Eur J Nutr (2012) 51:961–973
DOI 10.1007/s00394-011-0274-4

ORIGINAL CONTRIBUTION

Purple corn anthocyanins retard diabetes-associated glomerulosclerosis in mesangial cells and db/db mice

Jing Li · Min-Kyung Kang · Jin-Kyu Kim · Jung-Lye Kim · Sang-Wook Kang · Soon Sung Lim · Young-Hee Kang

Received: 26 August 2011 / Accepted: 1 November 2011 / Published online: 20 November 2011

Abstract
Purpose Diabetic glomerulosclerosis is the hardening of the renal glomeruli that can lead to kidney failure. In the early stage of glomerulosclerosis occur renal mesangial expansion and renal filtration dysfunction. Purple corn has been classified as a functional food and is rich in anthocyanins exerting potential disease-preventive activities. The in vitro study using human renal mesangial cells examined that anthocyanin-rich purple corn butanol fraction (PCB) can attenuate high glucose (HG)-promoted mesangial cell proliferation and matrix accumulation.
Methods Cells were cultured for 3 days in media containing 33 mM glucose in the presence of 1–20 μg/mL PCB. In the in vivo animal study, db/db mice were treated with 10 mg/kg anthocyanin-rich polyphenolic extracts of purple corn (PCE) for 8 weeks.
Results HG enhanced mesangial production of the fibrosis biomarkers of collagen IV and connective tissue growth factor (CTGF), which was markedly attenuated by adding PCB. Such mesangial fibrosis entailed interleukin-8 activation via eliciting Tyk2-STAT signaling pathway. PCB dampened HG-promoted mesangial hyperplasia that appeared to be attributed to increased expression of platelet-derived growth factor. The 8-week administration of PCE lowered plasma glucose level of db/db mice and ameliorated severe albuminuria. Moreover, PCE lessened collagen fiber accumulation in kidney glomeruli and CTGF expression via retarding TGF-β signaling. Protein expressions of nephrin and podocin, key proteins for filtration barrier function of the glomerular capillary wall, were repressed by treating mice with PCE.
Conclusion Purple corn may be a potent therapeutic agent for the treatment for diabetes-associated glomerulosclerosis accompanying proteinuria and kidney filtration dysfunction.

Keywords db/db mice · Glomerulosclerosis · High glucose · Mesangial cells · Purple corn anthocyanin · Renal fibrosis

Abbreviations

CTGF	Connective tissue growth factor
DN	Diabetic nephropathy
ECM	Extracellular matrix
HG	High glucose
HRMC	Human renal mesangial cells
IL	Interleukin
MMP	Matrix metalloproteinases
MT1-MMP	Membrane type-1 matrix metalloproteinase
PCB	Anthocyanin-rich purple corn butanol extract
PCE	Anthocyanin-rich polyphenolic extracts of purple corn
PDGF	Platelet-derived growth factor
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinases

Jing Li and Min-Kyung Kang contributed equally to this work.

J. Li · M.-K. Kang · J.-L. Kim · S.-W. Kang · S. S. Lim · Y.-H. Kang (✉)
Department of Food and Nutrition, Hallym University, Chuncheon, Kangwon-do 200-702, South Korea
e-mail: yhkang@hallym.ac.kr

J.-K. Kim
Institute of Natural Medicine, Hallym University, Chuncheon 200-702, Korea

Introduction

Diabetic nephropathy (DN) is characterized by renal hardening and interstitial fibrosis caused by extracellular matrix (ECM) accumulation [1]. The most distinctive diabetic lesion in glomeruli is mesangial expansion concurrently accompanying hyperplasia ultimately leading to diabetic nephrosclerosis [2, 3]. When nephrosclerosis occurs, glomerular arteries will become thick by myofibroblasts and dense collagen tissues [4]. Hyperglycemia-associated glomerular ECM accumulation can be caused by increased secretion of matrix-synthesizing growth factors and decreased formation of matrix-degrading proteases [5, 6]. DN induces the impaired renal function such as proteinuria, abnormal glomerular filtration rate and alterations in renal biomarker secretion [7]. In the nephrosclerosis process, connective tissue growth factor (CTGF) plays a vital role in the ECM-synthesizing process to enhance collagen accumulation [5, 8, 9]. In contrast, the key proteases are matrix metalloproteinases (MMP) responsible for the enzymatic breakdown of ECM [6, 10].

Numerous studies have investigated cellular mechanism(s) by which hyperglycemia or diabetes results in nephrosclerosis [11–13]. Hyperglycemia inflames intracellular signaling to stimulate induction of fibrogenic and prosclerotic cytokines activating ECM deposition [2, 14]. Transforming growth factor-β (TGF-β) is thought to be the key fibrogenic cytokine involved in the progression of DN [13]. It has been also demonstrated that DN is closely associated with mesangial inflammation [15, 16]. Chemokines such as monocyte chemotactic protein (MCP)-1 are responsible for diabetic ECM accumulation and early inflammation in diabetic kidney diseases [17]. However, the crosstalk between mesangial fibrosis and inflammation has not yet been well defined. Hyperglycemia may cause mesangial inflammation via the activation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling [11].

Purple corn has been utilized for centuries as a natural food colorant and drinks and cultivated in South America, mainly in Peru and Bolivia. Purple corn extract is rich in anthocyanins and bioactive phenolics [18]. Anthocyanins have been shown to possess anti-diabetic, anti-angiogenic and anti-carcinogenic activities as potential medicinal uses [18–21]. Several studies have well demonstrated that cyanidin 3-glucoside, the major purple corn pigment, is a chemopreventive and chemotherapeutic agent [22, 23]. However, there is little investigation into renal fibrosis–associated DN with anthocyanins. Recently, it is shown that administration of anthocyanin fraction can effectively improve liver fibrosis induced by dimethylnitrosamine in rats and may be used as a therapeutic agent [24]. Natural compounds such as puerarin and colchicine inhibit mesangial inflammation and ECM accumulation in DN through different inflammatory mechanisms [17, 25]. It is deemed that these compounds may retard renal fibrosis and glomerulosclerosis via disturbing fibrogenic pathways. Accordingly, the renoprotection of natural compounds against mesangial expansion may be specific therapies of renal fibrosis in DN.

Based on possible anti-diabetic functions of anthocyanin-rich purple corn extract as described in literatures [26, 27], this study attempted to determine whether purple corn anthocyanins may prevent diabetes-associated renal fibrosis and glomerulosclerosis in HG-exposed human renal mesangial cells (HRMC) and in db/db mice. The in vitro study demonstrated that the treatment with anthocyanin-rich purple corn butanol (BuOH) extract (PCB) markedly suppresses mesangial matrix expansion through collagen matrix accumulation and mesangial hyperplasia. In addition, the in vivo study employing db/db mice proved that anthocyanin-rich extracts of purple corn (PCE) retarded glomerulosclerosis with maintaining normal renal filtration barrier.

Materials and methods

Chemicals

Fetal bovine serum (FBS), trypsin–EDTA and penicillin–streptomycin were obtained from BioWhittaker (San Diego, CA, USA). 3-(4, 5-dimetylthiazol-yl)-diphenyl tetrazolium bromide (MTT) was purchased from DUCHEFA Biochemie (Haarlem, Netherlands). Dulbecco's modified eagle's media (DMEM), Nutrient Mixture F-12 Ham medium, mannitol and D-glucose were supplied by Sigma Chemical (St. Louis, MO, USA), as were all other reagents unless specifically stated otherwise. CTGF antibody was obtained from AbCam (Cambridge, UK) and antibodies of collagen IV, membrane type-1 MMP (MT-1 MMP), tissue inhibitor of metalloproteinases (TIMP)-2, nephrin and podocin were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies of TGF-β, phosphorylated (phospho)-tyrosine kinase 2 (Tyk2), phospho-signal transducers and activators of transcription (STAT)1, and phospho-STAT3 were obtained from Cell Signaling Technology (Beverly, CA, USA). PDGF-BB antibody was purchased from Peprotech (Rocky Hill, NJ, USA). Human recombinant protein IL-8 was provided by Sigma Chemical and IL-8 ELISA kit obtained from R&D systems (Minneapolis, MN, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse and donkey anti-goat IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Preparation of purple corn extracts

Powder of purple corn kernel was obtained from the Brilliant Project International (Seoul, Korea). The powder was applied to a glass open column (10.0 × 900 mm I.D.) packed with Diaion HP-20 (Mitsubishi Kasei Co., Tokyo, Japan) and eluted with water for washing of sugar or non-polyphenolic components, followed by 95% ethanol for PCE, the polyphenolic extracts of purple corn that was used for the in vivo animal study.

For solvent fractionation, PCE (60 g) was suspended in water and then partitioned sequentially with *n*-hexane (Hex), methylene chloride (MC), ethyl acetate (EA) and *n*-butanol (BuOH, PCB), leaving a residual aqueous fraction (Aq). Each fraction was then evaporated *in vacuo* to yield the fractions of Hex (0.33 g, 0.55%), MC (0.65 g, 1.08%), EA (5.57 g, 9.28%), BuOH (26.44 g, 44.07%) and Aq (22.98 g, 38.30%).

MC Culture and proliferation

Human renal MC (HRMC, Sciencell Research Laboratories, Carlsbad, CA, USA) was cultured at 37 °C humidified atmosphere of 5% CO_2 in air. Routine culture of HRMC was performed in DMEM/F12 (7:1) media containing 15% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. HRMC in passage of 6–10 was sub-cultured at 80% confluence and used for further experiments. To mimic diabetic renal fibrosis caused by chronic hyperglycemia, HRMC was incubated in 33 mM glucose-added DMEM containing 2% FBS and 8 μg/mL insulin for 3 days in the absence and presence of PCB. For osmotic control incubations, another set of HRMC was cultured in DMEM containing 2% FBS (+2 μg/mL insulin) and supplemented with 27.5 mM mannitol. PCB was supplemented at concentrations of 1–20 μg/mL for 3-day culture experiments. Culture media were collected and stored in −20 °C.

After the 3-day incubation period under the condition of HG and mannitol, the MTT assay was carried out for measuring cell proliferation [28]. After unconverted MTT was removed, the purple formazan product was dissolved in isopropanol with gentle shaking. Absorbance of formazan dye was measured at $\lambda = 570$ nm with background subtraction using $\lambda = 690$ nm.

Western blot analysis

Western blot analysis was conducted using whole-cell lysates and collected culture media prepared from HRMC at a density of 3.0×10^5 cells [29]. Whole MC lysates were prepared in a lysis buffer containing 1 M *β*-glycerophosphate, 1% *β*-mercaptoethanol, 0.5 M NaF, 0.1 M Na_3VO_4 and protease inhibitor cocktail. Cell lysates containing equal amounts of total proteins or equal volumes of culture medium were electrophoresed on 6-10% SDS-PAGE and transferred onto a nitrocellulose membrane. Non-specific binding was blocked by soaking the membrane in a TBS-T buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl and 0.1% Tween 20] supplemented 3% bovine serum albumin for 3 h. The membrane was incubated with an antibody to human CTGF, collagen IV, PDGF-BB and phosphorylated Tyk2. The membrane was then incubated with a secondary antibody of goat anti-rabbit IgG, goat anti-mouse IgG or donkey anti-goat IgG conjugated to horseradish peroxidase. Each protein level was determined by using Supersignal West Pico Chemiluminescence detection reagents (Pierce Biotechnology, Rockford, IL, USA) and Konica X-ray film (Konica Co., Tokyo, Japan). Incubation with mouse anti-human *β*-actin was conducted for the comparative control.

Western blot analysis was also carried out using tissue extracts prepared from mouse kidney. Whole-tissue extracts were prepared in 1 M Tris–HCl (pH 6.8) lysis buffer containing 1 M *β*-glycerophosphate, 10% SDS, 0.5 M NaF, 0.1 M Na_3VO_4 and protease inhibitor cocktail. The following procedure was described above.

Enzyme-linked immunosorbent assay (ELISA)

The cytokine secretion of IL-8 in HG-triggered HRMC was determined by using ELISA. Collected culture media were assayed for the secretion of IL-8 using ELISA kits (R&D System) according to the manufacturer's instructions.

In vivo animal experiments

Adult male db/db mice (C57BLKS/+Lepr^{db} Iar; Jackson Laboratory, CA) and their age-matched non-diabetic db/m littermates (C57BLKS/J; Jackson Laboratory) were used in the present study. Mice were kept on a 12-h light/12-h dark cycle at 23 ± 1 °C with 50 ± 10% relative humidity under specific pathogen-free conditions, fed a standard pellet laboratory chow diet (CJ Feed, Korea) and were provided with water ad libitum at the animal facility of Hallym University. This study included old db/db mice at 8 weeks of age because they develop diabetes (hyperglycemia) at the age of 7–8 weeks [30]. The animals were allowed to acclimatize for a week before beginning the experiments. Mice were divided into three subgroups ($n = 8$–10 for each subgroup). The first group of mice was non-diabetic db/m control mice and received drinking water as the PCE vehicle. Quantitative and qualitative compositions of anthocyanins as well as total phenolics were evaluated in extracts of purple corn kernel [31]. The other db/db mice were orally administrated drinking water or 10 mg/kg BW

PCE daily for 8 weeks. All experiments were approved by the Committee on Animal Experimentation of Hallym University and performed in compliance with the University's Guidelines for the Care and Use of Laboratory Animals. No mice were dead, and no apparent signs of exhaustion were observed during the experimental period.

Sampling of blood and urine

Tail blood samples were collected during the 8-week PCE supplementation, and blood glucose (3.5 μL blood samples) was measured with a blood glucose meter (ACCU-CHEK Performa, Roche diagnostics, Mannheim, Germany). After the 8-week treatment with PCE, glucose tolerance test was performed with tail blood samples for 120 min after an intraperitoneal challenge with 1 g/kg BW glucose [32]. The 24-h urine sample collection was carried out by using metabolic cages. In addition, fasting blood samples were drawn from the retro-orbital venous plexus using heparinized capillary tubes under anesthesia on the time of killing. Organs of kidney, liver, pancreas, spleen and heart were weighed just after killing. Blood hemoglobin A1C (10 μL blood samples) was also measured by using high-performance liquid chromatography. Urinary albumin and creatinine were measured using Albuwell M ELISA kit (Exocell, Philadelphia, PA, USA) according to a manufacturer's instruction [30]. Urinary albumin excretion was normalized to urinary levels of creatinine measured using Creatinine Companion Chemical Assay (Exocell). Urinary albumin excretion was expressed as urinary albumin-to-creatinine ratio.

Masson trichrome staining and microscopy

Animals were killed by cervical dislocation under anesthesia at the termination of experimental protocols. For the histological analyses, kidney specimens were obtained at the end of the experiments and fixed in 10% buffered formalin. The paraffin-embedded kidney specimens were sectioned at 5 μm thickness, de-paraffinized and stained with Masson trichrome for the light microscopic visualization of collagen fibers [33]. The stained tissue sections were examined using an Optical microscope AXIOIMAGER (Zeiss, Göttingen, Germany), and five images (200×) were taken for each section.

Immunohistochemical staining

For the immunohistochemical analysis, paraffin-embedded kidney tissue sections (5 μm thick) were employed. The sections were placed on glass slides, de-paraffinated and hydrated with xylene and graded alcohol. The sections were pre-incubated in a boiling sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for antigen retrieval. Specific primary antibody against mouse collagen IV or podocin was incubated with the tissue sections overnight. The tissue sections were incubated for 1 h with horseradish peroxidase-conjugated anti-goat IgG. For the collagen IV visualization, the sections were developed with 3,3′-diaminobenzidine as a substrate to produce brownish staining, counter-stained with hematoxylin and mounted in VectaMount mounting medium (Vector Laboratories, Burlingame, CA, USA) [33]. For the podocin visualization, the sections were incubated for 3 h with Cy3-conjugated anti-goat IgG.

Data analysis

The results are presented as mean ± SEM for each treatment group. Statistical analyses were performed using Statistical Analysis Systems statistical software package (SAS Institute Inc., Cary, NC, USA). Significance was determined by one-way ANOVA, followed by Duncan range test for multiple comparisons. Differences were considered significant at $P < 0.05$.

Results

Effect of PCB on HG-induced mesangial fibrosis and hyperplasia

HG elevated fibrogenic collagen IV secretion (Fig. 1a). Four fractions of Hex, MC, EA and BuOH (PCB) extracted from PCE were employed in this study. Among these fractions tested at 10 μg/mL, only PCB attenuated collagen IV production. In addition, this study evaluated whether HG-stimulated mesangial cell proliferation was demoted by adding the purple corn fractions. HG caused mesangial cell proliferation with ≈25% increase in cell viability (Fig. 1b). Treatment with 10 μg/mL PCB showed antiproliferative ability without any cytotoxicity, while other fractions did not show such effects.

CTGF is an important growth factor improving diabetic renal fibrosis through stimulating production of collagen and other ECM proteins [34]. Cellular expression and secretion of CTGF augmented by HG were concentration-dependently diminished in PCB-treated HRMC (Fig. 1c). Collagen IV secretion encumbered by PCB was attributed most likely to the reduction in CTGF production due to its supplement. Accordingly, it is deemed that PCB ameliorated mesangial expansion and hyperplasia in the DN pathogenesis.

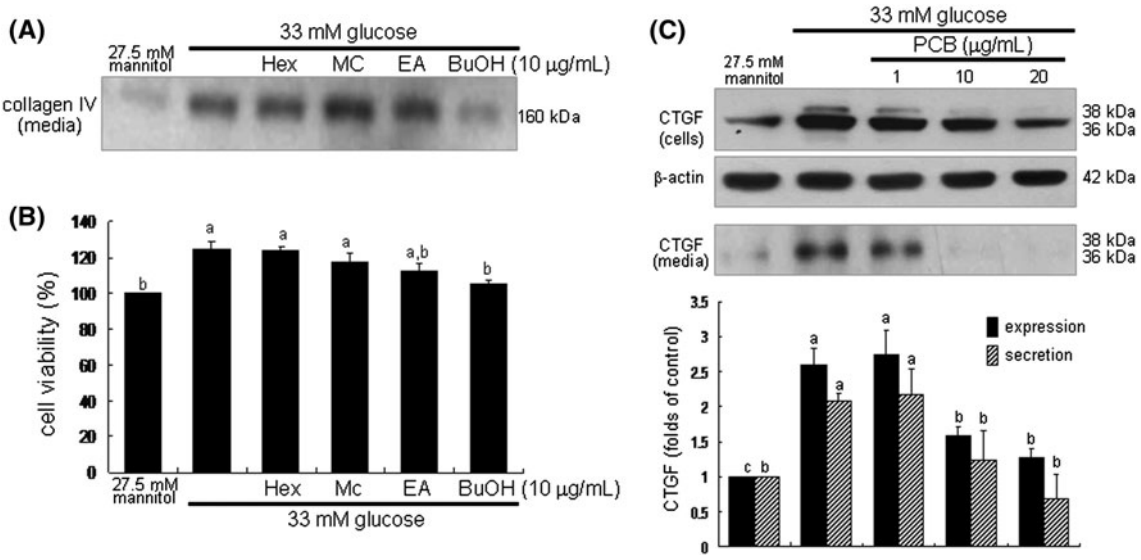

Fig. 1 Inhibition of high glucose–induced collagen IV secretion (**a**) and cell proliferation (**b**) by various purple corn fractions and CTGF production by PCB (**c**). See the fraction preparation in the "Materials and methods". Values of cell proliferation are mean ± SEM ($n = 6$) and expressed as percent cell survival relative to 5.5 mM glucose plus 27.5 mM mannitol controls (cell viability = 100%). β-actin protein was used as an internal control. Representative blots shown are typical of three independent experiments. The *bar* graphs (mean ± SEM, $n = 3$) in the *bottom panel* represent quantitative results obtained from a densitometer. Means without a common letter differ, $P < 0.05$

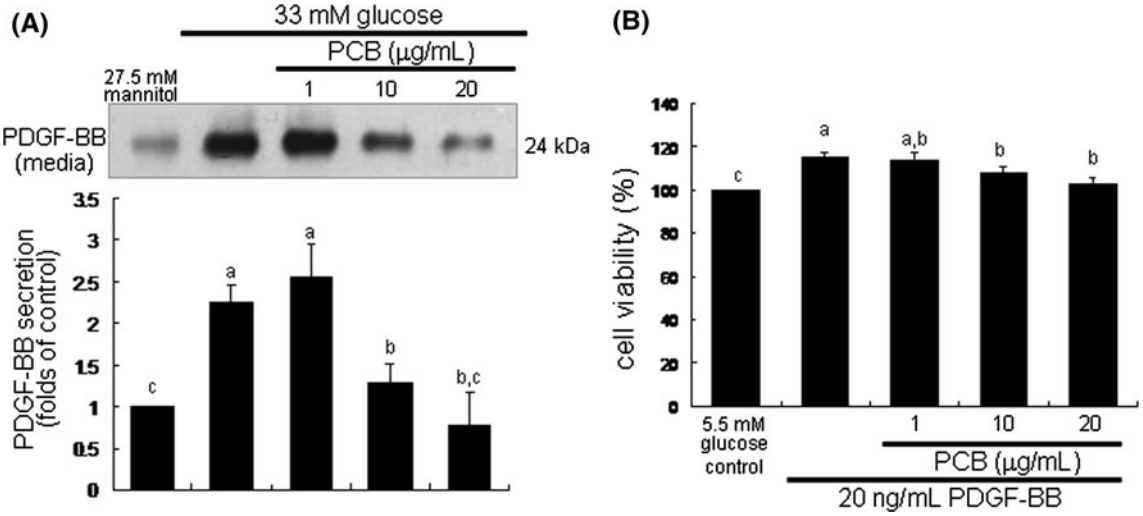

Fig. 2 Inhibitory effects of PCB on high glucose–induced PDGF-BB secretion in mesangial cell (**a**) and stimulation of mesangial cell proliferation by PDGF-BB (**b**). The bar graphs (means ± SEM, $n = 3$) in the *bottom panel* represent quantitative results obtained from a densitometer. Values of cell proliferation are mean ± SEM ($n = 5$) and expressed as percent cell survival relative to 5.5 mM glucose controls (cell viability = 100%). Means without a common *letter* differ, $P < 0.05$

Effect of PCB on HG-induced PDGF-BB production

This study found that HG stimulated PDGF-BB secretion from mesangial cells and that PCB inhibited this secretion (Fig. 2a). Interestingly, 20 μg/mL PDGF-BB per se triggered mesangial cell proliferation by ≈15%, which was blunted in a concentration-dependent fashion by adding PCB to cells (Fig. 2b). Thus, this could be a possible mechanism by which PCB prevented mesangial hyperplasia leading to glomerulosclerosis.

Effect of PCB on IL-8-induced hyperplasia and fibrosis

When mesangial cells were exposed to HG, the IL-8 secretion was markedly elevated (Fig. 3a). In contrast, the treatment of cells with PCB concentration-dependently

mitigated the secretion, as detected by ELISA. The local production of chemokines by mesangial cells has been linked to inflammatory processes within the glomerulus. This study revealed that HG induced mesangial inflammation and that PCB retarded such inflammation closely liked to the diabetes-instigated nephropathy [12, 24].

The chemokine IL-8 increased the production of CTGF and collagen IV, indicating that IL-8 was involved in mesangial ECM deposition (Fig. 3b). PCB suppressed collagen IV secretion elevated by IL-8 as well as CTGF production. Accordingly, PCB appeared to debilitate HG-induced mesangial expansion and hyperplasia by disturbing the inflammatory action of IL-8.

Blockade of activation of Tyk2-STAT signaling by PCBE

This study examined that the IL-8-associated mesangial inflammation was mediated by activation of Tyk2, the first member of the JAK family. The addition of 10 ng/mL IL-8 to mesangial cells phosphorylated Tyk2, which was dampened by 20 μg/mL PCB (Fig. 3c).

As with IL-8, the challenge with HG induced Tyk2 activation in mesangial cells, which was greatly attenuated by the supplementation of 20 μg/mL PCB (Fig. 4). It should be noted that mesangial cells exposed to HG increased IL-8 secretion by ≈eightfold (Fig. 3a). In addition, ≥10 μg/mL PCB disturbed the activation of Tyk2

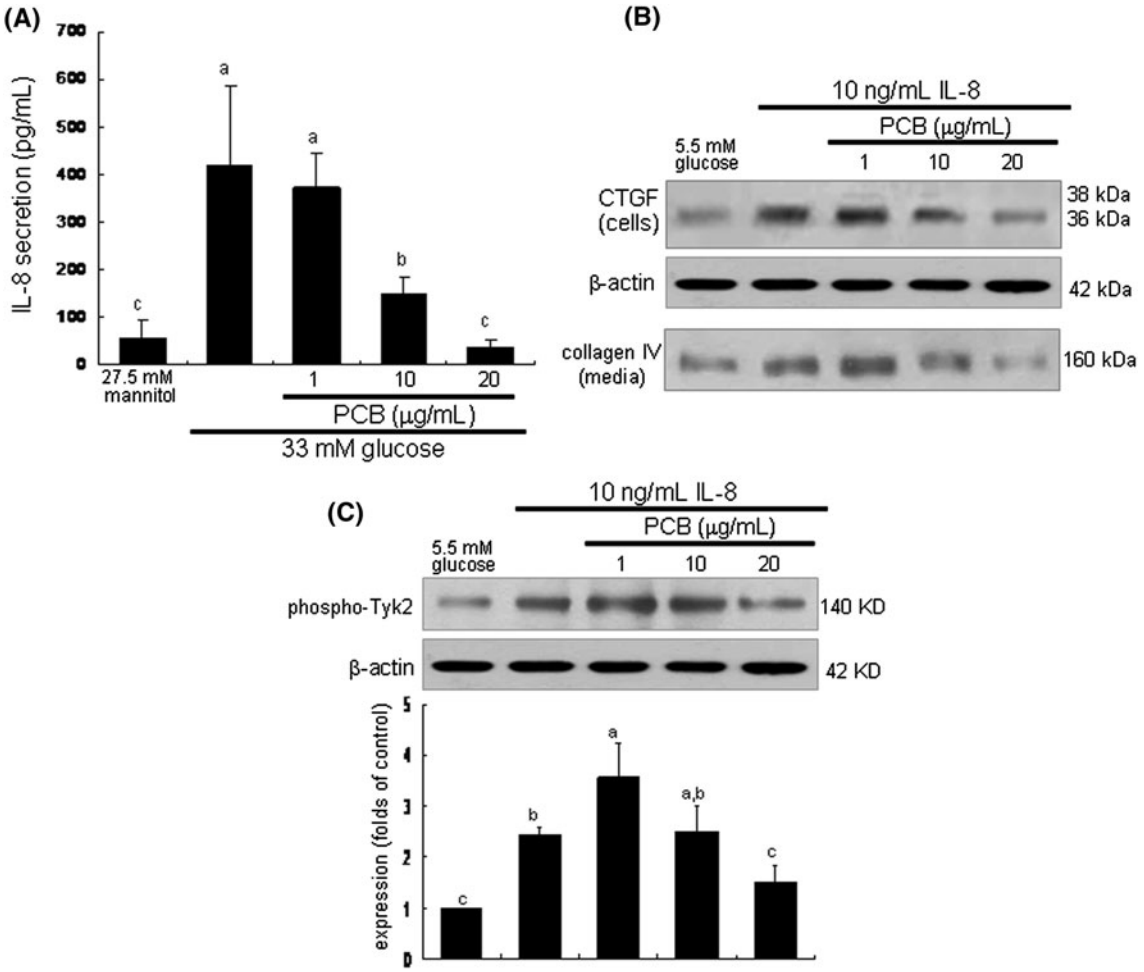

Fig. 3 Suppressive effects of PCB on high glucose–induced IL-8 secretion (**a**), IL-8-enhanced CTGF expression and collagen secretion (**b**) and IL-8-induced Tyk2 phosphorylation (**c**) in human renal mesangial cells. IL-8 secretion was determined by using a commercial ELISA kit, and respective data represent mean ± SEM from three independent experiments. *β*-Actin protein was used as an internal control. The *bar* graphs (means ± SEM, $n = 3$) in the *bottom panel* represent quantitative results obtained from a densitometer. Means without a common *letter* differ, $P < 0.05$

downstream proteins, STAT1 and STAT3 promoted by HG (Fig. 4). These results demonstrate that HG produced the chemokine IL-8 that activated Tyk2-STAT signaling in mesangial cells, resulting in mesangial inflammation. Therefore, PCB had capability to sever the IL-8-Tyk2-STAT downstream signaling that may instigate mesangial inflammation responsible for initiating diabetic renal fibrosis and glomerulosclerosis.

Effect of PCA on basic characteristics of db/db mice

Figure 5 depicted the alterations of basic characteristics of food intakes, drinking water intakes and body weights of db/db mice during the 8-week experimentation. The food intake of db/db mice was considerably higher (twofold) than that of db/m controls throughout the 8-week experimental period, while the food intake of PCE-administrated db/db mice decreased by ≈10%, compared to that of untreated db/db mice (Fig. 5a). The drinking water intake of db/m controls was less than ≈20 mL/day throughout the experimentation (Fig. 5b). In contrast, db/db mice gradually increased the water intake from the first week of the experimentation with the initial intakes of ≈30 mL/day, indicative of developing diabetes. The water intake of PCE-administrated db/db mice maintained to their initial intake.

The body weight of db/db mice was heavier by 20–60% than that of db/m controls (Fig. 5c). The body weight of db/db mice was lower by 10–40% than that of PCE-fed animals, which was most likely due to higher metabolic rate causing diabetic weight loss. The level of plasma HbA1c, a biomarker of diabetic complications, markedly increased in db/db mice, which was attenuated by PCE administration (data not shown).

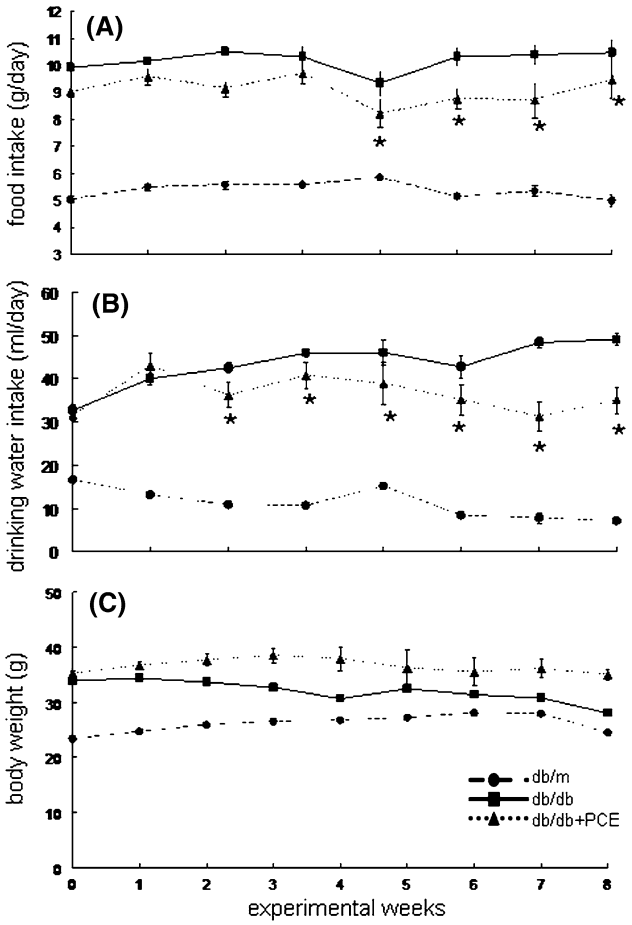

Fig. 5 Alterations of food intake (**a**), drinking water intake (**b**) and body weight (**c**) during the 8-week experimental period by PCE in diabetic db/db mice. Values are expressed as mean ± SEM ($n = 8$–10 in each group). *$P < 0.05$, relative to untreated db/db mice

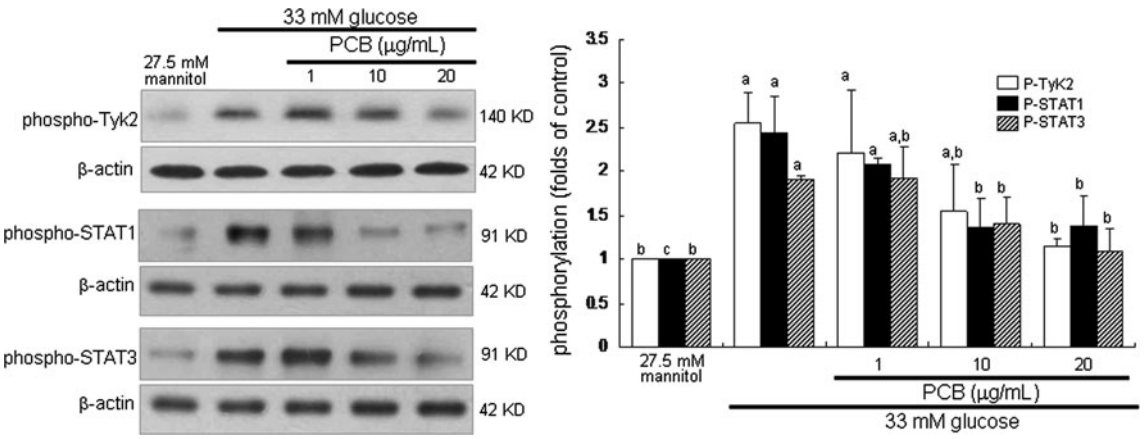

Fig. 4 Suppression of high glucose–induced Tyk2 phosphorylation by PCB in human renal mesangial cells. *β*-Actin protein was used as an internal control. The *bar* graphs (means ± SEM, $n = 4$) in the *right panel* represent quantitative results obtained from a densitometer. Values not sharing a common *letter* are different at $P < 0.05$

Alterations of plasma glucose level and urinary albumin excretion by PCE

This study examined blood glucose and urinary albumin excretion during the 8-week experimental period. The blood glucose level of db/db animals was much higher (three- to fourfold) than that of db/m controls (Fig. 6a). The blood glucose level of db/db mice declined continuously from the 4th week after PCE supplementation. In the 2-h glucose-tolerant test, the plasma glucose level was restored more rapidly to its initial level in db/db mice treated with PCE after the oral challenge with 1 g/kg glucose (Fig. 6b).

Following the 8-week PCE therapy, the severe albuminuria observed in db/db animals was alleviated with a significant decrease in urinary albumin excretion level normalized by urinary creatinine (Fig. 6c). PCE treatment recovered the reduced urinary creatinine level of db/db mice (0.321 mg/dL vs. 0.163 mg/dL).

Alleviation of diabetic glomerulosclerosis by PCE

The liver and kidney of db/db mice were much heavier than those of db/m controls, while there were no considerable differences in other organs (data not shown). In this study, pathological changes and collagen accumulation in the kidney glomeruli were examined in db/db mice experiencing 8-week experimental episode, as observed by using both Masson trichrome staining and immunohistochemical analysis. Histological staining with Masson trichrome for glomerular collagen fibers (blue color) showed that there was a heavy blue staining in db/db mouse glomerulus, compared with db/m control (Fig. 7a). This suggests that db/db mice accumulated collagen fibers in the glomerulus. In contrast, the PCE treatment retarded glomerular fibrosis in db/db mice. The blue collagen staining was not as strong as that of db/db mice, while the muscle fibers (red color) were predominated (Fig. 7a). Consistently with Masson trichrome

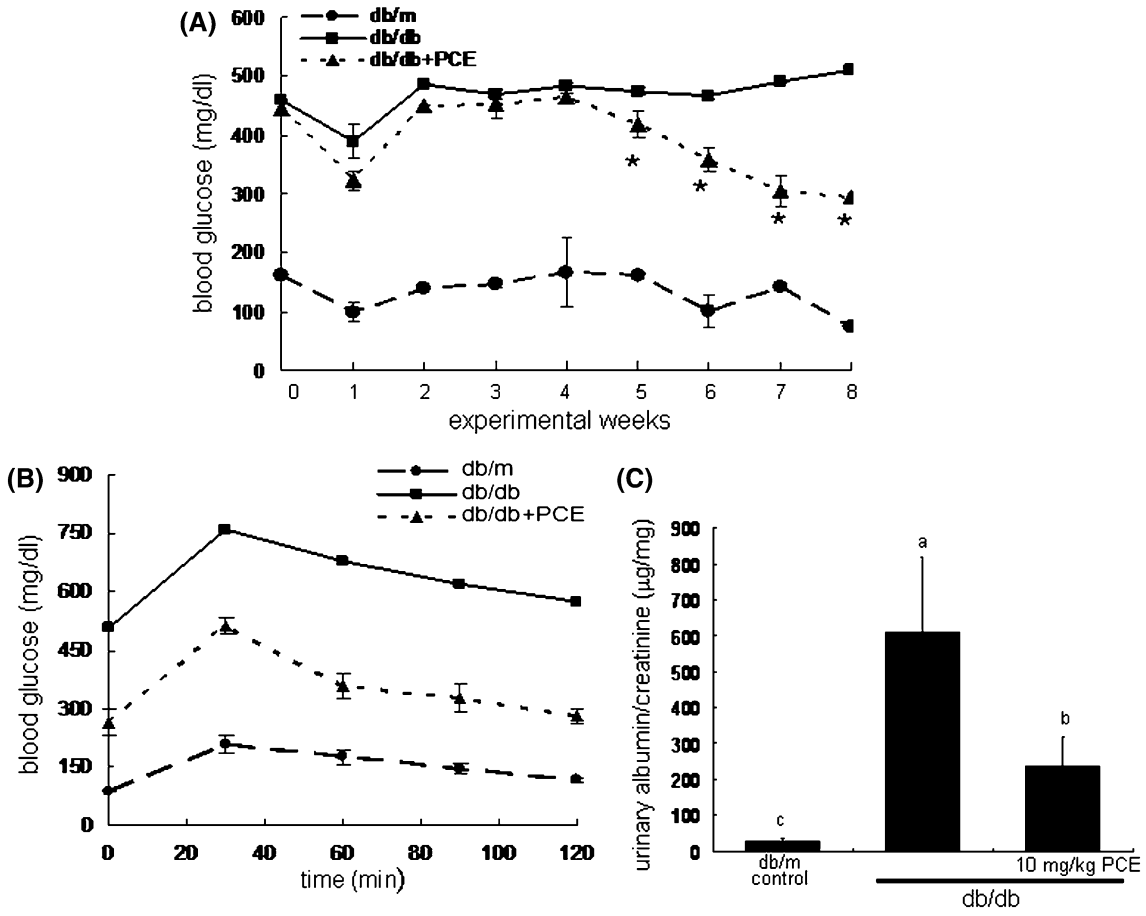

Fig. 6 Effect of PCE on plasma glucose (**a**, **b**) and urinary albumin excretion (**c**) in diabetic mice. Amount of urinary albumin excretion was normalized by urine creatinine (**c**). Values are expressed as mean ± SEM ($n = 8$–10 in each group). *$P < 0.05$, relative to untreated db/db mice. Values not sharing a common *letter* in bar graphs are different at $P < 0.05$

staining, the immunohistochemical data showed that there was a heavy collagen IV (brown color) expression in db/db mouse glomerulus, which was diminished by PCE treatment (Fig. 7b).

Fibrogenic expression of TGF-β and CTGF as well as collagen IV was minimal in db/m mouse renal tissues (Fig. 8a). However, these expressions were enhanced in the db/db mouse kidney, which may be responsible for the diabetic glomerulosclerosis detected in db/db mice (Fig. 7). In contrast, the PCE supplement blunted collagen IV expression in db/db mouse renal tissues accompanying attenuation of renal TGF-β and CTGF expression (Fig. 8a). On the other hand, this study examined expression of MT-1 MMP, a matrix-degrading MMP responsible for the enzymatic breakdown of ECM. The MT-1 MMP expression declined, but TIMP-2 expression elevated in the db/db mouse kidney (Fig. 8b). In db/db mice supplemented with PCE, the MT-1 MMP and TIMP-2 expressions were reversed, indicating that PCE ameliorated diabetic glomerulosclerosis observed in db/db mice.

Up-regulation of nephrin and podocin by PCE

In db/db mice, the renal tissue levels of nephrin and podocin dropped (Fig. 9a), indicating that the renal filtration barrier was damaged in diabetic animal kidney. However, the PCE treatment appeared to repair the damage through up-regulating nephrin and podocin expression. In addition, the nephrin and podocin expression in kidney glomerulus was evaluated by fluorescent microscopy using a specific antibody of podocin (Fig. 9b). Immunohistochemical fluorescence staining results supported the Western blot data of podocin expression. Heavy staining in the glomerulus of PCE-treated db/db mice was observed, which was comparable to, if not indistinguishable from, that in the glomerulus of db/m controls.

Discussion

Glomerulosclerosis is a kidney disease that is usually associated with hypertension, leading to kidney failure and

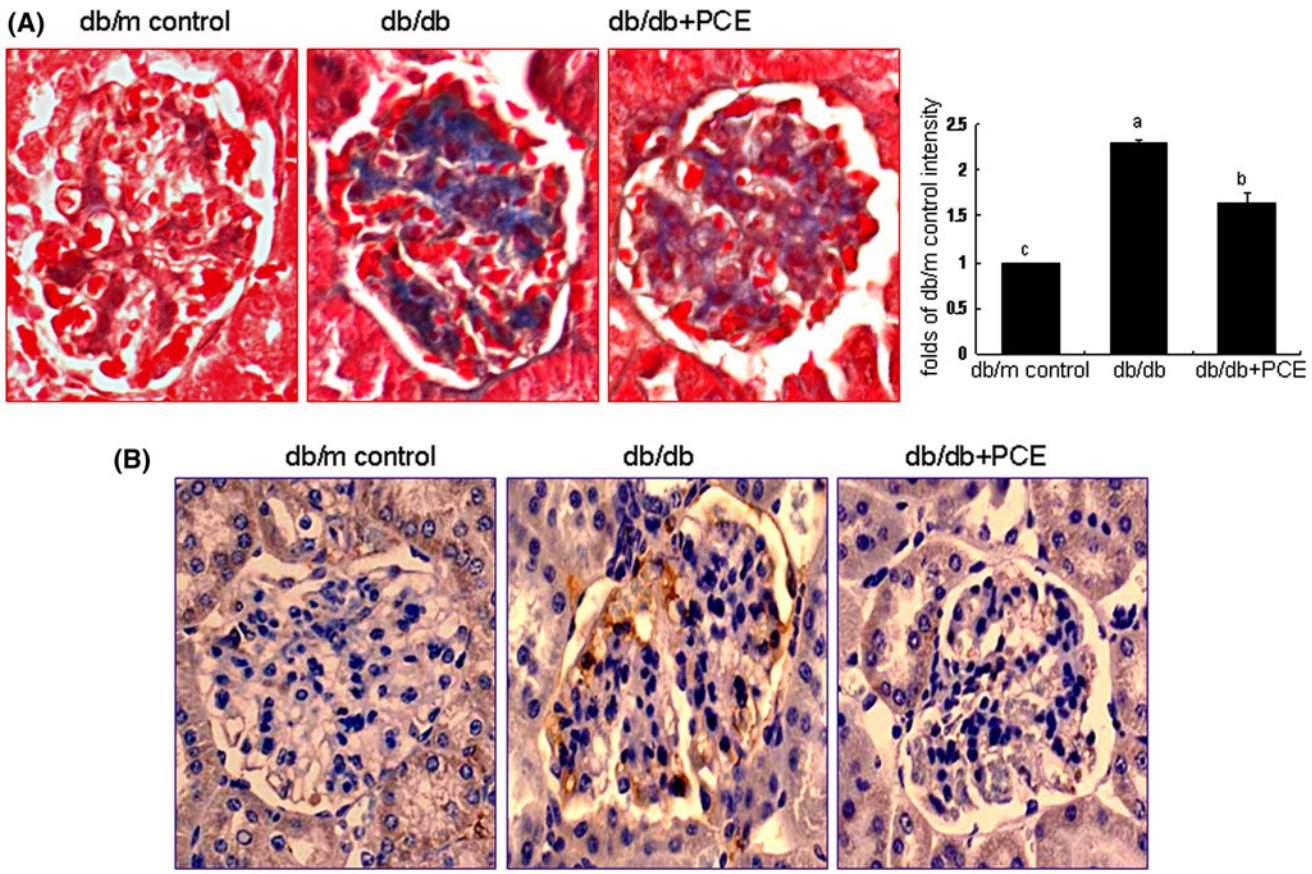

Fig. 7 Inhibition of high glucose–induced glomerular fibrosis by treatment with PCE in db/db mouse model. Pathological collagen IV changes in kidney glomeruli in different mouse groups observed by Masson trichrome staining (**a**). The collagen fibers were stained in blue, and muscle fibers were stained in *red*. The relative staining intensity of Masson trichrome was measured, and the respective values are mean ± SEM from six mice. Means without a common *letter* differ, $P < 0.05$. Immunohistochemical staining was performed for glomerular collagen fibers (**b**). Magnification: 200-fold

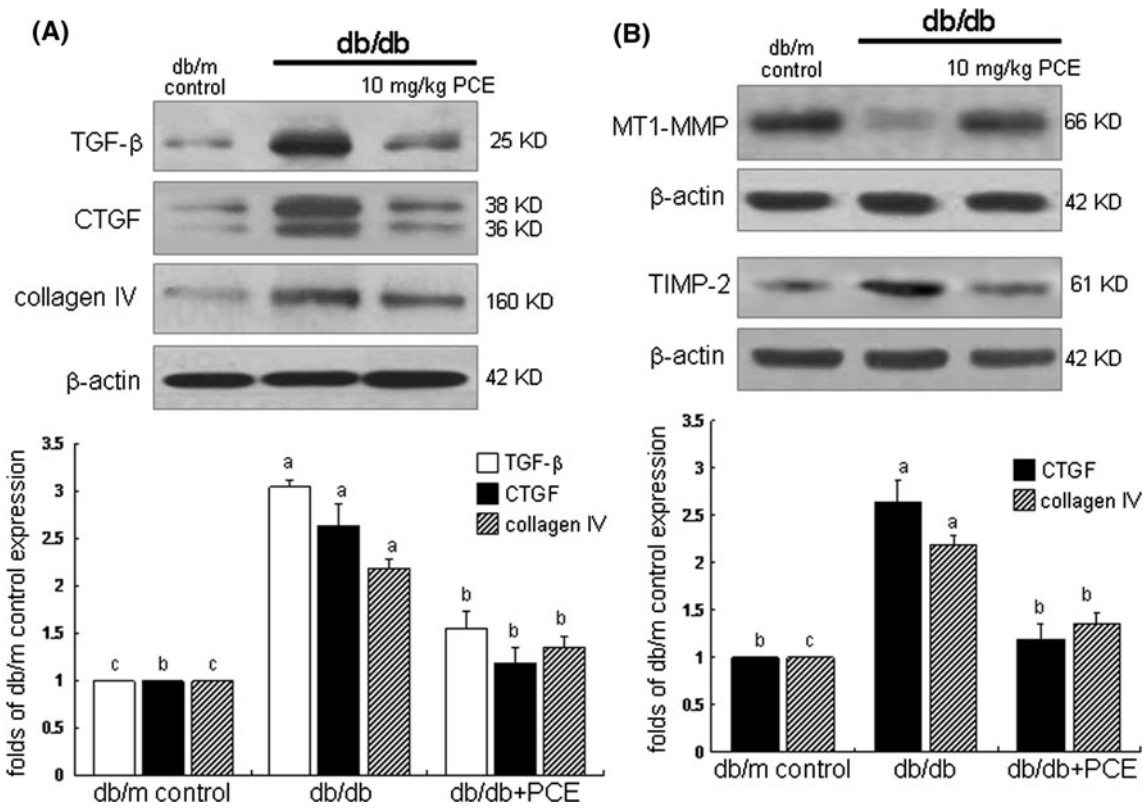

Fig. 8 Inhibitory effects of PCE on cellular expression of TGF-β, CTGF, collagen IV (**a**), MT-1 MMP and TIMP-2 (**b**) in db/db mice. The *bar* graphs (mean $\pm$ SEM, $n = 3$) in the *bottom panels* represent quantitative results obtained from a densitometer. Values not sharing a *letter* are different at $P < 0.05$

heart failure. The present study investigated whether purple corn anthocyanins exerted renoprotection by disturbing molecular events responsible for chronic diabetic glomerulosclerosis. To mimic the pathological environment of diabetic nephrosclerosis, mesangial cells were chronically exposed to HG. The collagen secretion into the glomeruli was attenuated by treating mesangial cells with PCB under HG condition. In addition, PCB suppressed HG-promoted CTGF induction leading to ECM accumulation. Simultaneously, HG-stimulated mesangial hyperplasia was mitigated by adding PCB to mesangial cells, and such inhibition appeared to be attributed to reduced mesangial production of PDGF-BB. Accordingly, purple corn was an agent targeting against chronic hyperglycemia-inflamed hyperplasia and renal sclerosis. However, it should be noted that PCB but not other fractions of Hex, MC and EA may be effective in blocking diabetes-associated glomerulosclerosis. The PCB was rich in anthocyanins, whereas EA extract contained some other phenolic compounds such as p-coumaric and ferulic acid [35]. Additionally, there were full of highly non-polar components in MC and Hex extracts. Phenolic compounds and non-polar components in PCE were not effective in antagonizing mesangial matrix accumulation. Therefore, the inhibitory activity of renal fibrosis and glomerulosclerosis was most likely ascribed to anthocyanins rich in PCB as a major bioactive agent.

Interest in anthocyanins has increased due to their potential health benefits rather than natural colorants. This study evaluated whether anthocyanins rich in purple corn color retarded chronic hyperglycemia-induced mesangial hyperplasia and renal fibrosis. Extracts of purple corn have a high content of anthocyanins and other phenolics. Some literature proved that cyanidin 3-glucoside inhibited proliferation of human colorectal adenocarcinoma HT29 cells with the other bioactive phenolics [23]. Purple corn color suppressed 7,12-dimethylbenz[*a*]anthracene-induced mammary carcinogenesis, indicating that this color may be a promising chemotherapeutic agent [20, 22]. This study evaluated whether anthocyanins in PCB possessed renoprotection against diabetes-associated nephropathy. Anthocyanins ameliorated insulin resistance and lowered the transcription of sterol regulatory element binding protein-1 in mouse white adipose tissue [27], indicating that they may have benefits for the prevention of obesity and diabetes. However, the renoprotective effect of anthocyanins on DN is little investigated.

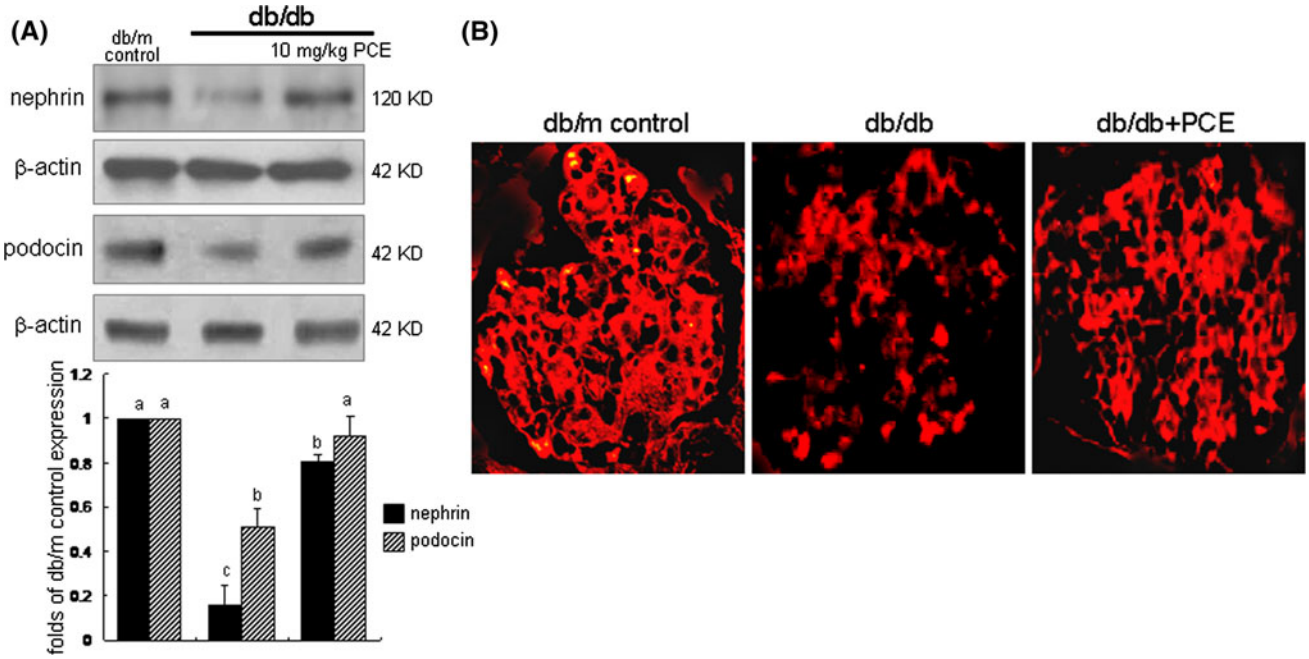

Fig. 9 Expression enhancement of nephrin and podocin by PCE in db/db mice. Western blot analysis was performed using antibodies of nephrin and podocin (**a**). The *bar* graphs (mean ± SEM, $n = 3$) in the *bottom panel* represent quantitative results obtained from a densitometer. Means without a common *letter* differ, $P < 0.05$. After the PCE treatment for 8 weeks, histological sections of mouse kidneys were immunohistochemically stained using anti-mouse podocin (**b**) and stained with a secondary antibody of Cy3-conjugated IgG. Each photograph is representative of at least four animals. Magnification: 200-fold

It has been reputed that diabetic nephropathy may be closely related to mesangial inflammation [12, 24]. Intracellular cell adhesion molecule-1 and MCP-1 recruiting inflammatory immune cells were up-regulated in diabetic patients with nephropathy [11, 12, 14–16]. It was assumed that MCP-1 appeared to be responsible for ECM accumulation and early inflammation in DN pathogenesis [36]. Mesangial cells were potent producers of a variety of chemokines, leading to specific attraction of inflammatory immune cells into the glomeruli [37, 38]. However, so far, there is limited knowledge about the responsiveness of mesangial cells to chemokines. Activated mesangial cells produce many inflammatory mediators resulting in amplification of glomerular injury [37]. In this study, IL-8 produced from glomerular mesangial cells was involved in mediating the mesangial expansion via CTGF induction triggering collagen IV deposition. The chemokine IL-8 was produced by mesangial cells exposed to HG, resulting in mesangial inflammation via activated Tyk2 signaling. Accordingly, HG evoked glomerular injury of fibrosis due to mesangial inflammation of IL-8 via activated Tyk2-STAT signaling pathway. The crosstalk between diabetes-associated mesangial fibrosis and chemokine-induced inflammation should be further defined.

Type 2 diabetes is an important cause of renal dysfunction and the most common cause of end-stage renal disease [39, 40]. In our in vivo study, db/db mice were employed for 8 weeks to investigate diabetic glomerular fibrosis and renal injury. Anthocyanin-PCE ameliorated glomerular fibrosis and reduced microalbuminuria detected in db/db mice, together with easing hyperglycemia, which were important therapeutic strategies for renal and vascular disease prevention in type 2 diabetes. There have been no reports dealing with protective effects of anthocyanins on DN. Nevertheless, other reports have stated anti-diabetic activity of anthocyanins in different standpoints [27, 41]. Collagen fibers mainly secreted by mesangial cells were accumulated in the glomeruli of db/db mice, indicative of the induction of mesangial fibrosis, the main feature of DN. The collagen IV accumulation, which is a crucial factor of mesangial expansion, was lessened by PCE administration. The mesangial expansion and glomerular fibrosis in db/db mice may result from these molecular changes within the renal tissue such as activation of various pro-inflammatory cytokines and growth factors [40]. In addition, the production of advanced glycation end-products and increased oxidative stress may further exacerbate renal fibrotic injury. It was deemed that the renoprotective effects of PCE associated with DN stemmed from targeting these molecular pathways.

Increased urinary albumin excretion is associated with renal damage of nephrin and podocin in diabetes [42]. In

type 2 diabetes with glomerular fibrosis, PCE administration ameliorated albuminuria possibly through preventing loss of nephrin and podocin proteins. PCE blocked diabetes-induced down-regulation of nephrin specifically located at the slit diaphragm of glomerular podocytes. In addition, the decreased level of podocin lining podocytes restored in PCE-treated db/db mice. Mutations of the genes encoding for nephrin and podocin lead to early onset of heavy proteinuria [43]. Thus, PCE supplementation maintained the integrity of the glomerular filtration barrier at the glomerular basement membrane and allowed the kidney to properly operate the urine filtration. This would allow somehow speculating some clinical relevance alleviating diabetes-associated renal dysfunction in human. Unfortunately, this study did not measure plasma urea nitrogen level. However, it was deemed that PCE reduced plasma urea nitrogen level and elevated creatinine clearance. Based on the observed anti-glycative and anti-inflammatory effects of PCE [36], the supplementation of PCE might be helpful for the clinical prevention or attenuation of diabetic kidney disease. It should be noted that elucidating anti-glomerulosclerotic and anti-fibrotic ability of PCE is of significance in human. However, there have been few investigations with dietary anthocyanins made in human [44].

In summary, the in vitro study revealed that anthocyanins rich in PCB encumbered HG-inflamed PDGF-BB-associated mesangial hyperplasia and IL-8-instigated CTGF expression and collagen IV deposition. The in vivo study ameliorated diabetes-associated glomerular fibrosis and renal filtration dysfunction. The capability of PCB to deter mesangial inflammation-linked renal fibrosis may be promising in hampering the progression to end-stage renal diseases. Furthermore, PCE treatment alleviated glomerulosclerosis and restored loss of nephrin and podocin proteins in db/db mice. PCE lessened heavy proteinuria with proper functioning of the renal filtration barrier. Therefore, the renoprotection of PCE against renal fibrosis and filtration barrier dysfunction may be specific therapies targeting glomerulosclerosis leading to DN.

Acknowledgments This study was supported by the grant from the Ministry for Food, Agriculture, Forestry and Fisheries through Korea Institute of Planning and Evaluation for Technology of Food, Agriculture, Forestry and Fisheries (109163-03-1-SB030) and by the Korea Research Foundation grant funded by the Korean Government (MEST), the Regional Research Universities Program/Medical & Bio-Materials Research Center.

References

1. Ban CR, Twigg SM (2008) Fibrosis in diabetes complications: pathogenic mechanisms and circulating and urinary markers. Vasc Health Risk Manag 4:575–596
2. Kanwar YS, Wada J, Sun L, Xie P, Wallner EI, Chen S et al (2008) Diabetic nephropathy: mechanisms of renal disease progression. Exp Biol Med (Maywood) 233:4–11
3. Schena FP, Gesualdo L (2005) Pathogenetic mechanisms of diabetic nephropathy. J Am Soc Nephrol 16:S30–S33
4. Zoccali C (2006) Endothelial dysfunction and the kidney: emerging risk factors for renal insufficiency and cardiovascular outcomes in essential hypertension. J Am Soc Nephrol 17:S61–S63
5. Pozzi A, Voziyan PA, Hudson BG, Zent R (2009) Regulation of matrix synthesis, remodeling and accumulation in glomerulosclerosis. Curr Pharm Des 15:1318–1333
6. Thrailkill KM, Clay Bunn R, Fowlkes JL (2009) Matrix metalloproteinases: their potential role in the pathogenesis of diabetic nephropathy. Endocrine 35:1–10
7. Sugaru E, Nakagawa T, Ono-Kishino M, Nagamine J, Tokunaga T, Kitoh M et al (2006) SMP-534 ameliorates progression of glomerular fibrosis and urinary albumin in diabetic db/db mice. Am J Physiol Renal Physiol 290:F813–F820
8. Ito Y, Aten J, Nguyen TQ, Joles JA, Matsuo S, Weening JJ, Goldschmeding R (2011) Involvement of connective tissue growth factor in human and experimental hypertensive nephrosclerosis. Nephron Exp Nephrol 117:e9–e20
9. Riser BL, Cortes P (2001) Connective tissue growth factor and its regulation: a new element in diabetic glomerulosclerosis. Ren Fail 23:459–470
10. McLennan SV, Martell SK, Yue DK (2002) Effects of mesangium glycation on matrix metalloproteinase activities: possible role in diabetic nephropathy. Diabetes 51:2612–2618
11. Banes AK, Shaw S, Jenkins J, Redd H, Amiri F, Pollock DM, Marrero MB (2004) Angiotensin II blockade prevents hyperglycemia-induced activation of JAK and STAT proteins in diabetic rat kidney glomeruli. Am J Physiol Renal Physiol 286:F653–F659
12. Fornoni A, Ijaz A, Tejada T, Lenz O (2008) Role of inflammation in diabetic nephropathy. Curr Diabetes Rev 4:10–17
13. Fukuda N, Tahira Y, Matsuda H, Matsumoto K (2009) Transforming growth factor-β as a treatment target in renal diseases. J Nephrol 22:708–715
14. Pohlers D, Brenmoehl J, Löffler I, Müller CK, Leipner C, Schultze-Mosgau S et al (2009) TGF-β and fibrosis in different organs-molecular pathway imprints. Biochim Biophys Acta 1792:746–756
15. Chen L, Zhang J, Zhang Y, Wang Y, Wang B (2008) Improvement of inflammatory responses associated with NF-κB pathway in kidneys from diabetic rats. Inflamm Res 57:199–204
16. Min D, Lyons JG, Bonner J, Twigg SM, Yue DK, McLennan SV (2009) Mesangial cell-derived factors alter monocyte activation and function through inflammatory pathways: possible pathogenic role in diabetic nephropathy. Am J Physiol Renal Physiol 297:F1229–F1237
17. Li JJ, Lee SH, Kim DK, Jin R, Jung DS, Kwak SJ et al (2009) Colchicine attenuates inflammatory cell infiltration and extracellular matrix accumulation in diabetic nephropathy. Am J Physiol Renal Physiol 297:F200–F209
18. Jing P, Giusti MM (2007) Effects of extraction conditions on improving the yield and quality of an anthocyanin-rich purple corn (*Zea mays* L.) color extract. J Food Sci 72:C363–C368
19. Crozier A, Jaganath IB, Clifford MN (2009) Dietary phenolics: chemistry, bioavailability and effects on health. Nat Prod Rep 26:1001–1043
20. Wang LS, Stoner GD (2008) Anthocyanins and their role in cancer prevention. Cancer Lett 269:281–290
21. Zafra-Stone S, Yasmin T, Bagchi M, Chatterjee A, Vinson JA, Bagchi D (2007) Berry anthocyanins as novel antioxidants in human health and disease prevention. Mol Nutr Food Res 51:675–683

22. Fukamachi K, Imada T, Ohshima Y, Xu J, Tsuda H (2008) Purple corn color suppresses Ras protein level and inhibits 7, 12-dimethylbenz[a]anthracene-induced mammary carcinogenesis in the rat. Cancer Sci 99:1841–1846
23. Jing P, Bomser JA, Schwartz SJ, He J, Magnuson BA, Giusti MM (2008) Structure-function relationships of anthocyanins from various anthocyanin-rich extracts on the inhibition of colon cancer cell growth. J Agric Food Chem 56:9391–9398
24. Choi JH, Hwang YP, Choi CY, Chung YC, Jeong HG (2010) Anti-fibrotic effects of the anthocyanins isolated from the purple-fleshed sweet potato on hepatic fibrosis induced by dimethylnitrosamine administration in rats. Food Chem Toxicol 48: 3137–3143
25. Lee HT, Kim M, Kim M, Kim N, Billings FT IV, D'Agati VD, Emala CW Sr (2007) Isoflurane protects against renal ischemia and reperfusion injury and modulates leukocyte infiltration in mice. Am J Physiol Renal Physiol 293:F713–F722
26. Ranilla LG, Apostolidis E, Genovese MI, Lajolo FM, Shetty K (2009) Evaluation of indigenous grains from the Peruvian Andean region for antidiabetes and antihypertension potential using in vitro methods. J Med Food 12:704–713
27. Tsuda T, Horio F, Uchida K, Aoki H, Osawa T (2003) Dietary cyanidin 3-O-beta-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. J Nutr 133: 2125–2130
28. Kang SW, Choi JS, Bae JY, Li J, Kim DS, Kim JL et al (2009) Blockade of vascular angiogenesis by *Aspergillus usamii* var. shirousamii-transformed Angelicae Gigantis Radix and *Zizyphus jujuba*. Nutr Res Pract 3:3–8
29. Li J, Lee YS, Choi JS, Sung HY, Kim JK, Lim SS, Kang YH (2010) Roasted licorice extracts dampen high glucose-induced mesangial hyperplasia and matrix deposition through blocking Akt activation and TGF-β signaling. Phytomedicine 17:800–810
30. Kolavennu V, Zeng L, Peng H, Wang Y, Danesh FR (2008) Targeting of RhoA/ROCK signaling ameliorates progression of diabetic nephropathy independent of glucose control. Diabetes 57:714–723
31. Li J, Lim SS, Lee JY, Kim JK, Kang SW, Kim JL, Kang YH (2011) Purple corn anthocyanins dampened high-glucose-induced mesangial fibrosis and inflammation: possible renoprotective role in diabetic nephropathy. J Nutr Biochem (in press)
32. Lee S, Kim W, Moon SO, Sung MJ, Kim DH, Kang KP et al (2007) Renoprotective effect of COMP-angiopoietin-1 in db/db mice with type 2 diabetes. Nephrol Dial Transplant 22:396–408
33. Bae JY, Choi JS, Kang SW, Lee YJ, Park J, Kang YH (2010) Dietary compound ellagic acid alleviates skin wrinkle and inflammation induced by UV-B irradiation. Exp Dermatol 19:e182–e190
34. Li J, Kang SW, Kim JL, Sung HY, Kwun IS, Kang YH (2010) Isoliquiritigenin entails blockade of TGF-beta1-SMAD signaling for retarding high glucose-induced mesangial matrix accumulation. J Agric Food Chem 58:3205–3212
35. Pedreschi R, Cisneros-Zevallos L (2007) Phenolic profiles of Andean purple corn (*Zea mays* L.). Food Chem 100:956–963
36. Li J, Lim SS, Lee ES, Gong JH, Shin D, Kang IJ, Kang YH (2011) Isoangustone A suppresses mesangial fibrosis and inflammation in human renal mesangial cells. Exp Biol Med (Maywood) 236:435–444
37. Gómez-Guerrero C, Hernández-Vargas P, López-Franco O, Ortiz-Muñoz G, Egido J (2005) Mesangial cells and glomerular inflammation: from the pathogenesis to novel therapeutic approaches. Curr Drug Targets Inflamm Allergy 4:341–351
38. Wada T, Matsushima K, Kaneko S (2008) The role of chemokines in glomerulonephritis. Front Biosci 13:3966–3974
39. Brown WV (2008) Microvascular complications of diabetes mellitus: renal protection accompanies cardiovascular protection. Am J Cardiol 102:10L–13L
40. Shah IM, Mackay SP, McKay GA (2009) Therapeutic strategies in the treatment of diabetic nephropathy—a translational medicine approach. Curr Med Chem 16:997–1016
41. Takikawa M, Inoue S, Horio F, Tsuda T (2010) Dietary anthocyanin-rich bilberry extract ameliorates hyperglycemia and insulin sensitivity via activation of AMP-activated protein kinase in diabetic mice. J Nutr 140:527–533
42. Barutta F, Corbelli A, Mastrocola R, Gambino R, Di Marzo V, Pinach S et al (2010) Cannabinoid receptor 1 blockade ameliorates albuminuria in experimental diabetic nephropathy. Diabetes 59:1046–1054
43. Huber TB, Simons M, Hartleben B, Sernetz L, Schmidts M, Gundlach E et al (2003) Molecular basis of the functional podocin–nephrin complex: mutations in the NPHS2 gene disrupt nephrin targeting to lipid raft microdomains. Hum Mol Genet 12:3397–3405
44. Giordano L, Coletta W, Tamburrelli C, D'Imperio M, Crescente M, Silvestri C, Rapisarda P, Reforgiato Recupero G, De Curtis A, Iacoviello L, de Gaetano G, Rotilio D, Cerletti C, Donati MB (2011) Four-week ingestion of blood orange juice results in measurable anthocyanin urinary levels but does not affect cellular markers related to cardiovascular risk: a randomized cross-over study in healthy volunteers. Eur J Nutr (in press)