetic mice (non-DM) and diabetic mice (DM) consuming a normal diet or 0.5, 1, or 2% naringenin at week 10. Data are the mean ± SD, n = 10. Means among bars without a common letter differ, $P < 0.05$.

as fibronectin and TGF-β1 in kidney. This compound also lowered renal PKC activity and suppressed NF-κB activation. Therefore, supplementation with this agent or foods rich in this compound might be helpful for the prevention or alleviation of diabetic nephropathy.

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