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

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Abbreviations: DN, diabetic nephropathy; ECM, extracellular matrix HG, high glucose; HRMC, human renal mesangial cells; ICAM-1, intracellular cell adhesion molecule-1; IL, interleukin; MCP-1 monocyte chemoattractant protein-1; PCA, anthocyanins-rich purple corn extract; STAT, signal transducers and activators of transcription; Tyk, tyrosine kinase; VCAM-1, vascular cell adhesion molecule-1

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ABSTRACT

Diabetic nephropathy (DN) is one of the major diabetic complications and the leading cause of end-stage renal diseases. In the early DN the renal injury and macrophage accumulation take place in the pathological environment of glomerular vessels adjacent to renal mesangial cells expressing pro-inflammatory mediators. Purple corns utilized as a daily food are 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 experienced 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 of β1 and β2 through blocking mesangial Tyk2 pathway. In the in vivo animal study, db/db mice were treated with 10 mg/kg PCA daily for 8 weeks. PCA attenuated the 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 MCP-1 expression and MIP-2 transcription in diabetic kidney, inhibiting the induction of the macrophage makers of CD68 and F4/80. These results demonstrate that PCA antagonized the infiltration and accumulation of macrophages in diabetic kidneys through disturbing mesangial IL-8-Tyk-STAT signaling pathway. Therefore, PCA may be a potential renoprotective agent treating diabetes-associated glomerulosclerosis.
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 leading to cause of end stage renal disease (1919,22). Chronic hyperglycemia has been implicated as a major contributor to several diabetic complications (1920,27). Intracellular signaling events in glomerular endothelial cells induced by hyperglycemia result in endothelial dysfunction, inflammation, and microvascular thrombosis (6,13,18,30). Diabetes-associated intrinsic factors such as cytokines induce atherosclerotic and inflammatory diseases (27). Hyperglycemia induces mesangial fibrosis that requires activation of interleukin (IL)-8 (17). In the kidney high glucose (HG) promotes mesangial production of monocyte chemoattractant protein-1 (MCP-1), IL-6, and tumor necrosis factor-α (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 as daily food and drinks and cultivated in South America, mainly in Peru and Bolivia. Food colors of purple corn
are rich in anthocyanins and functional phenolics (11). Anthocyanins have been reported to possess anti-diabetic, anti-angiogenic and anti-carcinogenic activities as potential medicinal uses (4,32,33). However, little investigation was made into the effects of anthocyanins on monocyte recruitment to glomerular endothelial cells and monocyte infiltration to 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 anti-diabetic functions of anthocyanin-rich purple corn extract as described in literatures (31), the 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 tyrosin kinase (Tyk) 2-signal transducers and activators of transcription (STAT) were explored in terms of cellular induction of cell adhesion molecules and integrins. In addition, the 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, the IL-8 activation was responsible for hyperglycemia-associated mesangial fibrosis, which was mediated via eliciting 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). Dulbecco’s modified Eagle’s media (DMEM), nutrient mixture F-12 Ham medium, mannitol, and D-glucose were obtained from Sigma-Aldrich Chemical (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), and E-selectin and MCP-1 antibodies were provided by R&D systems (Minneapolis, MN), phospho-Tyk2, phospho-STAT1 and phospho-STAT3 were purchased from Cell signaling Technology (Beverly, CA). CXCR2, CD11b and F4/80 antibodies were supplied from Abcam Biochemicals (Cambridge, UK). β-Actin antibody was obtained from Sigma-Aldrich Chemical. Horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse and donkey anti-goat IgG were purchased from Jackson ImmunoReserch Laboratories (West Grove, PA).

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 Company, Tokyo, Japan) and eluted with water for washing of sugar or non-polyphenolic components, followed by 95% ethanol for PCA.

Cell culture

Human renal MC (HRMC, Sciencell Research Laboratories, Carlsbad, CA) were cultured at 37°C humidified atmosphere of 5% CO₂ 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, 100 μg/ml streptomycin. HRMC in passage of 6-10 were sub-cultured 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 d. Subsequently, each culture medium was collected, centrifuged at 1500 rpm for 10 min to remove cellular debris, decanted into clean tubes, and the conditioned medium was stored at -20°C.

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 at 37°C humidified atmosphere of 5% CO₂ 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 a fresh phenol red-free DMEM containing 1 mg/ml 3-(4, 5-Dimetylthiazol-yl)-diphenyl tetrazolium bromide (MTT, DUCHEFA Biochemie, Haarlem, 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 culture media prepared from HUVEC at a density of 3.0 X 10⁵ cells (14). Mouse kidney tissue extracts were also prepared from mice that experienced PCA experimental episode. 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 Na₃VO₄ and 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) and Immunobilon Western Chemiluminescent Horseradish Peroxidase Substrate (Millipore Corp., Billerica, MA) and Agfa X-ray film (Agfa-Gevaert, Belgium). 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 in RPMI 1640 medium were grown on 24-well glass slides in different mesangial cell conditional media and treated with 1-20 μg/ml PCA. After 24 h incubation, 5 nM calcein AM was added onto glass slides for 30 min in 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 the microscopical observation (14). Fluorescent images were obtained by Axiomager optical fluorescence microscope (Zeiss, Germany). The quantitative results were obtained by using a Fluoroscan enzyme-linked immunosorbent assay (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/+Leprdb Iar; Jackson Laboratory, CA) and their age-matched non-diabetic db/m littermates (C57BLKS/J; Jackson Laboratory) were used in the present study (17). 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 8 week-old db/db mice because they develop diabetes (hyperglycemia) at the age of 7-8 weeks (16). 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 PCA vehicle. The other db/db mice were orally administrated drinking water or 10 mg/kg BW PCA 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.

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 using hematoxylin for counter-staining.

For the immunohistochemical analyses, paraffin-embedded integument sections (5 μm thick) were employed. The sections were placed on glass slides, deparaffinated and hydrated with xylene and graded alcohol. The sections were pre-incubated in boiling sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for the antigen retrieval. A specific primary antibody against ICAM-1, CD11b, CD68 or F4/80 was incubated with sections overnight. For the visualization, the sections were developed with 3,3'-dianinobenzidine to produce a brown color, counter-stained with hematoxylin, and mounted in mounting medium. The stained tissue sections were examined using an Optical microscope AXIOIMAGER (Zeiss, Göttingen, Germany) and five images (X400) were taken for each section. PAS staining and protein levels of CD68 and F4/80 were quantified by image analysis program of 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 System) according to the manufacturer’s instructions.

Data analysis

The results are presented as mean ± SEM. Statistical analyses were conducted using the

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 (Figure 1A). However, there was a heavy staining observed in THP-1 cells exposed to HG-HRMC conditioned media (with 33 mM glucose), showing increased THP-1 cell adherence (Figure 1B). When non-toxic PCA at 1-20 \( \mu \text{g/ml} \) 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 onto glass slides (Figure 1B). 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 the inflammatory cells 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 of endothelial cells were up-regulated by culturing them in HG-HRMC conditioned media (Figure 2A). In contrast, PCA suppressed the induction of endothelial VCAM-1 and E-selectin. 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 (Figure 2B). 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 the 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 the 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 of 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 (Figure 3A). The similar effects of Tyk2 inhibitor on integrin β1 induction of THP-1 cells were observed (Figure 3B). 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.

**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, the histological changes in the kidney glomeruli were examined in db/db mice having experienced 8 week-experimental episodes, as observed by using PAS staining. Histological staining with PAS for the detection of mesangial expansion showed that
there was a dark-reddish staining in the glomeruli of db/db mice, compared to db/m controls (Figure 4A). In contrast, the 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 Tyk2-STAT signaling pathway (17). This study investigated whether the activation and filtration of monocytes entailed renal IL-8 activation accompanying activation of Tyk2-STAT pathway. The induction of IL-8 receptor beta, known as CXCR2, was markedly enhanced in kidney tissues of db/db mice, while the CXCR2 induction was demoted by PCA supplementation (Figure 4B).

This study further elucidated that the activation of Tyk2-STAT pathway in db/db mice was disturbed by PCA. 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 (Figure 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 the glomerular adhesion and infiltration of inflammatory cells responsible for diabetic glomerulosclerosis.

**Inhibition of macrophage accumulation by PCA in kidney**

ICAM-1 is involved in cell to cell interaction and adhesion and promotes macrophage accumulation in diabetic kidney (28). Immunohistochemical analysis showed that the induction of ICAM-1 and integrin αM (CD11b) was enhanced in the glomerulus of db/db mice (Figure 6A). 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 (Figure 6B). 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 of 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 (Figure 7). In marked contrast, the
cellular levels of CD68 and F4/80 in db/db mice supplemented with 10 mg/kg PCA decreased, incomparable well with that of db/m (Fig. 6).

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

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 (13,18). 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 pro-inflammatory cytokine secretion of mesangial cells (21). Accordingly, the association of monocytes with mesangial cells may be important in migrating monocytes to the kidney in the 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 cultured in
conditioned media of mesangial cells exposed to HG. The HG-HRMC conditioned media up-regulated expression of endothelial adhesion molecules of VCAM-1 and E-selection. In addition, the monocyte expression of integrin \(\beta_1\) and \(\beta_2\) communicating with these adhesion molecules was induced by HG-HRMC conditioned media. Some mediators released from HG-experienced mesangial cells may favor the 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-\(\alpha\) 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 of purple corn (11). Anthocyanins exhibiting anti-diabetic, anti-angiogenic and anti-carcinogenic 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-\(\beta\)-D-glucoside-rich purple corn color 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 renal mesansium. In this study PCA down-regulated 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 the leukocyte adhesion key factors of ICAM-1 and CD11b were reduced by supplementing PCA. The kidney mesangium produce and respond to a variety of cytokines and growth factors, and play an important role in response to local injury (12,25). It should be noted that in this study endothelial cells and THP-1 monocytes would be exposed to various cytokines and growth factors originated from HG-experienced 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 in 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 the Tyk2 inhibition. In addition, PCA reduced the kidney tissue levels of Tyk2 and STAT1/3 elevated in db/db mouse kidney. Collectively, PCA can interrupt IL-8-Tyk2-STAT signaling that may instigate the 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 macrophages accumulation was exacerbated with the duration of diabetes and the severity of renal injury and loss of renal function (7). Macrophage accumulation in the glomeruli 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 of the monocyte/macrophage makers in diabetic kidney. PCA appreciably hampered macrophage infiltration and accumulation in the glomeruli of db/db mice. On the other hand, the MCP-1 expression and the MIP-2 transcription were up-
regulated in kidney tissues of db/db mice, revealing that monocyte chemotaxis and macrophage infiltration was 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 kidney 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 integrins of \( \beta_1 \) and \( \beta_2 \) 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 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.
REFERENCES


FIGURE LEGENDS

**Figure 1:** PCA suppression of monocyte adhesion by adding PCA to 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 (w) 5.5 mM glucose, w/ 27.5 mM mannitol and w/ 33 mM glucose] for 24 h, and were stained with calcein AM for 30 min. Microphotographs (three independent experiments) were obtained using fluorescence microscopy with fluorescent blue filter. Magnification: 200X. The bar graphs (B, bottom panel, n=3) represent 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 differ, $P<0.05$.

**Figure 2:** Inhibition of HUVEC expression of VCAM-1 and E-selectin (A) and THP-1 expression of integrin $\beta_1$ and integrin $\beta_2$ (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 mg/L PCA added to HRMC conditioned media [with (w) 27.5 mM mannitol and w/ 33 mM glucose] for 6 h. Cell lysates were subjected to Western blot analysis with a primary antibody of VCAM-1, E-selectin, integrin $\beta_1$, or integrin $\beta_2$. $\beta$-Actin protein was used as an internal control. The bar graphs (mean $\pm$ SEM, n = 3) in the bottom panels represent quantitative results obtained from a densitometer. Means not sharing a common letter differ, $P<0.05$.

**Figure 3:** Attenuation of HUVEC expression of VCAM-1 and E-selectin (A) and THP-1 expression of integrin $\beta_1$ (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 $\mu$M Tyk2 inhibitor added to HRMC conditioned media [with (w) 27.5 mM mannitol and w/ 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 $\beta_1$. $\beta$-Actin protein was used as an internal control. The bar graphs (mean $\pm$ SEM, n = 3) in the bottom panels represent quantitative results obtained from a densitometer. Respective values
not sharing a common letter are different at $P<0.05$.

**Figure 4:** Histological staining (A) of PAS 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 weeks. The 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 four animals (A). The PAS intensity was quantified and shown in right panel. Magnification: X400. 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 graphs (mean ± SEM, n = 9-10) in the right panel represent quantitative results obtained from a densitometer. Means not sharing a common letter differ, $P<0.05$.

**Figure 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 weeks. The 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 graphs (mean ± SEM, n = 9-10) in the bottom panel represent quantitative results obtained from a densitometer. Respective values not sharing a common letter differ, $P<0.05$.

**Figure 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 10 mg/kg PCA for 8 weeks. The 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 counter-stained with hematoxylin. The ICAM-1 and CD11b expression levels were identified as brown staining. Each photograph is representative of four animals. Magnification: X400. 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 graphs (mean ± SEM, n = 9-10) in the right panel represent quantitative results obtained from a densitometer. Respective values not sharing a letter are different at \( P<0.05 \).

**Figure 7:** Suppression of the macrophage markers of 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 weeks. The db/m mice were introduced as a control. For the measurements of the 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 counter-stained with hematoxylin. The CD68 and F4/80 levels were identified as brown staining. Each photograph is representative of four animals. Magnification: X400. The CD68 and F4/80 levels were quantified and shown in bottom panel. The bar graphs (mean ± SEM, n = 9-10) represent quantitative results. Means not sharing a common letter differ, \( P<0.05 \).

**Figure 8:** Inhibition of MCP-1 (A) and 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 weeks. The 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 (mean ± SEM, n = 9-10) in the bottom panel 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 differ, \( P<0.05 \).
Figure 1

(A) normal RPMI media

- w/ 5.5 mM glucose
- w/ 27.5 mM mannitol
- w/ 33 mM glucose

(B) HRMC conditioned media

- w/ 33 mM glucose

PCA (µg/ml)

- 1
- 10
- 20

HRMC conditioned media

- w/ 5.5 mM glucose
- w/ 27.5 mM mannitol

Staining intensity (fold of control)

- w/ 5.5 mM glucose
- w/ 27.5 mM mannitol
- 1
- 10
- 20

w/ 33 mM glucose
Figure 5

The figures show the expression levels of various proteins in db/db mice treated with 10 mg/kg PCA compared to controls.

**Tyk2**
- Control: 140 kDa
- 10 mg/kg PCA: 140 kDa

**phospho-Tyk2**
- Control: 91 kDa
- 10 mg/kg PCA: 91 kDa

**β-actin**
- Control: 42 kDa
- 10 mg/kg PCA: 42 kDa

**STAT1**
- Control: 91 kDa
- 10 mg/kg PCA: 91 kDa

**phospho-STAT1**
- Control: 86 kDa
- 10 mg/kg PCA: 86 kDa

**β-actin**
- Control: 42 kDa
- 10 mg/kg PCA: 42 kDa

**STAT3**
- Control: 86 kDa
- 10 mg/kg PCA: 86 kDa

**phospho-STAT3**
- Control: 42 kDa
- 10 mg/kg PCA: 42 kDa

The bar graph below depicts the expression levels of the proteins in db/db mice, with the expression fold of control indicated.