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Diabetic Nephropathy: Novel Molecular Mechanisms and Therapeutic Avenues

Lead Guest Editor: Sebastian Oltean
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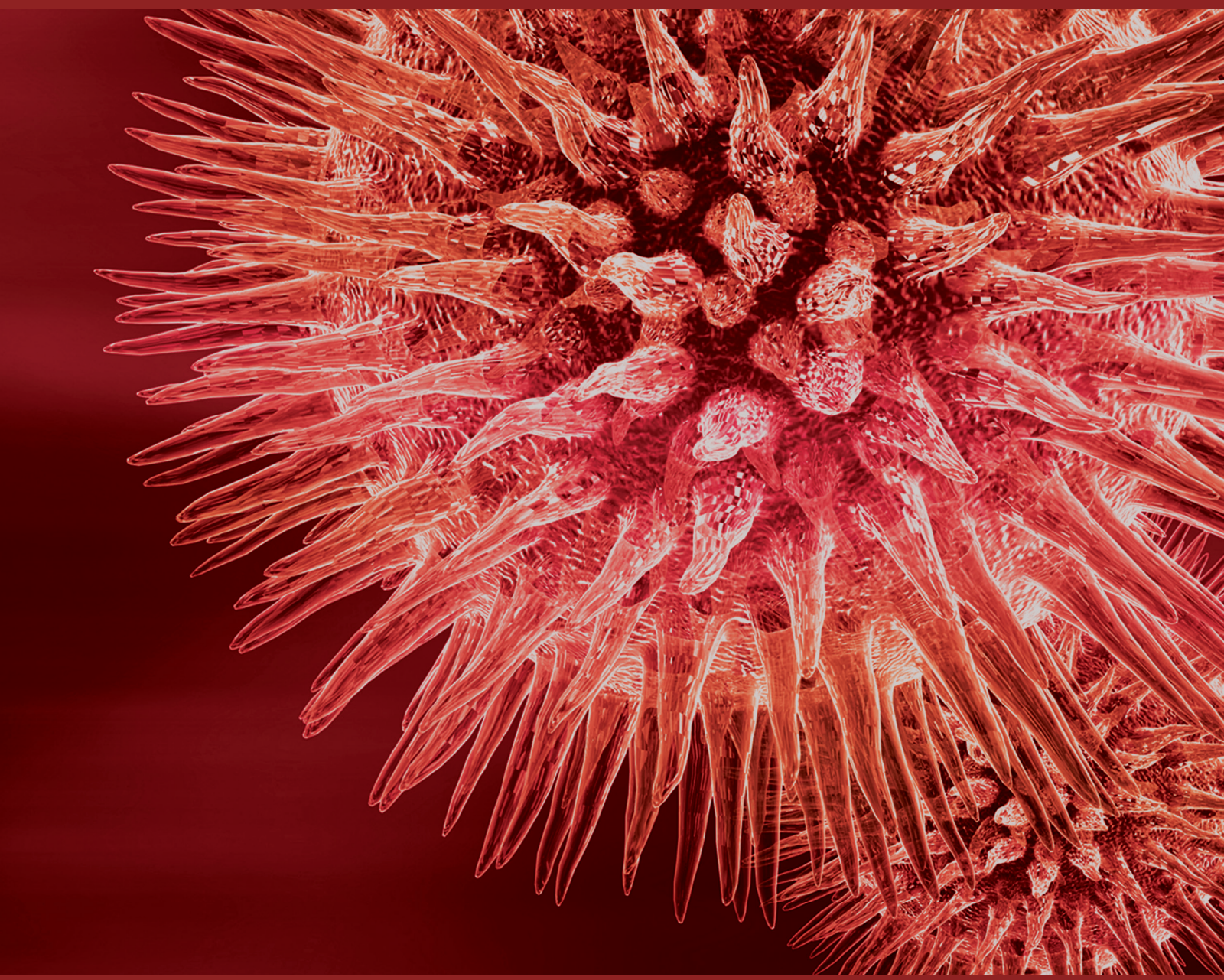

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Editorial

Diabetic Nephropathy: Novel Molecular Mechanisms and Therapeutic Avenues

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Chronic kidney disease (CKD) is a major health problem worldwide. In Europe it is estimated that ~16% of the general population has CKD stages 1–4 and in US the prevalence is similar at ~13%. CKD is associated with increased risk of cardiovascular disease and mortality. It is therefore important to further our understanding regarding the molecular determinants of CKD and find new ways to manipulate them for therapeutic benefit. A large proportion of CKD is due to diabetic nephropathy (DN), one of the microvascular complications of diabetes. In the Western world, DN is the leading cause of end-stage renal disease and kidney replacement therapy (almost half of these patients), an immense burden on healthcare costs. It is estimated that 40–45% of patients with type I diabetes and 30% of patients with type II diabetes have nephropathy.

Multiple molecular mechanisms of DN progression have been described in recent years; however, mechanism-derived treatments for DN are still lacking. This is partly due to poor understanding of the detailed molecular mechanisms underlying diabetic complications. The standard of care is to control glycaemia but most of the time this does not stop the occurrence of the nephropathy.

There is therefore an increasing need to better understand molecular mechanisms of progression in DN and to develop novel treatments that target specifically these mechanisms and are able to slow progression of the underlying CKD in DN.

This special issue is trying to address these goals by publishing both articles that describe novel molecular pathways implicated in DN and novel treatment ideas.

We do hope that the papers here collected may offer the readers an improved knowledge on innovative and original advances in DN pathogenesis and management.

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Research Article

CYLD Deubiquitinase Negatively Regulates High Glucose-Induced NF-κB Inflammatory Signaling in Mesangial Cells

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Nuclear factor-kappa B (NF-κB) is the key part of multiple signal transduction of inflammation in the pathogenesis of diabetic nephropathy (DN). The ubiquitin-proteasome system is extensively involved in the regulation of the NF-κB pathway. Cylindromatosis (CYLD) has deubiquitinase activity and acts as a negative regulator of the NF-κB signaling pathway. However, the association between CYLD and NF-κB inflammatory signaling in DN is unclear. In the present study, mouse glomerular mesangial cells (GMCs) and rat GMCs were stimulated by elevated concentrations of glucose (10, 20, and 30 mmol/L high glucose) or mannitol as the osmotic pressure control. CYLD was overexpressed or suppressed by transfection with a CYLD expressing vector or CYLD-specific siRNA, respectively. Our data showed that high glucose significantly inhibited the protein and mRNA expression of CYLD in a dose- and time-dependent manner (both $p < 0.05$). siRNA-mediated knockdown CYLD facilitated the high glucose-induced activation of NF-κB signaling and triggered the release of MCP-1, IL-6, and IL-8 (all $p < 0.05$). However, these high glucose-mediated effects were blunted by overexpression of CYLD ($p < 0.05$). The present results support the involvement of CYLD in the regulation of NF-κB inflammatory signaling induced by elevated glucose, implicating CYLD as a potential therapeutic target of DN.

1. Introduction

Diabetic nephropathy (DN) is a common and serious diabetic microvascular complication. Nuclear factor κB (NF-κB) plays a central regulatory role in the expression of various inflammatory cytokines and adhesion molecules involved in the occurrence of DN [1]. In unstimulated cells, NF-κB is conjugated to IκBα and kept in an inactive state in the cytosol [2]. Different physiological or pathological stimuli can activate and promote the phosphorylation of IκBα and its subsequent degradation by the ubiquitin-proteasome pathway (UPP), thereby exposing the nuclear-localization sequence (NLS) of NF-κB and leading to its translocation from the cytoplasm to the nucleus, where it activates the transcription of genes for the immune and inflammatory response [3].

Ubiquitin targets proteins for degradation by the 26S proteasome. Our previous research proved that ubiquitylation plays an important role in the activation of the NF-κB signaling pathway in the pathogenesis of DN [4]. Like phosphorylation, ubiquitination is a reversible reaction mediated by deubiquitinases (DUBs), which hydrolyze ubiquitin chains

and which are considered to oppose the functions of their counteractive ubiquitinases [5]. More than 90 DUBs exist in the human genome, and some DUBs are specific for distinct ubiquitin linkages, suggesting that DUBs participate in specific biological functions.

As one of the DUBs, cylindromatosis (CYLD) is the main negative regulatory factor and inflammation inhibiting factor in the NF-κB signaling pathway. CYLD is activated by different inducers, including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), cluster of differentiation 40 (CD40), and phorbol 12-myristate 13-acetate (PMA) [6]. However, subsequent studies have indicated that although CYLD targets NF-κB signaling factors, its function may depend on the cell type and stimulating receptor [7]. Furthermore, there is no evidence to support the association between CYLD and NF-κB signaling in the pathogenesis of DN.

In the present study, we observed the expressions of CYLD, IκBα, phosphorylated (p)-IκBα, NF-κBp65, and p-NF-κBp65 and the release of MCP-1, IL-6, and IL-8 in cultured glomerular mesangial cells (GMCs) stimulated by elevated concentrations of glucose, siRNA, or lentivirus vector constructed to realize silencing or overexpression of CYLD gene. These experiments were done to explore the role of CYLD in the regulation of NF-κB inflammatory signaling in the pathogenesis of DN.

2. Materials and Methods

2.1. Cell Culture and Treatment. Mouse GMCs (SV40 MES 13) and rat GMCs (HBZY-1) were purchased from the China Center for Type Culture Collection (CCTCC) and maintained in low glucose (5.6 mmol/L) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) at 37°C and 5% CO_2. The experimental groups included normal control group (NC; medium with 5.6 mmol/L glucose), high glucose treatment group (HG; culture medium with 10 mmol/L (HG1), 20 mmol/L (HG2), or 30 mmol/L (HG3) glucose), osmotic pressure control group (OP; medium with 5.6 mmol/L glucose + 24.4 mmol/L mannitol), and MG132 intervention group (with medium that contained 30 mmol/L glucose + 1 μmol/LMG132, in which MG132 was added to the culture medium to block protein ubiquitination). Cells were exposed to these treatments for 6, 12, 24, 48, and 72 h; protein and mRNA were extracted and culture supernatants were collected for further study.

2.2. CYLD Overexpression Vector Construction and Transfection. Mouse CYLD gene primers were designed and the *cyld* gene was obtained by polymerase chain reaction (PCR) amplification. A recombinant lentivirus vector harboring *cyld* was generated by restriction enzyme (Jikai, Shanghai, China) action on the specific domain and transformation between *cyld* and vector. Lentivirus was purified and packaged using a packaging mixture (Jikai). The HEK293 human embryonic kidney T cells were transferred with the packaged lentivirus and the multiplicity of infection (MOI) was detected by fluorescence microscopy to optimize the infection conditions. Stable overexpression of CYLD (LV-CYLD group) was achieved using medium containing 1×10^8 transducing units (TU)/mL of CYLD lentivirus (GMC MOI = 50). The blank transfection group (LV-CON235 group) used medium with 1×10^8 TU/ml CON235 virus (GMC MOI = 50). Both were stimulated using 30 mmol/L glucose for 24 h at 37°C. Cells and culture supernatants were collected for Western blotting and ELISA analyses.

2.3. siRNA Transfection. siRNA targeting CYLD (sense: 5′-GAG GAT CCC CGG GTA CCG GTC GCC ACC ATG AGT TCA GGC CTG TGG AGC CAA G-3′; anti-sense: 5′-TCC TTG TAG TCC ATA CCT TTG TAC AGG CTC ATG GTT GGA CTC-3′) were synthesized by RiboBio Biotechnology (Guangzhou, China). Transfection was done using Lipofectamine® 2000 (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. Experiments were performed at 48 h after transfection. siRNA-mediated knockdown of CYLD was achieved by growth of cells in medium containing 100 nmol/L CYLD siRNA. These cells were then stimulated by 30 mmol/L glucose. Cells and culture supernatants were collected for Western blotting and ELISA analyses.

2.4. Protein Extraction and Western Blot. Total proteins were isolated from GMCs using a total protein extraction kit (Beyotime, Beijing, China). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). Immunoblotting was performed using anti-CYLD goat polyclonal antibody (1 : 1,000; Abcam, Cambridge, MA), anti-IκBα mouse monoclonal antibody (1 : 1,000; CST, Boston, USA), anti-p-IκBα ser32/36 mouse monoclonal antibody (1 : 1,000; CST, Boston, USA), anti-NF-κBp65 mouse monoclonal antibody (1 : 2,000; CST, Boston, USA), anti-p-NF-κBp65 ser536 mouse monoclonal antibody (1 : 2,000; CST, Boston, USA), and anti-β-actin rabbit monoclonal antibody (1 : 3000; Beyotime, Beijing, China). Images were taken with a molecular imaging system (FUJI, Tokyo, Japan).

2.5. RNA Extraction and Reverse-Transcription- (RT-) PCR. Total RNA was extracted from GMCs using an RNA extraction kit (Tiangen Biotech, Beijing, China). Total RNA was reverse-transcribed using an RNA PCR kit (Baoshengwu, Dalian, China). cDNA was amplified in a gradient thermal cycler (Eppendorf, Hamburg, Germany) using PCR Master Mix (Baoshengwu). The results were determined using an ultraviolet transilluminator and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression. The primer sequences were CYLD (forward, 5′-CTT GCC TGA CTG GGA CT-3′; reverse, 5′-TTC TGA CCA CCA TCT CG-3′) and GAPDH (forward, 5′-TGG CCT TCC GTG TTC CTA C-3′; reverse, 5′-GAG TTG CTG TTG AAG TCG CA-3′).

2.6. Enzyme-Linked Immunosorbent Assay (ELISA). GMCs (1×10^4 per well) were seeded in 24-well plates and induced by high glucose as described above. MCP-1, IL-6, and IL-8 protein level in the culture supernatants were determined

using commercially available ELISA kits (Jikai, Shanghai, China) according to the manufacturer's protocols. MCP-1, IL-6, and IL-8 protein levels were determined by comparing the samples to the standard curve generated by the kit.

2.7. Statistical Analysis. All data are obtained from at least three independent experiments and are expressed as mean ± standard deviation (SD). Differences were statistically analyzed using one-way analysis of variance (ANOVA), followed by the Least Significant Difference post hoc test for multiple comparisons. A probability value of $p < 0.05$ was considered significant.

3. Results

3.1. High Glucose Inhibits the Expression of CYLD in GMCs. To determine whether CYLD is regulated by glucose in GMCs, we first detected CYLD proteins and mRNA by Western blot and RT-PCR. As shown in Figure 1(a), compared with the NC group, the relative CYLD mRNA and protein expressions in GMCs (SV40 MES 13 and HBZY-1) gradually decreased after treatment with 30 mmol/L glucose for 6 h to 72 h and were lowest at 72 h ($p < 0.05$). Compared with the NC group, the relative CYLD mRNA and protein expressions were inhibited by different concentrations of high glucose at 24 h, particularly in the 30 mmol/L glucose group ($p < 0.05$; Figure 1(b)). However, there was no significant change between the NC and OP groups, suggesting that osmotic pressure had little effect on the inhibited expression of CYLD in the high glucose medium. These data suggest that high glucose inhibited CYLD expression in GMCs (SV40 MES 13 and HBZY-1) in a time- and dose-dependent manner.

3.2. High Glucose Induced the Activation of NF-κB Inflammatory Signaling by Phosphorylation and Ubiquitination Degradation of IκBα. Compared with the NC group, the protein expressions of p-IκBα, NF-κBp65, and p-NF-κBp65 were significantly induced following 6, 12, 24, 48, and 72 h of exposure to 30 mmol/L glucose in a time-dependent manner in mouse GMCs (SV40 MES 13) ($p < 0.05$; Figure 2(a)). The protein levels of these signaling molecules were also significantly induced by the different concentrations of high glucose in a dose-dependent manner ($p < 0.05$; Figure 2(b)). However, as an important inhibitory protein of NF-κB pathway, the levels of IκBα protein were significantly decreased by high glucose in time- and dose-dependent manners ($p < 0.05$; Figures 2(a) and 2(b)). After the proteasome inhibitor MG132 treatment, IκBα, p-NF-κBp65 and NF-κBp65 protein levels were partially reversed compared with the HG3 group ($p < 0.05$), but p-IκBα protein levels were not changed significantly ($p > 0.05$). Moreover, no apparent differences were found between the NC group and NC + MG132 group, suggesting that MG132 partially reversed high glucose-induced IκBα ubiquitination degradation and NF-κB activation (Figure 2(c)). Next, we used ELISA to determine whether high glucose could stimulate the release of MCP-1, IL-6, and IL-8, which are downstream inflammatory cytokines of NF-κB signaling pathway. As shown in Figure 2(d), compared with the NC group, levels of MCP-1, IL-6, and IL-8 protein in culture supernatants were significantly increased by high glucose in time- and dose-dependent manners ($p < 0.05$). The collective data indicate that high glucose induced the activation of NF-κB inflammatory signaling by phosphorylation and ubiquitination degradation of IκBα.

3.3. siRNA-Mediated Knockdown CYLD Facilitates the Activation of NF-κB Induced by High Glucose. We then investigated the underlying mechanism by which CYLD deubiquitinase was involved in the regulation of NF-κB inflammatory signaling in mouse GMCs. siRNA were constructed to realize silencing CYLD gene; the results showed that compared with NC or the 30 mmol/L glucose group, the protein and mRNA expressions of CYLD were significantly decreased by siRNA-CYLD ($p < 0.05$), suggesting that high-induced downregulation of CYLD was significantly facilitated by transfecting siRNA-CYLD (Figure 3(a)). Meanwhile, Western blot revealed that, compared with the 30 mmol/L glucose group, the level of IκBα protein was decreased by siRNA-CYLD ($p < 0.05$), but the protein expressions of p-IκBα, p-NF-κBp65, and NF-κBp65 were increased ($p < 0.05$) (Figure 3(b)), suggesting that the high glucose-induced the activation of NF-κB was significantly facilitated by synergistically treating siRNA-CYLD.

3.4. Overexpression of CYLD Reverses High Glucose-Induced Activation of NF-κB Signaling Pathway. To assess the involvement of CYLD deubiquitinase in the regulation of NF-κB signaling, we established the lentivirus vector conferring CYLD overexpression upon transfection of mouse GMCs. Some mouse GMCs were also stimulated by 30 mmol/L glucose for 24 h. As shown in Figure 4(a), the mRNA and protein expression levels of CYLD were significantly increased by CYLD overexpression compared with blank load transfection group ($p < 0.05$). The high glucose-induced decrease of CYLD expression was reversed by overexpression of CYLD ($p < 0.05$), as was high glucose-inhibited IκBα protein expression ($p < 0.05$; Figure 4(b)). Conversely, high glucose-induced p-IκBα, NF-κBp65, and p-NF-κBp65 protein levels were significantly blunted by overexpression of CYLD (Figure 4(b)), and similar results for the downstream inflammatory cytokines MCP-1, IL-6, and IL-8 were observed by ELISA (Figure 4(c)). These results revealed that high glucose-mediated activation of NF-κB inflammatory signaling was blunted by overexpression of CLYD, suggesting that CYLD deubiquitinase negatively regulated NF-κB inflammatory signaling in GMCs.

4. Discussion

NF-κB inflammatory signaling has an important role in the occurrence and development of DN [8]. The activation of NF-κB is mediated by the polyubiquitylation of phosphorylated IκBα, followed by their proteasomal degradation [4, 9, 10]. We have shown that high glucose can activate NF-κB signaling through the phosphorylation and then ubiquitination of IκBα. Moreover, high glucose may be involved in the pathogenesis of DN by specifically impacting IκBα sumoylation [11]. The proteasome inhibitor, MG132, ameliorates kidney

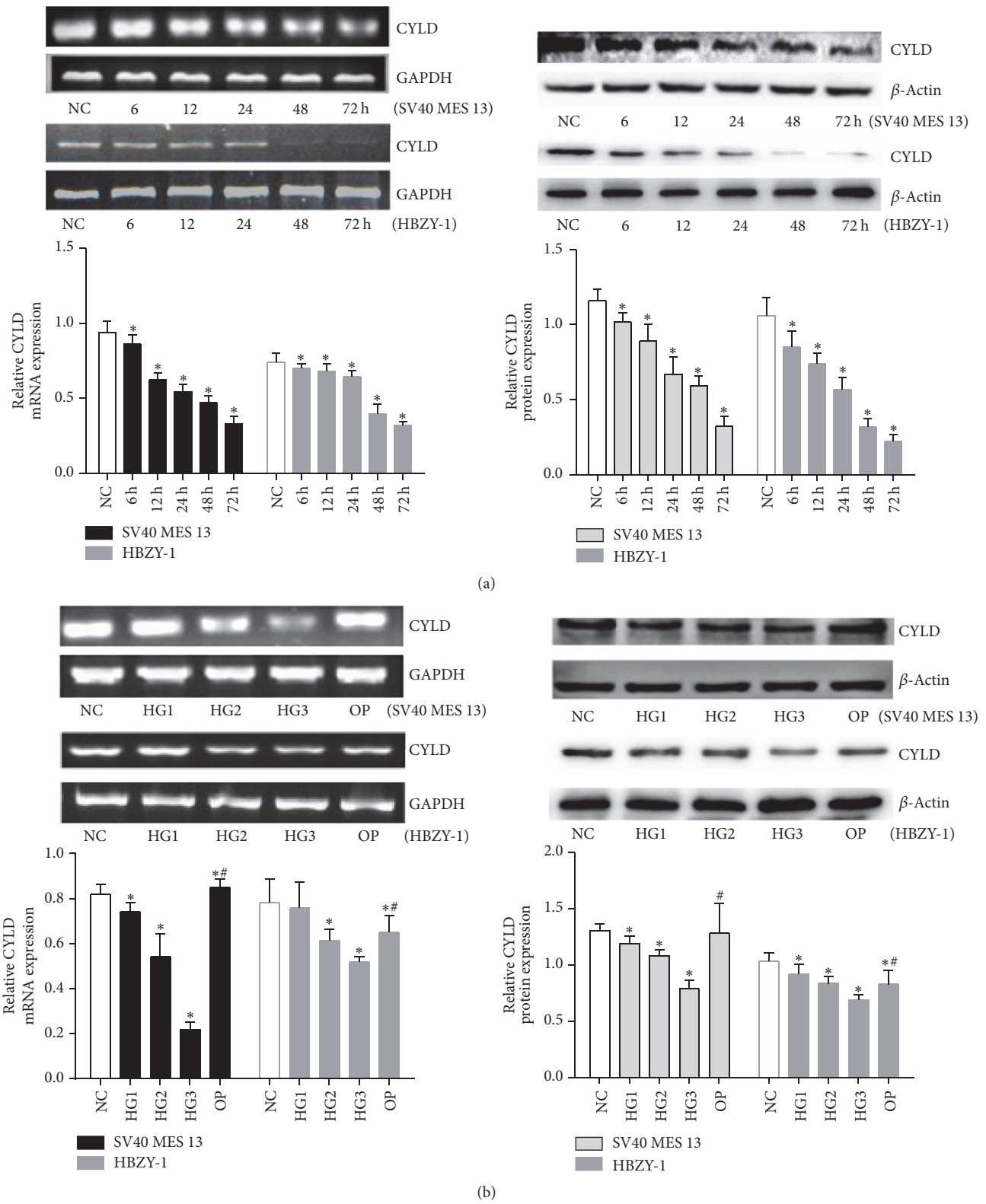

Figure 1: *CYLD protein and mRNA expression after high glucose challenge for various times and various glucose concentrations determined by Western blot and RT-PCR*. (a) GMCs (SV40 MES 13 and HBZY-1) were treated with 30 mmol/L glucose for 6, 12, 24, 48, and 24 h, and Western blot and RT-PCR were performed to detect the expression of CYLD. (b) GMCs (SV40 MES 13 and HBZY-1) were treated with the indicated concentrations of glucose or mannitol for 24 h. The gray graph shows the relative statistical values for CYLD protein and mRNA expression in each group. The data were normalized to GAPDH/β-actin and are expressed as mean ± SD; $^{*}p < 0.05$ compared with NC group; $^{\#}p < 0.05$ compared with 30 mmol/L glucose (HG3) group.

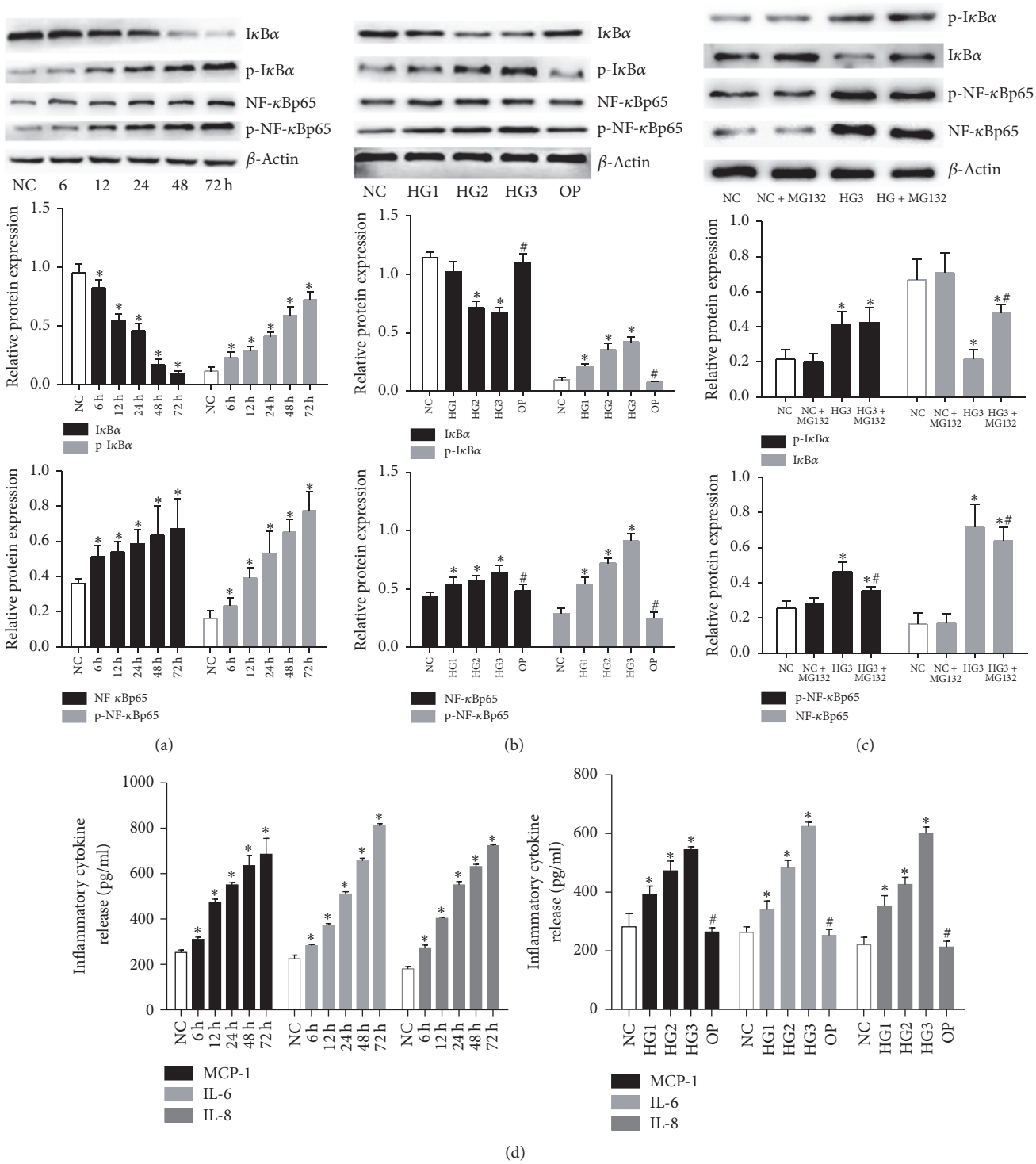

Figure 2: *High glucose induces the activation of NF-κB signaling and release of MCP-1, IL-6, and IL-8.* (a) Protein expressions of IκBα, p-IκBα, NF-κBp65, and p-NF-κBp65 in lysates of mouse GMCs treated with 30 mmol/L glucose for 6, 12, 24, 48, and 72 h were detected by Western blot. (b) Protein expressions of IκBα, p-IκBα, NF-κBp65, and p-NF-κBp65 in lysates of mouse GMCs treated with an indicated concentration of high glucose for 24 h as detected by Western blot. (c) MG132 partially reversed high glucose-induced IκBα ubiquitination degradation and NF-κB activation. (d) Release of the inflammatory cytokines MCP-1, IL-6, and IL-8 in the cell culture supernatant was quantified by ELISA. Data were normalized with respect to β-actin and are expressed as mean ± SD. The gray graphs show the relative statistical values in each group and confirmed these trends. $^{*}P < 0.05$ compared with NC group; $^{\#}P < 0.05$ compared with 30 mmol/L glucose (HG3) group.

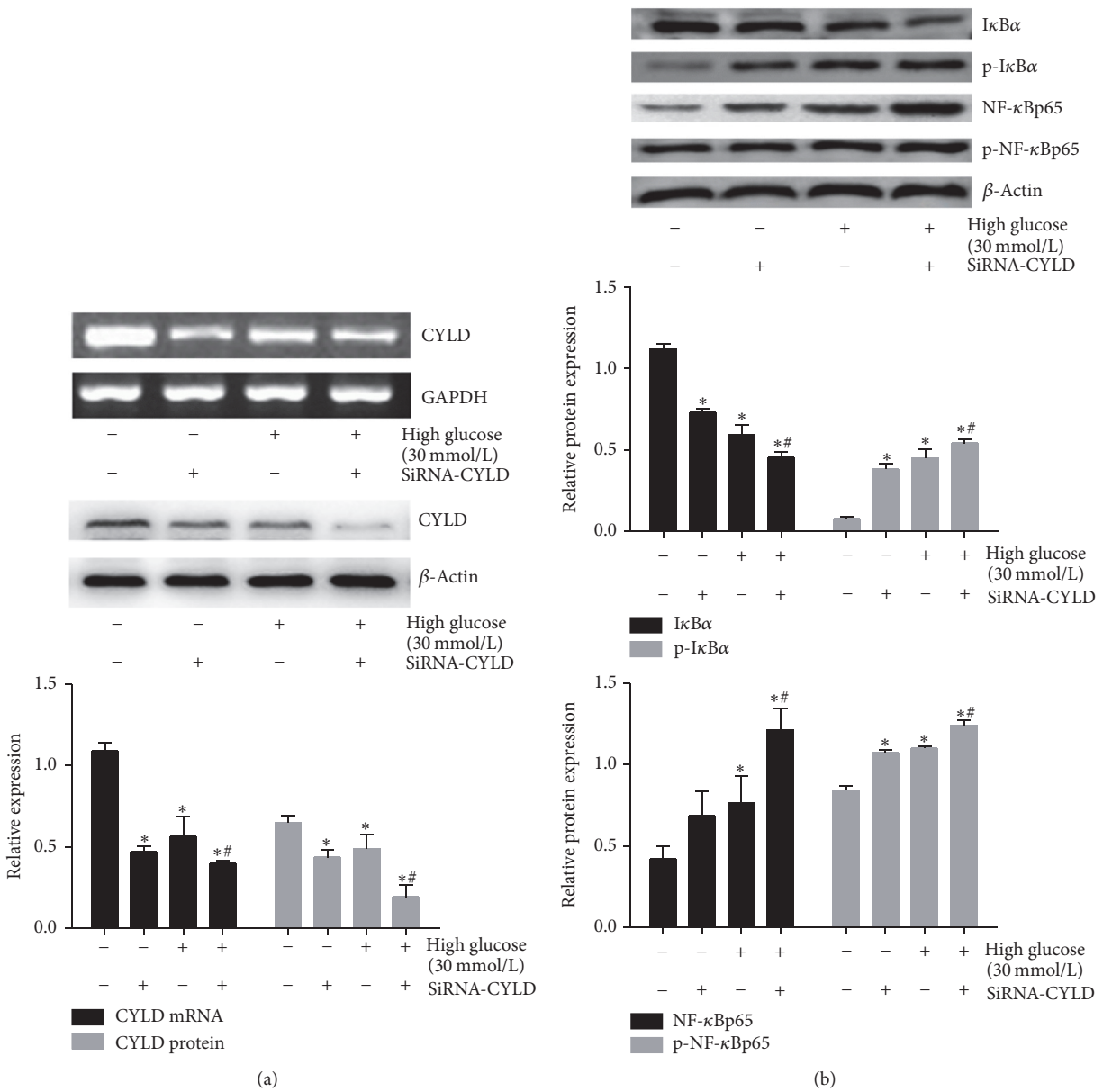

Figure 3: *siRNA-mediated knockdown CYLD facilitated the activation of NF-κB induced by high glucose.* (a) RT-PCR and Western blot detected the mRNA and protein expression of CYLD in mouse GMCs treated with 30 mmol/L glucose and (or) CYLD siRNA for 24 h. (b) Western blot detection of the expression of IκBα, p-IκBα, NF-κB, and p-NF-κB in mouse GMCs treated with 30 mmol/L glucose and (or) CYLD siRNA for 24 h. Data were normalized with respect to β-actin and are expressed as mean ± SD. The gray graphs show the relative statistical values in each group and confirmed these trends. $^{*}p < 0.05$ compared with the NC group; $^{\#}p < 0.05$ compared with the 30 mmol/L glucose stimulation group.

lesions and attenuates DN by inhibiting IκBα, SnoN, and Smad7 protein ubiquitination degradation [12, 13]. These studies suggest that targeting the UPP may be a potential target for the treatment of DN.

Ubiquitination is a dynamic process that can be counterbalanced by deubiquitinating enzymes including the tumor suppressor CYLD [6]. The human CYLD gene is located on chromosome 16q12.1 and encodes a protein of 956 amino acids; the C-terminal region of CYLD contains a catalytic domain with sequence homology to ubiquitin-specific proteases (USP) family members [14]. The initial clue to the signaling function of CYLD came from an RNA interference-based functional screening study, which identified CYLD as a DUB that negatively regulates NF-κB activation. Overexpression of CYLD leads to a decrease in NF-κB activity induced by several receptors including TNFR1, CD40, TLR4, EDAR, and LMP1 [15, 16]. The critical role of CYLD in NF-κB regulation suggests the involvement of this DUB in important biological

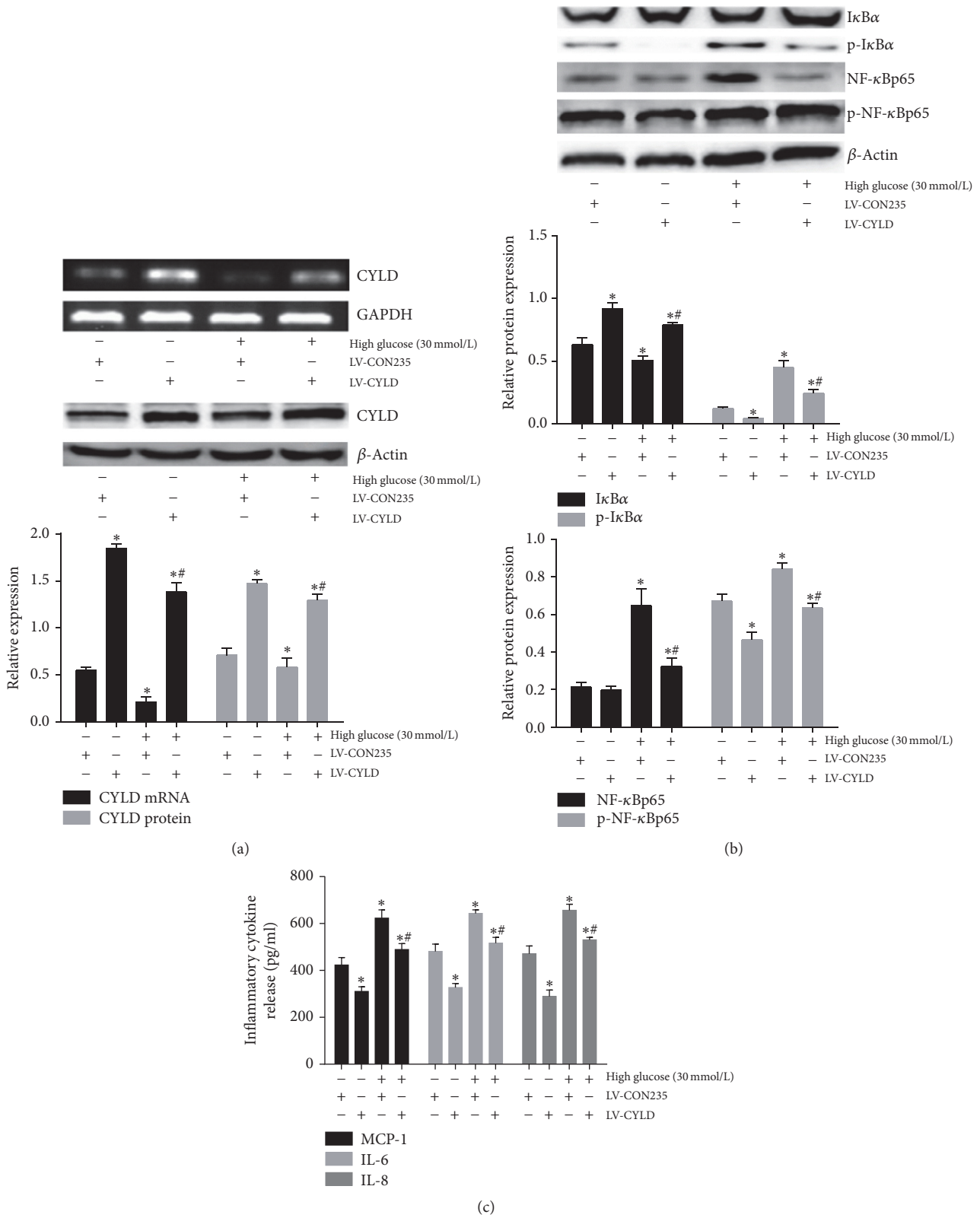

Figure 4: *Overexpression of CYLD reverses high glucose-induced activation of the NF-κB signaling pathway*. (a) RT-PCR and Western blot detection of the mRNA and protein expression of CYLD in mouse GMCs treated with 30 mmol/L glucose and (or) overexpression of CYLD for 24 h. (b) Western blot detection of the expression of IκBα, p-IκBα, NF-κBp65, and p-NF-κBp65 in mouse GMCs treated with 30 mmol/L glucose and (or) overexpression CYLD for 24 h. (c) ELISA detection of the release of the inflammatory cytokines MCP-1, IL-6, and IL-8 from mouse GMCs treated with 30 mmol/L glucose and (or) overexpression of CYLD for 24 h. The gray graphs show the relative statistical values in each group and confirmed these trends. $^{*}p < 0.05$ compared with the NC + blank load transfection (NC + LV-CON235) group; $^{\#}p < 0.05$ compared with 30 mmol/L glucose + LV-CON235 group.

processes. One prominent function of CYLD is the regulation of immune response and inflammation [17]. CYLD negatively regulates the induction of proinflammatory mediators by *Streptococcus pneumoniae* and *Escherichia coli* [18]. CYLD$^{-/-}$ mice do not develop spontaneous tumors; however, they are highly susceptible to dextran sulfate sodium-induced colitis and azoxymethane-induced tumor development [19, 20]. Thus, CYLD plays a critical role in the suppression of tumor proliferation [21]. In addition, CYLD is suggested to be involved in the induction of cell death [22]. Reduced CYLD expression is also shown to increase the survival of several cell types [23, 24]. However, the association between deubiquitinase CYLD and NF-κB signaling in DN remains unclear. In the present study, we found that high glucose dose- and time-dependently downregulated the protein and mRNA expressions of CYLD in GMCs (SV40 MES 13 and HBZY-1) and increased the expression levels of p-IκBα, NF-κBp65, and p-NF-κBp65, and furthermore induced the release of MCP-1, IL-6, and IL-8. These results showed that both CYLD and NF-κB inflammatory signaling pathway could be regulated by high glucose, suggesting that CYLD could be involved in the regulation of NF-κB inflammatory signaling under high glucose stress.

Since CYLD can cleave K63 in addition to linear linkages, its functions are not defined by the cleavage of linear chains alone. Previous studies reported that CYLD binds to IKKγ and its upstream signaling components including TAK1, TRAF2, TRAF6, and RIP1 [14–16]. In particular, CYLD activity interferes with the NF-κB signaling by catalyzing the specific K63-polyUb chains from IKKγ, TRAF2, and TRAF6, without affecting the K48-polyUb chains of IκBα [25]. However, recent research revealed that CYLD inhibited NF-κB signaling by deconjugating the polyubiquitylation of phosphorylated IκBα proteins and rendered resistance to murine hepatocyte death [26]. Whether CYLD inhibits the activation of NF-κB signaling through the deubiquitination of IκBα in high glucose conditions is unclear. In this study, high glucose-inhibited expression of IκBα was significantly reversed by the overexpression of CYLD, and p-IκBα, NF-κBp65, and p-NF-κBp65 proteins levels were significantly decreased. In accordance with NF-κBp65, similar profiles of proinflammatory cytokines MCP-1, IL-6, and IL-8 released from GMCs were reversed by overexpression of CYLD, suggesting that high glucose-induced activation of NF-κB inflammatory signaling was blunted by overexpression of CYLD. On the contrary, compared with high glucose group, the level of IκBα proteins was more obviously decreased by synergistically treating siRNA-CYLD, and p-IκBα, NF-κBp65, and p-NF-κBp65 were evidently increased, suggesting that high glucose-induced activation of NF-κB was facilitated by siRNA-mediated knockdown of CYLD. We speculate that CYLD deubiquitinase negatively regulates NF-κB inflammatory signaling by deconjugating the polyubiquitylation of phosphorylated IκBα, followed by inhibiting activation of NF-κB signaling under high glucose environments.

However, it must be pointed out that overexpression of CYLD did not totally reverse high glucose-induced activation of NF-κB signaling, suggesting that there must be other mechanisms involved in the cross-talk of inflammation induced by high glucose. Even so, our study suggest a potential therapeutic target for the inhibition of the NF-κB inflammatory signaling and treatment of DN. An increasing number of studies have reported that the target drugs of CYLD play an important role in the treatment of various disease. A study reported that, as novel MALT1 inhibitors, β-lapachone analogs exhibited potent antiproliferative activity and inhibited the cleavage of CYLD mediated MALT1; this may be a promising therapeutic target for the treatment of aggressive subtype of diffuse large B-cell lymphoma [27]. In another study, interference with CYLD completely restored glucocorticoid resistance in children with acute lymphoblastic leukemia (ALL), suggesting that targeting CYLD may be a pharmacological approach to treatments for patients with refractory ALL [28]. In view of the fact that the regulatory mechanisms of NF-κB signaling are extremely complex, the establishment of an animal model for DN and targeting renal CYLD intervention are necessary further studies, which will focus on the interaction among CYLD and inflammatory signaling to clarify the molecular mechanisms in the pathogenesis of DN and other diabetic complications.

In conclusion, the present study found that high glucose significantly inhibited the expression of CYLD and activated NF-κB inflammatory signaling in a dose- and time-dependent manner. These high glucose-induced effects were facilitated by siRNA-CYLD but blunted by overexpression of CYLD. The present results support the involvement of CYLD deubiquitinase in the regulation of NF-κB inflammatory signaling induced by high glucose, implicating CYLD as a potential therapeutic target of DN.

Disclosure

Wei Huang is the co-first author. Yang Long is the cocorrespondence author.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

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Research Article

Protective Effects of Pyridoxamine Supplementation in the Early Stages of Diet-Induced Kidney Dysfunction

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Pyridoxamine, a structural analog of vitamin B6 that exerts antiglycative effects, has been proposed as supplementary approach in patients with initial diabetic nephropathy. However, the molecular mechanism(s) underlying its protective role has been so far slightly examined. C57Bl/6J mice were fed with a standard diet (SD) or a diet enriched in fat and fructose (HD) for 12 weeks. After 3 weeks, two subgroups of SD and HD mice started pyridoxamine supplementation (150 mg/kg/day) in the drinking water. HD fed mice showed increased body weight and impaired glucose tolerance, whereas pyridoxamine administration significantly improved insulin sensitivity, but not body weight, and reduced diet-induced increase in serum creatinine and urine albumin. Kidney morphology of HD fed mice showed strong vacuolar degeneration and loss of tubule brush border, associated with a drastic increase in both advanced glycation end products (AGEs) and AGEs receptor (RAGE). These effects were significantly counteracted by pyridoxamine, with consequent reduction of the diet-induced overactivation of NF-kB and Rho/ROCK pathways. Overall, the present study demonstrates for the first time that the administration of the antiglycative compound pyridoxamine can reduce the early stages of diet-dependent kidney injury and dysfunction by interfering at many levels with the profibrotic signaling and inflammatory cascades.

1. Introduction

One of the most feared chronic microvascular complications of diabetes is diabetic nephropathy. In western world, diabetes is indeed the leading cause of end-stage renal disease, surpassing other etiologies, such as hypertension. One-third of type 1 diabetes mellitus patients and 20% of type 2 diabetes mellitus patients develop end-stage renal disease [1], a pathological condition that dramatically contributes to increasing mortality among diabetic patients if compared to healthy nondiabetic individuals [2].

An early sign of diabetic nephropathy is an increased protein release in urine, displayed as microalbuminuria, which is associated with the progression of renal damage, and is caused by glomerular hypertrophy, hyperfiltration, widening of basement membranes, tubule-interstitial fibrosis, glomerulosclerosis, and podocytopathy [3].

Currently established therapeutic approaches for diabetic nephropathy are primarily antihypertensives that act on renin angiotensin aldosterone system, such as angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers. These appear to reduce proteinuria and to delay, but not prevent, the onset of end-stage renal disease [4].

For this reason, one of the most actual challenging topics is to identify pharmacological treatments that prevent the onset of diabetic nephropathy by acting on the initial stages of diabetes related renal dysfunction. One of the most recently proposed pharmacological tools is pyridoxamine, a structural analog of vitamin B6 that exerts antiglycative effect [5]. Pyridoxamine has been demonstrated to be effective in reducing serum creatinine increase in patients with initial diabetic nephropathy impairment [4, 6]. Pyridoxamine solves its action by inhibiting the formation of advanced

glycation end products (AGEs) from glycated proteins and by trapping pathogenic reactive carbonyl compounds (Amadori product), the intermediates in the formation of AGEs [7]. AGEs are irreversible products of protein glycation (glycative stress) expressed ubiquitously and overproduced in case of sugar accumulation, such as during hyperglycemia.

We and others have previously contributed to demonstrating that fructose exposure evokes more AGEs than glucose, mainly because the anomerization equilibrium for fructose is shifted more to the reactive, open chain form of the sugar [8, 9].

Accordingly, accumulation of AGEs (mainly carboxymethyllysine and carboxyethyllysine, CML and CEL) due to chronic exposure to hypercaloric diets significantly contributes to the progression of metabolic, degenerative diseases and related cardiovascular complications [10–17].

Increasing AGEs levels can cause alterations of extracellular proteins, such as collagen and elastin as well as the activation, through the interaction with their receptor RAGE, of different inflammatory signaling pathways in the kidney. Among these, NF-κB is, indeed, the most influent inflammatory signal transduction molecule found to be activated by AGEs-RAGE interaction [18]. Interestingly, recent findings convincingly show that the activation of the proinflammatory NF-kB pathway is also involved in the induction of expression/activity of selective profibrogenetic signaling pathways [19].

However, so far, the potential beneficial effects of pharmacological modulation of AGEs production in the context of diet-related kidney dysfunction have been poorly investigated. To our knowledge, renoprotective effects of pyridoxamine supplementation have been tested only in strains of mice genetically programmed to develop diabetic nephropathy, with limited insights on molecular mechanisms [20–22]. Our study aims to investigate whether oral administration of pyridoxamine prevents the onset of diet-related kidney dysfunction, affecting selective inflammatory and profibrotic signaling pathways in a nongenetic animal model of diet-induced metabolic disease.

2. Materials and Methods

2.1. Animals and Treatments. Four-week-old male C57Bl/6j mice (Charles River Laboratories, Calco, Italy) were cared for in compliance with the European Council directives (number 86/609/EEC) and with the Principles of Laboratory Animal Care (NIH number 85-23, revised 1985). The scientific project was approved by the Ethical Committee of the Turin University. Mice were randomly allocated into the following dietary regimens: standard diet (SD, $n = 15$) and a high-fat high-fructose diet (HD, $n = 20$) for 12 weeks. Mice were provided with diet and water ad libitum. The HD contained 45% kcal fat (soybean oil and lard), 20% protein (casein), and 35% carbohydrate (fructose) (D03012907 diet, Research Diets). After three weeks of dietary manipulation, two subgroups of SD and HD began pyridoxamine supplementation in the drinking water for the remaining nine weeks (SD + P, $n = 5$; HD + P, $n = 10$). The pyridoxamine dosage (1 g/L, the equivalent of about 150 mg/kg/day) was chosen according to literature data [23] and calculated on the average daily water intake. During the 12 weeks of the experimental protocol, body weight, glycaemia, and food intake were strictly monitored.

2.2. Procedures and Plasma Analyses. Six-hour fasting glycaemia was measured at the start of the protocol and every 3 weeks by saphenous vein puncture using a conventional glucometer (GlucoMen LX kit, Menarini Diagnostics). After 12 weeks, mice were anesthetized and euthanized by cardiac exsanguination. Blood was collected and kidneys were rapidly removed and transversely sectioned. One-half was embedded in 4% PAF. The rest of the kidney was frozen in liquid N_2 and stored at −80°C for protein analysis. Plasma lipid profile and creatinine levels were determined by standard enzymatic procedures using reagent kits. Plasma insulin and urine albumin level were measured using an enzyme-linked immunosorbent assay (ELISA) kit.

2.3. Morphological Analysis. Medial sagittal sections of kidney were fixed in 4% formaldehyde solution in M 0.010 phosphate pH 7.4 for 18 h at 4°C. Then, dewaxed 5 μm sections were stained with PAS technique and examined under a light microscope Olympus BX41. Ten randomized pictures at ×200 were obtained to evaluate the degree of tubular damage. Accordingly, with previous publication in which rodents were fed with the same diet [24] tubular damage was expressed in terms of proximal tubules with vacuolar degeneration and loss of the brush border.

In order to evaluate the efficiency of the treatment, administered ad hoc score of damage was applied as follows.

Grade 1. There are up to twenty percent of proximal tubules with vacuolar degeneration and disruption of the brush border.

Grade 2. There are between twenty-one and forty percent of proximal tubules with vacuolar degeneration and disruption of the brush border.

Grade 3. There are more than forty-one percent of the proximal tubules with vacuolar degeneration and disruption of the brush border.

Vacuolar degeneration was defined by the presence of cytoplasmic vacuoles pushing toward the luminal surface of the proximal tubular cell, whereas brush border disruption consisted in a partial or complete loss of its continuity of solution.

2.4. Preparation of Tissue Extracts. Kidney extracts were prepared as previously described [25]. Briefly, mice kidneys were homogenized at 10% (w/v) in a Potter Elvehjem homogenizer (Wheaton, NJ, USA) using a homogenization buffer containing 20 mM HEPES, pH 7.9, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethyl sulphonyl fluoride (PMSF), and 1 ul/ml PIC. Homogenates were centrifuged at 4000 rpm at 4°C for

Table 1: Effects of HD and pyridoxamine administration on mouse body and tissue weight and blood chemistry at 12 weeks of dietary manipulation. Values are mean ± SEM. $^{*}p < 0.05$ versus SD; $^{\$}p < 0.05$ versus HD.

	SD ($n = 10$)	SD + P ($n = 5$)	HD ($n = 10$)	HD + P ($n = 10$)
Body weight (g)	25.4 ± 1.3	26.6 ± 3.0	32.8 ± 1.7*	34.7 ± 3.6*
Food intake (g/day)	2.25 ± 0.07	2.72 ± 0.12	2.30 ± 0.28	2.40 ± 0.14
Kidney weight (% BW)	1.02 ± 0.08	1.08 ± 0.11	0.94 ± 0.10	1.11 ± 0.18
Blood glucose (mg/dl)	72.8 ± 18.9	61.8 ± 16.2	138.8 ± 12.7*	104.6 ± 9.7$^{*\$}$
Plasma insulin (μg/l)	85.8 ± 5.3	78.1 ± 12.7	106.8 ± 6.8*	103.5 ± 11.0*
Plasma triglyceride (mg/dl)	37.1 ± 7.6	29.3 ± 5.5	50.5 ± 9.2*	34.7 ± 4.7$^{\$}$
Plasma cholesterol (mg/dl)	77.2 ± 6.1	84.8 ± 16.4	97.2 ± 8.2*	87.2 ± 2.2

5 min and supernatants and pelleted nuclei were separated. Supernatants were removed and centrifuged at 14,000 rpm at 4°C for 40 min. The supernatants thus obtained, containing cytosolic proteins, were carefully removed. The pelleted nuclei were resuspended in extraction buffer containing 20 mM HEPES, pH 7.9, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM EGTA, 20% glycerol, 420 mM NaCl, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethyl sulphonyl fluoride (PMSF), and 1 ul/ml PIC and centrifuged at 14,000 rpm for 20 min at 4°C. The amount of protein contained in cytosolic and nuclear fractions was determined using a BCA protein assay following the manufacturers' instructions. Samples were stored at −80°C until use.

2.5. Western Blotting. Semiquantitative immunoblot analyses of nuclear translocation of p65, expression of CML, CEL, RAGE, vimentin, and fibronectin, and the phosphorylation of IKKα/β, IκBα, RhoA, MYPT1, and Smad2 were carried out in mouse kidney tissue extracts. Equal amounts of proteins were separated by SDS-PAGE and electrotransferred to PVDF membrane. Membranes were incubated with primary antibodies (rabbit anti-NF-kB [1 : 1000], rabbit anti-IKKα/β [1 : 1000], rabbit anti-Ser$^{176/180}$IKKα/β [1 : 5000], mouse anti-IκBα [1 : 1000], mouse anti-Ser$^{32/36}$IκBα [1 : 1000], rabbit anti-RhoA [1 : 1000], rabbit anti-ser^{188}RhoA [1 : 1000], rabbit anti-MYPT1 [1 : 1000], rabbit anti-Thr^{853}MYPT1 [1 : 1000], rabbit anti-Smad2 [1 : 1000], rabbit anti-Ser$^{465/467}$Smad2 [1 : 1000], rabbit anti-Vimentin [1 : 1000] and rabbit anti-Fibronectin [1 : 5000], goat anti-RAGE, [1 : 1000], mouse anti-CML [1 : 500], and mouse anti-CEL [1 : 100]) followed by incubation with appropriated HRP-conjugated secondary antibodies. Proteins were detected with ECL detection system and quantified by densitometry using analytic software (Quantity-One, Bio-Rad, Hercules, CA, USA). Results were normalized with respect to densitometric value of tubulin for cytosolic proteins or histone H3 for nuclear proteins.

2.6. Immunohistochemistry Analysis. RAGE was analyzed by immunohistochemistry on 4 μm formalin-fixed, paraffin-embedded kidney. Slices were deparaffinized in xylol, rehydrated in a graded ethanol series, and subjected to retrieval (20 minutes, 100°C, in tris-EDTA buffer solution, pH 9). Endogenous peroxidase activity was blocked using 0.6% hydrogen peroxide. After blocking, sections were incubated overnight with primary antibody (goat anti-RAGE, 1 : 100, Chemicon International) and subsequently for 1 h with HRP-conjugated anti-goat secondary antibody. Immunohistochemical staining was performed using the Detection System Peroxidase/DAB. Nuclei were counterstained with hematoxylin.

2.7. Sirius Red Analysis. Slices were deparaffinized in xylol, rehydrated in a graded ethanol series, and incubated with a saturated aqueous solution of picric acid containing Sirius Red (1 mg/ml) for 1 h. After washing twice in acidified water (containing 0.2% glacial acetic acid), slices were dehydrated in 100% ethanol and cleared with xylol for microscope evaluation.

2.8. Statistical Analysis. One-way ANOVA followed by Bonferroni's post hoc test was adopted for comparison among the experimental groups. Data were expressed as mean ± SEM. Statistical tests were performed with GraphPad Prism 6.0 software package (GraphPad Software, San Diego, CA, USA). Threshold for statistical significance was set to $p < 0.05$.

2.9. Materials. All compounds were purchased from Sigma Chemical, unless otherwise stated.

3. Results

3.1. Effects of HD and Pyridoxamine Administration on Metabolic Parameters. Mice fed with the high-fat high-fructose diet (HD) for 12 weeks had greater body weights than control diet-fed littermates (SD) (Table 1). The body weight gain was not significantly reduced by pyridoxamine. The levels of fasting blood glucose and insulin were significantly increased in HD fed mice when compared to SD (Table 1; $p < 0.05$). The concentration of fasting serum glucose, but not insulin, was significantly reduced by daily administration of pyridoxamine (HD + P), remaining still higher than that recorded in the SD group. Chronic exposure to HD strongly increased serum levels of triglycerides and total cholesterol. Most notably, pyridoxamine treatment significantly normalized the changes in triglycerides contents and trendily reduced the levels of total cholesterol.

3.2. Effects of HD and Pyridoxamine Administration on Kidney Structure and Function. Representative images from the different experimental groups are shown in Figure 1. Mice fed with the HD exhibited a severe degree of vacuolar degeneration, as well as a complete loss of the brush border integrity. Damage was observed in S1-S2 and S3 parts of the tubule. Pyridoxamine administration prevented the diet-induced morphological alterations. Kidneys from groups fed with chow diet showed proximal tubules with a normal well-preserved histoarchitecture.

The HD-induced renal pathology correlated with decline in kidney function, as shown by increased levels of serum creatinine, which was significantly reduced by pyridoxamine administration (Figure 2(a)). Similarly, albumin levels were increased in the HD group compared with the control group and slightly reduced by pyridoxamine treatment, without reaching a statistical significance (Figure 2(b)).

3.3. Effects of HD and Pyridoxamine Administration on Kidney AGEs and RAGE. The causal role of the diet-related activation of the AGEs-RAGE system in evoking activation of proinflammatory and profibrotic pathways, thus contributing to the onset of diet-dependent kidney dysfunction, is well known. Here we measured local expression levels of the most known AGEs carboxymethyllysine/carboxyethyllysine (CML/CEL), showing a massive overproduction at 12 weeks of dietary manipulation (Figures 3(a) and 3(b)). As expected, the daily supplementation with the antiglycative pyridoxamine prevented AGEs accumulation. Immunohistochemistry analysis (Figure 4) demonstrated a parallelism between AGEs overproduction and increased expression of the AGE-receptor RAGE in kidney sections of HD fed mice. This evidence was confirmed and quantified by western blot analysis, showing a threefold increase in RAGE expression in HD group when compared to SD mice (Figure 3(c)). Interestingly, both western blot and immunohistochemical analysis demonstrated that pyridoxamine daily supplementation prevented renal AGEs accumulation as well as RAGE hyperexpression.

3.4. Effects of HD and Pyridoxamine Administration on Kidney NF-kB Pathway Activation. As shown in Figures 5(a) and 5(b), diet manipulation did not affect total expression of IKK α/β and IκBα, two core elements of the NF-kB signaling cascade. As NF-kB activation mainly occurs via IkB kinase-(IKK-) mediated phosphorylation of the inhibitory molecule, IkBα, we measured their levels of phosphorylation showing that HD evoked increased phosphorylation of IKK α/β on $\text{Ser}^{176/180}$ and of IκBα on $\text{Ser}^{32/36}$ when compared with mice under the SD, suggestive of an increased activation of this pathway.

This overactivation was associated with a marked increased translocation of the p65 NF-κB subunit from the cytosol to the nucleus (Figure 5(c)). In contrast, chronic administration of pyridoxamine to HD fed mice weakened both IKK α/β and IκBα phosphorylation and counteracted HD-induced p65 nuclear translocation (Figures 5(a), 5(b), and 5(c)), thus contributing to reducing local overactivation of a key proinflammatory pathway.

3.5. Effects of HD and Pyridoxamine Administration on Kidney Fibrosis. As shown in Figure 6(a), a robust increase in the phosphorylation of SMAD2 was detectable in the kidney of HD mice. SMAD2 is the canonical profibrotic transcriptional factor that contributes to transmitting TGF-β signals from cell surface to the nucleus promoting the production of profibrotic proteins such as vimentin and fibronectin. As expected, the increased activation of SMAD2 was associated with a higher expression of vimentin and fibronectin (Figures 6(b) and 6(c)).

Most notably, pyridoxamine supply significantly reduced the activity of the profibrotic pathway by halving the phosphorylation of SMAD2 as well as the following expression of vimentin and fibronectin.

TGF-β also activates the noncanonical pathway of RhoA, a member of the Ras superfamily of small GTP-binding proteins, which contributes to fibronectin production in diabetic kidney.

Here we demonstrated that the intensity of RhoA phosphorylation in Ser^{188} was halved in the kidneys of hypercalorically fed mice when compared to chow fed mice (Figure 7(a)), thus indicating a diet-dependent massive increase of RhoA pathway activity. This effect was associated with a significant increase of the Ser^{853} phosphorylation of MYPT1 (a downstream marker of RhoA/ROCK pathway) (Figure 7(b)). In contrast, treatment of mice exposed to HD with pyridoxamine abolished all the alterations in RhoA/ROCK pathway evoked by HD.

However, as shown in Figure 8, no significant difference in the collagen deposition was detectable with Sirius Red assay among the experimental groups, thus demonstrating that a marked fibrotic process was not yet fully developed at 12 weeks of dietary manipulation.

4. Discussion

In keeping with previously published papers [24, 26], we confirmed that 12 weeks of dietary manipulation with a high-fat high-fructose diet evoked metabolic derangements, leading to kidney injury, as shown here by alteration in tissue morphology and reduction in its functionality (increased serum creatinine and albuminuria). Most notably, we demonstrated that oral chronic administration of pyridoxamine, a structural analog of vitamin B6 that exerts antiglycative effect, at the dose of 150 mg/kg/die, significantly reduced the metabolic alterations by improving fasting glycaemia and lipid profile. These beneficial effects were associated with improvement in kidney function and morphology due, at least in part, to significant reduction in the local accumulation of CEL and CML (the two most studied AGEs).

In fact, the key role of AGEs accumulation and/or RAGE overexpression in the development of glomerular kidney injury has been recently documented by Yasuhiko and colleagues using transgenic mice overexpressing RAGE

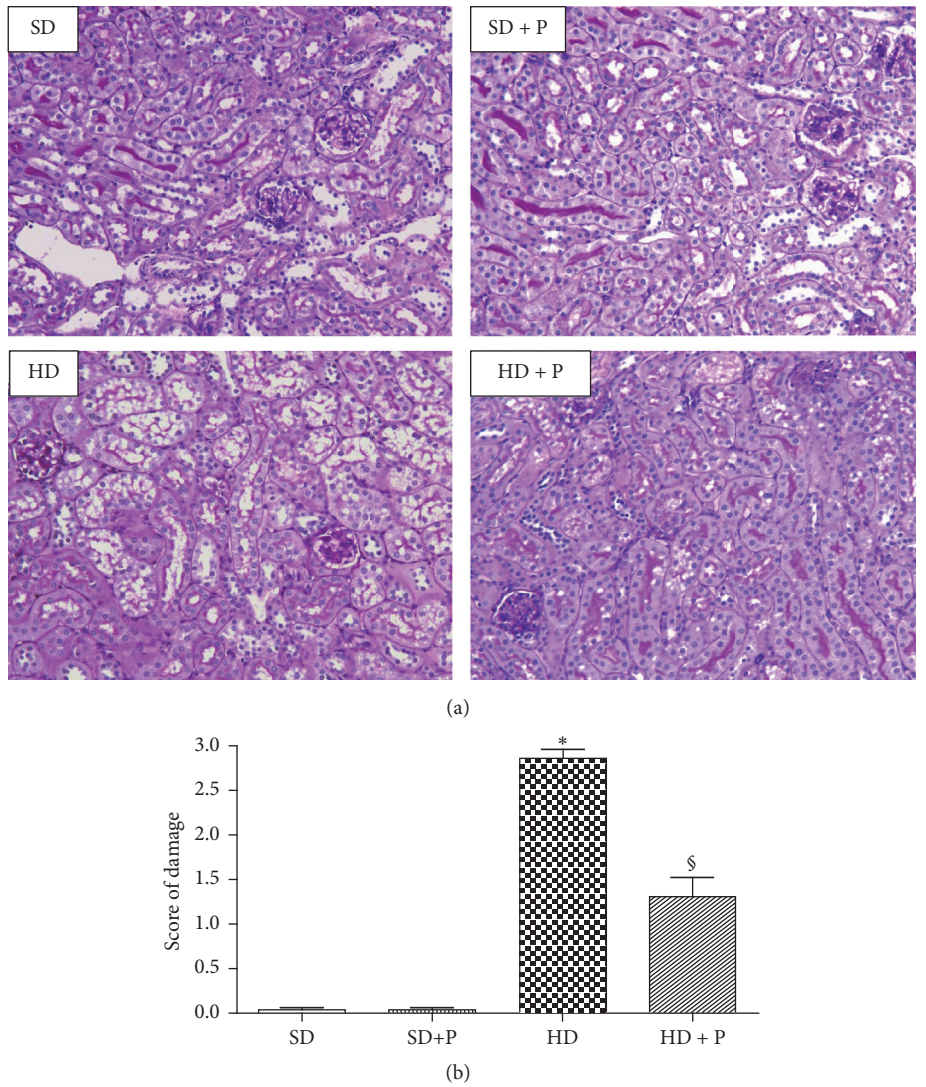

Figure 1: Effects of HD and pyridoxamine administration on kidney structure. (a) Representative kidney section from the experimental group stained with PAS (20x) and (b) score quantification. SD with or without pyridoxamine treatment (150 mg/kg/day): proximal tubules with a normal histoarchitecture. HD: severe degree of tubular vacuolar degeneration and loss of the brush border. HD + pyridox: proximal tubules with almost full protected histology. Values are mean ± SEM of five animals per group. $^{*}p < 0.05$ versus SD; $^{\S}p < 0.05$ versus HD.

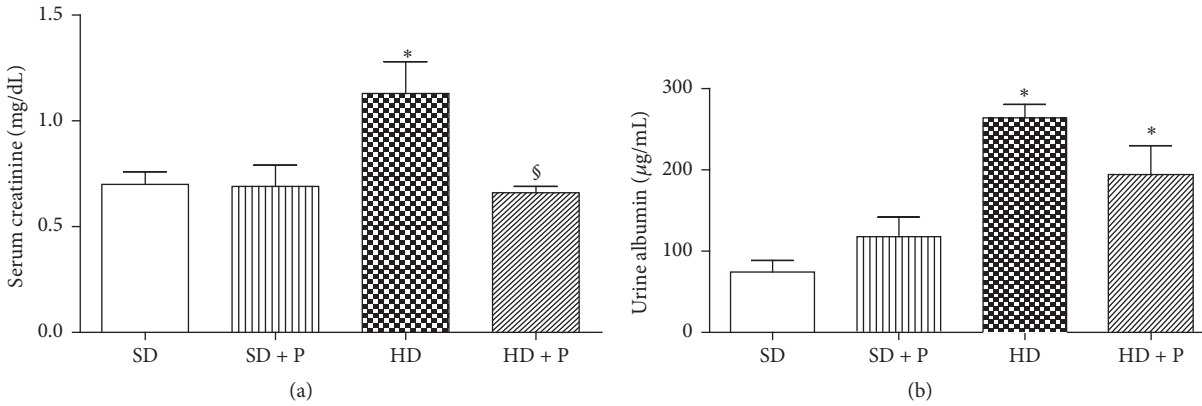

Figure 2: Effects of HD and pyridoxamine administration on kidney function. Serum creatinine (a) and urinary albumin (b) levels were measured in mice exposed to SD or HD in the absence or presence of pyridoxamine (150 mg/kg/day). Values are mean ± SEM of five-ten animals per group. $^{*}p < 0.05$ versus SD; $^{\S}p < 0.05$ versus HD.

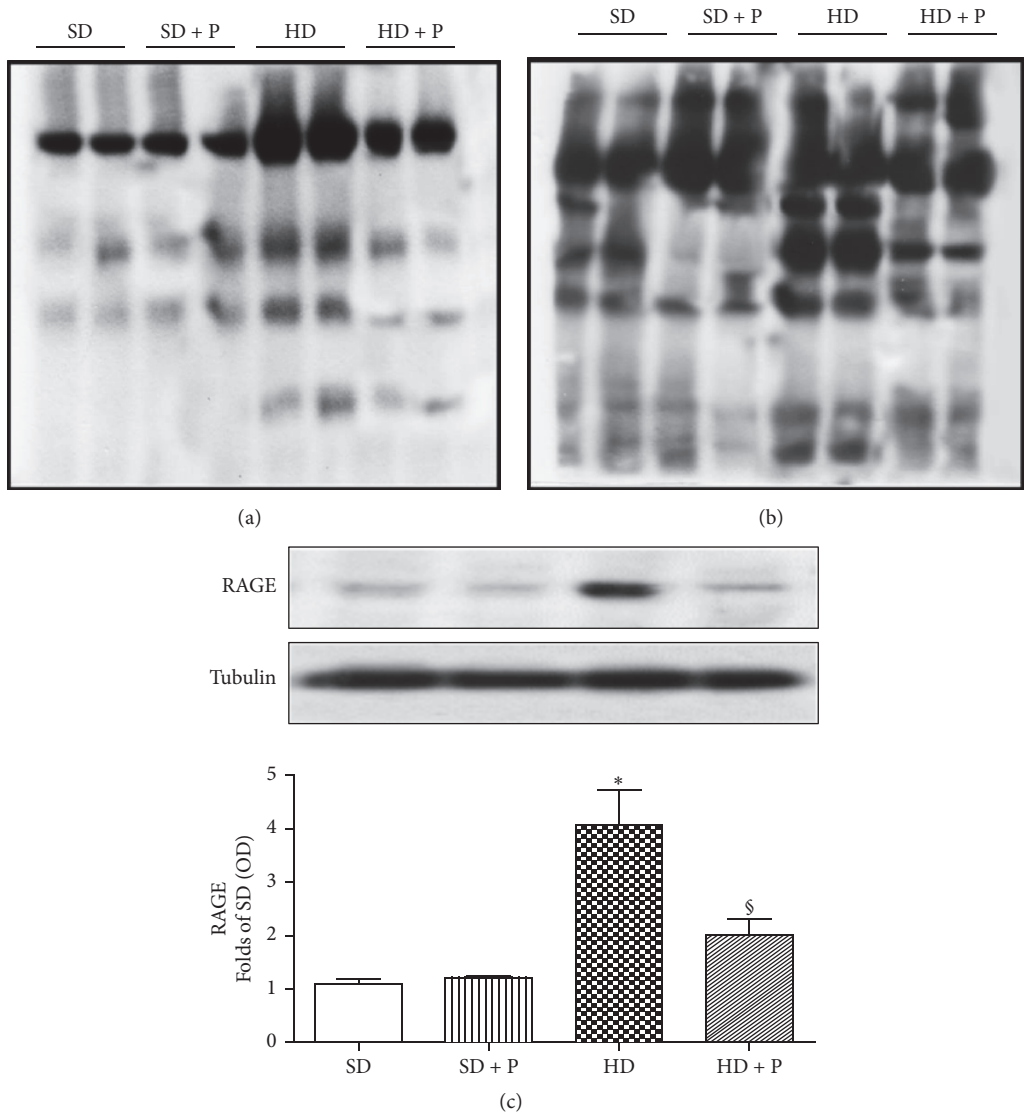

Figure 3: Effects of HD and pyridoxamine administration on kidney AGE and RAGE expression. CEL (a), CML (b), and RAGE (c) were analyzed by western blot on kidney homogenates obtained from mice exposed to SD or HD with or without pyridoxamine administration (150 mg/kg/day). Protein expression is measured as relative optical density (OD), corrected for the corresponding tubulin contents and normalized to the SD band. The data are means ± SEM, five animals per group. $^{*}p < 0.05$ versus SD; $^{\S}p < 0.05$ versus HD.

in vascular cells. As shown by the authors, RAGE overexpression led to renal dysfunction and advanced glomerulosclerosis and its selective inhibition reverted the renal deleterious effects [27]. Very recently, the involvement of AGEs accumulation in tubular injury and the protective effects of anti-RAGE antibody to block CML-RAGE pathway has been demonstrated in human renal tubular epithelial cell line [28]. According to these findings, the antiglycative compound pyridoxamine has been recently suggested as effective supplementary approach in patients with initial diabetic nephropathy impairment [4, 6]. However, so far, the molecular mechanism(s) underlying its protective role has been slightly examined.

Here we investigated the effects of pyridoxamine on the AGEs/RAGE signaling pathway and the following signaling cascades, focusing on the NF-kB inflammatory signaling pathway. AGEs/RAGE interaction, indeed, stimulates the activation of NF-κB, a key transcriptional factor which connects immune response to infection and inflammation. In its inactive form, NF-κB is sequestered by the inhibitor of κB (IκB) in the cytoplasm and this can be released upon phosphorylation by IκB kinase complex (IKK), which is formed by three subunits termed as IKKα, β, and γ. Once translocated in the nucleus, NF-κB binds to RAGE promoter and further enhances RAGE expression. Thus, the activation of RAGE by AGEs induces RAGE expression [29]. Moreover, NF-kB activation contributes to renal inflammation mostly by upregulating the renal expression of chemokines and cytokines involved in the increasing of vascular permeability such as monocyte chemoattractant

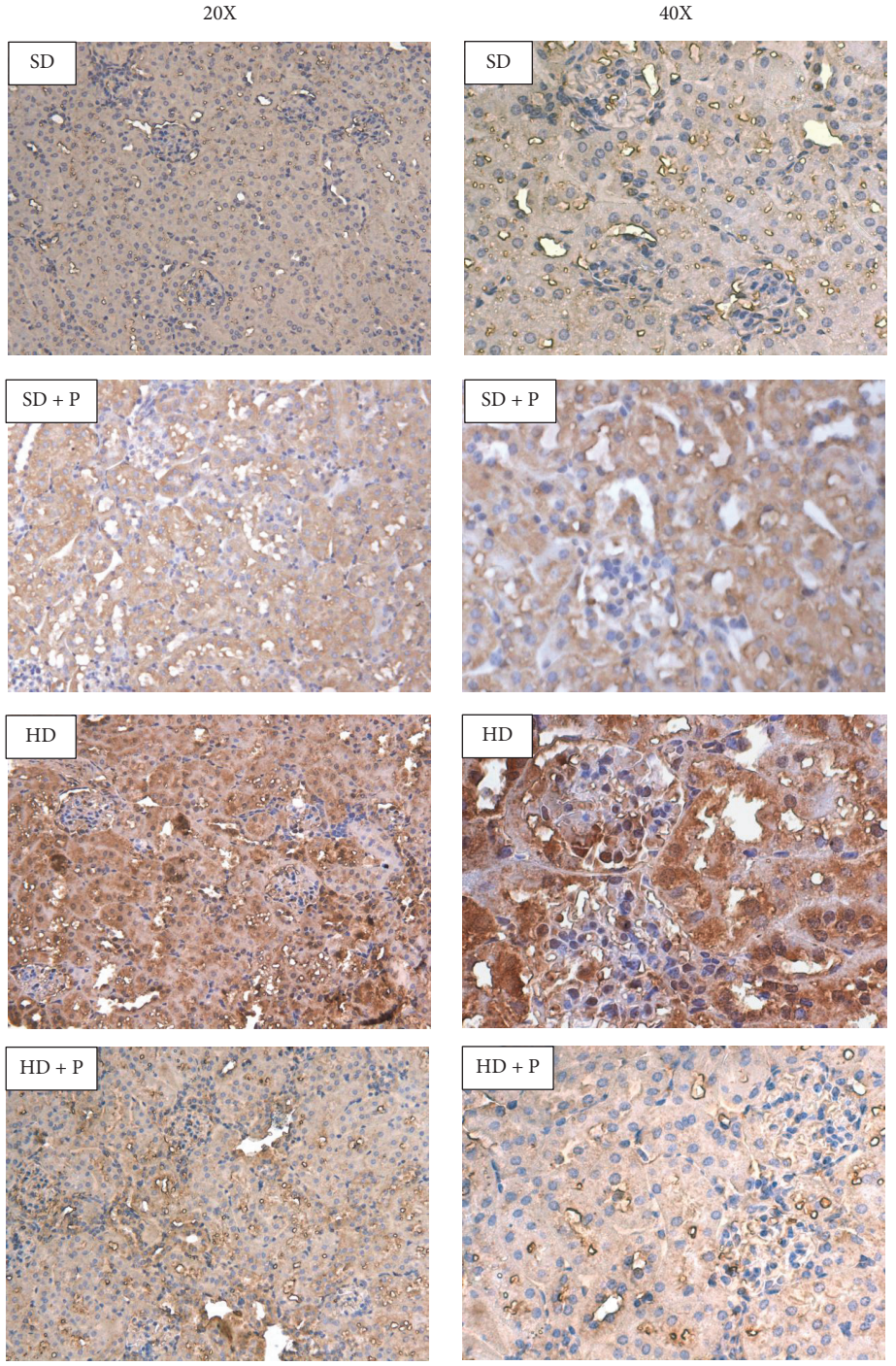

Figure 4: Effects of HD and pyridoxamine administration on kidney RAGE expression. Representative 20x and 40x magnification images of immunohistochemistry analysis for RAGE on kidney sections from mice fed with the SD or the HD, with or without pyridoxamine administration (150 mg/kg/day).

protein-1 (MCP-1), IL- (interleukin-) 1, IL-6, IL-18, and TNF (tumour necrosis factor), which are critically involved in kidney disease pathogenesis [19]. Finally, stimulation of RAGE by AGEs increases ROS levels through activation of NAPDH oxidase [30] enhancing levels of oxidative stress and consequently further contributing to an excessive inflammatory response. Our data strongly support the key role of this signaling pathway in the pathogenesis of diet-related kidney dysfunction and the crucial cross-talk mechanism between AGE-RAGE and NF-kB cascades, showing a parallelism between the AGE accumulation/RAGE overexpression and the robust activation and translocation of p65 subunit of NF-kB from the cytosol to the nucleus following dietary manipulation. Interestingly, both the cascades were blunted in their activation by pyridoxamine administration.

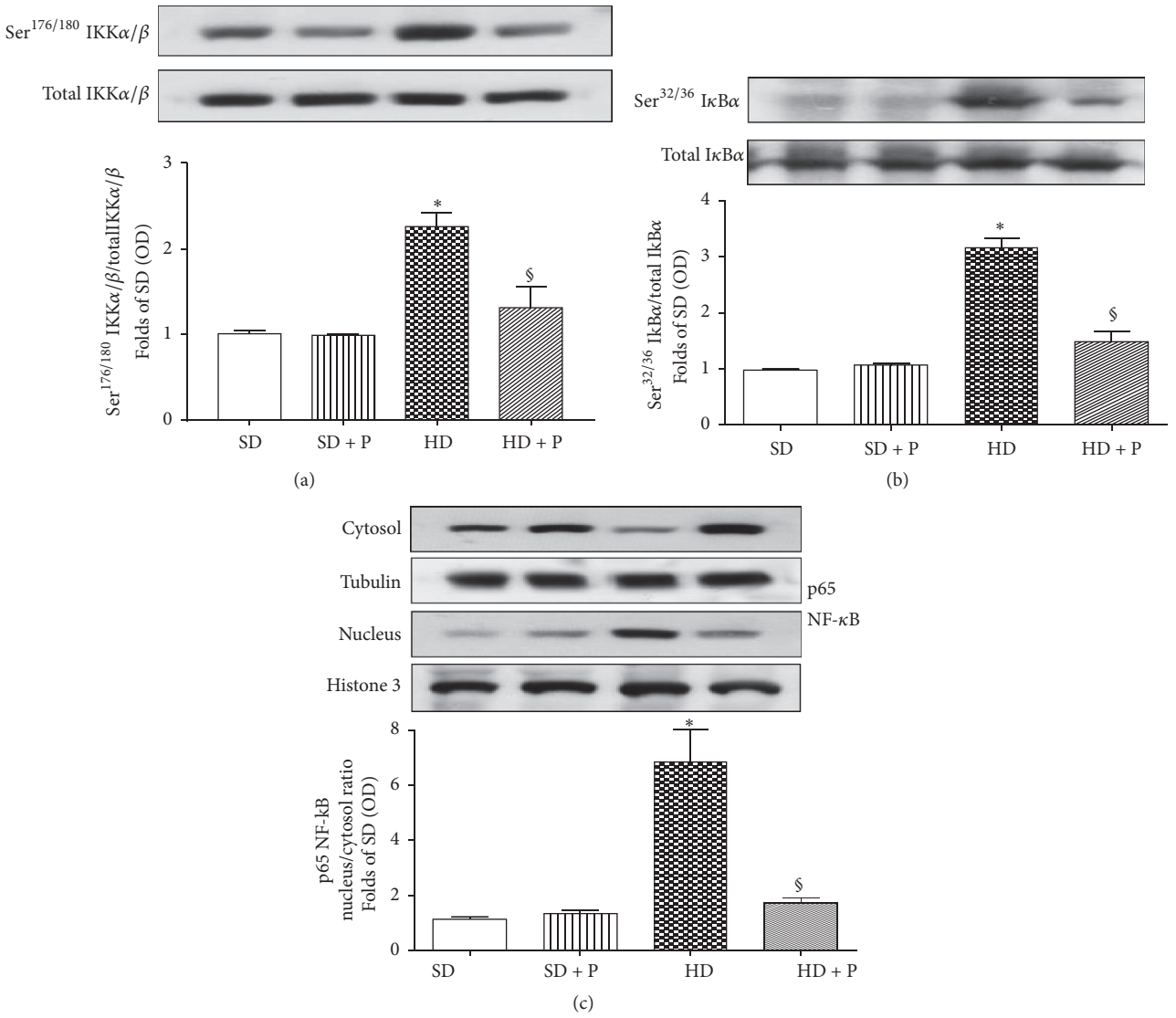

Figure 5: Effects of HD and pyridoxamine administration on kidney NF-kB pathway activation. Total protein expressions of IKK (a) and IkBα (b) and their related phosphorylated forms and NF-kB p65 subunit translocation in the nucleus (c) were analyzed by western blot on kidney homogenates obtained from mice exposed to SD or HD, with or without pyridoxamine administration (150 mg/kg/day). Protein expression is measured as relative optical density (OD), corrected for the corresponding tubulin or histone H3 contents and normalized to the SD band. Results are shown as phosphorylated/total protein ratio (a and b) or as nucleus/cytosol protein ratio (c). The data are means ± SEM of three separate experiments, five animals per group. $^{*}p < 0.05$ versus SD; $^{\S}p < 0.05$ versus HD.

Recent findings convincingly showed that the activation of the proinflammatory NF-kB pathway is also involved in the induction of expression and/or activity of selective profibrogenic signaling pathways, including the prosclerotic cytokine TGF-β (transforming growth factor-β) [19]. Oldfield and others [31] demonstrated that the activation of RAGE induced the overexpression of TGF-β, thus demonstrating the involvement of AGEs in the onset of tubulointerstitial fibrosis. Previously published i*n vitro* studies demonstrated that AGEs may evoke production and accumulation of collagen [32, 33].

Unfortunately, we could not confirm these findings, as our model of mild renal injury does not allow the detection of significant accumulation of extracellular collagen. Longer kinetics of dietary manipulation and/or more severe dietary insult would be requested to confirm *in vivo* these findings. However, our main focus was the study of the molecular mechanisms of protection evoked by pyridoxamine, which should be clinically used as supplementary approach to delay the development of diabetic nephropathy and not to counteract a drastic deposition of collagen and renal fibrosis. Thus, we decided to deepen our investigation on potential pharmacological modulation of the two most widely known TGF-β depending early profibrotic pathways, whose correlation with AGEs accumulation and pyridoxamine administration has not yet been tested and was not even proposed.

The first cascade is the canonical signaling pathway that involves the phosphorylation and activation of Smad2 and

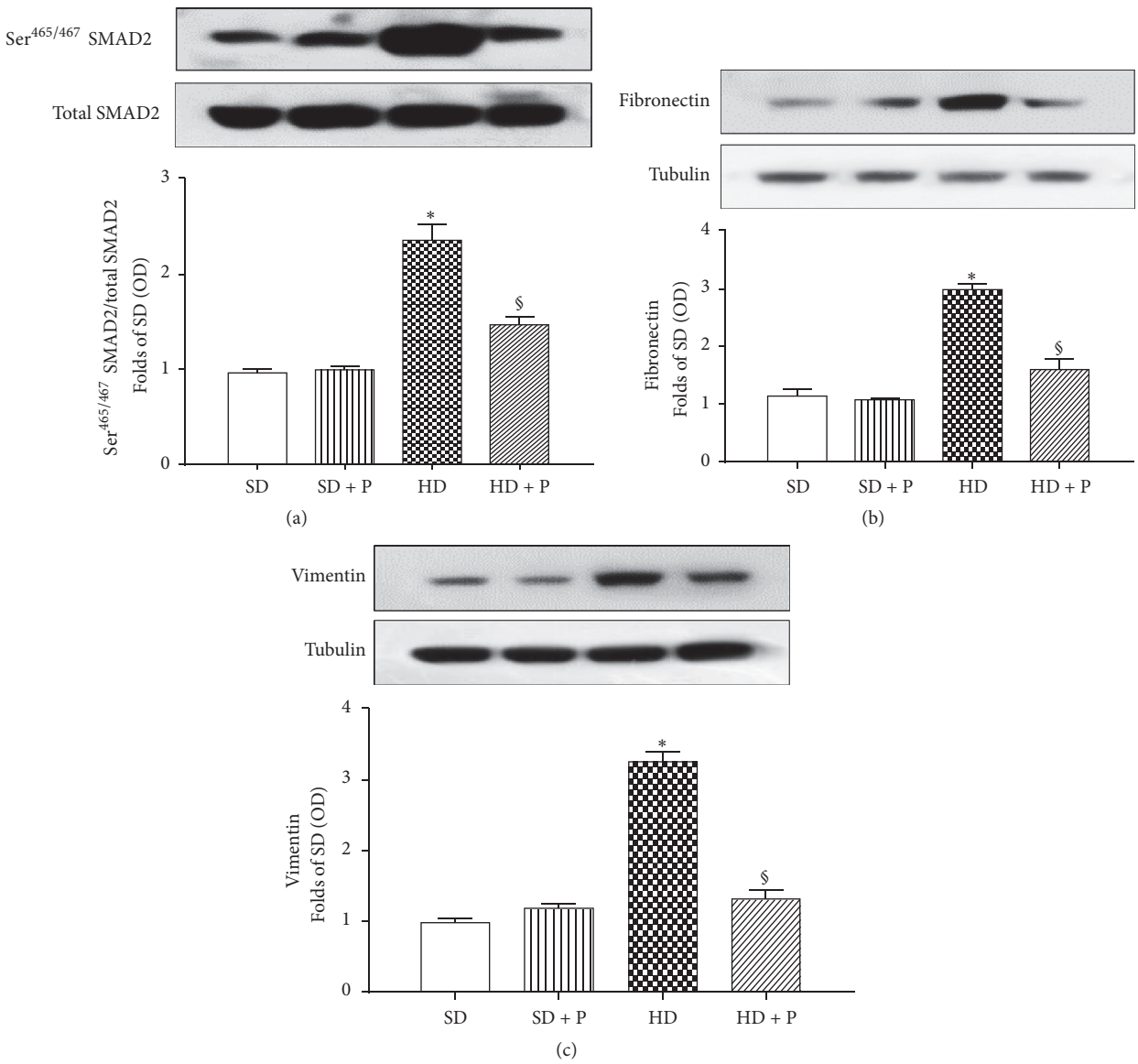

Figure 6: Effects of HD and pyridoxamine administration on profibrotic response. SMAD2 phosphorylation (a) and total protein expression of fibronectin (b) and vimentin (c) were analyzed by western blot on kidney homogenates obtained from mice exposed to SD or HD, with or without pyridoxamine administration (150 mg/kg/day). Protein expression is measured as relative optical density (OD), corrected for the corresponding total SMAD or tubulin contents and normalized to the SD band. The data are means ± SEM, five animals per group. $^{*}p < 0.05$ versus SD; $^{\S}p < 0.05$ versus HD.

Smad3. Smad4 then binds activated Smad2/3, which enables this complex to translocate to the nucleus and transcribe specific profibrotic genes such as fibronectin and vimentin [34]. Recent studies identified within the inflammation/fibrosis pathway SMAD2/3 an essential modulator of insulin sensitivity related to glomerular dysfunction. Specifically, Smad signaling has an essential role in the development and progression of HFD-induced kidney injury and progression of obesity-related glomerulopathy. Inhibition of Smad signaling, indeed, protects podocytes from metabolic stress through increasing mitochondrial activities [35].

The second cascade is the RhoA/ROCK pathway. RhoA is a member of the Ras superfamily of small GTP-binding proteins, which has been demonstrated to contribute to profibrotic signaling and fibronectin production in diabetic kidney and interfering with VEGF-mediated endothelial cell function [36–38].

Here we demonstrated, for the first time, that both the canonical and noncanonical profibrotic pathways are affected by pharmacological modulation of local AGEs accumulation. In fact, pyridoxamine administration resulted in robust reduction of the enhanced phosphorylation and thus activation of Smad2 and the overexpression of vimentin and fibronectin, two important profibrotic proteins upregulated by Smad2 pathway activation. At the same time, pyridoxamine treatment significantly reverted the activation of the

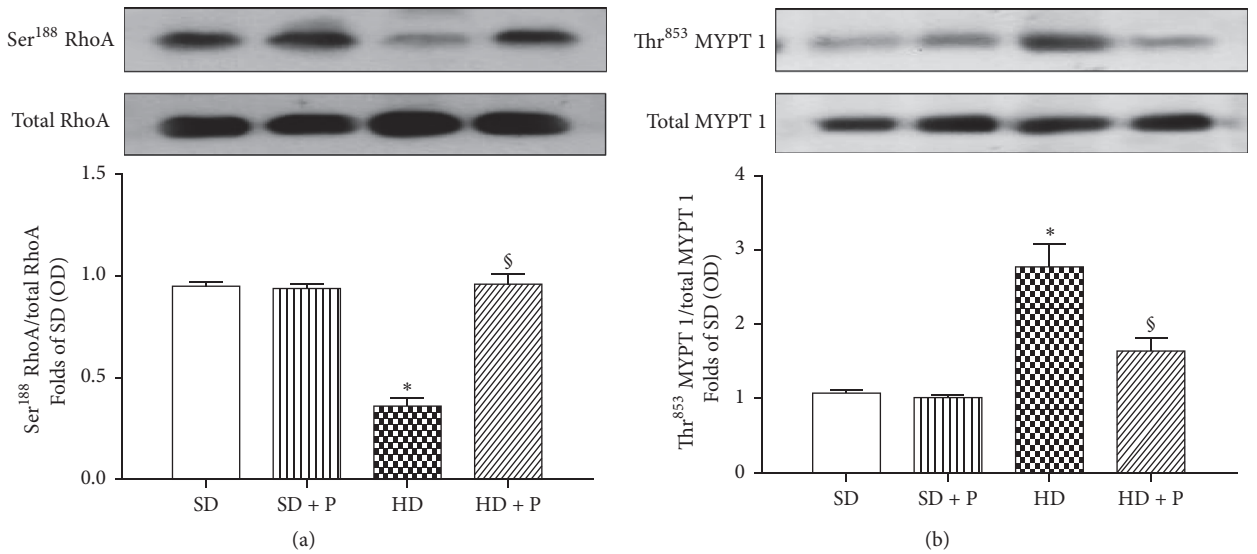

FIGURE 7: Effects of HD and pyridoxamine administration on kidney RhoA pathway activation. Total protein expressions of RhoA (a), MYPT1 (b), and their related phosphorylated forms were analyzed by western blot on kidney homogenates obtained from mice exposed to SD or HD, with or without pyridoxamine administration (150 mg/kg/day). Protein expression is measured as relative optical density (OD), corrected for the corresponding total protein contents and normalized to the SD band. The data are means ± SEM, five animals per group. $^{*}p < 0.05$ versus SD; $^{\S}p < 0.05$ versus HD.

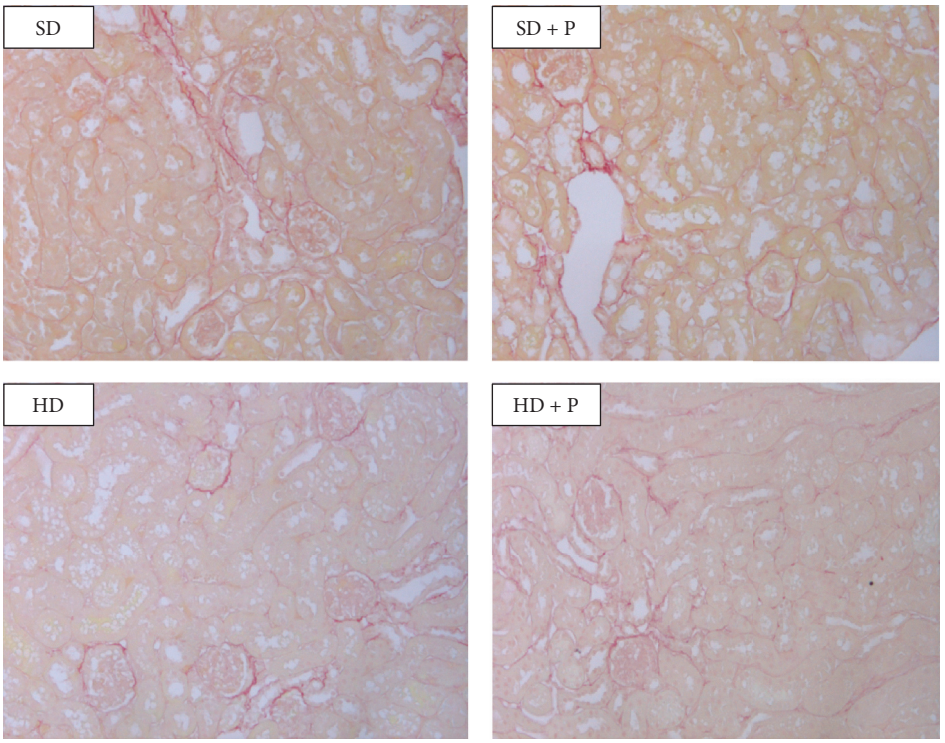

FIGURE 8: Effects of HD and pyridoxamine administration on kidney fibrosis. Representative kidney section from the experimental group stained with Sirius Red (20x).

RhoA pathway and the following phosphorylation of MYPT1, a RhoA downstream effector.

These modulatory effects of pyridoxamine on the two fibrotic cascades can be consequences of the reduction of the NF-kB pathway activation due to pyridoxamine interference with the AGEs/RAGE and NF-kB cross-talk mechanism. However, we cannot rule out direct interaction between AGEs and the RhoA/ROCK pathway. In fact, AGEs have been recently demonstrated to directly activate RhoA in endothelial cells, leading to increased hyperpermeability,

through a selective complex formation between RAGE and RhoA [39]. Further investigations are needed to better clarify the involvement of NF-kB independent mechanisms of activation of profibrotic pathways by AGEs.

To the best of our knowledge, this is the first study that analyzes from a mechanistic point of view the consequences of pyridoxamine chronic oral administration in a model of diet-induced kidney dysfunction, demonstrating that this antiglycative compound exerts protective beneficial nephrology effects by reducing AGEs levels, leading to interferences with selective inflammatory and profibrotic signaling pathways.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

R. Mastrocola and M. Collino are equally contributing authors.

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Review Article

Molecular Mechanisms and Treatment Strategies in Diabetic Nephropathy: New Avenues for Calcium Dobesilate—Free Radical Scavenger and Growth Factor Inhibition

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Diabetic nephropathy is one of the most important microvascular complications of diabetes mellitus and is responsible for 40–50% of all cases of end stage renal disease. The therapeutic strategies in diabetic nephropathy need to be targeted towards the pathophysiology of the disease. The earlier these therapeutic strategies can bring about positive effects on vascular changes and prevent the vasculature in patients with diabetes from deteriorating, the better the renal function can be preserved. Studies evaluating anti-inflammatory and antioxidative strategies in diabetic nephropathy demonstrate the need and value of these novel treatment avenues. CaD is an established vasoactive and angioprotective drug that has shown a unique, multitarget mode of action in several experimental studies and in different animal models of diabetic microvascular complications. On the molecular level, CaD reduces oxidative stress and inhibits growth factors such as fibroblast growth factor and vascular endothelial growth factors. Recent findings have demonstrated a strong rationale for its use in reducing urine albumin excretion rate and markers of inflammation as well as improving endothelial function. Its beneficial effects make it an attractive therapeutic compound especially in the early stages of the disease. These findings, although promising, need further confirmation in prospective clinical trials with CaD.

1. Introduction

Diabetes mellitus (DM) is a leading cause of morbidity and mortality. Vascular lesions from microvascular and macrovascular involvement lead to impaired blood flow and contribute to damage and dysfunction of one or more target organs, that is, the heart, kidneys, eyes, and nervous system. Diabetic nephropathy (DN) is one of the most important microvascular complications of DM and is responsible for 40–50% of all cases of end stage renal disease (ESRD) [1]. Among the patients with type 1 diabetes (T1D), as many as one-third develop serious renal complications, characterized by increasing urinary albumin excretion rates (AER) and decreasing kidney function, measured by eGFR, with 10%–20% of the subjects progressing to ESRD [1]. Diabetic nephropathy is therefore the leading cause of ESRD and when confounded by hypertension, it is the leading risk factor for cardiovascular disease [1].

2. Proteinuria and Diabetic Nephropathy

A marker of DN is albuminuria, arbitrarily defined as microalbuminuria (urine albumin excretion <300 mg/24 h) and macroalbuminuria (>300 mg/24 h). Compared to diabetic patients with normoalbuminuria (<30 mg/24 h), patients with persistent macroalbuminuria (overt DN) have an almost 10-fold higher risk of developing ESRD [2]. Histologically, DN manifests as diffuse or nodular mesangial expansion, tubular and glomerular basement membrane thickening, and interstitial fibrosis [3]. However, few diabetic patients

presenting with albuminuria are biopsied. Therefore, the hallmark of DN diagnosis remains as urinary protein excretion.

However, recently it has become evident that in a subgroup of diabetic patients nephropathy can occur without going through a phase of proteinuria but rather show a rise in the prevalence of reduced renal function as measured by eGFR without significant proteinuria. Indeed, whereas diabetic nephropathy is traditionally defined as the presence of proteinuria or progression to ESRD, there is now increasing utilization of decreased renal function, as reflected by declined eGFR, in the definition of diabetic kidney complications.

The detection and diagnosis of DN can be a challenge due to its insidiousness. The onset of microalbuminuria is the earliest clinical manifestation of renal injury in most patients with diabetes [2]. Without specific interventions, many patients with DM progress from microalbuminuria to macroalbuminuria. The presence of macroalbuminuria especially when it is associated with a decrease in renal function (i.e., decrease in glomerular filtration rate [GFR]) is considered diagnostic for overt DN. Patients with type 1 DM generally present with microalbuminuria 6–10 years after being diagnosed with diabetes. Of these patients, 20–45% progress to DN during the next 10 years. Overt DN occurs therefore about 20 years after the diagnosis of type 1 DM in many patients [2, 4].

In type 2 DM, the natural history of DN development is similar but due to delayed diagnosis of their diabetes, type 2 DM patients may already have overt proteinuria and impaired GFR at the time of diagnosis (or shortly after their diabetes diagnosis). In these patients the duration of diabetes is often not precisely known. This makes it difficult to prevent and to treat DN in a timely manner. Renal endpoints, such as ESRD or doubling of serum creatinine, occur mostly within ten years in approximately 20% of microalbuminuric patients and in more than 60% of macroalbuminuric patients. The incidence of renal complications is more or less similar in type 1 and 2 DM [5–7].

The cellular and molecular mechanisms involved in the pathophysiology of proteinuria in diabetes are worth exploring when evaluating therapeutic strategies that interfere with the development and progression of DN. Hyperglycemia can lead to hemodynamic changes in the glomerulus, endothelial cell dysfunction, changes in the basal membrane, and podocyte injury. All of these mechanisms are likely involved in DN development at different time points. Therapeutic strategies, however, are often directed at one or two of these pathophysiological changes. The best example of this is the blockade of the renin-angiotensin system (RAAS) in DN. Glomerular hyperfiltration is present from the onset of diabetes until macroalbuminuria presents. *During the rise of macroalbuminuria GFR declines rapidly*. This sequence of events is consistent with the hypothesis that glomerular hyperfiltration causes progressive glomerular damage and that the subsequent pathological changes in the interstitium are mediated by microinflammation followed by interstitial fibrosis. Inhibition of the RAAS improves glomerular hyperfiltration by dilating the efferent glomerular arterioles thereby reducing glomerular pressure and proteinuria. In addition to these hemodynamic effects RAAS inhibition has distinct molecular effects on cell functions such as hypertrophy, proliferation, and migration. Inhibition of angiotensin II affects endothelial cells, mesangial cells, and podocytes. In this review, the pathological mechanisms of DN will be explored further, as well as the therapeutic role that CaD, a molecule with anti-inflammatory, antioxidative, and antiangiogenic properties, may play in DN.

3. Histology of Diabetic Nephropathy

The first reports from renal biopsies of patients with diabetic kidney disease described the initial changes of the disease, that is, glomerular hypertrophy, mild mesangial expansion with matrix accumulation, and thickening of the glomerular capillary walls [8]. These changes have also been consistently observed by electron microscopy. With progression of the disease, there is further increase in mesangial expansion with accumulation of extracellular material in the mesangium. In subsequent years the glomerular volume may increase further and there may be formation of hyaline nodules in the glomerular tuft. This histological pattern is referred to as nodular diabetic glomerulosclerosis or Kimmelstiel-Wilson disease [9, 10]. In the past, Kimmelstiel-Wilson has been used as a synonym of DN. The presence of nodules of different size, at times laminated, with variable distribution in the glomeruli, is pathognomonic of DN. From this stage of the disease, progressive thickening of capillary walls and global glomerulosclerosis is observed [8]. Two other typical glomerular lesions are (i) capsular drop, a homogeneous hyaline deposit in Bowman's capsule and (ii) glomerular hyalinosis [11–13]. However, these changes have also been observed in other glomerular diseases [14]. In typical cases of DN, microaneurysms, produced by mesangiolysis, are evident.

The changes in the tubulointerstitium are more consistent. In tubules, increased reabsorption of protein, interstitial fibrosis, followed by tubular atrophy is observed [8, 9]. Most likely, these changes are associated with activation of tubular cells and induction of inflammatory mechanisms. Macrophages invade the interstitium thus contributing to interstitial inflammation and fibrosis. Over time, the tubules disappear and are replaced by fibrotic tissue.

Blood vessels of the diabetic kidney are characterized by intimal hyaline thickening of arterioles and rarefication of capillaries [15, 16]. Arteriolar lesions may involve any of the arterioles. Intimal fibrosis of the arteries is also common in DN, but this feature is not pathognomonic because intimal fibrosis occurs in other diseases as well. The most important is the presence of activated and damaged endothelial cells in the diabetic kidney [17]. It is hypothesized that endothelial cells are damaged and respond by activation of inflammatory mechanisms, recruit inflammatory cells from the circulation, and contribute to the inflammatory state of the diabetic kidney [18]. Despite the increased knowledge about the pathophysiological mechanisms of diabetic kidney disease, it is still unclear how metabolic and hemodynamic damage to endothelial and epithelial cells, inflammation, and progressive fibrosis with deterioration of the microcirculation

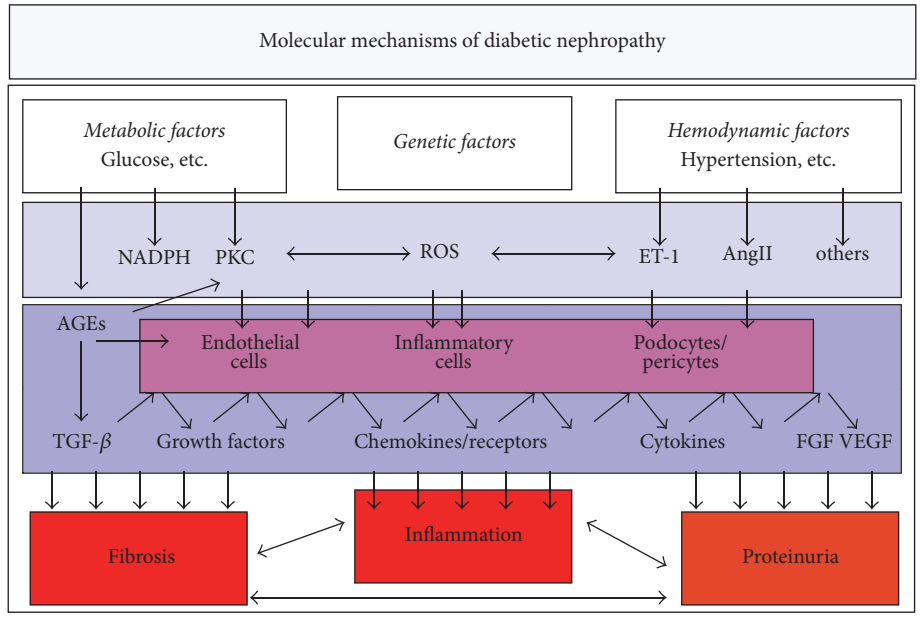


Figure 1: A schematic overview on structures and mechanisms in the pathogenesis of diabetic nephropathy. The pathogenesis of diabetic nephropathy involves several mechanisms over the course of the disease. Hyperglycemia is the leading cause of diabetic nephropathy; however, other metabolic factors such as hypercholesterolemia also play a role. Genetic factors are prominent since only ca. 30% of diabetic patients develop diabetic nephropathy. Importantly, hypertension and hemodynamic factors in the kidney, that is, hyperfiltration contribute significantly to the development of the disease. These external factors are translated by several intracellular pathways such as NADPH or PKC into cell activation. Different cell types respond in a specific fashion. Growth factors such as VEGF or TGF-b and chemokines such as MCP-1 are expressed and lead to inflammation and proteinuria followed by fibrosis. AngII, angiotensin II; ET-1, endothelin-1; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; PKC, protein kinase C; ROS, reactive oxygen species; TGF, transforming growth factor; VEGF, vascular endothelial growth factors; FGF, fibroblast growth factor.

are connected. However, the prevention of microvascular damage and the inhibition of inflammatory mechanisms would appear to be important therapeutic and preventative goals in DN.

4. Pathogenesis of Diabetic Nephropathy: A Comprehensive View

The pathogenesis of DN is complex and multifactorial (Figure 1). The metabolic disturbances of diabetes together with hyperglycemia, hyperlipidemia, and hypertension alter the endocrine homeostasis of the vascular wall with a shift in balance of regulatory systems such as nitric oxide (NO)/reactive oxygen species (ROS), vascular endothelial growth factors (VEGF)/VEGF-R, and angiopoietin/tie-2. These pathological changes are fueled by the toxic breakdown products such as oxidized and glycosylated molecules which in turn bind to specific receptors and impair cellular function further *(RAGE lit)*. One of the initial endothelial cell disturbances is an increase in permeability for albumin and other proteins. Over the years this "endothelial dysfunction" will contribute to hypertension and microinflammation with perivascular damage through the altered release of growth factors and cytokines as well as increased permeability for inflammatory cells and cytokines. Mesangial cells, podocytes, pericytes, and tubular epithelial cells are functionally altered and lead to nephropathy, with more inflammation, epithelial cell damage, and increased fibrosis. Over the years these mechanisms impair renal function by vascular rarefication, interstitial fibrosis, and nephron degeneration. The rate of progression of DN is influenced by complex interactions between genetic predisposition, dietary, and lifestyle factors. The patient's genetic profile is responsible for the cellular response to a metabolic stimulus; that is, some patients show an exaggerated endothelial response compared to others when exposed to the same metabolic stress. This may partially explain the spectrum of pathophysiology in diabetic kidneys. In addition, due to the sheer number of nephrons (>1 million/kidney) it takes 15–20 years for kidney failure and ESRD to develop. It is obvious that this process can either be slowed down by a lessened exposure to vascular risk factors or be enhanced if damaging factors are increased.

5. Endothelial Cells and Diabetic Vascular Disease

Alterations of endothelial cells and the vasculature play a central role in the pathogenesis of DN [17–19]. Endothelial cells have a key function in regulating the maintenance of capillary function and possibly its regeneration [20, 21]. They are the first cells to encounter metabolic disturbances, that is, alterations of glucose homeostasis and hemodynamic changes such as increase in blood pressure. Originally, the endothelium was believed to act as a "cellophane wrapper"

of the vascular tree, with the main function of selectively regulating permeability of water and electrolytes. We now understand that the endothelium embodies a wider range of homeostatic functions. Endothelial cells have the ability to act both as a sensor of metabolic and hemodynamic stress and as a responder with changes in endothelial cell function. Permeability, coagulation, inflammation, and vascular tone are the major mechanisms regulated by endothelial cells [20, 21]. Under physiological conditions, endothelial cells prevent thrombosis by different anticoagulant and antiplatelet adhesiveness and aggregation mechanisms. One important way in which endothelial cells control the clotting system is by regulating the expression of binding sites for anticoagulant and procoagulant factors in the glycocalyx on the endothelial cell surface. In the quiescent state, endothelial cells maintain blood fluidity by promoting the activity of numerous anticoagulant pathways, including the protein C and protein S pathway. After activation, which can be induced by cytokines and also by metabolic factors such as diabetes or hypertension, the balance of endothelial properties can be shifted in favor of clot formation through the coordinated induction of procoagulant and suppression of anticoagulant mechanisms. Inflammatory cytokines, such as tumor necrosis factor, suppress the formation of thrombomodulin, an endothelial anticoagulant cofactor, and induce the expression of tissue factor, which is an important procoagulant cofactor. This shift of balance between procoagulant and anticoagulant factors does not only influence thrombosis and coagulation in the microvasculature but also contributes, both directly and indirectly, to the inflammatory state of the microvasculature in diabetes.

6. Inflammation and Oxidative Stress

The endothelium is also a key player in the inflammatory response. Under healthy conditions the endothelium with its smooth glycocalyx provides a slippery surface where circulating blood cells do not adhere [22]. Leukocytes rolling via specific adhesion molecules on the endothelium represent the initial stage of a multistep process leading to extravasation of white blood cells to sites of inflammation or infection [23]. The recruitment of platelets and leukocytes at sites of vascular injury is a very rapid response and is mediated by the release of preformed components expressed and stored by the endothelium. These storage sites within the endothelial cells include Weibel-Palade bodies. Their major constituents are the multimers of von Willebrand factor and P-selectin, one of the initially important and most active promoters of platelet and leukocyte adhesion. In addition, the endothelial cells release angiopoietin-2 which interferes with its antagonist angiopoietin-1 and leads to endothelial cell activation with an inflammatory response and an increase in permeability [24, 25].

Endothelial cells play an important regulatory role in the circulation as a physical barrier controlling endothelial permeability [19, 22]. Permeability can vary between different vascular beds. For example, in the renal glomerulus, permeability is relatively high while, on the other hand, the blood-brain barrier and the blood retinal barrier are almost impermeable to circulating molecules. In diabetes, the permeability of the endothelium is increased. This pathological change leads to an increase in albumin excretion in the kidney nephron and also to enhanced flow of vasoactive substances into the surrounding tissue of organs such as the heart and brain [22].

Furthermore, endothelial cells are a source of vasoregulatory substances such as endothelium-derived NO and prostacyclin which are released in response to physical stimuli and hormones and induce vascular relaxation to reduce leukocyte adhesion and inhibit platelet function [25].

A disturbance of these endothelium-dependent regulatory systems plays an important role in the development of diabetic complications such as DN. Endothelial dysfunction is characterized by a shift in the actions of the endothelium towards reduced vasodilation, a proinflammatory and prothrombotic state. It occurs early in association with the metabolic syndrome but is also influenced by other risk factors such as smoking and hypertension. It appears physical inactivity may also influence endothelial cell function [26].

Endothelial dysfunction is prominent in hypertension and diabetes and may be important at different stages in the development of coronary artery disease, chronic heart failure, peripheral vascular disease, stroke, and chronic kidney failure. Microinflammation and disturbances of endothelial cell permeability are early signs of organ deterioration. Patients who develop diabetes usually consume a high-calorie diet rich in macronutrients which is able to induce vascular abnormalities. It has been shown that high-fat meals may lead to impaired endothelium-dependent vasodilation. Protein, lipid, and glucose loads are associated with a marked production of ROS and oxidative stress [27, 28]. In addition, diabetes is frequently associated with other known cardiovascular risk factors, including hypertension, obesity, and dyslipidemia. All these mechanisms aggravate endothelial dysfunction with increased permeability and low-grade systemic inflammation.

7. Vascular Endothelial Growth Factors and Diabetic Nephropathy

The complex relationship between endothelial cells, podocytes, and the VEGF/VEGF receptor system provides an important insight into the intricate network which is responsible for the proper functioning of the renal endothelium under healthy conditions and contributes to the pathogenesis of endothelial cell dysfunction in DN. VEGF-A is expressed by glomerular podocytes in large quantities [29]. The molecule acts by binding to its receptor (VEGF-R2) [30]. Interestingly, this receptor is abundantly expressed on glomerular endothelial cells [31]. VEGF-A crosses the barrier of the basement membrane and the cellular glycocalyx to bind to endothelial cells. Endothelial cells need VEGF-A for maintenance of their various functions (differentiation, permeability, and expression of NOS) and for their survival [31]. Sivaskandarajah et al. convincingly showed that it is deleterious for endothelial cells not to

have enough VEGF-A leading to endothelial cell death, loss of glomerular integrity, and glomerulosclerosis [32, 33]. However, too much of VEGF-A also induces endothelial cell damage, an increase in glomerular permeability and inflammation [34]. An appropriate balance of VEGF expression, binding, and signaling is necessary to keep glomeruli healthy and prevent glomerulosclerosis and rarefication. Under diabetic conditions this balance is impaired [35]. Early in the disease the expression of VEGF-A is enhanced and leads to endothelial cell dysfunction [36]. The VEGF system is delicately balanced by regulated expression of VEGF splicing variants [37] and regulated binding to its receptor. The VEGF-A is transported to the VEGF-R2 by specific ligand binding to glycosaminoglycans, providing both storage and adherence in the endothelial cell glycocalyx [38]. This complex of VEGF/heparan sulfates and possible other cofactors is presented to the VEGF-receptors where specific signal transduction is induced and cellular response manufactured. However, also the cellular response is dependent on the availability of effector molecules for the response mechanisms. This additional regulating mechanism has been well studied for the role of eNOS and VEGF [36]. The reduced NO response despite high concentrations of VEGF in the renal vasculature has been called "uncoupling" of eNOS and VEGF, resulting in an impaired cellular response and enhanced pathophysiology in diabetic nephropathy [30]. We suggest that CaD interferes with this mechanism by inhibiting the binding of VEGF (and other growth factors such as FGF) to heparin sulfates, thereby reducing the affinity of the VEGF/HS complex for receptor binding bodies. Such a mechanism would lead to a reduction of VEGF activity in states of enhanced VEGF expression or increased HS sulfate availability. It is also possible that other partners in this signaling complex would be inhibited by CaD. At the same time this modulating effect would not lead to a direct inhibition of VEGF signaling, thereby reducing the side effects observed by direct blockade of the VEGF receptor by antibodies.

The VEGF/VEGF-R system is not the only system where a cellular cross-talk, isoform-specific gene expression, directed molecular transport, and regulated binding and signaling form a complex network for the regulation of a physiological system. In the glomerulus the endothelin system [39] and the angiopoietin/tie-2–system have been characterized. However, in the pathogenesis of DN, the VEGF/VEGF-R system seems to be of critical importance [40]. It will be interesting to see whether novel therapeutic approaches will interfere with these mechanisms.

8. Therapeutic Strategies for the Treatment of Diabetic Nephropathy

The complex pathogenesis of DN makes the development of evidence-based therapeutic strategies difficult [41]. Two large studies, the Diabetes Control and Complications Trial conducted in type 1 DM and the United Kingdom Prospective Diabetes Study in type 2 DM, showed that intensive blood glucose control delays the onset and the progression of diabetic microvascular complications including nephropathy [42–44]. These studies strongly suggest that hyperglycemia, that is, the increase in blood glucose concentration, is a major factor in the pathogenesis of DN. However, treatment of hyperglycemia alone does not halt DN progression. In addition to hyperglycemia, enhanced activation of the RAAS is thought to contribute significantly to the pathogenesis of DN. Inhibitors of the RAAS have been shown to lower blood pressure and decrease albuminuria which leads to additionally protective renal and cardiovascular effects [45–47]. Although this approach has shown significant therapeutic effects on the diabetic kidney, it has still made only a small contribution to prevention of the disease [46].

Novel well-tolerated long term treatment strategies which work in the early stages of the diabetes-induced renal changes are urgently needed for preventing or delaying DN. These therapeutic regimens have to be directed at key pathogenic mechanisms and be well tolerated in the long term.

9. CaD and Diabetic Microvascular Disease

Calcium dobesilate (CaD) is a synthetic vasoactive drug indicated in the treatment of microangiopathies, in particular diabetic retinopathy. It is the calcium salt of 2,5-dihydroxybenzenesulfonic acid. CaD has been shown to reduce endothelial damage by acting via multiple pathogenic pathways involved in the progression of diabetic microvascular complications [48–51]. The extensive clinical experience with CaD and its favorable benefit/risk profile makes it a promising candidate for the treatment of early stages of diabetic kidney disease.

CaD has been used for more than 40 years in the treatment of diabetic vascular complications, showing consistent efficacy [48–51]. Nevertheless, the mechanism responsible for this effect has not been clearly delineated. CaD has been considered as an angioprotective agent and has been shown to decrease blood viscosity and vascular permeability in patients with diabetic retinopathy [49]. In addition, it has been reported to possess antioxidant capacity that could account for reduction of vascular permeability [50]

Through its unique, multitarget mode of action, CaD has interestingly shown multifaceted pharmacological effects in reducing diabetic microvascular complications. Several studies have demonstrated the effects of CaD on endothelial dysfunction, microinflammation, vasoconstriction, and hyperpermeability. Treatment strategies that include CaD may be useful for preventing or delaying the progression of diabetic retinopathy and nephropathy.

10. CaD and Endothelial Cell Function

A reduction in endothelium-dependent relaxation of diabetic vasculature has been associated with both a decrease in NO-mediated vasodilation and an increase in ROS production [48]. Superoxide (O_2^-) generation by dysfunctional mitochondria in diabetes has been postulated as the primary initiating event in the development of diabetic complications [52]. The endothelium under diabetic conditions seems to be more sensitive to free radical-induced injury. The abnormal endothelium-dependent relaxation in aorta from diabetic rabbits was restored to normal by superoxide dismutase,

suggesting a role for superoxide anions in the endothelial cell abnormality caused by DM [52]. Diabetes-induced vascular endothelial dysfunction can reduce NO production and elevate endothelin-1 (ET-1) levels, diminishing capillary diameter and restricting blood flow. In addition to its direct vasoconstrictor effects, elevated ET-1 may in turn contribute to endothelial dysfunction by inhibiting NO production. CaD exerts strong antioxidative effects in vitro [53]. Ruiz et al. showed that CaD enhanced the endothelium-dependent relaxation induced by acetylcholine in rabbit isolated aorta artery [54]. The effect was clearly endothelium-dependent, indicating that CaD may act on the endothelial derived relaxing factor, NO. A confirmation of these findings came from Suschek et al. In cell cultures obtained from normal Wistar rats and BB rats (a model of type 1 DM), CaD induced a dose dependent increase of NO synthase activity [48]. Additional studies both in vitro and in animal models have confirmed this positive effect of CaD [48, 53, 55].

11. eNOS Uncoupling

An enhancement of endothelial permeability is one of the first pathological changes in diabetic microvasculature disease. In vivo, an increase in capillary permeability can be induced by the intraperitoneal administration of prooxidants in rats and measured relative to the concentration in the peritoneal cavity of a dye (Evans blue) injected intravenously. CaD (100 mg/kg orally for 7 days) produced a significant reduction in capillary permeability demonstrating the ability to protect the peritoneal wall from oxidative insult and therefore decrease the induced permeability [51, 56].

CaD has also been shown to reduce the adhesion of inflammatory cells to surfaces [57] and exhibit several anti-inflammatory effects, including the inhibition of NF-KB- and p38 MAPK-mediated leukocyte adhesion to retinal vessels and reduction of adhesion molecules such as ICAM-1 [51]. In the same animal model, CaD showed that reduction of a proinflammatory state and leukocytes recruitment lead to a significant inhibition of tight junction alterations thus protecting the inner blood retinal barrier.

12. CaD and FGF/VEGF Inhibition

Vascular endothelial growth factor is a key factor in the pathogenesis of diabetic microvascular disease by inducing angiogenesis and vascular permeability. CaD has shown antiangiogenic activity and reduces vascular permeability under diabetic conditions [58, 59]. CaD significantly inhibited the proliferation of human umbilical vein endothelial cells (HUVEC) induced by VEGF (10 ng/ml), without significantly affecting HUVEC proliferation in the absence of VEGF and reduced also VEGF-induced angiogenesis in vivo [58]. Previous investigators had demonstrated that CaD interferes with heparin binding on fibroblast growth factor (FGF) and inhibits the signaling of FGF via its receptors FGFRs [60]. These experiments have demonstrated that CaD recognizes both growth factors, as a heparin antagonist within the binding site in these polypeptides, changes the three-dimensional structure of the growth factor at their receptor recognizing site, and is capable of dissociating the receptor-growth factor signaling complex. CaD seems therefore to be a member of a novel group of molecules which inhibit growth factors not only by interfering with their receptor binding but by regulating growth factor activity by specifically interfering with its interaction with glycoproteins. This mechanism explains not only the inhibitory effect on FGF and VEGF by CaD but also the low rate of side effects as compared to VEGF antibodies in diabetes [60, 61]. While VEGF antibodies completely block the effects of VEGF on the intracellular signaling pathways and thereby also block the VEGF-induced signals which are necessary for endothelial cell survival, interference with the heparin sulfate binding sites reduces the binding of VEGF and FGF to its coreceptor and therefore reduces its effects on endothelial cells but does not abolish the effect of VEGF on its specific membrane-bound receptor (Figure 2).

13. CaD and Oxidative Stress

Increases in oxidant production clearly have been shown to occur when vascular or glomerular cells are exposed to hyperglycemia. The metabolism of glucose via mitochondrial pathways and the activation of NADPH oxidases via PKC activation have been shown to contribute significantly to oxygen radical production. CaD has been described as having strong antioxidative effects both in vitro and in vivo. CaD has proven to be an oxygen free radical scavenger and to inhibit free radicals production both in vitro and in vivo animal models. In vitro, CaD has been shown to scavenge oxygen free radicals generated by reaction between xanthine and xanthine oxidase in absence or presence of DMSO (dimethylsulphoxide) and iron chloride ($FeCl_2$) in a dose dependent manner (human polymorphonuclear blood cells). Therefore it is believed to inhibit both oxidative damage in capillary cells and inflammatory cascades [28–32].

Increasing evidence indicates that the disruption of mitochondrial bioenergetics may be important in the development and progression of diabetic nephropathy. As the kidney relies on oxidative phosphorylation to provide the ATP for tubular reabsorption it is not surprising that mitochondrial homeostasis is strictly essential for an optimally functioning kidney. There is ample evidence that there is a disturbance of mitochondrial bioenergetics in the diabetic kidney.

14. CaD and Diabetic Nephropathy

Dong and coworkers have analyzed the effects of CaD on the expression of glomerular tissue inhibitor of metalloproteinase 1 (TIMP1), collagen IV, and ultrastructure of glomerular basement membrane in diabetic STZ rats [62, 63]. Their results showed that, after 12 weeks, kidney function in CaD-treated animals increased and was significantly improved compared to that in control diabetic animals. Electron microscopy showed that thickness of glomerular capillary basement membrane (GBM) was also improved. Podocyte foot processes were preserved and expression of TIMP1 and collagen IV were significantly less in treated rats. CaD may improve DN by inhibiting the overaccumulation of

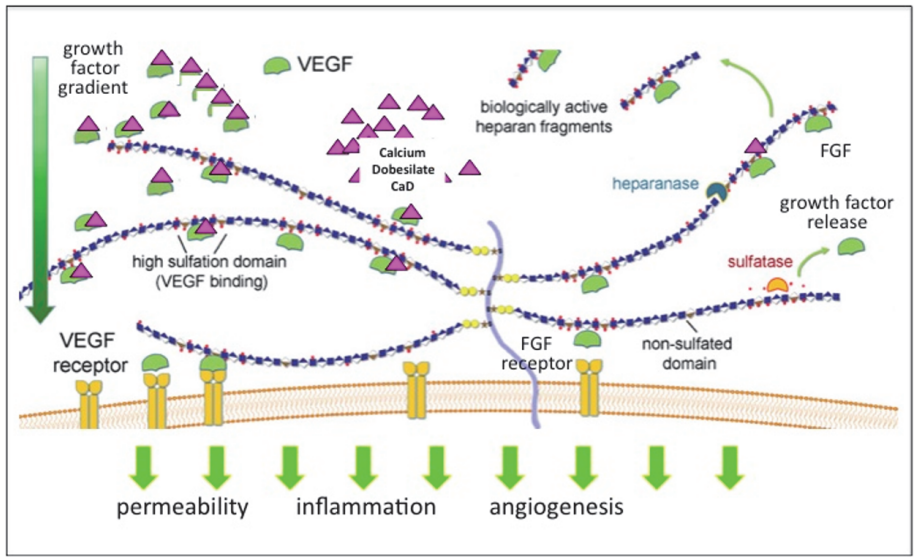


Figure 2: The FGF/VEGF system and its alterations in diabetes: the effects of CaD. In diabetic patients VEGF and its receptors are overexpressed. Circulating growth factors requires two binding sites in order to elicit a cellular response: (1) to heparan sulfate domains on extracellular proteoglycans and (2) to its specific membrane-bound receptor. The heparan sulfate domains provide a gradient for growth factors and allow coordinated binding to their receptors where intracellular pathways are activated which lead to endothelial dysfunction, albuminuria, and angiogenesis. The heparin binding sites are regulated physiologically by heparanases and sulfatases. CaD binds specifically to the negatively charged domain of growth factors thereby interfering with their binding to their receptors and thus reducing endothelial cell dysfunction, albuminuria, and angiogenesis under diabetic conditions. While VEGF antibodies completely block the effects of VEGF on the intracellular signaling pathways and thereby also blocking the VEGF-induced signals which are necessary for endothelial cell survival, interference with the heparin sulfate binding sites reduces the binding of VEGF and FGF to its coreceptor and therefore reduces its effects on endothelial cells but does not abolish the effect of VEGF on its specific membrane-bound receptor. VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor.

collagen IV and TIMP1. Recently Jafarey et al. showed that CaD is effective in protecting rats against gentamicin-induced nephrotoxicity. This protective effect of CaD has been considered probably dependent on its antioxidant properties [64].

Zhang and colleagues analyzed the effects of CaD in diabetic patients [65]. A total of 121 patients with type 2 DM and albuminuria received CaD (500 mg, 3 times/day) for 1, 2, or 3 months, respectively. Urinary albumin excretion, medium and low shear rate, and whole blood viscosity were significantly lower in the treated patients. The rate of microalbuminuria normalization after 12 weeks was 90%. In addition, the benefit was positively correlated with CaD treatment duration. They additionally measured the plasma concentrations of plasminogen inhibitor-1 (PAI-1) and described a decrease of PAI-1 during CaD treatment, which contributes to the therapeutic effect of CaD. Other positive effects of CaD, such as a decrease in endothelin, may be related to the molecular effects of the drug.

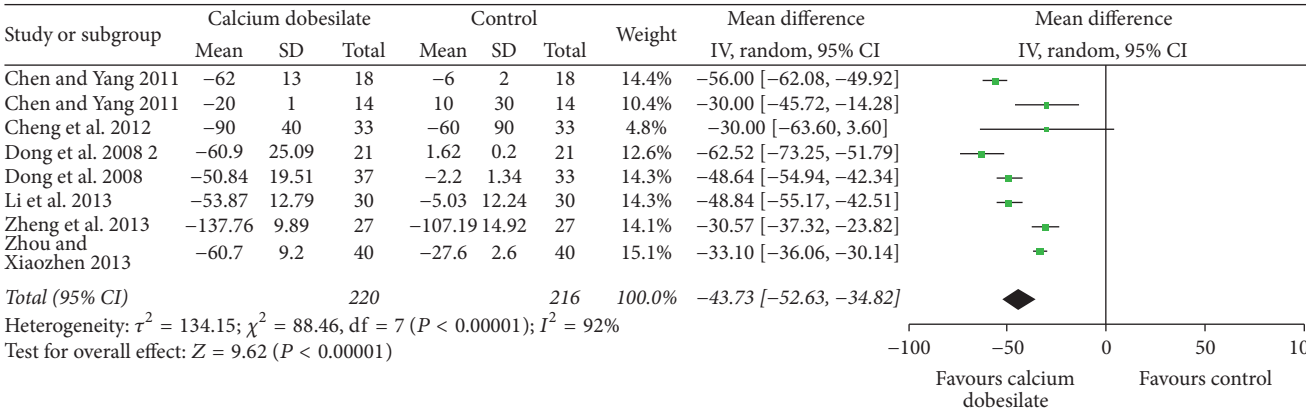
The specific mode of action of CaD, as compared to other antiproteinuric drugs such as RAAS blockade, has been demonstrated in the study by Dong et al. [66]. Patients were randomly assigned to three groups: placebo, CaD, and the angiotensin converting enzyme- (ACE-) inhibitor perindopril. There was a comparable decrease in albuminuria in the two treatment groups. However, a comparison of different markers of endothelial dysfunction such as endothelin and NO showed a clear difference in the action of CaD versus the ACE-inhibitor. In addition to decreasing PAI-1, CaD also reduced endothelin levels and increased NO. In other studies, in hemodialysis patients and in patients affected by early stages of DN, reduced serum levels of high-sensitivity C-reactive protein, improved micro-inflammatory state, decreased serum levels of ET-1, and increased levels of NO were observed after CaD administration [63, 67].

The hypothesis of additive effects of CaD to a standard therapy of RAAS blockade was analyzed in four smaller studies. In all four studies, an additional decrease in albuminuria after the combination treatment with CaD was observed when combined with benazepril, enalapril, perindopril, or losartan [68–71].

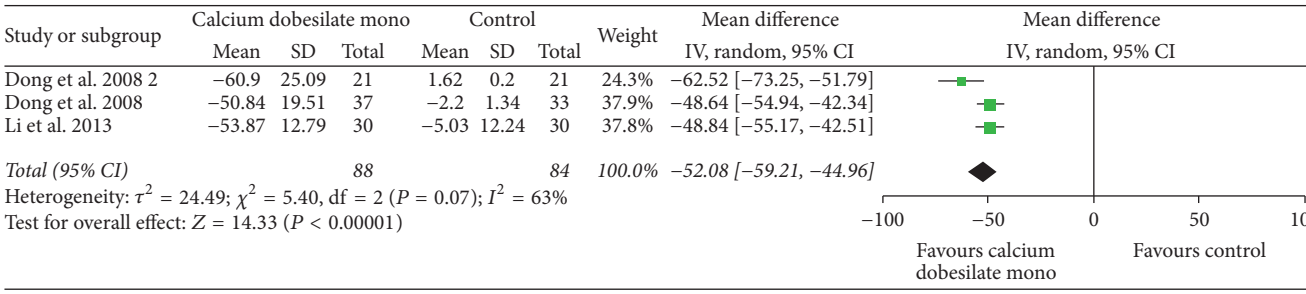
15. Meta-Analysis of CaD Treatment on Diabetic Nephropathy in Chinese Patients with Type 2 Diabetes

Meta-analysis of the data obtained in Chinese diabetic patients with albuminuria from seven studies [63, 66, 68–70, 72, 73] supports the hypothesis that CaD may reduce albuminuria in these patients and is a promising therapeutic strategy for microvascular disease not only in the retina but also in the kidney (Tables 1 and 2).

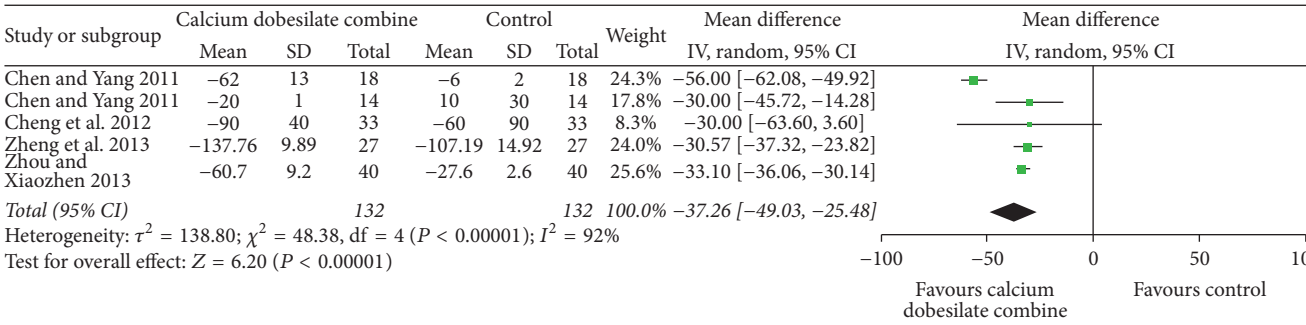
These seven studies were identified by a literature search of MEDLINE, EMBASE, and CENTRAL from the time



(a) Overall UAER changes from baseline between CaD and control treatment groups



(b) UAER changes from baseline between CaD monotherapy and control treatment groups



(c) UAER changes from baseline between CaD in combination with ACE-inhibitor/ARB treatment group compared to ACE-inhibitor/ARB monotherapy groups

Figure 3

Table 1: Baseline demographic and disease characteristics of patients included in meta-analysis.

	CaD	Control
N	220	216
Age (years)	59.3 ± 6.0	58.3 ± 7.1
Male (%)	41.5%	45%
DM duration (year)	8.1 ± 2.1	7.9 ± 2.0
Baseline HbA1c (%)	7.0 ± 0.7	7.2 ± 1.0

recording commenced until December 2015. The three study selection criteria used were (1) randomized placebo-controlled trials; (2) study duration ≥ 12 weeks; and (3) treatment of Chinese patients with type 2 diabetic nephropathy. The eligibility of all studies retrieved from the databases was screened independently by two reviewers based on those three predetermined selection criteria. Disagreements between reviewers were resolved by consultation with a third investigator. A validated 3-item scale was used to evaluate the overall reporting quality of the trials selected for inclusion in the meta-analysis. The meta-analysis was performed by computing the weighted mean difference (WMD) and 95% confidence interval (CI) for change from baseline to study endpoint for CaD versus control treatment groups. All statistical analyses were performed with the Review Manager statistical software package (Version 5.2).

Treatment with CaD was received by 220 patients while a control treatment group with placebo with standard diabetic treatment was received by 216 patients. Overall, when

Table 2: Comparisons between CaD treatment and control treatment groups in UAER changes from baseline.

	Number of studies	Number of subjects (CaD versus control)	WMD from baseline	95% CI	I^2
All	7	220/216	−43.73*	−52.63, −34.82	92%
Monotherapy	3	88/84	−52.08*	−59.21, −44.96	63%
Combination with ACEI/ARB treatment	4	132/132	−37.26*	−49.03, −25.48	92%

* $P < 0.001$.

compared to the control group, CaD treatment, either as monotherapy or in combination with ACE-inhibitor/angiotensin II receptor blocker (ARB), led to a significantly greater change from baseline in urine albumin excretion rate (UAER) (WMD, −43.73 mg/24 h; 95% CI, −52.63 to −34.82 mg/24 h, $p < 0.001$) (Figure 3(a)). As monotherapy only, CaD was associated with a significantly greater change in UAER (WMD, −52.08 mg/24 h; 95% CI, −59.21 to −44.96 mg/24 h, $p < 0.001$) (Figure 3(b)). When comparing CaD in combination with ACE-inhibitor/ARB to control treatment (ACE-inhibitor/ARB alone), the combination treatment was associated with a significantly greater change in UAER (WMD, −37.26 mg/24 h; 95% CI, −49.03 to −25.48 mg/24 h, $p < 0.001$) (Figure 3(c)).

16. Conclusion

New therapeutic strategies in DN need to be specifically targeted towards the pathophysiology of the disease. The earlier these therapeutic strategies can bring about positive effects on vascular changes and prevent the vasculature in diabetics from deteriorating, the better we will be able to preserve renal function in patients with diabetes. Studies evaluating anti-inflammatory and antioxidative strategies in DN demonstrate the need and value of these novel treatment avenues.

CaD is an established vasoactive and angioprotective drug that has shown a unique, multitarget mode of action in several experimental studies and in different animal models of diabetic microvascular complications. At the molecular level, CaD reduces oxidative stress and inhibits growth factors such as FGF and VEGF. Importantly, CaD has shown to be effective in the treatment of diabetic retinopathy (mainly type 2 DM).

Even though more evidence is needed to better understand the role of CaD in other diabetes-related microangiopathies such as DN, recent findings have demonstrated a strong rationale for its use in reducing UAER and markers of inflammation as well as improving endothelial function. Its effects on VEGF and on oxidative stress make it an attractive therapeutic compound especially in the early stages of the disease. These findings, although promising, need further confirmation in prospective clinical trials with CaD.

Conflicts of Interest

Hermann Haller has received honoraria as a speaker from VIFOR Pharma. The other authors declare that there are no conflicts of interest regarding the publication of this paper.

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Research Article

Urinary Neutrophil Gelatinase-Associated Lipocalin Is Complementary to Albuminuria in Diagnosis of Early-Stage Diabetic Kidney Disease in Type 2 Diabetes

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Background. Two clinical phenotypes of diabetic kidney disease (DKD) have been reported, that is, with or without increased albuminuria. The aim of study was to assess the usefulness of urinary neutrophil gelatinase-associated lipocalin (uNGAL) for the early diagnosis of DKD in the type 2 diabetes mellitus (T2DM). *Methods*. The study group consisted of 123 patients with T2DM (mean age 62 ± 14 years), with urine albumin/creatinine ratio (uACR) < 300 mg/g and eGFR ≥ 60 ml/min/1.73 m^2. The control group included 22 nondiabetic patients with comparable age, sex, and comorbidities. uNGAL, albumin, and creatinine were measured in the first morning urine samples. uACR and uNGAL/creatinine ratios (uNCR) were calculated. *Results*. In the control group, maximum uNCR was 39.64 μg/g. In T2DM group, 24 patients (20%) had higher results, with the maximum value of 378.6 μg/g. Among patients with uNCR > 39.64 μg/g, 13 (54%) did not have markedly increased albuminuria. Women with T2DM had higher uNCR than men ($p < 0.001$), without difference in uACR ($p = 0.09$). uNCR in T2DM patients correlated significantly with HbA1c. Sex, total cholesterol, and uACR were independent predictors of uNCR above 39.64 μg/g. *Conclusions*. Increased uNGAL and uNCR may indicate early tubular damage, associated with dyslipidemia and worse diabetes control, especially in females with T2DM.

1. Introduction

Diabetes is a group of metabolic diseases characterized by heterogenic pathophysiology and clinical manifestations. Type 2 diabetes (T2DM) is the most frequent type of carbohydrate metabolic disorders; it is estimated that T2DM comprises 90–95% of all cases. In most countries, T2DM prevalence is constantly increasing, and the increase is faster than the population growth [1]. In addition, a major medical problem is the increasing morbidity and mortality from complications of diabetes, affecting eyes, kidneys, heart, cardiovascular, and nervous system [2]. In most countries, the diabetic kidney disease (DKD) is the most common cause of nephropathies requiring renal replacement therapy [3]. Morphological changes in kidneys in diabetes are induced by disorders of kidney metabolism caused by increased glycemia, as well as by changes in renal hemodynamics, or activation of the renin-angiotensin-aldosterone system (RAA). In a

substantial proportion of T2DM patients, structural changes in kidneys as well as the structural-functional relationships differ from the classical Kimmelstiel-Wilson nodular sclerosis observed in type 1 diabetes (T1DM) [4]. In particular, histopathological studies suggest tubular involvement in about 40% of patients with DKD associated with T2DM, and tubular changes in these patients are unproportionate comparing with glomerular pathology [4–6]. Literature provides convincing evidence that changes in kidneys of T2DM patients are more heterogenic than in T1DM [4–7].

Neutrophil gelatinase-associated lipocalin (NGAL) was first identified in activated neutrophils. It belongs to the lipocalin protein family, and it is able to bind and transport small ligands [8]. Under physiological conditions NGAL is expressed at very low levels in kidneys, trachea, lungs, stomach, and colon. It is present in blood in low concentrations and it undergoes free glomerular filtration followed by nearly complete resorption in the mechanism of megalin-mediated endocytosis in the renal proximal tubule. Increased NGAL synthesis in response to a damaging factor in the distal convoluted tubule and urinary secretion of NGAL constitute the major fraction of urinary NGAL [9, 10]. NGAL as an early biomarker of kidney damage was identified in 2003 during studies searching for novel markers of ischemic and toxic kidney injury in patients undergoing cardiac surgery [9]. The urinary concentrations of NGAL (uNGAL) increase in a consequence of tubular dysfunction associated with acute kidney injury caused by ischemia and secondary tubular damage [9]. Studies suggest that uNGAL may be an appropriate biomarker of tubular changes in chronic kidney disease including DKD, both in T2DM and in type T1DM [11–13]. The studies of Fu et al. [14] and Kim et al. [15] suggested important role of uNGAL measurements in early diagnosis of DKD. Among patients with T2DM, an increase in uNGAL significantly correlated with a decrease in GFR [11, 16].

The aim of the study was to assess the function of renal tubules in patients with early-stage T2DM as reflected by uNGAL concentrations in a group of T2DM patients at the early stage of DKD, that is, with eGFR $\geq$ 60 ml/min/1.73 m^2 and urine albumin/creatinine ratio (uACR) < 300 mg/g.

2. Materials and Methods

The study was conducted in accordance with the Declaration of Helsinki and received permission from the Bioethics Committee of the Regional Medical Chamber in Rzeszów, Poland (number 70/2014/B).

The study recruited adult patients with T2DM who were referred to the ambulatory specialist nephrological care by their diabetologist. Between 2014 and 2015, 123 patients were enrolled in the study. Inclusion criteria were eGFR (2009 Chronic Kidney Disease, Epidemiology Collaboration, CKD-EPI equation) >60 ml/min/1.73 m^2 and no overt proteinuria (uACR < 300 mg/g). Only patients who signed the informed consent were included in the study. Exclusion criteria were treatment with nephrotoxic medications, other kidney diseases, urinary tract infections, systemic infections, cancer, allergy, systemic connective tissue diseases, anemia, pregnancy, and nonstable hypertension (≥130/90 mmHg in self-monitoring). Additionally, a control group included 22 nondiabetic patients with age, sex, and comorbidities similar to T2DM patients; this allowed comparison of laboratory results. The control group included adult patients of the nephrological ambulatory with eGFR CKD-EPI > 60 ml/min/1.73 m^2 and no overt proteinuria. These were mainly patients with stable arterial hypertension, or benign simple kidney cysts. Two control patients suffered in the past infections of the lower urinary tract; one patient was diagnosed with duplication of renal pelvis. The exclusion criteria were the same as for T2DM patients.

First morning urine samples were taken from patients and controls for the measurements of uNGAL, albumin, and creatinine concentrations, as well as for the general urine examination. The concentrations of uNGAL were measured with the automated chemiluminescent microparticle immunoassay on the ARCHITECT analyzer (Abbott Diagnostics, Abbott Park USA). Urine albumin was measured with immunoturbidimetry and urine creatinine with enzymatic method using Olympus AU680 biochemistry analyzer (Olympus, Center Valley, PA, USA). The results of the measurements were used to calculate uACR and uNGAL/creatinine ratio (uNCR). Other laboratory results were obtained as a part of routine patients' assessment performed in nephrology ambulatory.

2.1. Statistical Analysis. A number of patients (percentage of the group) are reported for qualitative variables. Mean ± standard deviation or median (lower-upper quartile) are shown for normally or nonnormally distributed quantitative variables, respectively. The distributions of the variables were assessed with Shapiro-Wilk's test. The t-test or Mann-Whitney's U test was used to study differences between the groups. The variables that differed significantly between patients with high and low uNCR were used as predictor variables in multiple regression analysis. Odds ratios with 95% confidence intervals were reported for multiple regression analysis. Spearman's correlation coefficients are reported for correlations. All the tests were two-tailed and the results at $p < 0.05$ were considered statistically significant.

3. Results

The group of T2DM patients did not differ from the control group participants in terms of age, sex, eGFR, or cardiovascular comorbidities, but they had higher BMI (Table 1). Also, the average concentrations of urine albumin and NGAL, as well as the values of uACR and uNCR, did not differ between diabetic patients and control subjects (Table 1). However, the maximum uNCR in the control group was 39.64 μg/g and, among studied T2DM patients, 24 (20%) had higher values, with the maximum of 378.6 μg/g.

T2DM patients with uNCR above the maximum control value were characterized by higher triglycerides, total cholesterol, and LDL-cholesterol, as well as higher urine albumin and uACR as compared to patients with lower uNCR (Table 2). The correlation between uNCR and uACR

Table 1: Characteristics of studied patients.

	Control patients ($N = 22$)	T2DM patients ($N = 123$)	p value
Age, years	57 ± 15	62 ± 13	0.1
Male gender, N (%)	9 (41)	57 (46)	0.7
BMI, kg/m^2	28 ± 6	32 ± 6	0.009
eGFR, ml/min/1.73 m^2	87 ± 15	90 ± 17	0.3
Hypertension, N (%)	15 (68)	98 (80)	0.2
Ischemic heart disease, N (%)	3 (14)	24 (19)	0.5
Heart failure, N (%)	3 (14)	9 (7)	0.3
Dyslipidemia, N (%)	19 (95)	111 (95)	1.0
Treatment with ACEI or ARB, N (%)	12 (55)	86 (70)	0.2
Urine albumin, mg/l	5.9 (3.0–22.9)	8.0 (3.2–18.0)	0.6
uACR, mg/g	6.0 (3.6–9.0)	7.3 (3.4–19.2)	0.3
Urine NGAL, μg/l	10.9 (6.0–38.2)	15.3 (6.4–29.6)	0.7
uNCR, μg/g	12.2 (5.9–27.9)	13.5 (6.5–31.4)	0.3

T2DM, type 2 diabetes mellitus; BMI, body mass index; eGFR, estimated glomerular filtration rate; ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; uACR, urine albumin/creatinine ratio; NGAL, neutrophil gelatinase-associated lipocalin; uNCR, urine NGAL/creatinine ratio.

Table 2: The differences between T2DM patients with urine NGAL to creatinine ratio (uNCR) below and above the maximum control value.

	T2DM patients with uNCR ≤ 39.64 μg/g ($N = 99$)	T2DM patients with uNCR > 39.64 μg/g ($N = 24$)	p value
Age, years	62 ± 12	62 ± 17	0.9
Men, N (%)	54 (55)	3 (12)	<0.001
BMI, kg/m^2	32 ± 5	32 ± 7	0.9
Treatment with ACEI or ARB, N (%)	67 (68)	19 (79)	0.3
T2DM duration, years	6 (1–10)	6 (5–12)	0.5
Newly diagnosed diabetes, N (%)	27 (27)	4 (16)	0.3
Ophthalmologic examination, N (%)	83 (84)	11 (46)	<0.001
Retinopathy, N (% of examined)	17 (20)	2 (18)	0.9
HbA1c, %/mmol/mol	6.50 (5.90–8.50)/47.5 (41.0–69.4)	6.95 (6.10–8.60)/52.5 (43.2–70.5)	0.4
eGFR, ml/min/1.73 m^2	90 ± 17	91 ± 20	0.9
Triglycerides, mmol/l	1.56 (1.20–2.03)	2.42 (1.40–3.39)	0.021
Total cholesterol, mmol/l	4.59 (3.83–5.72)	5.74 (4.55–7.14)	0.003
LDL-cholesterol, mmol/l	2.60 (1.95–3.61)	3.69 (2.40–4.47)	0.004
HDL-cholesterol, mmol/l	1.19 (0.96–1.46)	1.23 (1.06–1.40)	0.5
Urine albumin, mg/l	6.8 (3.0–12.9)	16.3 (10.7–35.9)	0.003
uACR, mg/g	4.8 (3.1–13.0)	16.0 (9.1–50.0)	<0.001
uACR < 30 mg/g, N (%)	84 (88)	13 (54)	<0.001

T2DM, type 2 diabetes mellitus; BMI, body mass index; eGFR, estimated glomerular filtration rate; ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; uACR, urine albumin/creatinine ratio; NGAL, neutrophil gelatinase-associated lipocalin; uNCR, urine NGAL/creatinine ratio.

was highly significant (Figure 1(a)). Still, in 13 (54%) of the 24 patients with high uNCR values, uACR was below 30 mg/g and ranged from 2.35 to 16.10 mg/g. Interestingly, the patients with high uNCR were mainly women ($N = 21$, i.e., 88%). Women with T2DM had significantly higher uNCR than men [24.23 (8.89–56.80) versus 11.40 (3.36–18.02) μg/g; $p < 0.001$], without significant difference in uACR [8.87 (3.41–33.45) versus 5.33 (3.15–13.28) mg/g; $p = 0.09$]. The average concentrations of uNGAL were also higher in DMT2 women than men, although the difference was not statistically significant [17.15 (7.60–43.90) versus 13.70 (6.10–23.80) μg/l; $p = 0.1$].

Among 123 T2DM patients, 94 (76%) underwent the ophthalmologic examination, including 83 with low uNCR and 11 with high uNCR. We did not observe significant associations between uNCR and the presence of diabetic retinopathy (Table 2). Age, eGFR, and BMI values, as well as known diabetes duration, did not differ between the groups with uNCR

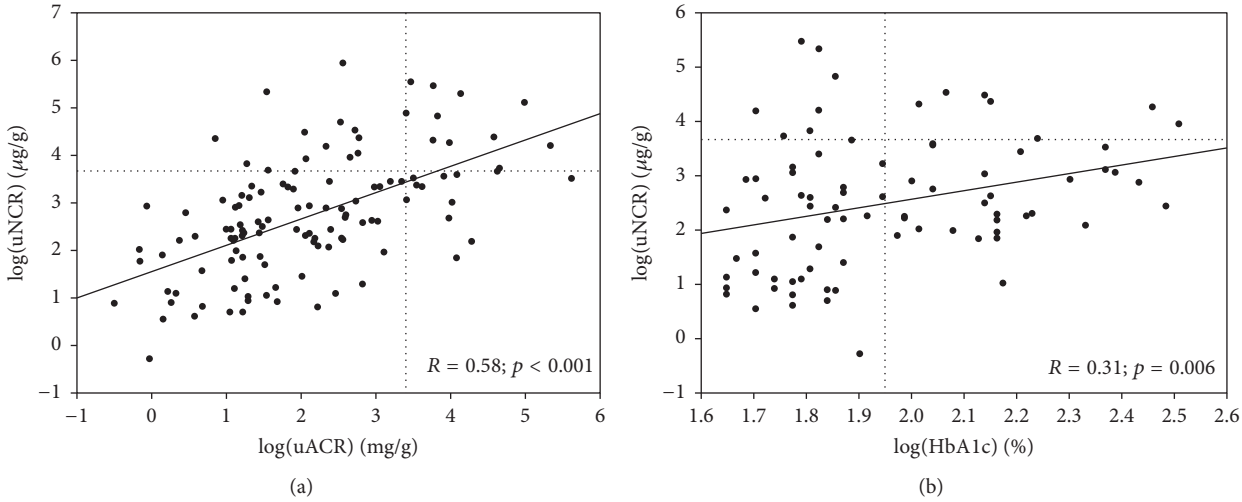

Figure 1: The associations between uNCR and uACR (a) and HbA1c (b) among T2DM patients. The reference lines denote uNCR = 39.64 μg/g (i.e., maximum in the control group), uACR = 30 mg/g, and HbA1c = 7%.

Table 3: Multiple linear regression to predict uNCR > 39.64 μg/g in patients with T2DM.

Dependent variables	Odds ratio (95% confidence interval)	p value
Women	7.98 (1.90–33.3)	0.004
Triglycerides, per 1 mmol/l	1.26 (0.73–2.16)	0.4
Total cholesterol, per 1 mmol/l	1.83 (1.15–2.91)	0.009
uACR, per 1 mg/g	1.03 (1.004–1.05)	0.022

T2DM, type 2 diabetes mellitus; uACR, urine albumin/creatinine ratio; uNCR, urine NGAL/creatinine ratio.

above and below 39.64 μg/g (Table 2). Although HbA1c was significantly correlated with uNCR (Figure 1(b)), it did not differ significantly between the patients with uNCR above and below the maximum control value (Table 2). uNCR did not correlate with eGFR ($R = -0.14$; $p = 0.1$), age ($R = 0.14$; $p = 0.1$), or time from T2DM diagnosis ($R = 0.13$; $p = 0.1$). uNCR was nonsignificantly higher among patients treated with angiotensin-converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARB) comparing with those not consuming the medications [15.87 (7.90–36.02) versus 9.72 (3.82–23.70) μg/g; $p = 0.053$], while uACR did not differ between the groups [7.89 (3.37–18.40) versus 4.58 (3.05–16.90); $p = 0.4$]. In multiple logistic regression (Table 3), sex, total cholesterol, and uACR were identified as the independent predictors of high uNCR (i.e., above the maximum control value of 39.64 μg/g).

In control group, no significant correlation was observed between uNCR and uACR. Also, there were no significant differences between control men and women in uNGAL and uNCR values. In contrast to DMT2 patients, uNGAL concentrations were nonsignificantly higher in control men [20.10 (7.40–48.40) versus 10.50 (6.00–20.30) μg/l; $p = 0.1$]; however, uNCR was nonsignificantly higher in control women [12.53 (6.42–28.81) versus 7.73 (5.92–13.69) μg/g; $p = 0.3$].

4. Discussion

In most cases, recognition of the diabetic kidney disease (DKD) is based on results of tests such as albuminuria, creatininemia with the estimation of eGFR, or renal imaging. Clinical symptoms of DKD appear late and are not characteristic. In T1DM, DKD coexists with diabetic retinopathy that can be detected in ophthalmologic examination. However, in T2DM, DKD may be present in patients without retinopathy [17, 18]. Diabetic retinopathy is less frequent in T2DM and is a poor predictor of type of nephropathy [18]. Our results are consistent with these observations, as we did not observe significant associations between uNCR and the presence of retinopathy. However, our observations regarding retinopathy must be treated with caution, as only a part of patients underwent ophthalmologic examination.

Renal biopsy is performed only in a relatively small number of T2DM patients. This usually happens during advanced stages of the disease when serum creatinine is elevated and overt proteinuria occurs. In T2DM patients, the morphological abnormalities in kidneys and the clinical course of DKD are varied. In this group of patients, not only classical glomerular changes but also changes in renal tubules and in the renal interstitium play an important role in kidney failure [5, 6, 19]. In nearly 40% of T2DM patients, renal biopsy does not reveal typical glomerular pattern as observed in T1DM. In a substantial proportion of such patients, biopsy results show disproportionately severe damage to the tubulointerstitial tissue as well as hyaline changes in small renal arteries [5, 6]. For these reasons, eGFR and albuminuria (proposed by Kidney Diseases Improving Global Outcomes initiative [20] for the clinical assessment and prediction of

CKD progression) may be insufficient in the early assessment of kidney function among T2DM patients. If albuminuria and uACR, together with eGFR, are considered markers of glomerular damage, then in the group of T2DM in our study the occurrence of CKD stages G1, G2, and A1 can only be suspected; we can, however, recognize stages G1, G2, and A2. In turn, when the uNCR above 39.64 μg/g (i.e., the maximum uNCR in the control group) is considered a marker of tubular and interstitial damage, a subgroup of 20% of patients with tubular damage can be distinguished. This tubular damage cannot be discovered during routine nephrological diagnostic tests. More than half of the patients in our study had normal uACR values according to the current diagnostic criteria [20], that is, uACR lower than 30 mg/g. In such patients, a clinicist may not become alert enough to be able to recognize early nephropathy connected with T2DM. Our study shows that especially in women with T2DM with abnormal lipid profile and inadequate diabetes control the diabetic kidney disease may be underdiagnosed. The United Kingdom prospective diabetes study (UKPDS) [21] indicates that in women with T2DM the decrease in glomerular filtration is frequently not accompanied by albuminuria. Similarly, in a study of Parving et al. [22], more than 50% of patients had no albuminuria. The study analyzed data from more than 24000 T2DM patients, nearly 80% of whom had glomerular filtration above 60 ml/min/1.73 m^2 [22].

Higher uNCR values in women with T2DM are partly due to lower urine creatinine excretion in women than in men. We have observed this both in T2DM patients and in controls. However, control women had lower uNGAL concentrations than control men while, among T2DM patients, uNGAL was higher in women than in men. Thrailkill et al. [23] observed higher uNGAL concentrations in females compared to males in subjects with T1DM [23]. Higher uNCR values in women with T2DM suggest that the early stages of DKD may be similarly common in both sexes or even more common in women, although end-stage renal disease is in fact more common in diabetic men [24–26]. Female gender is protective against the development of end-stage renal disease in nondiabetic renal disease [24, 27] but this gender-protective effect is probably diminished in diabetes mellitus [28, 29].

In our study, uNCR correlated positively with uACR in the T2DM patients. This is consistent with the results of Nielsen et al. [30], who observed correlation between uNGAL and albuminuria among 177 patients with T2DM and normal eGFR during 3.5 years of follow-up. Increased uNGAL predicted the increase in urinary albumin excretion ranging from "microalbuminuria" to "macroalbuminuria" and higher concentrations of uNGAL were associated with a more rapid deterioration of renal function [30]. Our study indicates that patients with uNCR above 39.64 μg/g had on average higher albuminuria and uACR. Several pathomechanisms may be listed as underlying this observation. If we assume DKD with primary glomerular involvement, the increased urinary excretion of NGAL may result from disrupted mechanisms of protein transport involving megalin and cubilin, caused by long-term, excessive reabsorption of albumin in tubules [31, 32]. Also, other substances that leak to primary urine through the damaged glomerular barrier may cause tubular cells' damage, hence initiating inflammation and the process of renal interstitial fibrosis. This, in turn, contributes to further kidney damage resulting in albuminuria cooccurring with tubular proteinuria and increased urinary excretion of NGAL [33]. However, as clearly indicated in practice guidelines on DKD [34], nephropathy other than early glomerular damage may be responsible for the increased urinary excretion of NGAL in diabetic patients. Hence, in our study, the increase in uNCR in the group of patients with normal albuminuria may also be linked with primary tubular damage. In diabetic patients, tubular cells are negatively affected by hyperglycemic environment. This leads to the development of inflammation in the tubulointerstitial tissue, increased production of extracellular matrix, and epithelial-mesenchymal transition of renal tubular cells [35]. In result of active inflammation, the tubulointerstitial tissue is infiltrated by leucocytes, including monocytes that differentiate into tissue macrophages and initiate the repair process and induce fibrosis. This may lead to an increase in the values of uNCR and/or uACR in some T2DM patients [36–38]. In our study, 20% of T2DM patients had elevated uNCR (above the maximum value in the control group), and less than half of those patients had elevated albuminuria. Fu et al. [14] observed that tubular damage defined by the increase in uNCR appears even in patients with diabetes of short duration, and the uNGAL may become a more promising and earlier marker of kidney damage in T2DM than uACR. Similarly, in the study by Kim et al. [15] nonalbuminuric proteinuria correlated significantly with uNGAL in patients with early-stage DKD (eGFR $\geq$ 60 ml/min/1.73 m^2).

The majority of our patients were treated with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers as part of nephroprotection in DKD [34]. We have not observed statistically significant differences in uNCR or uACR between patients treated with renin-angiotensin-aldosterone (RAA) system blockade and those not treated, although there was a tendency towards higher uNCR in patients who were on such treatment. These results may suggest poor protective effect of RAA blockade against tubular changes in T2DM. This observation is consistent with the results of Nielsen et al. [30] who reported no effects of angiotensin-converting enzyme inhibitors treatment on uNGAL concentration in patients with T2DM with normal glomerular filtration rate and "microalbuminuria."

In the present study, patients with better diabetes control and less atherogenic lipid profile had also lower uNCR values. There is evidence that, in T2DM patients with CKD stages 1 to 4, better glycemic control contributes to improved kidney function and brings benefits to the vascular system [22, 39]. Also, some studies suggest that lowering total cholesterol slows down the progression of renal disease in T2DM [40, 41]. As uNCR value may be considered a noninvasive indicator of renal tubules' function, the results of the present study lead us to hypothesis that better diabetes control together with the treatment of dyslipidemia may have a positive

influence on the tubule function and probably also renal interstitial changes. However, to validate this hypothesis, further prospective studies on a larger population of T2DM patients are required.

5. Conclusion

Combinations of biomarkers representing different mechanisms of DKD pathogenesis may be helpful in the determination of a pattern of changes in kidney function, especially in the heterogenic group of T2DM patients. Our results suggest that the determination of uNCR in addition to uACR and eGFR enables early detection of kidney disease in a part of patients with T2DM. Our results should be treated with caution, because of the limited numbers of T2DM patients and low number of controls recruited. However, our results suggest that uNCR values higher than a cut-off value (39.64 μg/g in our sample) may be an indicator of early damage to renal tubules, especially in T2DM women with dyslipidemia and worse diabetes control. The results, especially the cut-off value, should be validated in larger study.

Disclosure

Preliminary results of the study were presented in a poster session of the American Society of Nephrology in San Diego in 2015.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Antiangiogenic Therapy for Diabetic Nephropathy

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Angiogenesis has been shown to be a potential therapeutic target for early stages of diabetic nephropathy in a number of animal experiments. Vascular endothelial growth factor (VEGF) is the main mediator for abnormal angiogenesis in diabetic glomeruli. Although beneficial effects of anti-VEGF antibodies have previously been demonstrated in diabetic animal experiments, recent basic and clinical evidence has revealed that the blockade of VEGF signaling resulted in proteinuria and renal thrombotic microangiopathy, suggesting the importance of maintaining normal levels of VEGF in the kidneys. Therefore, antiangiogenic therapy for diabetic nephropathy should eliminate excessive glomerular angiogenic response without accelerating endothelial injury. Some endogenous antiangiogenic factors such as endostatin and tumstatin inhibit overactivation of endothelial cells but do not specifically block VEGF signaling. In addition, the novel endothelium-derived antiangiogenic factor vasohibin-1 enhances stress tolerance and survival of the endothelial cells, while inhibiting excess angiogenesis. These factors have been demonstrated to suppress albuminuria and glomerular alterations in a diabetic mouse model. Thus, antiangiogenic therapy with promising candidates will possibly improve renal prognosis in patients with early stages of diabetic nephropathy.

1. Introduction

Diabetic nephropathy has become a leading cause of end-stage kidney disease (ESKD) in developed countries. The global pandemic of obesity will further result in the increased prevalence of diabetic nephropathy. The current mainstay of the treatment of diabetic nephropathy is glycemic control, as well as lowering blood pressure with specific classes of antihypertensive agents that block renin-angiotensin-aldosterone system (RAAS). RAAS inhibitors have been demonstrated to have renoprotective effects in patients with diabetic nephropathy, but their efficacies have not always been determined to be sufficient in clinical practice. In the same way, intensive glycemic control resulted in inconsistent benefits in patient with nephropathy among large clinical trials. Thus, once overt diabetic nephropathy develops, specific therapies targeting the underlying mechanisms are required in order to prevent the progression to ESKD, in addition to blood pressure control with RAAS inhibitors and appropriate glycemic control.

Angiogenesis is one of the potential targets for the treatment of diabetic nephropathy. Vascular endothelial growth factor (VEGF) is a critical regulator of angiogenesis, and its glomerular expression is involved in the pathogenesis of diabetic nephropathy. Antiangiogenic (in particular, anti-VEGF) therapy for diabetic nephropathy has been shown to be a promising strategy in many animal experiments, but some recent evidence raises concerns about its use in clinical practice. In this review, we will outline abnormal angiogenesis and VEGF in the pathogenesis of diabetic nephropathy, explain the benefits and limitations of antiangiogenic therapy, and then finally propose alternative antiangiogenic strategies to address such concerns.

2. Roles of VEGF in Angiogenesis

Angiogenesis is the physiological and pathological process through which new blood vessels develop from preexisting vessels. It is involved in embryogenesis, in wound healing,

in tumor growth and metastasis, in atherosclerosis, and in the onset of inflammatory diseases in humans [1]. A number of proangiogenic and antiangiogenic factors are responsible for regulating angiogenesis, including VEGF, basic fibroblast growth factor (bFGF), angiopoietins, and ephrin.

VEGF is one of the most potent proangiogenic factors. The VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) in mammals [2]. VEGF-A is a prototype member of the family and is crucially involved in physiological and pathological angiogenesis. VEGF-A shows haploid insufficiency, as inactivation of a single copy of the gene resulted in embryonic lethality in mice due to immature organ development, including impaired blood vessel formation [3, 4], suggesting an essential role of VEGF-A in vasculogenesis and angiogenesis. VEGF-A has a variety of functions: though perhaps most importantly, it promotes angiogenesis through stimulation of the proliferation and migration of endothelial cells [5]. VEGF-A also has vascular permeability activity and monocyte chemotactic activity [6, 7], which are involved in inflammation in some pathological processes. There are several isoforms of VEGF-A through alternative splicing, such as VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{165}b, VEGF-A_{189}, and VEGF-A_{206} in humans [8–10]. Among isoforms of VEGF-A, VEGF-A_{165} is quantitatively and qualitatively predominant.

VEGF-A binds to and activates the tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) [2]. VEGFR-1 has a much higher affinity for VEGF-A, whereas VEGFR-2 has approximately 10-fold higher tyrosine kinase activity [11]. Therefore, angiogenic signals are mainly generated from VEGF-A bound to VEGFR-2, whereas VEGFR-1 could play as negative regulator of VEGF-A at least in some conditions, such as embryogenesis. VEGF-A-bound VEGFR-2 undergoes dimerization and tyrosine phosphorylation, and this reaction promotes the phosphorylation of several targets, including phosphoinositide 3-kinase (PI3K) and Ras GTPase-activating proteins [12]. Phospholipase Cγ is also activated in VEGF-A-bound VEGFR-2, followed by the activation of protein kinase C (PKC), especially PKCβ [13, 14]. PKC then activates Raf-MEK-extracellular signal-regulated (ERK) pathways for endothelial cell proliferation [14]. In addition, the activation of VEGFR-2 inhibits apoptosis of endothelial cells via the PI3K-Akt pathway [15]. VEGF-A also binds to neuropilin 1 (NRP1), which presents VEGF-A to VEGFR-2 and enhances VEGF-A-induced VEGFR-2 signaling [16]. Compared with VEGF-A_{165}, VEGF-A_{165}b does not fully activate VEGFR-2 and thus could act as a much weaker agonist for VEGFR-2 than VEGF-A_{165} [17]. This is potentially explained by the fact that VEGF-A_{165}b has distinct C-terminal amino acid sequence, which leads to insufficient interaction with NRP1 [18]. VEGF-A_{121} could bind to NRP1, with lower affinity than VEGF-A_{165} [19]. On the other hand, VEGF-A_{189} has higher affinity for NRP1 than VEGF-A_{165} [20]. However, secreted VEGF-A_{189} could be trapped by extracellular matrix through its highly basic sequences [21]. Therefore, VEGF-A_{165} has the most potent agonist activity for VEGFR-2 in vivo.

3. Angiogenesis in Diabetic Nephropathy

Diabetic nephropathy is clinically defined by the presence of microalbuminuria followed by a progressive increase in proteinuria and a decrease in glomerular filtration rate (GFR) in the setting of long-standing diabetes with or without other microangiopathies, such as retinopathy. An earlier finding of (preclinical) diabetic nephropathy is glomerular hyperfiltration seen as increased GFR, with no morphological changes or only glomerular hypertrophy. Histologically, glomerular alterations in diabetic nephropathy include glomerular basement membrane thickening and/or mesangial matrix accumulation in the early stages and Kimmelstiel-Wilson's nodular lesions with or without microaneurysm and mesangiolysis, eventually leading to glomerulosclerosis, in advanced stages.

Abnormal angiogenesis has long been implicated in the morphology and pathophysiology of diabetic nephropathy. Initially, new blood vessel formation in glomeruli representing aberrant angiogenesis was reported in patients with type 1 diabetes [22]. Other groups then reported similar findings in patients with type 2 diabetes [23, 24]. Such abnormal blood vessels were observed in the glomerular tuft area, Bowman's capsule, and the glomerular vascular pole [22, 25]. An interesting study using computer-aided reconstruction of three-dimensional images in patients with diabetic nephropathy demonstrated that these abnormal vessels are anastomosed to lobular structure of the intraglomerular capillary network and that the distal end of the vessels is anastomosed to the peritubular capillary [26]. In animal studies, type 1 and type 2 diabetic rodent models showed formation of new glomerular capillaries and the elongation of preexisting capillaries [27, 28], which are similar findings to those observed in human diabetic nephropathy. These abnormal new vessels have been considered to be associated with the increased glomerular filtration surface, leading to glomerular hypertrophy and hyperfiltration in the early stages of diabetic nephropathy.

VEGF-A expression is likely to be associated with the formation of abnormal new vessels. In an experimental diabetic model, renal protein and mRNA levels of VEGF-A and VEGFR-2 were upregulated in the early stage, and these increased levels persisted in the late stage [29]. Similarly, plasma and urinary levels of VEGF-A were elevated in patients with diabetic nephropathy [30, 31]. Recent clinical study revealed that increased circulating VEGF-A in type 2 diabetic patients was correlated with glycemic control, high-sensitive C-reactive protein, and albuminuria, suggesting the role of VEGF-A as a biomarker of inflammation and nephropathy in diabetes [32]. Another study involving type 2 diabetic patients showed a significant correlation between circulating VEGF-A and serum levels of hypoxia-inducible factor-1α (HIF-1α) and insulin-like growth factor-1 (IGF-1), which are considered to be involved in the pathogenesis of diabetic nephropathy [33]. However, some results of glomerular VEGF-A expression in human diabetic nephropathy have been controversial. Immunohistochemical analysis on renal biopsies revealed that glomerular VEGF-A was increased in the early stage of diabetic nephropathy [34], whereas oligonucleotide microarray analysis on human

kidneys demonstrated that glomerular VEGF-A mRNA levels were decreased in patients with diabetic nephropathy [35]. Considering the fact that the decreased VEGF-A was observed in severely injured glomeruli with reduced podocyte markers in the latter study, glomerular VEGF-A levels might in fact decrease in the advanced stage of diabetic nephropathy. Indeed, glomerular expression of VEGF-A was shown to be decreased in sclerotic areas and nodular lesions of human diabetic nephropathy [36]. Therefore, increased glomerular VEGF-A in the early stage is probably involved in the characteristic alterations, including abnormal angiogenesis, while decreased glomerular VEGF-A in the later stage of the disease may promote glomerular scarring.

4. Biology of Glomerular VEGF

VEGF-A plays important role not only in maintaining glomerular capillary structure but also in repairing it following glomerular endothelial injuries [37, 38]. VEGF-A is constitutively expressed in podocytes and, to lesser extent, in tubular epithelial cells [39]. Since the expression of VEGFR-2 is localized to endothelial cells in glomeruli [40], there are important interactions that occur between podocytes and glomerular endothelial cells via the VEGF-A-VEGFR-2 axis. Indeed, VEGF-A is considered to be transported via diffusion across the glomerular basement membrane from podocytes to endothelial cells, against the flow of glomerular filtration [41].

Pivotal roles of VEGF-A expression in podocytes were demonstrated via a series of elegant experiments using genetically modified mice. Podocyte-specific heterozygous VEGF-A deficient mice showed proteinuria and glomerular endothelial injury similar to preeclampsia, and podocyte-specific VEGF-A_{164}-overexpressing mice showed marked collapsing glomerulopathy [42]. In another report, transgenic rabbits that express human VEGF-A_{165} in both the kidneys and liver under the control of a α1-antitrypsin promoter also exhibited progressive proteinuria and renal dysfunction with prominent glomerular capillary proliferation and podocyte hypertrophy at the early stage and then glomerulosclerosis and tuft collapse at the later stage [43]. More recently, the importance of glomerular VEGF-A expression in an adult kidney was examined using conditional gene expression, or the deletion technique. Eremina et al. conditionally deleted VEGF-A gene from podocytes in adult mice and observed increased proteinuria as well as intracapillary thrombi and obliterated capillary loops with swollen endothelial cells, resembling renal thrombotic microangiopathy [44]. On the other hand, Veron et al. induced podocyte-specific overexpression of VEGF-A in adult transgenic mice and observed proteinuria, glomerulomegaly, glomerular basement membrane thickening, mesangial expansion, and podocyte effacement [45]. Taken together, these results suggested that a "normal" level of VEGF-A is essential for maintaining the glomerular capillary structure, including the glomerular filtration barrier in the adult kidneys, and both too much and too little VEGF-A in glomeruli can lead to significant renal pathology (Figure 1).

In diabetic nephropathy, VEGF-A is likely a crucial mediator, according to a number of publications. As described above, increased glomerular expression of VEGF-A is seen in patients with diabetic nephropathy as well as in its animal models. In addition, human VEGF-A_{165} transgenic rabbits developed microaneurysms [43], while podocyte-specific VEGF-A-overexpressing mice showed glomerular basement membrane thickening and mesangial expansion [45], both of which are similar to the histology of diabetic nephropathy. Furthermore, selective VEGFR-2 stimulation by overexpression of the mutant form of VEGF-A binding only to VEGFR-2 in mice resulted in mesangial matrix expansion with endothelial cell proliferation [48]. These findings suggest that increased glomerular expression of VEGF-A is sufficient to cause early glomerular alterations in diabetic nephropathy. However, hyperglycemia is necessary to develop advanced lesions. Veron et al. induced diabetes in podocyte-specific conditional VEGF-A_{164} transgenic mice and observed massive proteinuria as well as Kimmelstiel-Wilson-like nodular glomerulosclerosis, microaneurysms, and mesangiolysis in the glomeruli of the mice [46], which was consistent with advanced diabetic glomerulopathy (Figure 1).

Such synergistic effects of hyperglycemia and increased VEGF-A in diabetic glomerulopathy may be explained by the unique hypothesis of "uncoupling of VEGF-A with nitric oxide (NO)" [49, 50]. Normally, VEGF-A stimulates endothelial NO release, and NO is required for the actions of VEGF-A on endothelial cells. When hyperglycemia impairs normal endothelial function and reduces NO production, elevated levels of glomerular VEGF-A noted in diabetes could exert deleterious effects on endothelial cells, leading to diabetic glomerulopathy. Indeed, endothelial NO synthase- (eNOS-) deficient mice with streptozotocin-induced hyperglycemia (type 1 diabetic model) or those crossbred with obese db/db mice (type 2 diabetic model) both exhibited massive proteinuria and glomerular alterations identical to human advanced diabetic nephropathy [51, 52]. Diabetic eNOS deficient mice also developed profound podocyte injuries, possibly due to the impairment of crosstalk between glomerular endothelial cells and podocytes [53]. Similarly, db/db mice with VEGFR-1 inhibition, which enhanced VEGFR-2 activity, showed prominent albuminuria and mesangial expansion together with a loss of podocytes and endothelial cells [54]. Furthermore, even without hyperglycemia, podocyte-specific VEGF-A overexpression in eNOS-null mice resulted in nodular glomerulosclerosis, mesangiolysis, and microaneurysms that were associated with massive proteinuria [55]. Conversely, NO donor nicorandil ameliorated proteinuria and glomerular pathology, including podocyte injury, in diabetic eNOS knockout mice [56]. These findings emphasize the advantage of specific therapies targeting uncoupling of VEGF-A with NO in diabetic nephropathy by suppressing increased glomerular VEGF-A or supplementing endothelial NO.

VEGF-A_{165}b is also expressed in immature podocytes, but the expression is lower in matured glomeruli [57]. Recently, VEGF-A_{165}b was shown to be upregulated in renal cortical tissues taken from patients with early diabetic nephropathy [58]. Podocyte-specific overexpression of VEGF-A_{165}b in diabetic mice resulted in the amelioration of diabetic

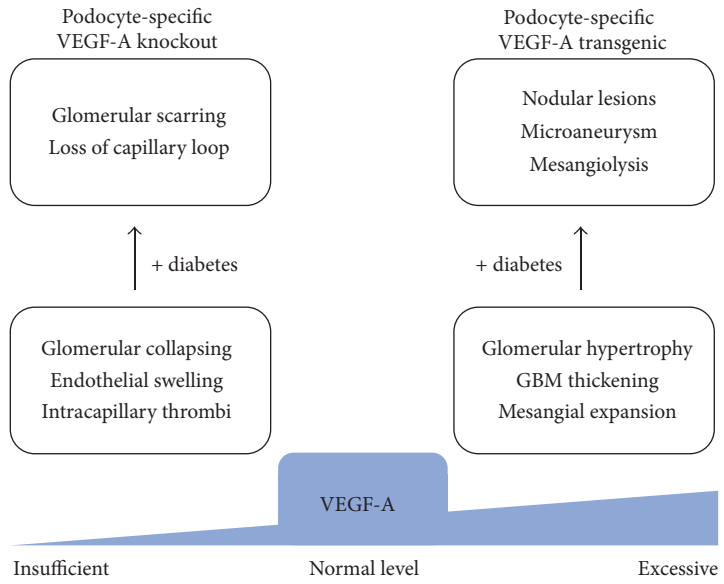

Figure 1: Histological alterations of glomeruli associated with excessive ("too much") or insufficient ("too little") glomerular vascular endothelial growth factor (VEGF)-A. In conditionally podocyte-specific VEGF-A transgenic mice, VEGF-A-overexpressing glomeruli become hypertrophic with glomerular basement membrane (GBM) thickening and mesangial expansion, similar to early stages of diabetic glomerulopathy [45]. Induction of diabetes in these transgenic mice results in Kimmelstiel-Wilson-like nodular lesions, microaneurysms, and mesangiolysis in the glomeruli [46], which are observed in advanced diabetic nephropathy. In contrast, conditionally podocyte-specific VEGF-A deficient mice show glomerular capillary thrombi and obliterated capillary loops with swollen endothelial cells, consistent with the findings of renal thrombotic microangiopathy [44]. Diabetes accelerates the dropout of glomerular capillaries in this conditional knockout mice, leading to glomerulosclerosis [47].

glomerulopathy [58], suggesting the protective role of increased VEGF-A_{165}b in diabetic nephropathy.

Unlike VEGF-A, involvement of the other VEGF family members in diabetic glomerulopathy has not been well-established. It has been shown that VEGF-B is mainly expressed in renal medullary tubular cells, but not in glomeruli, whereas expression of its receptor VEGFR-1 was found in endothelial cells [59]. However, a recent report did demonstrate that VEGF-B inhibition suppressed histological alterations and renal dysfunction in diabetic mice [60]. Although VEGFR-1 inhibition results in the exacerbation of diabetic glomerulopathy as above [54], VEGF-B inhibition was found to reduce lipotoxicity and improve insulin-resistance in podocytes in this study [60], suggesting that VEGF-B is probably involved in diabetic glomerulopathy through nonangiogenic mechanisms.

5. Anti-VEGF Therapies for Diabetic Nephropathy

As described above, the fact that the increased expression of VEGF-A in podocytes associated with hyperglycemia leads to characteristic glomerular alterations provides the rationale for anti-VEGF therapy against diabetic nephropathy. The landmark experiments revealed that the administration of neutralizing monoclonal anti-VEGF antibodies to type 1 and type 2 diabetic animals decreased albuminuria and glomerular hypertrophy [61, 62], indicating the efficacy of anti-VEGF therapy against diabetic nephropathy. Then, SU5416, a pan-VEGF receptor tyrosine kinase inhibitor, was also reported to reduce albuminuria in type 2 diabetic mice [63].

However, there have been emerging concerns about anti-VEGF therapy in humans. Soon after introduction of bevacizumab, a humanized monoclonal anti-VEGF antibody, in clinical practice to prevent cancer growth and metastasis, proteinuria and hypertension were reported to occur as common complications [64]. These clinical findings were subsequently reported in patients treated with multitargeted tyrosine kinase inhibitors (TKIs), small molecules that inhibit VEGFR intracellular intrinsic kinases, such as sunitinib and sorafenib [65]. Based on the observation of decreased urinary nitrite/nitrate excretion and serum levels of NO metabolites in patients treated with VEGF inhibitors [66], hypertension induced by anti-VEGF antibody may be involved in the disruption of the VEGF-A-endothelial NO axis as noted above. Proteinuria is probably caused as a result of impaired interaction between podocytes and glomerular endothelial cells. Eremina et al. first reported that bevacizumab treatment in some cancer patients led to a renal pathology of glomerular endothelial swelling, red blood cell fragmentation, and intracapillary thrombi, which were characteristics of thrombotic microangiopathy, and subsequently reproduced these findings in podocyte-specific VEGF-A-deficient mice as above [44]. Recently, the novel role of VEGF-A in the kidneys was revealed as a potential mechanism underlying bevacizumab-related renal thrombotic microangiopathy. VEGF-A inhibition decreased

Table 1: Candidates of endogenous antiangiogenic factors as therapeutic agents for diabetic nephropathy.

	Description	Target molecule	Effect on ECs	Clinical use
sFlt-1	Soluble form of VEGFR-1 that binds to circulating VEGF and prevents it from binding to VEGFR-2.	VEGF	Apoptosis	None
VEGF-A_{165}b	Inhibitory VEGF-A splice variant which induces insufficient phosphorylation of VEGFR-2.	VEGFR-2	Survival?*	None
Tumstatin	Protein fragment cleaved from type IV collagen that binds to endothelium via integrin and inhibits protein synthesis.	$\alpha v\beta 3$-integrin	Apoptosis	None
Endostatin	Protein fragment cleaved from type XVIII collagen which acts on endothelium to suppress cell cycle genes and antiapoptotic genes.	$\alpha 5\beta 1$-integrin (glypicans, VEGFR-2)	Apoptosis	Available in China
Angiostatin	Protein fragment cleaved from plasminogen which binds to potentially many proteins to induce its apoptosis.	Angiomotin and others**	Apoptosis	Under trial
Vasohibin-1	Endothelium-derived protein that causes negative feedback response in endothelial cells stimulated by VEGF-A and promotes its survival by inducing SOD2 and Sirt1.	Unknown	Survival	None

ECs, endothelial cells; sFlt-1, soluble fms-like tyrosine kinase-1; VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2; SOD2, superoxide dismutase-2; Sirt1, sirtuin-1. *VEGF -A_{165}b may attenuate endothelial survival effect of VEGF-A_{165}, but endothelial protective effect was also reported (see text). **Other representative molecules include surface ATP synthase, NG2 proteoglycan, c-Met, and annexin II.

the renal level of inhibitory complement factor H (CFH), in which genetic variants were known to be features of hereditary thrombotic microangiopathy, suggesting that VEGF-A is involved in local regulation of the complement system [67]. Therefore, anti-VEGF antibody therapy for diabetic nephropathy needs to eliminate only the "excess" glomerular VEGF-A but must not lower it to a subnormal level. However, considering that diabetes induces endothelial dysfunction and reduces NO bioavailability, administration of anti-VEGF antibodies in the diabetic condition is likely to result in proteinuria and renal dysfunction. Indeed, diabetes was a major risk factor for proteinuria in bevacizumab-treated patients [68, 69]. Furthermore, in conditionally podocyte-specific VEGF-A-deficient mice, diabetes accelerated proteinuria and apoptosis of glomerular endothelial cells, leading to profound glomerular scarring [47] (Figure 1). At present, anti-VEGF antibody or TKIs therapy for diabetic nephropathy is not warranted.

There are several endogenous antiangiogenic systems to prevent excessive angiogenesis in the body (Table 1). Important information regarding such systems has been derived from understanding the pathogenesis of preeclampsia. One of the endogenous antiangiogenic factors involved in preeclampsia is soluble fms-like tyrosine kinase (sFlt-1). sFlt-1 is a soluble form of VEGFR-1 capable of binding to VEGF-A, VEGF-B, and PlGF and acts as a potent VEGF antagonist. Conditionally podocyte-specific overexpression of sFlt-1 in mice ameliorated diabetic glomerulopathy as well as albuminuria [70], and adeno-associated virus transferred sFlt-1 overexpression in db/db mice resulted in reduced albuminuria and improved podocyte injury [71]. Furthermore, podocyte-specific overexpression of angiopoietin-1, which was a regulator for vascular stabilization, was recently shown to prevent albuminuria as well as glomerular endothelial proliferation with elevated levels of sFlt-1 [72]. However, in an earlier report, the intravenous injection of sFlt-1 in mice induced proteinuria [73]. In addition, adenoviral transfer of sFlt-1 in mice induced proteinuria and caused glomerular endotheliosis similar to VEGF-A-deficient glomeruli [74]. Thus, sFlt-1 therapy for diabetic nephropathy potentially has the same concerns as the use of anti-VEGF antibodies.

VEGF-A_{165}b acts as a weaker VEGFR-2 agonist (Table 1). Recently, therapeutic effects of recombinant human VEGF-A_{165}b in type 1 and type 2 diabetic mice were reported. In this study, VEGF-A_{165}b ameliorated albuminuria and glomerular basement membrane thickening and normalized VEGFR-2-mediated glomerular permeability [58]. In addition, VEGF-A_{165}b restored glomerular endothelial glycocalyx, which constitutes a barrier to glomerular permeability and is injured by diabetes [58], suggesting that VEGF-A_{165}b possibly provides

endothelial protection in diabetic nephropathy. Interestingly, podocyte-specific overexpression of VEGF-A_{165}b in mice reduced endothelial fenestrations [75] but did not result in increased urinary albumin excretion and renal thrombotic microangiopathy [58]. Moreover, VEGF-A_{165}b was reported to protect endothelial cells from cytotoxicity induced by serum starvation in vitro [76]. As such, VEGF-A_{165}b may be a promising therapy for those diagnosed as having diabetic nephropathy. However, given the weak agonist activity of VEGF-A_{165}b for VEGFR-2, an appropriate dosage regimen would need to be addressed in the future studies.

6. Alternative Antiangiogenic Therapies

Although anti-VEGF therapy could lead to the potential renal adverse effects in cancer treatment, a much lower dose regimen may be a possible option for use in those with diabetic nephropathy. However, the therapeutic efficacy of such low-dose regimen has not yet been investigated in diabetic animal experiments. Novel drugs targeting intracellular downstream signaling pathways of VEGFR-2 could be effective and safe therapeutic options in the future. Considering the limitations of current anti-VEGF therapies, alternative antiangiogenic therapies that do not serve as direct inhibitors of VEGF-A may have potential benefits for the treatment of diabetic nephropathy. Some extracellular matrix protein fragments are known to act as circulating endogenous antiangiogenic factors and have antitumor efficacies (Table 1). These antiangiogenic factors interfere with VEGF-A signaling processes but do not directly antagonize VEGF-A.

Tumstatin is derived from a type IV collagen α3 chain and inhibits pathological angiogenesis via suppression of endothelial cell proliferation [77]. It binds to the $\alpha v\beta 3$ integrin of endothelial cells [78]. The antiangiogenic activity of tumstatin is considered to be based on the inhibition of focal adhesion kinase (FAK), PI3 kinase, protein kinase B (PKB/Akt), and the mammalian target of rapamycin (mTOR) in endothelial cells [79]. The therapeutic effects of tumstatin-derived peptide on diabetic nephropathy in a type 1 diabetic mouse model were examined [80]. In the study, tumstatin peptide significantly suppressed albuminuria and glomerular histological alterations as well as increased the number of glomerular capillaries in diabetic mice. Tumstatin peptide also significantly suppressed the increase in renal VEGF-A and VEGFR-2 induced by diabetes.

Endostatin is derived from type XVIII collagen and possesses potent inhibitory effects on tumor growth [81]. It also inhibits VEGF-induced endothelial cell proliferation, migration, and tube formation in vitro [82]. Endostatin interacts with $\alpha 5\beta 1$ integrin, leading to the inhibition of FAK and subsequent inhibition of mitogen-activated protein kinases (MAPKs) [83]. The therapeutic potential of endostatin in nonneoplastic disorders with angiogenic processes has been reported [84–86]. In type 1 diabetic mice, endostatin peptides significantly suppressed albuminuria and histological alterations [87]. They also significantly suppressed the expansion of glomerular capillary area and increased VEGF-A and VEGFR-2 in diabetic mice. Because $\alpha 5\beta 1$ integrin is localized to endothelial cells in glomeruli and upregulated in diabetes [87], endostatin primarily acted on the glomerular endothelial cells. On the other hand, recent clinical study revealed that type 2 diabetic patients with nephropathy had a higher circulating level of endostatin, indicating the clinical usefulness of endostatin as a risk marker of diabetic nephropathy [88].

Unlike tumstatin and endostatin, angiostatin is a proteolytic fragment of plasminogen and inhibits tumor neovascularization [89]. Adenoviral delivery of angiostatin significantly ameliorated albuminuria and glomerular hypertrophy in a type 1 diabetic rat model [90]. It also suppressed the increased expression of VEGF-A in diabetic kidneys. In another report, however, adenoviral overexpression of angiostatin in a remnant kidney model resulted in a reduction in peritubular capillary density [91].

Taken together, these results suggest the therapeutic potential of tumstatin, endostatin, and angiostatin in diabetic nephropathy. However, their therapeutic effects may not necessarily be associated with antiangiogenic properties. For example, as $\alpha v\beta 3$ integrin, the receptor for tumstatin, is heavily expressed in podocytes [92], the primary target for tumstatin may not be endothelial cells but podocytes. Endostatin suppressed glomerular VEGF-A mainly produced by podocytes in diabetic mice [87], and thus it might act on podocytes rather than endothelial cells. Therefore, the mechanisms of their therapeutic efficacy in diabetic nephropathy, as well as antiangiogenic effects on endothelial cells, have not yet been convincingly and fully elucidated. This fact limits the clinical use of these fragments as antiangiogenic drugs for diabetic nephropathy. Among these fragments, endostatin has been already introduced in clinical practice as an anticancer drug to be combined with definitive chemotherapy. Recombinant human endostatin (Endostar®, Nanjing NingQi Medicine Science and Technology Co., Ltd., Nanjing, China) was developed and approved for lung cancer in China. Notably, recombinant human endostatin did not result in hypertension and proteinuria [93, 94] or did not significantly elevate the incidence of proteinuria [95] in clinical trials. Recombinant human angiostatin is undergoing clinical trial for anticancer efficacy in patients with lung cancer, but this trial has not been completed. Tumstatin has not been considered in clinical trials yet.

7. Vasohibin-1 as a Novel Therapeutic Agent

A novel endogenous angiogenesis inhibitor, Vasohibin-1 (VASH1), was identified in a microarray analysis performed to explore genes upregulated by VEGF-A in endothelial cells [96]. The gene for human VASH1 is located on chromosome 14q24.3 and consists of seven exons, which is highly conserved in vertebrates. Human VASH1 protein is composed of 365 amino acids and serves as an endothelial cell-derived negative feedback regulator of angiogenesis; that is, it is upregulated in endothelial cells in response to proangiogenic stimuli and acts on endothelial cells to inhibit its activation (Table 1). Functional analysis revealed that some basic amino acid residues at the C-terminus of VASH1 were important for heparin binding and antiangiogenic activity [97]. VASH1 is known to be a secretory protein, and coexpression of

small vasohibin-binding protein (SVBP) is required for the secretion and antiangiogenic activity of VASH1 [98]. An anticancer effect of VASH1 through its inhibition of tumor angiogenesis has been confirmed in several reports [96, 99, 100]. Although this protein was shown to induce prolyl hydroxylase-mediated degradation of hypoxia-inducible factor-1α [101], the precise mechanism for its antiangiogenic activity remains to be elucidated. The receptor(s) for VASH1 on endothelial cells and its intracellular signaling pathway have not yet been detected. Notably, VASH1 does not induce apoptosis but rather promotes survival in endothelial cells, unlike other antiangiogenic factors (Table 1). In vitro analyses, knockdown of VASH1 induced premature senescence of endothelial cells and those cells became highly vulnerable to death caused by cellular stress [102]. In contrast, the overexpression of VASH1 made endothelial cells resistant to premature senescence and stress-induced cell death with augmented expression of superoxide dismutase 2 (SOD2) and sirtuin 1 (Sirt1) [102], suggesting that VASH1 improved the stress tolerance of endothelial cells. In addition, adenoviral transfer of human VASH1 gene to mice inoculated with Lewis lung carcinoma cells not only inhibited tumor angiogenesis but also matured remaining tumor vessels [99]. Such VASH1-induced vessel maturation led to enhanced anticancer effect of cisplatin, probably due to the improved delivery of the agent to cancer cells. Therefore, VASH1 inhibits VEGF-A-induced "excessive" angiogenic response in endothelial cells, along with enhancing the tolerance to cellular stresses and prolonging its survival, leading to the protection and stabilization of vessels.

Increased expression of VASH1 has been observed in various human malignancies and correlated with poor prognosis [103–105]. Such malignancies possibly upregulate VASH1 in order to suppress the growth and metastasis. We recently reported the clinical significance of VASH1 in patients with kidney diseases. First, the correlation between plasma and urinary VASH1 and clinical parameters was evaluated [106]. Plasma levels of VASH1 were inversely correlated with age and blood pressure. Moreover, it was found that elevated plasma and urinary levels of VASH1 predicted worse renal prognosis in patients with kidney diseases. Second, the renal distribution of VASH1 in renal biopsy specimens taken from patients with kidney diseases was evaluated [107]. VASH1 was observed in endothelial cells and in glomerular crescentic lesions and interstitial inflammatory cells. The number of VASH1-positive cells in the glomeruli was correlated with glomerular VEGFR-2-positive area and crescent formation. These results suggest that increased systemic and renal expression of VASH1 is associated with the progression of kidney diseases. Given the same tendency that VASH1 expression is associated with poor prognosis of cancer [103–105], VASH1 may be upregulated in kidney diseases in order to counter cellular stress such as local inflammation.

Thus far, the therapeutic efficacies of VASH1 on several nonneoplastic disorders associated with angiogenesis, such as atherosclerosis, macular degeneration, and bronchiolitis obliterans, have been reported [108–110]. The potential role of VASH1 as a biomarker for rheumatoid arthritis was also demonstrated [111]. Based on both the antiangiogenic and the endothelial protective effects of VASH1, we evaluated the therapeutic effects of VASH1 in type 1 and type 2 diabetic nephropathy mouse models [112, 113]. These mice were given intravenous injections of adenoviral vectors encoding human VASH1 (Ad-VASH1) every two weeks. In both mouse models, VASH1 overexpression significantly ameliorated glomerular hypertrophy, glomerular hyperfiltration, and albuminuria and also expanded glomerular endothelial area in the diabetic mice. Diabetes-induced mesangial type IV collagen accumulation and glomerular monocytes infiltration were also suppressed by treatment with Ad-VASH1. In type 1 (streptozotocin-induced) diabetic mice, enhanced phosphorylation of VEGFR-2 was prevented in kidneys treated with Ad-VASH1. Recombinant human VASH1 (rhVASH1) also prevented the phosphorylation of VEGFR-2 induced by high glucose levels in cultured glomerular endothelial cells in a dose-dependent manner [112]. Thus, VASH1 inhibits the excess angiogenic response in diabetic glomeruli by preventing activation of VEGFR-2. In addition, rhVASH1 suppressed the increased transforming growth factor-β (TGF-β) and monocyte chemotactic protein-1 (MCP-1) induced by high glucose level in cultured mesangial cells. In type 2 diabetic (db/db) mice, adenoviral overexpression of VASH1 relieved a diabetes-induced podocyte injury. Treatment with rhVASH1 also restored the expression of epithelial markers and prevented the expression of mesenchymal markers in cultured podocytes. Therefore, VASH1 was likely to directly act on mesangial cells and podocytes in diabetic glomeruli.

Furthermore, the roles of endogenous VASH1 in diabetic nephropathy was underscored by our recent report using VASH1 heterozygous knockout (VASH1$^{+/-}$) mice [114]. In the recent study, streptozotocin-induced type 1 diabetic VASH1$^{+/-}$ mice exhibited increased albuminuria, glomerular hypertrophy, and mesangial matrix accumulation and decreased slit diaphragm density. Glomerular CD31-positive area and renal VEGF-A expression were enhanced in the diabetic VASH1$^{+/-}$ mice as compared with diabetic wild type mice. Glomerular monocytes infiltration and nuclear translocation of activated NF-κB were also exacerbated in the diabetic VASH1$^{+/-}$ mice [114]. Thus, endogenous VASH1 probably prevents both angiogenic and inflammatory responses in diabetic glomeruli, as similar anti-inflammatory effect of endogenous VASH1 was also confirmed in a unilateral ureteral obstruction model [115].

Taken together, these results suggest the therapeutic potential of VASH1 for early diabetic nephropathy through suppressing excessive angiogenic response in endothelial cells and protecting mesangial cells and podocytes from diabetic insult. Since VASH1 increases the stress tolerance of endothelial cells and promotes their survival [116], VASH1 therapy for diabetic nephropathy should not only eliminate the risk of endothelial injury as shown by anti-VEGF antibodies but also add the benefit of protecting endothelial cells from hyperglycemia.

8. Conclusion

Abnormal angiogenesis is involved in the pathogenesis of diabetic nephropathy, and VEGF-A is considered to be the

most important mediator. Although anti-VEGF therapy is a rational approach, recent evidence suggests that the suppression of VEGF-A to subnormal levels results in renal thrombotic microangiopathy, with leading to glomerular scarring especially in diabetic conditions with endothelial dysfunction. VEGF-A_{165}b has the potential to be a therapeutic agent. Matrix-derived antiangiogenic factors such as endostatin are likely to suppress increased angiogenic response in diabetic glomeruli without excessive VEGF-A inhibition. VASH1 possesses both antiangiogenic and endothelial protective efficacies, and, thus, it will likely become a favorable candidate for antiangiogenic therapy. Based on the experimental evidence, a novel antiangiogenic therapy that prevents abnormal angiogenic response in glomeruli but does not induces endothelial injury should be a promising approach for the treatment of diabetic nephropathy.

Conflicts of Interest

The authors have declared that there are no conflicts of interest regarding the publication of this article.

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Research Article

Curcumin Ameliorates Diabetic Nephropathy by Suppressing NLRP3 Inflammasome Signaling

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Diabetic nephropathy (DN) is the leading cause of end-stage renal disease, partly because of the lack of effective treatments for DN. Curcumin has been shown to exert strong antifibrotic effects in DN, but the underlying mechanisms are not well characterized. In this study, we sought to determine the effects of curcumin on diabetic renal disease in db/db mice and characterize the underlying mechanism of action. We administered curcumin to db/db mice for 16 weeks. In comparison to mock-treated db/db mice, curcumin-treated mice showed diminished renal hypertrophy, reduced mesangial matrix expansion, and a lower level of albuminuria. Furthermore, the upregulated protein and mRNA expressions of collagen IV and fibronectin in the renal cortices of the db/db mice were inhibited by curcumin treatment. Additionally, curcumin treatment was associated with significant reductions in mature interleukin-1β, cleaved caspase-1, and NLRP3 protein levels in the renal cortices of db/db mice as well as in HK-2 cells exposed to high glucose concentration. In summary, curcumin, a potent antifibrotic agent, is a promising treatment for DN, and its renoprotective effects appear to be mediated by the inhibition of NLRP3 inflammasome activity.

1. Introduction

The incidence of diabetes is rapidly increasing worldwide, and the condition is projected to affect 300 million people by 2025, approximately one-third of whom will develop diabetic nephropathy (DN) [1]. DN is manifested by the accumulation of extracellular matrix proteins and an irreversible decline in renal function and is the leading cause of end-stage renal disease (ESRD) [2]. The molecular mechanisms underlying DN are incompletely characterized, and, thus, few clinical therapies are available for this condition [3]. Currently, the main treatment methods for DN focus on lowering blood glucose levels and reducing hypertension by inhibiting the renin-angiotensin system [4–6]. However, these approaches merely delay DN progression and therefore the development of ESRD but do not prevent ESRD [7, 8]. Thus, novel therapies that target additional DN pathways are urgently required.

Curcumin, a major component extracted from the rhizome *Curcuma longa*, commonly known as turmeric, has been consumed by humans as a curry spice for centuries. Its chemopreventive effects have been extensively investigated and are well defined [9]. A recent experimental study showed that short-term curcumin treatment in high-fat diet-fed mice ameliorated muscular oxidative stress by activating Nrf2 and disrupting the Nrf2-Keap1 complex and led to increases in the expression and activity of heme oxygenase-1 in porcine renal epithelial proximal tubule cells [10, 11]. Moreover, curcumin treatment has been shown to decrease macrophage infiltration in the kidneys of chronic renal failure rats, indicating that the anti-inflammatory properties of curcumin may be responsible for alleviating disease in this animal model [12]. Because of its strong anti-inflammatory activity, curcumin has been used to treat various inflammatory diseases such as DN, Alzheimer disease, and major depression [13]. However, the precise mechanisms by which curcumin ameliorates DN remain unclear.

Tubulointerstitial inflammation is crucial in promoting the development and progression of DN [14]. The NOD-like receptor 3 (NLRP3) inflammasome is a molecular platform activated upon signs of cellular "danger" [15]. This

inflammasome is composed of the NLRP3 protein, caspase-1, and the adaptor protein apoptosis-associated speck-like protein containing a caspase-activating recruitment domain. Upon activation, the NLRP3 inflammasome triggers innate immune defenses through the maturation of proinflammatory cytokines such as interleukin-1β (IL-1β) and might contribute to the development of DN [15]. This inflammasome is activated in the kidneys of streptozotocin-induced diabetic rats, and the suppression of its activation can significantly reduce renal tissue inflammation and improve renal function in these rats [16]. Furthermore, NLRP3 knockout protects unilateral ureteral occlusion mice and renal ischemia/reperfusion-induced acute kidney injury mice from renal tubular damage and interstitial inflammation [17, 18]. In vitro experiments have shown that NLRP3 protein expression, cleavage of caspase-1 and IL-1β, and release of IL-1β, IL-18, and ATP in HK-2 cells significantly increased after high glucose stimulation [14]. These data show that the NLRP3 inflammasome plays a key role in the process of sterile kidney inflammation, which led us to hypothesize that IL-1β and the NLRP3 inflammasome may be responsible for the renoprotective effects of curcumin in DN.

In this study, we aimed to determine the effects of curcumin on diabetic kidney disease in db/db mice, which develop renal lesions similar to those seen in patients with DN [19]. Furthermore, we aimed to characterize the mechanisms underlying the action of curcumin by evaluating the changes in IL-1β expression levels and NLRP3 inflammasome activity in cultured HK-2 cells exposed to high glucose concentrations and treated with curcumin.

2. Materials and Methods

2.1. Reagents. Curcumin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies were obtained from the following sources: NLRP3 antibody, Adipogen (San Diego, CA, USA); IL-1β and caspase-1 antibodies, Santa Cruz Biotechnology (Santa Cruz, CA, USA); collagen IV and fibronectin antibodies, Abcam (Cambridge, MA, USA); and secondary antibodies and β-actin antibody, Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for mouse albumin were acquired from Assaypro (St. Charles, MO, USA). Amicon® Ultra-4 Centrifugal Filter Devices were purchased from Millipore (Billerica, MA, USA). All cell culture reagents were obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Animals and Experimental Protocol. Male C57BL/KsJ db/db (diabetic) mice and age-matched db/m (nondiabetic) mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). The mice were divided into three groups of 6 mice each: a control group of db/m mice and two groups of db/db mice subjected to a mock treatment or curcumin administration. Treatments were scheduled to begin at the age of 10 weeks and end at the age of 26 weeks. All treatments were performed daily by oral gavage. Nondiabetic (db/m) and mock-treated diabetic (db/db) mice received 1% sodium carboxymethyl cellulose (a vehicle), while the remaining db/db mice received 200 mg/kg/day curcumin.

The animals were housed in a pathogen-free environment with a 12-hour light-dark cycle and given unrestricted access to standard mouse chow and water ad libitum (Department of Laboratory Animals, Central South University, Changsha, China). We measured blood glucose levels in all experimental animals once a week during the experimental period. Serum samples were collected prior to animal sacrifice at 26 weeks of age. In addition, 24-hour urine samples were collected, and urinary albumin excretion was determined (micrograms per 24 hours) as reported previously [20, 21]. All mice were sacrificed by cervical dislocation while under gaseous anesthesia (isoflurane) at 26 weeks of age, and their kidneys were harvested. The protocols for animal experimentation and the care of animals were consistent with the licenses held by the Central South University, which fulfill and follow international rules and guidelines.

2.3. Measurement of Urine Albumin. Mice were individually housed in metabolic cages during the last 3 days of treatment, and after 3 h of habituation, 24-hour urine samples were collected. Animals continued to have free access to water and standard laboratory diet during this period. The 24-hour urine samples were stored at −70°C until analysis. Urinary albumin concentration was measured using a mouse albumin ELISA kit (Assaypro, USA) following the manufacturer's protocol.

2.4. Analysis of Blood Glucose and Renal Function. After a 12-hour fast, the mice were anesthetized with chloral hydrate, and blood was obtained from the tail vein. Blood glucose was measured in duplicate using a glucose meter (OneTouch Ultra, LifeScan, Milpitas, CA, USA). The serum creatinine (SCR) level and blood urea nitrogen (BUN) level were determined with an automatic analyzer (model 7170; Hitachi Co., Ltd., Japan).

2.5. Histological Evaluation. After the animals were sacrificed, their kidneys were rapidly dissected and weighed, and the renal cortices were separated. The left cortices were snap-frozen for Western blot and real-time polymerase chain reaction (PCR) analyses, and the right cortices were used to histologically evaluate renal lesions. The right cortices were fixed in 10% paraformaldehyde and embedded in paraffin, and 3 μm thick sections were prepared. The sections were stained with Periodic Acid-Schiff (PAS). All histological evaluations were performed in a blinded manner. Twenty glomeruli were evaluated for each mouse. The degree of damage in each glomerulus was assessed using a semiquantitative scoring method as follows: grade 0, normal glomeruli (no damage); grade 1, mesangial expansion area, up to 25% (minimal damage); grade 2, 26%–50% expansion (moderate damage); grade 3, 51%–75% expansion (moderate-to-severe damage); and grade 4, 76%–100% expansion (severe damage). The glomerular matrix expansion index (GMI) was then calculated by the following formula:

$$\mathrm{GMI} = \frac{(1 \times n_1 + 2 \times n_2 + 3 \times n_3 + 4 \times n_4)}{(n_0 + n_1 + n_2 + n_3 + n_4)}, \quad (1)$$

where n represents the number of glomeruli with the respective grades of damage [21].

2.6. Immunohistochemistry. Immunohistochemical analyses were conducted to determine the collagen IV and fibronectin levels in paraffin-embedded renal tissue sections. Pepsin-based antigen retrieval was carried out. Given the homogeneity of the target protein staining, the interstitial staining for collagen IV and fibronectin was measured using computerized morphometry (Image Pro-Plus 6.0 software, Bethesda, MD). The areas of collagen IV and fibronectin staining in 20 randomly selected fields at 400x magnification in the cortex and outer medulla were quantified as the percentage of the total measured area. The immunohistochemical assessments were performed by an observer who was blinded to the study groups.

2.7. Cell Culture and Stimulation. HK-2 cells were purchased from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in low-glucose Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin (Life Technologies) at 37°C in a humidified atmosphere containing 5% CO_2. In order to find the most appropriate culture conditions, HK-2 cells were grown in media with normal glucose concentration (5 mM), high glucose concentrations (15, 25, 35, and 50 mM), or high mannitol concentrations (5 mM glucose + 30 mM mannitol) for 12, 24, 48, and 72 h with and without curcumin (5, 10, and 15 μM). On the basis of the results obtained, HK-2 cells were incubated in media with 35 mM glucose with or without 10 μM curcumin for 48 h in subsequent experiments to detect fibronectin, IL-1β, caspase-1, and NLRP3 protein expression. Cell extracts and precipitated supernatants were analyzed using ELISA and Western blot. Each experiment was repeated three times, and the average of the three values was used.

2.8. Western Blot Analysis. Proteins from renal cortical tissues, cell-free supernatants (extracted using the Amicon Ultra-4 Centrifugal Filter Device), and cell lysates were separated using 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidinedifluoride membranes (Millipore, Bedford, MA, USA). Nonspecific binding was blocked by incubation with 5% skim milk for 1 h at room temperature. The membranes were incubated overnight at 4°C with primary antibodies against fibronectin (1 : 400), IL-1β (1 : 400), caspase-1 (1 : 200), and NLRP3 (1 : 1000) and subsequently hybridized with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The protein bands were visualized with an enhanced chemiluminescence kit and quantified using ImageJ software.

2.9. Quantitative Real-Time PCR. Total RNA was isolated from kidney tissues by using TRIzol reagent according to the manufacturer's instructions (Invitrogen). In total, 1 μg RNA was converted to single-stranded cDNA by using SuperScript Reverse Transcriptase II (Fermentas). The resulting cDNA was amplified using the PCR SuperMix kit (TaKaRa). The following primers were used: collagen IV, 5′-AGAAGCGAGATGTTCAAGAAG-3′ (forward) and 5′-GTTGTGACGGTGGCAGAG-3′ (reverse); fibronectin, 5′-TGTTATGGAGGAAGCCGAGGTT-3′ (forward) and 5′-CGATGCAGGTACAGTCCCAGA-3′ (reverse); and β-actin, 5′-TGACGTGGACATCCGCAAAG-3′ (forward) and 5′-CTGGAAGGTGGACAGCGAGG-3′ (reverse). Amplification reactions were performed as follows: one cycle of 94°C for 2 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, followed by one cycle of 72°C for 7 min. All reactions were performed in the CFX™ Real-Time System (Bio-Rad Laboratories, Inc.). The relative abundance of mRNA was standardized with β-actin mRNA as the invariant control.

2.10. Statistical Analyses. All data were expressed as means ± standard deviation. Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Comparisons among different groups were made using one-way analysis of variance. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of Curcumin on Renal Hypertrophy. Body and kidney weights were markedly greater in the mock-treated db/db (diabetic) mice than in the db/m (nondiabetic) control mice. The kidney : body weight ratio, however, was slightly but not significantly lower in the mock-treated db/db mice than in the db/m mice because the diabetic mice were much heavier than the nondiabetic mice. Body and kidney weights were lower in the curcumin-treated db/db mice than in the mock-treated db/db mice.

3.2. Effects of Curcumin on Blood Glucose Level. All db/db mice remained hyperglycemic throughout the experimental period (data not shown). Blood glucose levels were remarkably higher in the db/db mice than in the db/m mice but did not differ between the curcumin-treated and mock-treated db/db mice (Table 1).

3.3. Effects of Curcumin on Renal Function. The db/db mice exhibited macroalbuminuria. The urinary albumin excretion rate was 18-fold higher in the mock-treated db/db mice than in the db/m mice. The administration of curcumin was associated with a significant attenuation of albuminuria as compared to the level in the mock-treated db/db mice. In addition, the SCR level, a marker of glomerular filtration, was significantly lower in the curcumin group than in the mock treatment group. The BUN level was slightly, but not significantly, lower in the curcumin group than in the mock treatment group (Table 1).

3.4. Effects of Curcumin on Renal Histology. Mesangial matrix expansion was evident in the glomeruli of the mock-treated db/db mice (Figure 1). PAS-positive mesangial matrix areas were substantially larger in the mock-treated db/db mice than in the db/m mice. The GMI score was significantly lower in the curcumin-treated db/db mice than in the mock-treated db/db mice.

Table 1: Curcumin protects against the progression of diabetic nephropathy in db/db mice.

	db/m	db/db	db/db + Cur
Body weight, g	20.11 ± 1.38	44.91 ± 5.57$^{\#}$	35.54 ± 3.02*
Kidney weight, g	0.14 ± 0.02	0.23 ± 0.04$^{\#}$	0.19 ± 0.03*
Blood glucose, mmol/L	7.86 ± 0.98	27.44 ± 2.97$^{\#}$	26.34 ± 5.74
SCR, μmol/L	38.48 ± 7.39	49.48 ± 5.59$^{\#}$	41.90 ± 4.71*
BUN, mmol/L	8.89 ± 2.10	9.58 ± 2.13	9.34 ± 2.30
Urinary albumin, μg/24 h	38.08 ± 23.37	603.83 ± 234.22$^{\#}$	243.14 ± 50.27*

db/m, nondiabetic mice; db/db, diabetic mice (mock-treated); db/db + Cur, db/db mice treated with curcumin at a dose of 200 mg/kg body weight.
$^{\#}p < 0.05$, compared with the db/m mice (control); $^{*}p < 0.05$, compared with the mock-treated db/db mice ($n = 6$ per group).
Values shown are mean ± SD.
SCR, serum creatinine; BUN, blood urea nitrogen.

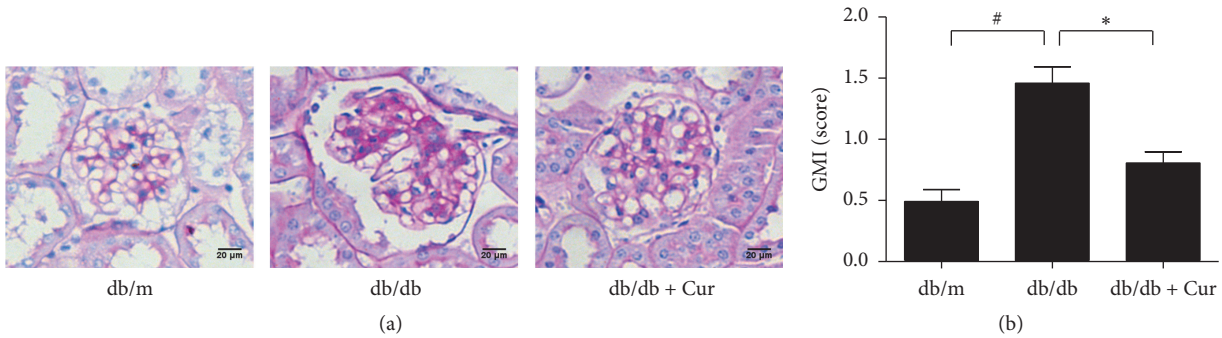

Figure 1: Curcumin offers protection against glomerular expansion in diabetic nephropathy. (a) Representative photomicrographs of Periodic Acid-Schiff- (PAS-) stained renal cortical sections from db/m mice, mock-treated db/db mice, and curcumin-treated db/db mice. (b) Glomerular matrix expansion index (GMI) scores calculated from the analysis of 20 glomeruli per mouse. db/m, nondiabetic mice; db/db, diabetic mice (mock-treated); db/db + Cur, db/db mice treated with curcumin at a dose of 200 mg/kg body weight. $^{\#}p < 0.05$, compared with the db/m mice; $^{*}p < 0.05$, compared with the mock-treated db/db mice ($n = 6$ per group). Magnification, ×400; scale bar, 20 μm for all micrographs.

3.5. Effects of Curcumin on Collagen IV and Fibronectin Expression. Collagen IV and fibronectin accumulation was observed in the mesangial area of the glomeruli in mock-treated db/db mice, and both proteins were expressed at lower levels in the glomeruli of curcumin-treated db/db mice than in the mock-treated mice. The semiquantitative scores for collagen IV and fibronectin were higher in the mock-treated db/db mice than in the db/m mice and significantly lower in the curcumin-treated db/db mice than in the mock-treated db/db mice (Figures 2(a) and 2(b)). Consistent with this, the mRNA levels of collagen IV and fibronectin in the renal cortex were higher in the mock-treated db/db mice than in the db/m mice, and curcumin administration was associated with a significant reduction in these levels as determined by real-time PCR (Figure 2(c)).

3.6. Effects of Curcumin on IL-1β Production and NLRP3 Inflammasome Activity. We found a significant increase in NLRP3 protein expression and cleavage of caspase-1 and IL-1β in the kidney tissues of mock-treated db/db mice as compared to db/m mice, and this upregulation was dramatically inhibited in the curcumin group (Figure 3).

3.7. Effects of High Glucose Levels on NLRP3 Inflammasome Activation in HK-2 Cells. To investigate the effect of high glucose levels on NLRP3 inflammasome activation, we analyzed NLRP3 protein expression and the cleavage of caspase-1 and IL-1β in HK-2 cells. NLRP3 expression and caspase-1 and IL-1β cleavage were enhanced by exposure to glucose in a dose-dependent manner and peaked at a glucose concentration of 50 mM (Figures 4(a) and 4(b)). However, at this concentration, we observed morphological alterations in HK-2 cells. We therefore used a glucose concentration of 35 mM in subsequent experiments. HK-2 cells were treated with 35 mM glucose for 72 h, and NLRP3 expression and caspase-1 and IL-1β cleavage were found to change in a time-dependent manner, with peaks at 48 h (Figures 4(c) and 4(d)).

3.8. Effect of Curcumin on Fibronectin Expression and NLRP3 Inflammasome Activation in HK-2 Cells Exposed to High Glucose Concentrations. HK-2 cells stimulated with 35 mM glucose were treated with different curcumin doses for 48 h. The high fibronectin expression induced by glucose stimulation was significantly reduced after curcumin treatment at doses of 10 and 15 μM; however, cell apoptosis was observed at the latter dose (Figure 5(a)). Western blot analysis showed that NLRP3 expression and caspase-1 and IL-1β cleavage were higher in HK-2 cells treated with 35 mM glucose than in cells treated with the same dose of mannitol. These changes were inhibited in cells treated with 10 μM curcumin (Figures

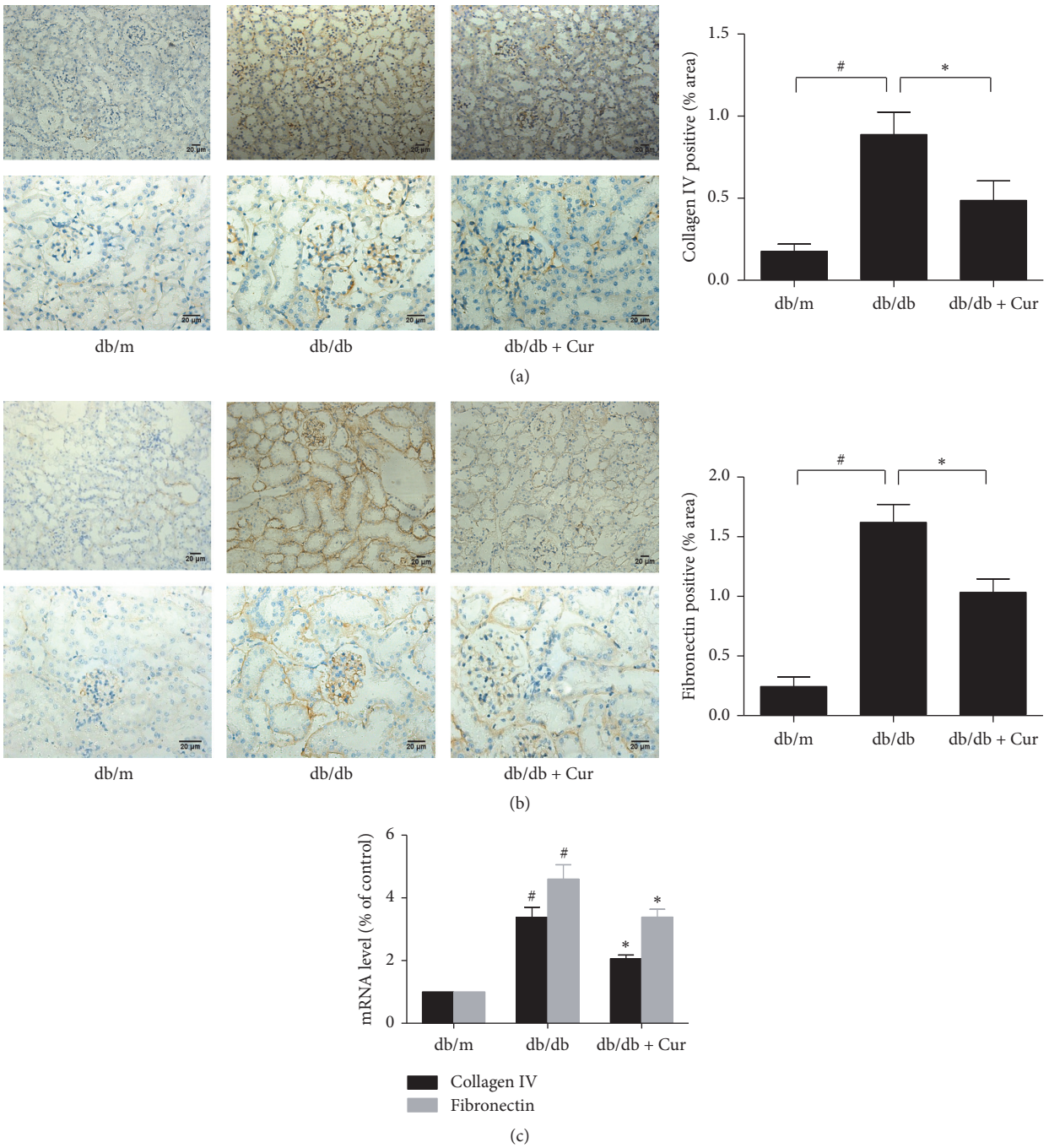

Figure 2: Curcumin partially reverses the upregulation of collagen IV and fibronectin expression in db/db mice. (a) Collagen IV and (b) fibronectin protein expression in the renal cortex as determined using immunohistochemical staining. Magnification, ×200 (top) and ×400 (bottom); scale bar, 20 μm. (c) The mRNA levels of collagen IV and fibronectin measured using real-time polymerase chain reaction and expressed as a percentage of the mRNA level in the db/m group. db/m, nondiabetic mice; db/db, diabetic mice (mock-treated); db/db + Cur, db/db mice treated with curcumin at a dose of 200 mg/kg body weight. $^{\#}p < 0.05$, compared with the db/m mice (control); $^{*}p < 0.05$, compared with the mock-treated db/db mice ($n = 6$ per group).

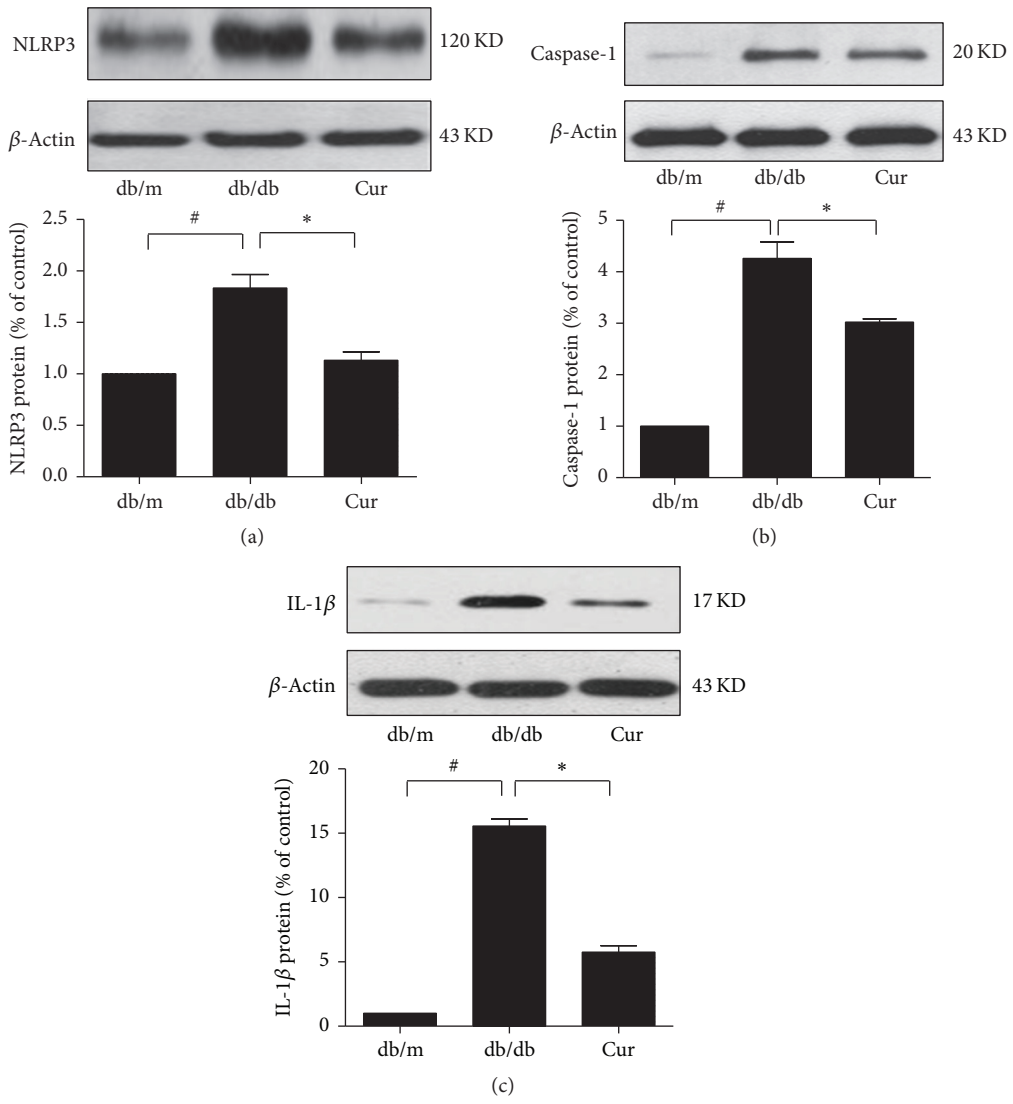

FIGURE 3: Curcumin reduced interleukin-1β (IL-1β) production and NLRP3 inflammasome activity in db/db mice. The expression of NLRP3 protein (a) and cleavage of caspase-1 (b) and IL-1β (c) as determined using Western blot analysis; β-actin was used as the internal loading control. db/m, nondiabetic mice; db/db, diabetic mice (mock-treated); db/db + Cur, db/db mice treated with curcumin at a dose of 200 mg/kg body weight. $^{\#}p < 0.05$, compared with the db/m mice (control); $^{*}p < 0.05$, compared with the mock-treated db/db mice ($n = 6$ per group).

5(b)–5(d)). Neither NLRP3 expression nor caspase-1 and IL-1β cleavage were affected by mannitol stimulation (Figure 5).

4. Discussion

The present study evaluated the effects of curcumin treatment on DN in db/db mice and investigated the underlying mechanisms in HK-2 cells. The results showed that curcumin treatment decreased renal hypertrophy, improved renal function (as assessed using the urinary albumin excretion rate), and ameliorated renal histological changes in db/db mice. In addition, the findings in HK-2 cells suggested that the renoprotective effects of curcumin were mediated by the inhibition of NLRP3 inflammasome activation.

Curcumin, the yellow pigment of the plant *Curcuma longa* (turmeric), is extensively used in food preparation and has attracted considerable research attention for its various bioactivities [22, 23]. Curcumin has been well established to exert a variety of pharmacological activities, in particular anti-inflammatory activity [13]. The renoprotective effect of curcumin in streptozotocin-induced DN rats has been confirmed [24, 25]. In these rats, curcumin alters posttranslational modifications of histone H3, heat-shock protein-27, and MAP kinase p38 expression [26] and prevents diabetes-associated abnormalities in the kidneys by inhibiting p300 and nuclear factor-κB [27]. However, streptozotocin-induced DN rats are a model of type 1 diabetes, and little is known about the role of curcumin in db/db mice, which are a model of type 2 diabetic mellitus. The db/db mice are a genetic model of obesity associated with hyperphagia and hyperglycemia. A major benefit of this model is the consistent temporal development of hyperglycemia (6–8 weeks), albuminuria

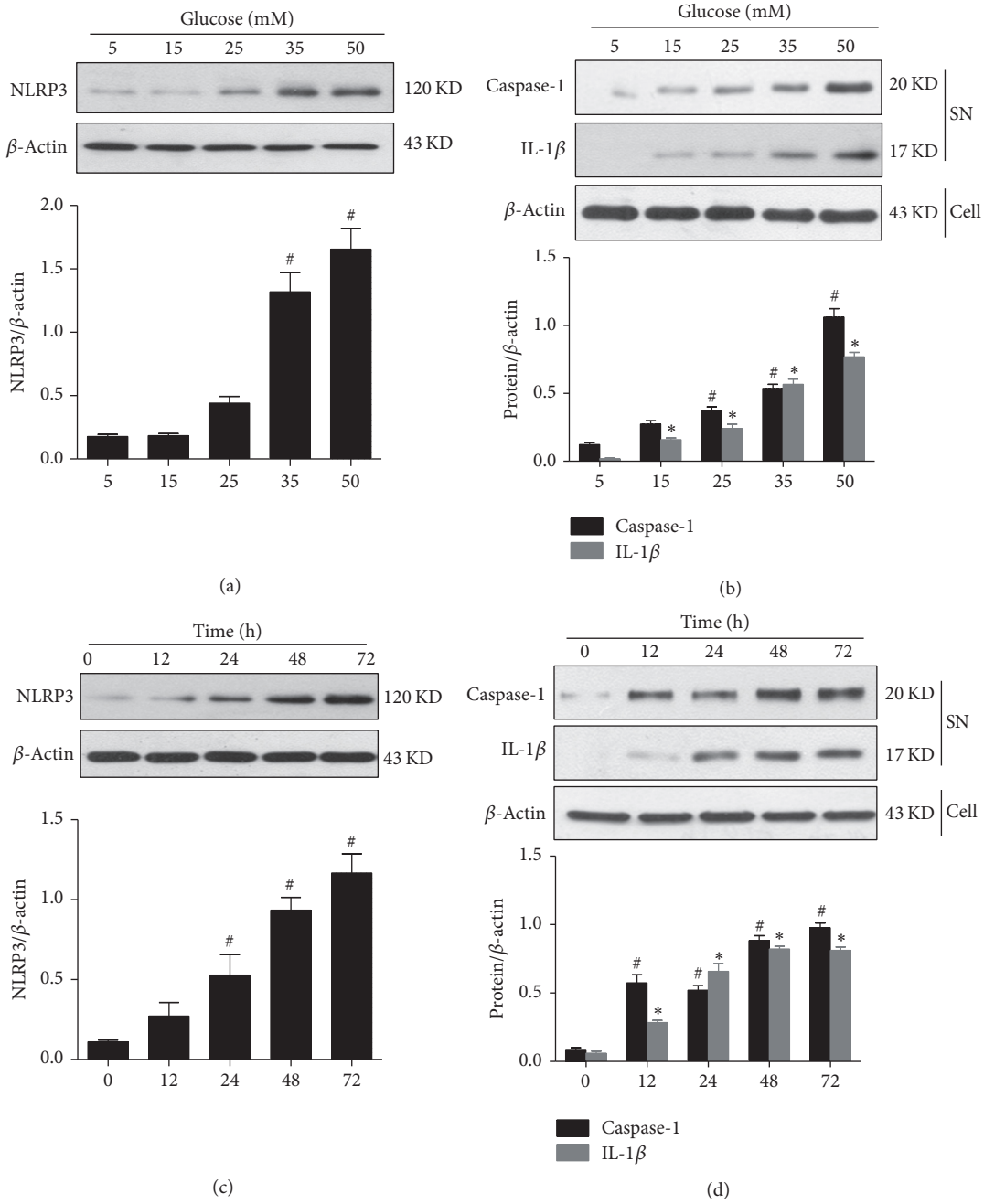

Figure 4: Time- and dose-dependent effects of glucose on NLRP3 inflammasome activation in HK-2 cells, as assessed using Western blot analysis. (a) NLRP3 protein expression in HK-2 cells exposed to different glucose concentrations for 48 h. (b) Cleaved caspase-1 p20 and 17 kD mature interleukin-1β (IL-1β) in culture supernatants. (c) NLRP3 protein expression in HK-2 cells treated with 35 mM glucose for different time periods. (d) Cleaved caspase-1 p20 and 17 kD mature IL-1β in cell culture supernatants. β-Actin was used as the internal loading control. (a, c) $^{\#}p < 0.05$, compared with cells treated with 5 Mm glucose or cells treated for 0 h. (b, d) $^{\#}p < 0.05$ and $^{*}p < 0.05$, compared with cells treated with 5 mM glucose or cells treated for 0 h. The average values from three independent experiments are shown.

(10–12 weeks), and mesangial matrix expansion (14–16 weeks) [28]. Moreover, the glomerular mesangial expansion and histological lesions in these mice resemble those found in human DN.

One of the hallmarks of DN is the development of proteinuria, which is usually followed by a progressive decline in renal function. Urinary albumin excretion is regarded as one of the earliest signs of glomerular damage in overt diabetic renal disease. This damage is characterized structurally by glomerular basement membrane thickening and mesangial expansion, which can be detected histologically as an increase in the PAS-positive mesangial matrix area, due to the accumulation of extracellular matrix proteins. In this study, we found that elevated urinary albumin excretion, mesangial

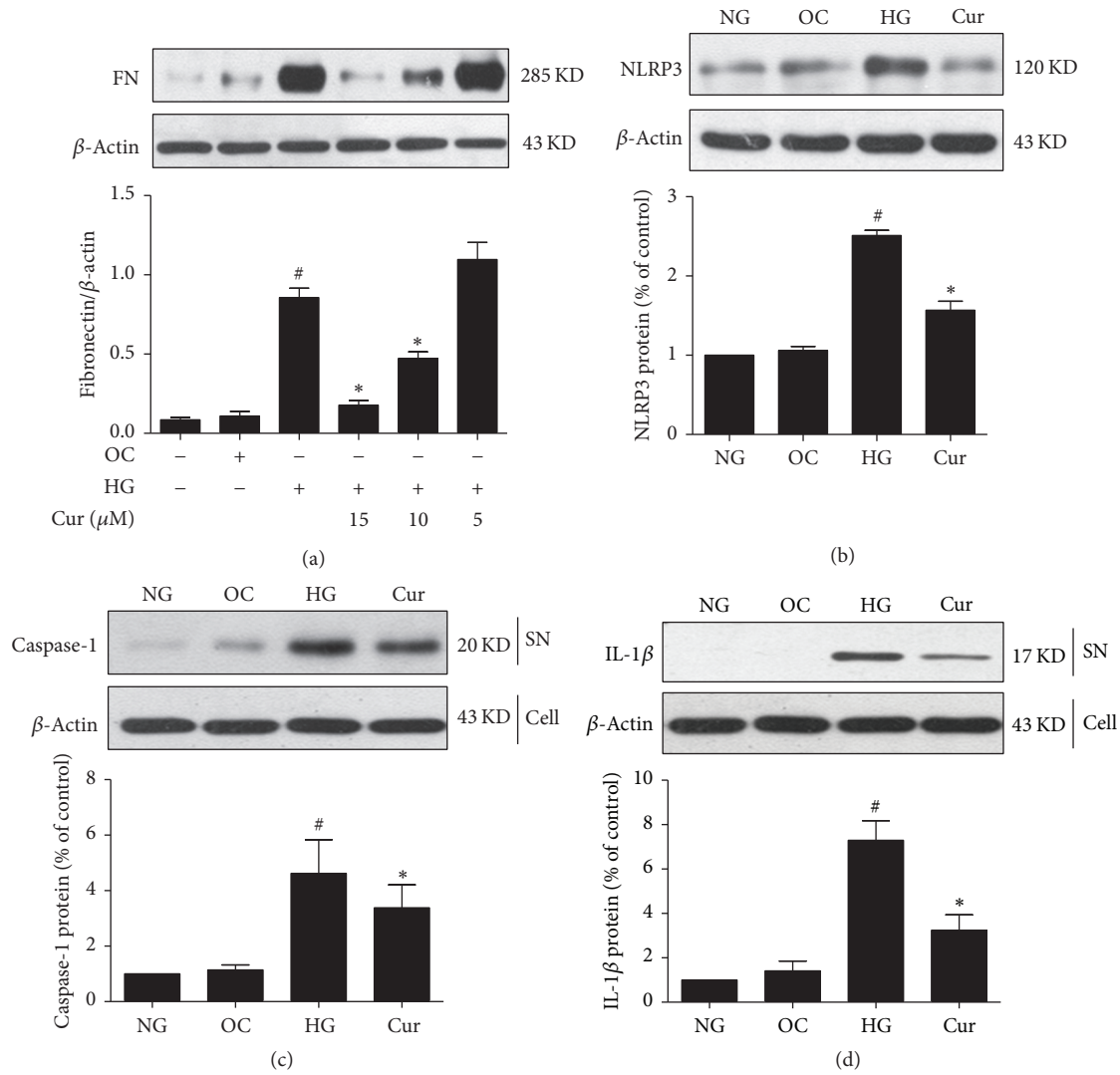

Figure 5: Curcumin inhibited the high glucose-induced increases in fibronectin and NLRP3 expression and NLRP3 inflammasome activation in HK-2 cells as assessed using Western blot analysis. HK-2 cells were assigned to four groups: (1) normal glucose (NG); (2) high glucose (HG); (3) curcumin (Cur); and (4) osmotic control with mannitol (OC). (a) Fibronectin protein expression in HK-2 cells exposed to 35 mM glucose for 48 h with or without different doses of curcumin. (b) NLRP3 protein expression in HK-2 cells treated with 35 mM glucose for 48 h and 10 μM curcumin. (c, d) Cleaved caspase-1 p20 and 17 kD mature interleukin-1β (IL-1β) in culture supernatants. β-Actin was used as the internal loading control. $^{\#}p < 0.05$, compared with the 5 mM glucose (NG) group; $^{*}p < 0.05$, compared with the 35 mM (HG) group. The average values from three independent experiments are shown.

matrix expansion, and the upregulated expression of collagen IV and fibronectin were lower in the curcumin group than in the mock treatment group. In addition, remarkable changes were observed in body weight, kidney weight, and SCR levels after curcumin treatment. These findings indicate that curcumin inhibited disease progression in the early stage of DN in db/db mice. Furthermore, our study demonstrated that blood glucose levels were not affected by curcumin treatment, indicating that the renoprotective effects of curcumin are independent of any hypoglycemic action in this animal model of DN.

We speculated that the renoprotective effects of curcumin may be mediated by the suppression of NLRP3 inflammasome activation and IL-1β production. This is because curcumin has been shown to reduce NLRP3 inflammasome activation and IL-1β production in lipopolysaccharide-induced septic shock [29], and both IL-1β (a proinflammatory cytokine) and NLRP3 inflammasomes have been shown to be involved in the development of DN [14, 17]. Furthermore, recent reports have implicated that the NLRP3 inflammasome is an important contributor to inflammation and tissue damage during acute kidney injury, chronic kidney disease [30], and kidney inflammation and fibrosis [31]. Correa-Costa et al. [32] confirmed the important roles of both Toll-like receptors and NLRP3 inflammasomes in an experimental model of tubulointerstitial nephritis and found that allopurinol downregulated individual components of the inflammasome pathway and diminished this injury. Together,

these results indicate that the NLRP3 inflammasome is a potential therapeutic target in DN.

Consistent with the aforementioned reports, our study showed that NLRP3 expression and caspase-1 and IL-1β cleavage were significantly inhibited in curcumin-treated db/db mice, suggesting that curcumin may attenuate DN progression by inhibiting NLRP3 inflammasome activation. To further evaluate this, we observed the effects of curcumin treatment on NLRP3 inflammasome activity in HK-2 cells exposed to high glucose levels. We selected HK-2 cells, which are immortalized proximal tubular epithelial cells, rather than mesangial cells because tubulointerstitial inflammation is crucial in promoting the development and progression of DN [33] and because NLRP3 protein is mainly expressed in renal tubular epithelial cells [14]. We found that NLRP3 expression and caspase-1 and IL-1β cleavage were inhibited after curcumin treatment in HK-2 cells exposed to high glucose levels, showing that curcumin inhibits the activation of NLRP3 inflammasomes.

Our study has some limitations. Curcumin is poorly absorbed when administered orally and has low solubility in water. Moreover, we did not assess the pharmacokinetics of curcumin or measure the plasma and tissue concentrations of curcumin in the mice. Nevertheless, we did obtain measurable changes in the parameters tested after curcumin treatment. It would be interesting to change the curcumin dosage or route of administration in further experiments in order to improve the bioavailability of curcumin and perhaps obtain better results.

In conclusion, curcumin displays renoprotective effects after disease onset in db/db mice despite sustained hyperglycemia. The protective effect of curcumin in DN may be attributable, at least in part, to the inhibition of NLRP3 inflammasome activation. Collectively, our findings may open a new avenue to explore the effects and molecular mechanisms of curcumin in DN.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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