Original Research

Isoangustone A suppresses mesangial fibrosis and inflammation in human renal mesangial cells

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Abstract

Development of diabetic nephropathy with fibrosis is associated with hyperglycemia-linked inflammation. Increased levels of proinflammatory factors have been found in diabetic patients with nephropathy. The present study was to test the hypothesis that isoangustone A, a novel compound present in licorice, can inhibit renal fibrosis and inflammation inflamed by high glucose (HG) in human mesangial cells through disturbing transforming growth factor β (TGF-β) and nuclear facor κB (NF-κB) pathways. Serum-starved mesangial cells were cultured in 33 mmol/L glucose media. Cells were treated with 1–20 μmol/L isoangustone A isolated from *Glycyrrhiza uralensis* licorice for three days. Exposure of cells to HG elevated connective tissue growth factor and collagen production, which was dose-dependently reversed by isoangustone A. Isoangustone A boosted HG-plummeted membrane type matrix metalloproteinase (MMP)-1 expression and diminished HG-elevated tissue inhibitor of MMP-2 expression. HG activated mesangial TGF-β1-SMAD-responsive signaling, which was repealed by $\geq$10 μmol/L isoangustone A. Furthermore, HG upregulated intracellular cell adhesion molecule-1 (ICAM-1) level and monocyte chemoattractant protein-1 (MCP-1) mRNA expression, and such increases were dose-dependently suppressed by isoangustone A most likely through hampering TGF-β signaling pathways. Blockade of NF-κB signaling appeared to be responsible for attenuating HG-triggered induction of ICAM-1 and MCP-1. Our findings provide the first evidence that isoangustone A dampens mesangial sclerosis associated with inflammation in response to HG through hindering TGF-β and NF-κB signaling.

Keywords: high glucose, isoangustone A, renal fibrosis, inflammation, mesangial cells

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Background

Hyperglycemia instigates structural alterations including extracellular matrix (ECM) accumulation leading to diabetic nephropathy (DN).[1] Mesangial expansion initiated by mesangial cell proliferation with ECM accumulation is one of the harbingers of glomerulosclerosis and tubulointerstitial fibrosis.[2,3] Diabetes-associated accumulation of ECM constituents such as collagen, fibronectin and laminin at the glomerular level can result from a stimulated production of matrix-synthesizing growth factors and a reduced expression of matrix-degrading proteases.[2,4,5] The key factor among the ECM-producing growth factors is thought to be connective tissue growth factor (CTGF) expressed in the mesangium.[1,6] In addition, matrix metalloproteinases (MMPs) have been described as the key matrix-degrading proteases responsible for degradation of ECM.[2,4] Hyperglycemia activated intracellular signaling pathways that induce fibrogenic and prosclerotic cytokines triggering ECM accumulation.[7,8] High ambient glucose (HG) stimulated resident renal cells to produce transforming growth factor β1 (TGF-β1) acting as a fibrogenic cytokine.[7] Furthermore, SMAD proteins that modulate the activity of TGF-β ligands played a role in the transcriptional expression of ECM constituents.[9]

In recent years, accumulating evidence demonstrates that DN pathogenesis is associated with renal inflammation.[10,11] Increased levels of certain proinflammatory factors such as intercellular adhesion molecule (ICAM)-1 and monocyte chemoattractant protein-1 (MCP-1) have been observed in diabetic patients with nephropathy.[10] ICAM-1 was characterized as possessing a role in the proinflammatory pathways, where ICAM-1 appeared to produce a recruitment of inflammatory immune cells such as macrophages and granulocytes.[12] Previous studies have demonstrated that MCP-1, a potent chemokine involved in the recruitment of macrophages, plays an important role in DN pathogenesis

in terms of inflammation.[13] However, the role of MCP-1 in ECM accumulation under diabetic conditions and the early inflammation with nuclear factor κB (NF-κB) activation in DN are not well defined.[14]

Much effort has been made to discover promising therapeutic drugs for DN treatment. There are few agents like angiotensin-converting enzyme inhibitors and angiotensin receptor blockers available to boost the function of diabetic kidneys.[15] Recently, pharmacotherapy with natural products targeting diabetes-associated mesangial hypertophy and fibrosis has been delineated.[16,17] Mangiferin, a naturally occurring glucosylxanthone, ameliorated ECM expansion and TGF-β1 overexpression in glomeruli of diabetic nephropathic rats.[18] (-)-Epigallocatechin gallate (EGCG) alleviated oxidative stress responsible for diabetic renal lesions.[19] In addition, resveratrol, phytoalexin produced naturally by several plants, attenuated renal hypertrophy in early-stage diabetes by activating AMP-activated protein kinase (AMPK).[20]

The present study tested the hypothesis that isoangustone A dampens excessive accumulation of mesangial matrix due to inflammation induced by HG. Licorice-containing novel bioactive compounds have been frequently employed as a traditional medicine for the treatment of vascular diseases and inflammatory processes.[21–23] Novel bioactive isoangustone A (Figure 1a) isolated from methylene chloride fractions of *Glycyrrhiza uralensis* licorice was employed in this study. In order to emphasize a cardinal role of isoangustone A in inhibiting ECM amassing, alterations in CTGF expression involved in collagen IV production and in MMP activity responsible for ECM degradation were assessed. In addition, it was tested that isoangustone A influenced proinflammatory ICAM-1 and MCP-1 induction through modulating TGF-β1-responsive signaling pathways and NF-κB-dependent mechanisms in mesangial inflammation. Furthermore, this study explored a crosstalk between mesangial matrix remodeling and diabetes-associated renal inflammation responsible for the antifibrotic efficacy of isoangustone A in diabetic glomerulosclerosis.

Materials and methods

Chemicals and materials

Fetal bovine serum (FBS), trypsin-ethylenediaminetetraacetic acid (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 medium (DMEM), nutrient mixture F-12 Ham's medium, RPMI 1640 medium, mannitol and D-glucose were provided by Sigma Chemical (St Louis, MO, USA), as were all other reagents unless specifically stated otherwise. Antibody of human CTGF was purchased from AbCam (Cambridge, UK). Antibodies of human collagen type IV, membrane type-1 matrix metalloproteinase (MT1-MMP), ICAM-1, MCP-1 and NF-κB were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies of human tissue inhibitor of matrix metalloproteinases-2 (TIMP-2) were obtained from R&D systems (Minneapolis, MN). Antibodies of human TGF-β1, TGF-β receptor I kinase (TGF-β RI), TGF-β receptor II kinase (TGF-β RII), phospho-SMAD2, SMAD2, SMAD4, inhibitory κBα (IκBα) and phospho-IκBα were supplied by Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse and donkey anti-goat IgG were provided by Jackson ImmunoResearch Laboratories (West Grove, PA, USA). TGF-β RI inhibitor (HTS 466284) was supported by Merck Chemicals (Darmstadt, Germany). Reverse transcriptase and Taq DNA polymerase were purchased from Promega (Madison, WI, USA).

Preparation of crude extracts and isoangustone A of *G. uralensis*

Our previous study showed procedures on rapid preparation and identification of isoangustone A in licorice.[24] Briefly, commercially available roots of licorice *G. uralensis* were obtained from Daekwang (Chuncheon, Korea). The dried and ground licorice roots (0.5 kg) were extracted three times (each for 1 h), each with 3 L of methylene chloride by sonication. The combined extracts were concentrated at 40°C with a Model EYELA N-1000 rotary evaporator (Tokyo Rikakikai, Tokyo, Japan), which yielded 125 g of crude extracts for the high-speed counter-current chromatography (HSCCC) separation.

The HSCCC method combined with an online high-performance liquid chromatography (HPLC) bioassay demonstrated rapid and efficient access to active constituents.[24] HSCCC was applied to separate peak fractions using *n*-hexane–ethyl acetate–methanol–water (6.5:5.5:6:4, v/v) as the solvent system. Each peak fraction from the HSCCC procedure was individually collected and analyzed by HPLC. All analyses were performed using an Agilent Zorbax SB-C18 reversed phase column coupled with an Agilent Zorbax Extend C18 guard column. In addition, structural identification of the HSCCC peak fractions was obtained by comparing with electrospray ionization mass spectrometry and ^{1}H- and ^{13}C-NMR spectra data. The HSCCC peak fractions were identified as isoangustone A and two other compounds, dehydroglyasperin C and dehydroglyasperin D. A total amount of 47.1 mg of isoangustone A was obtained from 700 mg of crude extracts. Isoangustone A was dissolved in dimethyl sulfoxide for live culture with cells in media; a final culture concentration of dimethyl sulfoxide was $<0.5\%$.

Culture of mesangial cells and cell viability

Human renal mesangial cells (HRMCs) (Sciencell Research Laboratories, Carlsbad, CA, USA) were incubated at 37°C, humidified atmosphere of 5% CO_2 in air. Routine culture of HRMCs was performed in DMEM plus F12 (7:1) media containing 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells in passage of 6–10 were subcultured at 80% confluence and employed for experiments.

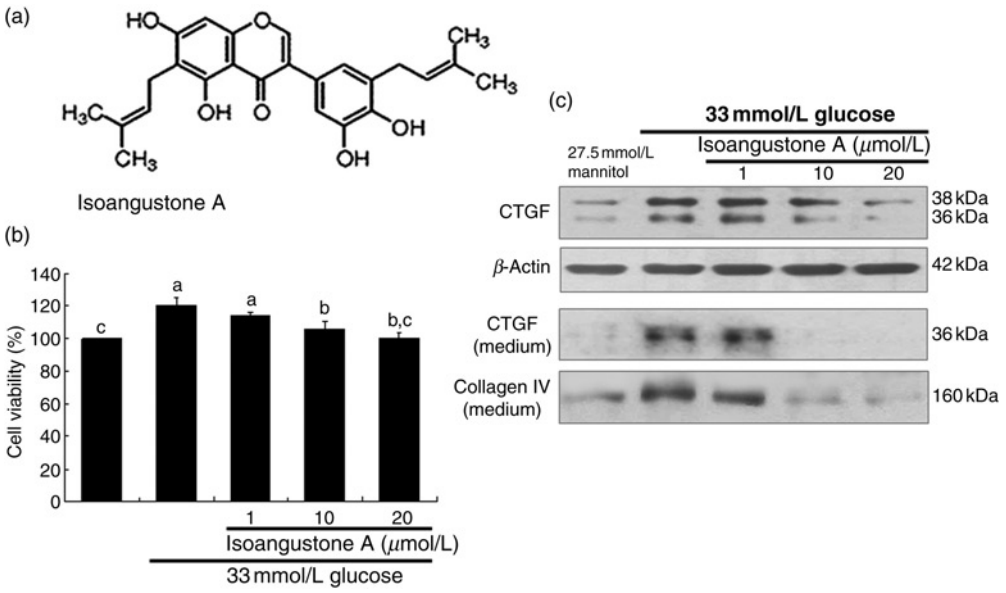

Figure 1 Chemical structure of isoangustone A (a), and cell viability (b) and connective tissue growth factor (CTGF)/collagen type IV production (c) of human renal mesangial cells (HRMCs) challenged with 33 mmol/L glucose in the absence and presence of isoangustone A. HRMCs were treated with 1–20 μmol/L isoangustone A for three days in the culture media of 33 mmol/L glucose. Cells were incubated with 5.5 mmol/L glucose and 27.5 mmol/L mannitol as osmotic controls. Values (b) are means ± SEM ($n = 5$) and expressed as percent cell survival relative to mannitol controls (cell viability = 100%). Values not sharing a letter are different at $P < 0.05$. For the production of CTGF and collagen type IV (c), cell lysates or culture media were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis with a primary antibody against CTGF or collagen type IV

To mimic hyperglycemia-induced glomerular sclerosis, HRMCs were incubated in 33 mmol/L glucose-added DMEM containing 2% FBS and 8 μg/mL insulin for three days in the absence and presence of submicromolar isoangustone A. For osmotic control incubations, another set of HRMCs was cultured in DMEM plus 27.5 mmol/L mannitol containing 2% FBS (+2 μg/mL insulin). The concentrations of isoangustone A used for three-day culture experiments were below 20 μmol/L.

After the three-day incubation period under conditions of HG and mannitol, the MTT assay was carried out for measuring cell viability.[25] HRMCs were cultured in a fresh phenol red-free DMEM containing 1 mg/mL MTT for three hours at 37°C. After unconverted MTT was washed out, formazan products were dissolved in 2-propanol 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 executed using whole-cell lysates prepared from HRMCs and collected culture media.[17] HRMC lysates were prepared in a lysis buffer containing 1% β-mercaproethanol, 1 mol/L β-glycerphosphate, 0.1 mol/L Na_3VO_4, 0.5 mol/L NaF and protease inhibitor cocktail. Equal amounts of total lysate proteins or equal volumes of culture medium were electrophoresed on 6% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred onto a nitrocellulose membrane. Non-specific binding was blocked by soaking the membrane in a TBS-T buffer (50 mmol/L Tris-HCl [pH 7.5], 150 mmol/L NaCl and 0.1% Tween-20) containing 3% bovine serum albumin for three hours. The membrane was incubated with anti-human rabbit polyclonal antibodies of cellular proteins to be tested and then was incubated with a secondary antibody, a goat anti-rabbit IgG, goat anti-mouse IgG or donkey anti-goat IgG conjugated to horseradish peroxidase. The protein levels were measured with Supersignal West Pico Chemiluminescence detection reagents (Pierce Biotechnology, Rockford, IL, USA) and Konica X-ray film (Konica, Tokyo, Japan). Incubation with polyclonal mouse anti-human β-actin antibody was performed for comparative control.

Reverse transcriptase-polymerase chain reaction analyses

Following culture protocols, total RNA was isolated from 6×10^5 HRMCs using a commercially available Trizol reagent kit (Invitrogen, Carlsbad, CA, USA). RNA (5 μg) was reversibly transcribed with 200 U of reverse transcriptase and 0.5 mg/mL oligo-$(dT)^{15}$ primer (Bioneer, Daejeon, Korea). The expression of mRNA transcripts of human MCP-1 (forward primer: 5′-AACTGAAGCTCGCACTCTCG-3′, reverse primer: 5′-TCAGCACAGATCTCCTTGGC-3′, 258 bp) and β-actin (forward primer: 5′-GACTACCTCATGAAGATC-3′, reverse primer: 5′-GATCCACATCTGCTGGAA-3′, 500 bp) were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). The PCR was performed in 25 μL of 10 mmol/L Tris-HCl (pH 9.0) containing 25 mmol/L $MgCl_2$, 10 mmol/L dNTP, 5 U of Taq DNA polymerase and 10 μmol/L of each primer, and started with five-minute denaturation at 94°C followed by 30 PCR cycles. Each cycle consisted of 60 s at 94°C, 60 s at 55°C, 60 s at 72°C and the final extension was for 10 min at 72°C. After themocycling, 15 μL

of PCR products was electrophoresed on 1% agarose-formaldehyde gel containing 0.5 μg/mL ethidium bromide. The bands were visualized using a TFX-20M model-UV transilluminator (Vilber-Lourmat, Marne-la-Vallee, France) and gel photographs were taken. The absence of contaminants was routinely checked by the RT-PCR assay with negative control samples without addition of a primer.

Realtime PCR

The levels of mRNA transcripts of MCP-1 (forward primer: 5′GATCTCAGTGCAGAGGCTCG 3′, reverse primer: 5′ TGCTTGTCCAGGTGGTCCAT 3′, 153 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward primer: 5′ GAAGGTGAAGGTCGGAGTC 3′, reverse primer: 5′ GAAGATGGTGATGGGATTTC 3′) were quantified by realtime RT-PCR using SYBR Green PCR kit (Qiagen, Valencia, CA, USA) and data analysis of realtime RT-PCR results and calculation of the relative quantization were performed using the Delta CT analysis by the Rotor-gene software version 6.0 (Corbett Research, Mortlake, Australia). The housekeeping GAPDH was used for internal normalization. The standard PCR conditions were 94°C for 10 min, then 40 cycles at 94°C (30 s), 60°C (30 s) and 72°C (60 s), followed by the melting curve analysis.

MCP-1 small interfering RNA transfection

This study attempted to determine the involvement of inflammatory MCP-1 in HG-triggered glomerular fibrosis. Human-specific siRNA kit (Santa Cruz Biotechnology) was used to specifically inhibit MCP-1 expression. Nucleofection of HRMCs was performed for the gene delivery according to the optimized protocols provided by the manufacturer (Amaxa Biosystem, Cologne, Germany). HRMCs ($0.5–1 \times 10^6$ cells) were pelleted, gently resuspended in the Amaxa cuvette with 100 μL of nucleofector solution (Amaxa nucleofector VPI-1004 kit), mixed with 300 nmol/L MCP-1 small interfering RNA (siRNA) and pulsed in the nucleofector device with the program A-33 for mesangial cells. Cells were immediately transferred into 500 μL prewarmed fresh DMEM/F12 medium (without FBS and antibiotics) into six-well plates. After 24 h of nucleofection, transfected cells were starved for 24 h and exposed to HG and cultured for three days.

Transcriptional reporter gene assay

To determine the promotor activity of the ICAM-1 gene, a promoter-reporter construct with pGL3-basic vector containing luciferase gene driven by the translucent ICAM-1-promoter (Cosmo Co, Seoul, Korea) was employed. A pGL3-basic vector reporter construct was used as a transfectional control. The nucleofection procedure of HRMCs with an ICAM-1-reporter construct was described above. Briefly, HRMCs were re-suspended in the Amaxa cuvette with 100 μL of nucleofector solution, mixed with 3 μg ICAM-1-reporter vector and pulsed in the nucleofector device (program A-33). Cells were immediately transferred into prewarmed fresh medium in six-well plates. After 24 h nucleofection, transfected cells were starved for 24 h and exposed to HG in the presence of 10 μmol/L isoangustone A. After three days of culture, the cells were washed and 100 μL of cell lysis reagent was added. Promoter activity of HRMCs nucleofected with a luciferase-harboring ICAM-1 promoter construct was measured using the dual-luciferase reporter kit (Promega Biosciences). For the measurement of luciferase activity,[26] 20 μL of lysate mixed with 100 μL of assay reagent was assayed using a luminometer (Lumat LB 9501, Berthold, Germany).

Nuclear extract preparation

Cytosolic and nuclear protein fractions were prepared by a detergent lysis procedure to determine the translocation of NF-κB. The cells were lysed in 10 mmol/L HEPES buffer (pH 7.9, 10 mmol/L KCl, 0.4 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 0.5% Nonidet P40, 20 μg/mL of each of aprotinin and leupeptin) and incubated on ice for one hour. Nuclei were pelleted by a centrifugation at 3000g for 20 min. Proteins were extracted from nuclear pellets by incubation in a high-salt buffer containing 420 mmol/L NaCl, 20 mmol/L HEPES ([pH 7.9], 0.4 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 20% glycerol, and 10 μg/mL of each of aprotinin, antipain and leupeptin) at 4°C with vigorous shaking. The nuclear debris was pelleted by a brief centrifugation at 20,000g for 30 min and the supernatant was stored for further experiments.

Data analysis

The data are presented as mean $\pm$ SEM. Statistical analyses were conducted using Statistical Analysis Systems statistical software package (SAS Institute, Cary, NC, USA). Significance was determined by one-way analysis of variance, followed by Duncan multiple range test for multiple comparisons. Differences were considered significant at $P < 0.05$.

Results

Effect of isoangustone A on HG-induced mesangial hypertropy and fibrosis

Mesangial cell proliferation and matrix accumulation have been described as important factors of renal fibrosis.[2,3] To determine whether HRMCs proliferated under HG condition and isoangustone A inhibited such proliferative activity, cell viability was measured by a MTT assay. High glucose caused mesangial cell proliferation with a 20% increase in cell viability, which was reversed by isoangustone A (Figure 1b). Isoangustone A *per se* was not cytotoxic (data not shown), indicating that it had antiproliferative activity in ambient HG-exposed mesangial cells.

This study investigated inhibitory effects of isoangustone A on HG-induced production of CTGF and collagen IV. Western blot data showed that cellular expression and secretion of CTGF were boosted in HRMCs exposed to HG, and that the CTGF production was suppressed by

$\geq$10 μmol/L isoangustone A (Figure 1c). In addition, the HG-elevated collagen IV secretion, a fibrosis biomarker, was retarded by adding 10 μmol/L isoangustone A (Figure 1c). Accordingly, the collagen IV secretion encumbered by isoangustone A was most likely due to its diminution of CTGF production.

Isoangustone A inhibition of MT-1 MMP and TIMP-2 induction triggered by HG

This study determined that HG modulated expression of MT-1 MMP and TIMP-2 and that such expression was influenced by isoangustone A. HG retarded MT-1 MMP expression of mesangial cells and augmented TIMP-2 expression (Figure 2). In contrast, isoangustone A reversed the HG-induced suppression of MT-1 MMP and upregulation of TIMP-2 in HRMCs, indicating that isoangustone A may ameliorate the disturbance of the MMP system associated with matrix accumulation.

Attenuation of HG-induced TGF-β1-SMAD2 signaling by isoangustone A

TGF-β plays a vital role to enhance CTGF expression by binding with TGF-β RII followed by TGF-β RI phosphorylation.[6,8] When HG was solely applied to HRMCs, HG induced cellular expression of TGF-β1 (Figure 3a). In addition, it was found that TGF-β RI and TGF-β RII were significantly induced by 24-h HG stimulation. However, isoangustone A reversed the HG-induced increase in cellular levels of TGF-β1 and its receptor kinases in a dose-dependent manner (Figure 3a).

TGF-β activates the receptor-regulated SMAD2, thereby allowing SMAD2 to phosphorylate, to bind with SMAD4 and to translocate into the nucleus for gene expression.[9] HG stimulated SMAD4 expression and activated SMAD2 phosphorylation at 24 h after stimulation (Figure 3b). When HRMCs were treated with 1–20 μmol/L isoangustone A and exposed to HG, it suppressed HG-enhanced SMAD4 expression and SMAD2 phosphorylation dose-dependently (Figure 3b). Accordingly, isoangustone A dampened the HG induction of TGF-β and subsequent activation of the downstream SMAD signal pathway.

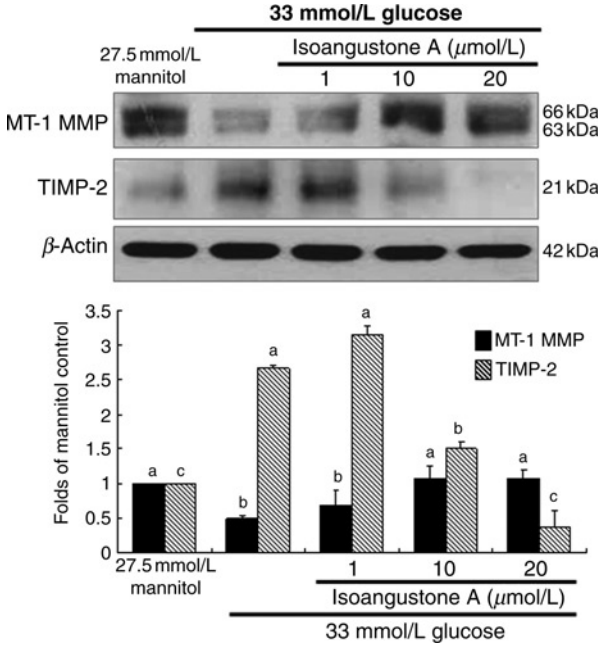

Figure 2 Western blot data showing expression of membrane type 1 matrix metalloproteinase (MT-1 MMP) and tissue inhibitor of MMP (TIMP)-2 in human renal mesangial cells (HRMCs) treated with isoangustone A under high glucose conditions. Cells were challenged with 1–20 μmol/L isoangustone A and then exposed to 33 mmol/L glucose for three days. Additionally, HRMCs were incubated for three days in 5.5 mmol/L glucose and 27.5 mmol/L mannitol as osmotic controls. After HRMC culture protocols, cell extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis with a primary antibody against MT-1 MMP and TIMP-2. β-Actin protein was used as an internal control. The bar graphs (means $\pm$ SEM, $n = 3$) in the right panel represent quantitative results obtained from a densitometer. Values not sharing a letter are different at $P < 0.05$

Inhibition of ICAM-1 and MCP-1 by isoangustone A in HG-stimulated mesangial cells

This study revealed if mesangial inflammation may be involved in renal firobrotic process. To test whether ICAM-1 expression and MCP-1 transcription were mediated through TGF-β signaling, a TGF-β RI inhibitor HTS 466284 was employed. HG enhanced ICAM-1 expression by approximately two-fold, and this was reversed to the normal level by 10 μmol/L HTS 466284 (Figure 4a). This finding indicates that the HG-elevated ICAM-1 expression was mediated via TGF-β signaling. Interestingly, the HG-elevated ICAM-1 expression was suppressed by isoangustone A in a dose-dependent manner (Figure 4b).

Reporter gene luciferase activity assay showed that HG enhanced promoter activity of ICAM-1, known as a target gene of the inflammatory signaling involved (Figure 4c). When transfected mesangial cells were treated with HG, the promoter activity of ICAM-1 was markedly enhanced. This elevation was blunted by treating the cells with 10 μmol/L isoangustone A. This reveals that ICAM-1 was an important factor involved in HG-induced mesangial inflammation.

MCP-1 induction possibly involved in diabetes-associated mesangial inflammation was examined in HG-exposed HRMCs. The RT-PCR assay showed that MCP-1 mRNA was upregulated by adding 33 mmol/L glucose to mesangial cells. Such transcriptional induction was dampened by treatment of cells with 10 μmol/L TGF-β RI inhibitor (Figure 5a), which was consistent with realtime PCR data (Figure 5b). This implies that TGF-β was an upstream regulator of these proinflammatory mediators. Furthermore, the CTGF expression and collagen IV secretion were examined in siRNA-transfected HRMCs exposed to HG. When MCP-1 siRNA was present, HG-promoted CTGF expression and collagen IV secretion were suppressed (Figure 5c), denoting that MCP signaling was responsible for mesangial fibrosis. In addition, isoangustone A at $\geq$20 μmol/L mitigated MCP-1 mRNA transcription elevated by HG (Figure 5d). Thus, it is deemed that isoangustone A inhibited mesangial fibrosis closely linked to inflammation.

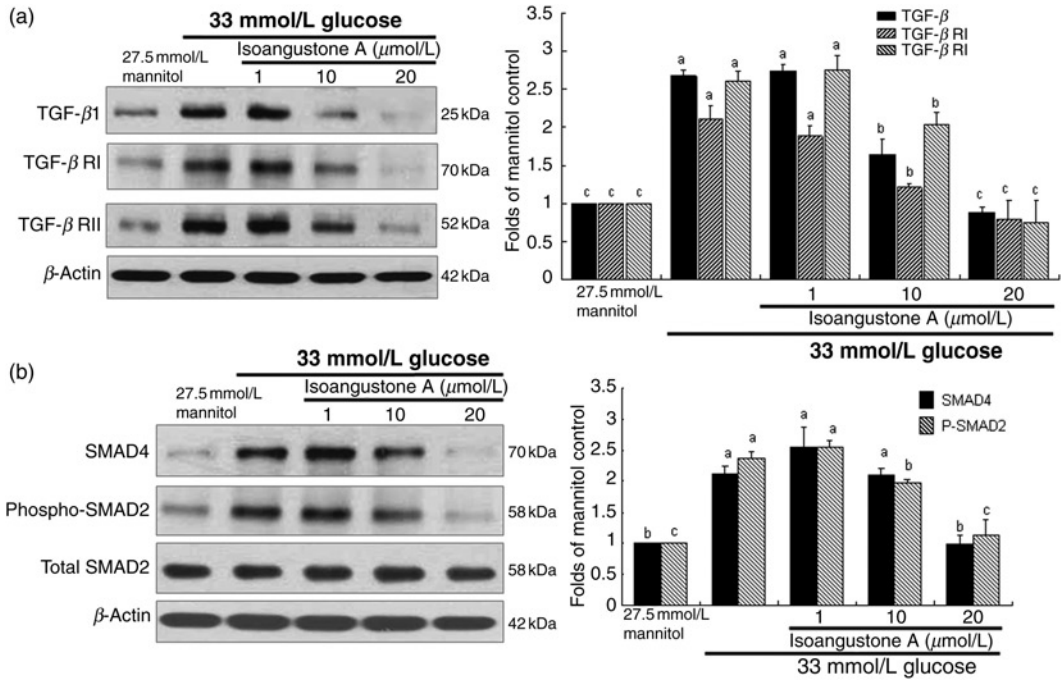

Figure 3 Inhibition of transforming growth factor β1 (TGF-β1) expression, TGF-β receptor kinase RI and RII activation and SMAD activation in human renal mesangial cells (HRMCs) treated with isoangustone A under high glucose conditions. HRMCs were treated with 1–20 μmol/L isoangustone A and then stimulated with 33 mmol/L glucose for three days. Cells were incubated with 5.5 mmol/L glucose and 27.5 mmol/L mannitol as osmotic controls. After HRMC culture protocols, cell extracts were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis with a primary antibody against TGF-β1, TGF-β RI and RII (a) and against SMAD4, SMAD2 and phosphorylated SMAD2 (b). Representative blot data were respectively obtained from three independent experiments and β-actin protein was used as an internal control. The bar graphs (means $\pm$ SEM, $n = 3$) in the right panel represent quantitative results obtained from a densitometer. Values not sharing a letter are different at $P < 0.05$

Disturbance of NF-κB signaling by isoangustone A

Western blot analysis revealed that HG triggered IκB phosphorylation in mesangial cells (Figure 6a), indicative of an increase in IκB dissociation from NF-κB complex. The treatment with isoangustone A reduced the HG-stimulated IκB phosphorylation. The nuclear translocation of NF-κB was further examined in HG-exposed mesangial cells. As expected, an increase of NF-κB p65 in the nucleus was observed following an exposure to 33 mmol/L glucose, showing that HG enhanced nuclear translocation of NF-κB (Figure 6b). Isoangustone A at $\geq$10 μmol/L inhibited nuclear translocation of NF-κB with disturbing IκB dissociation (Figure 6).

Discussion

Mesangial hyperplasia accompanying ECM accumulation is one of the harbingers of glomerulosclerosis and tubulointerstitial fibrosis leading to DN.[2,3] Hyperglycemia causes excessive amassing of ECM and thickening of glomerular and tubular basement membranes.[1] Diabetes-associated accumulation of ECM constituents such as collagen, fibronectin and laminin at the glomerular level can result from stimulated expression of matrix synthesis-related growth factors and reduced production of matrix-degrading proteases.[2,4,5] CTGF expressed in the mesangium is thought to be one of the ECM-producing growth factors.[1,6,8] In the HG-MC culture models mimetic to hyperglycemia-initiated mesangial hyperplasia and fibrosis, three-day-exposure of MC to ambient HG caused mesangial hyperplasia and matrix expansion, possibly converting quiescent MCs into ECM-overproducing myofibroblasts. HG-triggered CTGF production was responsible for elevated secretion of collagen IV, an intrinsic component of mesangial matrix. MMP is known to be one of the matrix-degrading proteases responsible for the enzymatic degradation of ECM.[2,4] The decreased level of mesangial MMP is accountable for excessive deposition of matrix in glomerulosclerosis and tubulo-interstitial fibrosis.[2] An abnormal matrix-degrading system involving MMP and TIMP was recognized in a model with diabetic renal fibrosis.[2] This study proved that HG suppressed the MT-1 MMP level and enhanced the TIMP-2 expression in MC, thus facilitating mesangial ECM accumulation in HG-inflamed MCs.

Much effort has been made to discover promising therapeutic drugs targeting diabetes-associated DN. Recently, pharmacotherapy with natural products is being delineated to alleviate mesangial hypertophy and fibrosis.[16,17] Mangiferin ameliorated glomerular ECM expansion and accumulation in glomeruli of DN rats.[18] EGCG assuaged oxidative stress responsible for diabetic renal lesions.[19] Additionally, resveratrol attenuated renal hypertrophy in early-stage diabetes by activating AMPK.[20] In this study, submicromolar isoangustone A retarded mesangial collagen IV deposition by diminishing CTGF production and augmenting MT-1 MMP expression with reduced TIMP-2 levels. Similarly, soy isoflavone genistein inhibited the secretion of ECM components and the expression of TGF-β in HG-cultured rat mesangial cells.[27] Isoangustone A

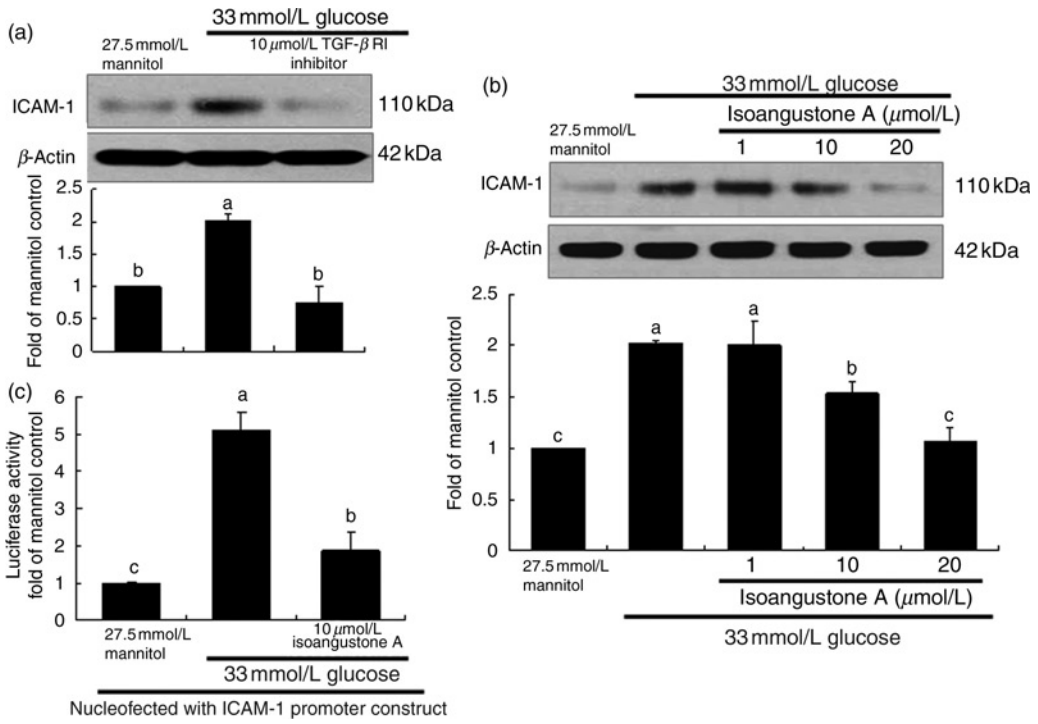

Figure 4 Suppression of inflammatory intracellular cell adhesion molecule-1 (ICAM-1) expression by transforming growth factor β receptor I kinase (TGF-β RI) inhibitor (HTS 466284, a) and isoangustone A (b) in human renal mesangial cells (HRMCs) treated with isoangustone A under high glucose conditions. Cells were incubated with 1–20 μmol/L isoangustone A for three days under conditions of high glucose at 33 mmol/L. Also, HRMCs were incubated with 5.5 mmol/L glucose and 27.5 mmol/L mannitol as osmotic controls. Cell extracts were employed for Western blot analysis with a primary antibody against ICAM-1. β-Actin protein was used as an internal control. The bar graphs (means ± SEM, $n = 3$) in the bottom panels represent densitometric results and respective blot data were obtained from three independent experiments. Inhibition of promoter activity of ICAM-1 (c) in high-glucose-exposed HRMCs treated with isoangustone A. HRMCs were nucleofected with a luciferase-harboring-ICAM-1 promoter construct. Transfected HRMCs were incubated with 33 mmol/L glucose for three days in the presence of 10 mmol/L isoangustone A. Transfected cells were incubated with 5.5 mmol/L glucose and 27.5 mmol/L mannitol as osmotic controls. Promoter activity was assessed using luciferase reporter gene assay. The bar graphs (means ± SEM, $n = 3$) represent quantitative results obtained from a luminometer. Values not sharing a letter are different at $P < 0.05$

is a novel bioactive compound found in licorice extract.[22] In our previous study, the isoflavone isoangustone A exhibited antioxidant activity scavenging 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) radical cation,[24] and showed potent antibacterial activity against methicillin-resistant *Staphylococcus aureus*.[28] In addition to such bioactive actions, isoangustone A may be a promising compound that displays renoprotection against diabetic renal fibrosis. However, our findings based on cell culture systems should be followed up by further *in vivo* studies and clinical trials to discern the specific benefits of isoangustone A on diabetic mesangial fibrosis and inflammation.

This study proposed that isoangustone A, effective in blunting HG-induced mesangial fibrogenesis, was a medicinal compound capable of targeting TGF-β-SMAD. Hyperglycemia activated intracellular signaling pathways to induce fibrogenic and prosclerotic cytokines triggering ECM accumulation.[7,8] It has been reported that TGF-β-stimulated mesangial fibrogenesis entails the TGF-β-specific SMAD signal transduction pathway.[9] Consistently, our previous study showed that TGF-β ligand-associated-SMAD signaling was involved in collagen IV accumulation along with enhanced cellular levels of CTGF and TIMP-2.[17] HG elevated cellular levels of TGF-β1 and RI/RII receptor kinases and promoted SMAD2 activation and SMAD4 expression. However, it is assumed that multiple interactions among a variety of pathways are necessary for maximal ECM expansion.

Inflammatory mechanisms including kidney macrophage accumulation contribute to the pathogenesis of DN.[13] Increased levels of ICAM-1 and MCP-1 have been found in diabetic patients with nephropathy.[10] Several studies showed that natural products were effective in alleviating mesangial inflammation.[29,30] EGCG attenuated inflammation in MRL/lpr mouse mesangial cells via the PI3K/Akt/mTOR pathway, suggesting a potential therapeutic role for the use of EGCG to regulate inflammation and control autoimmune disease.[29] Isoangustone A interrupted proinflammatory ICAM-1 and MCP-1 via blocking the cellular mechanisms of TGF-β and NF-κB. The renoprotection of isoangustone A against mesangial inflammation may be specific therapies of renal fibrosis. This study elucidated intracellular signaling mechanisms of isoangustone A modulating mesangial inflammation occurring in the HG pathogenesis. The TGF-β signaling was responsible for HG-triggered induction of mesangial ICAM-1 and MCP-1. It is deemed that isoangustone A attenuated mesangial inflammation and fibrosis through disturbing HG-inflamed TGF-β signaling. A close investigation on NF-κB signaling is still obligatory for mesangial inflammation. This study showed that the NF-κB signaling pathway was triggered by HG during ECM remodeling

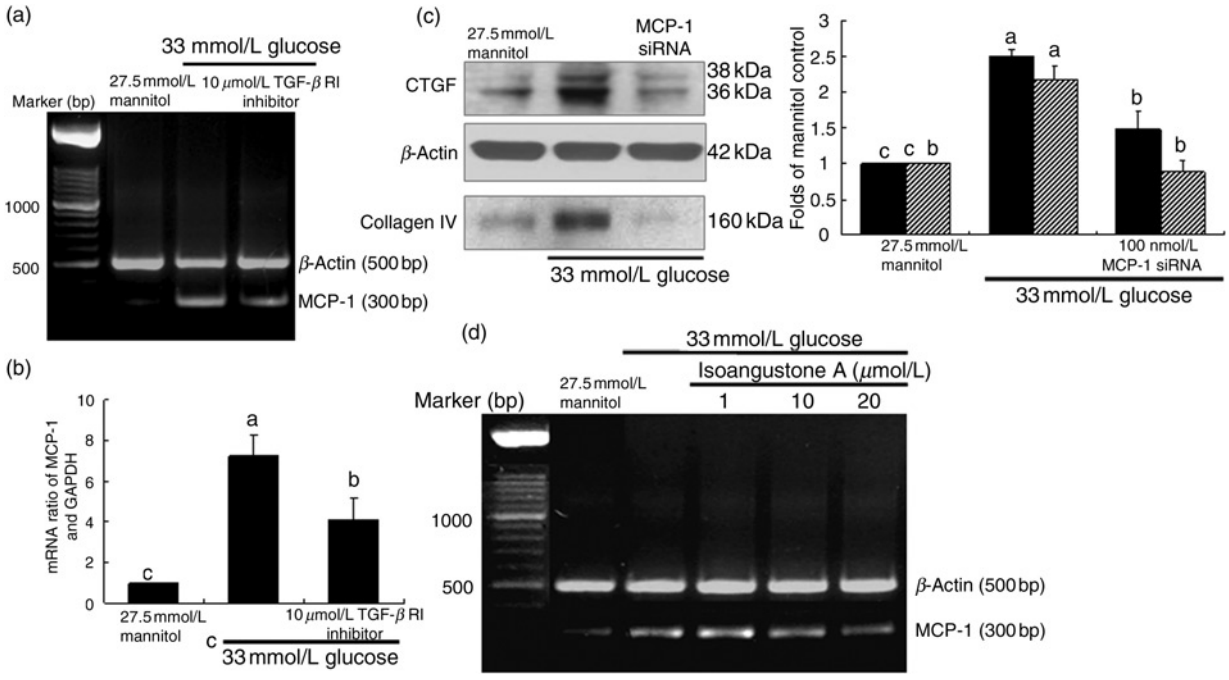

Figure 5 Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses (a and d) and realtime PCR (b) showing the steady-state mRNA transcriptional levels of monocyte chemoattractant protein-1 (MCP-1) in transforming growth factor β receptor I kinase (TGF-β RI) inhibitor-treated (a) or isoangustone A-treated (d) and 33 mmol/L glucose-stimulated human renal mesangial cells (HRMCs). HRMCs were incubated with 5.5 mmol/L glucose and 27.5 mmol/L mannitol as osmotic controls. β-Actin and glyceraldehyde-3-phosphate dehydrogenase genes were used as an internal control for the co-amplification with MCP-1 (3 separate experiments). For connective tissue growth factor expression and collagen IV secretion (c), cell extracts and culture media obtained from MCP siRNA-1-transfected mesangial cells were employed for Western blot analysis with a primary antibody against connective tissue growth factor and collagen IV. β-Actin protein was used as an internal control. The bar graphs (means $\pm$ SEM, $n = 3$) in the right panel represent quantitative results obtained from a densitometer. Values not sharing a letter are different at $P < 0.05$

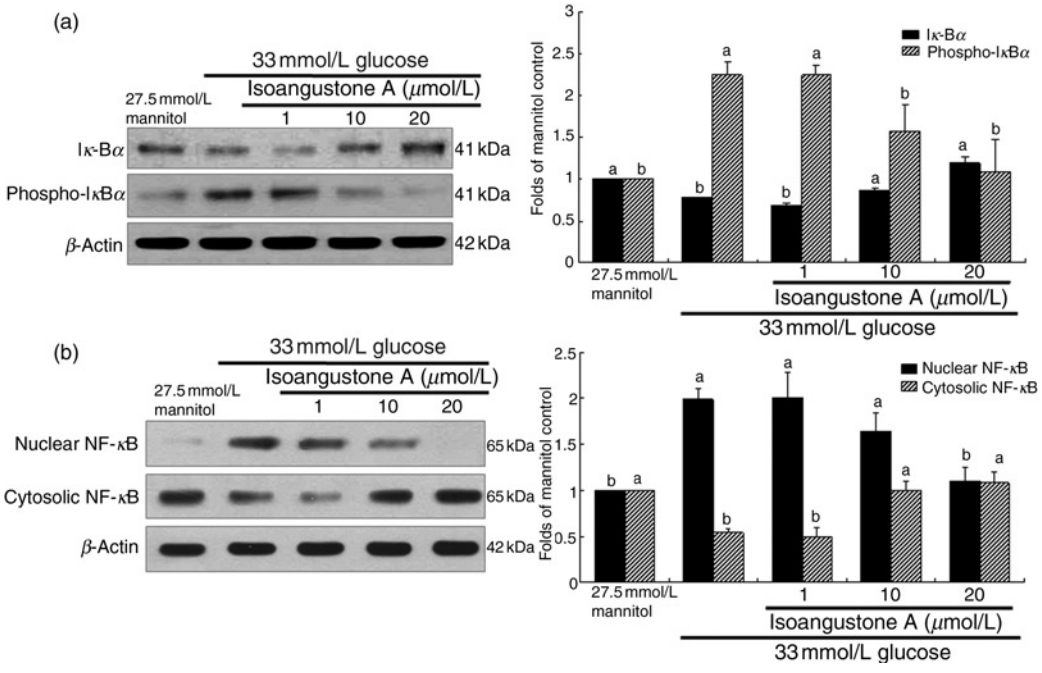

Figure 6 Blockade of IκB phosphorylation (a) and nuclear translocation of nuclear factor κB (NF-κB) (b) in human renal mesangial cells (HRMCs) treated with isoangustone A under high glucose conditions of 33 mmol/L. Confluent cells were incubated with 1–20 μmol/L isoangustone A for three days under conditions of high glucose. Also, HRMCs were also incubated with 5.5 mmol/L glucose and 27.5 mmol/L mannitol as osmotic controls. For the measurement of nuclear translocation of NF-κB (b), nuclear fraction of HRMCs was obtained. Western blot analysis was carried out using cell extracts and a primary antibody against IκB, phosphorylated IκB and NF-κB. β-Actin protein was used as an internal control. The bar graphs (means $\pm$ SEM, $n = 3$) in the right panel represent densitometric results and values not sharing a letter are different at $P < 0.05$. Respective blot data were obtained from three independent experiments

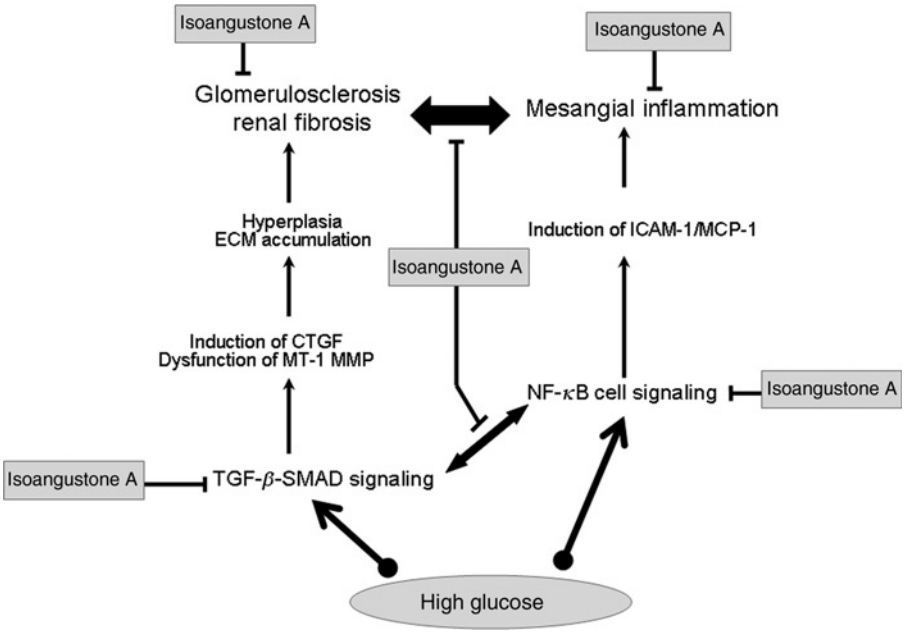

Figure 7 Schematic diagram showing actions of isoangustone A disturbing cross-link between transforming growth factor β (TGF-β) and nuclear factor κB (NF-κB) pathways responsible for mesangial fibrosis and inflammation. The symbol indicates stimulation ($\rightarrow$) due to high glucose or blockade ($\perp$) due to treatment with isoangustone A

and mesangial inflammation, leading to upregulation of ICAM-1 and MCP-1 expression. Furthermore, isoangustone A quenched this activated signaling of NF-κB and may suppress renal inflammation in DN. Honokiol, originally isolated from *Magnolia officinalis*, alleviated experimental glomerulonephritis by attenuating glomerular MCP-1 and ICAM-1, similar to collagen I and fibronectin mRNA levels of nephritic rats.[31]

Advanced glycation end products (AGEs) were shown to induce CTGF expression and ECM accumulation, which were predominantly mediated via a TGF-β1-independent pathway.[32] Unfortunately, this study did not examine mesangial formation of AGEs under HG culture condition. However, isoangustone A may suppress HG-triggered AGE formation or inhibit AGE receptor interaction, interrupting inflammation-associated signaling pathways. NF-κB is shown to promote inflammation and fibrosis in the aging glomerulus compared with young glomeruli, possibly leading to enhanced susceptibility to failure.[33] These results suggest that age-associated NF-κB-driven process has a pathogenic role in mediating chronic inflammation in chronic kidney disease.

In summary, this study showed that isoangustone A retarded HG-inflamed mesangial hyperplasis and matrix expansion through facilitating collagen IV accumulation (Figure 7). In addition, isoangustone A may retard diabetes-associated renal inflammation by attenuating inflammatory ICAM-1 expression and MCP-1 production in the mesangium. The capability of isoangustone A to deter renal fibrosis and inflammation may be promising in disturbing the progression of renal fibrosis to end-stage complicated renal diseases. The HG induction of mesangial CTGF and TIMP-2 entailed TGF-β1-SMAD-responsive pathways that were impeded by isoangustone A. In addition, renal fibrosis appeared to be augmented by the HG-stimulated mesangial inflammation most likely via NF-κB signaling that was disrupted by isoangustone A. Therefore, isoangustone A therapy targeting diabetes-associated DN appeared to be achieved by interrupting renal activation of TGF-β-SMAD and NF-κB signaling. However, the possibility that HG-induced mesangial fibrosis and inflammation are mediated directly by AGE formation cannot be ruled out.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. JL, E-SL, J-HG and DS conducted the experiments; SSL and I-JK prepared and analyzed isoangustone A in licorice; and JL and Y-HK wrote the manuscript.

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