JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 3205–3212 3205
DOI:10.1021/jf9040723

Isoliquiritigenin Entails Blockade of TGF-β1-SMAD Signaling for Retarding High Glucose-Induced Mesangial Matrix Accumulation

Jing Li,† Sang-Wook Kang,† Jung-Lye Kim,† Hye-Young Sung,† In-Sook Kwun,‡ and Young-Hee Kang*,†

†Department of Food and Nutrition, Hallym University, Chuncheon, Kangwon-do, 200-702 Korea, and ‡Department of Food Science and Nutrition, Andong National University, Andong, Kyungpook, 760-749 Korea

Diabetic nephropathy characterized as mesangial fibrosis and glomerulosclerosis results in renal failure and end-stage renal diseases. Enhanced expression and secretion of connective tissue growth factor (CTGF) play an important role in the expansion of glomerular mesangial matrix mostly composed of type IV collagen. Isoliquiritigenin can prevent various renal injuries via its anti-inflammatory action. However, the effect of isoliquiritigenin on diabetic nephropathy has never been explored. The present study was to investigate whether nontoxic isoliquiritigenin inhibited high glucose (HG)-induced mesangial fibrosis by retarding formation of type IV collagen as well as CTGF in human mesangial cells (HRMC). Serum starved cells were cultured in media containing 5.5 mM glucose plus 27.5 mM mannitol as an osmotic control or 33 mM glucose for 3 days with and without 1−20 μM isoliquiritigenin. Exposure of cells to HG caused marked increases in collagen secretion and CTGF expression, which was dose-dependently reversed by isoliquiritigenin at the transcriptional levels. Additionally, isoliquiritigenin boosted HG-plummeted type matrix metalloproteinase-1 (MT-1 MMP) expression and dampened HG-elevated tissue inhibitor of MMP-2 (TIMP-2) expression, facilitating the degradation of mesangial matrix. Isoliquiritigenin inhibited HG-upregulated CTGF and TIMP-2 expression via disturbing TGF-β1 signaling in HRMC, as evidenced by TGF-β receptor I kinase (TGF-β RI) inhibitor. HG-activated SMAD2 through autocrine TGF-β signaling was repealed by ≥10 μM isoliquiritigenin. HG induced SMAD4 expression of HRMC and obliterated antagonistic SMAD7, whereas isoliquiritigenin suppressed induction of TGF-β RII and TGF-β RI with blunting their downstream SMAD signaling. The results demonstrate that the bioactive isoliquiritigenin in licorice diminished mesangial matrix accumulation in response to ambient HG through retarding TGF-β1-SMAD signaling transduction. Therefore, isoliquiritigenin may be a potential therapeutic agent for the prevention and treatment of mesangial fibrosis and glomerulosclerosis leading to diabetic nephropathy due to longstanding diabetes mellitus.

KEYWORDS: High glucose; isoliquiritigenin; mesangial matrix; TGF-β; SMAD signaling

INTRODUCTION

Chronic hyperglycemia that persists even in fasting states is most commonly caused by diabetes mellitus and is the defining characteristic of the disease. Frequent or prolonged hyperglycemia results in damage to nerves, blood vessels and other body organs such as kidney and eye (*1*, *2*). Hyperglycemia causes structural alterations including predominant extracellular matrix (ECM) accumulation in diabetic nephropathy (*3*). Diabetic nephropathy involves excessive amassing of mesangial matrix and thickening of glomerular and tubular basement membranes leading to glomerulosclerosis and tubulointerstitial fibrosis (*3*, *4*). The key factors responsible for the regulation of ECM in diabetes are thought to be protease/antiprotease systems and pro-sclerotic cytokines (*3*). Low enzymatic degradation of extracellular matrix contributes to an excessive accumulation.

Growth factors play an important role in the pathogenesis of diabetic nephropathy (*2*, *3*). Hyperglycemia induced hemodynamic alterations stimulate resident renal cells and produce transforming growth factor (TGF)-β1, fibrogenic and inflammatory cytokine (*2*). TGF-β1 is known to augment deposition of ECM including collagen, laminin and fibronectin at the glomerular level (*5*). In addition, connective tissue growth factor (CTGF) plays a pivotal role in the fibrosis in various tissues including the kidney, promoting ECM accumulation on glomerular mesangial cells and podocytes and the tubulointerstitium (*3*). CTGF often described as an effector of TGF-β promotes TGF-β signaling through several different mechanisms (*6*, *7*). CTGF induces the expression of chemokines which themselves have pharmacological actions on cells (*6*). However, advanced glycation

*To whom correspondence should be addressed. Phone: 82-33-248-2132. Fax: 82-33-254-1475. E-mail: yhkang@hallym.ac.kr.

Published on Web 02/10/2010

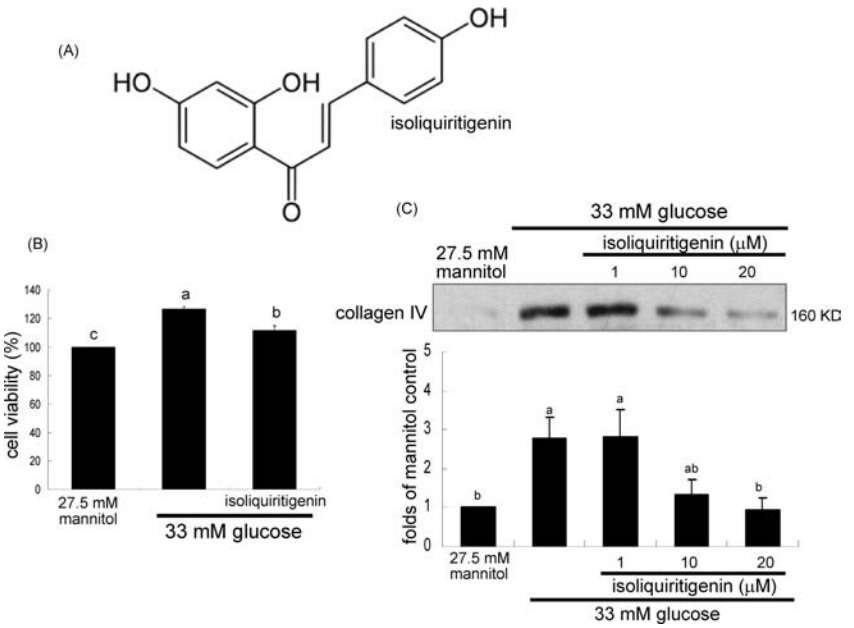

Figure 1. Chemical structure of isoliquiritigenin (**A**), and cell viability (**B**) and collagen type IV secretion (**C**) of human renal mesangial cells (HRMC) challenged with 33 mM glucose in the absence and presence of isoliquiritigenin. HRMC were treated with 1−20 μM isoliquiritigenin for 3 days in the culture media of 33 mM glucose. Cells were also incubated with 5.5 mM glucose and 27.5 mM mannitol as osmotic controls. Values (**B**) are means ± SEM ($n = 5$) and expressed as percent cell survival relative to mannitol controls (cell viability = 100%). For the secretion of collagen type IV (**C**), culture media were subjected to SDS−PAGE and Western blot analysis with a primary antibody against collagen type IV. The bar graphs (means ± SEM, $n = 3$) in the bottom panel represent quantitative results obtained from a densitometer. Values not sharing a letter are different at $P < 0.05$.

end-products (AGE) augmented mesangial production of ECM predominantly through a TGF-β1-independent pathway (*8*). Furthermore, it has been reported that SMAD proteins play a role in the transcriptional regulation of collagen and other ECM gene expression and in the development of fibrosis (*9*).

Fewer agents like angiotensin converting enzyme inhibitors and angiotensin receptor blockers are currently available to improve the function of diabetic kidney to some extent in patients with nephropathy. Thus, tremendous efforts are being made to explore promising therapeutic agents to treat diabetic nephropathy. Recently, pharmacotherapy with natural products has been attempted by targeting potential sites involved in the pathogenesis of diabetic nephropathy have been delineated. (−)-Epigallocatechin 3-*O*-gallate diminished renal damage caused by diabetic oxidative stress responsible for renal lesions of diabetic nephropathy (*10*). Polyphenols of *Hibiscus sabdariffa Linnaeus* retarded diabetic nephropathy in experimental type 1 diabetes (*11*). The flavone rutin effectively prevented experimental renal damage in streptozotocin-treated rats (*12*). In addition, the flavonoid astilbin ameliorated experimental diabetic nephropathy by inhibiting CTGF (*13*).

Isoliquiritigenin (**Figure 1A**) with a chalcone structure has been considered as a potent antioxidant with anti-inflammatory, antiatherosclerotic and cancer-preventing properties (*14*, *15*). When administered to diabetic rats, isoliquiritigenin inhibited sorbitol accumulation with a compelling aldose reductase inhibiting activity, whereby it may be effective in preventing diabetic complications (*16*). To explore whether isoliquiritigenin dampens excessive accumulation of mesangial matrix induced by high ambient glucose, the present study examined alterations in CTGF expression and collagen IV production in human glomerular mesangial cells treated with isoliquiritigenin. Activity of matrix metalloproteinases (MMP) responsible for enzymatic degradation of ECM was assessed to define a cardinal role of isoliquiritigenin in inhibiting ECM amassing in diabetic nephropathy. Furthermore, this study elucidated a potential role of TGF-β1-SMAD signaling in the matrix remodeling and glomerulosclerosis. Blockade of TGF-β1-SMAD signaling might be a novel therapeutic strategy of isoliquiritigenin for the treatment of diabetic renal injury and nephropathy.

MATERIALS AND METHODS

Chemicals and Materials. Fetal bovine serum (FBS), trypsin−EDTA, and penicillin−streptomycin were obtained from BioWhittaker (San Diego, CA). 3-(4,5-Dimethylthiazolyl)diphenyltetrazolium bromide (MTT) was purchased from DUCHEFA Biochemie (Haarlem, Netherlands). Dulbecco's modified Eagle's media (DMEM), Nutrient Mixture F-12 Ham medium, mannitol, D-glucose, and isoliquiritigenin were provided by Sigma Chemical (St. Louis, MO), as were all other reagents unless specifically stated otherwise. Isoliquiritigenin was solubilized in dimethyl sulfoxide; the final culture concentration was ≤0.5%. Antibody of human connective tissue growth factor (CTGF) was purchased from AbCam, Cambridge, U.K.). Antibodies of human collagen type IV and membrane type-1 matrix metalloproteinase (MT1-MMP) were provided by Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies of human tissue inhibitor of matrix metalloproteinases-2 (TIMP-2) and SMAD7 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 and SMAD4 were supplied by Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-rabbit IgG, goat anti-mouse and donkey anti-goat IgG were provided by Jackson ImmunoResearch Laboratories (West Grove, PA). Cyanine 3-conjugated goat anti-rabbit IgG was provided by Rockland (Gilbertville, PA). TGF-β RI inhibitor (HTS 466284) was supplied by Merck (Darmstadt, Germany). Reverse transcriptase and Taq DNA polymerase were purchased from Promega (Madison, WI).

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

To mimic hyperglycemia-induced glomerular sclerosis, HRMC were incubated in 33 mM glucose-added DMEM containing 2% FBS

and 8 μg/mL insulin for 3 days in the absence and presence of submicromolar isoliquiritigenin. For osmotic control incubations, another set of HRMC were cultured in DMEM plus 27.5 mM mannitol containing 2% FBS (+2 μg/mL insulin). The concentrations of isoliquiritigenin used for 3 day culture experiments were below 20 μM.

Cell Viability. After the 3 day incubation period under conditions of HG and mannitol, the assay of 3-(4,5-dimethylthiazolyl)diphenyltetrazolium bromide (MTT, DUCHEFA Biochemie, Haarlem, Netherlands) was carried out for measuring cell viability (*17*). HRMC were cultured in a fresh phenol red free DMEM containing 1 mg/mL MTT for 3 h 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 λ = 570 nm with background subtraction using λ = 690 nm.

Western Blot Analysis. Western blot analysis was executed using whole cell lysates prepared from HRMC and collected culture media (*18*). HRMC lysates were prepared in a lysis buffer containing 1% β-mercaptoethanol, 1 M β-glycerophosphate, 0.1 M Na_3VO_4, 0.5 M NaF and protease inhibitor cocktail. Equal amounts of total lysate proteins or equal volumes of culture medium were electrophoresed on 6% or 10% SDS−PAGE gels and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking the membrane in a TBS-T buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20] containing 3% bovine serum albumin for 3 h. 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) and Konica X-ray film (Konica, Tokyo, Japan). Incubation with polyclonal mouse anti-human β-actin antibody was performed for comparative control.

Immunocytochemistry. HRMC grown on glass slides were rinsed with phosphate buffered saline containing 0.2% Tween 20 (PBS-T), fixed with 4% ice-cold formaldehyde for 20 min and made permeable with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. For blocking any nonspecific binding, cells were incubated 1 h with 20% FBS in PBS-T. After being washed, fixed cells were incubated overnight with polyclonal anti-human CTGF in PBS-T at 4 °C. Cyanine 3-conjugated anti-rabbit IgG in PBS-T was added as a secondary antibody. Fluorescent images were taken with an Axiomager Optical fluorescence microscope (Zeiss, Germany).

Analyses of Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Following culture protocols, total RNA was isolated from 6 $\times$ 10^5 HRMC using a commercially available Trizol reagent kit (Invitrogen, Carlsbad, CA). RNA (5 μg) was reversibly transcribed with 200 units of reverse transcriptase and 0.5 mg/mL oligo-$(dT)^{15}$ primer (Bioneer, Korea). The expression of mRNA transcripts of human collagen IV (forward primer 5′-GGTGTTGCAGGAGTGCCAG-3′, reverse primer 5′-GCAAGTCGAAATAAAACTCACCAG-3′, 100 bp), human CTGF (forward primer 5′-AACTATGATTAGAGCCAACTGCCTG-3′, reverse primer 5′-TCATGCCATGTCTCCGTACATCTTC-3′, 477 bp), human SMAD4 (forward primer 5′-CCATTTCCAATCATCC-TGCT-3′, reverse primer 5′-ACCTTTGCCTATGTGCAACC-3′, 221 bp), β-actin (forward primer 5′-GACTACCTCATGAAGATC-3′, reverse primer 5′-GATCCACATCTGCTGGAA-3′, 500 bp) and GAPDH (forward primer 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse primer 5′-GAAGATGGTGATGGGATTTC-3′, 100 bp) were determined by RT-PCR. The PCR was performed in 25 μL of 10 mM Tris-HCl (pH 9.0) containing 25 mM $MgCl_2$, 10 mM dNTP, 5 units of Taq DNA polymerase and 10 μM of each primer, and started with 5 min denaturation at 94 °C followed by 30 PCR cycles. Each cycle consisted of 60 s at 94 °C, 60 s at 55 °C, and 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-20 M model UV transilluminator (Vilber-Lourmat, 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.

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

RESULTS

Effect of Isoliquiritigenin on HG Induced Mesangial Cell Proliferation. To determine whether HRMC proliferates and isoliquiritigenin has an antiproliferative effect under HG condition, MTT assay was carried out. HRMC incubation with 33 mM glucose for 3 days elevated cell viability by 20−30% increase compared to mannitol controls. When 10 μM isoliquiritigenin was treated with cells exposed to HG, HRMC proliferation was suppressed (**Figure 1B**). Isoliquiritigenin *per se* was not cytotoxic (data not shown), indicating that it has antiproliferative activity in ambient HG-exposed mesangial cells.

Blockade of Production of Collagen and CTGF by Isoliquiritigenin. This study investigated inhibitory effects of isoliquiritigenin on HG-induced production of collagen IV and CTGF. Western blot data showed that HG increased collagen IV secretion ($P < 0.05$, **Figure 1C**). HG-enhanced collagen IV secretion was encumbered by nontoxic isoliquiritigenin at 1−20 μM in a dose-dependent manner (**Figure 1C**).

Cellular expression and secretion of CTGF were boosted in HRMC exposed to HG (**Figure 2A**). However, CTGF production was suppressed by ≥10 μM isoliquiritigenin, confirmed by Western blot (**Figure 2A**). Immunocytochemical data for the cellular CTGF expression were consistent with Western blot data (**Figure 2B**). While there was a heavy cytoplasmic staining in HG-applied cells, the staining vanished in cells treated with ≥10 μM isoliquiritigenin. Accordingly, the decline of collagen IV secretion by isoliquiritigenin was most likely due to its diminution of CTGF production.

There were weak signals for the basal mRNA expression of collagen IV and CTGF in mannitol controls, which were elevated in HG-exposed cells (**Figures 3A** and **3B**). However, ≥10 μM isoliquiritigenin disturbed HG-induced mRNA accumulation of collagen IV and CTGF. The RT-PCR analysis revealed that HG enhanced mesangial production of collagen IV and CTGF at transcriptional levels.

Isoliquiritigenin Inhibition of MT-1 MMP and TIMP-2 Expression Upregulated by HG. This study attempted to determine that HG influenced mesangial expression of MT-1 MMP and TIMP-2, which was reversed by isoliquiritigenin. Exposure of mesangial cells to HG retarded MT-1 MMP expression, which was repealed by ≥10 μM isoliquiritigenin (**Figure 4**). In contrast, TIMP-2 expression was augmented in HRMC exposed to HG and the upregulated expression was abolished in ≥10 μM isoliquiritigenin-pretreated cells.

Isoliquiritigenin Blockade of TGF-β1-SMAD2 Signaling Upregulated by HG. TGF-β activates the receptor-regulated SMAD2, thereby allowing them to associate with SMAD4 and to translocate into the nucleus for gene expression (*9*). Western blot data showed that HG enhanced cellular levels of TGF-β1 and activated SMAD2 at 24 h after stimulation (**Figure 5A**). When HRMC were treated with 1−20 μM isoliquiritigenin and exposed to HG, isoliquiritigenin obliterated HG-enhanced TGF-β1 expression and SMAD2 phosphorylation in a dose-dependent manner. Intracellular localization of SMAD2 was evaluated by fluorescent microscopy using specific SMAD2 antibody (**Figure 5B**). Cytoplasmic immunofluorescence staining was observed in mannitol controls while an all-heavy nuclear staining in HG-alone-exposed HRMC was observed, indicative of nuclear localization of activated SMAD2 at single cell level. However, 20 μM isoliquiritigenin-treated cells markedly diminished the

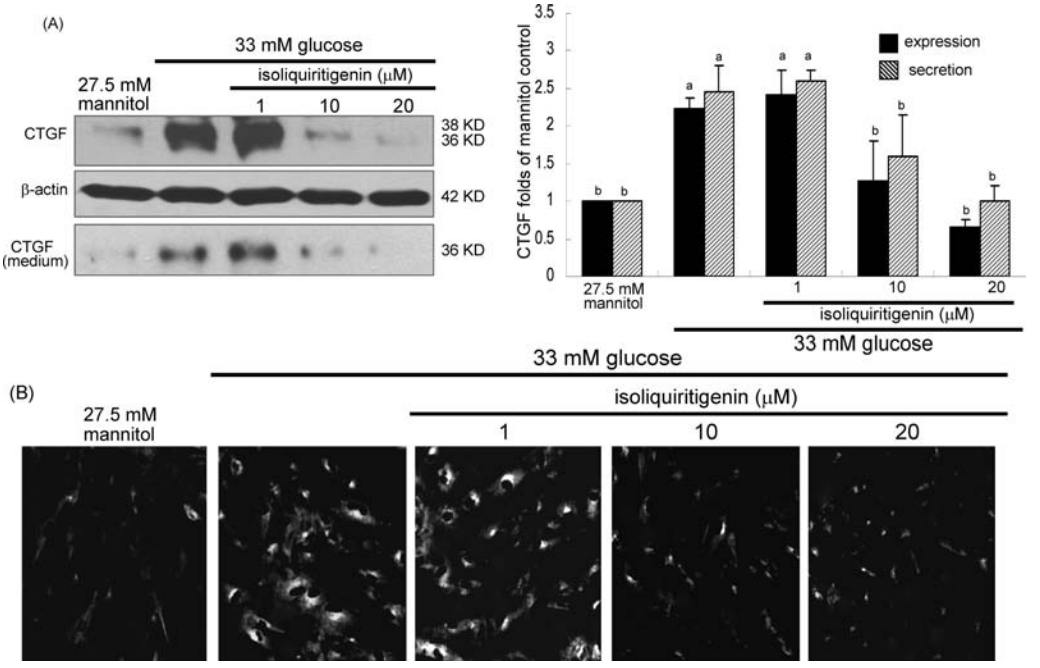

Figure 2. Inhibitory effects of isoliquiritigenin on levels of expression and secretion of connective tissue growth factor (CTGF) in high glucose (HG)-stimulated human renal mesangial cells (HRMC). HRMC were challenged with 1−20 μM isoliquiritigenin for 3 days in the culture media of 33 mM glucose. In addition, cells were incubated with 5.5 mM glucose and 27.5 mM mannitol as osmotic controls. After HRMC culture protocols, SDS−PAGE was performed with cell extracts or culture media and Western blot analysis with a primary antibody against CTGF (**A**). β-Actin protein was used as an internal control. The bar graphs (means ± SEM, $n = 3$) in the right panel represent quantitative results obtained from a densitometer. Respective values not sharing a letter are different at $P < 0.05$. Immunocytochemical analysis (**B**) showing cellular CTGF expression was carried out in cells treated with 1−20 μM isoliquiritigenin and exposed to HG. Antibody localization was detected with Cyanine 3-conjugated anti-rabbit IgG using a fluorescence microscopy with rhodamine green filter (3 separate experiments). Magnification: 200-fold.

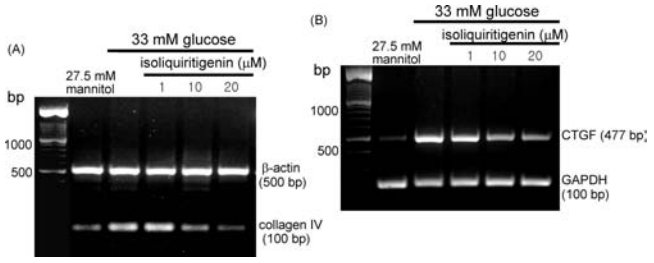

Figure 3. Reverse transcriptase-polymerase chain reaction analysis showing the steady state mRNA transcriptional levels of collagen type IV (**A**) and connective tissue growth factor (CTGF, **B**) in isoliquiritigenin-treated and 33 mM glucose-stimulated human renal mesangial cells (HRMC). Confluent cells were incubated with 1−20 μM isoliquiritigenin for 3 days under conditions of high glucose. Also, HRMC were also incubated with 5.5 mM glucose and 27.5 mM mannitol as osmotic controls. β-Actin or GAPDH gene was used as an internal control for the coamplification with collagen type IV or CTGF (3 separate experiments).

staining level of nuclear SMAD2 enhanced by HG, as shown in cells treated for 3 days with 10 μM type-I receptor serine/threonine kinase (TGF-β RI) inhibitor (HTS 466284).

Suppression of HG-Induced TGF-β-Receptor Kinase by Isoliquiritigenin. To initiate a particular TGF-β response, dimeric ligands of the TGF-β bind with high affinity to type-II receptor kinase (TGF-β RII) and transphosphorylate TGF-β RI on the cell surface. When HG was solely applied to HRMC, it was found that the cellular levels of TGF-β RI and TGF-β RII proteins were sharply upregulated within 24 h after the HG stimulation (**Figure 6**). HG may signal through heteromeric complexes of TGF-β RII and TGF-β RI receptors, which activate the downstream SMAD signal transduction pathway. When HRMC were treated with 1−20 μM isoliquiritigenin and exposed to HG, isoliquiritigenin at ≥1 μM substantially diminished the induction of TGF-β RII and TGF-β RI initiated by HG (**Figure 6**).

As shown in **Figures 2** and **4**, cellular expression of CTGF and TIMP-2 was boosted in HRMC exposed to HG. The elevated expression of CTGF and TIMP-2 was remarkably suppressed by ≥20 μM isoliquiritigenin, as observed in cells treated with 10 μM HTS 466284 (**Figure 7**). This indicates that isoliquiritigenin inhibited the HG induction of CTGF and TIMP-2 through dampening stimulation of TGF-β RI and subsequent activation of the downstream SMAD signal transduction pathway.

Expression Modulation of SMAD4 and SMAD7 by Isoliquiritigenin. Co-mediator SMAD4 participates in signaling by diverse TGF-β family members (*19*), where SMAD4 enters the nucleus constitutively and is immediately exported back to the cytoplasm. After TGF-β stimulation, SMAD4 first complexes with SMAD2 in order to become localized in the nucleus. There was relatively weak expression of SMAD4 protein in mannitol control cells, while HG enhanced cellular pool of SMAD4 at 24 h after stimulation (**Figure 8A**). When HRMC were treated with 1−20 μM isoliquiritigenin in presence of HG, ≥10 μM isoliquiritigenin dampened HG-upregulated SMAD4 expression.

RT-PCR data showed that there was low basal mRNA expression of SMAD4 in mannitol controls (**Figure 8B**). In contrast, the SMAD4 mRNA was greatly increased in HG-stimulated HRMC. However, the HG-instigated mRNA accumulation of SMAD4 was greatly downregulated at doses of ≥10 μM in isoliquiritigenin-treated cells (**Figure 8B**). This observation was consistent with a substantial attenuation of cellular SMAD4

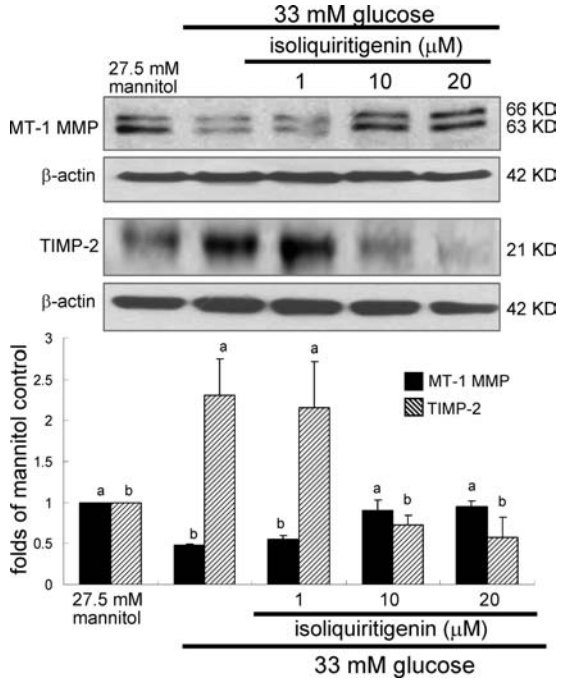

Figure 4. Western blot data showing expression of membrane type 1 matrix metalloproteinase (MT-1 MMP) and tissue inhibitor of MMP (TIMP)-2 in human renal mesangial cells (HRMC) treated with isoliquiritigenin under high glucose conditions. Cells were challenged with 1−20 μM isoliquiritigenin and then exposed to 33 mM glucose for 3 days. Additionally, HRMC were also incubated for 3 days in 5.5 mM glucose and 27.5 mM mannitol as osmotic controls. After HRMC culture protocols, cell extracts were subjected to SDS−PAGE and Western blot analysis with a primary antibody against MT-1 MMP or TIMP-2. β-Actin protein was used as an internal control. The bar graphs (means ± SEM, $n = 3$) in the bottom panel represent quantitative results obtained from a densitometer. Values not sharing a letter are different at $P < 0.05$.

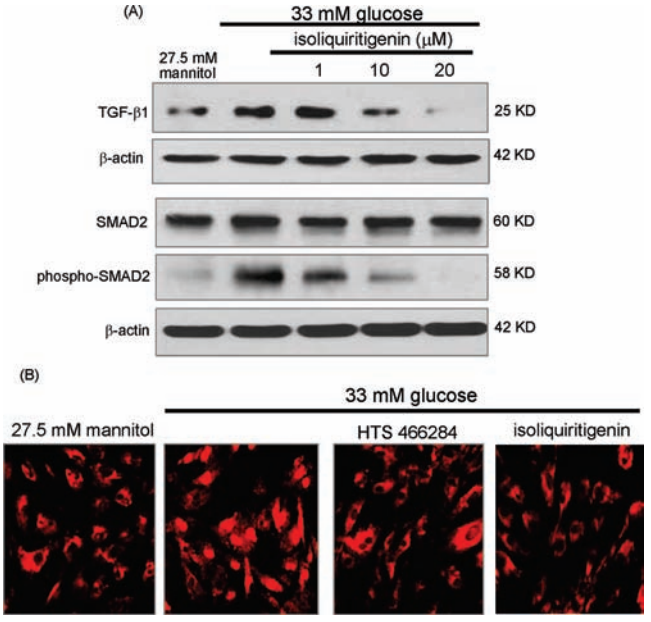

Figure 5. Inhibition of TGF-β1 expression and SMAD2 activation in human renal mesangial cells (HRMC) treated with isoliquiritigenin under high glucose (HG) conditions. HRMC were treated with 1−20 μM isoliquiritigenin and then stimulated with 33 mM glucose for 3 days. Cells were also incubated with 5.5 mM glucose and 27.5 mM mannitol as osmotic controls. After HRMC culture protocols, cell extracts were subjected to SDS−PAGE and Western blot analysis with a primary antibody against TGF-β1, SMAD2 and phosphorylated SMAD2 (**A**). Respective blot data were obtained from 3 independent experiments. β-Actin protein was used as an internal control. Immunocytochemical analysis (**B**) showing nuclear translocation of phospho-SMAD2 was carried out in cells treated for 3 days with 10 μM TGF-β RI inhibitor (HTS 466284) or 20 μM isoliquiritigenin under HG conditions. Antibody localization was detected with cyanine 3-conjugated anti-rabbit IgG using a fluorescence microscopy with rhodamine green filter (3 separate experiments). Magnification: 200-fold.

levels by isoliquiritigenin (**Figure 8A**). Accordingly, isoliquiritigenin may inhibit SMAD4 signaling through its transcriptional modulation.

The antagonistic SMAD7 that negatively regulates TGF-β pathways inhibits the recruitment and phosphorylation of the receptor-regulated SMAD2 (*19*). Western blot analysis revealed that SMAD7 levels were dropped in HG-exposed HRMC (**Figure 8A**), demonstrating that HG inhibited markedly the recruitment and phosphorylation of SMAD2. In contrast, isoliquiritigenin boosted HG-plummeted SMAD7 levels, being near-completely restored at ≥10 μM isoliquiritigenin. Since SMAD7 ubiquitinates the TGF-β receptors on the cell surface or endosomal membranes, it is assumed that the isoliquiritigenin-boosted SMAD7 may promote the ubiquitination of the TGF-β receptors.

DISCUSSION

Long-standing hyperglycemia is known to promote glomerulosclerosis and tubulo-interstitial fibrosis (*2*, *3*). Excessive amassing of matrix and thickening of glomerular and tubular basement membranes build up in the early phase of diabetic nephropathy (*4*). Conversion of normally quiescent mesangial cells into extracellular matrix-overproducing myofibroblasts is central to the pathogenesis of diabetic nephropathy. As expected, 3 day exposure of MC to ambient HG caused mesangial hyperplasia. In addition, HG promoted CTGF expression at the transcriptional levels, elevating secretion of collagen IV, an intrinsic component of mesangial matrix. The HG-MC culture outcomes are thought to be mimetic to glomerular hypertrophy and mesangial fibrosis instigated by hyperglycemia. Decreased enzymatic degradation of mesangial matrix is responsible for its excessive deposition in glomerulosclerosis and tubulo-interstitial fibrosis (*3*). Abnormal expression of MMP and TIMP was recognized in a model of diabetic renal fibrosis (*20*). This study found that HG dampened MT-1 MMP expression and elevated TIMP-2 expression in MC. Accordingly, there was an imbalance between ECM production and degradation in HG-inflamed MC. It was reported that the imbalance is an important factor in the process of ECM expansion in renal diseases (*20*). The relationship between CTGF, MT-1 MMP and TIMP-2 remains to be elucidated.

Modulation of CTGF and MMP involved in the matrix deposition is believed to prevent renal fibrosis (*3*). It becomes a major point of concern to develop natural materials from plants for preventing diabetic fibrosis and nephropathy. *Ginkgo biloba* extract retarded ECM accumulation by increasing levels of MMP and extracellular matrix metalloproteinase inducer and diminishing levels of TIMP and plasminogen activator inhibitor-1 in HG-inflamed rat MC (*21*). Natural salvianolic acid B inhibited HG-stimulated mesangial cell proliferation and ECM production through modulating cell-cycle progress and MMP activity (*22*). Rhein, an anthraquinone compound isolated from rhubarb, decreased the cellular hypertrophy and the ECM accumulation by reversing overactivity of hexosamine pathways in MCGT1 cells (*23*). This study revealed that isoliquiritigenin, a licorice

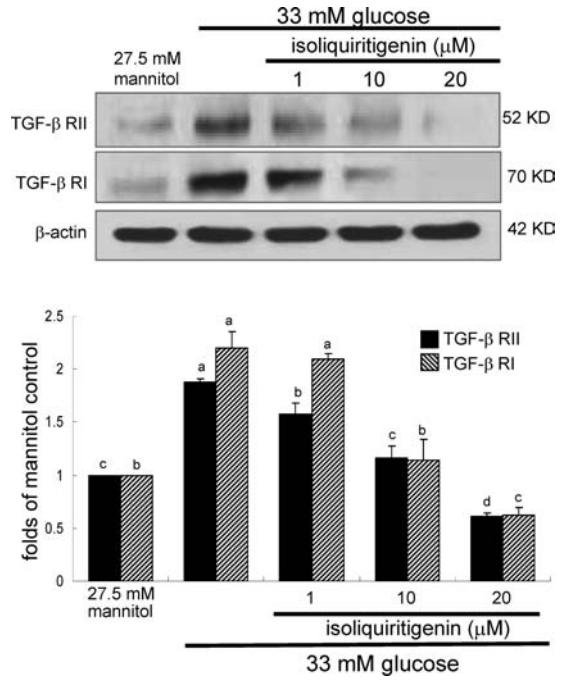

Figure 6. Inhibition of TGF-β receptor kinase RI and RII in human renal mesangial cells (HRMC) treated with isoliquiritigenin under HG conditions. HRMC were treated with 1−20 μM isoliquiritigenin and then stimulated with 33 mM glucose for 3 days. Cells were also incubated with 5.5 mM glucose and 27.5 mM mannitol for 3 days as osmotic controls. After HRMC culture protocols, cell extracts were subjected to SDS−PAGE and Western blot analysis with a primary antibody against TGF-β RI and RII. Representative blot data were respectively obtained from 3 independent experiments and β-actin protein was used as an internal control.

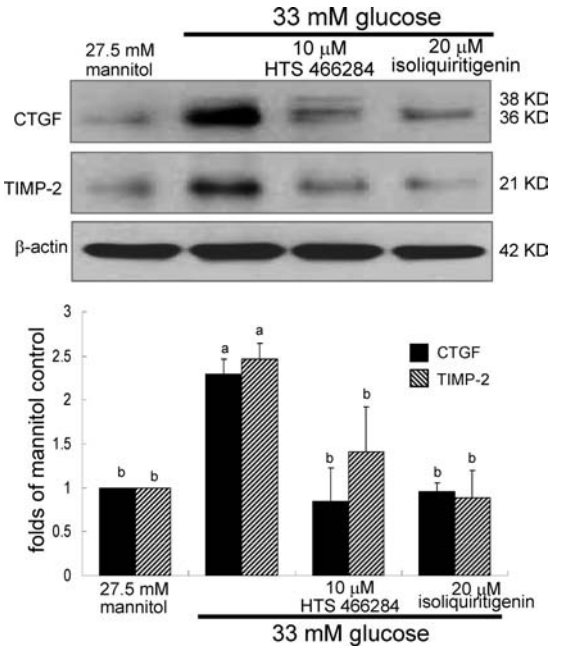

Figure 7. Obstruction of expression of connective tissue growth factor (CTGF) and tissue inhibitor of MMP (TIMP)-2 in human renal mesangial cells (HRMC) treated with TGF-β RI inhibitor (HTS 466284) or isoliquiritigenin in the presence of high glucose. Cells were challenged for 3 days with 10 μM HTS 466284 or 20 μM isoliquiritigenin, and then exposed to 33 mM glucose for 3 days. Additionally, HRMC were incubated for 3 days in 5.5 mM glucose and 27.5 mM mannitol as osmotic controls. Western blot analysis was carried out using cell extracts and a primary antibody against CTGF, TIMP-2 or β-actin. The bar graphs (means $\pm$ SEM, $n = 3$) in the bottom panel represent densitometric results and values not sharing a letter are different at $P < 0.05$.

component, might be a novel agent for alternative medicinal uses in diabetic nephropathy. Licorice has been used as a flavoring agent in foods, beverages and alternative medicines for the taste improvement (*24*). The chalcone isoliquiritigenin has been considered as a potent antioxidant with anti-inflammatory, antiatherosclerotic and cancer-preventing properties (*14*, *15*). When administered to diabetic rats, isoliquiritigenin inhibited sorbitol accumulation by inhibiting aldose reductase activity, whereby it may be effective in preventing diabetic complications (*16*). In addition to these bioactive actions, submicromolar isoliquiritigenin resolved mesangial collagen IV deposition with blunting CTGF production and boosting MT-1 MMP expression. Accordingly, isoliquiritigenin may be a promising natural compound that confers renoprotection against diabetic glomerulosclerosis and fibrosis.

This study attempted to elucidate possible mechanism(s) by which isoliquiritigenin suppressed mesangial proliferation and fibrosis. TGF-β was expressed in excess in areas of progressive ECM accumulation, and TGF-β blockade did not cause renal sclerotic and fibrotic changes (*5*, *7*). In this study, the cellular levels of CTGF and TIMP-2 were retracted in the presence of TGF-β RI inhibitor. TGF-β converges on the SMAD pathway involving receptor-regulated SMAD2 and common-mediated SMAD4 (*19*). SMAD2 inactivation or SMAD4 downregulation will terminate TGF-β signaling. Accordingly, the interruption of TGF-β-SMAD signaling might be a novel therapeutic strategy of isoliquiritigenin for the treatment of diabetic nephropathy. This study showed that isoliquiritigenin effective in blunting collagen amassing lowered HG-elevated cellular levels of TGF-β1 and RI/RII receptor kinases. Isoliquiritigenin repressed SMAD2 activation and SMAD4 expression, and enhanced inhibitory SMAD7 expression, proposing that isoliquiritigenin was a medicinal compound capable of targeting SMAD signaling.

It has been reported that advanced glycation end products (AGE) induced CTGF expression and promoted ECM accumulation, predominantly through a TGF-β1-independent pathway (*25*). Several studies have investigated different intracellular signaling mechanisms modulating diabetic hypertrophy and ECM accumulation in MC (*26*, *27*). The ERK1/2 signaling pathway was activated by AGE during ECM remodeling (*26*). The PI3K-PKCβ1-Akt signaling was responsible for HG-induced collagen I upregulation mediated by the transactivation of epidermal growth factor receptor (*27*). Likewise, HG-triggered mesangial proliferation may entail firing of PKCβ-Akt signaling, and isoliquiritigenin may quench this flamed signaling. The ERK1/2 signaling may be subsided by isoliquiritigenin possibly through dampening AGE formation. This study did not examine HG-induced formation of AGE that is known to modulate matrix proteins and remodeling enzymes. Nevertheless, it is deemed that isoliquiritigenin attenuates HG-triggered AGE formation or inhibits AGE receptor interaction, interrupting TGF-β-SMAD signaling pathways.

The close investigation on how isoliquiritigenin interrupted the TGF-β-SMAD signaling is still obligatory. Accumulation of intracellular sorbitol due to increased activity of aldose reductase has been implicated in the development of various secondary complications of diabetes. It was shown that aldose reductase was one of the TGF-β1 responsive genes in cultured MC (*28*). Thus, inhibition of aldose reductase by isoliquiritigenin may be useful to prevent ECM deposition in diabetic nephropathy.

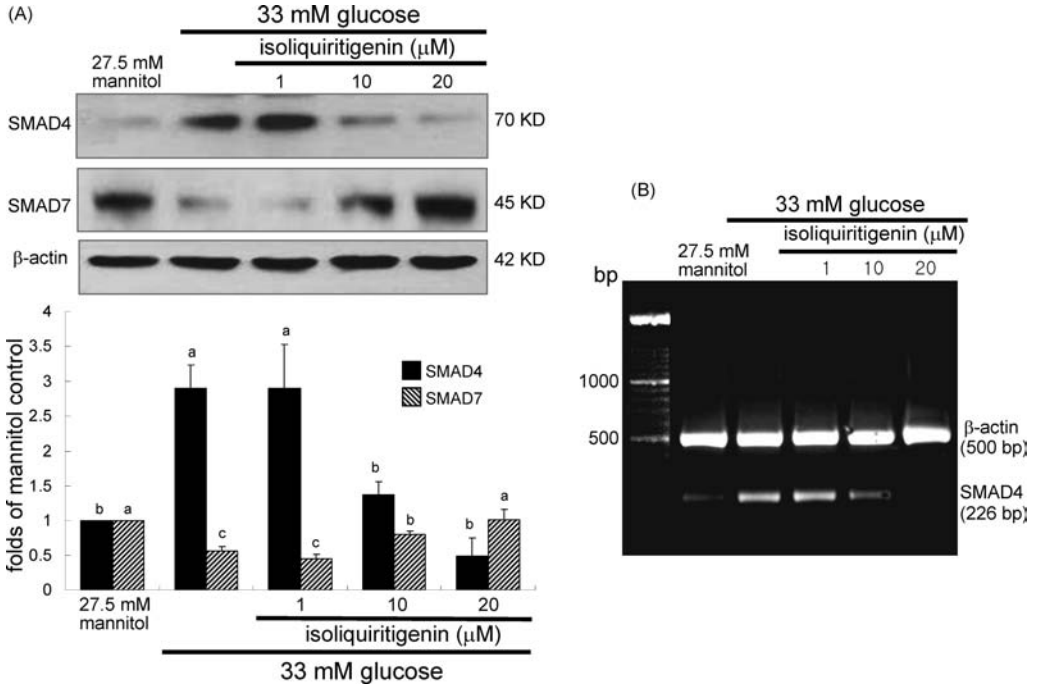

Figure 8. Alteration of cellular levels of SMAD4 and SMAD7 in human renal mesangial cells (HRMC) treated with isoliquiritigenin and exposed to high glucose. HRMC were treated with 1−20 μM isoliquiritigenin and then experienced with 33 mM glucose for 3 days. Cells were also incubated with 5.5 mM glucose and 27.5 mM mannitol as osmotic controls. For Western blot analysis (**A**), cell extracts were subjected to SDS−PAGE and immunoblotted with a primary antibody against SMAD4 and SMAD7. Respective blot data were obtained from 3 independent experiments. β-Actin protein was used as an internal control. The bar graphs (means ± SEM, $n = 3$) in the bottom panel represent quantitative results obtained from a densitometer. Values not sharing a letter are different at $P < 0.05$. Reverse transcriptase-polymerase chain reaction analysis (**B**) showing the steady state mRNA transcriptional levels of SMAD4 in isoliquiritigenin-treated and 33 mM glucose-stimulated HRMC. β-Actin gene was used as an internal control for the coamplification with SMAD4 (3 separate experiments).

Dietary curcumin suppressed aldose reductase and hence sorbitol accumulation in human erythrocytes under HG conditions, suggesting it as an agent to prevent or treat diabetic complications (*29*). Furthermore, it has been demonstrated that an inflammatory mechanism including kidney macrophage accumulation contributes to the pathogenesis of diabetic nephropathy (*30*, *31*). It was found that kidney chemokine monocyte chemoattractant protein-1 (MCP-1) was a major promoter of renal inflammation and fibrosis in diabetic nephropathy (*31*). Accordingly, the renoprotection of isoliquiritigenin against MCP-1 and inflammation may be specific therapies of renal fibrosis. Colchicine attenuated inflammatory cell infiltration via inhibition of enhanced expression of MCP-1 and intracellular adhesion molecule-1, and prevented ECM accumulation in diabetic nephropathy (*32*). Unfortunately, this study did not consider the specific anti-inflammatory actions of isoliquiritigenin for the treatment of mesangial matrix expansion.

In summary, this study showed that isoliquiritigenin retracted mesangial proliferation and matrix deposition leading to mesangial fibrosis. The ability to block mesangial matrix deposition argues for a therapeutic target of actions of isoliquiritigenin under pathological diabetic conditions. The HG induction of mesangial CTGF and TIMP-2 appeared to be mediated via TGF-β1-SMAD-responsive pathways that were disturbed by isoliquiritigenin. In addition, the induction levels of TGF-β RI/RII elevated by HG were downregulated by isoliquiritigenin. Therefore, isoliquiritigenin therapy that disrupted renal TGF-β1 activity may be promising in retarding the progression of fibrosis to end-stage renal diseases. However, the possibility that HG-induced mesangial hypertrophy and fibrosis are mediated via the TGF-β−independent ERK signaling pathway or the PI3K-PKCβ1-Akt signaling pathway cannot be ruled out. In addition, AGE formation and kidney inflammation may be crucial factors to be targeted by isoliquiritigenin

ABBREVIATIONS USED

AGE, advanced glycation end products; CTGF, connective tissue growth factor; DMEM, Dulbecco's modified Eagle's media; ECM, extracellular matrix; FBS, fetal bovine serum; HG, high glucose; HRMC, human renal mesangial cells; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinases; MT-1 MMP, membrane type-1 matrix metalloproteinase; MTT, 3-(4,5-dimetylthiazolyl)diphenyltetrazolium bromide; PBS-T, phosphate buffered saline-Tween 20; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; TBS-T, Tris buffered saline-Tween 20; TGF, transforming growth factor; TGF-β R, transforming growth factor-β receptor kinase; TIMP, tissue inhibitor of matrix metalloproteinases.

LITERATURE CITED

(1) Wilkinson-Berka, J. L. Vasoactive factors and diabetic retinopathy: vascular endothelial growth factor, cycoloxygenase-2 and nitric oxide. *Curr. Pharm. Des.* **2004**, *10*, 3331–3348.

(2) Schena, F. P.; Gesualdo, L. Pathogenetic mechanisms of diabetic nephropathy. *J. Am. Soc. Nephrol.* **2005**, *16*, S30–S33.

(3) Ban, C. R.; Twigg, S. M. Fibrosis in diabetes complications: pathogenic mechanisms and circulating and urinary markers. *Vasc. Health Risk Manag.* **2008**, *4*, 575–596.

(4) Kanwar, Y. S.; Wada, J.; Sun, L.; Xie, P.; Wallner, E. I.; Chen, S.; Chugh, S.; Danesh, F. R. Diabetic nephropathy: mechanisms of renal disease progression. *Exp. Biol. Med.* (*Maywood*) **2008**, *233*, 4–11.

(5) Ziyadeh, F. N. Mediators of diabetic renal disease: the case for TGFβ as the major mediator. *J. Am. Soc. Nephrol.* **2004**, *15*, S55–S57.
(6) Mason, R. M. Connective tissue growth factor (CCN2), a pathogenic factor in diabetic nephropathy. What does it do? How does it do it? *J. Cell. Commun. Signal.* **2009**, *3*, 95–104.
(7) Qi, W.; Chen, X.; Poronnik, P.; Pollock, C. A. Transforming growth factor-β/connective tissue growth factor axis in the kidney. *Int. J. Biochem. Cell Biol.* **2008**, *40*, 9–13.
(8) Zhou, G.; Li, C.; Cai, L. Advanced glycation end-products induce connective tissue growth factor-mediated renal fibrosis predominantly through transforming growth factor beta-independent pathway. *Am. J. Pathol.* **2004**, *165*, 2033–2043.
(9) Verrecchia, F.; Mauviel, A.; Farge, D. Transforming growth factor-beta signaling through the SMAD proteins: role in systemic sclerosis. *Autoimmun. Rev.* **2006**, *5*, 563–569.
(10) Yamabe, N.; Yokozawa, T.; Oya, T.; Kim, M.Therapeutic potential of (-)-epigallocatechin 3-O-gallate on renal damage in diabetic nephropathy model rats. *J. Pharmacol. Exp. Ther.* **2006**, *319*, 228–236.
(11) Lee, W. C.; Wang, C. J.; Chen, Y. H.; Hsu, J. D.; Cheng, S. Y.; Chen, H. C.; Lee, H. J. Polyphenol extracts from *Hibiscus sabdariffa Linnaeus* attenuate nephropathy in experimental type 1 diabetes. *J. Agric. Food Chem.* **2009**, *57*, 2206–2210.
(12) Kamalakkannan, N.; Stanely Mainzen Prince, P. The influence of rutin on the extracellular matrix in streptozotocin-induced diabetic rat kidney. *J. Pharm. Pharmacol.* **2006**, *58*, 1091–1098.
(13) Li, G. S.; Jiang, W. L.; Yue, X. D.; Qu, G. W.; Tian, J. W.; Wu, J.; Fu, F. H. Effect of Astilbin on experimental diabetic nephropathy in vivo and in vitro. *Planta Med.* [PMID: 19644810].
(14) Kwon, H. M.; Choi, Y. J.; Choi, J. S.; Kang, S. W.; Bae, J. Y.; Kang, I. J.; Jun, J. G.; Lee, S. S.; Lim, S. S.; Kang, Y. H. Blockade of cytokine-induced endothelial cell adhesion molecule expression by licorice isoliquiritigenin through NF-κB signal disruption. *Exp. Biol. Med.* (*Maywood*). **2007**, *232*, 235–245.
(15) Yamazaki, S.; Morita, T.; Endo, H.; Hamamoto, T.; Baba, M.; Joichi, Y.; Kaneko, S.; Okada, Y.; Okuyama, T.; Nishino, H.; Tokue, A. Isoliquiritigenin suppresses pulmonary metastasis of mouse renal cell carcinoma. *Cancer Lett.* **2002**, *183*, 23–30.
(16) Aida, K.; Tawata, M.; Shindo, H.; Onaya, T.; Sasaki, H.; Yamaguchi, T.; Chin, M.; Mitsuhashi, H. Isoliquiritigenin: a new aldose reductase inhibitor from glycyrrhizae radix. *Planta Med.* **1990**, *56*, 254–258.
(17) Choi, Y. J.; Lim, S. S.; Jung, J. Y.; Choi, J. S.; Kim, J. K.; Han, S. J.; Kang, Y. H. Blockade of nitroxidative stress by roasted licorice extracts in high glucose-exposed endothelial cells. *J. Cardiovasc. Pharmacol.* **2008**, *52*, 344–54.
(18) Kim, D. S.; Kwon, H. M.; Choi, J. S.; Kang, S. W.; Ji, G. E.; Kang, Y. H. Resveratrol blunts tumor necrosis factor-α-induced monocyte adhesion and transmigration. *Nutr. Res. Pract.* **2007**, *1*, 285–290.
(19) Moustakas, A.; Souchelnytskyi, S.; Heldin, C. H. SMAD regulation in TGF-β signal transduction. *J. Cell Sci.* **2001**, *114*, 4359–4369.
(20) Catania, J. M.; Chen, G.; Parrish, A. R. Role of matrix metalloproteinases in renal pathophysiologies. *Am. J. Physiol.* **2007**, *292*, F905–F911.
(21) Ji, L.; Yin, X. X.; Wu, Z. M.; Wang, J. Y.; Lu, Q.; Gao, Y. Y. Ginkgo biloba extract prevents glucose-induced accumulation of ECM in rat mesangial cells. *Phytother. Res.* **2009**, *23*, 477–485.
(22) Luo, P.; Tan, Z.; Zhang, Z.; Li, H.; Mo, Z. Inhibitory effects of salvianolic acid B on the high glucose-induced mesangial proliferation via NF-κB-dependent pathway. *Biol. Parm. Bull.* **2008**, *31*, 1381–1386.
(23) Zheng, J. M.; Zhu, J. M.; Li, L. S.; Liu, Z. H. Rhein reverses the diabetic phenotype of mesangial cells over-expressing the glucose transporter (GLUT1) by inhibiting the hexosamine pathway. *Br. J. Pharmacol.* **2008**, *153*, 1456–1464.
(24) Asl, M. N.; Hosseinzadeh, H. Review of pharmacological effects of Glycyrrhiza sp. and its bioactive compounds. *Phytother. Res.* **2008**, *22*, 709–724.
(25) Zhou, G.; Li, C.; Cai, L. Advanced glycation end-products induce connective tissue growth factor-mediated renal fibrosis predominantly through transforming growth factor beta-independent pathway. *Am. J. Pathol.* **2004**, *165*, 2033–2043.
(26) Berrou, J.; Tostivint, I.; Verrecchia, F.; Berthier, C.; Boulanger, E.; Mauviel, A.; Marti, H. P.; Wautier, M. P.; Wautier, J. L.; Rondeau, E.; Hertig, A. Advanced glycation end products regulate extracellular matrix protein and protease expression by human glomerular mesangial cells. *Int. J. Mol. Med.* **2009**, *23*, 513–520.
(27) Wu, D.; Peng, F.; Zhang, B.; Ingram, A. J.; Kelly, D. J. PKC-β1 mediates glucose-induced Akt activation and TGF-β1 upregulation in mesangial cells. *J. Am. Soc. Nephrol.* **2009**, *20*, 554–566.
(28) Huang, P.; Zhang, Y.; Jiang, T.; Zeng, W.; Zhang, N. Aldose reductase is a potent regulator of TGF-β1 induced expression of fibronectin in human mesangial cells. *Mol. Biol. Rep.* [PMID: 19847669].
(29) Muthenna, P.; Suryanarayana, P.; Gunda, S. K.; Petrash, J. M.; Reddy G. B. Inhibition of aldose reductase by dietary antioxidant curcumin: Mechanism of inhibition, specificity and significance. *FEBS Lett.* [PMID: 19850041].
(30) Qian, Y.; Feldman, E.; Pennathur, S.; Kretzler, M.; Brosius, F. C.3rd From fibrosis to sclerosis: mechanisms of glomerulosclerosis in diabetic nephropathy. *Diabetes* **2008**, *57*, 1439–1445.
(31) Tesch, G. H. MCP-1/CCL2: a new diagnostic marker and therapeutic target for progressive renal injury in diabetic nephropathy. *Am. J. Physiol.* **2008**, *294*, F697–F701.
(32) Li, J. J.; Lee, S. H.; Kim, D. K.; Jin, R.; Jung, D. S. Colchicine attenuates inflammatory cell infiltration and extracellular matrix accumulation in diabetic nephropathy. *Am. J. Physiol.* **2009**, *297*, F200–F209.

Received for review November 20, 2009. Revised manuscript received January 21, 2010. Accepted January 26, 2010. This study was supported by a grant from the Ministry of Knowledge Economy through Technological Development Project for Regional Strategic Planning, and by the Korea Research Foundation Grant funded by the Korean Government (MEST), the Regional Research Universities Program/ Medical & Bio-Materials Research Center.