

KMUP-1 attenuates high glucose and transforming growth factor- β 1-induced pro-fibrotic proteins in mesangial cells

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Abstract. We have previously demonstrated that KMUP-1, a xanthine-based nitric oxide enhancer, attenuates diabetic glomerulosclerosis, while increasing renal endothelial nitric oxide synthase expression in rats. However, the anti-fibrotic mechanisms of KMUP-1 treatment in diabetic nephropathy in terms of cell biology and transforming growth factor- β 1 (TGF- β 1) remain unclear. Therefore, the present study involved investigating the effects of KMUP-1 on high glucose (HG) or TGF- β 1-induced pro-fibrotic proteins in mouse mesangial (MES13) cells, and the effects of KMUP-1 on streptozotocin (STZ)-induced diabetic rats. It was identified that KMUP-1 (10 μ M) attenuated HG (30 mM)-induced cell hypertrophy while attenuating TGF- β 1 gene transcription and bioactivity in MES13 cells. In addition, KMUP-1 attenuated TGF- β 1 (5 ng/ml)-induced Smad2/3 phosphorylation while attenuating HG or TGF- β 1-induced collagen IV and fibronectin protein expression. Furthermore, KMUP-1 attenuated HG-decreased Suv39h1 and H3K9me3 levels. Finally, KMUP-1 attenuated diabetes-induced collagen IV and fibronectin protein expression in STZ-diabetic rats at 8 weeks. In conclusion, KMUP-1 attenuates HG and TGF- β 1-induced pro-fibrotic proteins in mesangial cells and attenuation of TGF- β 1-induced signaling and attenuation of HG-decreased Suv39h1 expression may be two of the anti-fibrotic mechanisms of KMUP-1.

Introduction

The worldwide prevalence of diabetes mellitus is expected to reach 642 million people by 2,040 (1). Diabetic nephropathy (DN), which develops in 40% of diabetic patients, is the leading cause of end-stage renal disease in the majority of developed countries (1). DN is characterized by renal hypertrophy, glomerulosclerosis and renal fibrosis, which involves extracellular matrix (e.g. collagen and fibronectin) accumulation (2,3). Hyperglycemia, transforming growth factor- β 1 (TGF- β 1) and deficient endothelial nitric oxide synthase (eNOS) are among the key pathogenic mechanisms of diabetic renal fibrosis (4). However, current supplementary pharmacological treatments, other than glycemic control and renin-angiotensin system blockade, have had limited success (1). Thus, the development of novel pharmacologic agents is required.

A previous study of the authors indicated that nitric oxide (NO)-cyclic guanosine monophosphate protein kinase (cGMP) inducers attenuate advanced glycation end-product-induced effects in renal fibroblasts (5). KMUP-1 is a synthetic xanthine-based derivative which enhances soluble guanylate cyclase (sGC), eNOS and cGMP (6). A previous study indicated that KMUP-1 attenuates rat diabetic glomerulosclerosis while increasing eNOS levels (7). However, the anti-fibrotic mechanisms of KMUP-1 in DN regarding cell biology and TGF- β 1 remain unclear.

As a result, the present study focused on elucidating the effects of KMUP-1 on high glucose (HG) or TGF- β 1-induced pro-fibrotic proteins in mouse mesangial (MES13) cells, as well as the effects of KMUP-1 on streptozotocin (STZ)-induced diabetic rats.

Materials and methods

Reagents. Cell culture media, Dulbecco's modified Eagle medium (DMEM) and F12, were obtained from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Recombinant human TGF- β 1 was obtained from PeproTech, Inc. (Rocky Hill, NJ, USA). KMUP-1 was synthesized in the laboratory of the authors, the stock solution (10 mM) was prepared by dissolving KMUP-1 in the solvent (10% absolute

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alcohol, 10% propylene glycol and 2% 1 N HCl), according to previous studies (8-10).

Cells. Mouse kidney mesangial cells (MES13) were purchased from the American Type Culture Collection (cat. no. CRL-1927; Manassas, VA, USA). Cells were grown in DMEM/F12 (3:1) medium (with 6.67 mM glucose) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified 5% CO₂ incubator at 37°C. Cells were starved in serum-free (0.5% FBS) media for 24 h prior to experiments in 5% FBS medium. All cell culture materials were obtained from Gibco (Thermo Fisher Scientific, Inc.).

Cell viability. MES13 cells were cultured in 24-well plates (5x10³/well). After 24 h, cells were treated with the control (KMUP-1 solvent at a final concentration of 1%) or KMUP-1 for 72 h. MTT (0.5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was incubated at 37°C for 4 h prior to harvesting. Following removal of the culture medium, cells were dissolved in dimethylsulfoxide (DMSO) and shaken for 10 min. Formazan was dissolved by DMSO and the assay was quantified by determining the absorbance at 540 nm using an ELISA reader (Dynex Technologies GmbH, Denkendorf, Germany).

TGF- β 1 promoter activity and bioactivity. Human TGF- β 1 promoter activity was detected using the pHGT5 plasmid, which was donated by Dr Jean-Louis Virelizier (Unité d'Immunologie Virale, Institut Pasteur, Paris, France) (11). The TGF- β 1 bioactivity reporter p3TP-lux, which contains the plasminogen activator inhibitor-1 promoter, was donated by Dr. Joan Massagué (Memorial Sloan Kettering Cancer Center, New York, USA) (12). Cells were cultured in 6-well plates at a density of 1.5x10⁴ cells/well and transfected with 1 μ g of the pHGT5 or p3TP-lux plasmid using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. Following 6 h of transfection, cells were treated with HG (30 mM) or combined with KMUP-1 (10 μ M) for 72 h. Luciferase activity was measured using the Dynatech ML1,000 luminometer (Dynatech Laboratories, Inc., Chantilly, VA, USA).

Immunoblotting. Briefly (13), MES13 cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM Na₃VO₄, 50 mM NaF and 10 mM β -glycerol phosphate) containing 0.1% protease inhibitor cocktail (Merck KGaA). Cell proteins were extracted and quantified with a DCTM protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). Proteins (50 μ g/well) were separated by 10% SDS-PAGE and transferred to PolyScreen polyvinylidene difluoride membranes (PerkinElmer, Inc., Waltham, MA, USA). Following blocking for 2 h at room temperature with 5% non-fat milk and rinsing with PBS, the membranes were probed with the primary antibodies overnight at 4°C and washed with 0.1% PBS-Tween-20 (PBST) 3 times (5 min each). The primary antibodies used were phosphorylated-Smad2/3 (p-Smad2/3; 1:1,000; cat. no. 8828), Smad2/3 (1:2,000; cat. no. 3102), Suv39h1 (1:1,500; cat. no. 8729) and H3K9me3 (1:2,000; cat. no. 9754), antibodies were obtained from Cell Signaling Technology, Inc.,

Danvers, MA, USA). Fibronectin (1:10,000; cat. no. AJ1297b) and collagen IV (1:2,000; cat. no. AP7369a) antibodies were obtained from Abgent, Inc. (San Diego, CA, USA), GAPDH (1:5,000; cat. no. sc-25778) and α -tubulin (1:5,000; cat. no. MS-581-P) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA) and Labvision/NeoMarkers (Thermo Fisher Scientific, Inc.), respectively. The membranes were then incubated in horseradish peroxidase (HRP)-conjugated anti-rabbit (1:5,000; cat. no. AP132P) or anti-mouse (1:5,000; cat. no. AP124P) secondary antibodies (Merck KGaA) for 1 h at room temperature and washed with 0.1% PBST 5 times (5 min each). The protein bands were detected by using the enhanced chemiluminescence ECL system (PerkinElmer, Inc.) and the bands were quantified by densitometric analysis (Image Studio Lite 5.25; LI-COR Biosciences, Lincoln, NE, USA).

Cell hypertrophy. Cell hypertrophy was determined by the ratio of whole cell protein lysates to total cell numbers (14). Briefly, MES13 cells were cultured in 6-well plates to 50% confluence and then treated with HG (30 mM) or HG + KMUP-1 (10 μ M) for 3 days. Following trypsinization, cells were washed twice with PBS and cell numbers were counted using a hemocytometer. Equal numbers of cells were lysed in RIPA buffer containing the protease inhibitor cocktail. Total protein content was measured by using the DCTM protein assay kit (Bio-Rad Laboratories, Inc.).

Animal experiments. Male Sprague-Dawley rats (n=23) weighing 200-250 g (aged 7 weeks) were purchased from BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan) and were housed (3 per cage) in a temperature-(22 \pm 2°C) and humidity (50 \pm 10%)-controlled room with a 12 h light/dark cycle in the Animal Center of Kaohsiung Medical University (Kaohsiung, Taiwan). Rats were divided into three groups: Control, streptozotocin (STZ)-diabetic and diabetic + KMUP-1. Briefly (15), after overnight fasting, rats received a single intraperitoneal injection of 55 mg/kg STZ (Sigma-Aldrich; Merck KGaA) in 0.1 M citrate buffer (diabetic, n=9; diabetic + KMUP-1, n=9) or citrate buffer (control, n=5). Thereafter, rats were given free access to food and water. Diabetic rats received Lantus insulin (Sanofi S.A., Paris, France) to maintain non-fasting blood glucose levels between 19.4 and 27.8 mmol/l. The diabetic + KMUP-1 treatment group was intraperitoneally injected with KMUP-1 (dissolved in distilled water, 5 mg/kg/day) daily (16). Rats were perfused with normal saline and anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg; Abbott Pharmaceutical Co., Ltd., Lake Bluff, IL, USA) at week 8. Kidneys were removed and kidney slices were immersed in 4% paraformaldehyde at room temperature for 24 h. Kidney slices were embedded in the paraffin block and cut into sections (thickness, 4 μ m) for immunohistochemical studies. All animal procedures were conducted in accordance with the national guidelines (17) and were approved by the Kaohsiung Medical University Animal Experiment Committee.

Immunohistochemistry. Briefly (15), tissue sections were rehydrated and deparaffinized in xylene and ethanol. The sections then underwent antigen retrieval in 10 mM sodium citrate buffer by microwaving for 30 min. Following this,

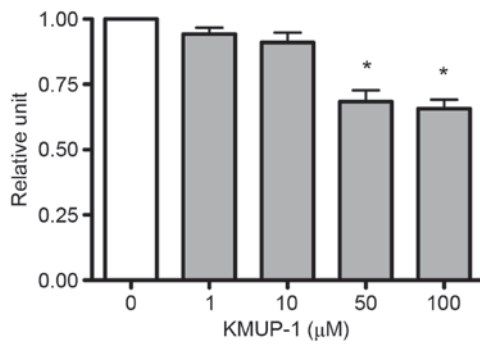


Figure 1. Dose-dependent effects of KMUP-1 on cell viability in MES-13 cells. Cells were treated with KMUP-1 in various doses (1-100 μ M, gray bars) for 72 h. Cell viability was measured by MTT assay. The results were expressed as the mean \pm standard error of four independent experiments. * P <0.05 vs. 0 μ M (blank bar).

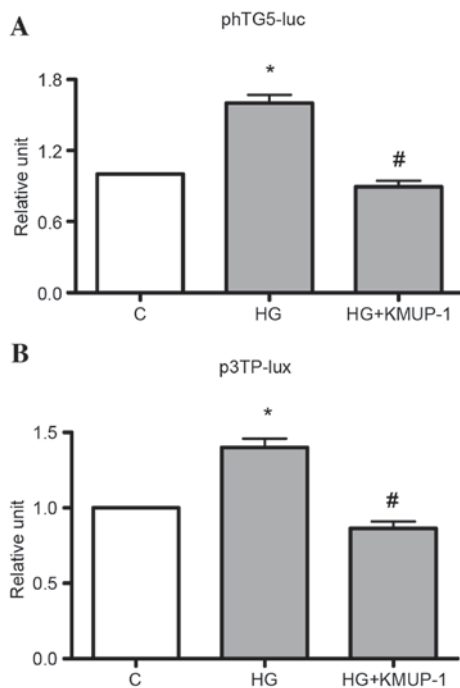


Figure 2. Effects of KMUP-1 on HG-induced TGF- β 1 transcriptional activity or bioactivity. Cells were transiently transfected with the phTG5-luc (TGF- β 1 transcriptional activity reporter) or 3TP-lux (TGF- β 1 bioactivity reporter) plasmids, cells were treated with HG (30 mM, gray bars) alone or in combination with KMUP-1 (10 μ M) for 72 h. (A) Effects of KMUP-1 on TGF- β 1 transcriptional activity. (B) Effects of KMUP-1 on TGF- β 1 bioactivity. The results are expressed as the mean \pm standard error, in three independent experiments. * P <0.05 vs. control. # P <0.05 vs. HG alone. HG, high glucose; KMUP-1; TGF- β 1, transforming growth factor- β 1; C, control group.

preincubation of tissue sections with the blocking buffer was conducted for 30 min prior to incubation with primary antibodies overnight at 4°C. Primary antibodies used were fibronectin (1:400, cat. no. AJ1297b) obtained from Abgent, Inc. and collagen IV antibodies (1:500, cat. no. ab6586) obtained from Abcam (Cambridge, UK). Following washing with PBST, sections were stained and incubated with DAB+ and the ready-to-use (undiluted) HRP-conjugated anti-rabbit secondary antibodies contained in the Dako REAL™ EnVision™ Detection system (cat. no. K5007; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 30 min at room

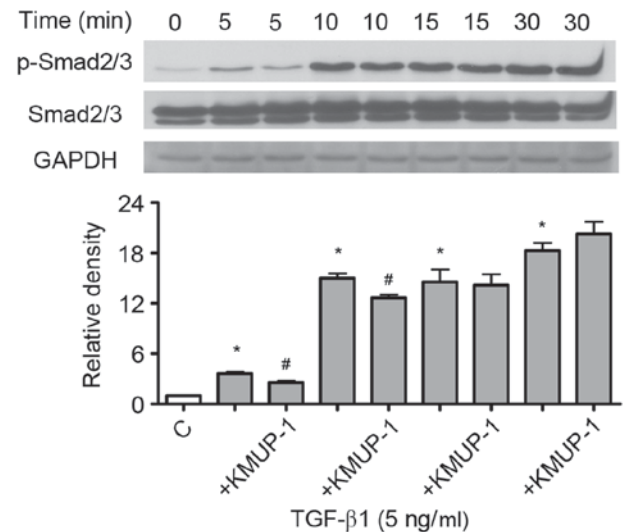


Figure 3. Effects of KMUP-1 on TGF- β 1-induced Smad2/3 signaling. Cells were treated with TGF- β 1 (5 ng/ml, gray bars) alone or in combination with KMUP-1 (10 μ M, pre-treated for 30 min) for 5-30 min. Smad2/3, p-Smad2/3 and GAPDH were measured by immunoblotting. The results were expressed as the ratio of p-Smad2/3 and Smad2/3 and expressed as the mean \pm standard error of three independent experiments. * P <0.05 vs. control, # P <0.05 vs. TGF- β 1 alone. TGF- β 1, transforming growth factor- β 1; p-, phosphorylated; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; C, control group.

temperature, according to the manufacturer's instructions, and counterstained with hematoxylin.

Statistical analysis. Statistical analyses were performed by using Stata 13.1 software (StataCorp LP, College Station, TX, USA). Data were expressed as the mean \pm standard error. Unpaired Student's *t*-tests were used for the comparison between two groups. P <0.05 was considered to be statistically significant.

Results

KMUP-1 attenuated HG-induced TGF- β 1 bioactivity and gene transcriptional activity in MES13 cells. The present study investigated the effects of KMUP-1 on cell viability of MES13 cells to determine the optimum concentration of KMUP-1. MES13 cells were treated with KMUP-1 (1-100 μ M) for 72 h and cell viabilities were measured using an MTT assay. The optimum concentration of KMUP-1 was determined to be 10 μ M (Fig. 1).

TGF- β 1 gene transcriptional activity or TGF- β 1 bioactivity was measured by transient transfection of phTG5 or p3TP-lux, respectively. It was identified that KMUP-1 (10 μ M) attenuated HG (30 mM)-induced TGF- β 1 gene transcriptional activity or TGF- β 1 bioactivity at 72 h (Fig. 2).

KMUP-1 attenuated TGF- β 1-induced smad2/3 phosphorylation. Since TGF- β 1 gene transcriptional activity and TGF- β 1 bioactivity were attenuated by KMUP-1, the present study examined whether KMUP-1 also attenuated TGF- β 1-Smad signaling pathway. TGF- β 1 (5 ng/ml) increased phosphorylation of Smad2/3 in a time-dependent manner (5-30 min; Fig. 3). Furthermore, KMUP-1 (10 μ M) attenuated TGF- β 1-induced p-Smad2/3 expression at 5-10 min (5 ng/ml; Fig. 3).

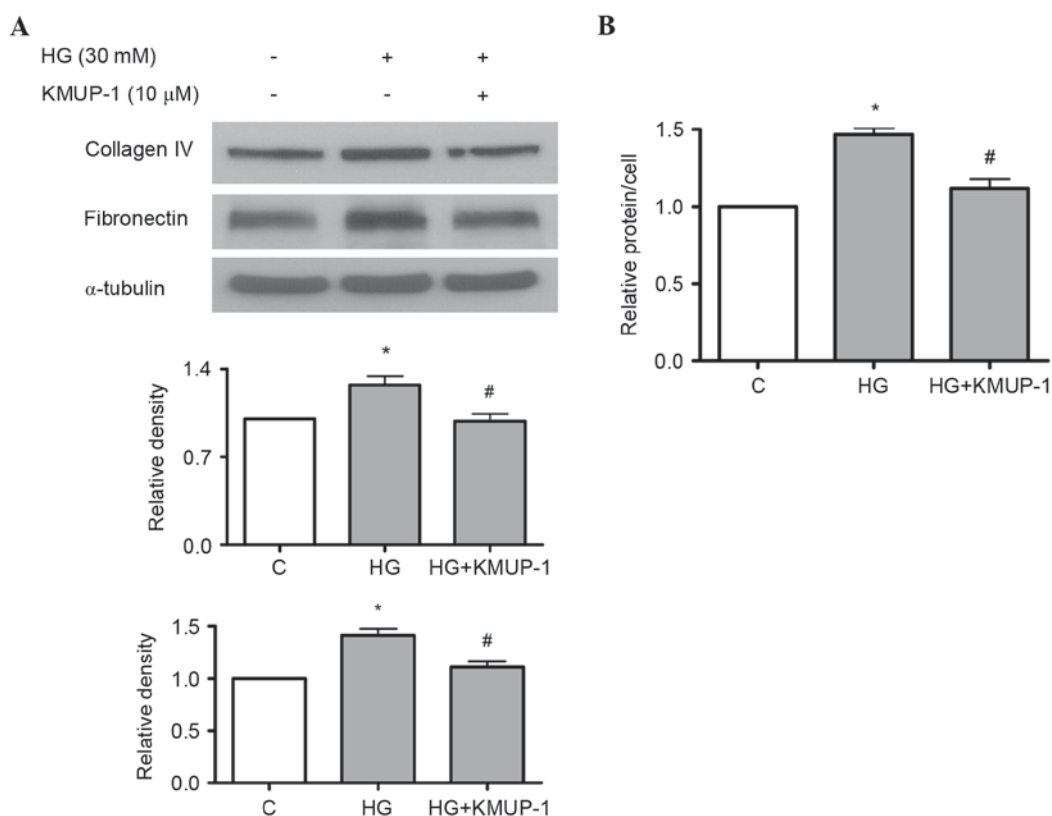


Figure 4. Effects of KMUP-1 on HG-induced collagen IV or fibronectin expression and cell hypertrophy. Cells were treated with HG (30 mM, gray bars) alone or in combination with KMUP-1 (10 μ M, pre-treated for 30 min) for 72 h. Collagen IV, fibronectin and α -tubulin levels were measured by immunoblotting. The results were expressed as the ratio of collagen IV and α -tubulin or as the ratio of fibronectin and α -tubulin. Cell hypertrophy was measured by the ratio of total protein to cell number. (A) Effects of HG on collagen IV or fibronectin protein expression. (B) Effects of HG on cell hypertrophy. The results were expressed as the mean \pm standard error of three independent experiments. * P <0.05 vs. control, # P <0.05 vs. HG group. HG, high glucose; C, control group.

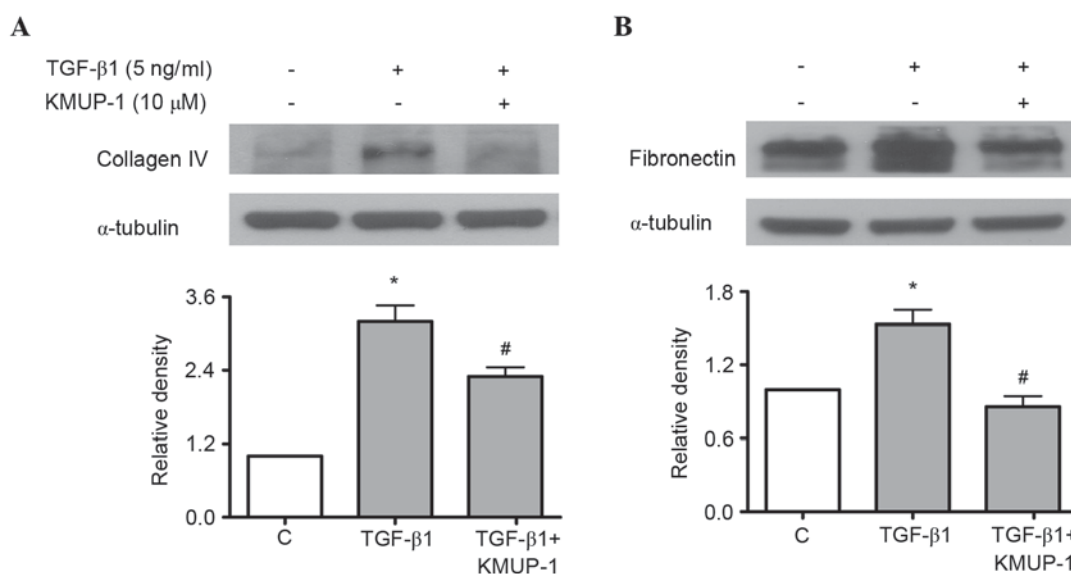


Figure 5. Effects of KMUP-1 on TGF- β 1-induced collagen IV or fibronectin expression. Cells were treated with TGF- β 1 (5 ng/ml, gray bars) alone or in combination with KMUP-1 (10 μ M, pre-treated for 30 min) for 72 h. Collagen IV, fibronectin and α -tubulin levels were measured by immunoblotting. (A) Effects of KMUP-1 on TGF- β 1-induced collagen IV protein expression. (B) Effects of KMUP-1 on TGF- β 1-induced fibronectin protein expression. The results were expressed as the ratio of collagen IV and α -tubulin or as the ratio of fibronectin and α -tubulin and expressed as the mean \pm standard error of the mean of three independent experiments. * P <0.05 vs. control, # P <0.05 vs. TGF- β 1 group. TGF- β 1, transforming growth factor- β 1; C, control group.

KMUP-1 attenuated HG or TGF- β 1-induced collagen IV or fibronectin protein expression. Both HG and TGF- β 1 have been previously reported to increase fibronectin and collagen

IV protein expression in mesangial cells (18,19). As a result, the present study investigated the effects of KMUP-1 on HG or TGF- β 1-induced fibronectin and collagen IV protein

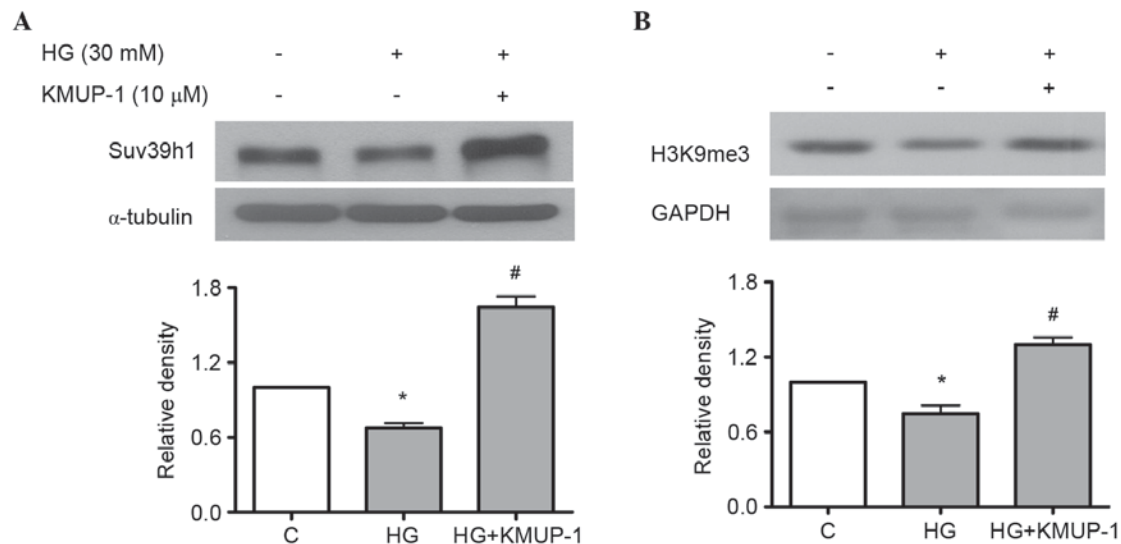


Figure 6. Effects of KMUP-1 on HG-decreased Suv39h1 and H3K9me3 protein expression. Cells were treated with high glucose (30 mM, gray bars) alone or in combination with KMUP-1 (10 μ M) for 72 h. Suv39h1, H3K9me3, α -tubulin and GAPDH levels were measured by immunoblotting. Suv39h1 was normalized to α -tubulin whereas H3K9me3 was normalized to GAPDH. (A) Effects of KMUP-1 on HG-decreased Suv39h1 protein expression. (B) Effects of KMUP-1 on HG-decreased H3K9me3 protein expression. The results were expressed as the mean \pm standard error of three independent experiments. * P <0.01 vs. control, # P <0.05 vs. HG group. HG, high glucose; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; C, control group.

expression. KMUP-1 (10 μ M) was demonstrated to attenuate HG (Fig. 4A) or TGF- β 1 (5 ng/ml)-induced fibronectin and collagen IV protein expression at 72 h (Fig. 5).

KMUP-1 attenuated HG-induced cell hypertrophy. A previous study identified that KMUP-1 decreased cardiac hypertrophy via the NO/cGMP pathway (20). In addition to extracellular matrix accumulation, DN is characterized by renal (including mesangial cell) hypertrophy (21). In addition, DN is associated with NO deficiency (22). For example, eNOS knockout mice develop diabetic renal hypertrophy while soluble guanylate cyclase enhancers attenuate DN (23,24). Therefore, the authors studied the effects of KMUP-1 on HG-induced cell hypertrophy. KMUP-1 (10 μ M) attenuated HG-induced cell hypertrophy at 72 h (Fig. 4B).

KMUP-1 attenuated HG-reduced histone methyltransferase Suv39h1. Suv39h1 is a histone lysine methyltransferase, which induces the gene-silencing H3K9me3, while HG increases pro-inflammatory genes concomitantly with decreased levels of H3K9me3 and Suv39h1 at their promoters in vascular smooth muscle cells (25). However, the roles of Suv39h1 in diabetic nephropathy remain unclear. As a result, the study investigated whether HG-induced pro-fibrotic collagen IV and fibronectin is associated with reduced Suv39h1 levels and the effects of KMUP-1 on HG-decreased Suv39h1 levels. KMUP-1 (10 μ M) was demonstrated to attenuate HG-decreased Suv39h1 (Fig. 6A) and H3K9me3 (Fig. 6B) levels at 72 h.

KMUP-1 attenuated collagen type IV and fibronectin expression in STZ-diabetic rats. To corroborate the *in vitro* findings, the effects of intraperitoneal KMUP-1 (5 mg/kg/day) treatment on STZ-diabetic rats were investigated at 8 weeks. Increased glomerular and tubular expression of collagen IV were both attenuated by KMUP-1 in diabetic rats (Fig. 7). Furthermore, increased peri-glomerular and tubulointerstitial

expression of fibronectin was attenuated by KMUP-1 in diabetic rats (Fig. 8).

Discussion

KMUP-1 was demonstrated to attenuate HG-induced TGF- β 1 and TGF- β 1-induced Smad2/3 signaling in mesangial cells. In addition, KMUP-1 attenuated HG or TGF- β 1-induced collagen IV and fibronectin expression and HG-induced cell hypertrophy while attenuating HG-decreased Suv39h1 expression in mesangial cells. In addition, KMUP-1 attenuated collagen IV and fibronectin expression in STZ-diabetic rats. The observations provide mechanistic insights into the role of KMUP-1 in the attenuation of diabetic rat glomerulosclerosis.

The finding that KMUP-1, an eNOS-NO-sGC-cGMP enhancer (6), attenuated HG-induced TGF- β 1 and its Smad2/3 signaling corroborates the notion that DN is associated with a deficiency of NO (22). Notably, it was identified KMUP-1 attenuated TGF- β 1-induced p-Smad2/3 expression only at 5-10 min. Similarly, NO has been identified to delay (however not abolish) TGF- β 1-induced p-Smad2/3 expression from 15-60 min in endothelial cells (26). Previous studies indicated that HG induces TGF- β 1 by decreasing NO and cGMP (27).

In the present study, KMUP-1 attenuated HG- or TGF- β 1-induced collagen IV and fibronectin expression while attenuating cell hypertrophy. These observations corroborate with a previous study demonstrating that overexpression of cGMP-dependent protein kinase attenuates HG-induced TGF- β 1 and expression of collagen or fibronectin in mesangial cells (28). In addition, sGC enhancers decrease DN in eNOS-knockout mice in combination with angiotensin II type I receptor blockade (29). Thus, sGC enhancers may be renoprotective in DN (24) by attenuating TGF- β 1-induced effects. Similarly, a previous study identified that eNOS deficiency induces diabetic renal hypertrophy and glomerulosclerosis in mice (23).

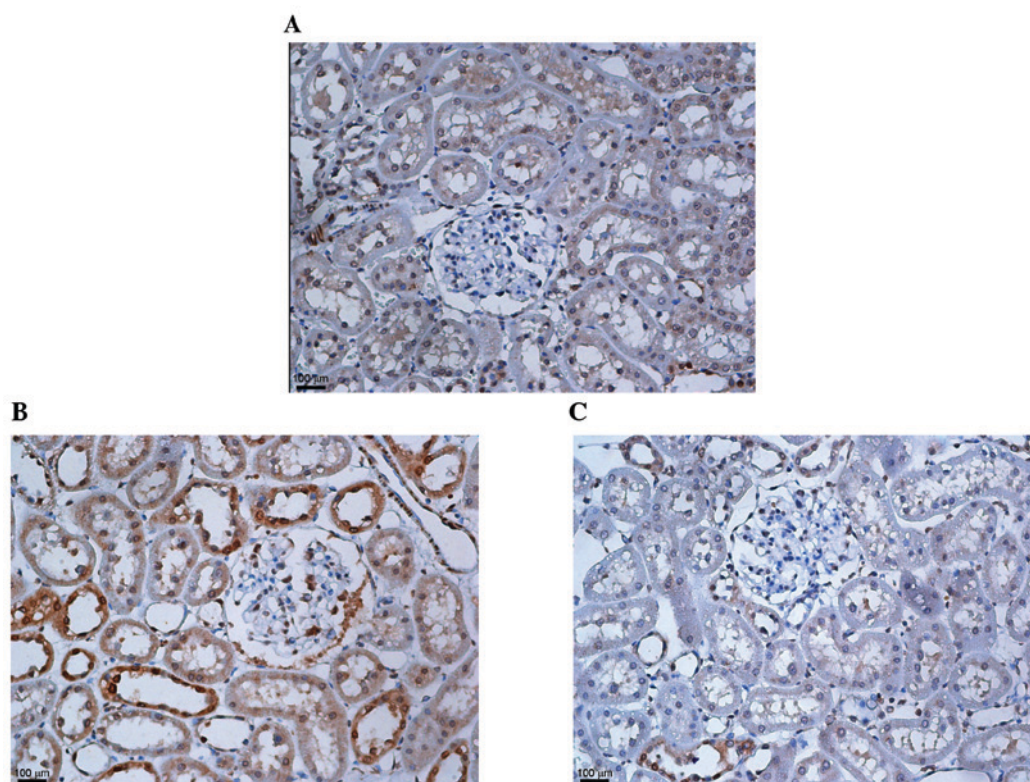


Figure 7. Effects of KMUP-1 on renal cortical collagen IV protein expression in STZ-diabetic rats. STZ-diabetic rats were intraperitoneally injected with KMUP-1 (5 mg/kg/day) daily for 8 weeks. Rats were perfused with normal saline and anesthetized on week 8. Kidneys were removed and immersed in 4% paraformaldehyde. Kidney slices were embedded in the paraffin block and cut into 4 μ m sections for immunohistochemical (collagen IV) studies. The studies conducted experimentation on (A) a control rat, (B) a diabetic rat and (C) a diabetic + KMUP-1 rat. STZ, streptozotocin.

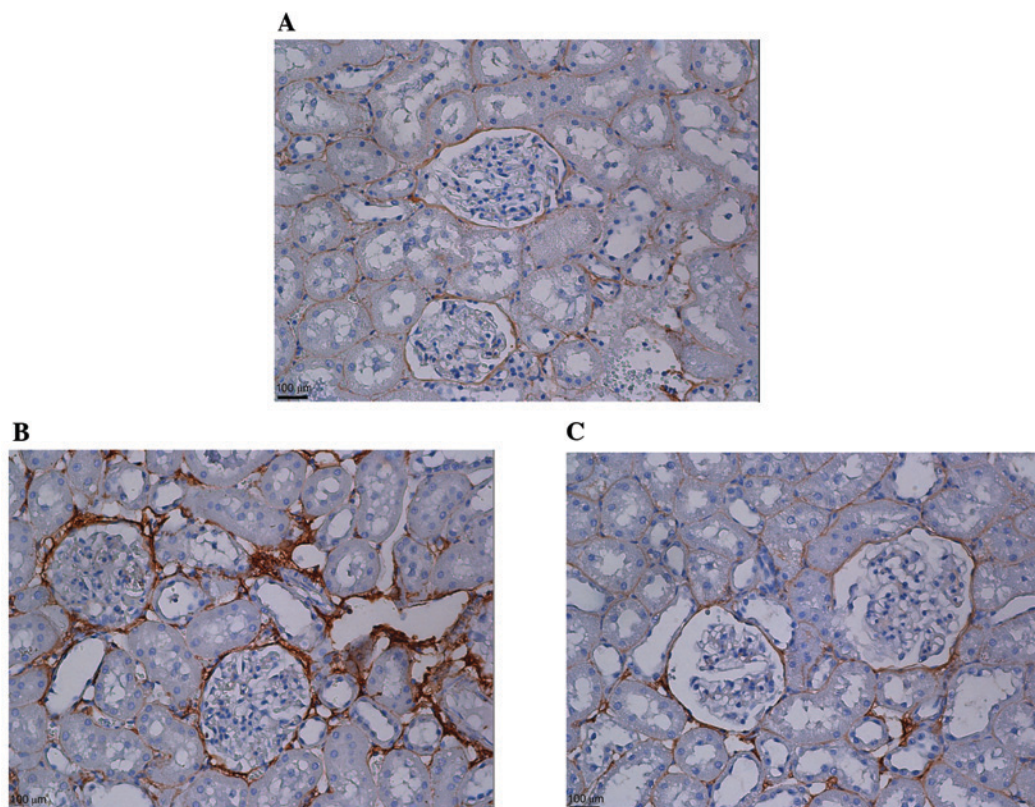


Figure 8. Effects of KMUP-1 on renal cortical fibronectin protein expression in STZ-diabetic rats. STZ-diabetic rats were intraperitoneally injected with KMUP-1 (5 mg/kg/day) daily for 8 weeks. Rats were perfused with normal saline and anesthetized on week 8. Kidneys were removed and immersed in 4% paraformaldehyde. Kidney slices were embedded in the paraffin block and cut into 4 μ m sections for immunohistochemical (fibronectin) studies on (A) a control rat, (B) a diabetic rat and (C) a diabetic + KMUP-1 rat. STZ, streptozotocin.

The present study presented that KMUP-1 attenuated HG-decreased Suv39h1 and H3K9me3 expression. Similarly, additional studies indicated that Suv39h1 is decreased in diabetic mouse vascular smooth muscle cells (30), while occupancy of H3K9me3 on some pro-fibrotic genes is decreased in diabetic mouse glomeruli (31). Thus, the decreased gene-silencing activity of Suv39h1 may be one of the pro-fibrotic mechanisms in DN. Notably, Suv39h1 protects from myocardial ischemia-reperfusion injury in diabetic rats (32).

The *in vitro* results were corroborated with the *in vivo* results that KMUP-1 attenuated increased expression of collagen IV and fibronectin in STZ-diabetic rats. This is in agreement with a previous study observing that KMUP-1 attenuates rat DN while increasing glomerular eNOS and decreasing matrix metalloproteinase-9 (MMP-9) expression (7). The attenuation of MMP-9 expression by KMUP-1 (7) may be an additional anti-fibrotic mechanism in that MMP-9 inhibitors attenuate HG-induced TGF- β 1 (33) and attenuate DN (34).

In conclusion, KMUP-1 attenuates HG- and TGF- β 1-induced pro-fibrotic proteins within mesangial cells. In addition, attenuation of TGF- β 1-induced Smad2/3 signaling and attenuation of HG-decreased Suv39h1 expression may be two of the anti-fibrotic mechanisms of KMUP-1. These observations provide novel mechanistic insight into the previously observed attenuation of diabetic rat glomerulosclerosis by KMUP-1.

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