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Purple corn anthocyanins inhibit diabetes-associated glomerular monocyte activation and macrophage infiltration

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Kang MK, Li J, Kim JL, Gong JH, Kwak SN, Park JH, Lee JY, Lim SS, Kang YH. Purple corn anthocyanins inhibit diabetes-associated glomerular monocyte activation and macrophage infiltration. *Am J Physiol Renal Physiol* 303: F1060–F1069, 2012. First published July 11, 2012; doi:10.1152/ajprenal.00106.2012.—Diabetic nephropathy (DN) is one of the major diabetic complications and the leading cause of end-stage renal disease. In early DN, renal injury and macrophage accumulation take place in the pathological environment of glomerular vessels adjacent to renal mesangial cells expressing proinflammatory mediators. Purple corn utilized as a daily food is rich in anthocyanins exerting disease-preventive activities as a functional food. This study elucidated whether anthocyanin-rich purple corn extract (PCA) could suppress monocyte activation and macrophage infiltration. In the in vitro study, human endothelial cells and THP-1 monocytes were cultured in conditioned media of human mesangial cells exposed to 33 mM glucose (HG-HRMC). PCA decreased the HG-HRMC-conditioned, media-induced expression of endothelial vascular cell adhesion molecule-1, E-selectin, and monocyte integrins-β1 and -β2 through blocking the mesangial Tyk2 pathway. In the in vivo animal study, *db/db* mice were treated with 10 mg/kg PCA daily for 8 wk. PCA attenuated CXCR2 induction and the activation of Tyk2 and STAT1/3 in *db/db* mice. Periodic acid-Schiff staining showed that PCA alleviated mesangial expansion-elicited renal injury in diabetic kidneys. In glomeruli, PCA attenuated the induction of intracellular cell adhesion molecule-1 and CD11b. PCA diminished monocyte chemoattractant protein-1 expression and macrophage inflammatory protein 2 transcription in the diabetic kidney, inhibiting the induction of the macrophage markers CD68 and F4/80. These results demonstrate that PCA antagonized the infiltration and accumulation of macrophages in diabetic kidneys through disturbing the mesangial IL-8-Tyk-STAT signaling pathway. Therefore, PCA may be a potential renoprotective agent treating diabetes-associated glomerulosclerosis.

db/db mice; glomerulosclerosis; macrophage infiltration; monocyte activation; purple corn anthocyanins

THE MESANGIUM IS AN INNER layer of the glomerulus, within the basement membrane surrounding the glomerular capillaries. Intraglomerular mesangial cells are specialized smooth muscle cells and pericytes located among the glomerular capillaries within a renal corpuscle of the kidney. These cells secrete the amorphous basement membrane-like materials known as mesangial extracellular matrix (ECM). The pathogenesis of diabetic nephropathy (DN) is histologically characterized by morphological and ultrastructural changes in the kidney, including expansion of mesangial cells and ECM and loss of the charge barrier on the glomerular basement membrane (10, 23). Mesangial cells produce and respond to a variety of cytokines and growth factors and also play an important role in responses to local injury (12, 18, 25).

DN is one of the most common microvascular complications of diabetes and a leading cause of end-stage renal disease (19, 22). Chronic hyperglycemia has been implicated as a major contributor to several diabetic complications (20, 27). Intracellular signaling events in glomerular endothelial cells induced by hyperglycemia result in endothelial dysfunction, inflammation, and microvascular thrombosis (6, 14, 18, 30). Diabetes-associated intrinsic factors such as cytokines induce atherosclerotic and inflammatory diseases (27). Hyperglycemia induces mesangial fibrosis that requires activation of IL-8 (17). In the kidney, high glucose (HG) promotes mesangial production of monocyte chemoattractant protein-1 (MCP-1), IL-6, and TNF-α, which, together with adhesion molecules, favor leukocyte recruitment and adhesion to endothelial cells (5, 21). The interaction of monocytes with mesangial cells is important in activating monocytes to migrate from the circulation to the kidney in the early stages of DN (21). Although the exact mechanism of monocyte/macrophage recruitment to the glomerulus is unknown, increased renal expression of MCP-1 is considered to be important in the initiation of this process (3, 5, 15, 21). In addition, the infiltrated macrophages may induce or accelerate the mesangial cell proliferation and injury in diabetic kidneys (8, 9, 29).

Purple corn, known as *Zea mays L.*, has been utilized for centuries for daily food and drink and cultivated in South America, mainly in Peru and Bolivia. The food colors in purple corn are rich in anthocyanins and functional phenolics (11). Anthocyanins have been reported to possess antidiabetic, antiangiogenic, and anticarcinogenic activities for potential medicinal use (4, 32, 33). However, little investigation has been made of the effects of anthocyanins on monocyte recruitment to glomerular endothelial cells and monocyte infiltration into the mesangium under hyperglycemic conditions. It is hypothesized that natural compounds such as anthocyanins may retard diabetes-associated recruitment and infiltration of monocytes onto glomerular endothelial cells.

Based on possible antidiabetic functions of anthocyanin-rich purple corn extract as described in the literature (31), an in vitro study attempted to determine whether anthocyanin-rich purple corn extract (PCA) prevented monocyte trafficking onto glomerular endothelium by glomerular mesangial cells exposed to HG. The in vitro study investigated cellular expression levels of cell adhesion molecules of endothelial cells and integrins of monocytes in conditioned media of human renal mesangial cells (HRMC). Intracellular signaling events of tyrosine kinase (Tyk) 2-signal transducers and activators of

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transcription (STAT) were explored in terms of cellular induction of cell adhesion molecules and integrins. In addition, an in vivo study employing *db/db* mice elucidated whether PCA retarded glomerular expansion and monocyte/macrophage infiltration. The mouse kidney tissues were histologically stained with periodic acid-Schiff (PAS) and immunohistochemically analyzed using antibodies of intracellular adhesion molecule-1 (ICAM-1), CD11b, CD68, and F4/80. In our in vitro experiments, IL-8 activation was responsible for hyperglycemia-associated mesangial fibrosis, which was mediated via eliciting the Tyk2-STAT signaling pathway (17). Accordingly, the involvement of IL-8-Tyk2-STAT signaling in monocyte/macrophage infiltration was examined in mouse kidney tissues.

MATERIALS AND METHODS

Materials. Fetal bovine serum (FBS), trypsin-EDTA, and penicillin-streptomycin were purchased from Lonza (Walkersville, MD). DMEM, nutrient mixture F-12 Ham medium, mannitol, and D-glucose were obtained from Sigma-Aldrich (St. Louis, MO), as were all other reagents, unless specifically stated elsewhere. VCAM-1, ICAM-1, integrin-β1, integrin-β2. and CD68 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); E-selectin and MCP-1 antibodies were provided by R&D Systems (Minneapolis, MN); and phospho-Tyk2, phospho-STAT1, and phospho-STAT3 were purchased from Cell Signaling Technology (Beverly, MA). CXCR2, CD11b, and F4/80 antibodies were supplied from Abcam Biochemicals (Cambridge, UK). β-Actin antibody was obtained from Sigma-Aldrich. Horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse, and donkey anti-goat IgG were purchased from Jackson ImmumnoResearch Laboratories (West Grove, PA).

Preparation of purple corn extracts. Purple corn kernel powder was obtained from the Brilliant Project International (Seoul, Korea). The powder was applied to a glass open column (10.0 × 900 mm ID) packed with Diaion HP-20 (Mitsubishi Kasei, Tokyo, Japan) and

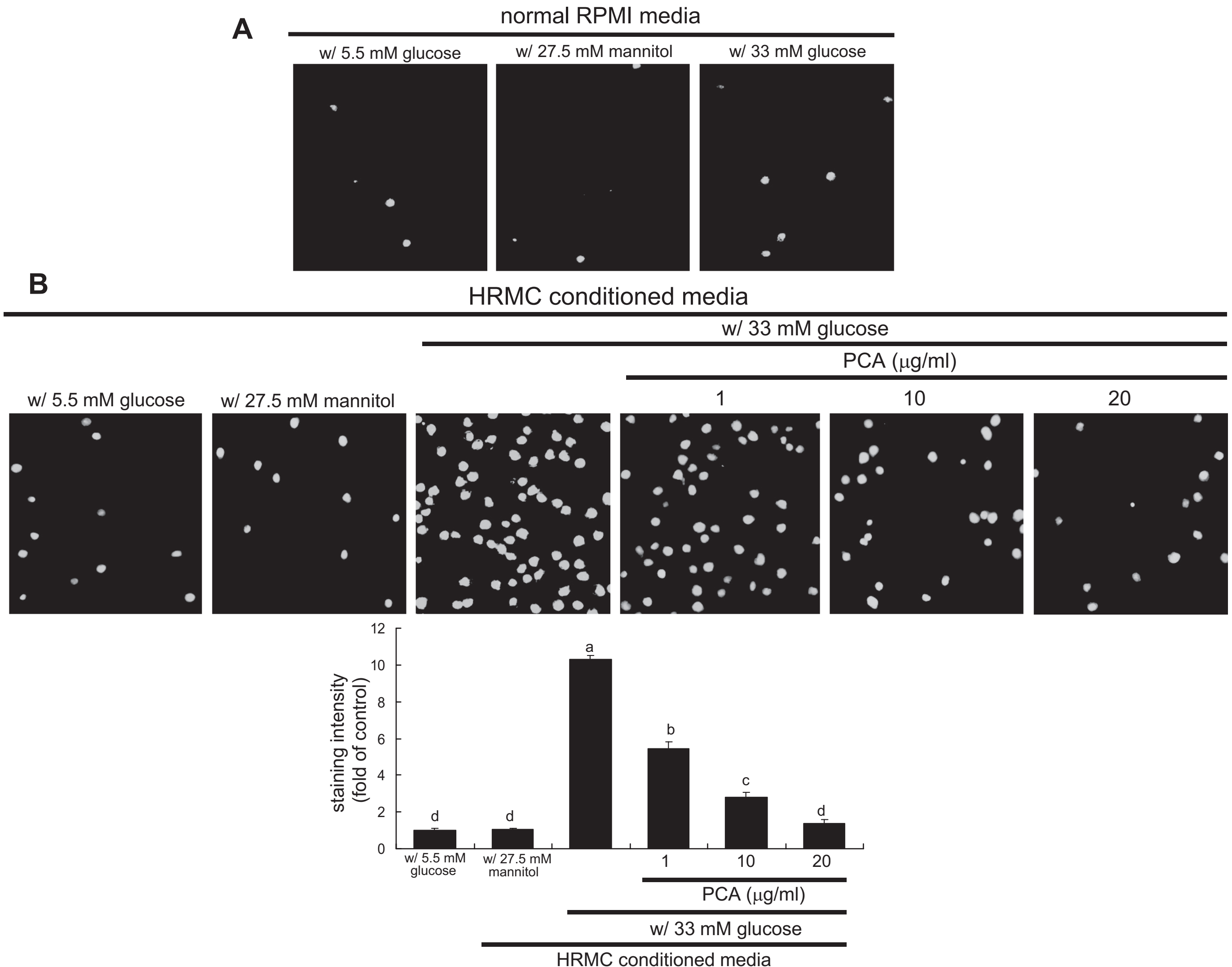

Fig. 1. Anthocyanin-rich purple corn extract (PCA) suppression of monocyte adhesion by adding PCA to human renal mesangial cell (HRMC)-conditioned media. THP-1 cells were cultured in normal RPMI media (*A*) and in conditioned media (*B*) collected from HRMC cultured in different media (with 5.5 mM glucose, 27.5 mM mannitol, and 33 mM glucose) for 24 h and then stained with calcein AM for 30 min. Microphotographs (3 independent experiments) were obtained using fluorescence microscopy with fluorescent blue filters. Magnification: ×200. The bar graph (*B*, *bottom*, $n = 3$) represents quantitative results obtained by using a Fluoroscan ELISA plate reader at $\lambda = 485$-nm excitation and $\lambda = 538$-nm emission. Means without a common letter (*a*–*d*) differ, $P < 0.05$.

eluted with water for washing of sugar or nonpolyphenolic components, followed by 95% ethanol for PCA.

Cell culture. HRMC (ScienCell Research Laboratories, Carlsbad, CA) were cultured in a 37°C humidified atmosphere of 5% CO_2 in air. Routine culture of HRMC was performed in DMEM plus F-12 (7:1) media containing 15% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. HRMC in *passages 6–10* were subcultured at 90% confluence and used for further experiments. To prepare different conditioned media, cells were incubated in serum-free media with 5.5 mM glucose, 5.5 mM glucose plus 27.5 mM mannitol, or 33 mM glucose for 3 days. Subsequently, each culture medium was collected, centrifuged at 1,500 rpm for 10 min to remove cellular debris, decanted into clean tubes, and the conditioned medium was stored at −20°C.

Human umbilical vein endothelial cells (HUVEC) isolated using collagenase were cultured in 25 mM HEPES-buffered M199 containing 10% FBS, 2 mM glutamine, 0.75 μg/ml human epidermal growth factor, and 75 μg/ml hydrocortisone in a 37°C humidified atmosphere of 5% CO_2 in air. HUVEC with cobblestone morphology were passaged at confluence and used within 10 passages. HUVEC were cultured in respective HRMC-conditioned media in the absence and presence of 1–20 μg/ml PCA for 6 h.

For the measurement of PCA toxicity, HRMC and HUVEC were incubated in fresh phenol red-free DMEM containing 1 mg/ml 3-(4, 5-dimetylthiazol-yl)-diphenyl tetrazolium bromide (MTT; DUCHEFA Biochemie, Haarlem, The Netherlands). After unconverted MTT was removed, the purple formazan product was dissolved in isopropanol with gentle shaking. Absorbance of formazan dye was measured at λ = 570 nm, with background subtraction at λ = 690 nm.

Western blot analysis. Western blot analysis was conducted using whole cell lysates collected from culture media prepared from HUVEC at a density of 5.0×10^5 cells (13). Mouse kidney tissue extracts were also prepared from mice that were subjected to the PCA experimental protocol. Whole cell lysates and kidney tissue extracts 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 a protease inhibitor cocktail. Cell lysates containing equal amounts of proteins or equal volumes of culture media were electrophoresed on 6–10% SDS-PAGE and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked with 3% bovine serum albumin for 3 h. The membrane was incubated overnight at 4°C with a primary antibody and washed in a TBS-T for 10 min. The membrane was then incubated for 1 h 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), Immunobilon Western Chemiluminescent Horseradish Peroxidase Substrate (Millipore, Billerica, MA), and Agfa X-ray film (Agfa-Gevaert). Incubation with anti-human β-actin was also performed for comparative control.

Cultured THP-1 monocytes and in vitro cell adhesion assay. Human monocytic THP-1 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium containing 10% FBS. Starved THP-1 cells in RPMI 1640 medium were grown on

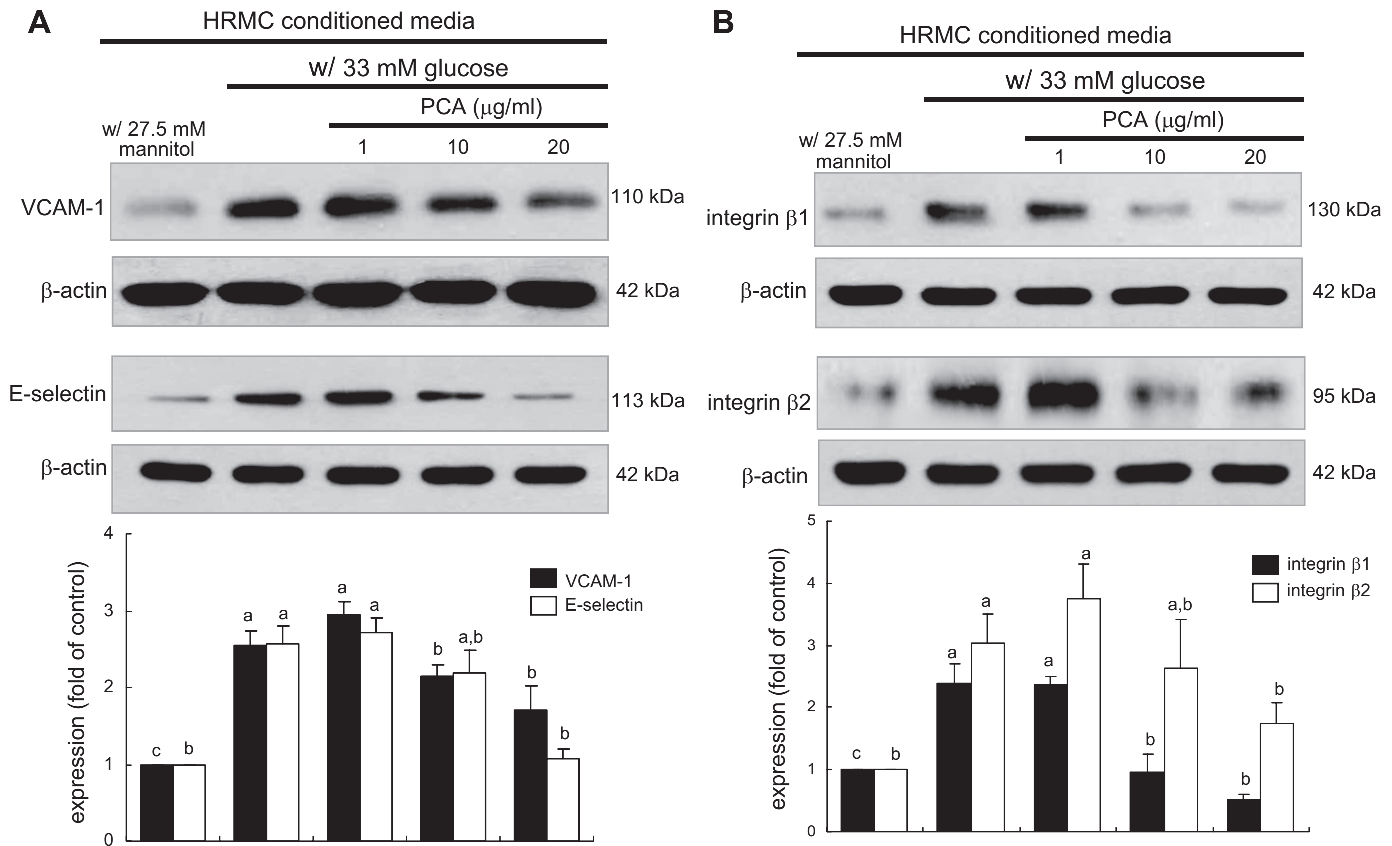

Fig. 2. Inhibition of human umbilical vein endothelial cell (HUVEC) expression of VCAM-1 and E-selectin (*A*) and THP-1 expression of integrin-β1 and integrin-β2 (*B*) by PCA. HRMC were incubated in 5.5 mM glucose plus 27.5 mM mannitol for osmotic control or 33 mM glucose. HUVEC and THP-1 monocytes were treated with 20 μg/ml PCA added to HRMC-conditioned media (with 27.5 mM mannitol and with 33 mM glucose) for 6 h. Cell lysates were subjected to Western blot analysis with a primary antibody of VCAM-1, E-selectin, integrin-β1, or integrin-β2. β-Actin protein was used as an internal control. The bar graphs (means ± SE, $n = 3$) at the *bottom* represent quantitative results obtained from a densitometer. Means not sharing a common letter (*a–c*) differ, $P < 0.05$.

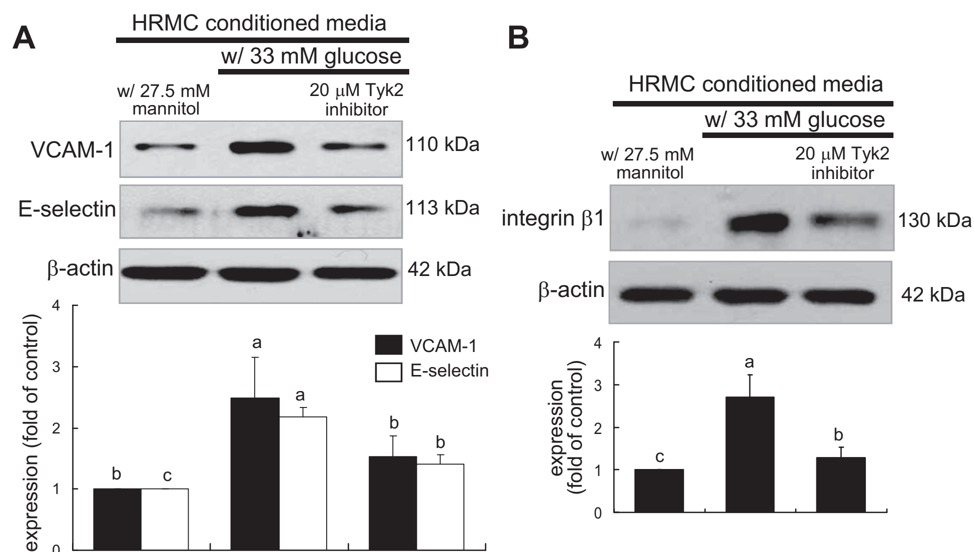

Fig. 3. Attenuation of HUVEC expression of VCAM-1 and E-selectin (*A*) and THP-1 expression of integrin-β1 (*B*) by Tyk2 inhibition in HRMC-conditioned media. HRMC were incubated in 5.5 mM glucose plus 27.5 mM mannitol for osmotic control or 33 mM glucose. HUVEC and THP-1 monocytes were treated with 20 μM Tyk2 inhibitor added to HRMC-conditioned media (with 27.5 mM mannitol and with 33 mM glucose) for 6 h. Cell lysates were subjected to Western blot analysis with a primary antibody of VCAM-1, E-selectin, or integrin-β1. β-Actin protein was used as an internal control. The bar graphs (means ± SE, n = 3) at the *bottom* represent quantitative results obtained from a densitometer. Respective values not sharing a common letter (*a–c*) differ, $P < 0.05$.

24-well glass slides in different mesangial cell-conditioned media and treated with 1–20 μg/ml PCA. After 24-h incubation, 5 nM calcein AM was added to the glass slides for 30 min in the dark. Glass slides were rinsed thoroughly with phosphate-buffered saline containing 0.2% Tween 20 (PBS-T). Cells were fixed with 4% ice-cold formaldehyde for 20 min and mounted for microscopic observation (13). Fluorescent images were obtained with an Axio Imager optical fluorescence microscope (Zeiss, Göttingen, Germany). The quantitative results were obtained by using a Fluoroscan ELISA plate reader (Bio-Rad Laboratories, Hercules, CA) at λ = 485-nm excitation and λ = 538-nm emission.

In vivo animal experiments. Adult male *db/db* mice (C57BLKS/+Lepr^{db} Iar; Jackson Laboratory) and their age-matched nondiabetic *db/m* littermates (C57BLKS/J; Jackson Laboratory) were used in the present study (17). Mice were kept on a 12:12-h light-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), and provided with water ad libitum at the animal facility of Hallym University. This study included 8-wk-old *db/db* mice because they develop diabetes (hyperglycemia) at the age of 7–8 wk (16). The animals were allowed to acclimate for 1 wk before the beginning of the experiments. Mice were divided into three subgroups (n =

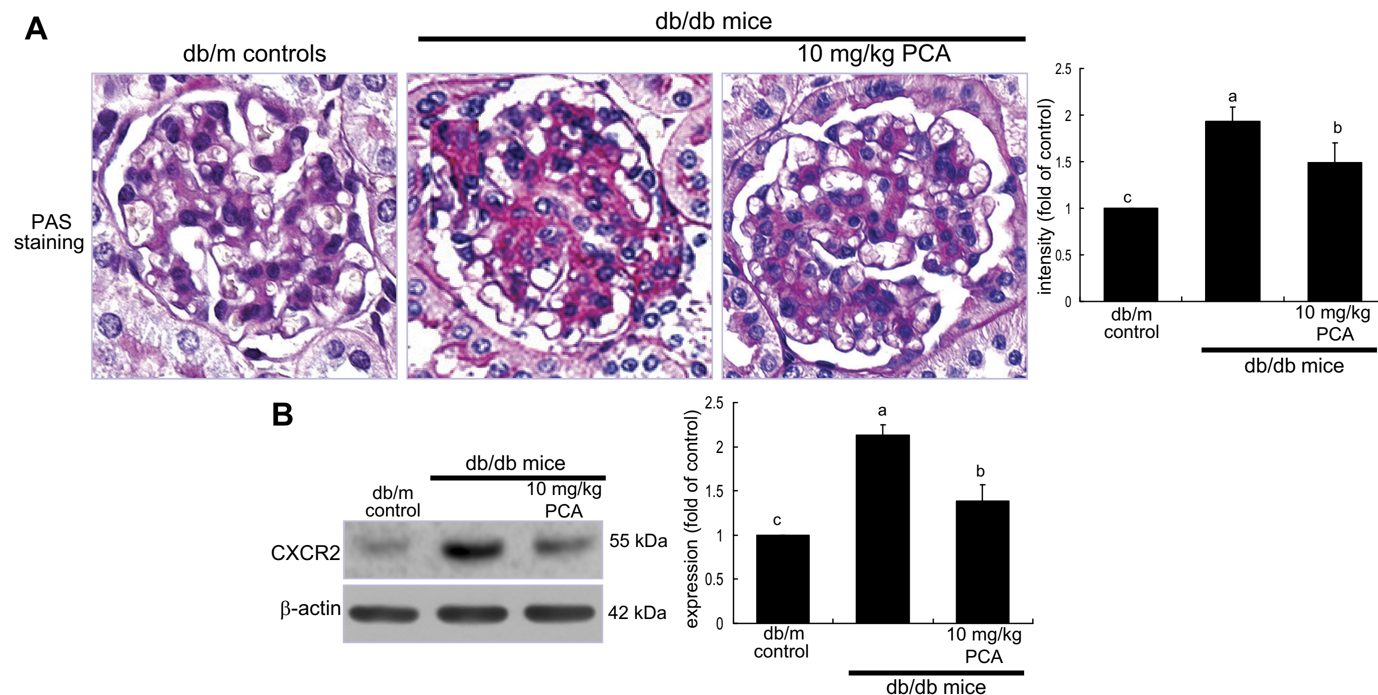

Fig. 4. Histological staining by periodic acid-Schiff (PAS; *A*) showing the diminution of mesangial expansion and Western blot analysis (*B*) showing inhibition of CXCR2 induction in *db/db* mice supplemented with PCA. The *db/db* mice were orally supplemented with 10 mg/kg PCA daily for 8 wk; *db/m* mice were introduced as control animals. Histological sections of mouse kidneys were stained by using PAS reagents and counterstained with hematoxylin. Each photograph is representative of 4 animals (*A*). The PAS intensity was quantified and is shown on the *right*. Magnification: ×400. For the measurement of CXCR2 levels, tissue extracts were subjected to Western blot analysis with a primary antibody against CXCR2. β-Actin protein was used as an internal control. The bar graph (means ± SE, n = 9–10; *right*) represents quantitative results obtained from a densitometer. Means not sharing a common letter (*a–c*) differ, $P < 0.05$.

8–10/subgroup). The first group of mice consisted of nondiabetic *db/m* control mice, which received drinking water as the PCA vehicle. The other *db/db* mice were orally administrated drinking water or 10 mg/kg body wt PCA daily for 8 wk. 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 died, and no apparent signs of exhaustion were observed during the experimental period.

Renal histology and immunohistochemical staining. The pathological kidney changes were examined with PAS reagent. Paraffin-embedded kidney tissues were cut into 5-μm sections and stained by using PAS reagent to identify kidney structure and hematoxylin for counterstaining.

For the immunohistochemical analyses, paraffin-embedded integument sections (5 μm thick) were employed. The sections were placed on glass slides, deparaffinized, and hydrated with xylene and graded alcohol. The sections were preincubated in boiling sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for antigen retrieval. A specific primary antibody against ICAM-1, CD11b, CD68, or F4/80 was incubated with sections overnight. For visualization, the sections were developed with 3,3′-dianinobenzidine to produce a brown color, counterstained with hematoxylin, and mounted in mounting medium. The stained tissue sections were examined using an Axio Imager optical microscope (Zeiss), and five images (×400) were taken for each section. PAS staining and protein levels of CD68 and F4/80 were quantified by an image-analysis program for the microscope system.

ELISA. The expression level of the chemokine macrophage inflammatory protein 2 (MIP-2) in mouse glomerulus was determined by using ELISA. Mouse kidney tissue extracts containing equal amounts of proteins were assayed to measure the tissue level of MIP-2 using an ELISA kit (R&D Systems) according to the manufacturer's instructions.

Data analysis. The results are presented as means ± SE. Statistical analyses were conducted using the Statistical Analysis Software package, version 6.12 (SAS Institute, Cary, NC). Significance was determined by two-way ANOVA, followed by Duncan's multiple-range test for multiple comparisons. Differences were considered significant at $P < 0.05$.

RESULTS

Inhibition of monocyte adhesion by PCA. This study attempted to examine whether HG-exposed mesangial cells facilitated monocyte adhesion to glomerular endothelial cells in a paracrine fashion, which was interrupted by PCA. THP-1 cells were incubated in normal RPMI 1640 media or HRMC-conditioned media for 24 h. Conditioned media were collected from HRMC cultured in DMEM/F-12 containing 5.5 mM glucose plus 27.5 mM mannitol or 33 mM glucose. THP-1 cells did not adhere onto well glass slides even in RPMI 1640 media containing 33 mM glucose (Fig. 1*A*). However, there was heavy staining observed in THP-1 cells exposed to HG-HRMC-conditioned media (with 33 mM glucose), showing increased THP-1 cell adherence (Fig. 1*B*). When nontoxic PCA at 1–20 μg/ml was supplemented to THP-1 cells in HG-HRMC-conditioned media, the adherence was dose dependently decreased. It should be noted that in low glucose-conditioned media (with 5.5 mM glucose) or mannitol-conditioned media (with 27.5 mM mannitol), small numbers of THP-1 cells were attached to glass slides (Fig. 1*B*). Accordingly, HRMC exposed to HG conditions promoted THP-1 monocyte adhesion to glomerular endothelial cells, facilitating the trafficking of inflammatory cells to the glomerular endothelium.

Suppressive effects of PCA on expression of adhesion molecules. The early stage of diabetic nephropathy involves inflammatory cell recruitment and transmigration from the circulation (1). Adhesion molecules may favor leukocyte recruitment and adhesion to glomerular endothelial cells. Expression of VCAM-1 and E-selectin in endothelial cells was up-

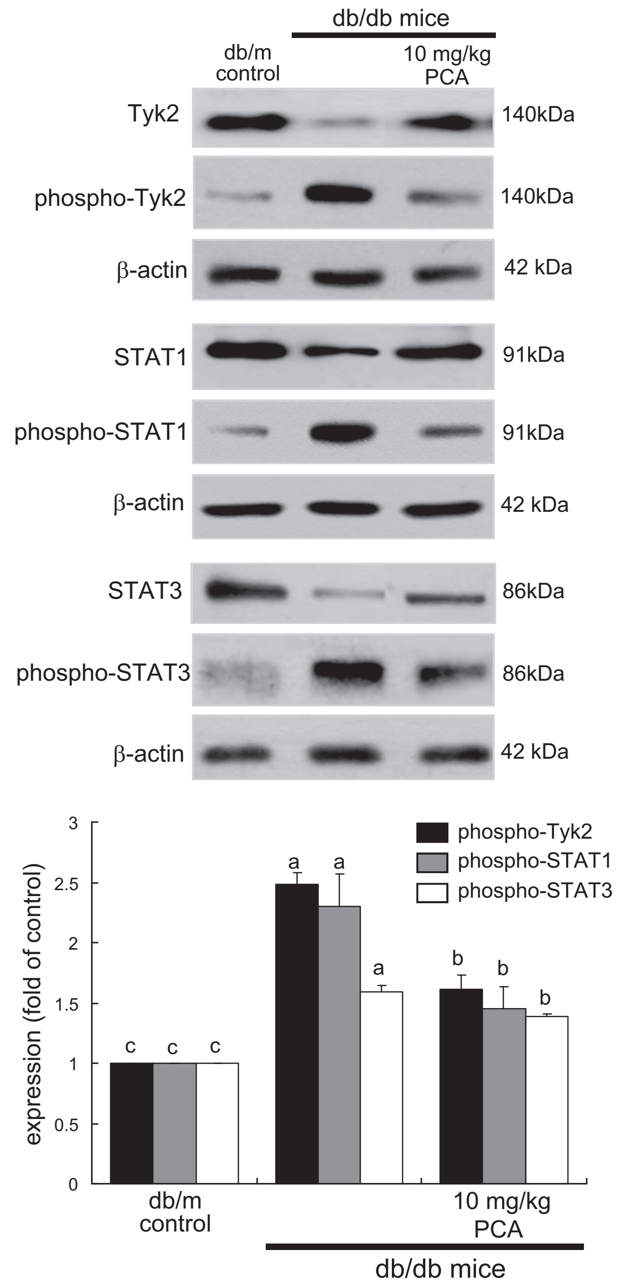

Fig. 5. Western blot analysis showing the inhibition of phosphorylation of Tyk2, STAT1, and STAT3 in *db/db* mice supplemented with PCA. The *db/db* mice were orally supplemented with 10 mg/kg PCA daily for 8 wk; *db/m* mice were introduced as a control. For the measurements of tissue levels of Tyk2, STAT1, STAT3, phospho-Tyk2, phospho-STAT1, and phospho-STAT3, tissue extracts were subjected to Western blot analysis with a primary antibody against Tyk2, STAT1, STAT3, phospho-Tyk2, phospho-STAT1, or phospho-STAT3. β-Actin protein was used as an internal control. The bar graph (means ± SE, $n = 9–10$) at the *bottom* represents quantitative results obtained from a densitometer. Respective values not sharing a common letter (*a*–*c*) differ, $P < 0.05$.

regulated by culturing them in HG-HRMC-conditioned media (Fig. 2*A*). In contrast, PCA suppressed the induction of endothelial VCAM-1 and E-seclectin. In addition, the expression of integrin-β1 and -β2, the monocyte receptors interacting with VCAM-1 and ICAM-1, was induced in THP-1 cells cultured in HG-HRMC-conditioned media (Fig. 2*B*). It should be noted that PCA was not significantly toxic to HUVEC and THP-1 cells in the range of ≤50 μg/ml concentrations (data not shown). Similarly, it was found that PCA diminished the induction of monocyte integrins. Thus PCA dampened the recruitment and transmigration of inflammatory cells by retarding the respective adhesion molecules and integrins of endothelial cells and monocytes during the process of HG-induced mesangial expansion.

Blockade of Tyk2 involvement in adhesion molecule expression by PCA. This study examined whether mesangial Tyk2 activation was responsible for the HG-HRMC-conditioned media-induced expression of VCAM-1, E-selectin, and integrin-β1 in HUVEC and THP-1 cells. When 20 μM Tyk2 inhibitor was added to HUVEC cultured in HG-HRMC-conditioned media, the increased expression of endothelial VCAM-1 and E-selectin was suppressed (Fig. 3*A*). Similar effects of the Tyk2 inhibitor on integrin-β1 induction of THP-1 cells were observed (Fig. 3*B*). Accordingly, the expression of endothelial cell adhesion molecules and monocyte integrins appeared to be mediated via the mesangial activation of Tyk2, a member of the JAK family.

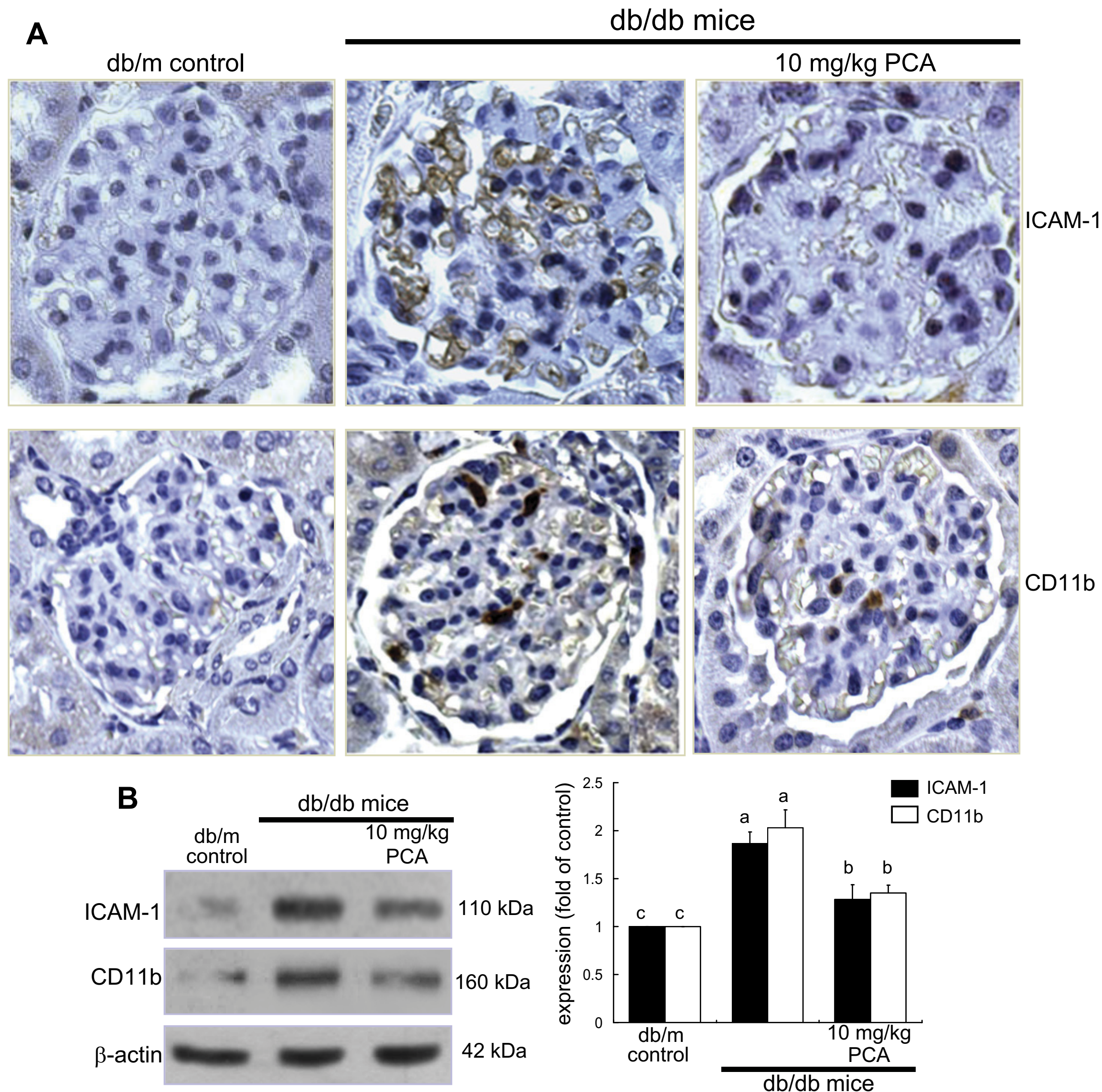

Fig. 6. Immunohistochemical staining (*A*) and Western blot analysis (*B*) showing the induction of ICAM-1 and integrin-αM CD11b in *db/db* mice supplemented with PCA. The *db/db* mice were orally treated with 10 mg/kg PCA for 8 wk; *db/m* mice were introduced as a control. For the measurements of the ICAM-1 and CD11b expression levels, histological sections of mouse kidneys were immunohistochemically stained using anti-mouse ICAM-1 and anti-mouse CD11b and stained with a secondary antibody of 3,3′-diaminobenzidine-conjugated IgG (*A*). The sections were counterstained with hematoxylin. The ICAM-1 and CD11b expression levels were identified as brown staining. Each photograph is representative of 4 animals. Magnification: ×400. Tissue extracts were subjected to Western blot analysis with a primary antibody against ICAM-1 or CD11b (*B*). β-Actin protein was used as an internal control. The bar graph (means ± SE, $n = 9–10$; *bottom right*) represents quantitative results obtained from a densitometer. Respective values not sharing a letter (*a–c*) differ, $P < 0.05$.

Improvement of histological changes and blockade of macrophage infiltration by PCA. Mesangial expansion and matrix accumulation are major features of diabetic glomerulosclerosis (2). In this study, histological changes in kidney glomeruli were examined in *db/db* mice having experienced 8-wk experimental periods, as observed by using PAS staining. Histological staining with PAS for the detection of mesangial expansion showed that there was dark-reddish staining in the glomeruli of *db/db* mice compared with in *db/m* controls (Fig. 4*A*). In contrast, PCA treatment diminished the staining, revealing the retardation of mesangial expansion in *db/db* mice.

We have previously shown that mesangial fibrosis required IL-8 activation via eliciting the Tyk2-STAT signaling pathway (17). This study investigated whether the activation and filtration of monocytes entailed renal IL-8 activation accompanying activation of the Tyk2-STAT pathway. The induction of IL-8 receptor-β, known as CXCR2, was markedly enhanced in kidney tissues of *db/db* mice, while CXCR2 induction was reduced by PCA supplementation (Fig. 4*B*).

This study further elucidated that the activation of the Tyk2-STAT pathway in *db/db* mice was disturbed by PCA.

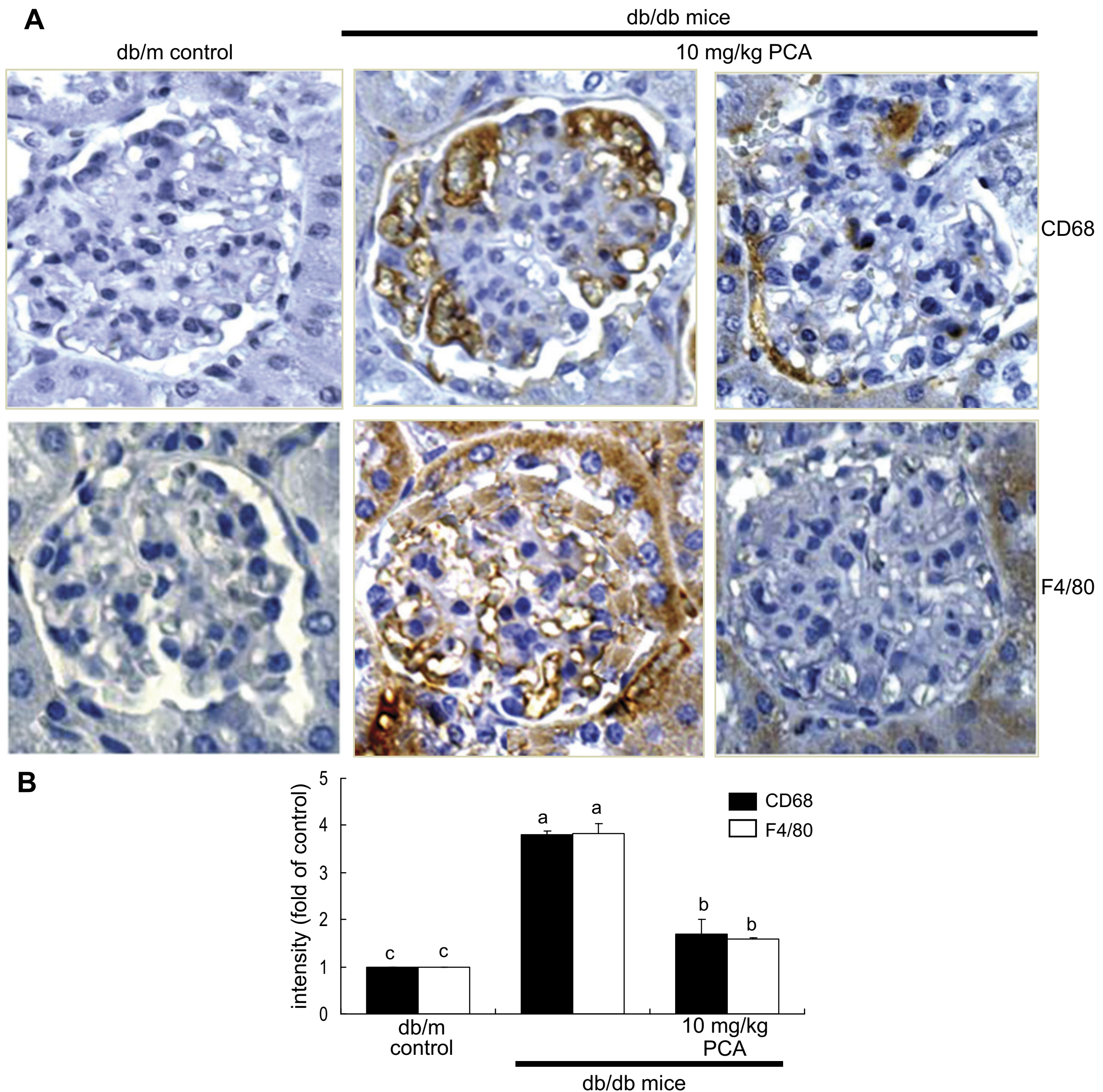

Fig. 7. Suppression of macrophage markers CD68 and F4/80 in *db/db* mice supplemented with PCA. The *db/db* mice were orally treated with 10 mg/kg PCA for 8 wk; *db/m* mice were introduced as a control. For the measurements of CD68 and F4/80 tissue levels, histological sections of mouse kidneys were stained using anti-mouse CD68 and anti-mouse F4/80 and stained with a secondary antibody of 3,3′-diaminobenzidine-conjugated IgG. The sections were counterstained with hematoxylin. CD68 and F4/80 levels were identified as brown staining. Each photograph is representative of 4 animals. Magnification: ×400. CD68 and F4/80 levels were quantified and are shown at the *bottom*. The bar graph (means ± SE, $n = 9–10$) represents quantitative results. Means not sharing a common letter (*a–c*) differ, $P < 0.05$.

Western blot analysis showed that the levels of phospho-Tyk2, phospho-STAT1, and phospho-STAT3 were elevated in *db/db* mice with a decrease in the levels of their total forms (Fig. 5). The activation of Tyk2 and of its downstream proteins of STAT1 and STAT3 was dampened in 10 mg/kg PCA-treated *db/db* mice. Accordingly, PCA can interrupt IL-8-Tyk2-STAT signaling that may instigate glomerular adhesion and infiltration of inflammatory cells responsible for diabetic glomerulosclerosis.

Inhibition of macrophage accumulation by PCA in the kidney. ICAM-1 is involved in cell-cell interaction and adhesion and promotes macrophage accumulation in the diabetic kidney (28). Immunohistochemical analysis showed that the induction of ICAM-1 and integrin-αM (CD11b) was enhanced in the glomeruli of *db/db* mice (Fig. 6*A*). In 10 mg/kg PCA-treated mice, the expression of ICAM-1 and CD11b was comparable to, if not indistinguishable from, that of *db/m* mice. The Western blot data supported the immunohistochemical data (Fig. 6*B*). Accordingly, PCA appeared to interfere with leukocyte recruitment and adhesion to glomerular endothelial cells.

This study tested whether PCA blocked macrophage infiltration in the glomeruli of *db/db* mice by examining the induction of the macrophage markers CD68 and F4/80. Immunostaining demonstrated that noticeable increases in CD68 and F4/80 levels were observed in *db/db* mouse glomeruli (brown color) compared with *db/m* control mice (Fig. 7). In marked contrast, the tissue levels of CD68 and F4/80 in *db/db* mice supplemented with 10 mg/kg PCA decreased compared with those in *db/db* mice (Fig. 7).

Renal levels of MCP-1 and MIP-2 involved in monocyte chemotaxis and macrophage infiltration were examined in *db/db* mice. MCP-1 was upregulated in kidney tissues of *db/db* mice, whereas 10 mg/kg PCA attenuated its induction (Fig. 8*A*). In addition, RT-PCR data showed that PCA inhibited MIP-2 transcription enhanced in *db/db* mice (Fig. 8*B*). Thus PCA may inhibit macrophage infiltration closely linked to renal inflammation.

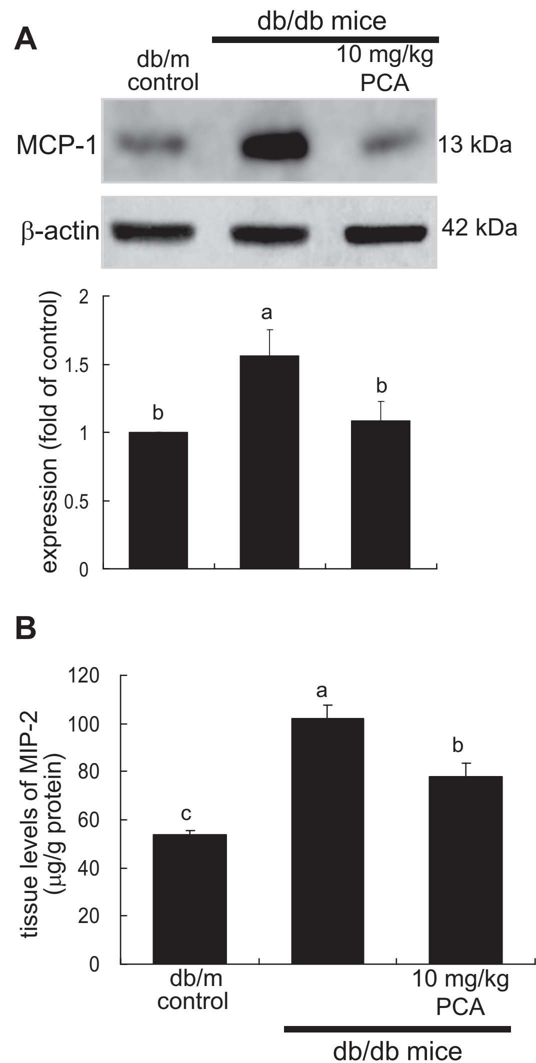

Fig. 8. Inhibition of monocyte chemoattractant protein-1 (MCP-1; *A*) and macrophage inflammatory protein 2 (MIP-2; *B*) induction in *db/db* mice supplemented with PCA. The *db/db* mice were orally supplemented with 10 mg/kg PCA daily for 8 wk; *db/m* mice were introduced as a control. Tissue extracts were subjected to Western blot analysis with a primary antibody against MCP-1 (*A*). β-Actin protein was used as an internal control. The bar graphs (means ± SE, n = 9–10) at the *bottom* represent quantitative results obtained from a densitometer. Tissue MIP-2 level was measured by using a MIP-2 ELISA kit (*B*). Respective values not sharing a common letter (*a*–*c*) differ, $P < 0.05$.

DISCUSSION

It is generally accepted that chronic hyperglycemia results in endothelial dysfunction, inflammation, and microvascular thrombosis (27) and leads to kidney failure like glomerulosclerosis. Hyperglycemia has been implicated as a major contributor to DN by inducing kidney hyperfiltration and mesangial expansion (14, 18). Simultaneously, pathological changes take place in the glomerular vessels, including monocyte adhesion provoked by the vascular pathological environment. HG augments monocyte activation and infiltration by increasing proinflammatory cytokine secretion by mesangial cells (21). Accordingly, the association of monocytes with mesangial cells may be important in migrating monocytes to the kidney in early DN. To mimic the molecular pathological environment of DN, this study applied HG-cultured mesangial cell-conditioned media to endothelial cells and THP-1 monocytes. Our study showed that the adhesion of THP-1 monocytes was elevated when they were cultured in conditioned media of mesangial cells exposed to HG. The HG-HRMC-conditioned media upregulated expression of endothelial adhesion molecules VCAM-1 and E-selection. In addition, the monocyte expression of integrin-β1 and -β2 communicating with these adhesion molecules was induced by HG-HRMC-conditioned media. Some mediators released from HG-exposed mesangial cells may favor monocyte activation and adhesion by enhancing induction of endothelial cell adhesion molecules and monocyte integrins. In the diabetic kidney, MCP-1, IL-6, and TNF-α produced by mesangial cells promotes leukocyte recruitment and adhesion to endothelial cells (21). Nevertheless, the exact mechanism of monocyte/macrophage recruitment to the glomerulus is unknown. Our previous study showed that mesangial fibrosis and matrix accumulation required IL-8 activation (17).

Anthocyanins and functional phenolics are major constituents of food colors in purple corn (11). Anthocyanins exhibiting antidiabetic, antiangiogenic, and anticarcinogenic activities

have been suggested for potential medicinal uses (4, 32). This may illuminate that anthocyanins could be a main biofunctional compound in purple corn to prevent renal vascular diseases. Some studies have proved that cyanidin 3-*O*-β-D-glucoside-rich purple corn prevents obesity and hyperglycemia in mice (31). However, there was little investigation made into the effects of anthocyanins on diabetes-associated monocyte activation and macrophage infiltration to the renal mesangium. In this study, PCA downregulated expression of endothelial cell adhesion molecules and monocyte integrins, possibly attenuating monocyte adhesion onto glomerular endothelial cells. In the renal tissues of *db/db* mice, the induction of key leukocyte adhesion factors ICAM-1 and CD11b was reduced by supplementing PCA. The kidney mesangium produces and responds to a variety of cytokines and growth factors and plays an important role in response to local injury (12, 25). It should be noted that in this study endothelial cells and THP-1 monocytes were exposed to various cytokines and growth factors originating from HG-exposed mesangial cells. Accordingly, PCA antagonized the effects of unknown mediators released in HG-HRMC-conditioned media on the induction of adhesion molecules and integrins required for glomerular monocyte interaction.

Macrophage accumulation and activation in diabetic *db/db* kidneys are associated with increased kidney chemokine production (21). Novel anti-inflammatory treatments to reduce macrophage-mediated injury in diabetic kidneys are considered, which has important implications for the management of patients with DN (7). This study attempted to explore the mechanisms of monocyte recruitment and macrophage infiltration and the process of macrophage-mediated injury and sclerosis in diabetic kidneys. Our previous study found that the cytokine IL-8 was one of mesangial cell-secreted factors that increased mesangial fibrosis (17). These results raise the possibility of specific therapies for targeting monocyte/macrophage infiltration. In the current study, the high level of CXCR2 was observed in kidney tissues of *db/db* mice. Thus the inflammatory responses and kidney macrophage infiltration linked to glomerulosclerosis were induced possibly due to mesangial IL-8 in *db/db* mice. This study found that the mesangial activation of VCAM-1, E-selectin, and integrin-β1 was attenuated by Tyk2 inhibition. In addition, PCA reduced the kidney tissue levels of Tyk2 and STAT1/3 elevated in *db/db* mouse kidneys. Collectively, PCA can interrupt IL-8-Tyk2-STAT signaling that may instigate glomerular adhesion and infiltration of inflammatory cells responsible for diabetic glomerulosclerosis.

Increased activation and infiltration of monocytes/macrophages has been demonstrated in renal biopsies in both experimental diabetes and patients with diabetic nephropathy (23, 24). Kidney macrophage accumulation was exacerbated with the duration of diabetes and the severity of renal injury and loss of renal function (7). Macrophage accumulation in the glomerulus and interstitium correlated with progressive glomerular and tubular injury (26). Further observations from *db/db* mouse models revealed that macrophages are the major immune cells infiltrating glomerular endothelial cells. CD68 is a typical marker demonstrating the macrophage lineage, and F4/80 is a transmembrane protein present on the cell surface of mouse macrophages and associated with mature macrophages. The present study showed that PCA attenuated the induction of CD68 and F4/80 monocyte/macrophage markers in the diabetic kidney. PCA appreciably hampered macrophage infiltration and accumulation in the glomeruli of *db/db* mice. On the other hand, MCP-1 expression and MIP-2 transcription were upregulated in kidney tissues of *db/db* mice, revealing that monocyte chemotaxis and macrophage infiltration were closely linked to renal inflammation. However, the stimulus for the increase in inflammation in diabetes is still under investigation (5). PCA suppressed the induction of these inflammatory mediators in diabetic mouse kidneys possibly by eliminating stimuli such as reactive oxygen species.

In summary, the in vitro study revealed that PCA disturbed the expression of endothelial cell adhesion molecules of VCAM-1 and E-selectin and the expression of monocyte integrin-β1 and -β2 enhanced by HG-HRMC-conditioned media. PCA blunted the mesangial Tyk-STAT signaling pathway responsible for the induction of endothelial adhesion molecules and monocyte integrins. In the in vivo study, PCA ameliorated diabetes-associated glomerular mesangial expansion and adhesion and infiltration of monocytes/macrophages. The capability of PCA to deter monocyte/macrophage infiltration into diabetic glomeruli may come from disturbing the renal IL-8-Tyk-STAT signaling pathway. Therefore, the renoprotection by PCA against mesangial activation of monocytes and infiltration of macrophages may be specific therapies targeting diabetes-associated diabetic glomerulosclerosis. In addition, PCA supplementation would be an important strategy for preventing renal vascular diseases in type 2 diabetes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: M.-K.K., S.-N.K., and Y.-H.K. provided conception and design of research; M.-K.K. and J.L. performed experiments; M.-K.K., J.L., J.-L.K., and J.-H.G. analyzed data; M.-K.K., J.L., J.-L.K., and J.-H.G. interpreted results of experiments; M.-K.K., J.L., and J.-L.K. prepared figures; M.-K.K., J.-L.K., S.-N.K., and Y.-H.K. drafted manuscript; J.H.Y.P., J.-Y.L., S.S.L., and Y.-H.K. edited and revised manuscript; J.-Y.L., S.S.L., and Y.-H.K. approved final version of manuscript.

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