Hydrogen sulfide alleviates diabetic nephropathy in a streptozotocin-induced diabetic rat model

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Running title: H2S alleviates diabetic nephropathy

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Keywords: diabetic nephropathy; hydrogen sulfide; inflammation; mesangial cell proliferation; oxidative stress; renin-angiotensin system

Background: H2S plays critical roles in the pathogenesis of chronic kidney diseases.

Results: H2S could improve renal function and attenuate glomerular basement membrane thickening, mesangial matrix deposition and renal interstitial fibrosis in diabetic rats.

Conclusion: H2S attenuates oxidative stress and inflammation, reduces mesangial cell proliferation, and inhibits the renin-angiotensin system in diabetic kidney.

Significance: H2S alleviates the development of diabetic nephropathy.

Abstract
Accumulating evidence has demonstrated that hydrogen sulfide (H2S) plays critical roles in the pathogenesis of chronic kidney diseases. The present study was designed to investigate whether H2S has protective effects against diabetic nephropathy. The diabetic rat model was established by intraperitoneal injection of streptozotocin and then administrated with H2S donor sodium hydrosulfide for 12 weeks. Rat glomerular mesangial cells were pretreated with NaHS or mitogen-activated protein kinase (MAPK) inhibitors (U0126, SP600125 and SB203580) prior to high glucose exposure and the cellular proliferation was determined. Our findings suggested that H2S could improve renal function and attenuate glomerular basement membrane thickening, mesangial matrix deposition and renal interstitial fibrosis in diabetic rats. H2S was found to reduce high glucose-induced oxidative stress via activating Nrf2 antioxidant pathway and exert anti-inflammatory effects via inhibiting NF-κB signaling. In addition, H2S could reduce high glucose-induced mesangial cell proliferation via blockade of MAPK signaling pathways. Moreover, H2S was also found to inhibit the renin-angiotensin system (RAS) in diabetic kidney. In conclusion, our study
demonstrates that H2S alleviates the development of diabetic nephropathy by attenuating oxidative stress and inflammation, reducing mesangial cell proliferation and inhibiting the RAS activity.

Introduction
Diabetic nephropathy (DN), defined as a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli, is the leading cause of end-stage renal disease and a major contributor to morbidity and mortality of diabetic patients throughout the world. DN is morphologically characterized by excessive accumulation of extracellular matrix with thickening of glomerular and tubular basement membranes and increased amount of mesangial matrix, which ultimately progress to glomerulosclerosis and tubulo-interstitial fibrosis (1). Various hyperglycemia-induced metabolic and hemodynamic derangements, including increased generation of advanced glycation end products (AGEs), enhanced production of reactive oxygen species (ROS), elevated levels of inflammatory cytokines, and activation of the renin-angiotensin system (RAS), are considered to be involved in the development and progression of DN (2, 3).

Hydrogen sulfide (H2S), which is recognized as the third gasotransmitter identified after nitric oxide and carbon monoxide, is endogenously generated by cystathionine γ-lyase (CSE), cystathionine β-synthase and 3-mercaptopyruvate sulfurtransferase. In recent years, accumulating evidence has demonstrated that H2S plays critical roles in the pathophysiology of chronic kidney diseases (CKDs). It has been reported that CKD is associated with significant reduction in plasma H2S concentration and expression of H2S-producing enzymes. Given the potent anti-oxidative, anti-inflammatory and cytoprotective properties of H2S, its deficiency may contribute to progression of CKD and the associated complications (4). H2S has also been found to exhibit anti-fibrotic effects in obstructed nephropathy and inhibit the proliferation and differentiation of renal fibroblasts. The anti-fibrotic mechanisms of H2S may involve its anti-inflammation as well as its blockade of transforming growth factor-β1 (TGF-β1) and mitogen-activated protein kinase (MAPK) signaling (5). In the present study, we established a streptozocin-induced diabetic rat model to investigate whether H2S has protective effects against DN.

Experimental procedures
Animal model and grouping
All experiments and procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Soochow University. Male Sprague-Dawley rats weighing 200-250 g were obtained from the Experimental Animal Center of Soochow University and were housed in a room at 22 ± 2 °C and 50 ± 5 % relative humidity with an alternating 12 h light/dark cycle. The diabetic rat model was induced with a single intraperitoneal injection of streptozotocin (65 mg/kg dissolved in 0.1 M citrate buffer). Blood glucose levels were measured three and five days after streptozotocin injection using a hand-held glucometer (Accu-Chek; Roche, Basel, Switzerland) by tail vein puncture blood sampling. Only rats with blood glucose levels ≥ 16.7 mM on both days were defined as diabetic and used in this study. Diabetic rats were then randomly assigned into two groups. One group was intraperitoneally administered with H2S...
donor sodium hydrosulfide (NaHS) at a dose of 14 μmol/kg/day for 12 weeks (DM+NaHS group). The other group was intraperitoneally injected with an equivalent volume of physiological saline for 12 weeks (DM group). In addition, two groups of sex- and age-matched normal rats intraperitoneally injected with NaHS solution or physiological saline were referred to as the NaHS group and control group.

**Cell culture and treatment**

The rat glomerular mesangial cell line (HBZY-1) was purchased from China Center for Type Culture Collection (Wuhan, China) and cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. To investigate the anti-proliferative effect of H₂S, mesangial cells were administered with 100 μM NaHS for 30 min prior to exposure to 30 mM glucose for 48 h. To further determine whether H₂S inhibits high glucose-induced mesangial cell proliferation via blockade of MAPK signaling, cells were preconditioned with 10 μM U0126 (ERK inhibitor) or 10 μM SP600125 (JNK inhibitor) or 20 μM SB203580 (P38 inhibitor) for 60 min followed by exposure to high glucose. MAPK inhibitors were purchased from Cell Signaling Technology (Beverly, MA, USA).

**Measurement of H₂S content**

H₂S levels were determined by the methylene blue method as previously described (5). H₂S concentration in the plasma was expressed as micromole per liter, while H₂S content in the kidney was expressed in nanomole per milligram of protein.

**Biochemical analysis**

Rat serum samples were used for the measurement of fasting blood glucose (FBG), blood urea nitrogen (BUN) and creatinine (Cr) with an automatic biochemistry analyzer (Olympus AU5400; Olympus, Tokyo, Japan). The 24-hour urine samples were collected using metabolic cages and the supernatants were used for examination of 24-hour urinary protein (24-h UP).

**Histological analysis and immunohistochemical staining**

Rat kidney samples were fixed in 10% buffered formalin, embedded in paraffin and sliced into 5-μm-thick sections. The slides were then stained with hematoxylin-eosin (HE) and Masson's trichrome and observed under a light microscope. The expression of types I and III collagen in renal tissue was examined by immunohistochemistry. After quenching endogenous peroxidase, achieving antigen retrieval, and blocking non-specific binding sites, the renal tissue sections were incubated with rabbit anti-rat collagen I and III antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA) at 4°C overnight, and then subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Invitrogen, Carlsbad, CA, USA) at room temperature for 30 min. Finally, the sections were exposed to diaminobenzidine peroxidase substrate for 5 min and counterstained with Mayers hematoxylin.

**Transmission electron microscopy (TEM)**

Rat kidney tissue was cut into small pieces, and then fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in an ascending series of alcohols, and embedded in epoxy resin. Ultrathin sections were cut and stained with...
uranyl acetate and lead citrate. Samples were observed and photographed under a Philips CM120 electron microscope (FEI, Eindhoven, The Netherlands).

Measurement of oxidative stress
Oxidative stress was evaluated by detecting malondialdehyde (MDA) level, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities, and ROS generation in renal tissue according to the instructions of detection kits (Jiancheng Biotech, Nanjing, China).

Enzyme-linked immunosorbent assay (ELISA)
The levels of angiotensin II (Ang II) and inflammatory markers (TNF-α: tumor necrosis factor-α; MCP-1: monocyte chemotactic protein-1; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1) in the renal tissue were measured using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.

Electrophoretic mobility shift assay (EMSA)
Nuclear protein extracts were prepared from kidney tissue using NE-PER extraction kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of nuclear protein were incubated with biotin-labeled oligonucleotide probes containing the specific recognition sequence for NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3') for 30 min at room temperature. The reaction mixtures were separated on a non-denaturing PAGE gel and then transferred onto a nylon membrane. The transferred DNA was cross-linked to the membrane, incubated with horseradish peroxidase-conjugated streptavidin, and then visualized with enhanced chemiluminescence.

Cell proliferation assay
Mesangial cell proliferation was assessed by MTT assay. Briefly, cells were seeded in a 96-well plate (5 × 10^3 cells/well) and incubated with 20 μl of 5 mg/ml MTT solution for 4 h. Subsequently, the cells were lysed using dimethylsulfoxide (150 μl/well). When the formazan crystals were completely dissolved, the optical density was measured at 490 nm using a microplate reader.

Western blot analysis
The rats were sacrificed by carbon dioxide asphyxiation, and the kidney tissues were removed and homogenized in ice-cold lysis buffer. Protein concentrations were quantified with the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein (80 μg) were loaded on 10% SDS-PAGE gels, transferred onto nitrocellulose membranes and blocked with 5% nonfat milk. The membranes were incubated with primary antibodies at 4°C overnight. The following primary antibodies were used: anti-CSE, anti-MMP-2, anti-TIMP-1, anti-TGF-β1, anti-Nrf2, anti-HO-1, anti-NQO1, anti-NF-κB p65, anti-ACE, anti-AT1R (Santa Cruz Biotech, Santa Cruz, CA, USA) and anti-ERK1/2, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-JNK, anti-phospho-JNK (Thr183/Tyr185), anti-p38, anti-phospho-p38 (Thr180/Tyr182) (Cell Signaling, Beverly, MA, USA). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The immunocomplexes were visualized with an enhanced chemiluminescence detection kit.
Statistical analysis

All data in this study are expressed as mean ± SD and differences between groups were analyzed using ANOVA with SPSS statistical software version 15.0 (SPSS, Chicago, IL, USA). Post hoc analysis was used if the ANOVA was significant. A value of \( P < 0.05 \) was considered statistically significant.

Results

As shown in Figure 1, H\(_2\)S levels in plasma and kidney tissue were reduced in the diabetic rats and elevated after treatment with NaHS. The protein expression of CSE was downregulated in the DM group compared with that in the control group. Administration of NaHS was not associated with significant changes in the CSE expression.

The biochemical test results are presented in Table 1. Serum levels of FBG, BUN and Cr were elevated in the DM group compared to the control group, whereas serum BUN and Cr levels were reduced in the DM+NaHS group compared to the DM group. 24-h UP was found to be increased in the DM group and decreased in the DM+NaHS group.

The pathological changes of kidney tissue were observed by HE staining, Masson staining and TEM (Figure 2). The major pathological alterations in diabetic kidney included glomerular basement membrane thickening, mesangial matrix deposition and renal interstitial fibrosis. Administration of NaHS alleviated renal pathological changes in diabetic rats, which could consequently attenuate the development of glomerular sclerosis.

The expression of types I and III collagen in renal tissue was determined by immunohistochemistry (Figure 3). Types I and III collagen expression was upregulated in the DM group, whereas NaHS treatment was found to downregulate the collagen expression in the DM+NaHS group.

The expression of matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-1 (TIMP-1) and TGF-β1, which are involved in the regulation of collagen production and degradation, was determined by Western blotting (Figure 4). The protein expression of TIMP-1 and TGF-β1 was upregulated and MMP-2 expression was downregulated in the DM group, whereas NaHS administration could downregulate the expression of TIMP-1 and TGF-β1 and upregulate MMP-2 expression in the DM+NaHS group.

Oxidative stress was evaluated by detecting MDA level, SOD and GSH-Px activities, and ROS generation in the renal tissue (Figure 5). There were marked increase in ROS and MDA levels and decrease in SOD and GSH-Px activities in the DM group. Conversely, NaHS treatment was associated with decreased ROS and MDA levels and increased SOD and GSH-Px activities in the DM+NaHS group.

The activity of NF-E2-related factor 2 (Nrf2), a key regulator of the anti-oxidative stress response, was determined by Western blotting (Figure 6). The nuclear expression of Nrf2 was significantly increased in the diabetic kidney following administration of NaHS. Consequently, the protein levels of two downstream targets of Nrf2, heme oxygenase-1 (HO-1) and NAD(P)H: quinone oxidoreductase 1 (NQO1), were elevated in the DM+NaHS group.

Inflammatory markers in renal tissue were measured by ELISA and the results are shown in Figure 7. The levels of TNF-α,
ICAM-1, VCAM-1 and MCP-1 were significantly elevated in the DM group compared to the control group, while these cytokine levels were reduced in the DM+NaHS group compared to the DM group.

NF-κB activity in the renal tissue was determined by EMSA and Western blotting (Figure 8). The DNA-binding activity and nuclear expression of NF-κB were increased in the DM group, whereas NaHS treatment was found to inhibit NF-κB activity in the DM+NaHS group.

As shown in Figure 9, the protein levels of phospho-ERK1/2, phospho-JNK and phospho-p38 MAPK were significantly elevated in the mesangial cells exposed to high glucose. However, pretreatment of the cells with 100 μM NaHS for 30 min could inhibit MAPK phosphorylation and reduce high glucose-induced mesangial cell proliferation. Similarly, preconditioning of the cells by 10 μM U0126 or 10 μM SP600125 or 20 μM SB203580 for 60 min prior to high glucose exposure also inhibited the proliferation of mesangial cells.

To investigate the effect of H2S on RAS activity in diabetic kidney, Ang II level was measured by ELISA and angiotensin converting enzyme (ACE) and Ang II type 1 receptor (AT1R) expressions were determined by Western blotting (Figure 10). Ang II level was found to be increased in the DM group and decreased in the DM+NaHS group. The protein expressions of ACE and AT1R were significantly upregulated in the diabetic rats and downregulated after treatment with NaHS.

Discussion
In the present study, we established a streptozocin-induced diabetic rat model to investigate the protective effects of H2S against DN. Our findings indicated that endogenous H2S generation and CSE protein expression were decreased in the diabetic rats, while exogenous administration of NaHS increased H2S contents in both plasma and renal tissue. The biochemical and histopathologic studies were carried out to evaluate the beneficial effects of H2S in the prevention of DN and the results revealed that H2S could improve renal function and attenuate glomerular basement membrane thickening, mesangial matrix deposition and renal interstitial fibrosis in diabetic rats.

There is growing evidence that oxidative stress and inflammation are both involved in the development and progression of DN (6-9). A number of ROS-generating pathways such as glycolysis, specific defects in the polyl pathway, uncoupling of nitric oxide synthase, xanthine oxidase, NAD(P)H oxidase, and advanced glycation have been identified as potentially major contributors to the pathogenesis of DN. In addition, mitochondrial production of ROS in response to chronic hyperglycemia may be the key initiator for each of these pathogenic pathways (10). Several components of the diabetic milieu such as hyperglycaemia, AGEs, and immune complexes can activate kidney cells via induction of stress-activated protein kinase signaling, resulting in the release of chemokines and upregulation of cell adhesion molecules. These events facilitate the kidney infiltration of monocytes and lymphocytes, which become activated in the diabetic kidney and secrete proinflammatory cytokines. This leukocyte activity amplifies the inflammatory response and promotes cellular injury and the development of fibrosis (11). In this study, our findings suggested that H2S could attenuate oxidative stress and inflammation in the kidney of diabetic rats, which might be two important
protective mechanisms against DN.

Nrf2, an antioxidative defense system of the body, modulates the expression of antioxidant genes through interaction with antioxidant stress element (ARE) (12). Under normal physiological conditions, Nrf2 is confined to the cytoplasm associated with the suppressor protein Keap1, and is degraded by the ubiquitin proteasome pathway. Oxidative and electrophilic stress factors stimulate dissociation of the Nrf2-Keap1 complex, thereby promoting the release and translocation of Nrf2 into the nucleus to upregulate expression of Nrf2/ARE-linked antioxidant genes (13). In the present study, H2S was found to activate Nrf2 and upregulate the protein expression of HO-1 and NQO1, which consequently enhanced resistance to oxidative stress in the kidney of diabetic rats.

NF-κB is a family of pleiotropic transcription factors that integrate an intricate network of extracellular and intracellular signaling pathways. NF-κB activation has been documented to be associated with renal inflammation (14). In the classical pathway of NF-κB activation, phosphorylation of IκB by the activated IκB kinase complex leads to proteolysis of the NF-κB-bound IκB. Free NF-κB dimers then translocate to the nucleus, where they bind NF-κB DNA sites and activate gene transcription (15). In this study, the DNA binding activity of NF-κB was increased in the kidney of diabetic rats, which suggested that NF-κB activation might be involved in the pathogenesis of DN. Moreover, H2S was found to alleviate the inflammatory response in diabetic kidney via negative regulation of NF-κB signaling.

Mesangial cell proliferation and excessive deposition of extracellular matrix proteins are the major pathologic features of DN and contribute to the development of chronic renal failure (16). MAPK signaling pathways, which promote cell cycle progression by regulating the expression of cyclin-dependent kinases, are critically involved in the proliferation of mesangial cells (17). In the present study, our findings indicated that ERK1/2, JNK and P38 MAPK signaling were activated in the mesangial cells exposed to high glucose, which might be an important molecular mechanism responsible for mesangial cell proliferation. Furthermore, H2S was found to reduce high glucose-induced mesangial cell proliferation by inhibiting phosphorylation of MAPKs.

It has been well documented that the RAS plays a central role in the pathogenesis of DN (18, 19). Ang II is the main effector of the RAS and exerts a vasoconstrictive effect predominantly on the postglomerular arterioles, thereby increasing the glomerular hydraulic pressure and the ultrafiltration of plasma proteins, which may result in the onset and progression of chronic renal damage. Ang II may also directly contribute to accelerate renal damage by sustaining cell growth, inflammation, and fibrosis (20). In this study, RAS was found to be activated in the diabetic rats and inhibited after treatment with NaHS, which suggested that H2S could attenuate the development of DN through suppressing the activity of RAS.

In conclusion, our study demonstrates that H2S alleviates the development of DN by attenuating oxidative stress and inflammation, reducing mesangial cell proliferation and inhibiting the RAS activity. H2S may reduce high glucose-induced oxidative stress via activating Nrf2 pathway and exert anti-inflammatory effects via inhibiting NF-κB signaling. Moreover, H2S may reduce high glucose-induced mesangial cell proliferation via blockade of MAPK signaling pathways.
References
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**Table 1. Biochemical analysis**

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<tr>
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<th>Control</th>
<th>DM</th>
<th>DM+NaHS</th>
<th>NaHS</th>
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<tr>
<td>FBG (mmol/L)</td>
<td>6.24 ± 0.95</td>
<td>28.72 ± 4.36*</td>
<td>26.50 ± 3.42</td>
<td>6.47 ± 0.89</td>
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<tr>
<td>BUN (mmol/L)</td>
<td>4.58 ± 0.73</td>
<td>10.95 ± 1.84*</td>
<td>7.32 ± 1.27**</td>
<td>4.92 ± 0.80</td>
</tr>
<tr>
<td>Cr (μmol/L)</td>
<td>38.65 ± 4.18</td>
<td>80.23 ± 6.54*</td>
<td>55.17 ± 5.62**</td>
<td>35.49 ± 5.07</td>
</tr>
<tr>
<td>24-h UP (mg)</td>
<td>12.74 ± 1.83</td>
<td>78.62 ± 4.70*</td>
<td>43.96 ± 3.85**</td>
<td>14.08 ± 2.16</td>
</tr>
</tbody>
</table>

FBG: fasting blood glucose; BUN: blood urea nitrogen; Cr: creatinine; 24-h UP: 24-hour urinary protein. Data are expressed as mean ± SD (n = 10). * P < 0.05, vs. Control; ** P < 0.05, vs. DM.
Figure 1. H$_2$S contents in plasma (A) and kidney tissue (B) measured by the methylene blue method; representative immunoblots (C) and densitometric analysis (D) of cystathionine $\gamma$-lyase (CSE). *P < 0.05, vs. Control; **P < 0.05, vs. DM. (n = 5)
Figure 2. Representative hematoxylin-eosin staining of kidney samples (A, arrows show mesangial matrix deposition in diabetic kidney); representative Masson's trichrome staining of kidney sections (B, arrows indicate collagen fibers stained blue in diabetic kidney); representative transmission electron micrographs of renal tissue (C, arrows show glomerular basement membrane thickening in diabetic kidney). Administration of NaHS attenuated mesangial matrix deposition, renal interstitial fibrosis and glomerular basement membrane thickening in diabetic rats (A-C).
Figure 3. Representative immunohistochemical staining of collagen I (A) and collagen III (B) in the renal tissue (collagens are stained brown); quantitative analysis of the positive staining of collagen I (C) and collagen III (D). * P < 0.05, vs. Control; ** P < 0.05, vs. DM.
Figure 4. Representative immunoblots (A) and densitometric analysis (B) of matrix metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-1 (TIMP-1) and transforming growth factor-β1 (TGF-β1). *P < 0.05, vs. Control; **P < 0.05, vs. DM. (n = 5)
Figure 5. Oxidative stress was evaluated by detecting MDA level (A), SOD and GSH-Px activities (B, C), and ROS generation (D) in the renal tissue. MDA: malondialdehyde; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; ROS: reactive oxygen species. *P < 0.05, vs. Control; **P < 0.05, vs. DM. (n = 10)
**Figure 6.** Representative immunoblots and densitometric analysis of NF-E2-related factor 2 (Nrf2) in nucleus and cytosol (A) and its downstream targets heme oxygenase-1 (HO-1) and NAD(P)H: quinone oxidoreductase 1 (NQO1) (B). *P* < 0.05, vs. Control; **P** < 0.05, vs. DM. (n = 5)
Figure 7. The concentrations of TNF-α (A), MCP-1 (B), ICAM-1 (C) and VCAM-1 (D) in the renal tissue measured by ELISA. TNF-α: tumor necrosis factor-α; MCP-1: monocyte chemotactic protein-1; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1. * P < 0.05, vs. Control; ** P < 0.05, vs. DM. (n = 10)
Figure 8. Detection of NF-κB-DNA binding activity by electrophoretic mobility shift assay (A); representative immunoblots and densitometric analysis of NF-κB in nucleus and cytosol (B). * P < 0.05, vs. Control; ** P < 0.05, vs. DM. (n = 5)
Figure 9. Western blot analysis of ERK1/2, JNK and p38 MAPK phosphorylation in mesangial cells (A-C); pretreatment of the mesangial cells with NaHS or MAPK inhibitors reduced high glucose-induced cellular proliferation (D). * P < 0.05, vs. cells exposed to normal glucose; ** P < 0.05, vs. cells exposed to high glucose. (n = 5)
Figure 10. The levels of angiotensin II (Ang II) in renal tissue measured by ELISA (A); representative immunoblots and densitometric analysis of angiotensin converting enzyme (ACE) and Ang II type 1 receptor (AT1R) (B). * P < 0.05, vs. Control; ** P < 0.05, vs. DM. (n = 5)
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