Experimental Diabetes Research

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Guest Editors: Yasuhiko Tomino, Mark E. Cooper, Theodore W. Kurtz, and Yoshio Shimizu

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Editorial Board

Contents

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Editorial

Experimental Models of Type-2 Diabetic Nephropathy

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Type 2 diabetic nephropathy is one of the major long-term microvascular complications occurring in nearly 40% of diabetic patients and also a major cause of end-stage kidney disease (ESKD) throughout the world. It is assumed that the number of type 2 diabetes and diabetic nephropathy patients is increasing and that more and more patients will experience progressive renal disease due to lack of effective treatments. The pathogenesis of type 2 diabetic nephropathy includes genetic, metabolic (hyperglycemic), and/or hemodynamic factors such as glomerular hypertension and associated renal hypertrophy. There are many progressive factors in patients with type 2 diabetic nephropathy, but few if any specific treatments for human diabetic nephropathy based on the mechanisms of disease initiation and progression have been clearly identified. Thus, it is important to investigate and determine pathogenesis (mechanisms of initiation and/or progression) and treatments using various experimental models of type 2 diabetic nephropathy.

This special issue contains 11 papers, based on studies of various animal models, cell cultures, and human samples.

In the paper entitled "*Dietary restriction ameliorates diabetic nephropathy through anti-inflammatory effects and regulation of the autophagy via restoration of Sirt1 in diabetic Wistar fatty (fa/fa) rats: a model of type 2 diabetes*," M. Kitada et al. examined the renoprotective effects of dietary restriction (DR) in Wistar fatty (*fa/fa*) rats (WFRs). DR ameliorated renal abnormalities including inflammation in WFRs. The decrease in Sirt1 levels, increase in acetylated-NF-κB, and impaired autophagy in WFRs were improved by DR. The authors concluded that DR exerted anti-inflammatory effects and improved the dysregulation of autophagy through the restoration of Sirt1 in the kidneys of WFRs, which resulted in the amelioration of renal injuries in type 2 diabetes.

In the paper entitled "*High glucose increases metallothionein expression in renal proximal tubular epithelial cells*," D. Ogawa et al. found that the renal tissues in adult male diabetic rats induced by streptozotocin were stained with antibodies for MT-1/-2. MT-1/-2 expression was also evaluated in mProx24 cells, a mouse renal proximal tubular epithelial cell line, stimulated with high glucose medium and pretreated with the antioxidant vitamin E. These observations suggest that MT-1/-2 is induced in renal proximal tubular epithelial cells as an antioxidant to protect the kidney from oxidative stress and that it may offer a novel therapeutic target against diabetic nephropathy.

In the paper entitled "*Targeted proteomics of isolated glomeruli from the kidneys of diabetic rats: sorbin and SH3 domain containing 2 is a novel protein associated with diabetic nephropathy*," S. Nakatani et al. examined the protein expression in the isolated glomeruli from spontaneous type 2 diabetic (OLETF) rats and their age-matched control littermates (LETO) in the early and proteinuric stages of diabetic nephropathy using QSTAR Elite LC-MS/MS. Sorbin and SH3 domain containing 2 (SORBS2) was significantly upregulated in both stages of diabetic nephropathy. Immunohistochemical and quantitative PCR analyses revealed upregulation of SORBS2 in the podocytes of glomeruli of OLETF rats. These findings suggested that SORBS2 may be associated with the development of diabetic nephropathy possibility by reorganization of actin filaments.

In the paper entitled "*Role of T cells in type 2 diabetic nephropathy*," C.-C. Wu et al. reviewed the current information concerning the role of T cells in the development and progression of type 2 diabetic nephropathy. Specific emphasis is placed on the potential interaction and contribution of the T cells to renal damage. The therapeutic

strategies involving T cells in the treatment of type 2 diabetic nephropathy are also reviewed. Improving knowledge of the recognition of T cells as significant pathogenic mediators in diabetic nephropathy reinforces the possibility of finding new potential therapeutic targets that may be translated into future clinical treatments.

In the paper entitled "*Involvement of F-Actin in chaperonin-containing t-complex 1 beta regulating mouse mesangial cell functions in a glucose-induction cell model*," J.-S. Chen et al. investigated the role of chaperonin-containing t-complex polypeptide 1 beta (CCT2) in the regulation of mouse mesangial cell (mMC) contraction, proliferation and migration with filamentous/globular- (F/G-) actin ratio under high glucose induction. Major results were as follows: (1) low CCT2 or high glucose showed the ability to attenuate F/G-actin ratio, (2) groups with low F/G-actin ratio all showed less cell contraction, and (3) suppression of CCT2 may reduce the proliferation and migration which were originally induced by high glucose. The authors concluded that CCT2 can be used as a specific regulator for mMC contraction, proliferation, and migration affected by glucose, in which the mechanism may involve the alteration of F-actin, particularly for cell contraction.

In the paper entitled "*Diabetic nephropathy amelioration by a low-dose sitagliptin in an animal model of type 2 diabetes (Zucker diabetic fatty rat)*," C. Mega et al. performed studies to assess the effect of chronic low-dose sitagliptin, a dipeptidyl peptidase 4 inhibitor, on metabolic profile and on renal lesions in a rat model of type 2 diabetic nephropathy, the Zucker diabetic fatty (ZDF) rat. The authors concluded that chronic low-dose sitagliptin treatment was able to ameliorate diabetic nephropathy, which might represent a key step forward in the management of type 2 diabetes and this serious complication.

In the paper entitled "*Role of mindin in diabetic nephropathy*," M. Murakoshi et al. suggested that certain inflammatory biomarkers should be useful for diagnosis or monitoring of diabetic nephropathy. Mindin (spondin 2) is a member of the mindin-/F-spondin family of secreted extracellular matrix (ECM) proteins. Recent studies have showed that mindin is essential for initiation of innate immune response and represents a unique pattern-recognition molecule in the ECM. The authors previously demonstrated that the levels of urinary mindin in patients with type 2 diabetes were higher than those in healthy individuals. Therefore, the authors suggest that urinary mindin may be a potent biomarker for the development of diabetic nephropathy.

In the paper entitled "*New experimental models of diabetic nephropathy in mice models of type 2 diabetes: Efforts to replicate human nephropathy*," M. J. Soler et al. explained that the generation of new experimental models of diabetic nephropathy created by crossing, knockdown, or knockin of genes continues to provide improved tools for studying diabetic nephropathy. These models provide an opportunity to search for new mechanisms involving the development of diabetic nephropathy, but their shortcomings should be recognized as well. Moreover, it is important to recognize that the genetic background has a substantial effect on the susceptibility to diabetes and kidney disease development in the various models of diabetes.

In the paper entitled "*Osmolarity and glucose differentially regulate aldose reductase activity in cultured mouse podocytes*," B. Lewko et al. examined whether aldose reductase (AR), the enzyme implicated in diabetic complications in different tissues, is modulated by high glucose and osmolarity in podocytes. Hyperosmolarity acutely stimulated AR expression and activity, with subsequent increase of AR expression but decrease of activity. High glucose also elevated AR protein level; however, this was not accompanied by respective enzyme activation. Furthermore, high glucose appeared to counteract the osmolarity-dependent activation of AR. The authors concluded that AR is modulated by high glucose and increased osmolarity in a different manner in podocytes. Posttranslational events may affect AR activity independent of enzyme protein amount. Activation of AR in podocytes may be implicated in diabetic podocytopathy.

In the paper entitled "*Signaling mechanisms in the regulation of renal matrix metabolism in diabetes*," M. M. Mariappan reviewed that mTOR-(mammalian target of rapamycin-) regulated pathways are pivotal in orchestrating high-glucose-induced production of extracellular matrix (ECM) proteins leading to functional and structural changes in the kidney culminating in adverse outcomes. Understanding signaling pathways that influence individual matrix protein expression could lead to the development of new interventional strategies. This review will highlight some of the diverse components of the signaling network stimulated by hyperglycemia with an emphasis on extracellular matrix protein metabolism in the kidney in diabetes.

In the paper entitled "*An angiotensin II type 1 receptor blocker, prevents renal injury via inhibition of the Notch pathway in Ins2 Akita diabetic mice*," M. Koshizaka et al. showed that telmisartan inhibited the angiotensin II-induced increased expression of transforming growth factor β and vascular endothelial growth factor A which could directly activate the Notch signaling pathway in cultured podocytes. The authors indicated that the telmisartan prevents diabetic nephropathy through the inhibition of the Notch pathway.

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Review Article

Signaling Mechanisms in the Regulation of Renal Matrix Metabolism in Diabetes

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Renal hypertrophy and accumulation of extracellular matrix proteins are among cardinal manifestations of diabetic nephropathy. TGF beta system has been implicated in the pathogenesis of these manifestations. Among signaling pathways activated in the kidney in diabetes, mTOR- (mammalian target of rapamycin-)regulated pathways are pivotal in orchestrating high glucose-induced production of ECM proteins leading to functional and structural changes in the kidney culminating in adverse outcomes. Understanding signaling pathways that influence individual matrix protein expression could lead to the development of new interventional strategies. This paper will highlight some of the diverse components of the signaling network stimulated by hyperglycemia with an emphasis on extracellular matrix protein metabolism in the kidney in diabetes.

1. Introduction

The importance of hyperglycemia in renal injury was confirmed by the Diabetes Control and Complications Trial [1] and the United Kingdom Prospective Diabetes Study [2], which demonstrated that diabetic kidney disease can be prevented by keeping blood sugar in target range; however, this is difficult to achieve. Diabetes, particularly type 2, is the most common cause of end-stage renal disease requiring chronic renal replacement therapy in the US. Despite its high prevalence, the mechanism of development and progression of diabetic nephropathy (DN) is still not fully understood, partly because of unrecognized and undiagnosed kidney changes that coexist during latent diabetes [3]. Much of our understanding of the mechanisms of injury in diabetes comes from studies on rodent models of diabetes. Although several such animal models of diabetes exist, no single animal model develops renal changes identical to those seen in humans. Brosius et al. [4] have compiled a report on the progress towards establishing and validating a murine model of human DN (http://www.diacomp.org/). It is likely to be difficult to generate a single mouse model that recapitulates all of the features of human DN.

Pathophysiology of DN involves an interaction of genetic, metabolic, and hemodynamic factors. Structural renal changes in diabetes start with glomerular hypertrophy, followed by glomerular basement membrane (GBM) thickening, mesangial matrix expansion, and development of sclerotic lesions [5]. Accumulation of extracellular matrix proteins is governed by a balance between increased synthesis regulated at the level of transcription and mRNA translation, and, degradation regulated by such processes as the balance between proteolytic activity of matrix metalloproteinases (MMPs) and their inhibitors, the tissue inhibitor of metalloproteinases (TIMPs). A variety of growth factors and cytokines participate in this pathology through complex signal transduction pathways in a cell-specific manner. In this review we will discuss the various mechanisms by which hyperglycemia can induce extracellular matrix synthesis and accumulation in diabetic kidneys.

2. Extracellular Matrix Components

An early sign of renal involvement in diabetes is an increase in basement membrane thickness that has been described as a prediabetic lesion [6]. The thickening of the renal basement membranes of the glomerulus (GBM) and tubules

(TBM) is due to a consequence of the hyperglycemia-induced metabolic perturbations resulting in augmented synthesis and accumulation of intrinsic ECM components at these sites [7, 8]. The major TBM components are type IV collagen, laminin, and entactin while collagen types I, V, and VI and fibronectin are generally considered components of the renal interstitium. The GBM is predominantly composed of laminin, collagen type IV alpha 3 to alpha 5, agrin, and perlecan. In diabetes it has been shown that collagen α3 through α5 (IV) chains, collagen V, laminin, fibronectin, and serum proteins contribute to thickened GBM [9]. Mesangial expansion is largely due to the accumulation of extracellular matrix (ECM) proteins such as collagen α1, α2 (IV) chains, collagens V and VI, laminin, and fibronectin [10, 11].

2.1. Laminins. Laminins are glycoproteins expressed primarily in basement membrane. Laminins are heterotrimeric structures consisting of combinations of five alpha, three beta, and three gamma chains that share a common domain and several globular and rod-like domains. The tissue-specific distribution of laminins is mainly determined by expression of the alpha chains; in particular, a number of alpha 5-chain mutations are associated with neonatal lethality and defective glomerulogenesis [12, 13]. Glomerular and proximal tubular epithelial cell laminin expression has been shown to increase in response to hyperglycemia and TGF-b coincident with increased thickening of the glomerular basement membrane [14, 15].

2.2. Collagen Type IV. Collagens serve as fibrotic markers in diabetic nephropathy, and type IV collagen provides the basic structural framework of the glomerular ECM. There are six genetically distinct alpha chains (α1 through α6), and all have similar domain structures. In the GBM alpha 3 through alpha 5 predominate whereas in the TBM alpha 1, 2, 3, and 5 are present [16, 17].

2.3. Fibronectin. Fibronectin is a glycoprotein that is found in the plasma as well as in the basement membrane and the mesangium of the glomerulus [18, 19]. Types V and VI collagen along with type IV, and fibronectin colocalize in a similar distribution in the glomerular subendothelial area and the mesangium. Entactin/nidogen (En/Nd) is an elongated approximately 150 kDa molecule containing three globular domains separated by two linear segments. Laminin, type IV collagen, and fibronectin are all capable of self-aggregation. Laminin also has additional binding sites for glycosaminoglycans and attaches to collagen via nidogen bridge. It serves as a link between the laminin and collagen IV networks in sub-endothelial, subepithelial and mesangial areas in the glomerular basement membranes [20, 21]. The networking pattern of these ECM components determine the pore size and the charge-selective properties of GBM [22].

3. Role of Transcription, mRNA Translation, and Ribosome Biogenesis in ECM Production

3.1. Transcription. Regulation of protein synthesis may occur at the level of transcription or mRNA translation. High glucose stimulates the transcription of matrix genes and represses matrix degradation leading to glomerulosclerosis [23–25]. Sanchez and Sharma [26] have extensively reviewed the mechanism of activation of transcription factors involved in the progression of diabetic kidney disease including upstream stimulatory factors (USF1 and 2), activator protein 1 (AP-1), cAMP-response element-binding protein (CREB), nuclear factor (NF)-κB, nuclear factor of activated T cells (NFAT), and stimulating protein 1 (Sp1). Until the beginning of the last decade transcription was the only studied regulatory mechanism for increased ECM protein synthesis induced by hyperglycemia in the renal tissues. Accumulating evidence from recent studies has established mRNA translation as another important and independent step in the regulation of protein synthesis [27–30].

3.2. mRNA Translation. The process of mRNA translation occurs in three steps: the initiation phase, which involves localization of the preinitiation complex containing the 40S ribosomal subunit and the initiator methionyl tRNA to the AUG (methionine) codon on the mRNA; the elongation phase, during which amino acids are added to the nascent peptide according the codon sequence of the mRNA; the termination phase, in which arrival at a stop codon leads to the release of the completed peptide chain. Of these three steps, initiation is the rate limiting step as it determines the recruitment of ribosomes to the specific mRNA. Elongation itself is composed of three traditionally defined steps: eEF1A-directed binding of the aminoacyl-tRNA to the A site (aminoacyl site) of the ribosome, peptide bond formation triggered by the enzymatic activity of the ribosome (the peptidyl transferase center), and eEF2- mediated translocation which moves the peptidyl-tRNA from the A site to the P site (peptidyl site) by precisely one codon (three nucleotides) [31–33]. Upregulation of these events result in augmented translational efficiency. Signaling pathways play a major role in regulation of translation. Among them the mTOR system regulates the initiation and elongation phases of translation of specific mRNAs to culminate in increased protein synthesis [31–33]. Such control is generally exerted through changes in the phosphorylation states of the translation initiation or elongation factors. Diabetic kidney tissues and renal cells treated with high glucose demonstrate activation of various initiation and elongation factors that are involved in regulation of mRNA translation [14, 15, 34]. Activation of these factors by high glucose and angiotensin II resulted in upregulation of selective proteins like laminin beta 1 chain and vascular endothelial growth factor (VEGF), respectively, in tubular epithelial cells [15, 35].

3.3. Ribosome Biogenesis. Ribosome biogenesis is a complex well-coordinated process in which hundreds of different proteins interact in the folding and processing of ribosomal RNA (rRNA) consisting of a small (40S) and large (80S) subunit in eukaryotic cells. The large subunit is composed of 5S, 28S, 5.8S rRNAs whereas the 40S subunit contains 18S rRNA. Furthermore, approximately 80 different ribosomal proteins (r-proteins) are found in eukaryotic ribosomes [36]. Ribosomes consisting of 80S and 40S ribosomal subunits and

ribosomal proteins are part of the translation machinery that aid in carrying out the process of peptide synthesis by the addition of amino acids through translation of the genetic code in mRNA. The smaller (40S) subunit of the ribosome serves as a platform to bring together messenger RNA, aminoacylated transfer RNAs, and translation factors. The larger (80S) ribosomal subunit provides peptidyl transferase activity to catalyze peptide bond formation in nascent polypeptides. Increased production of ribosome reflects enhanced capacity for translation. Ribosome biogenesis is so important for cell growth that a growing yeast cell synthesizes approximately 2000 ribosomes every minute, requiring 60% of total cellular transcription. In mammalian cells [37], this number is even higher; for example, a HeLa cell makes 7500 ribosomal subunits per minute [38]. Ribosome biogenesis is regulated by the activity of RNA polymerase I, which controls the rate of rRNA synthesis. The activity of RNA polymerase I at the ribosomal DNA promoter is modulated by a complex of proteins, which includes the nucleolar protein upstream binding factor (UBF) 1. UBF1 interacts with the protein complex TIF-1B (SL1 in humans), which consists of the TATA box-binding protein and three associated factors. The resulting complex promotes the binding of RNA polymerase I to the ribosomal DNA promoter [39, 40]. The activity of UBF1 is regulated, at least in part, by its phosphorylation at Serine 388 [41]. We observed increased UBF phosphorylation at Ser388 accompanied by increased rDNA transcription in glomerular epithelial cells treated with high glucose and in kidney tissues from type 2 diabetic mice model [42]. This eventually leads to increased rRNA molecules and ribosomal proteins thereby increasing translational capacity and sets the stage for increased matrix protein synthesis in renal tissues and cells in response to hyperglycemia.

4. Signaling Pathways Activators and Inhibitors of Protein Synthesis

4.1. mTOR. At the molecular level, mTOR is recognized as the mediator of signals from extracellular high glucose milieu to the nuclear contents of the cell. The complex signaling cascade regulated by high glucose to induce extracellular matrix protein synthesis is summarized in Figure 1. mTOR exists in two distinct physical and functional complexes, namely, mTORC1 and mTORC2 [43]. mTORC1 comprises mTOR, raptor, and mLST8; it phosphorylates the translation initiation regulators, p70S6 kinase and 4E-BP1, resulting in the changes in the activity of a number of initiation and elongation factors [44, 45]. In the resting cell, eukaryotic initiation factor 4E (eIF4E) is held inactive by its binding protein, 4E-BP1 [46]. When a stimulus for protein synthesis is received, mTORC1 is activated and it phosphorylates 4E-BP1. Phosphorylation of 4E-BP1 results in dissociation of eIF4E-4E-BP1 complex and release of eIF4E which then binds to the cap of the mRNA [47–50]. This augments the efficiency of translation. Phosphorylation of p70S6 kinase by mTORC1 affects both the initiation and elongation phases of mRNA translation. Activated p70S6 kinase phosphorylates ribosomal proteins and regulates ribosomal function. It also phosphorylates eukaryotic elongation factor 2 kinase (eEF2 kinase) which inhibits its activity [51, 52]. Decreased activity of eEF2 kinase contributes to reduced phosphorylation of eEF2 which results in activation of the latter [53]. As mentioned above, activated eEF2 facilitates the movement of aminoacyl tRNA from the A site to the P site on the ribosome during elongation phase of translation [51]. Thus, activation of p70S6 kinase facilitates the addition of amino acids to the newly synthesized peptide. Kidney tissues from type 2 diabetic *db/db* mice showed activation of mTORC1 that coincides with renal hypertrophy and matrix expansion. The constituents of the mesangial matrix expansion in the *db/db* mouse kidney consist of increased type IV collagen, fibronectin, and laminin [54, 55]. We have reported increase in laminin content in glomeruli and tubules by immunohistochemistry and morphometry in *db/db* mice kidneys when compared to *db/m* control mice; these diabetes-associated changes were inhibited by rapamycin. Ameliorative effect of rapamycin was shown to be due to inhibition of mTORC1 and its downstream pathways regulating the elongation phase of mRNA translation [34].

The mTORC2 complex contains mTOR, rictor, SIN1, and mLST8. Recent work has revealed that it controls the phosphorylation of the antiapoptotic proteins Akt/PKB and serum and glucocorticoid inducible kinase (SGK) and may promote cell survival [56–58]. Translation and processing of nascent polypeptides are highly coupled events that result in the production of mature and functional proteins. Recent investigations show that while mTORC2 activation of Akt and SGK1 can modulate translation, this complex also becomes recruited to the translating ribosome in order to process the newly synthesized polypeptide [59, 60]. The ribosome serves as a platform for cotranslational processing, folding, and transporting proteins to their target sites [61].

Most studies so far have been based on pharmacological inhibition of mTORC1 by rapamycin. Systemic administration of rapamycin, a specific and potent inhibitor of mTORC1, ameliorated pathological changes and renal dysfunction in diabetes [14, 62–65]. Thus, inactivation of mTORC1 is protective and reduced the effect of Erk- and TGF-beta-mediated prosclerotic pathways. However, cell-specific role of mTOR in renal hypertrophy induced by high glucose remained to be explored. Recent work by Gödel et al. [66] and Inoki et al. [67] shows that genetic reduction of mTORC1 activity by eliminating 1 *Raptor* allele prevents podocyte injury and ameliorates the progression of common glomerular diseases such as diabetic nephropathy; mTORC1 activation induced by ablation of an upstream negative regulator Tsc1 recapitulated many DN features, including podocyte loss, glomerular basement membrane thickening, mesangial expansion, and proteinuria in nondiabetic mice. Thus, mTORC1 remains an attractive target for potential therapeutic target to prevent DN.

Increase in protein synthesis occurs not only by stimulation of transcription and translation by also by inhibition of molecules that inhibit these processes. Signaling mechanisms augmenting protein synthesis have received much attention [14, 34, 62, 68, 69]. In contrast, constitutive signaling mechanisms that counteract the prohypertrophic signaling mechanisms and inhibit protein synthesis are not well

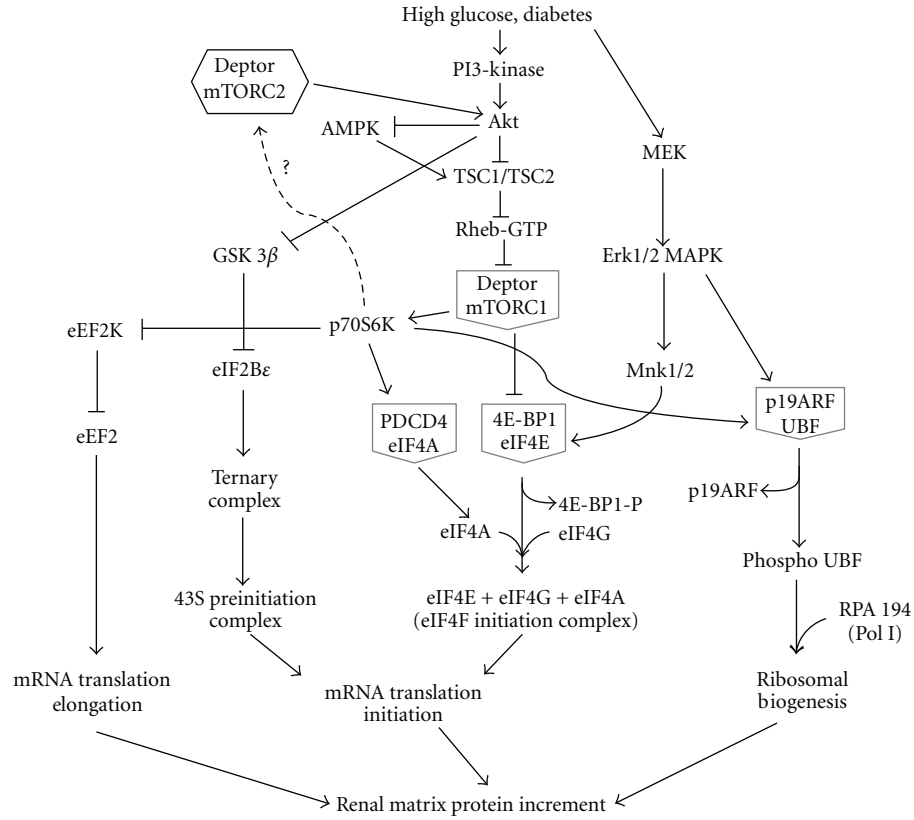

Figure 1: Intracellular signaling cascades regulated by high glucose leading to activation of promoters and suppression of intrinsic inhibitors of protein synthesis. Grey pentagons show the positive regulators held in an inactive repressor complex with an inhibitory protein. mTORC2 role in high glucose-induced protein synthesis has to be determined.

understood. There are at least three important constitutive inhibitors of protein synthesis. They are AMP-activated protein kinase (AMPK) and Deptor that inhibit the activity of mTOR and glycogen synthase kinase 3 beta (GSK 3β) that inhibits the activity of eukaryotic initiation factor 2B epsilon (eIF2Bε).

4.2. AMPK. AMPK plays a dual role in cell metabolism. It serves as an energy sensor and as a part of AMPK-TSC pathway, it inhibits Rheb/mTORC1 and keeps protein synthesis in check. Hyperglycemia reduced AMPK activity by phosphorylation at Thr172 on the catalytic alpha subunit resulting in activation of mTORC1 in glomerular epithelial cells [14]. Activation of mTORC1 contributes to the renal changes characteristic of DN, including glomerular hypertrophy, glomerular basement membrane (GBM) thickening, and the accumulation of mesangial matrix [14, 70–72]. Inhibition of AMPK by high glucose is required for high glucose-induced hypertrophy and ECM protein increment. Treatment of neonatal rat cardiomyocytes and renal glomerular epithelial cells with metformin, AICAR, or resveratrol activated AMPK and inhibited the development of hypertrophy induced by agents such as high glucose or phenylephrine [14, 71, 73]. Thus, AMPK could be a potential target for intervention in diabetic nephropathy.

4.3. GSK 3β. GSK 3β is a ubiquitously expressed, highly conserved serine/threonine protein kinase found in all eukaryotes. Unlike most protein kinases involved in signaling, GSK 3β is active in unstimulated, resting cells and it is inactivated upon phosphorylation at Serine 9. GSK 3β is inactivated during hypertrophy of skeletal myotube [74], heart [75, 76] and pulmonary artery smooth muscle [77]. GSK 3β phosphorylates its substrate eIF2B epsilon in the resting cell [78]. Activity of eIF2B epsilon is important for the formation of the preinitiation complex during the initiation phase of mRNA translation [79]. We observed that GSK 3β inhibits high glucose-induced protein synthesis in renal proximal tubular epithelial cells and renal tissues by inhibiting the activity of eIF2Bε [80]. Type 2 diabetic *db/db* mice showed increased phosphorylation of renal cortical GSK 3β and decreased phosphorylation of eIF2Bε, which correlated with renal hypertrophy at 2 weeks, and increased laminin β1 and fibronectin protein content at 2 months. These data raise the possibility that renal hypertrophy and laminin β1 accumulation induced by type 2 diabetes could be rescued by the activation of GSK 3β or by overexpression of an active form of GSK 3β in the kidney in the *db/db* mouse. In transgenic mice overexpressing activate form of GSK 3β in the heart, the hypertrophic response to calcineurin activation was severely impaired [81]. However, it is interesting to note that transgenic mice overexpressing

constitutively active form of GSK 3 ($GSK3^{S9A/S21A}$ knockin mice) exhibit glomerular injury with proteinuria [82].

4.4. Emerging Target in Signaling. Deptor (DEPDC6-DEP domain-containing and mTOR-interactive protein) is a novel mTOR regulatory protein that interacts with mTOR in both mTORC1 and mTORC2 and negatively regulates mTOR activity [83, 84]. As discussed earlier, mTORC1 is a central regulator of protein synthesis, ribosome biogenesis and cell growth during diabetic kidney. A series of elegant experiments based on loss-of-function strategy by Peterson et al., [85] showed that Deptor interacts directly with both mTOR complexes and inhibits downstream pathways regulated by both complexes. Liu et al., [86] have shown that enhanced interaction between mTOR and Deptor by resveratrol, a known inhibitor of mTORC1 [70], negatively regulated leucine-induced mTORC1 signaling in C2C12 myoblasts. Finally Deptor knockdown in vivo largely prevented the atrophic response produced by immobilization and, in part, this response was mediated by an increased muscle protein synthesis [87]. Given its powerful role as mTOR regulator, investigating the role of Deptor in diabetic kidney disease may provide a new avenue for preventing renal matrix accumulation.

5. Micro RNA (miR) in ECM Synthesis and Accumulation

miR microarray identified five miRs (192, 194, 204, 215, and 216) that were highly expressed in human and mouse kidney [88]. A seminal report by Kato et al. demonstrated that the expression of miR 192, one of the highly expressed miRs in the mouse kidney, is increased in the glomeruli from *db/db* type 2 diabetic mice when compared to control mice [89, 90] and in mesangial cells treated with high glucose [91]. Upregulated expression of miR 192 occurs as a consequence of TGF beta increment in diabetic glomeruli which in turn increased the expression of Col1a2. Wang et al. [91] demonstrated that in cultured human MCs exposed to high glucose or TGF-beta, as well as in mouse DN models in vivo, there was a significant upregulation of miR-377 that indirectly led to enhanced fibronectin production. Long et al. [92] identified miR-29c expression in the kidney glomeruli obtained from *db/db* type 2 diabetic mice in vivo and in kidney microvascular endothelial cells and podocytes treated with high glucose in vitro that has been found to enhance ECM protein accumulation.

Recent studies have elucidated the role of miRs in controlling translation [93]. miRs regulate gene expression by inhibiting translation and/or by inducing degradation of target messenger RNAs [94]. miRs bind directly to 3′ untranslated regions of specific transcripts and most often directly repress translation; furthermore, an mRNA can be simultaneously repressed by more than one miRNA species or one miRNA can modulate more than one transcript [95]. Programmed cell death 4 (PDCD4), an endogenous inhibitor of translation, has been identified as a target of miR 21, and it will be interesting to investigate the role of miR 21 in regulating ECM protein synthesis induced by diabetes. Dey et al. [96] reported that high glucose and TGFβ increase miR-21 and miR-214 in mesangial and proximal tubular epithelial cells. These microRNAs target downregulation of PTEN, an endogenous inhibitor of PI3 kinase dependent downstream Akt activity, for translational repression. The field of miRs and their role in diabetic kidney disease are an emerging field of investigation, and further studies will unravel the regulatory mechanism of these so-called "junk" DNA sequences in the genetic code [97].

6. Epigenetic Modification in ECM Production

Epigenetics is defined as mechanisms that affect chromatin structure and gene expression and dysregulation of the epigenome can also lead to disease. Major pathologic mediators of diabetes such as hyperglycemia, inflammatory factors, cytokines, and growth factors can lead to dysregulation of epigenetics [98]. Epigenetic changes include DNA methylation (covalent attachment of methyl groups at CpG dinucleotides), histone modifications (acetylation, methylation, phosphorylation, and ubiquitination), and RNA-based silencing. In the context of diabetic nephropathy, the altered state of the epigenome may be the underlying mechanism contributing to a "metabolic memory" that results in chronic inflammation and vascular dysfunction in diabetes even after achieving glycaemic control [99–101]. Identification of genetic and epigenetic risk factors that modulate ECM protein expression individually could provide the basis for the development of novel treatments and newer animal models of diabetic nephropathy.

7. Renal ECM Metabolism in Animal Models of Type 2 Diabetes

A higher proportion of individuals with type 2 diabetes are found to have microalbuminuria and overt nephropathy shortly after the diagnosis of their diabetes, because diabetes is actually present for many years before the diagnosis. This is why animal models of type 2 diabetes are very important so that newer and specific markers of early kidney injury could be identified before clinical diagnosis of the disease. However, these animals could only recapitulate some of the features of diabetic kidney disease seen in humans [102, 103]. T2DM is a complex genetic disease comprising many metabolic disorders with a common phenotype of glucose intolerance. Cohen et al. [104] have documented that glomerular pathology in type 2 diabetic *db/db* mice is accompanied by definable alterations in renal function, which are similar in chronology and nature to those found in human diabetes. Studies in *db/db* mice with type 2 diabetes have shown that accumulation of the renal matrix protein laminin-beta 1 is not associated with increase in its mRNA, suggesting potential regulation by mRNA translation [54]. Since hyperglycemia is associated with hyperinsulinemia, coinciding with the onset of laminin accumulation in the kidney in *db/db* mice, augmented laminin mRNA translation could be due to either elevated glucose or high insulin.

If hyperinsulinemia was to be implicated in laminin regulation in type 2 diabetes, the renal parenchyma would have to be responsive to insulin, unlike the liver which is insulin resistant. This was investigated by Feliers and colleagues; they employed a series of tests examining the status of insulin receptor activation and reported that kidney is responsive to insulin at the same time when liver is resistant to insulin in diabetic *db/db* mice [105]. These observations steered a series of investigations that identified a novel regulatory mechanism for ECM protein increment, mRNA translation [14, 15, 32], and also raised the possibility that hyperinsulinemia could participate in renal injury in type 2 diabetes.

There are two other models of type 2 diabetes which show progression of diabetic kidney disease that resemble human disease. The KKA^{y}/Ta mice produced by transfection of the yellow obese gene (Ay) into KK/Ta mice are obese, diabetic mice that manifest hyperglycemia, hypertriglyceridemia, hyperinsulinemia, and microalbuminuria. KKAy mice developed hyperglycemia, hyperinsulinemia, and obesity after 16 weeks, with proteinuria, mesangial matrix accumulation, GBM thickening, and tubular dilation. It was considered a good animal model for the early pathology changes of DN [106–108]. The MKR mice which transgenically express mutant IGF-1R specifically in skeletal muscle develop insulin resistance in fat and liver with rapidly progressive beta-cell dysfunction and type 2 diabetes [109]. They exhibit early onset of the disease phenotype as seen by insulin resistance (as early as 4 weeks), fasting hyperglycemia (from 5 weeks), and abnormal glucose tolerance (at 7–12 weeks), and they develop kidney disease characterized by ECM accumulation [110].

8. Management of Diabetic Renal Disease

Currently available therapies are not completely effective in arresting progression of diabetic kidney disease, especially at more advanced stages of disease. Consistent with investigations discussed above [35], studies have shown that ACE inhibitors and ARBs are beneficial in reducing the progression of albuminuria in patients with type 2 diabetes [5, 72, 111]. Treatment with an ACE inhibitor has been shown to normalize expression of laminin in murine mesangial cells [112]. A recent report shows the use of antifibrogenic drugs that block TGF beta to be effective in restoring kidney function [113]. Although the angiotensin-converting enzyme inhibitors and angiotensin receptor blockers retard the progression of diabetic nephropathy, they are not able to halt the eventual development of end-stage renal disease [114, 115]. One reason could be that pathological changes in the kidney may already be in place preceding the clinical diagnosis of diabetic nephropathy owing to the cumulative effects of postprandial hyperglycemic excursions, metabolic syndrome, and insulin resistance in type 2 diabetes. We need to take into consideration that several pathologic processes work in consort to result in kidney injury in diabetes. To date the usual investigational approach has been linear, having adopted the traditional one-variable-at-a-time model. Future investigations should apply a systems biology approach to understand how multiple pathogenetic events occurring simultaneously result in renal injury in diabetes.

9. Conclusion

Deregulation of protein synthesis, processing, and degradation underlie the development of renal matrix changes induced by hyperglycemia in type 2 diabetes. Thus, attenuating ECM accumulation and/or enhancing ECM degradation is considered a prime target in the preventive treatment of diabetic renal complications. In order to achieve this objective identifying the molecular mechanisms by which high glucose stimulates matrix protein synthesis is of paramount importance. Understanding these mechanisms may help develop early detection strategies and help identify those subjects at risk of progressing to advanced kidney derangement. While optimal control of hyperglycemia is a highly desirable approach in the treatment of diabetic complications including nephropathy, the difficulty in achieving this goal due to inability to adhere to therapeutic regimens and adverse effects of intensive glucose control regimens require us to find additional therapeutic avenues. Such interventions can only be developed by truly understanding the pathogenesis of kidney injury in diabetes and identifying viable therapeutic targets.

Abbreviations

ECM: Extracellular matrix protein
DN: Diabetic nephropathy
GBM: Glomerular basement membrane
TBM: Tubular basement membrane
4E-BP: 4E binding protein
AICAR: 5-aminoimidazole-4-carboxamide 1-β-ribofuranoside
AMPK: AMP-activated protein kinase
eEF: Eukaryotic elongation factor
eIF: Eukaryotic initiation factor
GβL: G proteinβ-subunit-like protein
GEC: Glomerular epithelial cells
TGFβ: Transforming growth factor beta
IGF: Insulin-like growth factor
mTOR: Mammalian target of rapamycin
PTEN: Phosphatase and tensin homolog on chromosome ten
Raptor: Regulatory associated protein of TOR
Rheb: Ras homolog enriched in brain
TSC: Tuberous sclerosis complex
UTR: Untranslated region
UBF1: Upstream binding factor 1
VEGF: Vascular endothelial growth factor
PDCD4: Programmed cell death 4.

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Review Article

New Experimental Models of Diabetic Nephropathy in Mice Models of Type 2 Diabetes: Efforts to Replicate Human Nephropathy

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Diabetic nephropathy (DN) is the leading cause of end-stage renal disease. The use of experimental models of DN has provided valuable information regarding many aspects of DN, including pathophysiology, progression, implicated genes, and new therapeutic strategies. A large number of mouse models of diabetes have been identified and their kidney disease was characterized to various degrees. Most experimental models of type 2 DN are helpful in studying early stages of DN, but these models have not been able to reproduce the characteristic features of more advanced DN in humans such as nodules in the glomerular tuft or glomerulosclerosis. The generation of new experimental models of DN created by crossing, knockdown, or knockin of genes continues to provide improved tools for studying DN. These models provide an opportunity to search for new mechanisms involving the development of DN, but their shortcomings should be recognized as well. Moreover, it is important to recognize that the genetic background has a substantial effect on the susceptibility to diabetes and kidney disease development in the various models of diabetes.

1. Introduction

Diabetic nephropathy is one of the major long-term microvascular complications and is the major cause of morbidity and premature mortality in individuals with type 2 diabetes mellitus. End-stage renal disease (ESRD) in patients with type 2 diabetes has increased dramatically worldwide during the last few decades, and diabetes is associated with worse survival among patients undergoing dialysis [1–3]. The pathogenesis of diabetic nephropathy includes both metabolic and hemodynamic factors [4]. A large number of candidate genes have been analyzed, both regarding initiation and progression but still are weak predictors of nephropathy in patients with type 2 diabetes. Experimental models of type 2 diabetes with nephropathy may offer a key to a better understanding of this complication in a multifactorial disease such as type 2 diabetes. Several experimental models have been developed to try to mimic human type 2 diabetes [5]. It seems that the ideal animal model for DN research in type 2 diabetes should have human-like kidney anatomy, hemodynamics, and physiology; develop the human type 2 characteristics and complications; allow studies in chronic stable DN, and allow measurement of relevant hemodynamic and biochemical parameters. However, it is difficult to generate a single-mouse model that recapitulates all of the features of established human diabetic nephropathy [6]. In 2003, the lack of reliable mouse models for studying severe DN prompted the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) to fund a consortium for the development and phenotyping of new diabetic mouse models that would resemble human DN more closely.

The Animal Models of Diabetic Complications Consortium (AMDCC) defined criteria for validating murine models of human diabetes and diabetic complications: >50% decline in GFR over the lifetime of the animal, >10-fold increase in albuminuria compared with controls for that

strain at the same age and gender, and pathology of kidneys (advanced mesangial matrix expansion, ± nodular sclerosis mesangiolysis, any degree of arteriolar hyalinosis, glomerular basement membrane thickening by >50% over baseline and tubulointerstitial fibrosis) [7]. The purpose of this paper is to summarize the features of the well-established and newer models of experimental nephropathy and their similarities to DN in patients with type 2 diabetes. There are many similarities in kidney disease findings in animal models of type 1 and type 2 diabetes, like in humans, which will not be discussed in the paper. Exacerbation of diabetic kidney disease by manipulations of the renin-angiotensin system such as renin overexpression [8], knockout of the bradykinin B2 receptor [7], or decorin deficiency [9] that have been studied so far in models of type 1 diabetes will not be discussed here, but the approaches are likely to be useful for type 2 diabetes as well.

2. Diabetic Nephropathy in Humans

In 1959, Gellman et al. [10] first reported findings in renal biopsies from patients with diabetic kidney disease and their clinical correlates. Before this was studied, the renal pathology in patients with diabetes mellitus had only been described systematically from autopsy findings. The initial changes of DN are glomerular hypertrophy, mild mesangial expansion (matrix), and thickening of the glomerular capillary walls (Figure 1). These changes are more evident by electron microscopy. When the disease progresses, there is also an increase in mesangial expansion; this mesangial matrix increase is defined as an increase in extracellular material in the mesangium such that the width of the interspace exceeds two mesangial cell nuclei in at least two glomerular lobules. As the disease progresses, there may be formation of nodules in the glomerular tuft. The nodules have variable size in a same glomerulus and affect some portions of the glomeruli, a pattern referred to as nodular diabetic glomerulosclerosis or *Kimmelstiel-Wilson nodules* (Figure 1). The presence of these nodules, large and small, some laminates, with variable size and distribution in the glomeruli, are "virtually" pathognomonic of DN, although similar nodules have been described as "idiopathic" in nondiabetic patients [11–13]. In DN, the glomeruli present increase of the mesangial intercapillary matrix, with progressive thickening of capillary walls and later evolution to global glomerulosclerosis.

Other two glomerular lesions, called "insudative lesions" (similar to arteriolar hyalinosis), are *capsular drop* and *glomerular hyalinosis*. The first is a homogenous hyaline deposit, in the Bowman's capsule. Usually this deposit is rounded or elongated, and its presence is highly suggestive of DN [14].

Glomerular hyalinosis is caused by a formation of a hyalin cap or a fibrin cap by accumulated plasma components in the peripheral segments of the tuft. In many typical DN cases, microaneurysms, produced by mesangiolysis, are evident. In tubules, there are nonspecific changes: reabsorption of protein droplets, interstitial fibrosis, and tubular atrophy. In vessels, usually there are notorious changes; the most characteristic lesion is intimal hyaline thickening of arterioles. Arteriolar lesions may involve any arteriole. Demonstration of arteriolar hyalinosis in both the afferent and efferent arterioles is virtually pathognomic of DN. Intimal fibrosis of the arteries is typical of DN, but this feature is not pathognomic because intimal fibrosis occurs in other diseases as well [15].

3. Classical Models of Nephropathy in Type 2 Diabetes

3.1. $LepR^{db/}LepR^{db}$ (db/db). The first described mutation in mice that resembled diabetes mellitus in humans occurred in an inbred mouse strain (C57BL/Ks) in 1966 [16]. The diabetic mutant is similar to the obese mutant in appearance [17], and the diabetic gene is transmitted as an autosomal recessive trait resulting from a mutation of the leptin receptor [16], leading to abnormal splicing and defective signaling of the adipocyte-derived hormone leptin [18]. Kidney function in mice with the db/db mutation on the C57BL/KS background has been intensively investigated and exhibits some features similar to early human diabetic nephropathy, class I to III. This model is widely used as a model of kidney disease and morbid obesity in type 2 diabetes (Figure 2). DN in the C57BL/KsJ(db/db) mouse is initially expressed as increased urinary albumin excretion at the age of 8 weeks without evidence of glomerular lesions by light microscopy [19].

Kidney hypertrophy has been noted in *db/db* mice at the age of 16 wk [20–22]. Glomerular hypertrophy has been measured by a digital planimeter using standard measurements of glomerular tuft areas at various ages of the *db/db* and *db/m* mice [23, 24]. Glomerular surface area was increased at 8 wk of age and remained increased at 25 wk of age. The time course of mesangial matrix expansion was described by Cohen et al. [23]. At 12 wk of age and after 4–6 wk of hyperglycemia, a twofold increase in mesangial matrix was noted. After 16 wk of age, a consistent threefold increase in mesangial matrix expansion was reported. At 5-6 mo of age, diabetic mice had larger glomeruli with increased mesangial matrix by periodic acid-Schiff (PAS) staining (Figure 2). By 18–20 mo of age, the mesangial matrix and glomerular enlargement became more pronounced and thickening of the glomerular basement membrane (GBM) was notable. In the oldest diabetic mice studied (16–22 mo of age), strikingly large subepithelial nodular densities were observed along with foot process fusion [24]. Albuminuria according to some studies is not very progressive in this model [24]. However, in the C57 background, which is generally resistant to kidney disease, albumin-creatinine ratio, in our experience, increases substantially as the animals age from 8 to 32 weeks of age [19].

3.2. $Lep^{ob/}Lep^{ob}$. The ob/ob recessive obese mouse carries a mutation in leptin, the ligand for the leptin receptor [26]. The Lep^{ob} mutation exists in different strains such as DBA2/J and C57BL/6J and FVB strains [27]. Renal structure and function in C57BL/6J ob/ob mice is said to be relatively mild. Chua et al. observed increased mesangial matrix expansion in FVB ob mice [27]. This model is not often used currently.

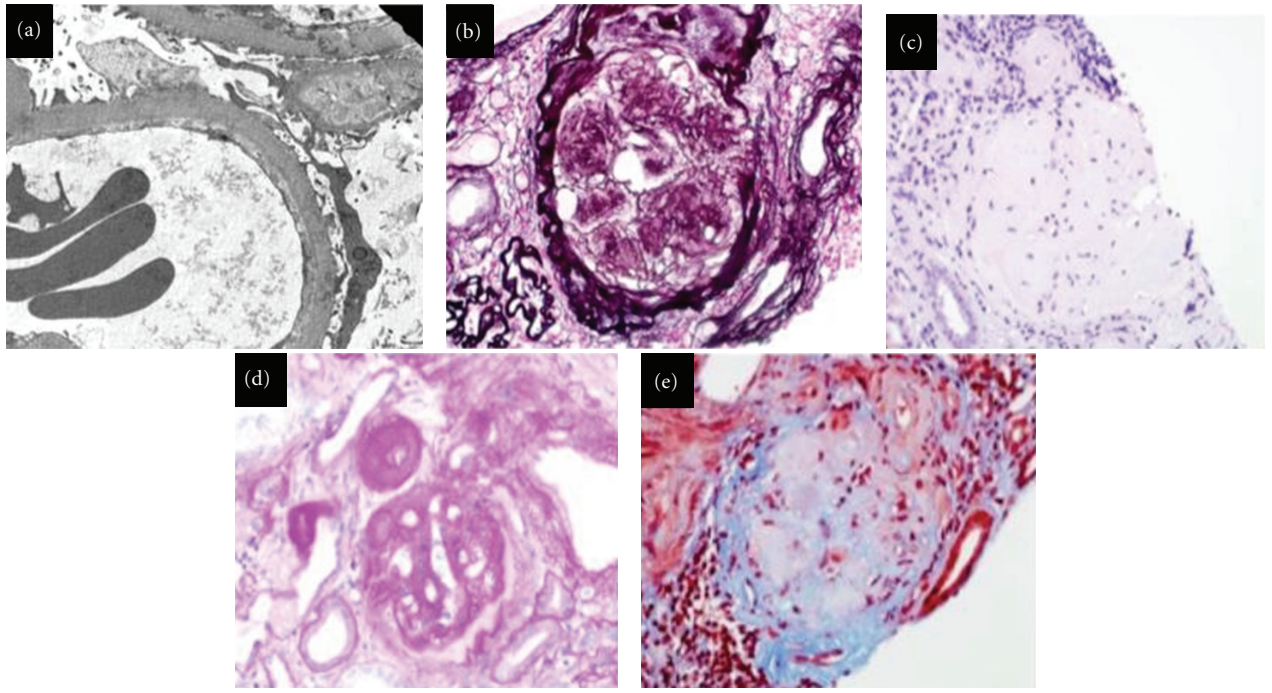


Figure 1: Established DN in humans: (a) glomerular basement membrane thickening by electron microscope. (b) Glomerular nodules in DN with intensively positive staining by methenamine silver. Nodular glomerulosclerosis: (c) hematoxilin eosine staining, (d) periodic acid-schiff stain demonstrates the mesangial nodules and esclerosis glomerular. (e) nodular diabetic glomerulosclerosis by Masson's trichrome staining, (Magnification ×400) in collaboration with Dr. Javier Gimeno.

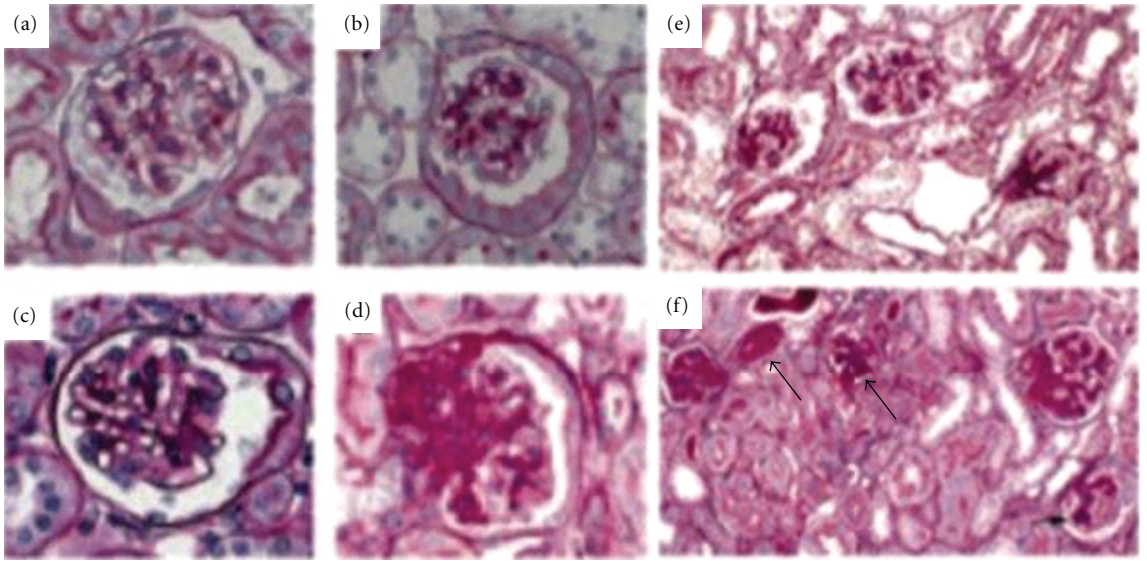


Figure 2: Glomerular histopathology in eNOS$^{-/-}$ db/db mice. Representative glomerular lesions of diabetic mouse kidneys at 24–26 wk: control (a); db/db (b and e); eNOS$^{-/-}$ (c) eNOS$^{-/-}$ db/db (d and f). (f) Arteriolar hyalinosis (big arrow) and early nodular glomerulosclerosis (small arrow) in eNOS$^{-/-}$ db/db mice (periodic acid-Schiff). Reprinted with permission from the Journal of the American Society of Nephrology [25].

3.3. New Zealand Obese Mice. New Zealand obese (NZO) mice are an inbred strain originally selected in New Zealand for polygenic obesity, and it is known to have obesity/diabetes as a result of QTLs on chromosomes 1, 2, 4, 5, 6, 7, 11, 12, 13, 15, 17, and 18 [28–30]. Mice are also prone to autoimmune disease as the kidneys exhibit light microscopic features that resemble lupus nephritis, not just diabetes lesions. The changes include glomerular proliferation, me-

sangial deposits, mild basement membrane thickening, and glomerulosclerosis [31, 32]. Eosinophilic nodules may be seen in some glomeruli, with occasional hyalinization of the glomerular arterioles and healing arteriolar inflammation [31, 32].

3.4. Agouti Mutation. The *agouti* gene is expressed during the hair-growth cycle in neonatal skin where it functions as a paracrine regulator of pigmentation [33, 34]. The secreted agouti protein antagonizes the binding of the a-melanocyte-stimulating hormone to its receptor (melanocortin 1 receptor) on the surface of hair bulb melanocytes, causing alterations in intracellular cAMP levels [33, 34]. Widespread, ectopic expression of the mouse *agouti* gene is central to the yellow obese phenotype, as demonstrated by the molecular cloning of several dominant *agouti* mutations and the ubiquitous expression of the wildtype *agouti* gene in transgenic mice. The hypothalamus and adipose tissue are biologically active target sites for *agouti* in the yellow obese mutant lines [33, 34]. Reports of albuminuria in diabetic KK-Ay mice suggest this mutation may be useful for the study of nephropathy. Moreover, the pathological changes in glomeruli from KK-Ay/TA mice are consistent with those in the early stage of human diabetic nephropathy [35]. This animal model of type 2 diabetes has, therefore, been proposed to examine the early stages of diabetic nephropathy [35]. It should be noted, however, that with aging diabetic KK-Ay mice develop spontaneous hydronephrosis, extremely dilated pelvis, and thinned atrophied parenchyma [36].

3.5. High-Fat Diet. A high-fat diet (HFD) provides a commonly used approach to induce obesity and insulin resistance in C57BL6 mice [37] and is also useful to study accelerated atherosclerosis associated with diabetes [38, 39]. Surwit et al. showed that genetic differences in the metabolic response to fat are more important in the development of obesity and diabetes than the increased caloric content of a high-fat diet [37]. HFD to C57BL/6 mice induces major systemic alterations compatible to human metabolic syndrome, including obesity, hyperglycemia, hyperinsulinemia, hypertriglyceridemia, and hypertension [40]. After the onset of metabolic syndrome, mice on an HFD developed increased UAE and glomerular lesions with the accumulation of extracellular matrix protein. Furthermore, renal pathophysiological alterations, including lipid accumulation, macrophage infiltration, increased oxidative stress, and impaired sodium handling were observed in the mice on an HFD [40].

4. New Models of DN

Previous animal models of diabetic kidney disease have manifested albuminuria and early renal pathologic changes such as glomerular basement thickening and mesangial expansion but with only minimal or inconsistent expression of other characteristic histopathologic features such as arteriolar hyalinosis and nodular glomerulosclerosis. The failure of previous models to manifest these lesions prompts the development of new models of DN (Table 1).

4.1. Db/db eNOS$^{-/-}$. Targeting Nos3, the gene encoding eNOS, or endothelial nitric oxide synthase was found to result in nephropathic changes in mouse models of type 1 and type 2 diabetes that mimic many aspects of human disease due to inhibition of nitric oxide formation [25]. C56BL/KS Db/db eNOS$^{-/-}$ mice developed striking albuminuria and characteristic pathologic changes of DN such as mesangiolysis, microaneurysms, and increased mesangial matrix expansion. In addition, nodular lesions (nodular glomerulosclerosis) and globally sclerotic glomeruli (diffuse glomerulosclerosis) were reported at 26 weeks [25, 41]. This model also exhibited remarkably decreased GFR on the basis of inulin clearance and serum creatinine measurements [25]. Studies in eNOS$^{-/-}$ mice demonstrated that this KO model develops moderate systemic hypertension. The increase in SBP was slightly greater in diabetic db/db eNOS$^{-/-}$ mice, although the results did not reach statistical significance [25]. These studies demonstrated that db/db eNOS$^{-/-}$ mice exhibit significant albuminuria and glomerular pathology that parallel the later phase of DN in patients with type 2 diabetes including arteriolar hyalinosis, mesangial expansion, thickening of GBM, and focal segmental and early nodular glomerulosclerosis [25, 41] (Figure 2). This model should prove useful for studying the role of endothelial dysfunction in development of DN and in facilitating the development of new diagnostic and therapeutic interventions.

4.2. NONcNZO10/LtJ. A NONcNZO10/LtJ is an inbred congenic strain derived from a cross between the Nonobese Nondiabetic (NON/LtJ) strain and the New Zealand Obese (NZO/HlLt) mouse, which provides a model of polygenic type 2 diabetes [29, 30, 42]. After approximately 8 mo of age, these mice also develop significant and progressively increasing albuminuria, with urine albumin-creatinine ratios >1000 μg/mg after 1 yr. Glomerular histopathology is impressively abnormal. In addition to glomerulosclerosis, however, there were features that are atypical of diabetic nephropathy. These included intraglomerular capillary thrombi and lipid deposition, nephritis, and evidence of Ig deposition. These features suggest that this may not be a good model for studying DN [29, 30, 42].

4.3. BTBR$^{ob/ob}$. Clee et al. characterized a mouse model of insulin resistance that develops in the progeny of the BTBR (black and tan, brachyuric) mouse strain crossed with C57BL/6 mice [43, 44]. BTBR mice are naturally hyperinsulinemic when compared with C57BL/6 mice, and BTBR × C57BL/6 F1 mice are substantially insulin resistant [44, 45]. When the *ob/ob* mutation is placed on a BTBR background, the mice are initially insulin resistant with elevated insulin levels, pancreatic islet hypertrophy, and marked hyperglycemia by 6 weeks of age [46].

The BTBR ob/ob mouse model of DN comes close to meeting all of the proposed criteria of the AMDCC (albuminuria, pathologic changes) and offers several important advantages compared with existing DN models. The most important of these is the degree to which it supposedly reproduces the essential structural and functional features of

Table 1: Some new models studied for diabetic nephropathy.

Animal model	Strain	Kidney pathology	References
Db/db eNOS$^{-/-}$	BKS	Significant albuminuria, decreased GFR, markedly increased mesangial matrix expansion, glomerular basement membrane thickening, arteriolar hyalinosis, mesangiolysis, nodular glomerulosclerosis, and tubulointerstitial injury	Zhao et al. [25] and Mohan et al. [41]
NONcNZO10/LtJ	NON/LtJ + NZO/HlLt	Albuminuria, glomerulosclerosis, intraglomerular capillary thrombi and lipid deposition, nephritis, and Ig deposition	Reifsnyder and Leiter [29, 30]
BTBR$^{ob/ob}$	BTBR	Albuminuria, loss of podocytes, extensive mesangial expansion, mesangiolysis, basement membrane thickening, and interstitial fibrosis	Hudkins et al. [46]
GIPR^{dn} transgenic	CD1	Renal, podocyte and glomerular hypertrophy, mesangial expansion, and matrix accumulation, glomerulosclerosis, proteinuria, and tubulointerstitial lesions	Herbach et al. [49]
GLUT1 transgenic	C57BL6/J	Albuminuria, glomerular hypertrophy, mesangial expansion, and glomerulosclerosis	Wang et al. [50]
podIR knockout (podocin or nephrin promoter)	Mixed genetic background (C57BL/6, 129/SV, and FVB)	Albuminuria, loss of foot process structure, podocyte apoptosis, increased glomerular matrix, thickened GBM, glomerulosclerosis, and kidney sclerosis	Welsh et al. [52]

human diabetic glomerular injury. Glomerular hypertrophy, marked expansion of mesangial matrix, mesangiolysis, and capillary basement membrane thickening have been identified in this model (Figure 3). Loss of podocytes (Figure 4) is also present in the BTBR ob/ob model [46].

The functional consequence of these changes in humans—marked proteinuria—also is present in this mouse model with a 10-fold increase in urinary protein excretion compared with controls [46]. Second, the model is robust and progressive: BTBR ob/ob mice uniformly develop features of DN and do so in a predictable time course in which podocyte loss is already detectable by 8 weeks of age and persists throughout the disease. Significant proteinuria is detectable as early as 8 weeks of age, corresponding with detectable podocyte loss, although it can be detected in some mice at even earlier ages, albeit without achieving statistical significance, when comparing 4-week-old cohorts with controls.

Mesangiolysis is also an early feature of the disease, detectable in approximately 10% of glomeruli at 8 weeks of age and coincides with detectable expansion of the mesangial matrix. These mesangial alterations are progressive with detectable expansion of the mesangial matrix. The BTBR *ob/ob* mouse is among the very few models in which pronounced mesangial expansion and mesangiolysis resembling advanced human DN predictably develop [46]. Third, DN develops more rapidly in BTBR *ob/ob* mice compared with models of leptin receptor deficiency (*db/db* mice) or most other mouse models currently used to study DN [7], which often require from 30 to 50 weeks or more to develop relevant lesions. The relatively rapid onset allows opportunities for testing therapeutic strategies aimed at halting or ameliorating DN in a much shorter time span. BTBR ob/ob mice develop a constellation of abnormalities that closely resemble advanced human DN more rapidly than most other murine models, making this strain particularly attractive for testing therapeutic interventions [46].

4.4. GIPR^{dn} Transgenic. Transgenic mice, expressing the mutated human glucose-dependent insulinotropic polypeptide receptor (GIPR), were generated under the control of the rat proinsulin 2 gene promoter in pancreatic beta cells [47]. These GIPR^{dn} transgenic mice exhibit an early disturbance in pancreatic islet development (severe reduction of beta-cell mass, disturbed composition of islets, and decreased islet neogenesis), diminished insulin secretion, and early-onset diabetes mellitus, without obesity or insulin resistance. In type 2 diabetic patients, a major abnormality is reduced insulinotropic action of GIP [48], as well as reduced volume density and mass of beta cells in the pancreas [47]. Therefore, GIPR^{dn} transgenic mice resemble important aspects of human type 2 diabetes mellitus.

GIPR^{dn} transgenic mice develop progressive diabetes-associated kidney lesions with many parallels to the human

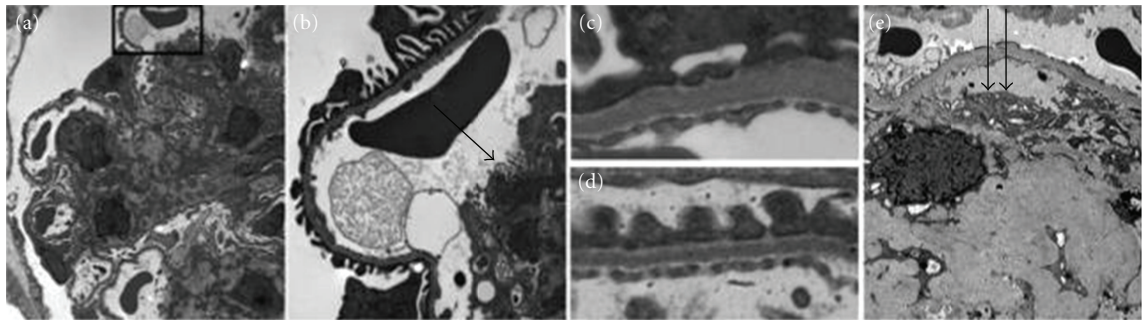
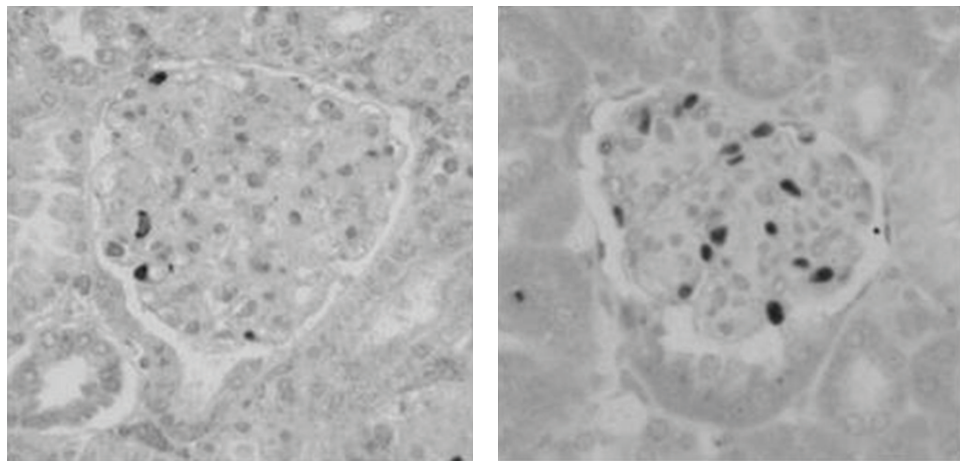


Figure 3: Ultrastructural changes in BTBR ob/ob mice resemble human DN. (a and b) Electron microscopy of glomeruli of 22-week-old BTBR ob/ob mice shows qualitatively good preservation of foot processes overall. There is an increased mesangial matrix and evidence of mesangiolysis with fraying of the mesangial/capillary interface (arrows) in (b). (c and d) Basement membranes are thickened, and there is focal effacement of foot processes in BTBR ob/ob mice (c) when compared with BTBR WT mice (d). There is no evidence of immune deposits. (e) Advanced human DN, occurring after one or more decades of diabetes, also shows marked mesangial matrix accumulation with similar fraying of the mesangial/capillary interface as seen in BTBR ob/ob mice (double arrows). Reprinted with permission from the Journal of the American Society of Nephrology [46].



(a) (b)

Figure 4: Podocyte loss in BTBR ob/ob mice. BTBR ob/ob mice have reduced podocyte number. (a and b) There is a reduction in podocyte number assessed by WT-1 staining, in BTBR ob/ob (a) compared with BTBR WT (b) mice. Reprinted with permission from the Journal of the American Society of Nephrology [46].

disease, that is, renal, glomerular, and podocyte hypertrophy, thickening of the GBM, reduction of the glomerular density of podocytes, progressive glomerulosclerosis albuminuria, and tubulointerstitial changes. This experimental model, may serve as an excellent new model for studying the pathogenesis of DN, the sequence of structural, functional, and molecular changes of glomerular cells, and for testing the efficacy of new therapies [49].

4.5. GLUT-1 Transgenic. Heilig's group recently published the phenotype of transgenic GLUT1-overexpressing mice (GT1S). This is an intriguing model designed to characterize the roles of GLUT1 and intracellular glucose in the development of glomerular disease without diabetes [50]. Kidneys of GT1S mice overexpressed GLUT1 in glomerular mesangial cells and small vessels without increased expression in renal tubules. GT1S mice were neither diabetic nor hypertensive. Glomerular GLUT1, glucose uptake, mean capillary diameter, and mean glomerular volume were all increased in the GT1S mice. The transgenic glomeruli revealed diffuse mesangial expansion on light microscopy, occasionally with nodular features, although the nodules were more cellular and do not really replicate the human Kimmelstiel Wilson nodules. Moderately severe glomerulosclerosis (GS) was found by 26 weeks of age in GT1S mice, with increased glomerular Type IV collagen and fibronectin. Modest increases in GBM thickness and albuminuria were detected with podocyte foot processes largely preserved, in the absence of podocyte GLUT1 overexpression [50]. Activation of glomerular PKC, along with increased TGFβ1, VEGFR1, VEGFR2, and VEGF was all detected in glomeruli of GT1S mice, likely contributing to GS. The transcription factor NF-κB was also activated. Overexpression of glomerular GLUT1, mimicking the diabetic GLUT1 response, produced numerous features typical of diabetic glomerular disease,

without diabetes or hypertension. This suggested that the GLUT-1 transporter overactivity plays an important role in the development of DN possibly by locally increasing glucose uptake at the glomerular level. In this respect, this model offers a strong evidence for intracellular glucose in the development of glomerulosclerosis [50].

4.6. PodIR Knockout. To determine whether insulin signaling in podocytes affects glomerular function in vivo, Welsh's group generated mice with specific deletion of the insulin receptor from their podocytes (podIRKO). PodIRKO mice were generated by crossing floxed insulin receptor mice with podocyte-specific Cre recombinase mice driven by both the nephrin and podocin promoters [51, 52]. Detailed renal evaluation at 3 weeks of age was normal in podIRKO mice with no abnormality identifiable using either light or electron microscopic analysis. At 5 weeks of age, both podIRKO models (nephrin and podocin promoter) started to develop albuminuria accompanied with loss of the podocyte foot process structure detected by electron microscopy [52]. At 8 weeks of age, significant levels of albuminuria and histological changes such as extensive loss of foot process structure and podocyte apoptosis were present in podIRKO. Furthermore, at 13 weeks of age, podIRKO mice had increased amounts of glomerular matrix, thickened GBM, and increased levels of glomerulosclerosis [52]. As they aged, these pathological features became more prominent, with some of the podIRKO mice developing macroscopically shrunken and sclerosed kidneys. These animals develop significant albuminuria together with histological features that recapitulate DN, but in a normoglycemic environment. These novel findings reveal the critical importance of podocyte insulin sensitivity for kidney function [52].

5. Genetic Background as a Modifier of Diabetic Kidney Disease

Gurley and others have emphasized the importance of the genetic background in kidney disease development in diabetic mice [7]. Their initial studies focus on STZ-induced diabetes but they are relevant to most models of diabetes. They reported among five common inbred mouse strains a hierarchical response of blood glucose levels to STZ-induced diabetes (DBA/2>C57BL/6>MRL/Mp>129/SvEv>BALB/c). In all five strains, males demonstrated much more robust hyperglycemia with STZ than females. STZ-induced diabetes was associated with modest levels of albuminuria in all of the strains, but was greatest in the DBA/2 strain, which also had the most marked hyperglycemia [53]. Observed renal structural differences between the strains were limited to mesangial expansion, but the strong correlation between high blood glucose and mesangial size expansion suggests that the size differences were caused by the differences in blood glucose levels. The differences in the responses to STZ-induced diabetes suggest that DBA/2 is the most susceptible to diabetic nephropathy and is most likely the most useful platform for model development [53]. It is important to emphasize the relative resistance to diabetic nephropathy of the widely used C57BL/6 mouse [7]. The Mouse Phenome Database is a useful source for comparative data of basal metabolic parameters distinguishing the more commonly used inbred strains (http://phenome.jax.org/). This website contains albumin-creatinine ratio data for males and females of 30 inbred strains [7].

6. Conclusions

The use of experimental animal models of DN has provided valuable information regarding some aspects of DN. Classical experimental models of type 2 DN have been available. However, for some time these models have not been able to reproduce features of advanced DN such as nodules in the glomerular tuft or glomerulosclerosis. Moreover, information on progressive decline in GFR is often missing in these models. In particular, through the efforts of the AMDCC investigators as well as others, using genetic breeding and other means to enhance disease severity, the characteristic features of experimental diabetic nephropathy are becoming more apparent. The importance of genetic background on susceptibility and resistance to kidney disease is increasingly recognized as a key factor. Data from the Diabetic Complications Consortium group has been very helpful for the researchers by creating a website that includes a protocol for validation of mouse models of diabetic nephropathy and updated characterization of different diabetic models.

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Research Article

An Angiotensin II Type 1 Receptor Blocker Prevents Renal Injury via Inhibition of the Notch Pathway in Ins2 Akita Diabetic Mice

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Recently, it has been reported that the Notch pathway is involved in the pathogenesis of diabetic nephropathy. In this study, we investigated the activation of the Notch pathway in Ins2 Akita diabetic mouse (Akita mouse) and the effects of telmisartan, an angiotensin II type1 receptor blocker, on the Notch pathway. The intracellular domain of Notch1 (ICN1) is proteolytically cleaved from the cell plasma membrane in the course of Notch activation. The expression of ICN1 and its ligand, Jagged1, were increased in the glomeruli of Akita mice, especially in the podocytes. Administration of telmisartan significantly ameliorated the expression of ICN1 and Jagged1. Telmisartan inhibited the angiotensin II-induced increased expression of transforming growth factor β and vascular endothelial growth factor A which could directly activate the Notch signaling pathway in cultured podocytes. Our results indicate that the telmisartan prevents diabetic nephropathy through the inhibition of the Notch pathway.

1. Introduction

The worldwide prevalence of diabetes in all age groups was 2.8% in 2000 and is estimated to be 4.4% in 2030 [1]. The total number of people with diabetes mellitus (DM) is expected to rise from 171 million in 2000 to 366 million in 2030. Diabetic nephropathy, a major microvascular complication of DM, is the most common cause of end-stage renal disease (ESRD) [2]. The number of ESRD cases is expected to increase mainly as a result of the increasing incidence of obesity and type 2 DM.

A number of pathways such as the protein kinase C pathway [3] and the polyol pathway [4] as well as advanced glycation end products [5] have been reported to play important roles in the development of diabetic nephropathy. It has also been reported that the renin-angiotensin system (RAS) plays a potent role in the initiation and progression of diabetic nephropathy [6].

A number of clinical evidences have suggested that the blockade of the RAS by angiotensin-converting enzyme (ACE) inhibitors (ACEIs) and/or angiotensin II type1 receptor (AT1R) antagonists (ARBs) could improve renal function or slow down disease progression in diabetic nephropathy [7]. Furthermore, it has been reported that ACEIs and/or ARBs inhibit the RAS and have pleiotropic effects, which improve renal prognosis.

Recently, Niranjan et al. reported that the Notch pathway was activated in diabetic nephropathy and in focal segmental

glomerulosclerosis (FSGS) [8]. The activation of the Notch pathway in podocytes has been studied in genetically engineered mice. These mice developed glomerulosclerosis due to the activation of p53, which induced apoptosis in podocytes. The same group also showed that pharmaceutical and genetic blockade of the Notch pathway prevented mice from developing diabetic and puromycin-aminonucleoside-(PAN-) induced glomerulosclerosis.

The Notch signaling pathway is a signaling pathway that determines cell fate [9]. Further, it is regulated by cell-cell communication during the formation of various internal components such as the nerves, blood, blood vessels, heart, and hormonal glands. Notch is a transmembrane receptor protein that interacts with ligands of the Jagged and Delta families [10].

The aim of this study was to examine the activation of the Notch pathway in Akita mice as well as the effects of telmisartan on the Notch pathway both *in vivo* and *in vitro*.

2. Materials and Methods

2.1. Reagents. Telmisartan was obtained from Nippon Boehringer Ingelheim Co., Ltd. (Tokyo, Japan). Candesartan was purchased from Tronto Research Chemicals (North York, Canada). Angiotensin II was obtained from Sigma-Aldrich (St. Louis, MO). Recombinant human TGF-β1 (#240-B) and recombinant human VEGF-A (#293-VE) were purchased from R&D systems (Minneapolis, MN). GSI was purchased from Calbiochem (San Diego, CA). Hoechst 33342 was from Dojindo laboratories (Kumamoto, Japan).

2.2. Animals. Male heterozygous Ins2 Akita diabetic mice (C57BL/6) and C57BL/6 controls were obtained from Japan SLC Inc. (Shizuoka, Japan). Eight-week-old Akita mice and control mice received telmisartan ($5\,\text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$) or no treatment for 15 weeks ($n = 8$ in each group). The blood glucose level, body weight, blood pressure, and urinary albumin excretion were measured every two weeks. The blood glucose level was examined using Medisafe-Mini (TERUMO Corporation, Tokyo, Japan), and the blood pressure was determined by the tail cuff method using Softron BP-98A (Softron, Tokyo, Japan). In order to estimate albuminuria, mice were individually housed in metabolic cages for 24 h. Urine was collected, and urinary albumin concentrations were measured with a Lebis Albumin assay kit (Shibayagi, Gunma, Japan). The blood creatinine levels, BUN, fasting blood glucose levels, and HbA1c were measured at the time of sacrifice. All experiments in this study were performed in accordance with the Guidelines of the Animal Care and Use Committee of Chiba University, Japan, which follows the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1985). The ethics committee for animal research at Chiba University approved all animal experiments.

2.3. Immunohistochemistry. The following commercially available antibodies were used: rabbit anti-Jagged1 (1 : 200 dilution, sc-11376) and rabbit antihuman TGF-β1 (1 : 50, sc-146) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-cleaved Notch1 antibody (1 : 100, Val1744, no. 2421S) was purchased from Cell Signaling (Danvers, MA). Rat anti-podocalyxin monoclonal antibody (0.5 μg/mL, MAB1556) was from R&D systems. Mice kidneys were embedded in OCT compound and frozen, and 10 μm sections were made. The sections were air dried, fixed in methanol (10 min on ice), rinsed in phosphate-buffered Tween (PBT), and blocked for 30 min with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). Primary antibodies were diluted in PBS containing 1% BSA and were incubated with the sections overnight at 4°C. The slides were rinsed with PBT for several times. The fluorophore-conjugated secondary antibodies were applied for 2 h. The sections were again rinsed with PBT for several times, mounted (Vectashield Mounting Medium with DAPI; Vector Laboratories, Inc., Burlingame, CA), and viewed under a fluorescence microscope (Axio Observer; Leica) or a confocal laser scanning microscope (Leica LSM5 PASCAL). The images were processed using Adobe Photoshop.

2.4. Cell Culture. Mouse podocytes, conditionally immortalized with a temperature-sensitive variant of the SV40 large T-antigen, were kindly provided by Dr. Peter Mundel (Albert Einstein College of Medicine, NY, USA). The preparation and characterization of these cells have been described elsewhere [11]. Podocytes were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco/Life Technologies, Grand Islands, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich), 100 U/mL penicillin, and 100 U/mL streptomycin (Sigma Aldrich). To propagate podocytes, cells were cultivated at 33°C and incubated with 10 U/mL of murine recombinant γ-interferon (Pepro Tech EC Ltd, London, UK) to enhance the expression of the T-antigen (permissive conditions). To induce differentiation, podocytes were cultured at 37°C without γ-interferon in RPMI 1640. Cells were cultured under nonpermissive conditions for at least 11 d before they were used in the experiments. The medium was changed every 3 d to induce full differentiation. Cells at passages 12 to 18 were used for the experiments in this study.

2.5. Reverse Transcriptase-Polymerase Chain Reaction. The expression of mRNA in podocytes was analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. After treatment with DNase, 1 μg of total RNA was reversely transcribed using oligo dT primer, pd(T)12–18 (Invitrogen, Carlsbad, CA), to avoid genomic contamination. The cDNA was generated using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Gene-specific oligonucleotides for the PCR analyses were designed according to the predicted cDNA sequences (http://www.ensembl.org/). The PCR was performed in a 25 μL PCR reaction containing 1 μL of complementary DNA (cDNA), Taq reaction buffer

Table 1: Characteristics of the experimental groups of mice.

	Wild control	Wild telmisartan	Akita control	Akita telmisartan
Blood glucose (mg/dL)	250 ± 34	284 ± 58	1216 ± 130*	955 ± 137*,†
HbA1c (%)	4.3 ± 0.3	4.2 ± 0.3	10.8 ± 1.4*	11.8 ± 0.5*
Body weight (g)	36.4 ± 3.4	40.7 ± 9.0	20.8 ± 0.8*	23.2 ± 1.4*,†
Systolic blood pressure (mmHg)	109.3 ± 4.7	96.1 ± 7.3	126.4 ± 5.9*	110 ± 5.1*,†
Urinary albumin (mg/day)	21.2 ± 9.4	10.9 ± 2.51	51.4 ± 11.6*	33.8 ± 8.5*,†

Data are expressed as the mean ± standard deviation (SD). *$P < 0.01$ versus wild-type control, †$P < 0.01$ versus Akita control.

(Go Taq, Promega, Madison, WI), and 10 μM of dNTPs. The primer sequences and sizes of the expected PCR products are as follows: Hes1, 5′-CCCTGTCTACCTCTCTCCTT-3′, 5′-AGGTGCTTCACAGTCATTTC-3′, 472 bp; TGF-β, 5′-TCCAAGAAAAAGAAAATGGA-3′, 5′-CTCTGAATCAGGTTGTGGAT-3′, 452 bp; VEGF-A, 5′-GTGGACATCTTCCAGGAGTA-3′, 5′-ATCTGCAAGTACGTTCGTTT-3′, 382 bp; β-actin, 5′-TCGTGCGTGACACATCAACATCAAAGAG-3′, 5′-TGGACAGTGAGGCCAGGATG-3′, 411 bp. PCR was performed for 25–30 cycles. Each cycle consisted of denaturation at 94°C for 2 min, annealing at 50°C for 30 s, and extension at 72°C for 30 s. PCR amplification was followed by a final extension step at 72°C for 7 min. An aliquot of 10 μL of each PCR product was subjected to electrophoresis on a 2% agarose gel (Ronza), followed by staining with an ethidium bromide solution (Sigma). The signals were photographed with a charge-coupled device (CCD) camera system (Printograph, ATTO). Densitometric analyses of the fluorograms were performed using an image scanner (EPSON GT-X900) with ImageJ software (http://rsbweb.nih.gov/ij/download.html).

2.6. Morphometric Analysis. Five glomeruli ($n = 3$, in each) were randomly selected from each specimen. The extent of extracellular mesangial matrix was determined by quantification of the periodic-acid-Schiff-staining- (PAS-) positive area in the mesangium and divided by the glomerular tuft area. The extracellular mesangial matrix area and glomerular tuft area were quantified by ImageJ.

2.7. Detection of Apoptosis by Hoechst Staining and Flow Cytometric Assays. Podocytes were treated with AII in the presence or absence of telmisartan for 72 h. After the treatment, apoptosis was defined as the presence of nuclear condensation on Hoechst staining. Alternatively, the cells were collected, washed twice with cold phosphate-buffered saline (PBS), and centrifuged at 1,000 g for 5 minutes. Subsequently, the Annexin V/propidium iodide assay was carried out to determine apoptosis according to the manufacturer's instructions (BD Pharmingen) and analyzed by flow cytometry (FACSCalibur; BD Immunocytometry Systems, San Jose, CA).

2.8. Statistical Analysis. Results are expressed as the mean ± standard error of the mean (SEM). Experimental points were performed in triplicates with a minimum of three independent experiments. An unpaired Student's t-test was used for comparison of two groups. $P < 0.05$ was considered significant.

3. Results

3.1. Telmisartan Reduces the Urinary Albumin Excretion in Akita Mice. First, we evaluated the effect of telmisartan on blood pressure in mice. Table 1 shows that Akita mice had a higher blood pressure than the controls. As expected, administration of telmisartan significantly lowered the blood pressure. Compared to the controls, Akita mice also had considerably higher levels of blood glucose and HbA1c, which eventually led to loss of body weight. Telmisartan decreased the blood glucose level and led to an increase in body weight in Akita mice (Table 1). The urinary albumin excretions were significantly increased in untreated Akita mice compared to wild-type controls, and administration of telmisartan significantly reduced urinary albumin excretion (Table 1).

Next, we investigated the effect of telmisartan on the glomerular morphology. Expansion of the mesangial areas was observed in Akita mice; however, telmisartan had no profound effect on the glomerular morphology as determined by light microscopy (Figure 1).

3.2. Telmisartan Inhibits the Notch Pathway and the Expression of TGF-β, Which Are Activated in the Glomeruli of Akita Mice. Recently, it has been reported that the Notch pathway is activated in podocytes in DM. Therefore, we examined the Notch pathway in Akita mice. ICN1 staining in kidneys revealed that the number of ICN1-positive cells in the glomeruli was significantly higher in Akita mice (Figures 2(a) and 2(b)). We could not observe ICN1-positive cells other than in the glomeruli. This indicated that the Notch pathway was activated in Akita mice, and the activation of the Notch pathway seemed to be restricted to the glomeruli. In order to identify cell types that were activated by the Notch pathway within the glomeruli, we also carried out coimmunostaining with an anti-ICN1 antibody and an anti-podocalyxin antibody (a marker for podocytes). We localized ICN1 proteins to the nuclei of the cells which were positive for podocalyxin within the cytoplasm (Figure 2(c)). Therefore, Notch pathway was activated in podocytes in diabetic conditions. Administration of telmisartan significantly reduced the number of ICN1-positive cells in the glomeruli (Figures 1(a) and 1(b)). Next, we investigated the expression of Jagged1, which is a ligand for the Notch

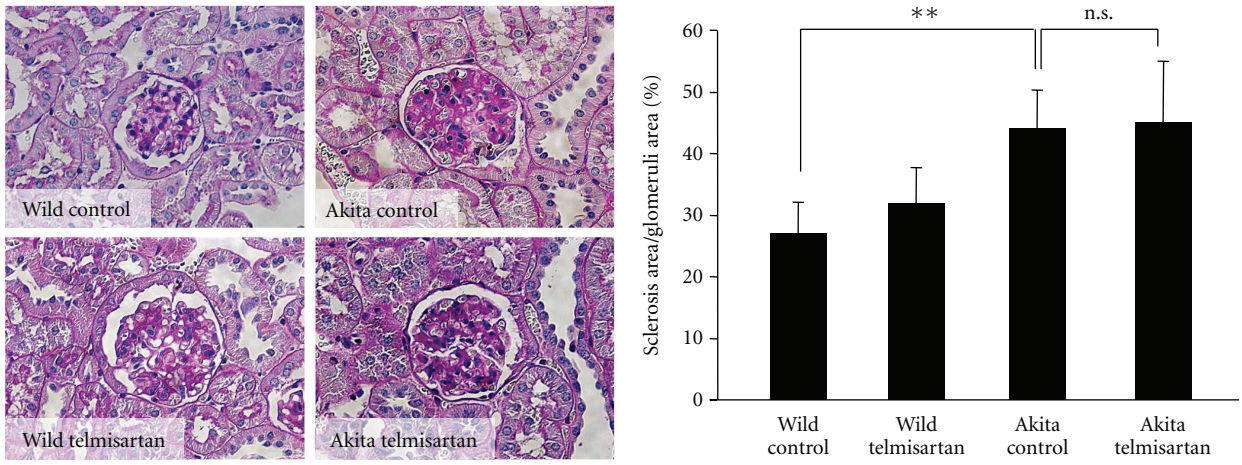

Figure 1: Morphometric analyses of the glomeruli of Akita mice. (a) Eight-week-old Akita mice and control mice received telmisartan ($5\,mg \cdot kg^{-1} \cdot day^{-1}$, in their drinking water) or no treatment, respectively, for 15 weeks ($n = 8$ in each group). After 15 weeks, the mice were sacrificed, the kidneys were harvested, and periodic acid-Schiff staining was performed. (b) Quantification of sclerosis per glomerular area was performed with the ImageJ software. $^{**}P < 0.01$, *n.s.*: not significant.

receptor. The expression pattern of Jagged1 was quite similar to that of ICN1 (Figure 2(d)). These results indicated that telmisartan inhibited the Notch pathway *in vivo* either directly or indirectly. It has been reported that the Notch pathway in podocytes was activated by TGF-β signaling [8]. Therefore, we investigated the expression of TGF-β by immunohistochemistry. We observed upregulated TGF-β expression in the glomeruli of Akita mice (Figure 2(e)), especially in podocytes (Figure 2(f)). Administration of telmisartan also suppressed the expression of TGF-β in the glomeruli (Figure 2(e)).

3.3. Angiotensin II Activates the Notch Signaling Pathway through Increased Expression of TGF-β and VEGF-A in Cultured Podocytes. Telmisartan lowered the blood pressure and improved the blood glucose level in Akita mice. From these findings, we were not able to completely exclude the possibility that the inhibitory effect of telmisartan on the Notch pathway *in vivo* was due to a systemic effect. Therefore, we used cultured mouse podocytes that were conditionally immortalized in order to not only rule out the influence of blood pressure and glucose levels but also elucidate the mechanism by which telmisartan inhibits the Notch pathway. Telmisartan is an AT1R blocker. For this reason, we studied the effect of angiotensin II (AII), a ligand for AT1R, on the activation of the Notch pathway. As shown in Figure 3(a), the mRNA expression of hairy enhancer of split homolog-1 (Hes1), which was a target gene of the Notch signaling pathway, increased considerably in the presence of 10^{-6} M AII. In addition, telmisartan inhibited the AII-induced mRNA expression of Hes1 (Figure 3(a)). The expression of Jagged1 mRNA was also increased in the presence of AII, and telmisartan inhibited AII-induced mRNA expression of Jagged1 (data not shown). We also examined the effect of candesartan, another type of AT1R blocker, and found that candesartan inhibited the AII-induced mRNA expression of Hes1 same as telmisartan (Figure 3(b)). It has been reported that TGF-β and VEGF-A activate the Notch pathway [12]; therefore, the effect of AII on the expression of TGF-β and VEGF-A was investigated. As shown in Figures 3(c) and 3(d), incubation with AII significantly increased the expression of both TGF-β and VEGF-A. Telmisartan reversed this effect.

Finally, we observed the effects of TGF-β and VEGF-A on the activation of the Notch pathway and found that these growth factors could activate the Notch pathway. However, telmisartan had no effect on the Notch pathway in the presence of TGF-β or VEGF-A (Figure 4).

3.4. Telmisartan Suppresses the Podocyte Apoptosis Induced by Angiotensin II. It has been reported that the activated Notch pathway induces apoptosis to the glomerular podocytes which eventually causes glomerulosclerosis. Therefore, we investigated whether telmisartan could prevent podocyte apoptosis. As shown in Figures 5(a) and 5(b), flow cytometer studies using annexin V and propidium iodide showed that apoptotic cells were increased in the podocytes treated with AII ($12.56 \pm 1.9\%$ versus $7.09 \pm 1.4\%$ in the control group, $P < 0.01$), and telmisartan treatment significantly decreased the AII-induced apoptotic cells ($8.51 \pm 2.0\%$ versus $12.56 \pm 1.9\%$ in the AII group, $P < 0.01$). We also examined the apoptosis by the use of Hoechst 33342 staining as shown in Figures 5(c) and 5(d). Nuclear condensation was observed in the podocytes in the presence of AII, and those changes were significantly decreased when the podocytes were treated with telmisartan. We also examined the effects of γ-secretase inhibitor (GSI) on the AII-induced apoptosis and found that GSI, an inhibitor of Notch signaling, was able to inhibit the AII-induced apoptosis (Figure 4). Collectively, these results indicated that the AII induced podocytes apoptosis via the activating Notch signaling pathway, and telmisartan

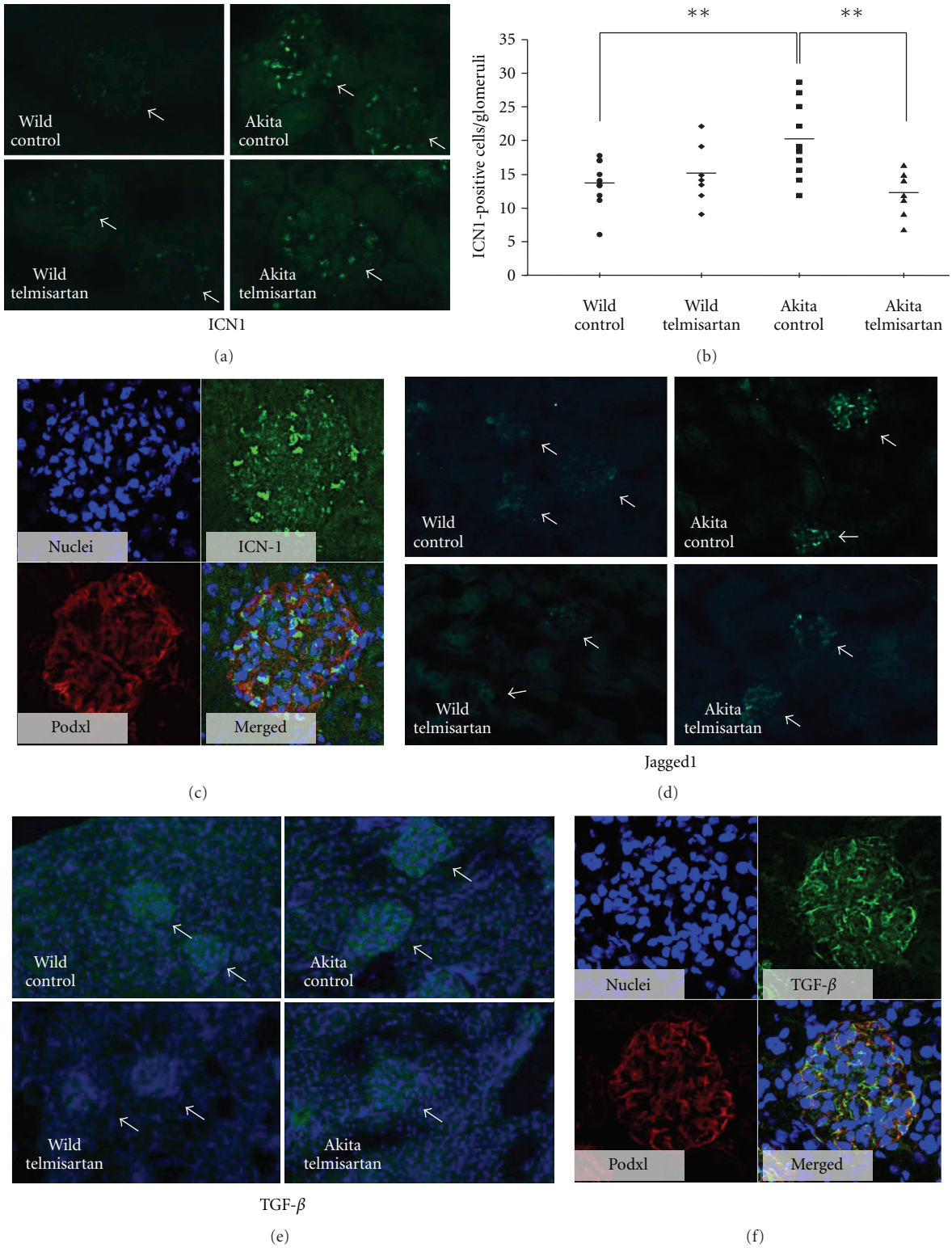

Figure 2: Notch pathway was activated in the glomeruli of Akita diabetic mice and telmisartan inhibited its expression. The expression of the intracellular domain of Notch1 (ICN1) (a and c), Jagged1 (d), and transforming growth factor β (TGF-β) (e and f) were examined by immunohistochemistry. Anti-podocalyxin (Podxl) antibody was used as a marker for podocyte. ICN-1 was localized to podocyte nuclei (c), while TGF-β was localized to podocyte cytoplasm, respectively (f). Quantification of ICN1-positive cells per glomeruli was performed (b). Ten glomeruli of each specimen were randomly selected. The ICN1-positive cells within the glomeruli were counted under a fluorescence microscope. Statistical significance was analyzed using Student's t-test. Arrows indicated the glomerulus. Bars indicated the mean value. $^{**}P < 0.01$.

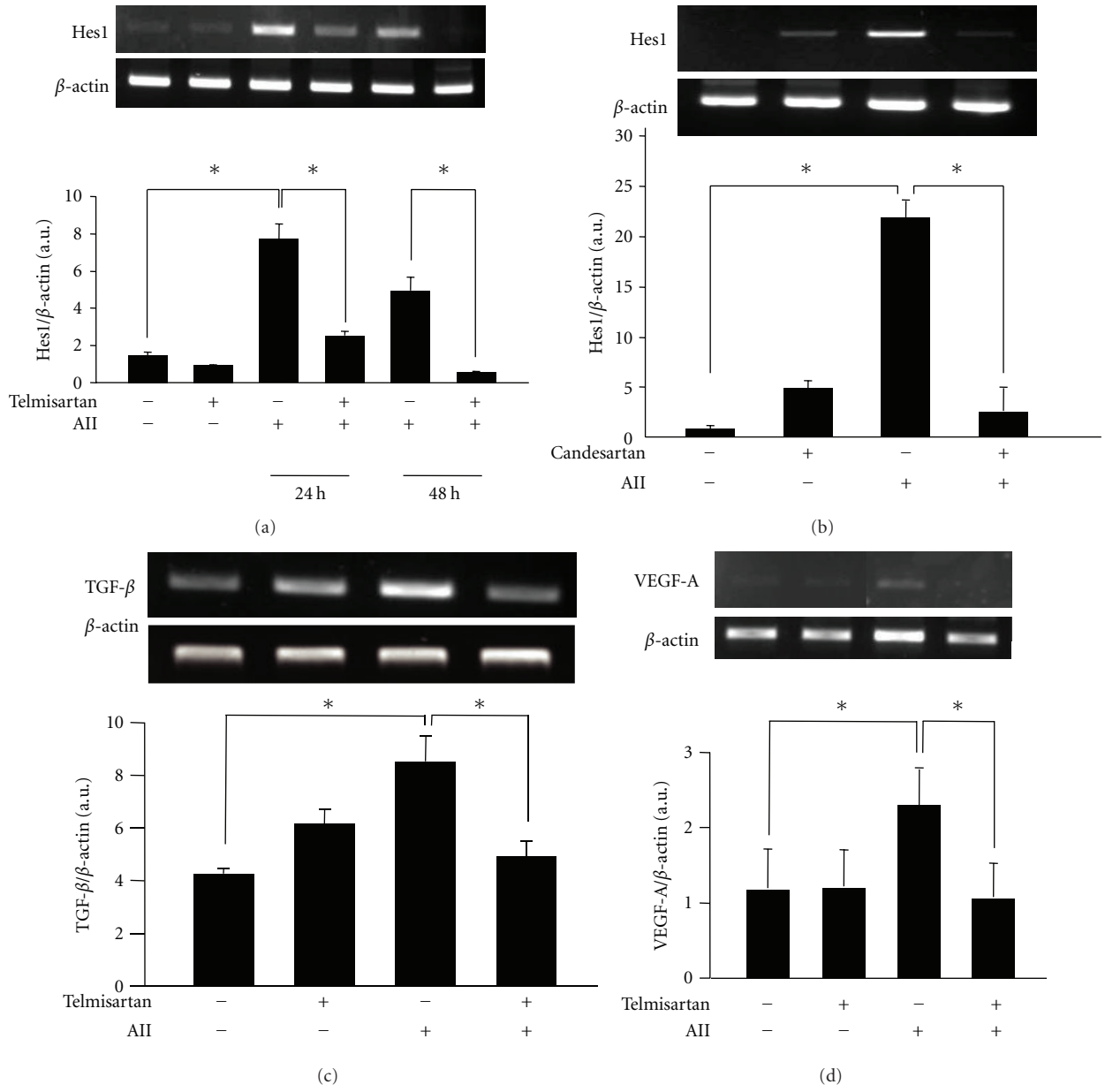

Figure 3: Telmisartan suppressed the activation of the Notch signaling pathway through inhibition of the angiotensin II type 1 receptor. The mRNA expression of Hes1, one of the Notch target genes; transforming growth factor β (TGF-β); vascular endothelial growth factor-A (VEGF-A) were examined by reverse transcriptase-polymerase chain reaction. (a) The podocytes were stimulated with 10^{-6} M Angiotensin II (AII) for 24 to 48 h. The mRNA expression of Hes1 increased in the presence of AII and peaked at 24 h. On the other hand, 10^{-6} M telmisartan suppressed the AII-induced mRNA expression of Hes1 (upper panel). Quantification of the Hes1 mRNA expression compared to the internal control (β-actin) (lower panel). (b) The podocytes were treated with 10^{-6} M AII in the presence or absence of 10^{-8} M candesartan for 24 h. Candesartan also suppressed the AII-induced mRNA expression of Hes1. (c) AII increased the TGF-β mRNA by 2.5-fold within 12 h. Telmisartan (10^{-6} M) suppressed the expression of TGF-β significantly. (d) AII increased the VEGF-A expression by 2.0-fold. Telmisartan suppressed the expression of VEGF-A significantly. $^*P < 0.05$.

inhibited podocytes apoptosis through the inhibition of Notch signaling pathway (Figure 5(e)).

4. Discussion

In the present study, we investigated the activation of the Notch pathway in the glomeruli (especially in the podocytes) of Akita mice. Treatment with telmisartan significantly reduced not only the urinary albumin excretion which was usually seen as an early manifestation of diabetic nephropathy but also the activation of the Notch pathway. We also confirmed that AII induced the activation of the Notch pathway in cultured podocytes. Incubation with AII increased the expression of TGF-β and VEGF-A, and telmisartan reversed

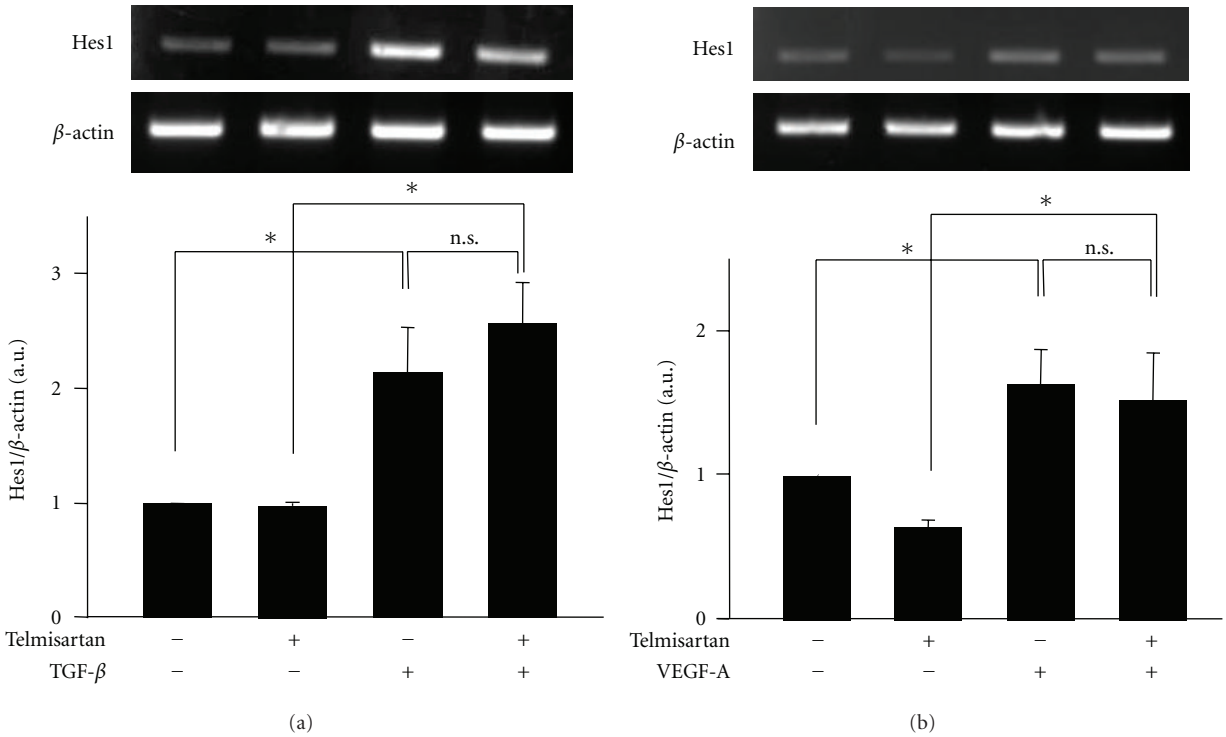

Figure 4: TGF-β and VEGF-A directly activated the Notch pathway. The podocytes were stimulated with 5 ng/mL transforming growth factor β (TGF-β) or 10 ng/mL vascular endothelial growth factor-A (VEGF-A) in the presence or absence of 10^{-6} M telmisartan. The mRNA expression of Hes1 was examined by reverse transcriptase-polymerase chain reaction. (a) TGF-β increased the expression of Hes1 irrespective of the presence or absence of telmisartan (upper panel). Quantification of Hes1 expression compared to the internal control (β-actin). TGF-β significantly increased the Hes1 expression within 2 h by 2.1-fold (lower panel). (b) VEGF-A increased the expression of Hes1 irrespective of the presence or absence of telmisartan (upper panel). Quantification of the Hes1 expression compared to the internal control (β-actin). VEGF-A significantly increased the Hes1 expression within 2 h by 1.6-fold (lower panel). $^*P < 0.05$, *n.s.*: not significant.

this effect. TGF-β and VEGF-A could directly activate the Notch pathway.

Diabetic nephropathy, the leading cause of ESRD in the western world and Asia, is a considerable socioeconomic burden. Investigation of the pathophysiology and establishment of a treatment for diabetic nephropathy is urgently needed. AII is a potent vasoconstrictor hormone that is cleaved from angiotensinogen by renin and ACE. In addition to its known vital role in both cardiovascular and blood pressure homeostasis, several lines of evidence implicate a role in diabetic nephropathy. Durvasula and Shankland have reported that high glucose activates the local RAS in podocytes (independent of ACE activity), which led to injury of the podocytes [13]. Therefore, RAS are locally and systemically activated under diabetic conditions. It has also been reported that the injury of podocytes, referred to as podocytopathy, is a hallmark not only in diabetic nephropathy but also in virtually all glomerular diseases [14]. There are not many pharmacological options to treat diabetic nephropathy; ACEIs and/or ARBs are currently the only drugs that effectively slow the progression of diabetic nephropathy [15]. Furthermore, clinical trials demonstrated that ARBs also lower the risk of type 2 DM compared with other antihypertensive therapies. These observations indicate that ARBs can potentially be used to induce effects other than blood pressure lowering effects. Indeed, ARBs have recently been proven to attenuate inflammation and oxidative stress and inhibit apoptosis [16]. These effects are known as pleiotropic effects. In addition to the previously reported pleiotropic effects, in the present study, we identified that telmisartan inhibited the activation of the Notch pathway. The Notch pathway is known to control a number of cell-fate-specific events in multiple organisms, especially during development, and it also plays a crucial role in diseases such as cancers and autoimmune diseases [17]. It has been recently reported that the Notch pathway is activated in mouse models of DM such as $Lpr^{db/db}$ mice (which mimics type 2 DM), in streptozotocin-treated mice (which leads to type 1 DM), and in kidney specimens from patients with DM [8]. It has also been reported that high glucose activated Notch pathway and increased the expression of VEGF in cultured podocyte [18]. We confirmed the activation of the Notch pathway in another diabetic animal, the Akita mouse. Our findings support the idea that the Notch pathway is generally activated in podocytes in DM. In recent years, GSIs received significant attention as drug candidates for the treatment of Alzheimer's disease and cancers [19]. Since GSIs are capable of inhibiting the Notch signaling pathway, they can be used in the treatment of diabetic nephropathy in the future. In addition to GSIs, our data also suggest that telmisartan inhibits the Notch pathway. To the best of our knowledge, this is the first report that describes the ARB-induced

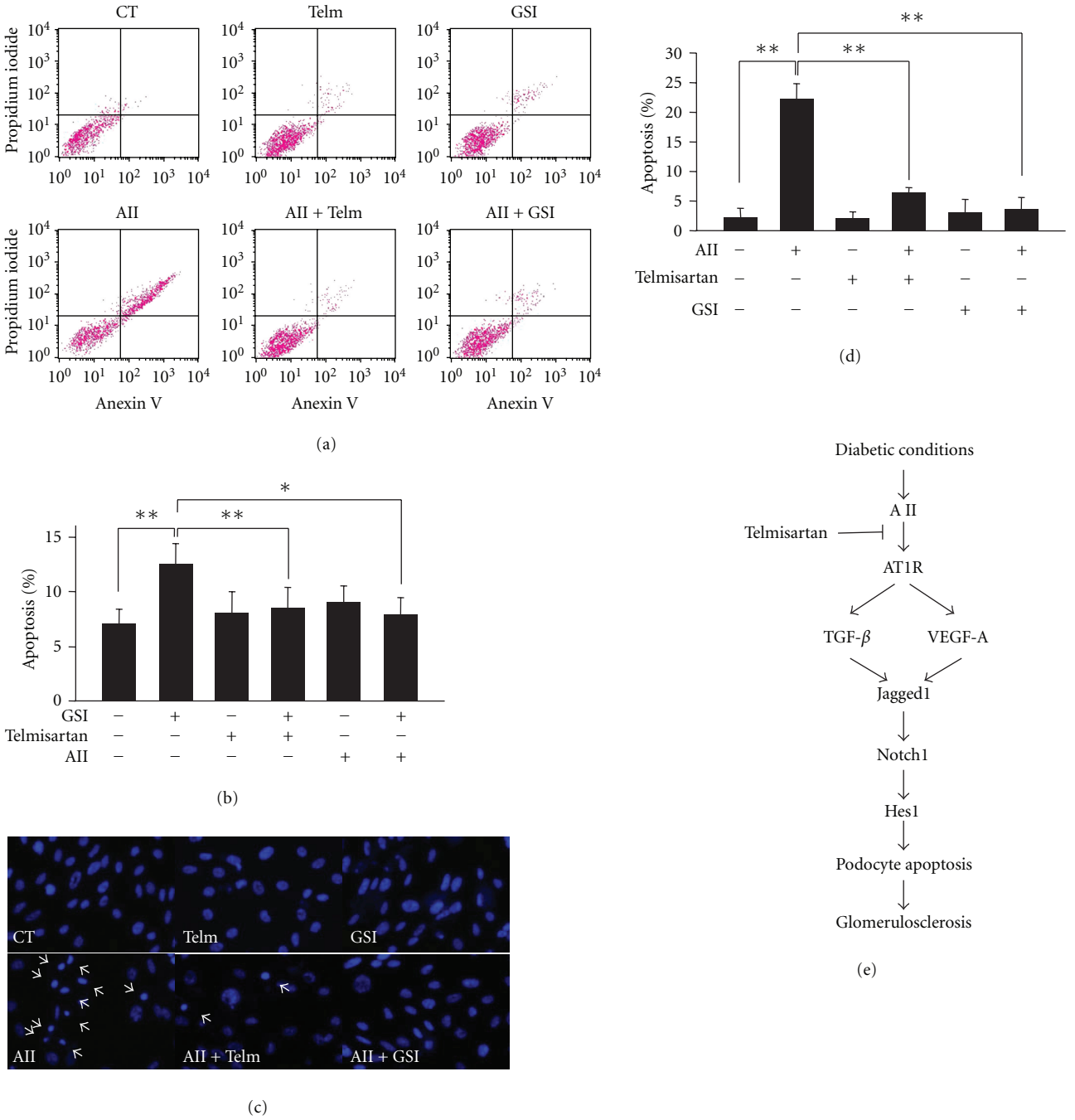

Figure 5: Telmisartan suppressed the podocyte apoptosis which was induced by angiotensin II. The effects of AII as well as telmisartan on the podocytes apoptosis were examined by the flow cytometry or by the Hoechst staining. (a, b) The podocytes were treated with 10^{-6} M AII in the presence or absence of 10^{-6} M telmisartan or 5 mM γ-secretase inhibitor (GSI) for 72 h. Apoptosis in podocytes was determined by low propidium iodide staining and prominent annexin V labeling using the flow cytometry. AII significantly induced podocytes apoptosis compared to the controls (12.56 ± 1.9% versus 7.09 ± 1.4%). Telmisartan significantly suppressed AII-induced apoptosis in podocytes (8.51 ± 2.0% versus 12.56 ± 1.9%). GSI also significantly suppressed that (7.89 ± 1.6% versus 12.56 ± 1.9%). Representative results of three independent experiments were presented. $^{*}P < 0.05$, $^{**}P < 0.01$. (c) The apoptosis in podocytes was examined by Hoechst staining. The podocytes were treated with 10^{-6} M AII, 10^{-6} M telmisartan, and 5 mM GSI as indicated in the figures for 72 h. Apoptosis was determined by nuclear condensation pattern and expressed as the percentage of apoptotic cells per high-power field. A total of 5 high-power fields in a pericentric distribution were quantitated per well. (d) Telmisartan and GSIs suppressed the podocyte apoptosis (CT 2.3 ± 1.5%, AII 22.3 ± 2.54%, Telm + AII 6.3 ± 0.9%, and GSI + AII 3.6 ± 2.0, resp.). *Telm*: telmisartan, $^{**}P < 0.01$. (e) Schematic illustration of the effects of telmisartan on the Notch pathway in podocytes.

inhibition of the Notch pathway both *in vivo* and *in vitro*. Telmisartan is a potent and highly selective AT1R antagonist. Furthermore, telmisartan exerted effects other than the blockade of AT1R, such as PPARγ activation [20]. Our data showed that telmisartan improved the levels of blood glucose, which might indicate that telmisartan functioned as a PPARγ agonist and improved insulin resistance in Akita mice. Although telmisartan significantly reduced urinary albumin excretion, we were not able to detect profound histological improvement. There might be some time difference between the improvement in urinary albumin excretion and the improvement histologically. Telmisartan lowered the blood pressure and improved the blood glucose level in Akita mice. From these findings, we were not able to completely exclude the possibility that the inhibitory effect of telmisartan on the Notch pathway *in vivo* was due to a systemic effect. However, we also used cultured podocytes in order to rule out the influence of blood pressure and glucose levels. Therefore, we argue that telmisartan could directly affect podocytes in order to inhibit the Notch pathway. We also investigated whether candesartan, another ARB, could suppress the Notch pathway and found that candesartan also inhibited Notch signaling pathway. Therefore, the inhibitory effect of Notch pathway by telmisartan seems to be a class effect of ARB.

It has been reported that the genetically activated Notch pathway in podocytes in mice activated p53 and induced apoptosis, which led to decreased expression of the slit diaphragm-related protein such as nephrin, causing proteinuria and renal dysfunction [8]. We tried to detect apoptosis by terminal deoxyribonucleotidyl transferase dUTP nick-end labeling (TUNEL) staining and by staining for activated caspase 3. However, we could not observe apoptosis in the glomeruli of Akita mice, and this could be attributed to technical reasons.

There are some limitations to this present study. First, we were not able to completely exclude the possibility systemic effects of telmisartan for reducing Notch signal *in vivo*. Second, we are not able to explain the reason why telmisartan did not improve the glomerulosclerosis which was seen in Akita mice. Third, we still do not completely understand the biological significance of activated Notch pathway in diabetic condition.

In summary, we showed that the Notch pathway was activated in podocytes of Akita mice and that administration of telmisartan inhibited the Notch pathway. Our data might indicate that telmisartan inhibits the Notch pathway. In addition to its blood pressure lowering effect, which leads to reduced cardiovascular morbidity and mortality, telmisartan might improve the renal prognosis, especially in diabetic subpopulations. Further investigations are needed to prove this hypothesis in the future.

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Research Article

Osmolarity and Glucose Differentially Regulate Aldose Reductase Activity in Cultured Mouse Podocytes

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Podocyte injury is associated with progression of many renal diseases, including diabetic nephropathy. In this study we examined whether aldose reductase (AR), the enzyme implicated in diabetic complications in different tissues, is modulated by high glucose and osmolarity in podocyte cells. AR mRNA, protein expression, and activity were measured in mouse podocytes cultured in both normal and high glucose and osmolarity for 6 hours to 5 days. Hyperosmolarity acutely stimulated AR expression and activity, with subsequent increase of AR expression but decrease of activity. High glucose also elevated AR protein level; however, this was not accompanied by respective enzyme activation. Furthermore, high glucose appeared to counteract the osmolarity-dependent activation of AR. In conclusion, in podocytes AR is modulated by high glucose and increased osmolarity in a different manner. Posttranslational events may affect AR activity independent of enzyme protein amount. Activation of AR in podocytes may be implicated in diabetic podocytopathy.

1. Introduction

Aldose reductase (AKR1B1, EC.1.1.1.21, abbreviated as AR), a member of the aldoketo reductase superfamily, catalyzes the reduction of broad spectrum of aldehydes, using NADPH as a cofactor. Specific AR substrates comprise saturated and unsaturated aldehydes such as progesterone, isocorticosteroids, aldehydes derived from biogenc amines, methylglyoxal, and other harmful metabolites [1–3]. The enzyme is distributed with varying abundance within different tissues [4]. The diversity of AR substrate types and its wide tissue distribution indicates that one of the physiological roles of AR may be detoxification of endogenous and xenobiotic aldehydes. Further postulated roles of this enzyme include cell protection from oxidative and osmotic stresses [5]. The antioxidant defense involves reduction of highly reactive aldehydes produced by lipid peroxidation [6, 7] as well as the reduction of glutathione conjugates of unsaturated aldehydes [8]. The osmoprotective role of AR is associated with reduction of glucose to sorbitol whose intracellular concentration counteracts extracellular osmotic pressure. Physiological importance of this mechanism has been particularly well documented in renal medullary cells in which AR activity and protein synthesis were induced by high extracellular NaCl concentration [9, 10].

Glucose, with its apparent Km of 50–200 mM [3], is a poor substrate for AR. Abnormally high intracellular sugar is required to trigger aldose reductase pathway. In consequence, glucose is converted to sorbitol and further to fructose, with extensive NADPH consumption. Major cytotoxic effects of this pathway include oxidative stress induced by a diminished pool of GSH, intracellular sorbitol accumulation, and increased levels of fructose and its metabolites. Finally, evidence is growing that AR may be involved in inflammatory responses by affecting the NF-κB-dependent expression of cytokines and chemokines [11, 12].

Within the kidney, the highest expression and activity of AR is found in the medullary region. In renal cortex abundance of the enzyme is very low as compared to medulla, as well as to various other tissues [4, 13]. Nevertheless, detailed studies have revealed the presence of AR in mesangial cells (MCs) and in podocytes of the glomeruli [14–16]. Increased expression of the enzyme has been demonstrated in the glomeruli of diabetic patients [17], while both mRNA and activity of AR were elevated in rat mesangial cells cultured in high glucose [18].

Whereas it is accepted that aldose reductase is implicated in pathogenesis of diabetic glomerulopathy [19–22], its role in podocytes has not yet been investigated. Podocytes are terminally differentiated cells, covering glomerular basement membrane with interdigitating foot processes connected by slit diaphragms. They play a critical role in maintaining the glomerular filter and in producing growth factors for both mesangial and endothelial cells. Due to their limited ability to proliferate and to replenish lost cells, podocyte impairment is considered to play a central role in the development of a majority of glomerular diseases [23]. In view of recent findings it seems likely that in diabetic patients the upregulation of AR pathway could contribute to deleterious changes in podocytes. The present study was designed to investigate the effect of high glucose and osmolarity, the two major factors affecting glomerular cells in diabetes, on the expression and activity of AR in podocytes.

2. Materials and Methods

2.1. Cell Cultures and Experimental Protocols. Conditionally immortalized mouse podocytes (Clone SVI, generous gift from Dr. N. Endlich, Greifswald University, Germany) were cultured as described previously [24]. Differentiated cells were grown in a standard RPMI1640 medium containing 5% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Sigma-Aldrich). At zero time podocytes were switched for 6, 12, 24, 48 hours, or 5 days to experimental media: NG-Nosm (normal glucose, normal osmolarity) containing 5.5 mM glucose and 285 mOsm/L, NG-Hosm (normal glucose, high osmolarity) containing 5.5 mM glucose and 385 mOsm/L, HG Nosm (high glucose, normal osmolarity) containing 30 mM glucose and 285 mOsm/L, and HG-Hosm (high glucose, high osmolarity) containing 30 mM glucose and 385 mOsm/L. Experimental media were based on RPMI1640 without glucose (Sigma-Aldrich) and osmolarity was adjusted by adding mannitol. All media were supplemented with 5% FBS and antibiotics, as indicated above. Osmolarity was checked using Vapor Pressure Osmometer (VAPRO 5520, Wescor Biomedical Systems, France).

It has been shown previously that cultured human embryonic kidney (HEK) cells rapidly utilize glucose, which within 24 hours may result in glucose starvation, endoplasmatic reticulum stress, and underglycosylation of numerous proteins [25]. Therefore, using a glucometer (Accu-Chek, Roche Applied Science) we have estimated changes in glucose content in the NG and HG media from podocytes cultured for 0, 24, and 48 hours (Table 1). Based on the obtained results, 48-hour interval has been chosen for replacement of experimental media.

Table 1: Estimation of glucose content (mM) in the media from podocytes cultured for 0, 24, and 48 hours in the presence of normal (NG) and high (HG) glucose. Results are expressed as means ± SE from 5 independent measurements.

	0 hrs	24 hrs	48 hrs
NG	5.8 ± 0.2	5.0 ± 0.4	4.7 ± 0.5
HG	30.0 ± 0.4	27.3 ± 0.9	26.7 ± 1.3

2.2. Isolation and Determination of Aldose Reductase Activity. At indicated time, culture flasks were placed on ice, experimental media were discarded, and 400 μL ice-cold lysis buffer containing (in mM) 50 HEPES (pH 7.2), 2 DTT, 5 EDTA, and 1 tablet/10 mL protease inhibitor cocktail (Complete Mini, Roche Applied Science) was added to each flask. The cells were scrapped, transfered into eppendorf tubes, and centrifuged at +4°C, 14,000 rpm for 30 minutes (Eppendorf Centrifuge 5810R). Resulting supernatant was stored at −80°C, except for an aliquot of 25 μL that was used for immediate protein assay according to the Bradford method [26]. Bovine serum albumin was used as a standard.

Activity of AR was determined spectrophotometrically (Ultrospec 3000) at 37°C as described previously [10]. In all tested groups, steady decrease in absorbance was observed up to 10 min after reaction was started (data not shown). Therefore, measurements were conducted for 6 minutes in the presence and absence of D,L-glyceraldehyde to correct for unspecific NADPH reductase activity [27]. AR activity was normalized to protein content and expressed in mU/μg (nmol NADPH oxidized per minute per μg protein), based on a molar absorption coefficient of 6220 $M^{-1} \cdot cm^{-1}$.

2.3. Immunoblot Analysis. Immunobloting was performed using standard techniques. In brief, trypsynized cells were centrifuged at 2,000 rpm for 10 minutes in +4°C; resulting pellet was lysed on ice in a buffer (pH 8.0) containing 1% Nonidet P-40, 20 mM Tris, 140 mM NaCl, 2 mM EDTA, 10% glycerol, and protease inhibitor cocktail (Complete Mini, Roche Applied Science) and centrifuged for 20 minutes at 14,000 rpm in +4°C. Fifteen micrograms of total protein were subjected to SDS-PAGE and transfer electrophoresis. The proteins on PVDF Immobilion membranes (Millipore, Bedford, MA, USA) were probed with primary antibodies to AR (1 : 400, rabbit polyclonal, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and to α-smooth muscle actin (1 : 2000, mouse monoclonal, Sigma-Aldrich, St. Louis, MO, USA) followed by alkaline phosphatase-labeled secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG, Santa Cruz Biotechnology Inc). The complexes were visualised with 5-bromo-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT; Sigma-Aldrich) and photographed in UVP BioImaging GDS-8000 system (UVP Inc., Upland, CA), using LabWorks 4.0 Image Acquisition and Analysis Software.

Table 2: PCR conditions for aldose reductase and β-actin genes. Previously published primer sequences were verified by NCBI BLAST. Amplification was conducted for 26 cycles.

Gene	Sequence of primers (5′ to 3′)	Amplicon (bp)	Annealing temp. (°C)
Aldose reductase	Sense: CCCAGGTGTACCAGAATGAGA Antisense: TGGCTGCAATTGCTTTGATCC	580	53.1
β-actin	Sense: CCGTAAAGACCTCTATGCCA Antisense: AAGAAAGGGTGTAAAACGCA	299	50.8

2.4. RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from each culture sample using TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions. RNA purity and concentration were determined by measuring the optical densities at 260 nm and 280 nm. Two micrograms of RNA were reverse transcribed at 37°C for 60 min using 0.1 μM antisense primer, 5 U/μL MuLV reverse transcriptase (Promega, Madison, WI, USA), 0.5 mM deoxynucleotide triphosphate mixture (dNTP), and 1 U/μL RNase inhibitor (Sigma-Aldrich). The PCR step was performed using 2.5 U Maxima Hot Start Taq DNA Polymerase (Fermentas Int Inc.), with 20 μL RT product and 80 μL PCR mix containing 0.1 μM sense primer. Primer sequences and PCR amplification conditions are shown in Table 2. Controls containing water in place of RNA were included in all experiments and gave negative results. The PCR products (18 μL/lane) were resolved on ethidium bromide-stained 1.4% agarose gels, viewed and photographed on a UV transilluminator (UVP Inc., Upland, CA).

2.5. Densitometry. Semiquantitative analysis of the band optical densities was performed using Quantity One software (Bio-Rad, Hercules, CA, USA) and normalized to β-actin (RT-PCR) or to α-smoth muscle actin (immunobloting).

2.6. Flow Cytometry. Following incubations, the cells were washed with PBS, trypsinized, suspended in PBS, and centrifuged two times for 7 minutes at 400 × g at room temperature. The pellets were then resuspended in 1 mL of a cold permeabilizing buffer (Ebioscience, USA), incubated for 30 minutes in room temperature, centrifuged and washed with 1 mL washing buffer (WB) (Ebioscience, USA). Finally, 1.5×10^5 cells/tube were stained for 60 minutes at room temperature with 1 : 100-diluted rabbit antibodies to AR (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Antigen-bound antibodies were visualized by 40 minutes incubation with 1 : 100 diluted Alexa488-conjugated donkey anti-rabbit IgG (Molecular Probes, Eugene,OR, USA). Stained cells were washed with 1 mL WB, resuspended in 500 μL PBS and analyzed in flow cytometer (Canto II Flow Cytometer, BD, USA). Basing on measurements of the single-cell fluorescence intensities, arbitrary selection of AR-positive cells was performed. For each selected population, the mean fluorescence index (MFI) was determined using FACS Diva software (BD, USA). Negative controls displayed negligible fluorescence of permeabilized podocytes.

2.7. Immunocytochemistry. For immunocytochemical analysis, the podocytes were grown on collagen-coated round glass cover slips in 24-well culture plates containing experimental media, as indicated before. Immunocytochemistry was performed as described previously [28]. Briefly, the cells were fixed (2% paraformaldehyde), permeabilized (0.3% Triton X-100), and incubated in blocking solution (2% fetal bovine serum, 2% bovine serum albumin, 0.2% fish gelatine, in PBS) for 60 minutes. This was followed by incubations, 60 min with primary rabbit antibody to AR (1 : 100, Santa Cruz Biotechnology, Inc.) and 45 min with secondary anti-rabbit IgG conjugated with Cy3 (1 : 200, Rockland Immunochemicals Inc Gilbertsville, PA, USA). Nonspecific staining was controlled by replacing primary antibody with blocking solution alone. F-actin was stained using 1 : 200 Alexa 488-phalloidin conjugate (Molecular Probes, Eugene, OR, USA). Apoptotic nuclei were detected by DNA condensation, using 1 μg/mL 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Merck Chemicals). All antibodies and stains were diluted in blocking solution. The cover slips were mounted on microscope slides using 15% Mowiol solution (Calbiochem, La Jolla, CA), and stained cells were analyzed under fluorescence microscope (Olympus IX51), using cellSens v.1.3 imaging software (Olympus).

2.8. Statistical Analysis. All of the data are presented as means ± SEM from 3 to 5 independent experiments. The statistical analyses were performed using SigmaStat (version 3.0. for Windows; SPSS Inc., Chicago, IL, USA). Data were analysed using Student's t-test for comparisons. Statistical significance is indicated by $P < 0.05$.

3. Results

3.1. Time Course of AR Activity in Podocytes. In NG-Nosm cells, AR activity remained constant during the whole incubation time (Figure 1). In contrast, hyperosmolar conditions stimulated the activity of AR in NG cells reaching peak level (0.213 ± 0.004 versus 0.117 ± 0.004 mU/μg in NG-Nosm, $P < 0.05$) already after 6 hours of incubation. Thereafter, a gradual decrease was observed, reaching the Nosm level after 48 hours. On the other hand, in HG medium after 6 hours only slight increase of AR activity was observed. After 5 days it reached the level that was only 1.2-fold higher than in the NG-Nosm group. Time course of changes in enzyme activities was similar in HG podocytes from both, normo- and hyperosmolar media which suggests that high

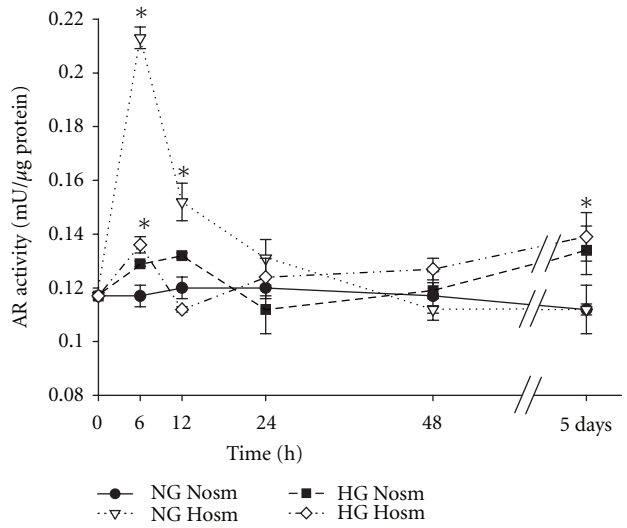

Figure 1: Time course of the changes in podocytic aldose reductase (AR) activity. Podocytes were cultured for indicated time periods in media with normal (5.6 mM, NG) or high (30 mM, HG) glucose content, and/or normal (285, Nosm) or high (385, Hosm) osmolarity. Cell lysates were prepared as indicated in Materials and Methods. Enzyme activities were calculated from the rate of the decrease in NADPH absorbance and are shown as absolute values. Data are expressed as means ± SE from 3–5 independent experiments, $^*P < 0.05$ versus NG-Nosm cells.

glucose counteracted the stimulatory effect elicited by high osmolarity in NG cells.

3.2. Effect of Increased Osmolarity on AR Protein Expression and Activity. Semiquantitative immunoblot analyses of the cells at each incubation time were performed to check whether observed changes in AR activity were related to changes in enzyme protein expression. The effects of short-time (6 hours) and prolonged (5 days) exposition to experimental conditions were further checked by flow cytometry. Densitometric quantification of obtained 37 kDa bands revealed that in hyperosmolar conditions, after 6-hour incubation AR expression in NG podocytes was markedly elevated (by 70 ± 13%, $P < 0.05$ versus NG-Nosm) (Figure 2(a)). Quantitative flow cytometric analysis (Figure 2(c)) yielded similar results, showing that AR increased by 86 ± 2% ($P < 0.01$). Respectively, activity of the enzyme increased by 82 ± 2%, $P < 0.05$ as compared to the NG-Nosm cells (Figure 2(b)). However, while AR protein expression in NG-Hosm cells remained increased during entire time activity of the enzyme dropped to the basal level after 24 hours. Conversely, no significant changes in expression or activity of AR were observed in HG podocytes exposed to increased osmolarity.

Immunofluorescent staining after 5 days of incubation confirmed that expression of AR in cytosol of NG-Hosm podocytes was upregulated, as compared to NG-Nosm and HG-Hosm cells (Figure 3(b)). NG-Nosm cells expressed evident perinuclear pools of AR with relatively weak staining in the rest of cytosolic regions (Figure 3(a)). In NG-Hosm podocytes, AR strongly stained in a diffuse pattern throughout the cell body. High osmolarity had no apparent effect on the F-actin cytoskeleton in NG cells. However, NG-Hosm podocytes displayed more delicate stress fibers, which was particularly noticeable after 5 days of incubation (Figure 3(d)).

3.3. Effect of Glucose Concentration on AR Protein Expression and Activity. In podocytes cultured in HG-Nosm media, AR protein was markedly increased (by 37 ± 4%, $P < 0.025$ versus NG-Nosm) as early as 6 hours after shifting to high glucose (Figures 4(a) and 4(c)). The enzyme remained elevated during the whole incubation period. However, this increase was not accompanied by significant changes in enzyme activity (Figure 4(b)). Conversely, in podocytes cultured in HG- Hosm media, high glucose suppressed the expression of AR and this effect could be observed over the whole period of incubation. After 5 days, AR protein decreased by 55 ± 15%, $P < 0.05$ as compared to NG-Hosm cells. During the first 12 hours of incubation in HG, activity of AR was also suppressed (by 26 ± 2%, $P < 0.05$ versus NG-Hosm at 6 h), while longer incubation seemed to reverse this effect.

3.4. Expression of AR mRNA in Podocytes. To verify whether the changes in enzyme protein expression reflect the mRNA levels for AR, we have reverse transcribed the total RNA from all experimental groups. Results of RT-PCR analysis revealed that already in basal (normal glucose and osmolarity) conditions, the expression of AR mRNA in podocytes was relatively high, with abundance comparable to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, data not shown) and β-actin (Figure 5(c)). Since high ambient glucose concentration may affect the expression of GAPDH [29], for our further analyses we have used β-actin as a housekeeping gene. Comparison of optical density ratios (AR to actin) of respective immunoblots to the RT-PCR products showed that expressions of AR mRNA and protein were correspondingly modulated. Similar to the enzyme activity in NG-Nosm cells, AR mRNA and protein expression remained at a constant level up to 5 days of incubation. Enhanced (NG-Hosm and HG-Nosm cells) or suppressed (HG-Hosm cells) expression of AR protein (Figure 5(b)) was accompanied by respective changes in mRNA levels (Figure 5(d)). This may suggest that in our experimental conditions, transcription and translation processes were synchronized, while modulations of AR activity might be ascribed to posttranslational modifications of the enzyme.

4. Discussion

In the present study we show that in cultured mouse podocytes, expression and activity of aldose reductase (AR) are modulated by high glucose and hyperosmolarity in a different manner. High osmolarity alone induced a rapid but transient increase of AR activity (Figures 1, and 2(b)), while only slight activation of the enzyme by high glucose was observed. As compared to the NG-Hosm cells from respective incubation time points, during the first 12 hours

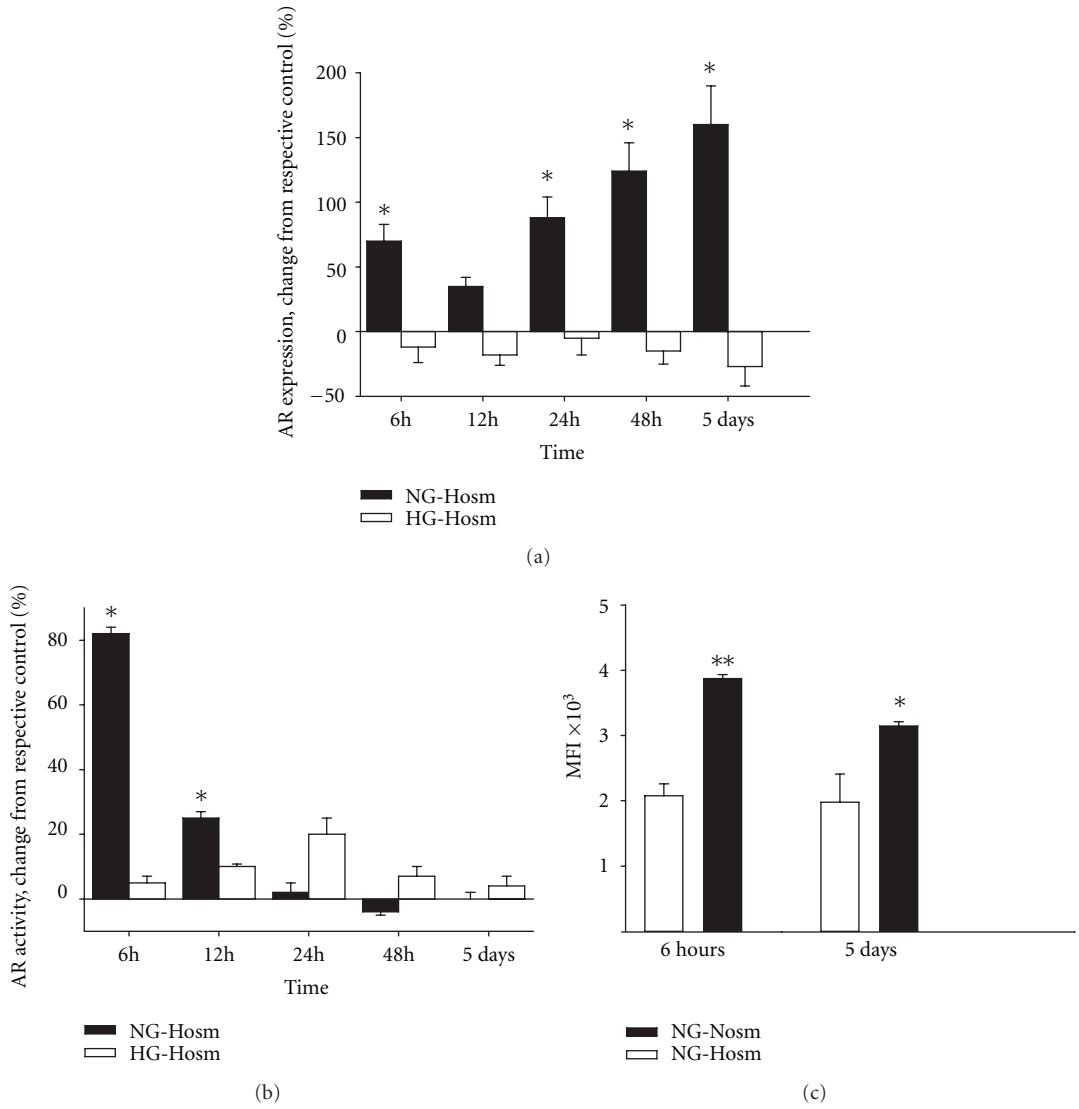

Figure 2: Effect of high osmolarity on (a, c) protein expression and (b) activity of AR in podocytes cultured in normal (NG) and high (HG) glucose. Protein expression was quantified by densitometry of immunoblot bands (a) and by flow cytometry (c) as indicated in Materials and Methods. Data are shown as percent changes of respective normoosmolar (Nosm) control values at the indicated time points (a, b) or as a mean fluorescence index, MFI (c). Results are expressed as means ± SE from 3–5 independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ versus Nosm cells from respective groups.

of incubation activity of AR was even suppressed by high glucose (Figure 4(b)) with parallel decline in protein expression (Figures 4(a) and 4(c)). In a variety of cells, hyperglycemic conditions have been reported to upregulate aldose reductase activity both *in vivo* and *in vitro* [2, 30, 31]. Apart from numerous deleterious effects associated with its activation [5], the beneficial roles ascribed to AR include protection of the cells against glucose-derived osmotic [32] and oxidative [33, 34] stresses. Nevertheless, similar to results observed in our study, reduction of AR protein has been reported in mouse podocytes cultured in hyperosmolar HG medium for two weeks [35] and in arterial endothelium [36]. In turn, high glucose-induced loss of AR activity was found in epithelium from thick ascending limb of Henle's loop [37]. In our experiments, high glucose not only counteracted the activation of AR in HG-Hosm cells but also blunted the AR activity in HG-Nosm podocytes, despite upregulated enzyme protein (Figures 1, 4(a), and 4(c)). The mechanism underlying this paradoxical effect is not clear. It could be possible that AR activity was suppressed in response to intracellular sorbitol accumulation. However, while some authors have observed such suppression [9], others state that this enzyme does not exhibit feedback inhibition [38]. Another possibility is that glucose-induced cytotoxicity [39] that was observed in podocytes *in vivo* and *in vitro* [40] accounted for the drop in measured AR activity. In our experiments, however, nuclear DAPI staining did not reveal any signs of apoptosis in either of tested cell groups. It is also possible that in high glucose milieu the enzyme was affected by posttranslational modifications.

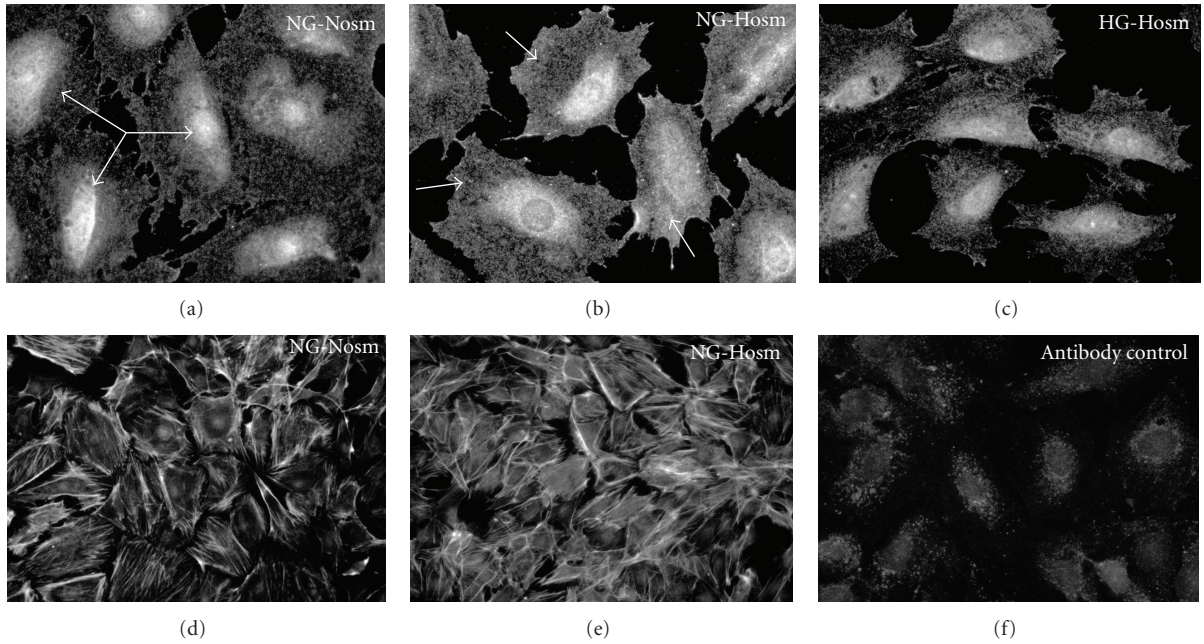

Figure 3: In podocytes cultured in normal glucose (NG), 5-day exposition to hyperosmolar conditions (Hosm) noticeably increased and redistributed AR immunostaining (b), as compared to the cells from normoosmolar (NG-Nosm) (a) or high glucose (HG-Hosm) (c) conditions. In the NG-Nosm cells, immunoreactivity was localized predominantly in perinuclear regions (arrows), while in NG-Hosm cells, intensive staining was diffuse throughout the cytoplasm (arrows). HG-Hosm cells stained to AR in a diffuse manner, with less pronounced perinuclear regions as compared to NG-Nosm. In response to high osmolarity, rearrangements of F-actin cytoskeleton were observed in NG cells. Thick transcellular stress fibers observed in NG-Nosm cells (d) were replaced by thin, more delicate F-actin filaments (e). Nonspecific staining control (f) was prepared as described in Materials and Methods. Magnification ×400 (a–c, f) and ×200 (d, e).

One of the major molecular mechanisms implicated in hyperglycemia cell damage is induction of protein kinase C (PKC) isoforms [41]. Multiple interrelationships between PKC and AR have been described, including phosphorylation and translocation of AR to the mitochondria [42]. In high glucose milieu, PKC mediates also activation of NF-κB, which, in turn, can regulate the transcription of AR gene [43]. Since PKC isoforms have been found in podocytes [44, 45], this mechanism could also be responsible for increased AR expression in HG-Nosm cells. On the other hand, enzymatic activity of AR is redox-sensitive due to the presence of cysteine residue (Cys-298) near the active site [46]. Both oxidation and S-thiolation of this residue modulate kinetics and activity of the enzyme [5, 47, 48]. It is therefore possible that high glucose-induced oxidative stress in podocytes [49, 50] could perturb the intracellular redox status leading to suppression of AR. Posttranslational modifications of AR may also involve an interplay between activatory S-nitrosylation of Cys-298 by nitric oxide (NO) and inhibitory glutathiolation of S-nitrosylated AR [51]. In addition, exposition to high glucose has been shown to inhibit NO synthesis *in vivo* and *in vitro* [52, 53]. Therefore, depletion of bioavailable NO could be another reason for loss of AR activity in podocytes incubated in HG medium. It has been reported previously that podocytes express neuronal [54] as well as inducible [55] forms of nitric oxide synthases; therefore such autocrine/paracrine regulation of AR by NO cannot be excluded.

In contrast to high glucose, hyperosmolarity appeared to markedly stimulate AR activity in NG podocytes (Figures 1 and 2(b)). This was not surprising, as osmotic stress is considered to be a major regulator of AR expression, and aldose reductase is called "a hypertonicity stress protein" [56]. In the sequences of rat and human AR promoter regions, osmotic response element (ORE) sequences have been found [57, 58], and induction of aldose reductase in response to increased osmolarity was reported in numerous renal and nonrenal cells [10, 15, 59, 60]. Moreover, similar to podocytes, in renal tubular epithelium and in Schwann cells induction of AR occurred in response to osmotic stress but not to high glucose *per se* [61, 62]. However, in our experiments, the effect seemed to be relatively short-lived. Although AR protein increased with duration of incubation in the Hosm conditions (Figure 2), activity of the enzyme declined simultaneously. While positive correlation between AR expression and activity was observed in some cell types [62, 63], the discrepancies were found in other [64, 65] which suggests that activation of the enzyme by exogenous factors could play a role independent of protein level. There are two forms of native AR, one of them reduced (not active) and the other one oxidized (active) [66–69]. Since mannitol is known to exert antioxidant properties, prolonged incubation of podocytes in mannitol-containing hyperosmolar medium might favour the reduced form of the enzyme. It is also tempting to speculate that hyperosmolarity-induced intracellular glutathione depletion

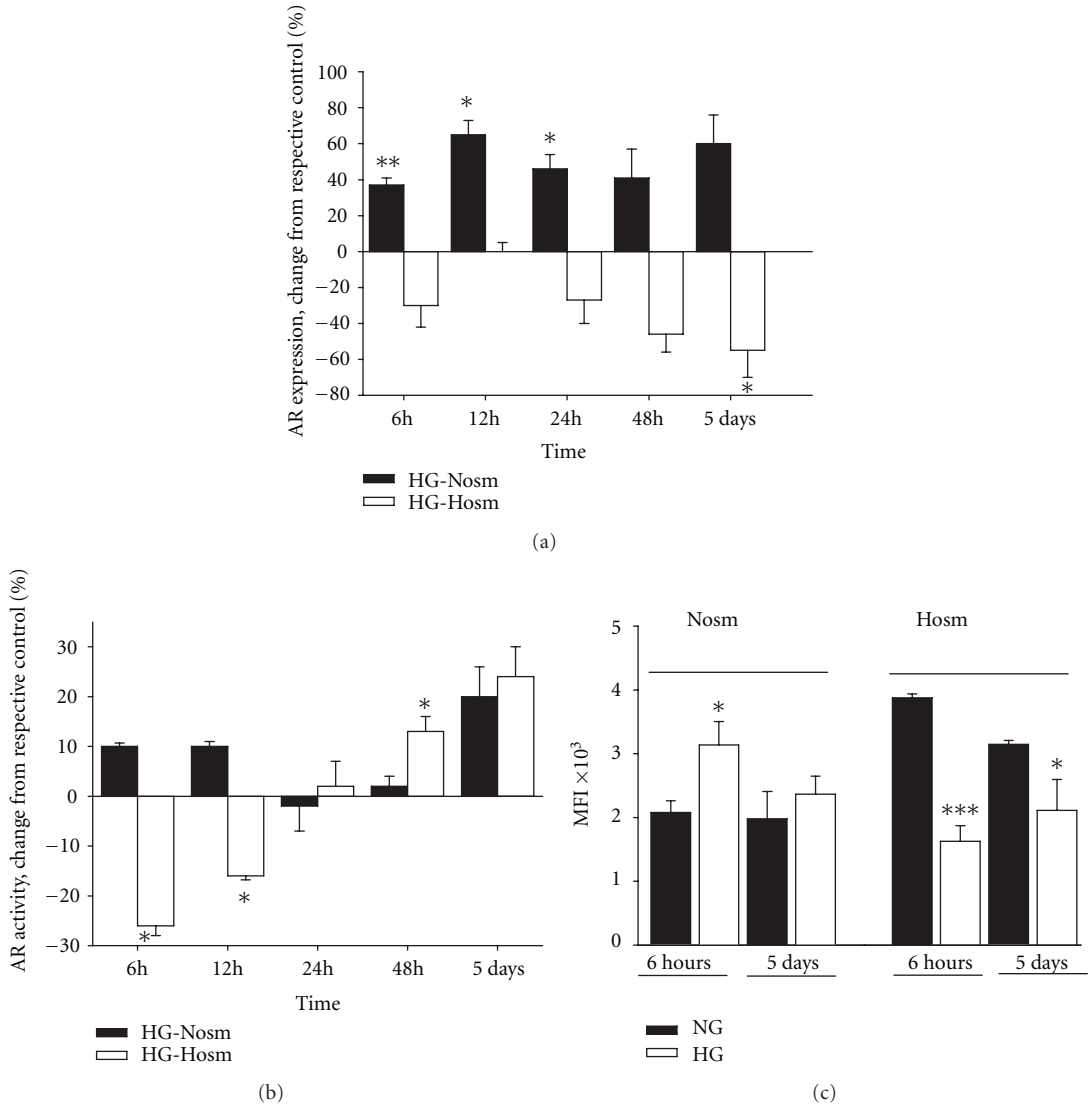

Figure 4: Effect of high glucose on (a, c) protein expression and (b) activity of AR in podocytes cultured in normo- (Nosm) and high- (Hosm) osmotic medium. Protein expression was quantified by densitometry of immunoblot bands (a) and by flow cytometry (c) as indicated in Materials and Methods. Data are shown as percent changes of respective normoglycemic (NG) control values at the indicated time points (a, b) or as a mean fluorescence index, MFI (c). Results are expressed as means ± SE from 3–5 independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus NG cells from respective groups.

could stimulate a transmembrane cystine transport into the podocytes. Uptake of L-cystine in order to replenish intracellular glutathione levels has been shown in various cells and organs, including the kidney [70, 71]. However, cystine, by binding to Cys-298, could also directly inactivate aldose reductase [72]. Transmembrane cystine transport occurs either via glutamate transporters (Na^+-dependent transport) or by glutamate/cystine antiporters [71] that are particularly abundant in brain tissue. By now, none of these transporters were found in podocytes. Nevertheless, podocytes have been shown to possess a differentiated amino acid transport system [73] including vesicular glutamate transporter [74, 75], and recent findings reveal more and more similarities between podocytes and neuronal cells. It seems therefore plausible that glutamate-dependent cystine transport may also be present in podocytes. However, this concept has not been proven yet.

Relatively high basal AR mRNA expression (Figure 5(c)) may suggest that podocytes are well equipped to metabolize not only toxic aldehydes formed inside the cells but also diverse plasma-derived endogenous and xenobiotic aldehydes that may cross the cell membrane during filtration process. Prominent expression of AR protein has been previously observed in rat and ox podocytes [76, 77]. Such constitutive expression of the enzyme may allow the podocytes to quickly adjust their AR system accordingly to current solute composition and/or osmolarity. However, in hyperglycemia, modulation of AR expression and activity may contribute

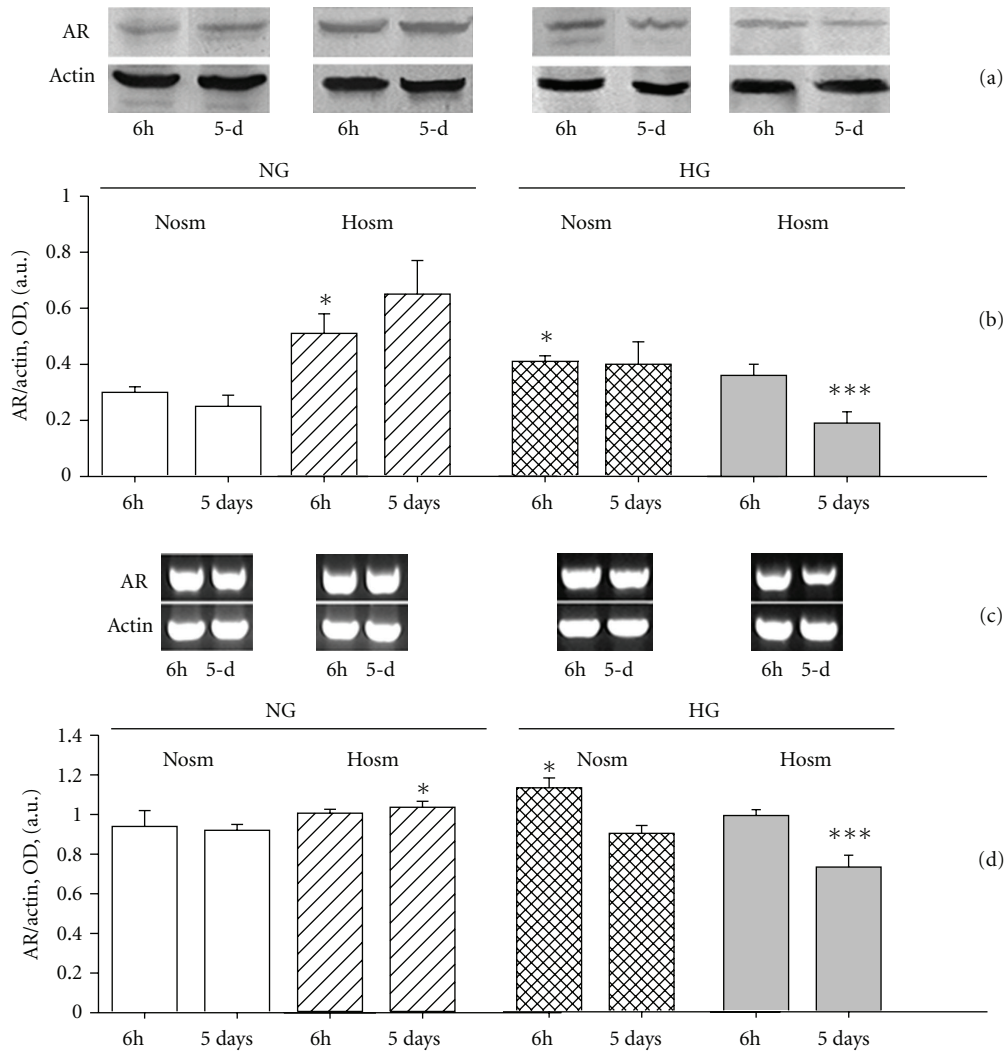

Figure 5: Expression of AR protein (a, b) and mRNA (c, d) in podocytes cultured in experimental media for 6 hours and 5 days, as described in Materials and Methods. (a) 15 μg protein from cell lysates was immunoblotted using rabbit polyclonal anti-AR antibody and visualized with BCIP-NDT, yielding ~37 kDa bands for AR and ~47 kDa bands for α-smooth muscle actin. (b) Results of densitometric quantification of corresponding immunoblot bands are expressed as optical density (OD) ratios of AR to actin bands. (c) 2 μg total RNA was reverse transcribed and resulting cDNAs were amplified in 26 cycles, using specific primers for AR and for β-actin (see Table 2). 18 μL of PCR products were electrophoresized in 1.4% agarose gel and stained with ethidium bromide. (d) Results of densitometric quantification of corresponding RT-PCR products are expressed as optical density (OD) ratios of AR to actin bands. $^{*}P < 0.05$ versus NG-Nosm, $^{**}P < 0.05$ versus NG-Nosm and HG-Nosm 5-d, $^{***}P < 0.025$ versus NG-Nosm and HG-Hosm 6 h. a.u., arbitrary units.

to the pathogenesis of glomerular impairment. In diabetes, increased plasma glucose results in elevated plasma osmolality. According to our HG-Hosm results, activity of podocytic AR may be then suppressed, which could impair antiosmotic defence and lead to podocyte loss. On the other hand, increased AR protein expressed by the podocytes appeared to be one of the major antigens in patients with membranous nephropathy [16]. Therefore, osmolality-dependent elevation of AR protein in podocytes may trigger the inflammatory response within the glomerulus. Aldose reductase has also been shown to modulate production of cytokines such as tumor necrosis factor α (TNF α) or vascular endothelial growth factor (VEGF) in the kidney and vascular smooth muscle cells [5, 21, 48, 78]. In glomeruli, podocytes are a prominent source of VEGF [79], TNF α [80], as well as of other cytokines. Their expression not only contributes to the podocyte integrity and survival but also regulates the functions of other glomerular and tubular cells. It seems likely that in diabetic conditions, modulation of AR in podocytes may be essential for production of factors responsible for regulation of glomerular barrier structure and permeability. Additionally, nonimmune effects of AR should be considered, such as oxidative damage, sorbitol accumulation, or overproduction of fructose [5, 48].

In summary, these results show that in cultured podocytes aldose reductase is regulated by both high glucose

and high osmolarity. Furthermore, changes in AR protein expressed by the cells do not necessarily correspond to changes in activity of the enzyme. High glucose alone elevated AR protein level, which was not accompanied by respective enzyme activation. High osmolarity in turn stimulated both activity and expression of AR in podocytes already within the first hours of exposition. However, while AR protein remained increased over the whole time of incubation, the activity of the enzyme concomitantly decreased. This suggests that in podocytes posttranslational events may affect AR activity independent of the enzyme protein content. Modulation of AR-dependent metabolic pathway in podocytes may be implicated in the pathomechanisms of diabetic podocytopathy.

Acknowledgments

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Review Article

Role of Mindin in Diabetic Nephropathy

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A number of studies have shown that proinflammatory cytokines have important roles in determining the development of microvascular diabetic complications, including nephropathy. Inflammatory biomarkers should be useful for diagnosis or monitoring of diabetic nephropathy. Mindin (spondin 2) is a member of the mindin -/F-spondin family of secreted extracellular matrix (ECM) proteins. Recent studies showed that mindin is essential for initiation of innate immune response and represents a unique pattern-recognition molecule in the ECM. Previously, we demonstrated that the levels of urinary mindin in patients with type 2 diabetes were higher than those in healthy individuals. We propose that urinary mindin is a potent biomarker for the development of diabetic nephropathy.

1. Introduction

Diabetic nephropathy is a major cause of end-stage kidney disease (ESKD) in the United States, Japan, and most of Europe [1]. Although the etiology of this insidious disorder is not well understood, hyperglycemia and hypertension may play pivotal roles in the pathogenesis of diabetic nephropathy. Actually, almost 30% of diabetic patients develop diabetic nephropathy despite strict blood glucose and/or blood pressure control [2]. Chronic low-grade inflammation (so-called microinflammation) has been found to play roles in the pathogenesis of diabetes [3, 4] and has been identified as a risk factor for the development of diabetes [5, 6]. Moreover, diabetes has been proposed as a disease of the innate immune system [7]. In addition, the studies in recent years have shown that inflammation and inflammatory cytokines are determinants in the development of microvascular diabetic complications such as neuropathy, retinopathy, and nephropathy [8–11]. In 1991, Hasegawa et al. reported that glomerular basement membranes from diabetic rats induced significantly greater amounts of tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) in cultured peritoneal macrophages than when these cells were incubated with basement membranes from nondiabetic rats [12]. Based on these findings, the authors suggested for the first time that inflammatory cytokines may participate in the pathogenesis of diabetic nephropathy [12]. At present, a number of clinical studies have suggested relationships between inflammatory cytokines and diabetic nephropathy [13, 14]. Inflammatory cytokines, that is, IL-1, interleukin-6 (IL-6), and interleukin-18 (IL-18) [15, 16], vascular endothelial growth factor (VEGF) [17, 18], monocyte chemoattractant protein-1 (MCP-1) [19, 20], and transforming growth factor-β (TGF-β) [21], as well as TNF-α [22–24], are involved in the development and progression of diabetic nephropathy. It has been demonstrated that endothelial, mesangial, glomerular, and tubular epithelial cells can synthesize inflammatory cytokines, which have significant renal effects.

To survey the glomerular gene expression profile in type 2 diabetes, we performed a microarray analysis using isolated glomeruli from diabetic KK/Ta mice [25]. We observed significant differences in the expression of immune response and inflammatory-related genes. Among them, mindin (also called spondin 2, SPON2) mRNA was significantly upregulated. The present paper focuses on mindin as a urinary marker in diabetic nephropathy.

Table 1: Roles of mindin in immune response and inflammation.

Authors	Year	Role of mindin	Reference
He et al.	2004	Pattern recognition molecule for microbial pathogens	[26]
Jia et al.	2005	Integrin ligand for inflammatory cell recruitment	[27]
Li et al.	2006	Regulating Rho GTPase expression in DCs and DC priming of T lymphocytes	[28]
Jia et al.	2008	Immune-enhancing agent in influenza infection	[29]
Li et al.	2009	Trafficking of normal eosinophils into the airspace and the pathogenesis of allergic airway disease	[30]
Guleng et al.	2010	Activation of NF-kappaB in a TLR-9 mediated manner during colitis	[31]

2. Molecular Structure of Mindin

Mindin is a highly conserved extracellular matrix (ECM) protein that is abundantly expressed in the spleen and lymph nodes [26]. It is a member of the mindin-F-spondin (FS) family of secreted ECM proteins that also includes mammalian FS, zebrafish mindin 1 and mindin 2, and *Drosophila* M-spondin [32–37]. Mindin was initially identified in zebrafish and was observed to accumulate selectively in the basal lamina [33]. Subsequently, the genes encoding rat and human mindin were cloned [35, 36]. Mouse Spon2 cDNA encodes an open reading frame of 330 amino acids with a calculated molecular mass of 36 kDa. Rat mindin is a secreted protein that promotes adhesion and outgrowth of hippocampal embryonic neurons *in vitro* [35]. All members of the mindin-FS family share three structural domains. Two domains, FS1 and FS2 (for F-spondin), are unique to this family. A third domain, called thrombospondin-type 1 repeats (TSRs), is found in a large group of proteins including thrombospondins, the semaphoring 5 family, and the ADAM (disintegrin and metalloproteinase) protein family [38]. The crystal structure of the FS domain of human mindin was demonstrated to be the domain that mediates integrin binding [39]. Remarkably, mindin also functions as a pattern recognition molecule for microbial pathogens [26] and as an integrin ligand for inflammatory cell recruitment and T-cell priming [27, 28, 30].

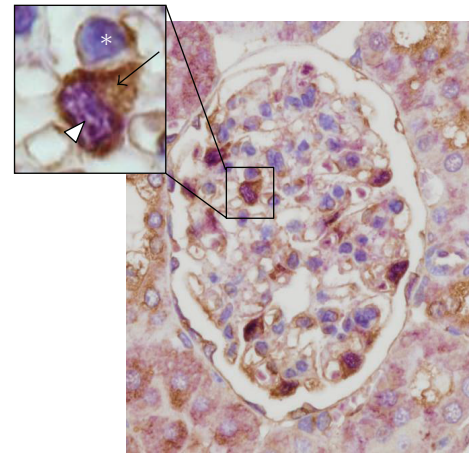

Figure 1: Mindin protein expression in glomeruli of 20-week-old KK/Ta mice. Double-immunohistochemical staining for mindin (brown; arrow) and WT1 (purple; arrowhead, podocyte nucleus marker) in kidney sections from mice with HD at 20 weeks of age. Counterstaining with hematoxylin (blue; asterisk) was used to visualize nuclei.

3. Role of Mindin in Inflammatory Disease

As shown in Table 1, mindin has been studied as essential factor in immune response. He et al. measured serum concentrations of TNF and IL-6 to assay cytokine concentrations in mindin-deficient mice treated with lipopolysaccharide (LPS) [26]. The serum concentrations of these cytokines in control mice treated with LPS were substantially increased. In contrast, the serum concentrations of these cytokines in the mindin-deficient mice were only slightly increased after LPS challenge, indicating that mindin is essential for the *in vivo* production of inflammatory cytokines during endotoxin-induced septic shock. Macrophages and mast cells from mindin-deficient mice show defective responses to a broad spectrum of microbial stimuli. Mindin recognizes LPS through its TSR domain [39]. Mindin also functions as an opsonin for macrophage phagocytosis of bacteria [26]. Mice lacking mindin exhibit defective clearance of influenza virus, whereas mindin-deficient macrophages show impaired activation following influenza infection [29]. Thus, mindin is a pattern recognition molecule that is critical for initiating innate immune responses such as Toll-like receptor 4 (TLR4) [29, 40] (Figure 2). Guleng et al. demonstrated that mRNA expression of mindin is upregulated during dextran sulfate sodium-induced acute intestinal inflammation and is also upregulated by CpG-ODN (a known TLR-9 ligand) stimulation *in vitro*. Moreover, mindin induces nuclear-factor-(NF-) κB promoter activation in a TLR-9 mediated manner [31].

As mentioned above, our microarray data suggested a relationship between mindin and diabetic nephropathy. We focused on mindin expression in the glomeruli and attempted to determine whether an increase in urinary mindin was associated with the development of diabetic nephropathy.

4. Mindin and Diabetic Nephropathy

In contrast to the immune reaction in response to acute infection or inflammation, the immune processes in chronic diseases such as diabetic nephropathy can be smoldering processes that are difficult to detect. Development of a diagnostic test, more convenient and reliable than those currently used, would therefore be highly desirable. Urine is easily accessible,

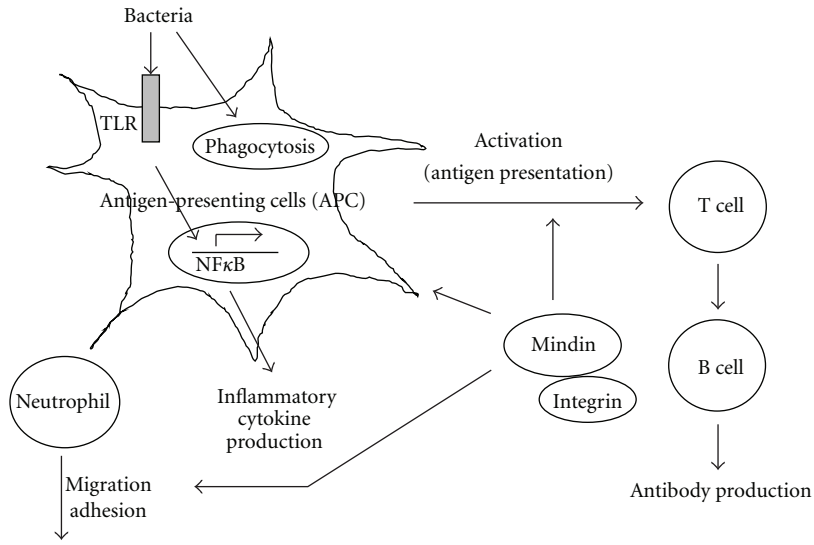

Figure 2: Mindin as an essential factor in immune response. Mindin has an important role in both innate and adaptive immunity.

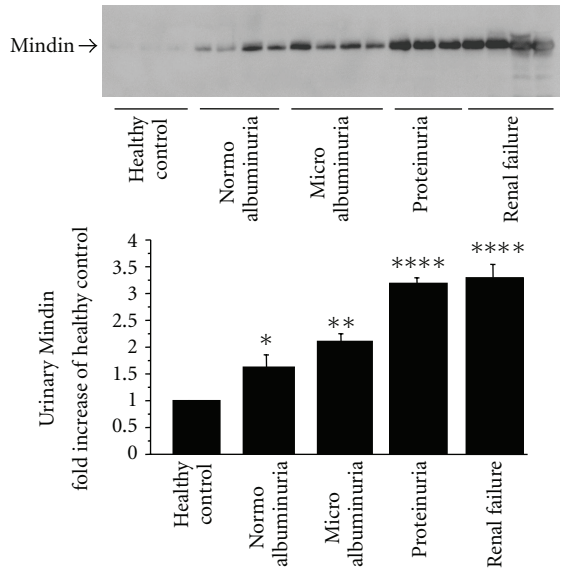

Figure 3: Urinary mindin secretion in patients with diabetic nephropathy by western blot analysis. Healthy control ($n = 3$), Normoalbuminuria ($n = 4$): <30 mg albumin/g creatinine, Microalbuminuria ($n = 4$): 30–300 mg albumin/g creatinine, Proteinuria ($n = 3$): >300 mg albumin/g creatinine, Renal failure ($n = 4$); $^{****}P < 0.0001$ versus healthy control; $^{**}P < 0.01$ versus healthy control; $^{*}P < 0.05$ versus healthy control.

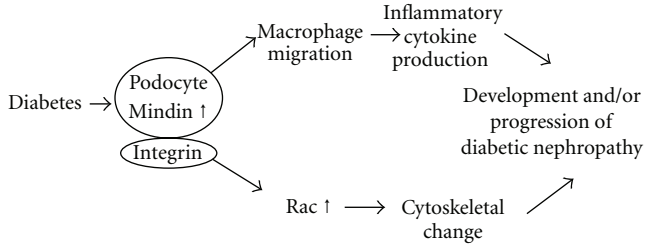

Figure 4: The hypothesis concerning the role of mindin in diabetic nephropathy. Mindin might be related to podocyte injury in diabetic nephropathy and regulate not only inflammation but also cytoskeletal change via integrin.

and therefore, as a diagnostic medium allows for noninvasive detection of biomarkers. With 70% of urinary proteins originating from the kidney and urinary tract, and 30% being filtered by the kidney, urinary biomarkers are likely to be linked to renal dysfunction or systemic changes [41]. Urinary albumin, the current gold standard, is known that significant structural changes have already appeared even at the stage of microalbuminuria in type 2 diabetes patients. Thus, it is necessary to develop a more sensitive measurement for detecting the early stage of renal injury in patients with diabetic nephropathy. Inflammatory markers should be useful biomarkers for diagnosis or monitoring of diabetic complications, particularly kidney disease. However, the sensitivity of these markers compared with albumin requires further investigation. We dissected isolated glomeruli from three 20-week-old KK/Ta mice fed a high-calorie diet (HC) or a standard diet (SD). The inbred mouse strain KK/Ta, established as a diabetic strain in Japan, spontaneously exhibits characteristics of type 2 diabetes associated with fasting hyperglycemia, glucose intolerance, hyperinsulinemia, mild obesity, dyslipidemia, and albuminuria. Renal lesions in KK/Ta mice closely resemble those in the early stage of human diabetic nephropathy. The albumin/creatinine ratio (ACR) in male KK/Ta mice at 16 weeks of age is 150–200 mg/gCr. KK/Ta mice glomeruli show diffuse hyperplasia in the glomerular mesangial areas with mild mesangial cell proliferation [42]. Immunohistological studies show an intense, specific fluorescence for albumin and γ-globulin along the glomerular capillary walls. Therefore, KK/Ta mice are considered to provide a suitable model for type 2 diabetes and the early stage of diabetic nephropathy in humans. Mindin expression was examined using real-time PCR, western blot analysis, and immunohistochemical staining of the glomeruli (Figure 1), as well as cultured podocytes and urine samples of both mice and humans. The mindin protein expression levels in mice were localized in the podocytes, and their levels in the glomeruli were increased in the HC group compared with the SD group [25]. We also examined podocyte cultures incubated in high glucose (HG). Mindin was detected in podocyte culture supernatants and its expression levels under HG stimulation were significantly higher than those under normal glucose stimulation. Therefore, we examined the urinary secretion of mindin. Urinary mindin expression levels in the HC group were already significantly higher than those in the SD group at 16 weeks of age. The urinary mindin level seems to be increased earlier compared with the ACR. Furthermore, mindin was also detectable in human urine. Urinary mindin expression in patients with type 2 diabetes increased compared with that in healthy individuals, reflecting the stage of diabetic nephropathy (Figure 3).

Podocytes cover the outer aspect of the glomerular basement membrane (GBM) and form the final barrier to protein loss [43]. The podocyte foot process is fixed to the GBM via $\alpha_3\beta_1$ integrin and α/β dystroglycans, and $\alpha_3\beta_1$ integrin is the major integrin expressed by podocytes [44–46]. Jia et al. reported that mindin serves as an integrin ligand [27]. We also found that β_1 integrin protein expression increased in the cultured podocytes stimulated under HG conditions (data not shown). Moreover, mindin was reported as a key regulator of Rho GTPase expression. Signaling through integrins activates Rho GTPases [28]. Cytoskeletal changes of podocytes regulated by the Rho family are critically involved in the pathogenesis of glomerular diseases. Mindin should be produced by damaged podocytes under high glucose conditions and serve as a biomarker of the progression of diabetic nephropathy (Figure 4).

5. Conclusion

Increasing evidence indicates that inflammatory and immune response mechanisms may contribute significantly to the development and progression of diabetic nephropathy. Recent studies showed that mindin is essential for the initiation of immune response and represents a unique pattern-recognition molecule in the ECM. We focused on the role of mindin and examined the urinary secretion of mindin in mice and humans. Mindin could be an early biomarker of the progression of diabetic nephropathy.

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Research Article

Diabetic Nephropathy Amelioration by a Low-Dose Sitagliptin in an Animal Model of Type 2 Diabetes (Zucker Diabetic Fatty Rat)

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This study was performed to assess the effect of chronic low-dose sitagliptin, a dipeptidyl peptidase 4 inhibitor, on metabolic profile and on renal lesions aggravation in a rat model of type-2 diabetic nephropathy, the Zucker diabetic fatty (ZDF) rat. Diabetic and obese ZDF (fa/fa) rats and their controls ZDF (+/+) were treated for 6 weeks with vehicle (control) or sitagliptin (10 mg/kg/bw). Blood/serum glucose, HbA1c, insulin, Total-c, TGs, urea, and creatinine were assessed, as well as kidney glomerular and tubulointerstitial lesions (interstitial fibrosis/tubular atrophy), using a semiquantitative rating from 0 (absent/normal) to 3 (severe and extensive damage). Vascular lesions were scored from 0–2. Sitagliptin in the diabetic rats promoted an amelioration of glycemia, HbA1c, Total-c, and TGs, accompanied by a partial prevention of insulinopenia. Furthermore, together with urea increment prevention, renal lesions were ameliorated in the diabetic rats, including glomerular, tubulointerstitial, and vascular lesions, accompanied by reduced lipid peroxidation. In conclusion, chronic low-dose sitagliptin treatment was able to ameliorate diabetic nephropathy, which might represent a key step forward in the management of T2DM and this serious complication.

1. Introduction

Type 2 diabetes mellitus (T2DM) is an increasing health problem, with increasing prevalence and incidence, according all the estimates worldwide [1]. The core pathophysiology of type 2 diabetes (T2DM) has been attributed to the classic triad of decreased insulin secretion, increased insulin resistance, and elevated hepatic glucose production. Further mechanisms have also key relevance, including those related with the fat cell (accelerated lipolysis), the gastrointestinal tract (incretin deficiency/resistance), the pancreatic α-cell (hyperglucagonemia), the kidney (increased glucose reabsorption), as well as the brain (insulin resistance), now referred to as the "ominous octet" [2]. The main problem of T2DM management is its serious micro- and macrovascular complications, which include, among others, diabetic nephropathy [3]. The incidence of T2DM is rapidly increasing, as is the prevalence of cardiovascular disease (CVD) and chronic kidney disease (CKD) resulting from diabetic complications [4, 5]. Diabetes remains the single most important cause of kidney failure, and diabetic nephropathy is a major microvascular complication of diabetes and progression to end-stage renal disease (ESRD) in different regions of the world [6, 7], accounting for approximately one-third of all cases of end-stage renal disease.

There is emerging evidence that microvascular disease begins prior to the onset of diabetes, and this occurs with microalbuminuria and decreased renal function. Experimental and clinical studies showed an adaptive response by the kidney to conserve glucose, which is essential to meet the energy demands of the body [8–11]. In the diabetic patient, instead of dumping glucose in the urine to correct hyperglycaemia, the kidney chooses to hold on to glucose. Even worse, the ability of the diabetic kidney to reabsorb glucose appears to be augmented by an absolute increase in the renal reabsorptive capacity for glucose [12, 13]. The hyperglycaemic profile is

aggravated by oxidative stress damage and inflammation, as well as by overactivity of the renin-angiotensin-aldosterone system (RAAS) and alteration of the extracellular matrix protein synthesis by glomerular epithelial cells, which contributes to further aggravate diabetic nephropathy [14–17].

Evidence is available that long-term maintenance of normal or near-normal glucose levels using pharmacological means is protective in diabetic patients, improving microvascular disease and reducing both morbidity and mortality [18–20]. Traditionally, noninsulin-dependent T2DM is pharmacologically managed with oral antidiabetic agents from several different classes, which includes agents that increase insulin secretion, improve insulin action, and delay absorption of carbohydrates. The more recent incretin-based therapies address a previously unmet need in the diabetic therapeutic approach by modulating glucose supply [21–23]. Their pharmacological action is based on gut incretin hormones, the glucose-dependent insulinotropic peptide (GIP), and the glucagon like peptide-1 (GLP-1), which appear to be malfunctioning in T2DM and have important effects on insulin and glucagon secretion [24, 25]. Sitagliptin is one of the best known incretin enhancers (or gliptin), which increase incretin contents due to the inhibition of dipeptidyl peptidase-4 (DPP-4) activity, which is responsible for the degradation of GLP-1 [23, 26–28]. Even though there is a patent association in observational studies between hyperglycaemia and diabetic complications, the benefits of a strict glycaemic control on micro- and macrovascular complications have been questioned. Therefore, the benefits of glucose reduction seem to be, at least partly, minimized by the side effects of the glucose-lowering antidiabetic agents, including hypoglycaemia, weight gain, and fluid retention. In this context, new therapeutic options with fewer side effects are advisory, and the appearance of incretin-based therapies is a hope. However, clinical studies with renal end points using these agents are lacking as well as animal studies assessing the influence of these drugs on renal function and lesion.

Rodent models of T2DM are frequently used to clarify the mechanisms responsible for the pathophysiology of diabetes evolution, as well as its complications. The Zucker diabetic fatty (ZDF) rat has a mutation in the gene coding the leptin receptor (fa/fa) that results in obesity, insulin resistance, reduced glucose tolerance, hypertension, and renal and cardiovascular (CV) disease, thus developing a phenotype very similar to humans with T2DM, including the existence of diabetes nephropathy [29–31]. Our group has previously reported that a chronic low-dose sitagliptin treatment promotes not only a reduction of hyperglycaemia, but also other protective actions (including antioxidant and anti-inflammatory actions) [32]. Considering the extra-pancreatic effects of incretins, namely, the GLP-1 ability to positively modulate the function of other tissues [33, 34], it seems important to evaluate the effects of sitagliptin in diabetic nephropathy as well as to characterize the nature of the putative benefit.

Concerning the management of diabetic nephropathy, the ability of antidiabetic drugs to ameliorate renal microvascular disease might be as important as their capability to control glucose. While the lowering glucose effects of incretin-dipeptidyl peptidase-IV (DPP-4) are well known, the effects on the kidney remain to be elucidated. Thus, the present study aimed to evaluate whether sitagliptin can prevent the development of renal dysfunction in diabetic ZDF rats.

2. Materials and Methods

2.1. Animals and Experimental Design. Male ZDF rats (ZDF/Gmi, fa/fa) and their littermates (ZDF/Gmi, +/+) were purchased from Charles River Laboratories (Barcelona, Spain) at 6 weeks of age. Rats were properly housed, handled daily, and kept in a controlled standard temperature (22-23°C), humidity (60%), and light-dark cycles (12/12 h). Throughout the experiment, the animals were provided with distilled water *ad libitum* and rodent maintenance chow (A-04 Panlab, Barcelona, Spain, containing 15.4% of protein and 2.9% of lipids). The chow was adapted to the animal's body weight (BW): 100 mg/g. Animal experiments were conducted according the European Council Directives on Animal Care and the National Laws.

When aged 20 weeks, eight lean control ZDF/Gmi (+/+) and eight obese diabetic ZDF (fa/fa) rats were sacrificed for tissue collection. The remainder diabetic ZDF (fa/fa) rats were divided the following two subgroups ($n = 8$ rats each) for a treatment period between 20 (T0) and 26 (Tf) weeks of age: a control and a treatment group, receiving, respectively, by oral gavage, once a day (6:00 PM), for 6 weeks, the vehicle (orange juice) and sitagliptin (10 mg/kg/BW). The same procedures were adopted with the lean nondiabetic ZDF (+/+) control rats. The ZDF (+/+) control group under sitagliptin treatment showed no relevant differences when compared with the ZDF (+/+) control rats under vehicle, and thus, the results were excluded from tables and figures in order to facilitate data comparison and interpretation. Animals of the same type were compared at the ages of 20 and 26 weeks to assess aging effects in the control lean ZDF (+/+) rats and disease progression in the obese diabetic ZDF (fa/fa) ones. Comparisons were made between lean (+/+) and obese diabetic (fa/fa) ZDF rats, at 20 and 26 weeks, to differentiate diabetic features from normal (20 weeks) or ageing characteristics (26 weeks). After these prior comparisons, our main group of interest, the chronic sitagliptin-treated obese diabetic (fa/fa) ZDF rats of 26 weeks, were compared to its untreated counterparts. Food intake and BW were measured each day before treatment and expressed as weekly average values.

2.2. Sample Collection and Preparation

Blood. When aged 20 weeks (T0) and at the end of the experiment (26 weeks-Tf), the rats were subjected to intraperitoneal anesthesia with a 2 mg/kg BW of a 2 : 1 (v : v) 50 mg/mL ketamine (Ketalar, Parke-Davis, Lab. Pfeizer Lda, Seixal, Portugal) solution in 2.5% chlorpromazine (Largactil, Rhône-Poulenc Rorer, Lab. Vitória, Amadora, Portugal), and blood samples were immediately collected by venipuncture from the jugular vein into syringes without anticoagulant (for serum samples) or with the appropriate anticoagulant:

ethylene-diaminetetraacetic acid (EDTA)-2K for Glycosylated haemoglobin (HbA_1c) measurement.

Tissues. The rats were sacrificed by anesthetic overdose. The kidneys were immediately removed, placed in ice-cold Krebs' buffer and carefully cleaned of extraneous fat and connective tissue. Subsequently, the organ was cross-sectioned, fixed and processed for paraffin embedding in accordance with the subsequent histological protocols.

2.3. Glycaemic, Insulinaemic and Lipidic Profile Assays. Serum glucose levels were measured using a Glucose oxidase commercial kit (Sigma, St. Louis, Mo, USA). Considering the variability of serum glucose levels in the rat, glycosylated haemoglobin (HbA_1c) levels were used as an index of glucose control, through the DCA 2000+ latex immunoagglutination method (Bayer Diagnostics, Barcelona, Spain). Plasma insulin levels were quantified by using a rat insulin ELISA assay kit from Mercodia (Uppsala, Sweden). The steady state beta cell function of individual animals was evaluated using the previously validated homeostasis model assessment (HOMA) of β-cell function [35]. The formula used was as follow: [HOMA-β%] = 360 × fasting serum insulin (mU/L)/fasting serum glucose (mg/dL) − 63. The values used (insulin and glucose) were obtained after an overnight of food deprivation. Serum total cholesterol (Total-c) and triglycerides (TGs) were analysed on a Hitachi 717 analyser (Roche Diagnostics) using standard laboratorial methods. Total-c reagents and TGs kit were obtained from bioMérieux (Lyon, France).

2.4. Kidney Function (Creatinine and Urea) and Trophism (Weight). Serum creatinine and blood urea nitrogen (BUN) concentrations were used as renal function indexes, through automatic validated methods and equipments (Hitachi 717 analyser, Roche Diagnostics Inc., Mass, USA). The weights of kidneys (KW) and the ratio KW/BW were measured in all the rats under study in order to be used as renal trophy indexes.

2.5. Kidney Lipid Peroxidation. Kidney lipid peroxidation was assessed by the thiobarbituric acid reactive-species (TBARs) assay, measuring the malondialdehyde (MDA) content, according to that previously described in [32]. Samples were analysed spectrophotometrically at 532 nm using 1,1,3,3-tetramethoxypropane as external standard. The concentration of lipid peroxides (in MDA) was expressed as μmol/L.

2.6. Histopathological Analysis

Haematoxylin and Eosin Staining. Samples were fixed in Bock's fixative and embedded in paraffin wax, and 3 μm thick sections were stained for routine histopathological diagnosis with haematoxylin and eosin (HE).

Periodic Acid of Schiff Staining. Periodic acid of Shiff (PAS) was used to evaluate and confirm the levels of mesangial expansion, thickening of basement membranes and sclerotic parameters. Samples were fixed in neutral formalin 10%, embedded in paraffin wax, and 3 μm thick sections were immersed in water and subsequently treated with a 1% aqueous solution of periodic acid, then washed to remove any traces of the periodic acid, and finally treated with Schiff's reagent. All samples were examined by light microscopy using a Microscope Zeiss Mod. Axioplan 2. The degree of injury visible by light microscopy was scored in a double-blinded fashion by two pathologists. Lesions were evaluated on the total tissue on the slide.

Histopathology. Glomerular damage was assessed by evaluating mesangial expansion, glomerular basement membrane and capsule of Bowman thickening, nodular sclerosis, glomerulosclerosis, atrophy, and hyalinosis of the vascular pole. Analysed tubulointerstitial lesions comprised inflammation, presence of hyaline cylinders, tubular basement membrane irregularity, tubular calcification, and the association of interstitial fibrosis and tubular atrophy (IFTA). The evaluation of vascular lesions was concentrated on arteriolar hyalinosis and arteriosclerosis. A semiquantitative rating for each slide ranging from normal (or minimal) to severe (extensive damage) was assigned to each component. Severity was graded as absent/normal, mild, moderate, and severe. Scoring was defined according to the extension occupied by the lesion (% area): normal: <25%; mild: 25–50%; moderate: 50–75%; severe: >75%. The final score of each sample was obtained by the average of scores observed in individual glomeruli in the considered microscopic fields. Tubulointerstitial damage was evaluated and graded by the same semiquantitative method, with the exception of IFTA, which was graded as normal, if absent, as mild, moderate, and severe, if present in <25%, between 25–50%, and over 50% of affected area. Regarding vascular lesions, arteriolar hyalinosis was scored as 0 if absent, as 1 if one arteriole with hyalinosis was present, and as 2 if more than one arteriole was observed in the entire slide. Arteriosclerosis was scored as 0 if no intimal thickening was present, as 1 if intimal thickening was less than the thickness of the media, and as 2 if intimal thickening was more than the thickness of the media and considering the worst artery on the slide. When using PAS, the rating was set for intensity and extension of staining, ranging from 0 (no staining) to 3 (intense and extensive staining), respecting tissue specificity scoring when adequate.

2.7. Statistical Analysis. The categorical variables are counts of renal lesions severity in scores. Quantitative values are reported as mean ± SEM. Significance level was accepted at 0.05. Data were analyzed using SPSS Statistics 18 (2009). Chi-square test with Monte Carlo simulation or exact test (when contingency tables are 2 × 2) was used to find out the differences of severity score distributions in renal lesions at the beginning of the study (20 weeks old) between lean control and obese diabetic ZDF (fa/fa) rats and at the end of the study (26 weeks old), between diabetic ZDF (fa/fa) rats vehicle-treated and diabetic ZDF (fa/fa) sitagliptin-treated and lean control rats. To get an overview of the influence of sitagliptin treatment in renal lesions after 6 weeks of chronic treatment with sitagliptin (final time 26 weeks), we generated two quantitative variables, by averaging the scores of two types of renal lesions: global glomerular lesions comprising

mesangial expansion, thickening of GBM, thickening of CB, nodular sclerosis, glomerulosclerosis, glomerular atrophy, and hyalinosis of the vascular pole and global tubulointerstitial lesions comprising hyaline cylinders, TBM irregularity, tubular calcification, IFTA, and tubular degeneration. On these two variables was performed an ANOVA and subsequent LSD post hoc test to find out the differences between diabetic ZDF (fa/fa) rats vehicle treated, diabetic ZDF (fa/fa) rats sitagliptin treated, and lean control rats.

3. Results

3.1. Effects of Sitagliptin Treatment on Body Weight and Glycaemic and Lipidic Profiles. Concerning the body weight, no significant differences were encountered between the diabetic and the lean control rats in the beginning of treatments (T0: week 20) despite the obese profile encountered in the diabetic ZDF (fa/fa) rats between the 8th and the 14th week (data not shown). At the end of the study (26 weeks), the control diabetic ZDF (fa/fa) rats exhibit an 8.7% reduction in their BW ($P < 0.001$); nevertheless, the lean control group gained weight. Sitagliptin treatment, during 6 weeks, stabilized the loss of weight in the diabetic ZDF (fa/fa) rats even preventing part of the BW loss when compared with the rats without treatment (Table 1).

At the T0 (when animals aged 20 weeks), the diabetic group showed a hyperglycaemic and a hyperlipidemic profile, also seen at the final time (Table 1). The values of HbA1c were higher in the diabetic rats when compared with those of the control animals, confirming the glycaemic deregulation. The diabetic ZDF (fa/fa) rats have also presented higher levels of Total-c and TGs versus the control ZDF (+/+) animals, in both times (Table 1). After 6 weeks of sitagliptin treatment (Tf: 26 weeks), a significant ($P < 0.001$) improvement in glycaemic control was observed in diabetic ZDF (fa/fa) rats, when compared with the vehicle-treated counterparts. This pattern of changes is also expressed by the HbA1c levels. TGs were significantly reduced ($P < 0.001$) in the diabetic rats treated with sitagliptin during 6 weeks versus the diabetic vehicle-treated animals (Table 1).

3.2. Effects of Sitagliptin Treatment on Insulin Levels and HOMA-Beta. At the beginning of the study (Ti: 20 weeks age), insulin levels were already significantly lower in diabetic animals when compared to lean control ($P < 0.01$) together with a significant different value of HOMA-beta ($P < 0.001$) (Table 1). At the final time, the vehicle-treated diabetic ZDF (fa/fa) rats exhibit relative insulinopaenia, when compared to vehicle-treated ZDF (+/+), accompanied by a further decrease ($P < 0.001$) of HOMA-beta. The insulinopaenic profile of the diabetic rats, as well as the decrease of HOMA-beta value, were partially, significantly prevented ($P < 0.001$) in the diabetic rats treated with sitagliptin when compared with those untreated (Table 1).

3.3. Effects of Sitagliptin Treatment on Kidney Function (Creatinine and Urea) and Trophism (Weight). At the beginning of the study (T0), urea contents were already significantly higher ($P < 0.001$) in the diabetic ZDF rats when compared with the control animals, without significant changes of creatinine (Table 2). The diabetic rats treated with sitagliptin showed urea values identical to those found for the control animals at the final time (26 weeks), contrasting with the higher value ($P < 0.01$) encountered in the diabetic ZDF without treatment (Table 2). Concerning the kidney trophism, we found that at week 20 (T0), there was already kidney hypertrophism, viewed by increased value ($P < 0.05$) of KW and of KW/BW in the diabetic rats when compared with the control animals, which was even increased in the final time (Table 2). Sitagliptin treatment did not changed kidney trophism parameters in the diabetic animals (Table 2).

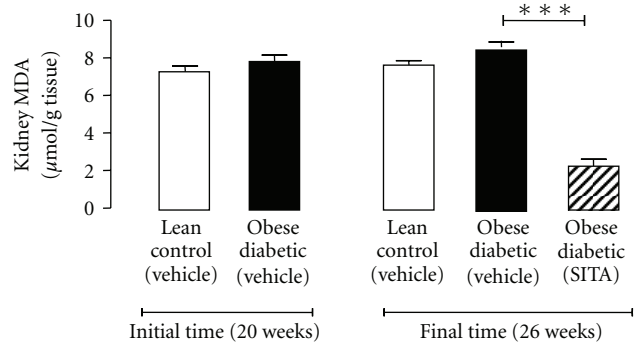

Figure 1: Kidney lipidic peroxidation (MDA) for the lean control and obese diabetic ZDF rats, in the initial and final times (6 weeks of vehicle or 10 mg/kg BW/day sitagliptin treatment). Data is expressed as mean ± sem of 8 rats/group: $^{***}P < 0.001$. MDA, malondialdehyde; SITA, sitagliptin.

3.4. Effects of Sitagliptin Treatment on Kidney Lipidic Peroxidation. At the initial time (20 weeks), MDA contents were unchanged between the lean control and the diabetic animals. A trend to higher values in the diabetic rats was found at the final time (Tf). This profile was completely reversed by sitagliptin treatment, since the kidney MDA values were substantially ($P < 0.001$) lower than those found in the diabetic untreated animals (Figure 1).

3.5. Effects of Sitagliptin Treatment on Renal Lesions Evolution

3.5.1. Glomerular Lesions. Comparative analysis between lean control and obese diabetic ZDF rats of 20 weeks of age revealed a significantly ($P < 0.001$) increased mesangial expansion, nodular sclerosis, glomerulosclerosis, and glomerular atrophy in the obese diabetic animals, accompanied by a significant thickening of glomerular basement membrane and capsule of Bowman ($P < 0.01$) (Figures 2(a) and 2(c)). When aged 26 weeks, the obese diabetic rats showed aggravated glomerular basement membrane thickening and glomerular atrophy ($P < 0.001$), when compared with the lean control animals, accompanied by a significantly more intense expression of mesangial expansion and capsule of Bowman thickening ($P < 0.01$). Glomerulosclerosis was also significantly more obvious in diabetic subjects ($P < 0.05$) (Figures 2(b) and 2(d)). Concerning ageing effects from 20 to 26 weeks in the lean rats, the most noted alterations were

Table 1: Body weight, glycaemic, insulinaemic and lipidic profile in the lean control and diabetic ZDF rats at the initial and final time (6 weeks of vehicle or sitagliptin treatment).

Time	Initial time (20 wks)		Final time (26 wks)		
Rat group	Lean control	Obese diabetic	Lean control	Obese diabetic	
Parameters	($n = 16$)	($n = 16$)	Vehicle ($n = 8$)	Vehicle ($n = 8$)	SITA ($n = 8$)
BW (g)	406.70 ± 6.83	388.10 ± 8.87	445.70 ± 8.16	354.40 ± 8.85^{aaa}	380.00 ± 14.46
Glucose (mg/dL)	133.30 ± 1.20	523.30 ± 3.60^{aaa}	133.30 ± 1.20	633.1 ± 15.70^{aaa}	546.33 ± 19.30^{bbb}
HbA1c (%)	3.16 ± 0.12	10.38 ± 0.50^{aaa}	3.20 ± 0.14	10.96 ± 0.20^{aaa}	9.18 ± 0.75^{bbb}
Insulin (mU/L)	15.00 ± 5.90	13.70 ± 0.90^{aa}	15.80 ± 3.00	7.60 ± 1.50^{aaa}	10.60 ± 1.80^{bbb}
HOMA-Beta (%)	76.80 ± 4.05	13.84 ± 1.50^{aaa}	80.90 ± 7.56	4.80 ± 1.12^{aaa}	7.89 ± 0.97^{bbb}
Total-c (mg/dL)	77.50 ± 1.50	155.50 ± 3.50^{aaa}	93.00 ± 2.96	193.00 ± 9.79^{aaa}	193.10 ± 4.62
TGs (mg/dL)	115.00 ± 11.00	374.50 ± 4.95^{a}	154.00 ± 19.14	400.20 ± 27.00^{aaa}	237.10 ± 22.54^{bbb}

Values are means ± SEM of n rats. [a]Lean control (vehicle) versus obese diabetic (vehicle) rats; [b]diabetic SITA-treated versus diabetic untreated rats. One, two, or three letters for $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. BW: body weight; HbA1c: glycosylated haemoglobin; HOMA: homeostasis model assessment; SITA: sitagliptin; Total-c: total-cholesterol; TGs: triglycerides; ZDF: Zucker diabetic fatty.

Table 2: Assessment of kidney function (serum creatinine and BUN) and weights (trophism) in the lean control and diabetic ZDF rats at the initial and final time (6 weeks of vehicle or sitagliptin treatment).

Time	Initial time (20 wks)		Final time (26 wks)		
Rat group	Lean control	Obese diabetic	Lean control	Obese diabetic	
Parameters	($n = 16$)	($n = 16$)	Vehicle ($n = 8$)	Vehicle ($n = 8$)	SITA ($n = 8$)
Creatinine (mg/dL)	0.55 ± 0.03	0.55 ± 0.06	0.53 ± 0.03	0.54 ± 0.08	0.49 ± 0.04
BUN (μg/L)	14.35 ± 0.47	18.15 ± 0.84^{aaa}	15.05 ± 0.54	18.03 ± 1.20^{aa}	15.16 ± 0.61^{b}
KW (g)	2.39 ± 0.08	3.25 ± 0.26^{a}	2.56 ± 0.04	3.02 ± 0.09^{a}	3.15 ± 0.05
KW/BW (g/Kg)	6.11 ± 0.15	8.82 ± 0.73^{a}	5.71 ± 0.07	8.42 ± 0.42^{aaa}	8.42 ± 0.40

Values are means ± SEM of n rats. Comparisons between groups: [a]lean control (vehicle) versus obese diabetic (vehicle) rats; [b]diabetic SITA-treated versus diabetic untreated rats. One, two, or three letters for $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. BUN: blood urea nitrogen; BW: body weight; KW: kidney weight; SITA: sitagliptin; ZDF: Zucker diabetic fatty.

capsule of Bowman thickening ($P < 0.01$) and increase in nodular sclerosis ($P < 0.01$) (Figures 2(a) and 2(b)). In the obese diabetic rats, between 20 and 26 weeks, there was a statistically significant increase in glomerular basement membrane ($P < 0.05$) and capsule of Bowman thickening ($P < 0.05$). Hyalinosis of the vascular glomerular pole was absent in all lean rats but was present in the obese diabetic ZDF rats, as soon as 20 weeks of age, with a tendency for aggravation in the 26 weeks (data not shown).

Concerning the sitagliptin effects in the diabetic rats at 26 weeks old, there was a reduction of severity of fibrosis, demonstrated by the significant decrease of global glomerulosclerosis ($P < 0.01$), which is in agreement with the less severe nodular sclerosis ($P < 0.01$) (Figure 3(a)). Hyalinosis of the vascular glomerular pole was also significantly decreased (Table 3). Mesangial expansion, glomerular atrophy, and glomerular basement membrane thickening showed a trend to improvement in the sitagliptin-treated diabetic rats versus the untreated (Figures 3(b), 3(c), and 3(d) and Table 3). Therefore, mesangial expansion showed a 37.5% reduction in the most severe grade; glomerular atrophy and glomerular basement membrane presented a 25% and 12.5% reduction, respectively, in grade 2 and 3 of lesion severity (Table 3). When considering all the glomerular lesions, the diabetic rats presented a notorious pattern of lesion ($P < 0.001$), when compared with the lean animals, which was significantly ameliorated ($P < 0.05$) by chronic sitagliptin treatment (Figure 4).

3.5.2. Tubulointerstitial Lesions. When aged 20 weeks, the obese diabetic rats already presented a significant increase in tubular degeneration ($P < 0.01$), tubular basement membrane irregularity, and IFTA ($P < 0.01$), when compared with the lean controls animals. The differences between these groups were more pronounced when aged 26 weeks, in which the obese diabetic subjects showed marked aggravation of hyaline cylinders, tubular basement membrane irregularity and IFTA ($P < 0.001$), together with significant increase in tubular degeneration ($P < 0.01$) (Table 4). The most significant ageing alterations found in the lean rats were tubular basement membrane irregularity ($P < 0.01$) and IFTA ($P < 0.01$), while in the obese diabetic animals, these were mainly IFTA ($P < 0.001$) and hyaline cylinders ($P < 0.01$) aggravation (Figure 2(d)).

Sitagliptin significantly prevented the appearance of hyaline cylinders in chronically treated diabetic rats ($P < 0.001$), together with a trend to decreased basement membrane irregularity (by 50%), tubular degeneration, and IFTA (by 37.5%) in grade 3 of lesion severity (Figure 3 and Table 4). Calcification of tubular epithelium was only present in diabetic rats, which did not suffer any mentionable recovery with sitagliptin treatment (Table 4). When considering all the

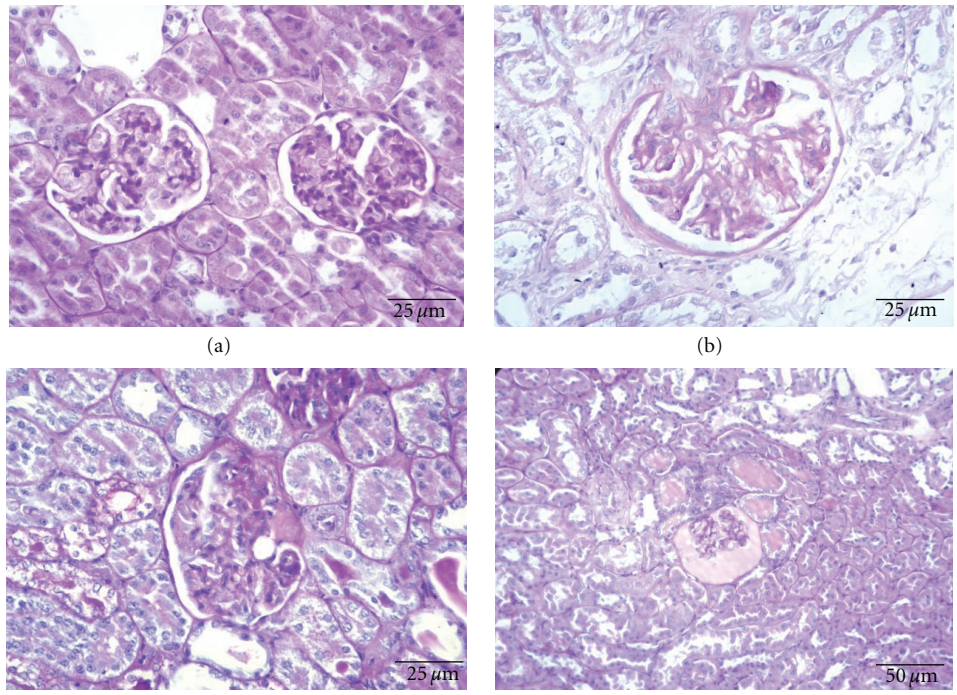

Figure 2: Evolution of renal lesions with ageing in lean control and obese diabetic ZDF rats: (a) normal renal histology in a lean control rat at 20 weeks of age (PAS, 400x); (b) a glomerulus presenting grade 1 mesangial expansion and thickening of the capsule of Bowman in a lean control rat at 26 weeks of age (PAS, 400x); (c) nodular glomerulosclerosis with sinequia of the tuft to Bowman's capsule, mesangial expansion and arteriolar sclerosis in a diabetic rat of 20 weeks (PAS, 400x); (d) atrophic, sclerosed glomerulus, exhibiting filtrate fluid in Bowman's space. Note the presence of hyaline cylinders and the irregularity of tubular basement membranes, diabetic rat of 26 weeks (PAS, 200x).

tubulointerstetial lesions, the diabetic rats presented a pattern of lesion ($P < 0.001$), when compared with the lean animals, which was significantly ameliorated ($P < 0.001$) by chronic sitagliptin treatment (Figure 4).

3.5.3. Vascular Lesions. Arteriolar hyalinosis was only found in the diabetic rats, which aggravated between 20 and 26 weeks ($P < 0.05$). Arteriosclerosis was only detected in lean animals when aged 26 weeks but was present in the diabetic rats at 20 weeks, which also exhibited aggravation of sclerosis at the final time, with 62.5% of the animals exhibiting grade 1 and 25% grade 2 lesions, in comparison to its lean counterparts, which showed 50% of animals in grade 1 and none in grade 2 (Table 5). Sitagliptin promoted a 50% improvement in the most severe form of hyalinosis (grade 2) and reduced the incidence of arteriosclerosis in the treated diabetic rats by 12.5% (Table 5).

4. Discussion

Diabetic nephropathy has emerged as the leading cause of end-stage renal disease (ESRD), and thus, preventing or delaying it, has been a major goal in biomedical research. The development of innovative therapeutic alternatives, such as the incretin enhancers (including sitagliptin), able to target not only hyperglycaemia, but also multiple risk factors, seems more likely to be beneficial as shown by recent approaches [27, 32]. Our present study reports the progression of renal disease in ZDF rats and demonstrates that a daily chronic administration of low-dose sitagliptin markedly reduces renal injury in this model.

It is well known that a commonly accepted animal model for type 2 diabetic nephropathy has not been available. The ZDF rat is characterized by hyperglycaemia, hyperinsulinaemia, hyperlipidaemia, moderate hypertension and obesity, and progressive renal injury [29]. These rats develop nephropathy by 12 wks of age, earlier than in most of other models of type 2 diabetes, characterized by focal segmental glomerulosclerosis (FSGS), associated with glomerulomegaly and mesangial expansion [36]. Thus, this animal model seems to be useful for preclinical evaluation of novel pharmacological compounds in human diabetic nephropathy. In the present study, the animal's ages were selected according to moment of initiation of relative insulinopenia (20 weeks) and of presence of significant diabetic complications (26 weeks). Although the literature describes in this animal model an earlier nephropathy, our animals were fed with normal rodent maintenance chow (with 2.9% of lipids) for developing all the different stages of T2DM in latter times than those described for this animal model. Therefore, if we intend to analyse renal lesions when rats presented lower insulin levels, those are the proper animal's ages. In order to achieve a better correlation between our animal observations and the

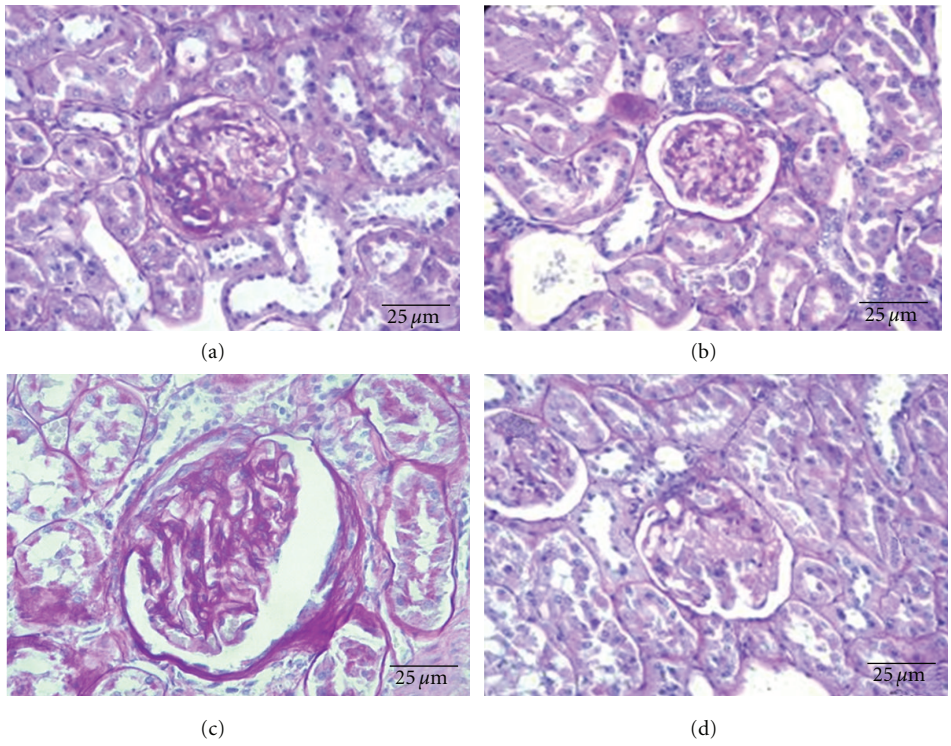

Figure 3: Effects of chronic sitagliptin treatment on renal lesions in obese diabetic ZDF rats; (a) regression of glomerulosclerosis, with more glomeruli presenting the more benign nodular form of sclerosis; (b) reduction in capsule of Bowman thickness and absence of sclerosis; (c) although there is persistence of grade 2 capsular thickening, there is absence of sclerosis and only the presence of grade 1 mesangial expansion; (d) presence of light mesangial expansion and hyalinosis of the vascular pole. Note in all figures the absence of hyaline cylinders and a more regular contour of the tubular basement membranes PAS, 400x.

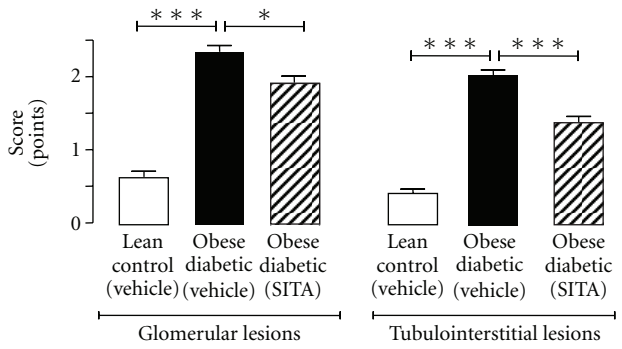

Figure 4: Effects of chronic sitagliptin treatment on renal glomerular and tubulointerstitial lesions in obese diabetic ZDF rats, at the final time (26 weeks). Data is expressed as mean ± sem of 8 rats/group: $^{*}P < 0.05$ and $^{***}P < 0.001$. SITA, sitagliptin.

human nephropathy process, we decided to adapt a recent human pathologic classification for diabetic nephropathy [37]. Despite the fact that our untreated diabetic ZDF presented lower body weight (BW) than their lean counterparts, our data show that along with the metabolic changes occurring over time in these rats, the nephropathy resembles human diabetic nephropathy in terms of morphology. The significant body weight loss of ZDF diabetic rats corresponds to the time of significant depletion of serum insulin levels compared with age-matched lean ZDF rats, which was an expected profile and is in agreement with the aggravation of the disease.

We must empathize that the administration of 10 mg/kg/day of sitagliptin, used in the current study, may be considered a low dose, as others have used higher doses or the administration of 10 mg/kg/BW twice a day [38, 39]. Nevertheless, we took in consideration that renal toxicity is very likely related to the extremely high urinary concentrations that result from rapid renal elimination of the drug in rodents. Since sitagliptin is virtually completely absorbed following an oral dose in rodents [40], the initial body burden of the drug is likely to be more directly related to the dosage on a mg/kg body weight basis than on a plasma AUC.

The nephropathy in this model has previously been described as focal segmental glomerulosclerosis (FSGS) associated with glomerulomegaly and mesangial expansion, findings characteristically seen in patients with obesity and metabolic syndrome [41, 42] associated with T2DM. In the literature, we found the descriptions of the tubulointerstitial lesions are mentioned only in passing and as secondary pathology [36, 43]. Renal vascular pathology has not been described. The data presented herein provides morphologic characterization of progressive nephropathy, including the glomerular, tubulointerstitial, and vascular lesions in the kidney of ZDF rats.

Table 3: Scoring and distribution of glomerular lesions in lean control and obese diabetic ZDF rats kidneys at the final time, 26 weeks of age (6 weeks of vehicle or sitagliptin treatment).

Glomerular lesion	Rat group ($n = 8$ each)	Scoring and distribution of glomerular lesions (n of rats)			
		Normal	Mild	Moderate	Severe
Mesangial expansion	Lean control (vehicle)	3	3	2	0
	Obese diabetic (vehicle)	0	0	3	5^{aaa}
	Obese diabetic (SITA)	0	1	5	2
Thickening of GBM	Lean control (vehicle)	3	5	0	0
	Obese diabetic (vehicle)	0	0	2^{aa}	6^{aa}
	Obese diabetic (SITA)	0	2	1	5
Thickening of CB	Lean control (vehicle)	1	6	1	0
	Obese diabetic (vehicle)	0	0	4	4
	Obese diabetic (SITA)	0	4	0	4
Nodular sclerosis	Lean control (vehicle)	2	4	2	0
	Obese diabetic (vehicle)	0	5	3	0
	Obese diabetic (SITA)	0	0	2^{bb}	6^{bb}
Glomerulosclerosis	Lean control (vehicle)	2	3	3	0
	Obese diabetic (vehicle)	0	0^{a}	3	5^{aaa}
	Obese diabetic (SITA)	0	4^{bb}	4^{b}	0^{bbb}
Glomerular atrophy	Lean control (vehicle)	6	2	0	0
	Obese diabetic (vehicle)	0	0	4^{aaa}	4^{aaa}
	Obese diabetic (SITA)	0	4^{bb}	2^{b}	2^{b}
Hyalinosis of the vascular pole	Lean control (vehicle)	8	0	0	0
	Obese diabetic (vehicle)	2	1	2	3
	Obese diabetic (SITA)	0	7^{bbb}	1^{b}	0^{b}

[a] Lean control (vehicle) versus obese diabetic (vehicle) rats; [b] diabetic SITA-treated versus diabetic untreated rats. One, two, or three letters for $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. CB: capsule of Bowman; GMB: glomerular basement membrane; SITA: sitagliptin. Scoring was defined according to the extension occupied by the lesion (% area of the glomerulus): normal: <25%; mild: 25–50%; moderate: 50–75%; severe: >75%.

Table 4: Scoring and distribution of tubular lesions in lean control and obese diabetic ZDF rats kidneys at the final time, 26 weeks of age (6 weeks of vehicle or sitagliptin treatment).

Tubular lesion	Rat group ($n = 8$ each)	Scoring and distribution of tubular lesions (n of rats)			
		Normal	Mild	Moderate	Severe
Hyaline cylinders	Lean control (vehicle)	6	2	0	0
	Obese diabetic (vehicle)	0	0	7^{aaa}	1
	Obese diabetic (SITA)	0	8^{bb}	0	0
TBM irregularity	Lean control (vehicle)	2	5	1	0
	Obese diabetic (vehicle)	0	0	1	7^{aaa}
	Obese diabetic (SITA)	0	3^{bb}	2	3^{bb}
Tubular calcification	Lean control (vehicle)	8	0	0	0
	Obese diabetic (vehicle)	5	3	0	0
	Obese diabetic (SITA)	4	4	0	0
IFTA	Lean control (vehicle)	2	6	0	0
	Obese diabetic (vehicle)	0	0	3^{aa}	5^{aaa}
	Obese diabetic (SITA)	1	2	3	2^{bb}
Tubular degeneration	Lean control (vehicle)	4	4	0	0
	Obese diabetic (vehicle)	0	1^{aa}	4^{aaa}	3^{aaa}
	Obese diabetic (SITA)	0	3^{b}	5^{b}	0^{bb}

[a] Lean control (vehicle) versus obese diabetic (vehicle) rats; [b] diabetic SITA-treated versus diabetic untreated rats. One, two, or three letters for $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. TMB: tubular basement membrane; IFTA: interstitial fibrosis and tubular atrophy. Scoring was defined according to the extension occupied by the lesion (% area of the tubulus): normal: <25%; mild: 25–50%; moderate: 50–75%; severe: >75%. SITA, sitagliptin.

Table 5: Scoring and distribution of vascular lesions in lean control and obese diabetic ZDF rats kidneys at the final time (26 weeks of age). Diabetic ZDF rats with versus without chronic sitagliptin.

Vascular lesion	Rat group ($n = 8$ each)	Scoring and distribution of vascular lesions (n of rats)		
		Normal	Mild/moderate	Severe
Arteriolar hyalinosis	Lean control (vehicle)	8	0	0
	Obese diabetic (vehicle)	1[aa]	1	6[aa]
	Obese diabetic (SITA)	3	3[b]	2[b]
Arteriosclerosis	Lean control (vehicle)	4	4	0
	Obese diabetic (vehicle)	1	5	2
	Obese diabetic (SITA)	3	4	1

[a]Lean control (vehicle) versus obese diabetic (vehicle) rats; [b]diabetic SITA-treated versus diabetic untreated rats. One, two or three letters for $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Scoring was defined according to the following criteria: arteriolar hyalinosis was scored as 0 if absent, as 1 if one arteriole with hyalinosis was present, and as 2 if more than one arteriole was observed in the entire slide. Arteriosclerosis was scored as 0 if no intimal thickening was present, as 1 if intimal thickening was less than the thickness of the media, and as 2 if intimal thickening was more than the thickness of the media. SITA, sitagliptin.

The lean ZDF rats demonstrated at 20 wk thickening of GBM, mesangial expansion, nodular sclerosis, interstitial fibrosis, and tubular atrophy (IFTA), which further aggravates with age. These observations are in accordance with Vora et al. (1996) [44] and could be classified as nondiabetic renal lesions attributed to aging in this strain. All the obese diabetic ZDF rats presented significant glomerular, tubulointerstitial and vascular lesions compared with lean ZDF controls in both ages analysed (20 and 26 wks). In the obese diabetic ZDF rats, the severity of the lesions aggravates with diabetes progression, confirming a link between diabetes (hyperglicaemia and hyperlipidaemia) and progressive renal injury.

In patients with diabetic nephropathy, the initial physiological change is glomerular hyperfiltration, while the initial morphological change is glomerular hypertrophy. At 26 wks old, the obese ZDF rats exhibit an aggravation of the lesions described for 20 wks, including mesangial expansion, glomerular basement membrane thickening, and glomerular hypertrophy. We observed that tubulointerstitial lesions are dependent of glomerulosclerosis, which is suggested by the aggravation of both (glomeruli and interstitium). Vascular pole hyalinization and arthrosclerosis also suffer aggravation with age. All of these histological alterations were accompanied by an augmentation of kidney weight. In the obese diabetic ZDF rats, a glomerular hypertrophy, expansion in the mesangial area related to the mesangial matrix, and renal hypertrophy was noted. In the present study, we did not evaluate the progression of proteinuria, but it is well documented by others [45, 46]. We measured blood urea nitrogen (BUN), and the results showed a significant increase the obese diabetic ZDF rats when compared to the lean control, suggesting a deficient kidney function. Nevertheless, serum creatinine levels were unchanged between groups, which is in accordance with others [47].

Chronic sitagliptin (low-dose) treatment ameliorated all lesions (glomerular, tubulointerstitial, and vascular), except the tubular epithelium calcification, in the diabetic-treated rats. Chronic sitagliptin administration was able to decrease BNU to levels analogous to those observed in lean controls, suggesting an amelioration of kidney function. The mechanism by which a low-dose of sitagliptin, which was unable to completely normalize the hyperglycaemic profile of the diabetic rats, is able to positively modulate kidney function is unknown. We may hypothesize that significant improvement of circulating levels of TG result in the attenuation of renal injury in treated diabetic ZDF rats. One explanation for this is that the augment of insulin levels by sitagliptin inhibits adipose tissue hormone-sensitive lipase (HSL) activity and, thus, adipose tissue fatty acid release. In addition, insulin and the augment of GIP induced by DPP-4 inhibition may enhance adipose tissue fatty acid reesterification and, thus, increase adipose tissue triacylglycerol (TAG) deposition. In the present work, we did not measure fat pads in ZDF rats, we did not evaluated lipids in kidney, and, thus, we cannot confirm our hypothesis. Nevertheless, in future studies, we intend to perform oil red staining in the kidney in order to assess lipotoxicity and the putative effects of sitagliptin. However, some previous data from our studies should be mentioned. We have demonstrated that this low-dose chronic sitagliptin treatment is able to promote a favorable impact on chronic inflammation and oxidative stress, which are key players of diabetes pathophysiology and may precede and further potentiate tissue damage [32]. Despite the lower dose used, we have previously demonstrated beneficial effects of sitagliptin on metabolic profile and reduction in inflammatory markers, as well as an amelioration of fibrosis, vacuolization, and congestion in endocrine pancreas and preservation of pancreatic islets were previously suggested [32]. The histomorphological observations were in accordance with the improvement in pancreatic beta-cell function, as suggested by the sitagliptin-evoked augment in HOMA-beta. The effects of chronic DPP-4 inhibition in increasing β-cell mass and function over time may occur, at least in part, by the augmentation of glucose-stimulated insulin secretion. This effect is believed to be primarily mediated via stabilization of the incretin hormones contents, including of GLP-1 [48]. We also observed a weight gain of treated diabetic animals that could be attributed to the amelioration induced by sitagliptin in the dysmetabolism and thus to an improvement in general

condition. This metabolic improvement by sitagliptin in diabetic ZDF rats was accompanied by a reduction in inflammatory markers (CRP and IL-1 beta) and pancreatic oxidative stress, as previously documented by our group [32]. Our results agree with those performed by others, which have been suggesting an antioxidant and anti-inflammatory effect of incretin modulators, due to attenuation of the deleterious effects of AGEs-RAGE-oxidative stress axis and to protection against the cytokine-induced apoptosis and necrosis [49–51].

Although large body of evidence indicates that oxidative stress is involved in the progression of fibrosis and end-stage renal disease, in experimental and human diabetic nephropathy [52], we failed to demonstrate it, at least when comparing kidney lipid peroxidation between diabetic untreated ZDF rats their lean mach control. However, further studies should better address this aspect, namely, by assessing other relevant kidney markers of oxidative stress, including levels of AGEs, as well as contents of antioxidants. However, our work suggests a favourable impact of sitagliptin treatment on kidney oxidative stress profile, expressed by reduced amount of lipid peroxidation, which might be further confirmed with additional parameters, but that is in agreement with recent studies from Vaghasiya et al. (2011) which have reported a significant decrease in renal lipidic peroxidation by sitagliptin in diabetic rats with renal damage [53].

Experimental evidence linking hyperlipidaemia to renal injury and progression of renal fibrogenesis has been well documented; lipids can modulate the progression of chronic renal diseases and may even be primary factors in the pathogenesis of renal tissue injury [54]. Additionally, the synergistic effects of hyperlipidaemia and diabetes on the development of renal injury have been recently observed in several animal models [55, 56]. In ZDF rats, Chander et al. (2004) and Suzaki et al. (2006) suggested that hyperlipidaemia, in concert with hyperglycaemia, may be responsible for the increased oxidative stress and initiation and aggravation of injury in the kidneys of these animals [57, 58]. Thus, we may hypothesize that the ability of sitagliptin to lower plasma lipids, as well as to promote a more favorable redox status in the kidney, as confirmed in the present study by the reduction of lipid peroxidation products, may have contributed to its renoprotective effects. Furthermore, the positive effects demonstrated in peripheral insulin resistance and pancreas lesions, as well as the antihypertensive effect [32], might be viewed as probable contributors to the renoprotection described in this study. On the other hand, we could not exclude the possible effects of the expected sitagliptin-induced inhibition of DPP-4 and consequent increment of GLP-1, since these effects have been associated by others to a protection of mesangial cells and to an amelioration of sodium, acid-base, and fluid homeostasis that contributes to the renoprotection [59, 60]. In any case, future studies should confirm the effects of sitagliptin on DPP-4 activity/expression, as well as on GLP-1 and glucagon levels, in order to have a more detailed picture of how the incretin pathway is affected and its relative contribution for the effects of sitagliptin here reported.

To our knowledge, this is the first report on the amelioration of diabetic nephropathy, and specifically of glomerulosclerosis, tubulointerstitial and vascular kidney lesions, by a chronic administration of a low dose of sitagliptin that does not reduce hyperglycaemia below a rather high level (partial, but significant, correction), indicative of noncompensated diabetes. The present study demonstrated that sitagliptin delays the development of nephropathy in ZDF rats, concomitantly with hypoglicaemic, hypolipidaemic and antioxidant effects. Although, further studies are required to elucidate the nature of the protective effects of sitagliptin on the diabetic kidney, the obtained results are consistent with pleiotropic effects of this new antidiabetic drug, which might underlie the renoprotective properties.

5. Conclusions

Chronic administration of a low dose of sitagliptin was able to ameliorate diabetic nephropathy in this model of obese type 2 diabetes/nephropathy, viewed by significant reduction of glomerulosclerosis and tubulointerstitial and vascular kidney lesions, which might be partial due to its benefits on correction of diabetes dysmetabolism (hyperglicaemia, dyslipidaemia, and insulin production/sensitivity), and due to a favorable impact on kidney lipid peroxidation. Further studies are required to assess the cellular/molecular nature of these effects. However, the beneficial and novel profile of this incretin modulator could prove crucial in the prevention of diabetic nephropathy evolution and might represent a key step forward in the management of T2DM and this serious complication.

Declaration of Interests

The authors report no conflict of interests.

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Research Article

Involvement of F-Actin in Chaperonin-Containing t-Complex 1 Beta Regulating Mouse Mesangial Cell Functions in a Glucose-Induction Cell Model

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The aim of this study is to investigate the role of chaperonin-containing t-complex polypeptide 1 beta (CCT2) in the regulation of mouse mesangial cell (mMC) contraction, proliferation, and migration with filamentous/globular-(F/G-) actin ratio under high glucose induction. A low CCT2 mMC model induced by treatment of small interference RNA was established. Groups with and without low CCT2 induction examined in normal and high (H) glucose conditions revealed the following major results: (1) low CCT2 or H glucose showed the ability to attenuate F/G-actin ratio; (2) groups with low F/G-actin ratio all showed less cell contraction; (3) suppression of CCT2 may reduce the proliferation and migration which were originally induced by H glucose. In conclusion, CCT2 can be used as a specific regulator for mMC contraction, proliferation, and migration affected by glucose, which mechanism may involve the alteration of F-actin, particularly for cell contraction.

1. Introduction

Functions of mesangial cell contraction, migration, and proliferation have been reported to be correlated with the development of diabetic nephropathy (DN). Mesangial cell contraction regulating intraglomerular pressure contributes to the occurrence of glomerular hyperfiltration in early DN and then progresses to end-stage renal disease (ESRD) [1]. Alteration in mesangial cell migration may limit repair following mesangiolysis and thereby contribute to the loss of kidney function in diabetic nephropathy [2, 3]. Aberrant proliferation of mesangial cells is commonly observed in DN that can lead to ESRD [4]. As the integrity of cytoskeletons is changed during mesangial cell contraction, migration, and proliferation, actin, the most important cytoskeletal protein, should play a role in the development of DN [5].

Actin is often the most abundant protein in a cell, comprising up to 15% of total protein. The function of actin in eukaryotic cells is ubiquitous, including regulation of cell contraction, adherence, movement, and phagocytosis. Actin in cells is generally present interchangeably between a monomer and a polymer, in which globular-(G-) actin subunits assemble into long filamentous polymers called F-actin. Some cellular functions involving cytoskeletons are regulated by this process of actin polymerization [6]. The chaperonin family may play an important role in maintaining the normal function of actin as the function of chaperone-assisted protein folding in cell allows many cytosolic proteins to attain the correct folded states and functional conformations during protein synthesis or during recovery from their denatured states [7].

The two most abundant classes of molecular chaperones are the heat shock protein (HSP)60 and HSP70. HSP60 chaperones, also termed chaperonins, are found in all organisms, and are classified into two distinct types. Type I is discovered in prokaryotic cells and endosymbiotic organelles. Type II is found in eukaryotic cytosol and is termed chaperonin-containing t-complex polypeptide 1 (CCT) [8]. CCT is composed of 8 different subunits (CCT $\alpha\beta\gamma\delta\varepsilon\zeta\eta\theta$, the equivalent of CCT1, 2, 3, 4, 5, 6, 7, and 8), but their functions are still not well understood.

Up to present, available data [9–12] suggests that CCT is the only chaperonin known to be abundant in the eukaryotic cytosol [11] and the primary substrate for cytoskeletal proteins, tubulins, and actins. Therefore, it is of interest to know how CCT interacts with cytoskeletal proteins and involves in cell functions, such as contraction, migration, and proliferation.

Of all CCT subunits, CCT2 has been proven to correlate with the function of actin [9]. Furthermore, CCT2 is suggested to have a relationship with mesangial cell contraction in our own proteomic study [13]. Therefore, in order to investigate the effect of CCT2 on the cytoskeletal functions of mesangial cells, an *in vitro* cell model capable of attenuating CCT2 expression by small interfering RNA (siRNA) was established. The aim of this study is to investigate if a relationship exists between CCT2 and F/G actin on various cell functions, including contraction, migration, and proliferation.

2. Materials and Methods

2.1. siRNA Transfection and Experimental Protocol. As our previous study found mouse mesangial cells (mMCs) incubating with high glucose has the upregulation of CCT2, the prime work of this study is to find the optimal amount of siRNA to establish a low CCT2 model. In brief, mycoplasma-free SV 40-transformed mMCs were prepared and kept as described previously [13, 14]. These mMCs (1×10^5/well) were maintained in DMEM+F12 containing 5% FBS and 21.25 mM D-glucose in 6-well plates. Once the cells were subconfluent, the medium was changed to Transfection Medium (Santa Cruz, sc-36868, USA), Transfection Reagent (TR, Santa Cruz, sc-29528, USA), and CCT2 siRNA suspension (Santa Cruz, sc-36625, USA), according to manufacturer's instructions. The volume of TR was fixed at 6 ul per the instructions. The optimal siRNA amount was determined by mMCs transfecting with the different volume of CCT2 siRNA 2, 4, and 6 ul per well. After 12 hrs, the whole medium was changed to 21.25 mM glucose with 1% fetal bovine serum (FBS) for 24 hrs. Cells from these different mixes were harvested for protein extraction. Western blotting was used to analyze the expression of CCT 2. Optimal ratio between the volume of TR and siRNA (TR-to-siRNA) was established.

Once the optimal ratio of TR-to-siRNA was determined, the subsequent experiments were as follows. First, mesangial cells were treated with TR containing CCT2 siRNA, scramble siRNA (CsiRNA, Santa Cruz, sc-37007, USA), and TR only. After 12 hrs, the concentration of culture medium among the above three groups was changed into 6.6 mM (N) and 21.25 mM (H) D-glucose for another 24 hrs. To validate the sequence specificity of gene knockdown *in vitro*, six groups, TR *p*N, CsiRNA *p*N, CCT2siRNA *p*N, TR *p*H, CsiRNA *p*H, and CCT2siRNA *p*H, were designed for the subsequent experiments assaying the protein expression of CCT2 and cell viability. Due to similar results in groups TR *p*N and CsiRNA *p*N, groups CsiRNA *p*N, CCT2siRNA *p*N, CsiRNA *p*H, and CCT2siRNA *p*H were selected for further experiments concerning the F/G-actin ratio, the arrangement of F-actin, and cell contraction, migration, and proliferation.

2.2. Cell Viability. To test the cell viability, MTT assay was used. Six groups were designed as described above. The MTT method was followed as previously described [14].

2.3. Western Blot (WB) for the Amount of CCT2 and F/G-Actin Ratio. To determine F/G-actin ratio and CCT2, mMCs were cultured in 10 cm Petri dishes at a density of 1×10^6cells per well for protein extraction.

For CCT2, in brief, the cells were washed with ice-cold PBS and lysed *in situ* with 0.5 mL ice-cold lysis buffer (2 M thiourea, 7 M urea, 4% CHAPS, and 0.5% ampholyte) at 4°C for 15 mins. For whole-cell preparations, supernatants were collected after centrifugation at 13 000 rpm for 20 mins. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Hercules, CA, USA). Samples were stored at −70°C until use. The protocol for WB was similar to the method previously described. The membranes were incubated overnight at 4°C with antibodies against CCT2 (Santa Cruz, CA, USA) in a ratio of 1 : 500 in TBST containing 1% BSA. Horseradish Peroxidase-(HRP-) labeled secondary antibody (Jackson ImmunoResearch, PA, USA) was used to detect CCT2 proteins.

For the F/G-actin ratio, protein extracts from mMCs were subjected to an F/G-actin *in vivo* assay kit (Cytoskeleton, CO, USA) based on the manufacturer's protocol. Briefly, cells were lysed with a cell lysis and F-actin stabilization buffer and homogenized using 26G syringes. Cell lysates were centrifuged at 100 000 g for 60 min at 37°C. Then supernatants (G-actin) were separated from the pellets (F-actin) and were immediately placed on ice. Pellets were resuspended to the same volume as the supernatants using ice-cold ddH_2O containing 1% cytochalasin D and were incubated on ice for 60 mins. Equal amounts of the samples (supernatant and pellet) were loaded into each lane and analyzed by WB with 1 : 500 dilution of antiactin antibody (Cytoskeleton, CO, USA) incubated in a blocking buffer for 1 hr at room temperature (RT). Blots were washed three times, and then the membranes were incubated in a 1 : 10000 dilution of goat anti-rabbit HRP (Jackson Immunoresearch, PA, USA) in TBST for 30 mins at RT. Membranes were washed three times, and the membrane-bound antibody detected was incubated with WB luminol reagent kit (Santa Cruz biotechnology, CA, USA) and captured on X-ray film.

2.4. Immunofluorescence for the Localization of F-Actin. Immunofluorescence (IF) staining of F-actin for cells indicated a cytoskeletal rearrangement in the cells [15, 16]. To localize

the expression of F-actin, mMCs were subjected to IF. mMCs were plated on 22 mm glass coverslips, washed with PBS, and then fixed with 3.7% paraformaldehyde for 10 min at RT. Cells were then permeabilized with PBS + 0.1% Triton X-100 for 5 min at RT, blocked with PBS + 0.1% BSA for 20 min at RT, then thoroughly rinsed with PBS and stained with Phallacidin conjugate with the green fluorescent Alexa Fluor 488 (Molecular Probes Inc., Cat. No. A12379, Ore, USA). A 1 : 500 dilution in blocking buffer 10 mins was used to label the F-actin. After incubating with phallacidin, the cells were treated with 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes Inc., Cat. No. D1306, Ore, USA)-dilactate 2 mins to label the nuclei. The developed sections were visualized using an optical photomicroscope (Olympus, Tokyo, Japan). Negative controls, from which primary antibodies were omitted, were included in the assay.

2.5. Cell Contraction. Phorbol 12-myristate 13-acetate (PMA, Merck Chemicals Darmstadt, Germany) was used to induce mMC contraction, which was assessed from changes in the planar surface area as described previously [14]. mMCs were cultured in 24-well plates at a density of 1×10^3 cells per well. Cells from study groups for cell contraction were assayed and analyzed. For detecting the role of F-actin, the F/G-actin ratio and the arrangement of F-actin in mMC before and after PMA stimulation were evaluated by WB and IF, respectively.

2.6. Cell Migration. For cell migration, mMCs were cultured in a 6-well plate at a density of 1×10^6cells per well. mMCs from study groups were applied to the assay of cell migration as previously described [17]. At the time point of 0 hr, a single wound was created in the center of the cell monolayer by gentle removal of the attached cells with a sterile plastic pipette tip. The debris was removed by washing with serum-free medium. Subsequently, at the time point of 12 hr, the wound was observed; the cells which migrated into the wounded area or protruded from the border of the wound were visualized and photographed under an inverted microscope. Each experiment was performed at least three times independently. For analysis, an Image J file was opened at 0 and 12 hr and the rate of wound closure was calculated. Data are expressed as percentage wound closure relative to the width of control wounds photographed at 0 hr. The wound closure area of the cells cultured in normal glucose was set at 100% [18]. In addition, mMCs at 0 hr and 12 hr were harvested for determining the F/G-actin ratio and the arrangement of F-actin.

2.7. Cell Proliferation. For the evaluation of cell proliferation, mMCs were cultured in a 96-well plate at a density of 1×10^3cells per well. The study groups were applied to the assay of cell proliferation using MTT assay. The protocol for MTT was performed as previously described [14]. According to our experimental design, cells from study groups were checked with MTT at the time points 0 and 12 hr. At 0 hr, MTT data was analyzed, so the effect of CCT2 siRNA on mesangial cell proliferation was determined. Further, the growth rate of mesangial cell from 0 to 12 hr was calculated. The formula for the growth rate is 12 hr–0 hr/0 hr. Each experiment was performed at least three times independently. In addition, mMCs at 0 and 12 hr were harvested for determining the F/G-actin ratio and the arrangement of F-actin.

2.8. Statistical Analysis. All experiments were repeated at least three times. Results within groups are expressed as the mean ± SEM. Unpaired t-tests were used to assess the statistical significance of differences between two groups, and paired t-tests were used for within-group comparisons. ANOVA with Tukey's multiple comparison test was used for comparisons between more than two groups. A P value of less than 0.05 was considered significant.

3. Results

3.1. Optimal Ratio of TR-to-siRNA for Establishing a Low CCT2 Cell Model. As seen in Figures 1(a) and 1(b), groups 6 : 2, 6 : 4, and 6 : 6, all with different volume, showed the attenuating effect on the expression of CCT2 in mMCs when compared to group TR *p*N. The group with the ratio of 6 : 2 showed the most significant effect over the other groups, yielding around 50% reduction of CCT2 level. We suggest that the ideal ratio of TR-to-siRNA is 6 : 2 for blocking CCT2. A low CCT2 cell model is thereby established for subsequent study.

3.2. Cell Viability, CCT2, and F/G-Actin Ratio in Groups with and without Low CCT2 after Glucose Stimulation. Using the ratio 6 : 2 of TR-to-siRNA for conducting CCT2 knockdown experiments, six groups, TR *p*N, CsiRNA *p*N, CCT2siRNA *p*N, TR *p*H, CsiRNA *p*H, and CCT2siRNA *p*H, were designed to test the cell viability and to verify the effect of CCT2siRNA. First, there were no differences among six groups for cell viability (data not shown), suggesting that the six groups had a similar outcome for cell viability. Second, as for the expression of CCT2, as Figures 2(a1) and 2(a2) show, mMCs from groups TR *p*H and CsiRNA *p*H both had statistically higher expression than did counter groups TR *p*N and CsiRNA *p*N. There were no differences between TR *p*H and CsiRNA *p*H or between TR *p*N and CsiRNA *p*N, suggesting that scramble siRNA did not lead to degradation of cellular CCT2. Groups CCT2siRNA *p*N and CCT2siRNA *p*H showed a significant attenuating effect when compared to those of other groups. We confirmed that, under the ratio of 6 : 2 between TR and amount of siRNA, the expression of CCT2 decreased significantly in mMCs incubating in normal or high glucose, irrespectively. As groups with and without scramble siRNA showed equal effect on the expression of CCT2, groups CsiRNA *p*N, CCT2 siRNA *p*N, CsiRNA *p*H, and CCT2 siRNA *p*H were chosen for subsequent experiments.

Protein extracts from four groups, CsiRNA *p*N, CCT2siRNA *p*N, CsiRNA *p*H, and CCT2siRNA *p*H, were investigated for F/G-actin ratio. As Figures 2(b1) and 2(b2) show, the F/G-actin ratio in groups CCT2siRNA *p*N, CsiRNA *p*H, and CCT2siRNA *p*H was statistically lower than that of group CsiRNA *p*N. There was no difference

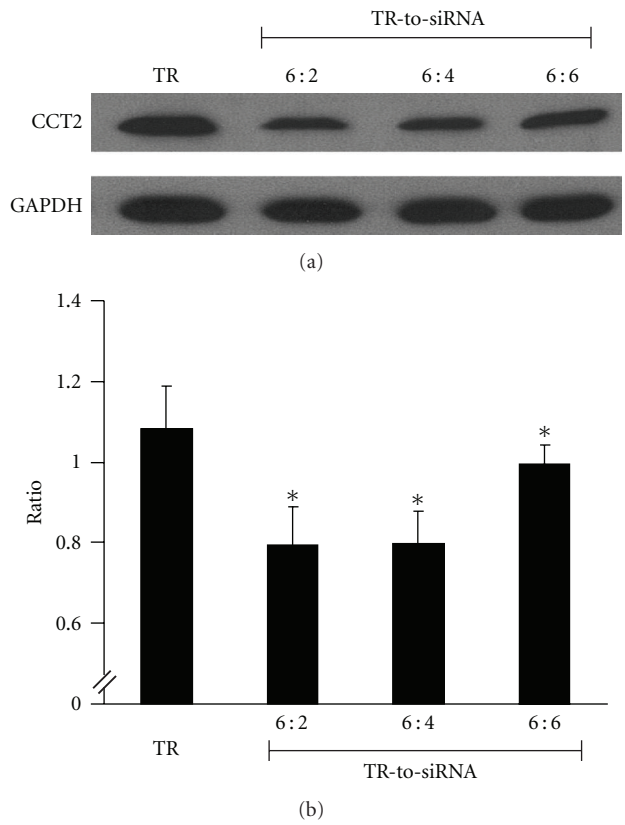

Figure 1: Optimal ratio between transfection reagent (TR) and volume of siRNA (TR-to-siRNA) for a low CCT2 mouse mesangial cell (mMC) model. The volume of TR was fixed at 6 ul, and transfection was performed with varying volumes of CCT2 siRNA. Representative plots show (a) western blotting used for the expression of CCT2 in mMC treated by TR only and TR plus CCT2siRNA at the ratios of 6 : 2, 6 : 4, and 6 : 6 and (b) the normalized CCT2 expression against GAPDH for above groups, in which group 6 : 2 displays an ideal suppressive effect on CCT2 and was chosen for subsequent experiments. $^*P < 0.05$ versus group TR.

among those three groups, suggesting that high glucose and CCT2siRNA both had a similar effect on mMC attenuating F/G-actin ratio. However, there was no synergetic effect in high glucose plus CCT2siRNA. In summary, our findings suggest that H glucose and low CCT2 may decrease the F/G-actin ratio in mMC.

3.3. Change of Cell Contraction, F/G-Actin Ratio, and Arrangement of F-Actin in the Groups with and without Low CCT2 after Glucose Stimulation. As shown in Figures 3(a) and 3(b), changes in the planar surface areas of mMCs in response to 1 μM PMA stimulation every 10 min were observed for groups CsiRNA *p*N, CCT2siRNA *p*N, CsiRNA *p*H, and CCT2siRNA *p*H. Differences in the baseline planar areas of these four groups were not statistically significant before the addition of PMA (data not shown). After PMA stimulation, mMC planar areas of the group CsiRNA *p*N decreased to 40%–50% of their original areas. However, groups CsiRNA *p*H, CCT2siRNA *p*N, and *p*H showed the smallest change in planar surface area when compared to that of group CsiRNA *p*N, and no significant difference in contractility was observed among these three groups. Our findings suggest that mMCs treated by H glucose or CCT2siRNA both showed a similar effect on the attenuating of PMA-stimulated contraction, and there was no synergetic effect for H glucose plus CCT2siRNA.

Taking the results of the expression of CCT2, F/G-actin ratio, and cell contraction together, groups with low CCT2 had an associated low F/G-actin ratio before PMA stimulation, and then these groups displayed significantly less cell contraction than that of normal F/G-actin ratio.

As for the expression of F/G-actin ratio and the arrangement of F-actin in mMCs before and after PMA stimulation, as Figure 3(c) shows, the change of F-actin distribution in mMC had obviously redistributed from bundle to aggregation after PMA 60′ stimulation. As Figure 3(d) shows, the expression of F/G-actin ratio in all groups remained a similar pattern before and after PMA stimulation.

In summary, our findings suggest that mMCs with low CCT2 or treated by H glucose may decrease the F/G-actin ratio and also show less cell contraction in PMA-induced cell contraction and F-actin aggregation after PMA, but maintain a similar F/G-actin ratio before and after PMA stimulation.

3.4. Change of Cell Proliferation, Migration, F/G-Actin Ratio, and Arrangement of F-Actin in Groups with and without Low CCT2 after Glucose Stimulation. For the change in mesangial cell proliferation under treated conditions, the data of MTT at time points 0 and 12 hr from groups CsiRNA *p*N, CCT2siRNA *p*N, CsiRNA *p*H, and CCT2siRNA *p*H were analyzed. At the point of 0 hr, group CsiRNA *p*H showed a significant increase over that of groups CsiRNA *p*N, CCT2 siRNA *p*N, and *p*H, suggesting that H glucose increased mMCs proliferation, but CCT2 siRNA blocked this effect (data not shown). Furthermore, as Figure 4(c) shows, the rate of mMC proliferation between 0 hr and 12 hr was calculated, and no significant changes among these four groups were found. Taken together, our findings suggest that H glucose increases mMC proliferation, but CCT2 siRNA reverses it.

For the observation of mMC migration, the percentage of wound closure at the time points 0 and 12 hr from groups CsiRNA *p*N, CCT2 siRNA *p*N, CsiRNA *p*H, and CCT2siRNA *p*H was analyzed. Group CsiRNA *p*H showed significantly enhanced ability of mMC migration when compared to that of groups CsiRNA *p*N, CCT2siRNA *p*N, and *p*H (Figures 4(a) and 4(b)). Among CsiRNA *p*N, CCT2siRNA *p*N, and *p*H, there was no difference in the effect on cell migration, suggesting CCT2siRNA may have a reversing effect, particularly in mMCs incubating in H glucose.

As for the arrangement of F-actin and expression of the F/G actin ratio in the process of mMC proliferation and migration, the distribution of F-actin and F/G-actin ratio in mMC at 0 and 12 hr showed no significant change (Figures 4(d) and 4(e)).

Taking the data of migration and proliferation together, our findings suggest that H glucose activates mMC proliferation and enhances the ability of mMC migration, which can

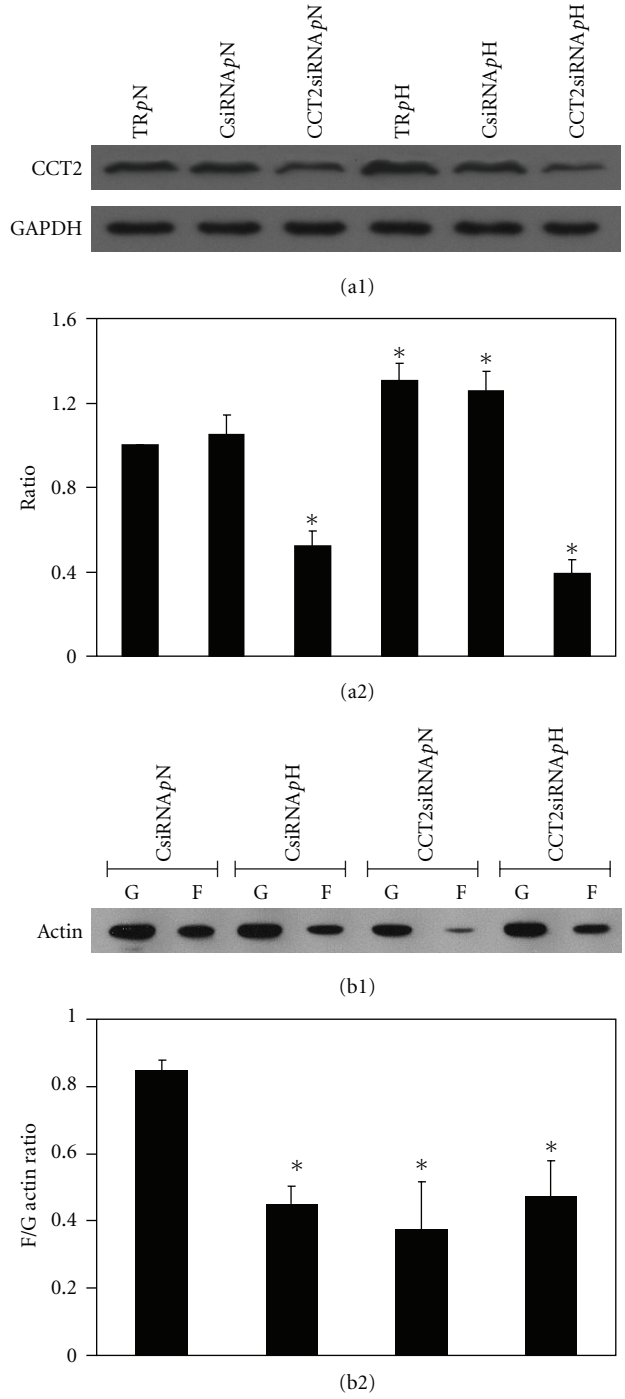

Figure 2: CCT2 and F/G-actin ratios in the CCT2 cell model with and without high glucose induction. The TR-to-siRNA ratio of 6 : 2 was used for the CCT2 knockdown experiment with evaluation of F/G-actin ratio on six designated groups. Representative plots show (a1) western blotting CCT2 expression and (a2) the normalized CCT2 expression against GAPDH for the six groups, in which induction of CCT2 expression was observed with high glucose group of transfection reagent (TR *p*H) and scramble siRNA (CsiRNA *p*H) and reduction of CCT2 expression was with normal glucose group (CCT2siRNA *p*N) and high glucose group (CCT2siRNA *p*H) of siRNA as compared to control group (TR *p*N). *Denotes $P < 0.05$ versus group TR *p*N. Under the same conditions, changes of the F/G-actin ratio were investigated with four subtly designated groups. As representative plot (b1) shows the expression of F- and G-actins responding to the four conditions, and plot (b2) shows the ratios of F-actin and G-actin expressed in the four conditions. Compared to control group CsiRNA *p*N, the decrease of F/G-actin ratio was observed for all experimental groups $^*P < 0.05$.

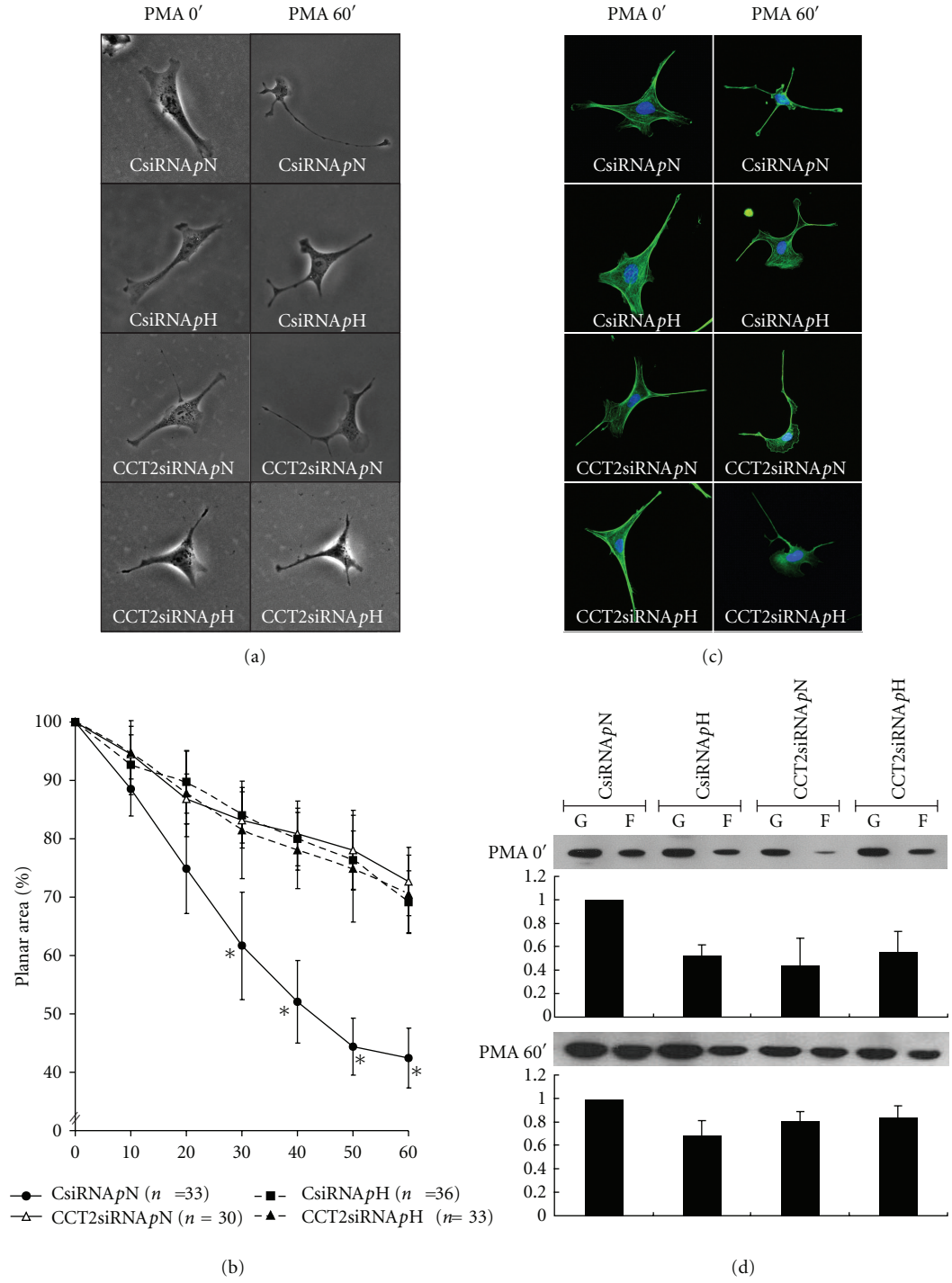

Figure 3: Change of cell contraction, F/G-actin ratio, and arrangement of F-actin in the CCT2 cell model with and without glucose stimulation. Four designated conditions varying in culture medium containing CCT2 or scramble siRNA were used to study the changes of the planar areas of mMC in response to 1 μM PMA stimulation. Representative plots show (a) the morphological changes of mMC (magnification 250x) before (0 min) and after (60 min) PMA treatment and (b) the degrees of cell contraction recorded at 10-min intervals. As group CsiRNA *p*N exhibited the highest contractility with 40–50% planar area reduction at 60 min, the other groups disclosed lower contractility 30 min after PMA stimulation, whereas *n* denotes the number of cells and $*$ represents $P < 0.05$ versus CsiRNA *p*N. Changes in actin were studied in the same conditions. Representative pictures show (c) the immunohistochemical blotting of actin in mMC with highlighting F-actin (green) and nuclei (blue) and (d) the change of F/G-actin ratio before and after PMA stimulation, in which a similar pattern persisted for the four groups.

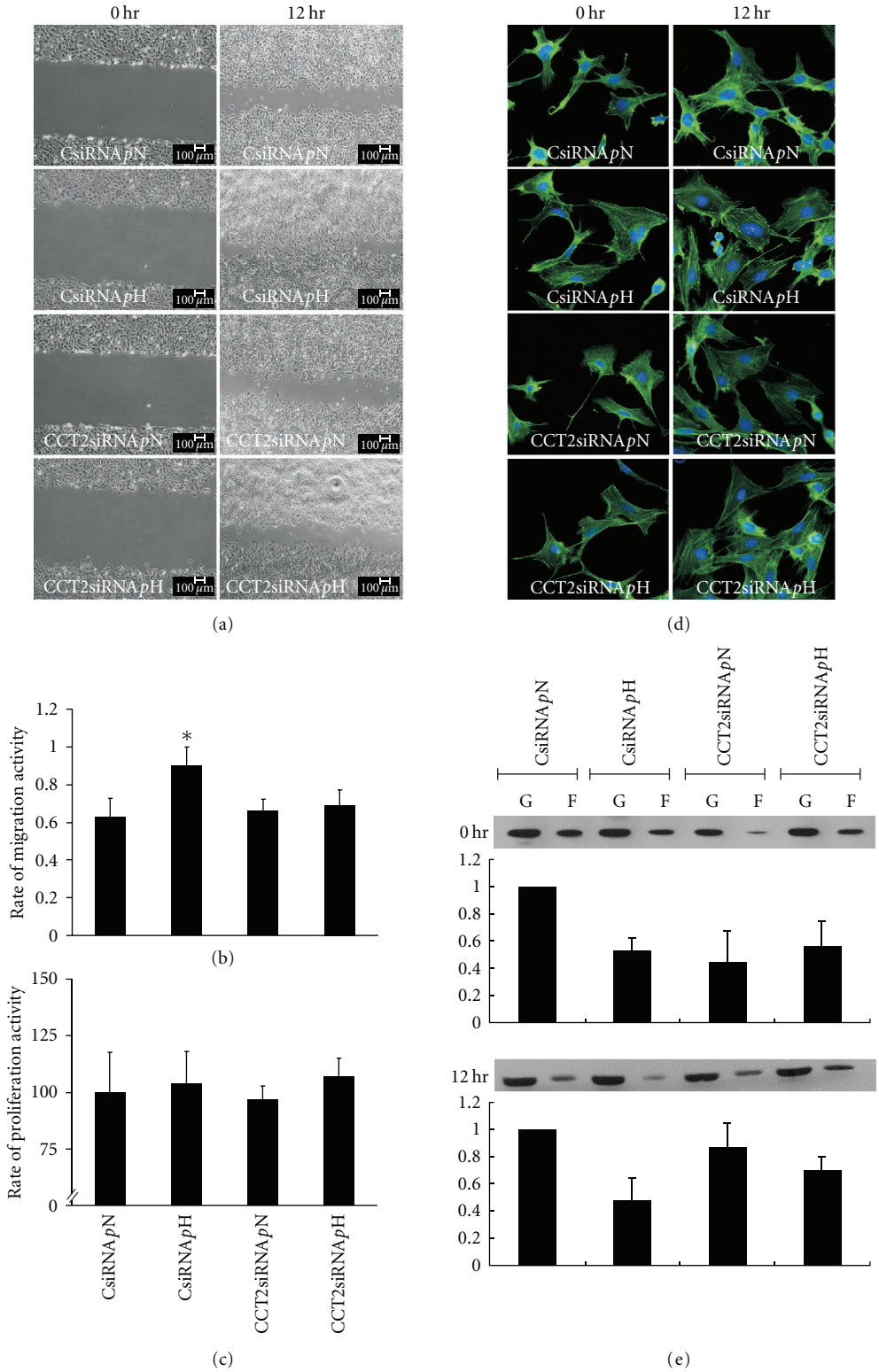

Figure 4: Change of cell proliferation, migration, F/G-actin ratio, and arrangement of F-actin in CCT2 cell model with and without high glucose stimulation. The same four designated groups in previous experiments were studied with high and normal glucose cultured for 24 hrs. Then, wound healing assay was performed to evaluate mMC migration. Representative pictures show (a) the images of mMC migration to close the wound, (b) the rate of migration, and (c) the rate of proliferation during the 12 hr testing interval. Group CsiRNA *p*H exhibited the highest migration activity among these groups. These experiments were performed in triplicate, and the rate of migration activity was calculated as % = (area at 0 hr–area at 12 hr)/area at 0 hr. Changes in actin were also studied. Representative pictures show (d) the immunohistochemical blotting of actin with highlighting F-actin (green) and nuclei (blue) and (e) the change of F/G-actin ratio during the migration testing interval. As seen, a similar pattern of no significant change was observed across the four groups.

be blocked by CCT2siRNA, but is not related to the change of F-actin.

4. Discussion

In our study, a low CCT2 cell model was established, and we found that the ideal ratio of TR-to-siRNA was 6 : 2 for blocking CCT2 in mMC. Second, a novel finding was that mMCs treated by H glucose or with low CCT2 showed less cell contraction induced by PMA, which mechanism involves decreasing F-actin. Third, H glucose activated mMC proliferation and enhanced the ability of mMC migration, which can be blocked by CCT2siRNA, but is not related to the change of F-actin. In summary, CCT2 is a regulator of mMC functions, which mechanisms may involve F-actin, particularly for cell contraction.

A new low CCT2 mMC model is established by siRNA in our study. The method of siRNA has been used to silence gene expression for studying gene function in cultured cells since 1998 [19]. However, the knockdown efficiency is dependent on the cell line, culture condition, amount of transfection reagent, siRNA quantity and quality, and exposure time of transfection agent to cell. Determination of the transfection efficiency is recommended when using a new cell line. In our study, the ratio of 2 : 6 may not completely block the function of CCT2, but it appears to be adequate for downregulating CCT2 in mMC.

The relationship between CCT2 and kidney cells has never been studied in the past; our findings firstly demonstrate that CCT2 is a regulator of mMC functions affected by glucose. CCT is a mammalian cytosolic chaperonin required for proper folding of proteins involved in cytoskeletal formation and contractility activity [20–22]. CCT has eight subunits, but the subunits of CCT differ appreciably among cell types [23], and the levels of different CCT subunits may have distinct expression in response to different stresses [24, 25]. Of eight subunits, CCT2 was assigned in this study based on our previous study [13]. We hypothesized that CCT2 would play a major role regulating mMC functions affected by glucose, also taking into account previous findings that CCT2 is related to regulation of cell cycle, neuronal differentiation, and stimulation of chemical stress [23]. The novel finding of our study is that blocking CCT2 in mMC may inhibit cell proliferation, migration, and contraction induced by H glucose stress.

The relationship between CCT and cell cytoskeleton has been proved by considerable evidence, but there has been no study focusing on CCT or CCT2 and kidney cells. Actin regulating cell movement and division [26] has been known to fold by CCT for 20 years [23, 27–29]. Evidence suggests that disruptions to actin dynamics can lead to changes in cell morphology and cytoskeletal assembly [26, 30]. Of CCT's 8 subunits, individual CCT subunits play a role in maintaining the normal function of actin [26]. Based on our data, knockdown of the expression of CCT2 in mMCs can lead to the significant decrease of F-actin, resulting in the alteration of cell contraction induced by PMA.

Accordingly, attenuating the expression of CCT2 by siRNA in mMC has a similar effect as that of mMCs treated by H glucose, showing a decrease in F/G actin ratio and the lessening of PMA-induced cell contraction. It is known that cell contraction is modulated by actin polymerization, which involves a number of signal transduction pathways [6]. In this regard, the relationship among H glucose, F-actin, and mMC contraction may be rationalized as follows. In 1998, Zhou et al. have suggested that high glucose alters actin assembly in mesangial cell *in vivo* and *in vitro*, which could account for lack of response to vasopressor agents [31]. Our data was consistent with this observation in mMC. Next, in terms of the relationship among CCT2, F-actin, and mesangial cells, Brackley et al. demonstrated that individual CCT plays a role in the expression of F-actin and then influences cytoskeletal organization [32, 33]. Also, they suggest that CCT monomers have a function independent of the CCT oligomer to indicate that some CCT subunits may carry some forms of chaperone activity. In our study, the real role of CCT2 played by monomers or oligomers was not investigated, but available data strongly suggested that, in mMCs, attenuating the expression of CCT2 could lead to the decrease of F/G actin ratio, subsequently lessening the PMA-induced cell contraction. Furthermore, it was not observed that H glucose plus CCT2 siRNA has a synergetic effect on decreasing cell contraction and F/G actin ratio. As the observation is hard to prove with our data, our hypothesis is that both factors may share the same pathway to influence cell contraction.

In the study, another novel finding is that CCT2siRNA lessens proliferation and migration in H glucose-induced mMCs. In terms of CCT and cell proliferation, Grantham et al. have demonstrated that siRNA-treated human cell lines with a reduced CCT level show growth arrest [33], and the degree of growth arrest highly correlates with the extent of CCT depletion. However, which CCT subunits respond to cell proliferation is not elucidated. In this regard, in our study, the role of CCT2 in the proliferation of mMC was established. On the other hand, regarding CCT and cell migration, although Satish et al. suggest that downregulating CCT6 blocks the migration of fibroblast induced by epidermal growth factor and platelet derived growth factor [34], our findings show that downregulating CCT2 blocks H glucose-induced mMC migration. Nevertheless, this finding is different from that of Satish et al., suggesting the chaperone activity of individual CCT monomers is not conserved over different cell lines.

In conclusion, the reduction of CCT2 with siRNA significantly reduced the contraction, proliferation, and migration of mMC, involving the alteration of F-actin, particularly in contraction. CCT2 appears to have a significant biological effect on the process of cellular motion.

Acknowledgments

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Review Article

Role of T Cells in Type 2 Diabetic Nephropathy

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Type 2 diabetic nephropathy (DN) is the most common cause of end-stage renal disease and is increasingly considered as an inflammatory disease characterized by leukocyte infiltration at every stage of renal involvement. Inflammation and activation of the immune system are closely involved in the pathogenesis of diabetes and its microvascular complications. Macrophage has been well recognized to play an important role in type 2 DN, leukocyte infiltration, and participated in process of DN, as was proposed recently. Th1, Th2, Th17, T reg, and cytotoxic T cells are involved in the development and progression of DN. The purpose of this review is to assemble current information concerning the role of T cells in the development and progression of type 2 DN. Specific emphasis is placed on the potential interaction and contribution of the T cells to renal damage. The therapeutic strategies involving T cells in the treatment of type 2 DN are also reviewed. Improving knowledge of the recognition of T cells as significant pathogenic mediators in DN reinforces the possibility of new potential therapeutic targets translated into future clinical treatments.

1. Introduction

Diabetes mellitus (DM) is a complex syndrome characterized by absolute or relative insulin deficiency leading to hyperglycemia and an altered metabolism of glucose, fat, and protein. These metabolic dysfunctions are pathologically associated with specific microvascular diseases and various characteristic long-term complications, including diabetic neuropathy, nephropathy, and retinopathy. Diabetic nephropathy (DN), affecting more than one third of patients with type 1 DM and up to 25% of all patients with type 2 DM, is an extremely common complication of DM that profoundly contributes to patient morbidity and mortality [1–4]. Diabetic nephropathy is a leading cause of chronic kidney disease, resulting in end-stage renal disease (ESRD) which has became a major problem facing human health worldwide [1–4]. Rapi dly increasing rates of DM with profound consequences of DN are the primary reason for this worldwide increase. Diabetic nephropathy (DN) is characterized as pathological findings of hypertrophy of glomerular structures, thickening of the basement membrane, and accumulation of extracellular matrix (ECM) components. Multiple mechanisms contribute to the development and outcomes of DN, such as an interaction between metabolic abnormalities, hemodynamic changes, genetic predisposition and inflammatory milieu, and oxidative stress, constituting a continuous perpetuation of injury factors for the initiation and progression of both of DM and DN [5]. Traditionally, metabolic and hemodynamic factors are the main causes of renal lesions in patients with type 2 DM and DN, both considered nonimmune diseases [6–8]. However, recent studies have shown that chronic inflammation is associated with the development and progression of type 2 DM, implying that immunologic and inflammatory mechanisms may play a pivotal role in the disease process [9–11]. Furthermore, increased infiltration of monocytes/macrophages and activated T lymphocytes, as well as augmented expression of inflammatory cytokines in the kidneys have also been found in patients with DN [9–11]. Serial research has demonstrated that DN is a metabolic and hemodynamic disorder, with inflammation playing a vital role in the process [12, 13]. Type 1 DM is an autoimmune disease, and the role of T cell has been well recognized in the

disease process. However, the role of T cells in type 2 DM is still debated. Several animal models are ideal for type 2 DM and DN that are extremely similar to that of humans and provide the tools necessary to investigate associated mechanisms [14–16]. The purpose of this review is to assemble current information concerning the role of T cells in the development and progression of type 2 DN, as evidenced from animal models related to human disease. Specific emphasis is placed on the potential interaction and contribution of the T cells to kidney damage. In addition, we also review the therapeutic strategies involving T cells in the treatment of type 2 DN.

2. T Cells, Metabolic Syndrome, and Type 2 DM

2.1. Adaptive T Cell Immune Response. The immune system is composed of innate and adaptive immunity. Innate immune system activation associated with chronic inflammation has been revealed to participate in the pathogenesis of type 2 diabetes and its complications [11]. It is widely recognized that adaptive immunity $CD4^+$ T cells can be differentiated into T-helper 1 (Th1), Th2, Th17, and Treg according to their cytokine profiles [17, 18]. Th1 cells produce large quantities of interferon-γ (IFN-γ), induce delayed hypersensitivity reactions, activate macrophages, and promote cell-mediated immunity. Th2 cells produce mainly interleukin-4 (IL-4), induce IgE production, suppress Th1 cell activation, and contribute to humoral immunity [17, 18]. Th17, the recently discovered $CD4^+$ effector T cell lineage distinct from Th1 and Th2, is a third distinct subset of T helper cells preferentially producing interleukin-17, but not IFN-γ or IL-4. We now have three types of effector helper T cells: Th1, Th2, and Th17 which are regulated reciprocally to maintain a balance in immune-mediated disease [19]. Regulatory T cells control adaptive immune responses by suppressing T cells, NK cells, NKT cells, B cells, and dendritic cells [20]. $CD8^+$ T cells recognize antigen in association with MHC class I molecules and are predominantly cytotoxic. Cytotoxic T cells use various mechanisms to kill their targets, including direct cell-cell signaling via surface molecules and indirect signaling via cytokines.

2.2. Type 1 versus Type 2 DM. Type 1 diabetes is an organ-specific autoimmune disease characterized by a progressive cell-mediated destruction of pancreatic beta cells, leading to an absolute deficiency of insulin. Both activation of the T-cell-mediated immune system leading to insulitis and humoral B cell response producing immunoglobulins against beta cell autoantigens participate in the pathogenesis of type 1 DM [21, 22]. Developing a more aggressive T-cell phenotype and changing the Th1-to-Th2 balance towards a more proinflammatory milieu (Th1 dominant) may be associated with the progression towards overt diabetes. Furthermore, evidence demonstrating the association of the Th17 subset with pathogenesis of type 1 diabetes is rapidly accumulating [23–25]. By contrast, type 2 diabetes is a nonautoimmune form of diabetes characterized by insulin resistance and relative (rather than absolute) insulin deficiency. At present, however, little is known about the role of T cells in the process of insulin resistance, metabolic syndrome, or type 2 DM [26].

2.3. T Cells in Insulin Resistance, Metabolic Syndrome, and Type 2 DM. Adipose tissue inflammation is now recognized as a crucial process leading to the metabolic syndrome, diabetes and atherosclerotic cardiovascular disease [27–29]. However, how adipose inflammation is initiated and maintained is still unclear. Macrophage infiltration of adipose tissue has been described in both animal models and human diseases [27]. Accumulation of other immune cells, such as T cells, has been observed in obese adipose tissue recently [30–32]. T lymphocytes are known to interact with macrophages and regulate the inflammatory cascade [33]. Nishimura et al. performed studies to investigate the functional role of T lymphocytes in adipose inflammation [34]. In mice fed a high-fat diet, larger numbers of $CD8^+$ effector T cells infiltrated obese epididymal adipose tissue, whereas the numbers of $CD4^+$ helper and regulatory T cells were diminished. The phenomenon of infiltration by $CD8^+$ T cells precede macrophage accumulation, and once $CD8^+$ T cells were depleted using specific antibodies, macrophage infiltration, adipose tissue inflammation, and systemic insulin resistance were ameliorated [34]. Nishimura et al. also found that obese adipose tissue activates $CD8^+$ T cells, which then promote the recruitment and activation of macrophages in this tissue [34]. These findings, indicating that systemic insulin resistance is ameliorated by CD8 depletion and aggravated by adoptive transfer of $CD8^+$ cells, strongly suggest that $CD8^+$ T cells play essential roles in the initiation and maintenance of adipose tissue inflammation and systemic insulin resistance [34].

Accumulation of $CD8^+$ T cells in obese epididymal fat pads was not accompanied by the presence of greater numbers of $CD8^+$ T cells in the systemic circulation, suggesting that $CD8^+$ T cells are activated by endogenous stimuli localized in the adipose tissue [34]. Coincubation with $CD8^+$ T cells plus lean adipose tissue may induce macrophage differentiation, suggesting that the interactions among $CD8^+$ T cells, macrophages, and adipose tissue may activate and propagate a local adipose inflammatory cascade [34]. In contrast to $CD8^+$ T cells, numerous $CD4^+$ T cells and regulatory T cells were lower. The predominant T-cell effect on glucose homeostasis, revealed by conducting $CD4^+$ T-cell reconstitution studies in lymphocyte-free mice, was the improvement of glucose tolerance, enhanced insulin sensitivity, and lessening of weight gain [35]. Regulatory T cells and subsets of $CD4^+$ Th2 cells are known to secrete anti-inflammatory cytokines that can inhibit macrophage recruitment and activation [36]. Whether the reducing numbers of $CD4^+$ and regulatory T cells augment the inflammatory response during the inflammatory cascades in obese adipose tissue requires further study to elucidate the detail mechanisms [34].

2.4. Effects of Hyperglycemia and Type 2 DM on T Cells. Elements of DM can directly or indirectly activate T cells.

High-glucose concentrations may induce macrophage production of IL-12, which can stimulate CD4 cell production of IFN-γ [37]. By contrast, hyperglycemia may activate nuclear factor kB (NF-kB) through PKC and reactive oxygen species to rapidly stimulate the expression of cytokines [38, 39]. T lymphocytes from patients with diabetes which have an activated phenotype and TNF-α-expressing Th1 cells are prevalently detected [40–42]. The expression of IL-1, TNF-α, and macrophage migration inhibitory factor (MIF) is markedly upregulated in the injured kidney [43, 44]. Furthermore, longer disease duration results in increased advanced glycosylation end (AGE) products and AGE-modified proteins, which could bind to the receptor for AGE on macrophages and T cells, stimulating synthesis and release of proinflammatory cytokines in DM [45–47]. IFN-γ secretion by T cells can initiate and induce further inflammation and oxidative stress within renal tissues [47]. Advanced glycosylation end (AGE) induces synthesis of IFN-γ that further accelerates the inflammation by the activation of macrophages and vascular cells with renal tissues [42, 47].

3. Leukocyte Recruitment and Renal Injury in DN

Although DN has not been considered an inflammatory disease in the past and although metabolic or hemodynamic factors are the major causes contributing to DN, recent studies have suggested that DN is an inflammatory process, and immune cells might be involved in its development and progression [13, 48]. Diabetic nephropathy (DN) is an inflammatory disease with prominent leucocytes infiltratiing the kidneys. Most research has focused on the contribution of macrophages because they are the foremost infiltrating immune cells in diabetic kidneys [43, 49]. Inflammation induced by macrophages may constitute important mechanisms in the progression of DN [43, 49, 50]. The importance of macrophages in diabetic renal injury has been clearly demonstrated; however, little is known about the role of lymphocytes. The levels of circulating activated lymphocytes were higher in type 1 diabetic patients with proteinuria than those in nonproteinuria patients [40, 51]. This suggests that activated T lymphocytes may also be associated with the development of type 1 DN. Whether T cells are associated with the development of type 2 DN is still unknown.

3.1. Lymphocyte Recruitment in Diabetic Kidney. In patients with type 1 diabetes, T-cell influx and accumulation in the juxtaglomerular apparatus are the factors that exacerbate diabetes and correlate with glomerular filtration surface and albumin excretion rate [51]. Previous investigations have also shown folds increasing in glomerular and interstitial $CD4^+$ and $CD8^+$ T cells, as well as in interstitial $FOXP3^+$ regulatory T cells in diabetic compared with non-diabetic wild-type mice [40, 51]. The development of early diabetic renal injury is associated with significant lymphocyte infiltration. There is no doubt that immune cells participate in the renal injury under the conditions of DN, and their migration into the kidney is a crucial step in the progression of this disease. Although the detailed mechanisms of leukocyte migration into renal tissues in DN are not completely understood nor is the functional role of T cells within this compartment, it has been reported that adhesion molecules and the chemokines are involved in this recruitment [42, 44, 52].

Recruitment of leukocyte is a key event in the disease progression of DN. Previous studies have demonstrated that mice deficient in intercellular adhesion molecule-1 (ICAM-1) may cause defects in macrophages and leukocytes homing into renal tissues, resulting in substantial reduction of renal injury [53]. The $CD4^+$ T cells homing into glomeruli of diabetic kidney were decreased in ICAM-1-deficient-db/db mice, as compared with those of db/db mice [53]. Because naive and effector T cells constitutively express LFA-1, and ICAM-1 expression is found on renal endothelial, epithelial, and mesangial cells, it is likely that this interaction will plays a significant role during T-cell migration into the kidney [54–56]. Chemotactic cytokines are also major factors that induce the recruitment of inflammatory cells into the kidney, subsequently amplifying the immune-mediated damage [57]. Once macrophage is infiltrated within the diabetic kidney, the macrophages and macrophage-derived products can induce further inflammation [43]. Monocyte chemoattractant protein-1 (MCP-1), an important chemokine regulating macrophage recruitment, is upregulated in patients with DN [58]. Moreover, constitutive RANTES expression directs subset-specific homing of $CD4^+$ T cells in the kidney [59]. The role of RANTES in directing T lymphocyte homing into the diabetic kidney is not yet clear.

Compared to Type 2 diabetic patients without DN, significantly lower plasma concentrations of sCTLA-4 and higher concentrations of sCD28 were noted in Type 2 diabetic patients with DN [60]. Furthermore, plasma sCD28 and sCD80 were found to be positively correlated with the fasting urine albumin, creatinine ratio in DN patients. The disease severity of DN related with elevated soluble adhesion molecule vascular cell adhesion molecule-1 and P-selectin were also found [60]. Costimulatory molecules, together with leukocyte adhesion molecules, are crucial for T lymphocyte and leukocyte-mediated inflammatory responses. The aberrant expression of soluble costimulatory molecules and adhesion molecules may be related to the activation of T cells and leukocytes in the progression of inflammation in type 2 DN [60].

3.2. Leukocytes and Diabetic Nephropathy: Cause or Consequence? Increased infiltration of monocytes/macrophages and activated T lymphocytes, as well as augmented expression of inflammatory cytokines in the kidneys, have been found in patients with DN [9–11]. Longer disease duration of DM results in an increase of advanced glycosylation end (AGE) products and AGE-modified proteins that may bind to leukocytes, stimulating the synthesis and release of proinflammatory cytokines in DM [45, 46]. By contrast, an activated renin-angiotensin-aldosterone system (RAAS) and endothelial dysfunction, well noted in patients with DM, have also been proven to be a crucial determinants of leukocyte activation and cytokine expression in generating proinflammatory and proliferative effects [61–63]. Thus, it is highly possible that metabolic or hemodynamic factors in DN

may trigger the immune-mediated inflammatory responses and cytokine production. Furthermore, prominent leukocyte infiltration in models of remnant kidneys or unilateral ureter obstruction, models considered to be nonimmunologically mediated, was also illustrated. Hence, the accumulation of leukocyte in the kidneys of DN can be the results of lesions from high glucose or glomerular hyperperfusion (secondary to hyperglycemia or hypertension), rather than the cause of DN. Therefore, leukocytes may be either the cause or the consequence of DN.

3.3. Interaction and Mechanisms of T-Cell-Mediated Renal Damage. The scale of $CD4^+$ and $CD8^+$ T cells accumulated in the diabetic kidneys was much smaller than that of macrophages in rodent models of both Type 1 and Type 2 DN [43, 64], suggesting that T cells may interact with macrophages to regulate inflammation and renal injury. Activated T cells can cause injury directly through cytotoxic effects and indirectly by recruiting and activating macrophages. Proinflammatory cytokines secreted by T ($CD4^+$, $CD8^+$) cells could activate neighboring macrophages directly or by stimulating mesangial cell production of colony stimulating factor-1 and MCP-1 indirectly [65]. Once macrophages have activated, they can release nitric oxide, reactive oxygen species, IL-1, TNF-α, complement factors, and metalloproteinases, all of which promote renal injury [49, 50]. T cells express the receptor for AGEs and can respond to AGEs [47]. The activation of $CD4^+$ and $CD8^+$ T cells by AGE can initiate IFN-γ secretion by T cells [47], which could induce further inflammation and oxidative stress within the diabetic kidney. In addition, $CD8^+$ cells may perform a cytolytic function in the diabetic kidney. All of these cytokines and molecules promote inflammation and induce further expression of macrophage colony-stimulating factor and ICAM-1 in renal cells, further contributing to renal injury [48, 66].

Nevertheless, infiltrating macrophages and T lymphocytes are the most probable sources of many cytokines mediating the renal injury in DN and its progression. The intrinsic renal cells (endothelial, mesangial, glomerular, and tubular epithelial cells) are able to synthesize many pro-inflammatory cytokines [67, 68]. At high glucose levels, podocytes are considered the major sources of IL-1α and IL-1β, and they may also produce MCP-1 [69, 70]. Increased secretion of TGF-β by peripheral blood mononuclear cells has been reported in patients with DN and seems to be responsible for fibrogenic and proliferative effects on fibroblasts [71–73]. Furthermore, the TGF-β is also a crucial pleiotropic cytokine associated with the development of Tregs and Th17 cells [19]. Collectively, induction of proinflammatory and profibrogenic molecules influences renal damage in diabetes.

The aberrant production of inflammatory cytokines and chemokines, as well as differential activation of MAPK in different leucocytes (T helper (Th) cells and monocytes), are the underlying immunopathological mechanisms of type 2 DM patients with DN [74]. An increasing body of evidence indicates that immigrated blood leukocytes might considerably alter the phenotype of endothelial cells and increase inflammation of the vascular bed [75]. Endothelial dysfunction is associated with most forms of cardiovascular disease, such as coronary artery diseases, chronic renal failure, and diabetes [76]. Interaction of renal tissue macrophages and T cells produces various reactive oxygen species, proinflammatory cytokines, metalloproteinases, and growth factors, which modulate the local response and increase inflammation within the diabetic kidney [5, 8, 77, 78].

4. Roles of T Cells in Type 2 Diabetic Nephropathy

4.1. Th1 Cells. The circulating lymphocytes trafficking through tissues may interact with tissue AGEs. Exposure of activated T lymphocytes to AGE may enhance the expression of interferon gamma (IFN-γ) indicating that the T cell AGE-receptor system might be linked to lymphokine production involving in renal damage. Under conditions of excessive AGE-protein and AGE lipid accumulation (e.g., aging and diabetes), enhanced production of AGE-induced IFN-γ may accelerate immune responses that contribute to tissue injury.

It is well recognized that T helper-1 (Th1) response precedes and accompanies type 1 diabetes [21]; hence, it is possible that Th1 cells are prevalent in type 1 diabetic kidney. Elevated levels of ICAM-1 and P-selectin within the diabetic kidney, combined with increased levels of IFN-γ and MIF, were associated with the homing of effector Th1 cells in glomeruli [79]. However, little is known about the mechanisms of Th1 cell migration in the type 2 DN model during the development and progression of kidney diseases. Higher serum IFN-γ levels, a Th1 cytokine, and positive correlations between plasma IFN-γ, proteinuria, and estimate glomerular filtration rate (eGFR) were found in type 2 diabetic patients with overt nephropathy [80]. Plasma IL-2R levels found in type 2 DM patients with overt DN were higher than those without overt nephropathy. Furthermore, a significantly positive correlation was determined between plasma IL-2R and proteinuria [80]. These results indicate that Th1 cellular immunity in conjunction with Th1 and proinflammatory cytokines may mediate tissue injury in patients with DN [80].

4.2. Th2 Cells. Th2 cells, producing IL-4 cytokines, can contribute to humoral immunity, suppress Th1 cell activation, and function as an inhibitory cytokine of autoimmunity and inflammations [17]. No significant change of serum IL-4 level in type 2 DN patients, as compared to those without nephropathy [80]. Specific polymorphisms within the IL4R locus and by specific genotypes at the IL4R, IL4, and IL13 loci were strongly associated with susceptibility to type 1 DM [81]. Furthermore, an association of interleukin (IL)-4 intron-3 polymorphism with susceptibility to end-stage renal disease was discovered [82]. However, the role of IL-4 gene polymorphisms in type 2 diabetic nephropathy still requires further evaluation.

IL-10, another important Th2 cytokine, exerts predominantly anti-inflammatory and immunosuppressive effects [83]. Low production capacity of IL-10 associated with the metabolic syndrome and type 2 DM [84]. Some studies have revealed elevated IL-10 levels in the sera of diabetic patients

with nephropathy, and a positive correlation between IL-10 levels and albuminuria has been suggested to participate in the DN pathogenesis [85–87]. IL-10 promoter variants and haplotypes (GTA and GTC) have predictive value in determining the susceptibility to nephropathy in Tunisian T2DM patients [88, 89].

4.3. Th17 Cells. Th17 is a third distinct subset of T helper cells and have been found to play vital roles in the pathogenesis of several autoimmune diseases such as multiple sclerosis and rheumatoid arthritis [19]. Increasing evidence demonstrates that the Th17 cell in type 1 DM in murine model and human type 1 DM [23–25]. Recently, T cell in type 2 DM patients has been revealed to be skewed toward a proinflammatory phenotype, requiring monocytes for maintenance and promoting chronic inflammation through elevated IFN-γ and IL-17 production [90]. However, IL-17A cannot be concluded to be associated with nephropathic complications of type 2 DM, due to an increased serum level of IL-17A found in patients with nonnephropathy [91].

4.4. Treg Cells. The expression rate of $CD4^+CD25^+Foxp3^+$ Treg cells between the control group and type 2 diabetic patients yielded no significant difference [92]. In type 2 diabetic patients with microalbuminuria and macroalbuminuria, the expression of $CD4^+CD25^+Foxp3^+$ Treg cells was significantly lowered, as compared with that of the control group, and patients with macroalbuminuria showed significantly lower expression of $CD4^+CD25^+Foxp3^+$ Treg cells than did the microalbuminuric patients. Significant inverse correlations were noted between the disease course and the expression of $CD4^+CD25^+Foxp3^+$ Treg cells between the urinary albumin excretion rate (UAER) and the expression of $CD4^+$ $CD25^+Foxp3^+$ Treg cells. Whether the $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) play role in type 2 DN still requires further investigation [92].

4.5. Cytotoxic T Cells. Adipose tissues can activate $CD8^+$T cells, which then promote the recruitment and activation of macrophages resulting in the metabolic syndrome, which can be significantly attenuated by a specific CD8 antibody [34]. In the diabetic kidneys of NOD mice, accumulation of $CD8^+$ cells is associated with increased expression of genes encoding perforin and granzyme B, as well as with colocalization in immunostaining for perforin [93]. This suggests that $CD8^+$ cells may perform a cytolytic function in the diabetic kidney. In streptozocin-induced diabetic nephropathy, previous studies have shown that the difference in expression of $CD4^+$ T cells in control and diabetic kidneys is more significant at 1 month than at 8 months, whereas expression of $CD8^+$ T cells is more significant at 8 months. It is speculated that DN is probably initiated and driven by a Th1 process. The function of $CD8^+$ T cells, however, becomes more significant at later stages of the disease when tissue loss is evident [94].

5. Type 2 DN and Therapeutic Strategies Involving T Cells

Due to the pathogenic complexity of DN, protecting diabetic patients from the development and progression of renal injury remains a challenge for physicians. The accumulation of inflammatory cells in renal biopsies of diabetic patients is associated with tissue damage and a progressive decline in renal function [95]. In animal models, the use of immunosuppressants, neutralizing antibodies, and genetic deficiencies has shown that reducing leucocyte accumulation and activation in diabetic kidneys suppresses the development of renal injury [96]. Many previous studies have used anti-inflammatory strategies that also suppress accumulation of lymphocytes in diabetic kidneys, indicating that lymphocytes may also contribute to disease progression. Inflammation plays an important role in the pathogenesis of proteinuria in DN. Inhibiting renal macrophage recruitment by immunosuppressant therapy, or modulating of MCP1 or ICAM-1 expression in diabetic mice, demonstrate antiproteinuric and renoprotective effects [13, 69, 97–99].

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that plays a pivotal role in mediating cell size and mass, proliferation, and survival [100]. High activation of mTOR within the kidney has been reported to occur in DN [101, 102]. Systemic administration of rapamycin, a specific and potent inhibitor of mTOR, markedly ameliorated pathological changes and renal dysfunctions in *db/db* mice [102]. Rapamycin markedly inhibited the influx of inflammatory cells, predominantly lymphocytes, and macrophages, associated with DN [100–104]. This effect is likely attributable to rapamycin-induced inhibition of the proliferation and clonal expansion of B and T lymphocytes [103–105]. Within the kidney, rapamycin also ameliorates the release of proinflammatory cytokines and chemokines, such as monocyte chemoattractant protein-1, RANTES, IL-8, and fractalkine, exacerbating the inflammatory process in DN [103–105]. These results indicate that mTOR activation plays a pivotal role in the development of DN and that rapamycin could be as a strong therapeutic potential agent for DN.

Depletion of $CD8^+$ T cells using specific antibodies could ameliorate macrophage infiltration, adipose tissue inflammation, and systemic insulin resistance. Whether depleting $CD8^+$ T cells could be applied in treatment of DN is still undetermined. Anti-inflammatory or immunosuppressive treatment has been applied to treat DN in animal models [106–109]; however, therapeutic approaches targeting T cells are still limited.

6. Conclusion

Beyond traditional metabolic and hemodynamic risk factors, type 2 DN is now increasingly considered as an inflammatory disease. The inflammatory process is not only due to an innate immune response dominated by macrophage-mediated effects, but also by the adaptive immune response mediated by leukocytes. T cells participate in the development of type 2 DN from processes of insulin resistance, metabolic syndrome, and type 2 DM into type 2 DN. The recruitment of leukocyte is a key event in the disease progression of DN. Diverse immune cells and cytokines exert important roles in the pathogenic complexity of development and progression during the DN process. Interaction of renal tissue

macrophages and T cells produces various reactive oxygen species, proinflammatory cytokines, metalloproteinases, and growth factors, which modulate the local response and increase inflammation within the diabetic kidney. A better understanding of the role of T cells in the context of DN will create several new opportunities for therapeutic intervention that may benefit patients with DN.

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Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Targeted Proteomics of Isolated Glomeruli from the Kidneys of Diabetic Rats: Sorbin and SH3 Domain Containing 2 Is a Novel Protein Associated with Diabetic Nephropathy

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To evaluate proteins associated with the development of diabetic nephropathy, a major cause of the end-stage renal disease, we analyzed protein expression in isolated glomeruli from spontaneous type 2 diabetic (OLETF) rats and their age-matched control littermates (LETO) in the early and proteinuric stages of diabetic nephropathy using QSTAR Elite LC-MS/MS. Among the 191 and 218 proteins that were altered significantly in the OLETF rats, twenty-four were actin cytoskeleton-associated proteins implicated in the formation of stress fibers, and the impairment of actin polymerization, intermediate filaments and microtubules. Importantly, sorbin and SH3 domain containing 2 (SORBS2), which is involved in the formation of stress fibers, was significantly upregulated in both stages of diabetic nephropathy (1.49- and 1.97-fold, resp.). Immunohistochemical and quantitative-PCR analyses revealed upregulation of SORBS2 in podocytes of glomeruli of OLETF rats. Our findings suggested that SORBS2 may be associated with the development of diabetic nephropathy possibility by reorganization of actin filaments.

1. Introduction

Diabetes mellitus accounts for more cases of end-stage renal disease than any other cause of chronic kidney disease [1]. While glomerular hypertrophy, mesangial matrix expansion, and glomerular basement membrane (GBM) thickening are the classical hallmarks of diabetic glomerular lesions, studies of diabetic patients and animal models have revealed that the onset of proteinuria is most closely associated with podocytopathies, such as podocyte apoptosis, hypertrophy, detachment from the GBM, and foot process effacement [2]. Indeed, diabetic nephropathy is now recognized as one of the major podocyte-associated diseases [3]. The podocyte is an excellent model system for studying actin cytoskeleton dynamics in a physiological context because changes in actin dynamics transfer directly into changes of kidney function [4]. Previous investigations have shown that the cytoskeleton on the GBM side in podocytes during foot process effacement is comprised of highly-ordered, actin-based bundles that run parallel to the longitudinal axis of the foot processes [4] and that actin fibers gather to form the stress fibers [4, 5]. Therefore, reorganization of the actin filaments is indispensable for foot process effacement.

Sorbin and SH3 domain containing 2 (SORBS2), alpha-actinin 1 (ACTN1), alpha-actinin 4 (ACTN4) and Rho GDP dissociation inhibitor alpha (ARHDGDIA) are proteins associated with stress fiber formation [6–11]. The relationship between these proteins and diabetic nephropathy has not been elucidated, although some of these proteins have been reported to be important in stress fiber formation in podocytopathies or proteinuria [4, 8, 9, 12]. The underlying cytoskeletal components that initiate and regulate the dynamic changes of these foot processes remain unclear.

Recently, proteome analysis has increasingly been used in the discovery of disease-specific proteins and biomarkers of kidney diseases [13, 14]. Proteome analysis of diabetic glomeruli from renal biopsy specimens of diabetic patients is difficult, due to the fact that renal biopsy is clinically limited in diabetic patients, and only small (often insufficient) quantities of glomeruli can be obtained from renal biopsy specimens. From these concerns, in order to reveal which proteins are involved in the diabetic glomerular alterations, including podocytopathies, we conducted a proteome analysis of isolated glomeruli from spontaneous type 2 diabetic (Otsuka Long-Evans Tokushima Fatty (OLETF)) rats and their age-matched control littermates (Long-Evans Tokushima Lean (LETO)) rats at 27 (early stage of diabetic nephropathy) and 38 (proteinuric stage of diabetic nephropathy) weeks of age using QSTAR Elite liquid chromatography with tandem mass spectrometry (QSTAR Elite LC-MS/MS) and iTRAQ technology.

2. Materials and Methods

2.1. Animals. All experimental procedures were conducted after obtaining approval of the Animal Care and Use Committee of the Osaka City University Medical School and in accordance with the Guide for Laboratory Animals. OLETF and LETO rats ($n = 20$, resp.) were provided by Otsuka Pharmacology Co., Ltd. (Tokushima, Japan). The diabetic phenotype of the OLETF rat has been extensively evaluated: (i) 25-week-old rats develop diabetes (hyperglycemia, hyperlipidemia, etc.) at nearly 100% incidence and (ii) 30-week-old rats develop proteinuria [15]. Therefore, we assumed that OLETF rats develop diabetic nephropathy with the early and the proteinuric stages at 27 and 38 weeks of age, respectively. 10 OLETF rats and 10 LETO rats were used for analysis at each time point. All animals were housed individually in each cage in an animal facility maintained on a 12-h (7:00–19:00) light/dark cycle, at a constant temperature of $23 \pm 1^{\circ}$C and relative humidity of $44 \pm 5\%$ for 21 and 32 weeks, respectively, from the start of the experiment and were provided tap water and food (rodent pellet diet MF 348 kcal/100 g, containing 4.2% crude fat; Oriental Yeast Co., Tokyo, Japan) ad libitum.

2.2. Biochemical Characterization. Total cholesterol and creatinine in serum specimens ($n = 10$/group), hemoglobin A1c, fasting plasma glucose concentrations in plasma specimens ($n = 10$/group), and protein and creatinine concentrations in spot urine samples ($n = 5$/group) were measured using an autoanalyser (Mitsubishi Chemical Medience Co., Ltd., Osaka, Japan).

2.3. Histopathological Examination. Renal tissues were fixed in 10% neutral formalin solution, embedded in paraffin, and cut into 3 μm sections using conventional techniques. Sections were stained with hematoxylin and eosin and periodic acid-Schiff (PAS) reagent and examined histopathologically by light microscopy.

2.4. Glomerular Isolation. Rats were anesthetized with intraperitoneal injection of pentobarbital (60 mg/kg) for euthanasia and necropsy. After laparotomy, the kidneys were perfused with ice-cold phosphate-buffered saline (PBS) until they were blanched. Glomeruli were isolated by a sieving technique, as described previously [16, 17]. Isolated glomeruli were collected under an inverted microscope to minimize tubular contamination (less than 5% tubular fragments) and centrifuged at 453 g for 10 min. The pellets were collected and used for proteome analysis.

2.5. Lysis and Digestion, iTRAQ Labeling, and LC-ESI MS/MS Analysis. The lyophilized samples were dissolved in 1000 μL tissue protein extraction reagent lysis buffer (Pierce, IL, USA) with protease inhibitor (p8340, Sigma-Aldrich). The glomerular lysates were ultrasonicated and insoluble material was removed by centrifugation at 13,000 g for 15 min at 10°C. Protein concentrations were quantified using the BCA Protein Assay kit (Pierce, Ill, USA). Protein reduction, alkylation, digestion and subsequent peptide labeling were performed using the AB Sciex iTRAQ Reagent Multi-Plex Kit (AB Sciex, Foster City, Calif, USA) according to the manufacturer's instructions with minor modifications [18, 19]. Briefly, 50 μg samples of protein were incubated at 60°C for 60 min in 20 μL dissolution buffer (0.5 M triethylammonium bicarbonate, 0.2% SDS) with 2 μL reducing reagent (50 mM tris(2-carboxy-ethyl)phosphine). Free cysteine sulfhydryl groups were blocked by incubation with 1 μL cysteine blocking reagent (20 mM methyl methanthiosulfonate) at room temperature for 10 min. Ten μL of trypsin solution (AB Sciex, Foster City, Calif, USA) was added, and each sample was incubated overnight at 37°C. Samples from OLETF and LETO rats were labeled with iTRAQ114 and iTRAQ115, respectively, and then mixed into one tube and fractionated using six concentrations of KCl solutions (10, 50, 70, 100, 200, and 350 mM) on an ICAT cation exchange cartridge (AB Sciex, Foster City, Calif, USA). After desalting and concentrating, peptides in each fraction were quantified by a DiNa-AI nano LC System (KYA Technologies, Tokyo, Japan) coupled to a QSTAR Elite Hybrid MS/MS spectrometer through a Nanospray ion source (AB Sciex, Foster City, Calif, USA), as described previously [19].

2.6. Identification of Proteins by IPA. Protein Pilot 2.0 software with the Paragon Algorithm (AB Sciex, Foster City, Calif, USA) was used for the identification and relative quantification of proteins. Tandem mass spectrometry data were compared against the rat protein database from Swiss-Prot 57.4 (20,400 sequences). We report only protein identifications with >95% statistical confidence in the Protein Pilot 2.0 software.

The Ingenuity Analysis (IPA; Ingenuity Systems, Mountain View, Calif, USA) was utilized to identify networks of interacting proteins, functional groups, and pathways. Information regarding the function and cellular localization of the identified proteins was obtained from IPA.

2.7. Immunohistochemistry for SORBS2. Immunohistochemical staining of the kidney sections was performed according to the avidin-biotin complex method, as described

previously [20], using primary mouse monoclonal anti-rat SORBS2 (clone S5C, Sigma-Aldrich), After deparaffinization with xylene and gradual dehydration, antigen retrieval was undertaken by microwaving in sodium citrate buffer (pH 6) for 25 min and endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 5 min. Sections were incubated with 1.5% normal horse or goat serum in PBS for 15 min and then with diluted primary antibody (1 : 500), overnight at 4°C. Biotinylated horse anti-mouse antibodies (diluted 1 : 200) were applied as the secondary antibodies for 30 min, and the slides were then incubated with the avidin-biotin peroxidase complex for 30 min. The peroxidase reaction was developed using 0.02~0.033% 3,3-diaminobenzidine tetrahydrochloride (DAB) and 0.03% hydrogen peroxide in tris-buffered saline for 1–5 min. Hematoxylin was used for counterstaining.

2.8. Immunofluorescence for SORBS2 and Synaptopodin. Double immunofluorescence of SPRBS2 and synaptopodin, a podocyte marker proteins was performed as previously described [21–23]. Four-μm-thick frozen kidney tissue sections were fixed with ice-cold acetone at −20°C for 5 min, followed by permeabilization with 1% Tween 20 PBS for 5 min at room temperature. After rinsing with 1% Tween 20 PBS, unspecific binding sites were blocked with horse anti-mouse and goat anti-rabbit serum in PBS for at least 30 min. Primary antibodies (prediluted in blocking solution) for SORBS2 (1 : 250) and synaptopodin (clone ab 101883, Abcam) (1 : 300) were applied for 60 min at room temperature, followed by incubation with the secondary antibody fluorescein red-conjugated horse anti-mouse IgG (Alexa Fluor 594) (Life technologies, Calif, USA) and fluorescein green-conjugated goat anti-rabbit IgG (Alexa Fluor 488) (Life technologies, Calif, USA) for 30 minutes at room temperature. Spatial colocalization of SORBS2 immunoreactivity (red fluorescence) with synaptopodin (green fluorescence), resulting in yellow, was obtained by overlaying separately recorded images on a color image. The immunofluorescence was analyzed by confocal microscopy with the help of the Fluoview software (Olympus Optical, Tokyo, Japan).

2.9. Validation of SORBS2 mRNA Expression by Real-Time Quantitative PCR (Q-PCR)

2.9.1. RNA Preparation. Glomeruli from OLETF and LETO rats at the proteinuric stage ($n = 3$, resp.) were laser-microdissected using the ZEISS PALM MB4 Microdissection System (ZEISS, Munich, Germany), according to the manufacturer's instructions. Total RNA was isolated from glomeruli using 4 M guanidine thiocyanate, 25 mM sodium citrate with 0.5% sarkosyl buffer with the phenol-chloroform-isoamyl alcohol extraction method, using glycogen as a carrier, as described previously [24]. Reverse transcription of total RNA was performed with Oligo-dT primer, and cDNA samples were stored at −20°C until assayed.

2.9.2. Real-Time Q-PCR. PCR amplicons were used to confirm SORBS2 gene expression using real-time Q-PCR. Primer sequences were designed with the Primer Express software (Applied Biosystems, USA). The probes and primers were as follows: TaqMan probe and primer set Rn00587190_m1 for SORBS2 (NM_053770.1) and TaqMan probe 5′-TGA GAC CTT CAA CAC CCC AGC CAT G-3′, and primers: forward 5′-TCA AAT AAG CCA CAG CGT C-3′, reverse 5′-AAC CAG CCG TCAT CACA C-3′ for GAPDH, cytoplasmic (NM_ 017008.3). The cDNA generated from each sample was used for Q-PCR according to the manufacturer's instructions, with GAPDH as an internal control.

2.10. Ultrastructural Examination. Separate portions of the kidneys from the OLETF and LETO rats ($n = 5$, resp.) at 38 weeks of age were also prepared for electron microscopy. Specimens were obtained from the renal cortex, fixed in 0.1 M cacodylate buffer solution (pH 7.4) containing 3% glutaraldehyde, and postfixed in the same buffer containing 1% osmium tetroxide at 4°C, as previously described [25]. Seventy nm sections were stained with uranyl acetate and lead citrate for examination using a JEM 1200 EXII electron microscope (JEOL, Tokyo, Japan).

2.11. Statistical Analysis. Statistical calculations were performed using Graph-Pad Prism version 5.0 for Windows (Graphpad Software, San Diego, Calif). For normally distributed data, statistical significance ($P < 0.05$) was evaluated using the unpaired t-test followed by an analysis of variance (F-test). In the case of statistically significant differences regarding variances, the Welch test was used to confirm the differences between groups. For nonparametric testing, the Mann-Whitney U-test was applied. The results are presented as box plots/dot plots. All values are expressed as the means ± SD. For analysis of protein expression, statistical analysis with Protein Pilot 2.0 software was employed.

3. Results

3.1. General Observations. The OLETF rats exhibited polyphagia and obesity from the very early stages of life. At 27 weeks of age (early stage of diabetic nephropathy), the mean body weight of the OLETF rats (642±40.9 g) was significantly higher than that of the LETO rats (491 ± 34.6 g). At 38 weeks of age (proteinuric stage of diabetic nephropathy), the body weights of the LETO rats were increased, although the final values for the OLETF (621 ± 69.4 g) and the LETO (580 ± 80.7 g) rats were not significantly different. At both time points, the kidney-to-body weight ratio of the OLETF rats (27 weeks: 0.703% ± 0.10%, 38 weeks: 0.780% ± 0.12%) was significantly higher than that of the LETO rats (0.597% ± 0.054% and 0.592% ± 0.050%, resp.).

The mean blood glucose, hemoglobin A1c and total cholesterol levels in the OLETF rats (27 weeks: 183 ± 58.9 mg/dL, 4.4 ± 0.85%, 123 ± 23.1 mg/dL, 38 weeks: 248 ± 53.6 mg/dL, 5.7% ± 1.2%, and 153 ± 34.6 mg/dL, resp.) were increased significantly compared with those in the LETO rats (27 weeks: 135 ± 34.2 mg/dL, 3.3% ± 0.11%, 97.4 ± 9.5 mg/dL, 38 weeks: 129 ± 25.3 mg/dL, 3.3% ± 0.18%, and 101 ± 9.37 mg/dL, resp.). The serum creatinine levels were

Table 1: Biological parameters from OLETF and LETO rats at 27 and 38 weeks of age.

	OLETF ($n = 10$)	LETO ($n = 10$)	P value	OLETF ($n = 10$)	LETO ($n = 10$)	P value
	27 weeks			38 weeks		
Body weight (g)	642 ± 40.9	491 ± 34.6	<0.0001	621 ± 69.4	580 ± 80.7	0.2089
Food intake (g/day)	34.9 ± 6.1	19.1 ± 1.5	<0.0001	39.3 ± 6.1	29.7 ± 2.0	<0.0001
Kidney-to-body weight ratio (%)	0.703 ± 0.10	0.597 ± 0.054	0.0091	0.780 ± 0.12	0.592 ± 0.050	0.0008
Fasting plasma glucose (mg/dL)	183 ± 58.9	135 ± 34.2	00388	248 ± 53.6	129 ± 25.3	<0.0001
Hemoglobin A1c (%)	4.4 ± 0.85	3.3 ± 0.11	0.0021	5.7 ± 1.2	3.3 ± 0.18	0.0001
Total cholesterol (mg/dL)	123 ± 23.1	97.4 ± 9.5	0.0081	153 ± 34.6	101 ± 9.37	0.0009
Creatinine (mg/dL)	0.24 ± 0.04	0.36 ± 0.04	<0.0001	0.33 ± 0.05	0.46 ± 0.1	0.0031
Urinary protein-to-creatinine ratio (mg/mg)	2.73 ± 2.10 ($n = 5$)*	0.60 ± 0.08 ($n = 5$)*	<0.0001	5.65 ± 2.36 ($n = 5$)*	0.94 ± 0.38 ($n = 5$)*	<0.0001

Values are expressed as the mean ± SD.
*Urinary specimens were obtained from 5 rats per group to measure urinary protein and creatinine levels.
OLETF: Otsuka Long-Evans Tokushima Fatty, LETO: Long-Evans Tokushima Lean.

significantly higher at both nephropathy stages for LETO rats (27 weeks: 0.36 ± 0.04 mg/dL and 38 weeks: 0.46 ± 0.10 mg/dL) compared with the OLETE rats (0.24 ± 0.04 mg/dL and 0.33 ± 0.05 mg/dL). The urinary protein to creatinine ratio in the OLETF rats was also elevated significantly at both time points examined (27 weeks: 2.73 ± 2.10 mg/mg and 38 weeks: 5.65 ± 2.36 mg/mg) compared with the LETO rats (0.60 ± 0.08 mg/mg and 0.94 ± 0.38 mg/mg) (Table 1).

3.2. Histopathological Examination. In the OLETF rats, histopathological examination demonstrated both focal and segmental glomerular changes. Slight expansion of the mesangial matrix was observed with mesangial cell proliferation at 27 weeks of age (Figure 1(a)). At 38 weeks of age, in addition to the mesangial area, a few glomeruli exhibited segmental lesions with PAS-positive deposits in the mesangium or capillary that resembled the fibrin caps commonly observed in exudative lesions in human diabetic nephropathy (Figure 1(c)). In the LETO rats, there were no obvious histopathological changes at both time points (Figures 1(b) and 1(d)).

3.3. Alterations of Protein Expression in Glomeruli from Diabetic Rats. The results of QSTAR Elite LC-MS/MS and Protein Pilot analyses are summarized in Table 2. Altered expression of 191 (91 up- and 100 downregulated) and 218 (121 up- and 97 downregulated) proteins was observed in isolated glomeruli from OLETF rats at the early and proteinuric stages of diabetic nephropathy, respectively. These proteins were involved in glycolysis, oxidative stress, and podocyte injury, based upon the IPA findings (Figure 2).

Eighty-seven proteins were differentially expressed in isolated glomeruli from OLETF rats compared with those from LETO rats at both stages of diabetic nephropathy. Among these 87 proteins, 24 were involved in actin cytoskeleton reorganization, that is, formation of stress fibers (SORBS2, ACTN1, ACTN4 and ARHGDIA), polymerization of actin filaments (actin-related protein 2/3 complex subunit 1 beta (ARPC1B), actin-related protein 2/3 complex subunit 5 (ARPC5), actin-related protein 3 homolog (ACTR3), myristoylated alanine rich kinase C substrate (MARCKS), and adducin 1 alpha (ADD1)), microtubules (tubulin alpha 1c (TUBA1C) and dynein cytoplasmic 1 (DYNC1)) intermediate filaments (vimentin (VIM), lamin A/C (LMNA), desmin (DES), nestin (NES) and plectin1 (PLEC1)), formation of GBM (integrin beta 1 (INTGB1), vinculin (VCL) and agrin (AGRN)), and other actin-binding proteins (plastin 3 (PLS3), spectrin alpha non-erythrocytic 1 (SPTAN1), calponin 3 (CNN3), tropomyosin 3 (TPM3) and ezrin (EZR)). Among these proteins, Table 2 presents the actin cytoskeleton-associated proteins with high- or low-fold changes of more than 20% (average iTRAQ ratio >1.20 or <0.83) and P values less than 0.05. SORBS2 was the only upregulated protein in glomeruli from OLETF rats at both the early and the proteinuric stages of diabetic nephropathy.

3.4. Confirmation of SORBS2 Expression by Immunohistochemistry. Figures 1(e), 1(f), 1(g), and 1(h) show representative immunostaining results for the SORBS2. There were no clear differences in the expression of SORBS2 between the OLETF and LETO rats at 27 weeks of age (Figures 1(e) and 1(f)). However, SORBS2 was clearly observed in podocytes from OLETF rats at 38 weeks of age (Figures 1(g) and 1(h)).

Immunofluorescence of SPRBS2 and synaptopodin are shown in Figure 3. SORBS2 was observed in glomeruli from OLETF rats at 38 weeks of age (Figure 3(a)). Synaptopodin was observed in glomeruli from OLETF rats at 38 weeks of age (Figure 3(b)). When the stainings of SORBS2 and synaptopodin were merged, they showed as a capillary pattern in glomeruli from OLETF rats at 38 weeks of age (Figure 3(c)).

3.5. SORBS2 mRNA Expression in Isolated Glomeruli. To determine whether SORBS2 localizes within glomeruli and to assess changes of its expression, real-time Q-PCR analyses were also performed. Consistent with the QSTAR proteome analysis results, a tendency towards increased SORBS2

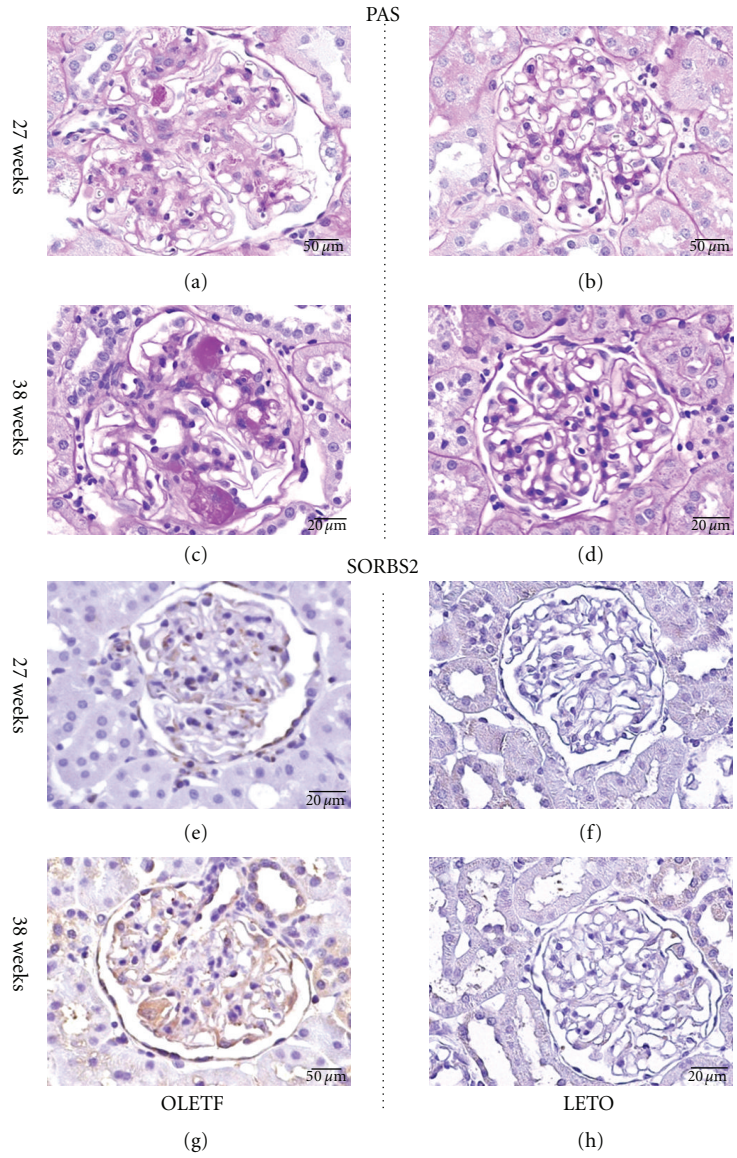


Figure 1: Periodic acid Schiff (PAS) and immunohistochemical staining in the kidneys from OLETF (a, c, e, and g) and LETO (b, d, f, and h) rats at 27 and 38 weeks of age. Slight expansion of the mesangial matrix with mesangial cell proliferation (a). Normal glomeruli (b). Exudative and sclerotic lesion (c). Normal glomeruli (d). SORBS2 positivity in podocytes from OLETF rats (e, f) and negativity in those from LETO rats (f, h). Scale bar = 20 μm.

mRNA expression in glomeruli from OLETF rats was observed compared to those from LETO rats (1.76 ± 0.15 versus 1.40 ± 0.19, $P = 0.06$).

3.6. Ultrastructural Examination Using Electron Microscopy. To validate the podocyte foot process effacement in the OLETF rats, ultrastructural examination was performed at 38 weeks of age. At this time, foot process effacement was not observed in the LETO rats, but was obvious in the OLETF rats (Figures 4(a) and 4(b)). These results and the urinary protein-to-creatinine ratio (5.65 ± 2.36 mg/mg) indicated that 38 weeks of age is appropriate for the detection of the proteinuric stage of diabetic nephropathy in the OLETF rats.

4. Discussion

In the present study, we performed a targeted proteome analysis of glomeruli isolated from rats in both the early (27 weeks of age) and the proteinuric (38 weeks of age) stages of diabetic nephropathy. It is reported that proteins quantified with a fold change of more than 20% (average iTRAQ ratio >1.20 or <0.83) and a P value less than 0.05 were identified as differentially expressed proteins [26, 27]. We demonstrated changes of many kinds of proteins in isolated glomeruli from diabetic rats; these proteins participate in glycolysis, citric acid cycle, formation of oxidative stress, and other intracellular processes, as shown in Figure 2. The results of IPA demonstrated that 17 actin cytoskeleton-associated

Table 2: Differentially expressed actin cytoskeleton-associated proteins in glomeruli isolated from OLETF and LETO rats, identified by QSTAR Elite LC-MS/MS and IPA.

Protein	GI number	Mass (Da)	Location	Function	Foldchange (27 weeks)	*P* value	Fold change (38 weeks)	*P* value
Stress fiber formation								
Sorbin and SH3 domain containing 2 (SORBS2)	205831248	124108	C	AD	1.49	<0.0001	1.97	<0.0001
Alpha-actinin 1 (ACTN1)	13123942	103058	C	ST, CL	1.02	0.764	1.40	<0.0001
Alpha-actinin 4 (ACTN4)	182705246	104654	C	ST, CL	0.93	0.46	1.22	<0.0001
Rho GDP dissociation-inhibitor alpha (ARHGDIA)	21759130	23207	C	RI	1.28	0.041	0.87	0.0413
Actin-filament polymerization								
Actin-related protein 2/3 complex subunit 1 beta (ARPC1B)	12229626	40950	C	AP, EC	0.65	0.0363	0.73	0.0136
Actin related protein 2/3 complex subunit 5 (ARPC5)	3121767	16320	C	AP, EC	0.72	0.0014	0.74	0.0002
Myristoylated alanine-rich protein kinase C substrate (MARCKS)	266495	31555	UN	AP	0.60	0.0004	0.71	0.002
Microtubules formation								
Tubulin alpha 1c (TUBA1C)	55976169	49895	C	MT	0.74	0.012	0.90	0.17
Intermediate filaments formation								
Lamin A/C (LMNA)	1346413	74139	N	IF	0.89	0.0076	1.29	<0.0001
Desmin (DES)	1352241	53536	C	IF	0.94	0.143	1.32	<0.0001
Nestin (NES)	146345465	177439	C	IF	0.83	<0.0001	1.03	0.32
Plectin 1 (PLEC1)	1709655	531791	C	IL	0.82	<0.0001	0.96	0.12
GBM (glomerular basement membrane)								
Integrin beta 1 (INTGB1)	1352494	15105	PM	APG	0.82	0.0015	0.92	0.0094
Agrin (AGRN)	399021	214846	PM	LB	0.62	0.042	0.90	0.490
Others								
Plastin 3 (PLS3)	226693553	70811	C	CL	0.82	0.02	0.82	0.0005
Calponin 3 (CNN3)	584956	36414	C	AB	0.60	0.0013	0.78	0.088
Tropomyosin 3 (TPM3)	148840439	32819	C	AS	0.71	<0.0001	0.80	<0.0001

AB: actin binding; AD: adaptor protein; AP: actin filament polymerization; APG: anchoring podocyte and GBM (glomerular basement membrane); AS: actin filaments stabilization; C: cytoplasm; CL: crosslinking actin filaments into bundles or networks; EC: endocytosis; IF: intermediate filaments; IL: interlinks; LB: laminin binding; LETO: Long-Evans Tokushima Lean; MT: microtubules; N: nuclear; OLETF: Otsuka Long-Evans Tokushima Fatty; PM: plasma membrane; RI: Rho GDP-dissociation-inhibitor activity; ST: stress fibers; UN: unknown.

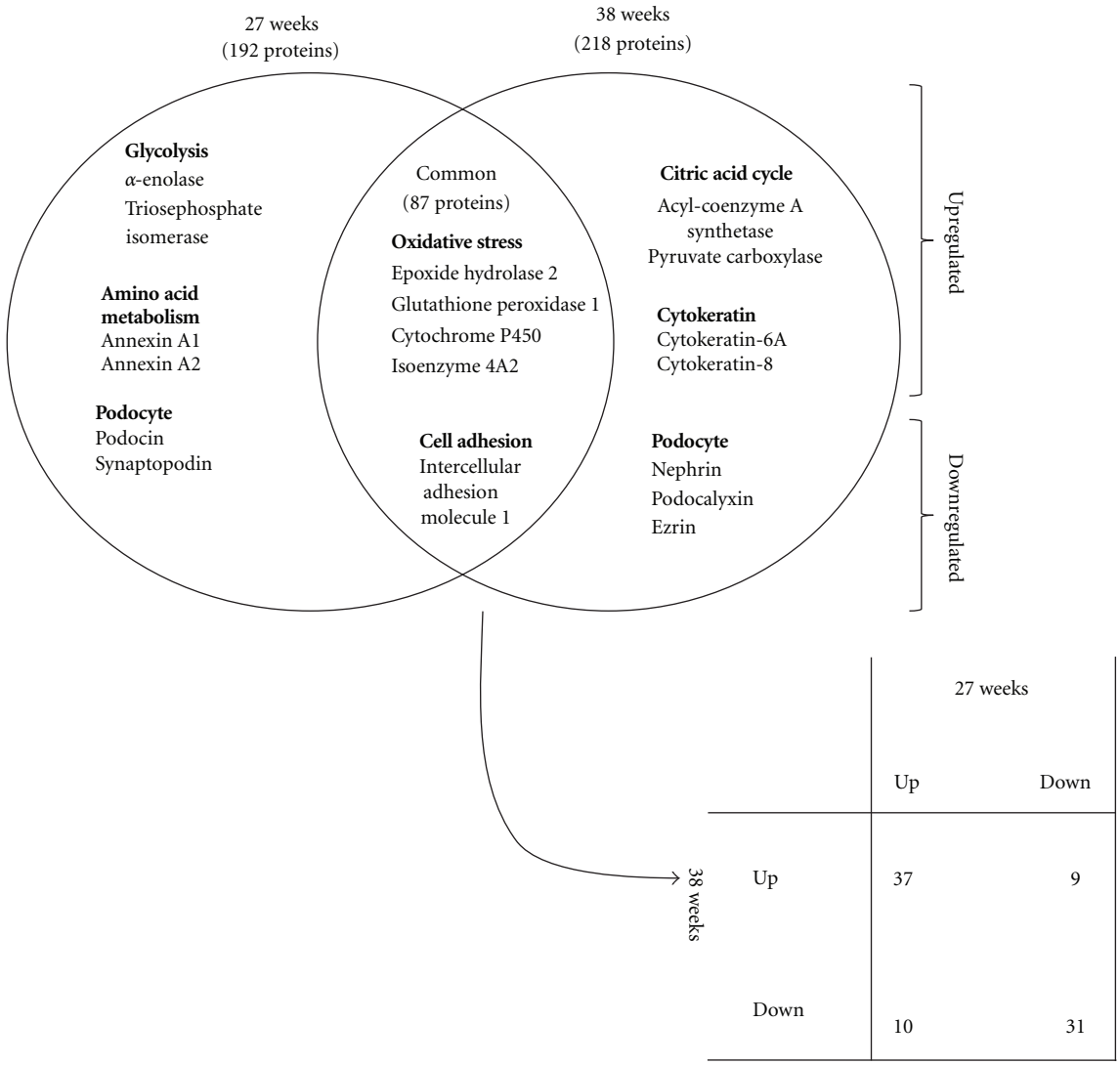
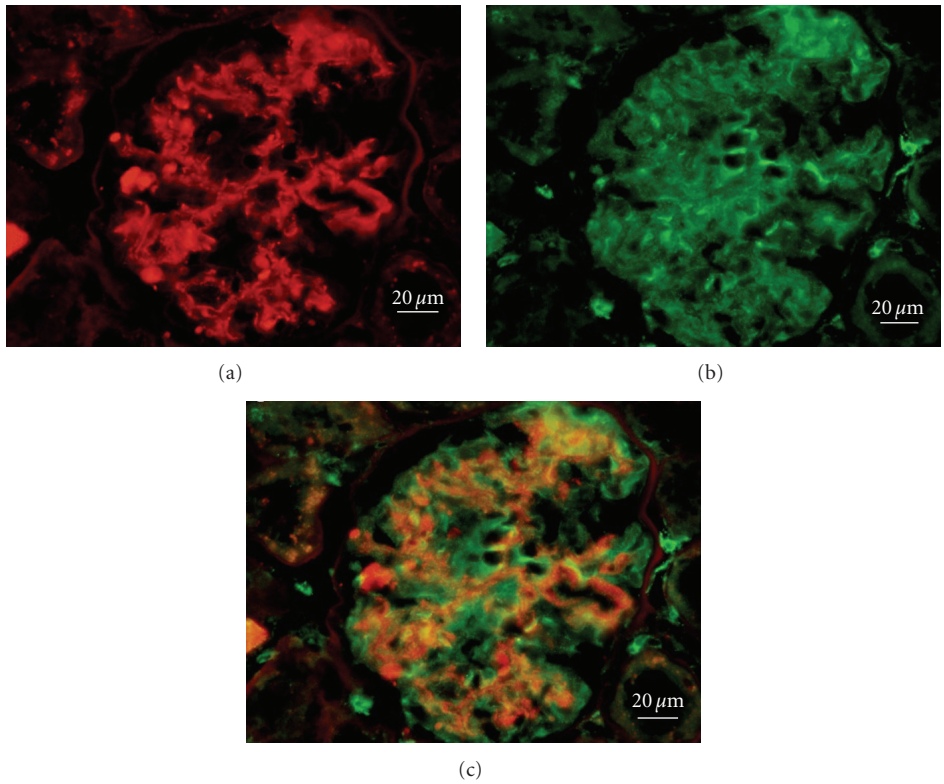


Figure 2: Comparative analysis of subclasses of differentially expressed proteins, excluding actin cytoskeleton-associated proteins, in the kidneys from OLETF and LETO rats at 27 and 38 weeks of age, by the Ingenuity Pathway Analysis (IPA).

proteins were significantly and differentially expressed between OLETF and LETO rats.

Among these proteins that were differentially expressed in isolated glomeruli from OLETF and LETO rats at both 27 and 38 weeks of age were SORBS2 (upregulated), ARCP1B (downregulated), ARPC5 (downregulated), MARCKS (downregulated), PLAS3 (downregulated), CNN3 (downregulated), and TPM3 (downregulated). SORBS2 was the only up-regulated protein in glomeruli from OLETF rats at both the early and the proteinuric stages of diabetic nephropathy, compared to those from LETO rats. There have been no previous reports suggesting any relationship between SROBS2 and diabetic nephropathy.

SORBS2 is an Arg/Abl-binding protein that contains three COOH-terminal Src homology 3 domains, a serine/threonine-rich domain, and several potential Abl phosphorylation sites. It is widely expressed in human tissues, such as heart, brain, spleen, pancreas, and kidney. In epithelial cells, SORBS2 is located in stress fibers [28]. In addition, SORBS2 has been reported to function as an adapter protein in the assembly of signaling complexes in stress fibers and as a potential link between the Abl family kinases and the actin cytoskeleton [8, 9]. In the present study, SORBS2 was up-regulated in isolated glomeruli from OLETF rats based upon proteome analysis using the QSTAR Elite LC-MS/MS. Although there was a tendency towards increased levels of SORBS2 mRNA in microdissected glomeruli from OLETF rats, we confirmed its localization in diabetic glomeruli, especially in podocytes of OLETF rats at the proteinuric stage using immunohistochemistry. Considering the previously reported functions of SORBS2 and the alterations of SORBS2 expression observed in the present study, SORBS2 may be associated with the development of diabetic nephropathy by reorganization of actin filaments, including actin stress fiber formation.



(a) (b)

(c)

Figure 3: Immunofluorescence for SORBS2: red (a), synaptopodin: green (b), and merge SORBS2 and synaptopodin: yellow (c) in OLETF rats at 38 weeks of age. SORBS2 was expressed as a capillary pattern in glomeruli from OLETF rats at 38 weeks of age. Scale bar = 20 μm.

ARCP1B, ARPC5, and MARCKS are actin filament polymerization-related proteins. During polymerization of actin filaments, binding of the Arp2/3 complex to the sides of actin filaments is important for its actin nucleation and branching activities [29]. MARCKS substrate is located at glomeruli, specifically to podocytes, and controls both actin polymerization and actin cytoskeleton binding to the membrane [30]. Alterations of crosslinking proteins that organize actin filaments into bundles or networks, that is, PLS3 (I-plastin), were also detected in the present study. PLS3 is an actin-binding protein expressed in the kidney, that is, known to be located in stress fibers [31] and has been reported to be related to minimal change nephritic syndrome [32]. Moreover, CNN3 plays a direct role in cell contractility in vivo and controls the cytoskeletal composition of podocytes [33]. TPM3 binds to actin filaments and has been implicated in their stabilization [34]. These protein changes may be related to the collapse of actin filaments and the disentanglement of actin bundles or networks of diabetic glomeruli in the early and proteinuric stages of diabetic nephropathy.

ACTN4 is widely expressed in podocyte foot processes and is colocalized with actin stress fibers [6]. The Upregulation of ACTN4 observed during the proteinuric stage in the present study is consistent with a previous report [10]. ACTN1 is present in multiple subcellular regions, including cell-cell and cell-matrix contact sites, cellular protrusions, lamellipodia, and stress fiber dense regions [35], and cross-links actin filaments within stress fibers [11]. ACTN1 was also up-regulated in glomeruli from OLETF rats at 38 weeks of age. ARHGDIA maintains the Rho family members (Rac1, Cdc42, and RhoA), which promote the assembly of actin-myosin filaments and cell stress fibers in the GDP-bound inactive form. Mice lacking ARHGDIA are initially viable and healthy but develop massive proteinuria and glomerulosclerosis later in life [7, 10]. Upregulation of ARHGDIA in the early stage might indicate suppression of the Rho family members. Coincident with the Upregulation of SORBS2 at both stages of OLETF rats in the present study, changes in these protein expression patterns may be associated with reorganization of actin filaments, leading to foot process effacement and the progression of diabetic nephropathy. In the present study; however, we could not validate the expression of ACTN4 and ARGDIA in OLETF rats.

In addition to increasing stress fibers, the impairment of polymerization of actin filaments, intermediate filaments and microtubules, disentanglement of actin filaments, and podocyte detachment from the GBM are also key events for podocytopathies. Intermediate filaments and microtubules are known to form the scaffold of major podocyte processes and the central cell body [36]. NES and PLEC1 were down-regulated in the kidneys from OLETF rats at 27 weeks of age, indicating the collapse or the disentanglement of intermediate filaments and microtubules in the early stage of diabetic nephropathy. Furthermore, podocytes are attached

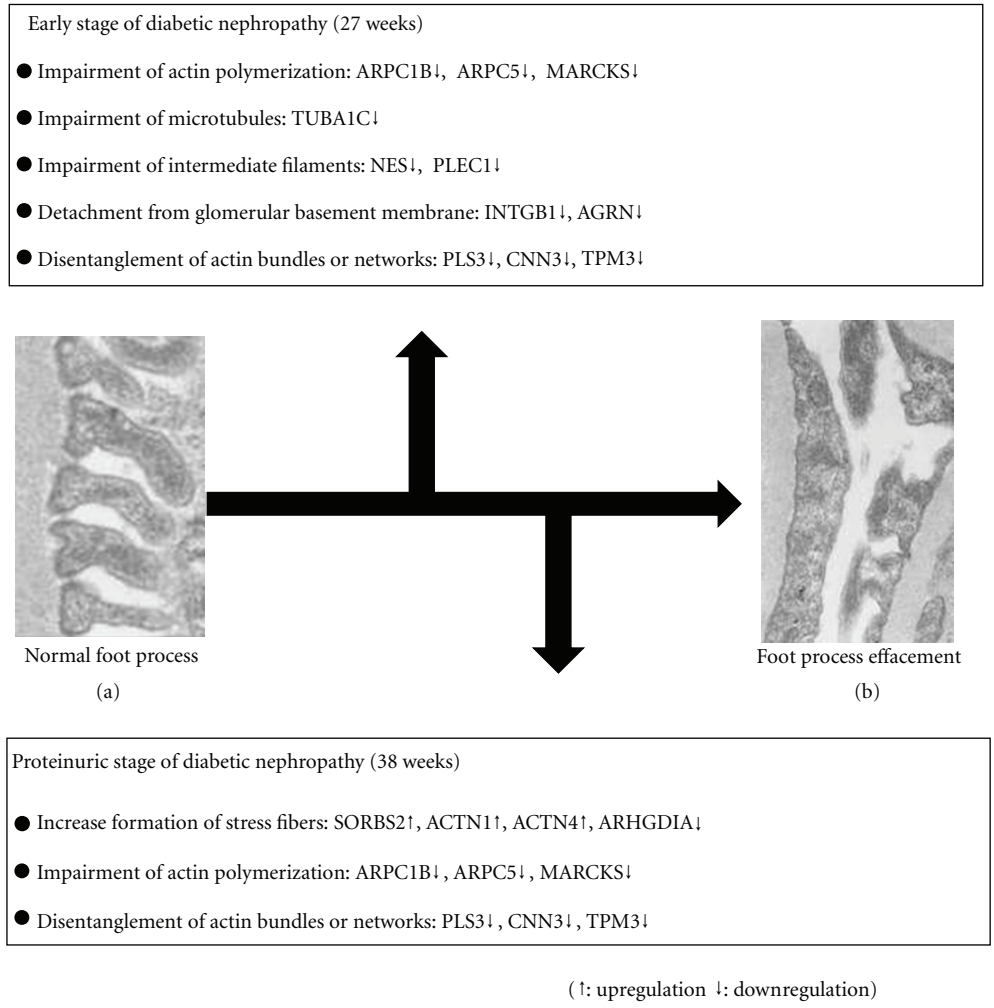


Figure 4: Alterations in protein expression during podocyte foot process effacement in diabetic nephropathy. Electron micrograph of podocytes from OLETF and LETO rats at 38 weeks of age. LETO rats (×40,000) (a), OLETF rats (×40,000) (b).

to the outer aspect of the GBM and their foot processes are connected to the GBM [37]. Downregulation of INTGB1 and AGR may indicate that podocytes are detached from the GBM, resulting in foot process effacement and proteinuria.

The alterations of proteins observed in this study are summarized in Figure 4. Based on proteome analysis of isolated glomeruli from diabetic rats, the following changes of the cytoskeleton at the early and proteinuric stages of diabetic nephropathy could occur: (1) increased formation of stress fibers during the proteinuric stage of diabetic nephropathy, (2) impairment of actin polymerization at both time points, suggesting collapse or dysfunction of actin filaments, (3) decreased expression of proteins associated with microtubules and intermediate filaments during both the early and the proteinuric stages, (4) decreased GBM cytoskeleton-associated proteins at both stages, suggesting podocytopathies, and (5) collapse or disentanglement of actin filaments at both stages. Our findings suggested that impairment or collapse of actin filaments may cause podocyte foot process effacement (Figure 4(b)) and the emergence of proteinuria in diabetic nephropathy. As observed in the present study, increases in stress fibers at the proteinuric stage may be related to reorganization of actin filaments [4, 5]. Proteome analysis demonstrated that numerous cytoskeleton-associated proteins could contribute to the onset and/or progression of diabetic nephropathy.

There are some limitations in the present study. First, in the glomerular isolation, we used a sieving method to minimize tubular contamination. Despite the implementation of this technique and effort, it is impossible to completely avoid tubular contamination. Indeed, one of the mitochondrial proteins, that is, mitochondrial import inner membrane translocase subunit 44 (TIM44), which is activated in diabetic nephropathy [38] was up-regulated in OLETF rats at 27 weeks of age (fold change 1.34, $P = 0.04$). A second limitation is the protein detection using the QSTAR Elite LC-MS/MS. Although, some podocyte-related proteins, such as podocin and synaptopodin, were downregulated in OLETF rats at 27 weeks of age (fold change 0.83 $P = 0.001$; fold change 0.63, $P = 0.0001$, resp.), these proteins were not detected in OLETF rats at 38 weeks of age, due to mechanical problems or problems with

reproducibility of protein identification using the QSTAR Elite LC-MS/MS. Lastly, although proteome analysis is one of the most powerful and useful tools in the detection of novel proteins for various kidney diseases, it requires validation or further mechanical analysis of the results of proteome analysis. Although there are some limitations in the present methodology, our findings demonstrated the usefulness of proteome analysis of isolated glomeruli, which allows the direct and comprehensive investigation of protein alterations of diabetic glomeruli.

In conclusion, the present proteome analysis demonstrated that numerous cytoskeleton-associated proteins contribute to the onset and/or progression of diabetic nephropathy. This proteome study also demonstrated, for the first time, that SORBS2 expression was increased in diabetic glomeruli and suggested that SORBS2 may be associated with the development of diabetic nephropathy by reorganization of actin filaments, along with other actin cytoskeleton-associated proteins. Further investigation is necessary to ascertain the significance of SORBS2 and these actin cytoskeleton-associated proteins in diabetic nephropathy.

Conflict of Interests

The authors declared that they have no conflict of interests.

Abbreviations

ACTN1:	Alpha-actinin 1
ACTN4:	Alpha-actinin 4
AGRN:	Agrin
ARHGDIA:	Rho GDP dissociation-inhibitor alpha
ARPC1B:	Actin-related protein 2/3 complex subunit 1 beta
ARPC5:	Actin-related protein 2/3 complex subunit 5
CNN3:	Calponin 3
DES:	Desmin
GBM:	Glomerular basement membrane
INTGB1:	Integrin beta 1
LMNA:	Lamin A/C
MARCKS:	Myristoylated alanine rich protein kinase C substrate
NES:	Nestin
PLEC 1:	Plectin 1
PLS3:	Plastin 3
SORBS2:	Sorbin and SH3 domain containing 2
TPM3:	Toropomyosin 3
TUBA1C:	Tubulin alpha 1c.

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Research Article

High Glucose Increases Metallothionein Expression in Renal Proximal Tubular Epithelial Cells

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Metallothionein (MT) is an intracellular metal-binding, cysteine-rich protein, and is a potent antioxidant that protects cells and tissues from oxidative stress. Although the major isoforms MT-1 and -2 (MT-1/-2) are highly inducible in many tissues, the distribution and role of MT-1/-2 in diabetic nephropathy are poorly understood. In this study, diabetes was induced in adult male rats by streptozotocin, and renal tissues were stained with antibodies for MT-1/-2. MT-1/-2 expression was also evaluated in mProx24 cells, a mouse renal proximal tubular epithelial cell line, stimulated with high glucose medium and pretreated with the antioxidant vitamin E. MT-1/-2 expression was gradually and dramatically increased, mainly in the proximal tubular epithelial cells and to a lesser extent in the podocytes in diabetic rats, but was hardly observed in control rats. MT-1/-2 expression was also increased by high glucose stimulation in mProx24 cells. Because the induction of MT was suppressed by pretreatment with vitamin E, the expression of MT-1/-2 is induced, at least in part, by high glucose-induced oxidative stress. These observations suggest that MT-1/-2 is induced in renal proximal tubular epithelial cells as an antioxidant to protect the kidney from oxidative stress, and may offer a novel therapeutic target against diabetic nephropathy.

1. Introduction

Diabetic nephropathy is a leading cause of end-stage renal disease, and many mechanisms have been proposed to explain the pathogenesis of renal injury in diabetes [1]. Recent studies have shown that hyperglycemia may induce oxidative stress by increasing reactive oxygen species (ROS) generation in the diabetic kidney [2–4] and that overexpression of the antioxidant superoxide dismutase 1 attenuated diabetic nephropathy in streptozotocin (STZ)-induced and *db*/*db* diabetic mice [5, 6]. Therefore, ROS could be an important mediator of diabetic nephropathy, and protection from ROS might offer a valuable therapeutic strategy to treat diabetic nephropathy.

Metallothionein (MT) is an intracellular metal-binding protein with a low-molecular mass (6-7 kDa) and a high cysteine content (20 of 61-62 amino acids). Its major isoforms, MT-1 and -2 (MT-1/-2), are widely distributed

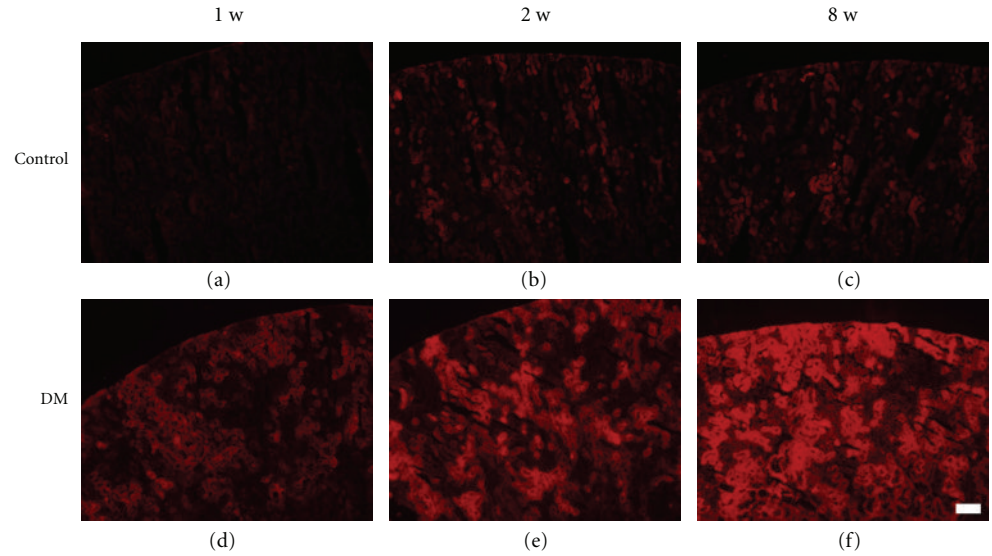


Figure 1: MT-1/-2 expression in the kidney. Diabetes was induced by injection of streptozotocin, and kidneys were obtained at 1 (a and d), 2 (b and e), or 8 (c and f) weeks after inducing diabetes. Immunofluorescent staining was performed as described in *Materials and Methods*. MT was strongly expressed in the renal cortex of diabetic rats (d, e, f) and hardly expressed in control rats (a, b, c). The expression of MT-1/-2 was greater at week 8 than at weeks 1 and 2 after diabetes induction. Scale bar: 100 μm.

throughout the body [7, 8]. Since MT-1/-2 expression is significantly upregulated by overload of essential trace metals (e.g., Zn and Cu), it plays an important role in heavy metal detoxification and essential metal homeostasis [9, 10]. In addition, MTs have been shown to act as nonspecific free radical scavengers [11, 12], suggesting that they exert antioxidant activities in various diseases, including diabetic nephropathy.

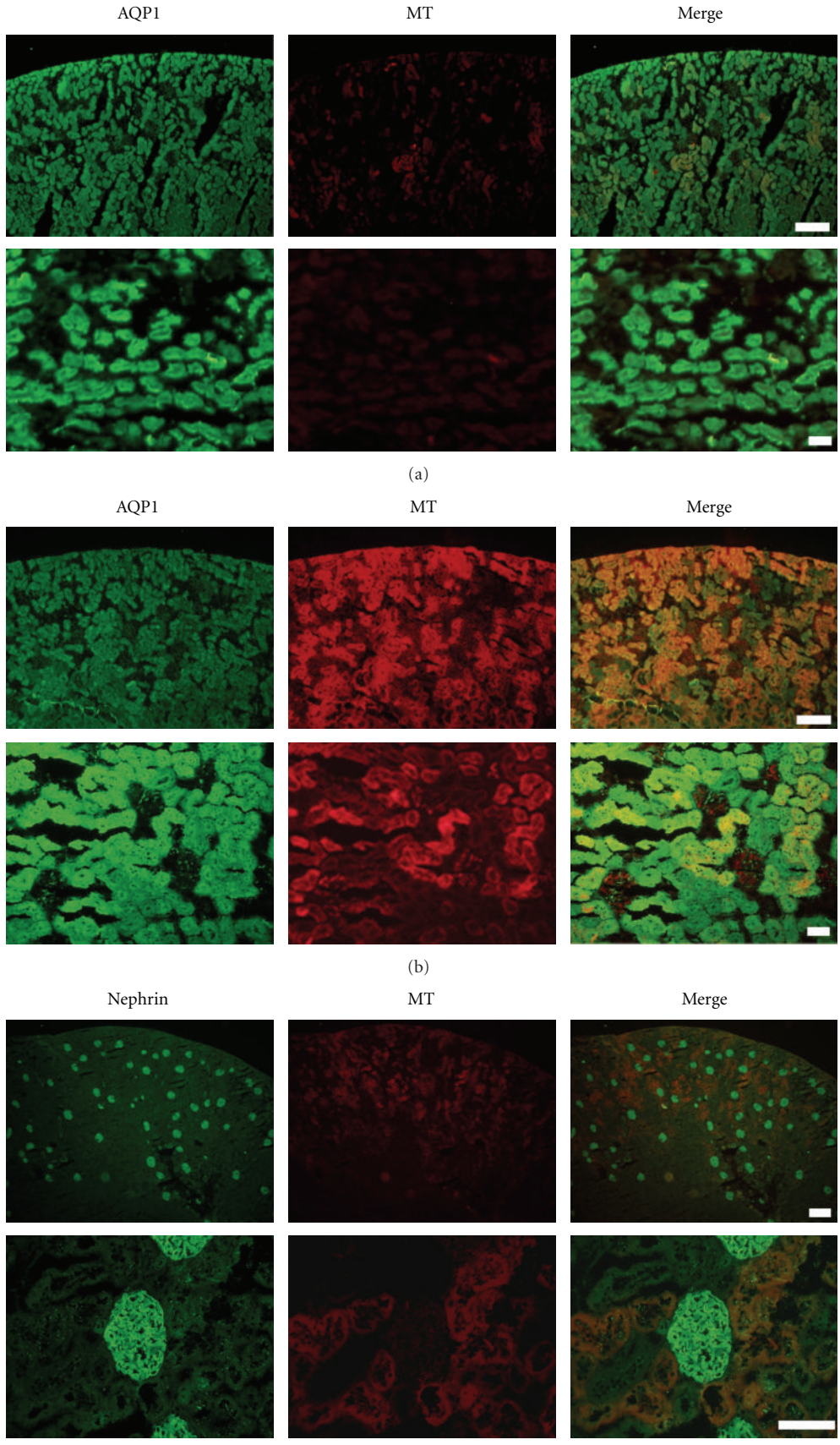
We and other investigators have demonstrated that MTs have neuroprotective effects in mouse models of Parkinson's disease [13–15]. In contrast, the role of MTs in the pathogenesis of diabetic nephropathy is poorly understood. Several studies reported that renal expression of MT is increased in STZ-induced diabetic rats [16], diabetic BB rats [17], and *ob*/*ob* diabetic mice [18]. However, the distribution of MTs in the diabetic kidney and the mechanisms by which MTs are induced in diabetes are poorly understood. Therefore, in the present study, we investigated the expression and localization of MT-1/-2 during the development of diabetic nephropathy and explored the mechanism by which MT-1/-2 expression was induced by high glucose in the kidney.

2. Materials and Methods

2.1. Experimental Protocol. Male Sprague Dawley rats were purchased from Charles River (Yokohama, Japan). Five-week-old rats were divided into two groups: (1) nondiabetic control rats (control; $n = 6$) and (2) STZ-induced diabetic rats (DM; $n = 6$). Diabetes was induced by peritoneal injection of 200 mg/kg STZ (Sigma-Aldrich Corp., MO) in citrate buffer (pH 4.5). Blood glucose was measured by the glucose oxidase method at 3 days after STZ injection and only rats with blood glucose concentrations >16 mmol/L were used in the study. All rats had free access to standard diet and tap water. All procedures were performed according to the Guidelines for Animal Experiments at Okayama University Medical School, Japanese Government Animal Protection and Management Law (No. 105) and the Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6). Rats were sacrificed at 1, 2, or 8 weeks after inducing diabetes. We measured body weight, hemoglobin A1c (HbA1c), and 24-h urinary albumin excretion (UAE) at 1, 2, and 8 weeks. The kidneys were removed, weighed, and fixed in 10% formalin for periodic acid—methenamine silver (PAM) staining, and parts of the remaining tissues were embedded in optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) and frozen immediately in acetone cooled on dry ice.

2.2. Immunofluorescent Staining of MT-1/-2 in Rat Kidney. Immunofluorescent staining was performed as previously described [19]. Renal expression of MT-1/-2 was detected using mouse anti-MT-1/-2 antibody (Dako, Carpinteria, CA) followed by Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA). To determine whether MT-1/-2 was localized in podocytes or proximal tubular epithelial cells, the sections were counterstained with guinea pig antinephrin antibody (Fitzgerald, Concord, MA) or rabbit antiaquaporin 1 antibody (Millipore, Billerica, MA), followed by Alexa Fluor 488 goat anti-guinea pig IgG or anti-rabbit IgG (Invitrogen), respectively. Fluorescence images were obtained using a fluorescence microscope (BX51; Olympus, Tokyo, Japan).

2.3. Cell Culture and Treatment. mProx24 cells, a murine renal proximal tubular epithelial cell line derived from



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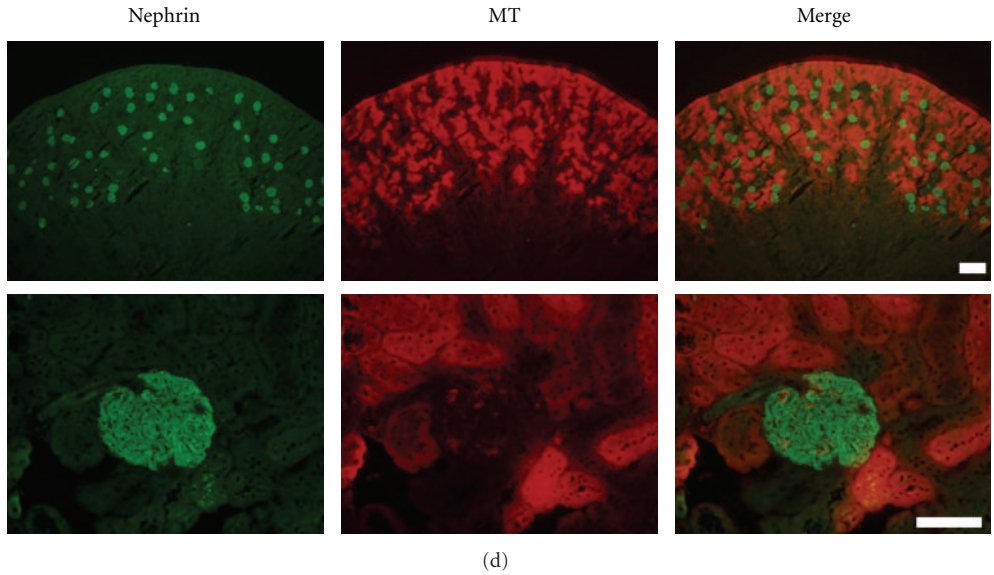

Figure 2: MT-1/-2 expression in podocytes and proximal tubular cells of the kidney. Immunofluorescent staining was performed as described in *Materials and Methods*. Eight weeks after inducing diabetes, MT-1/-2 was predominantly expressed in the proximal tubular epithelial cells of the kidney (b) and weakly expressed in podocytes (d) in the kidney of diabetic rats. In control rats, MT-1/-2 was weakly expressed in proximal tubular epithelial cells (a), but hardly in the podocytes (c). AQP1: aquaporin 1, MT: MT-1/-2. Scale bar: upper panels, 200 μm; lower panels, 50 μm.

C57BL/6J adult mouse kidney [20], were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich Corp.) supplemented with 1000 mg/L D-glucose, 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in 5% CO_2. To evaluate the effect of high glucose on MT expression, the cells were serum-starved by culture in 0.5% FBS for 24 h, then stimulated with 4500 mg/L D-glucose (high glucose) or D-mannitol (Sigma-Aldrich Corp.) for 24 h. For antioxidant treatment, the cells were pretreated with vitamin E (Sigma-Aldrich Corp.) at concentration ranges from 20 to 200 nM for 24 h, then stimulated with high glucose for 24 h. Individual experiments were repeated at least three times with different lots or preparations of cells.

2.4. Quantitative Analyses of MT-1 Gene and MT-1/-2 Protein Expression in mProx Cells. RNA was isolated from mProx cells using an RNeasy Mini kit (Qiagen, Valencia, CA). Single-strand cDNA was synthesized from the extracted RNA using a RT-PCR kit (Perkin Elmer, Foster City, CA). To evaluate the mRNA expression of MT-1 in mProx24 cells, quantitative RT-PCR (qRT-PCR) was performed using StepOnePlus (Applied Biosystems, Tokyo, Japan) and Fast-Start SYBR Premix Ex Taq II (Takara Bio Inc., Otsu, Japan). The primers for the MT-1 gene (upstream 5′-TCTAAGCGTCACCACGACTTCA-3′ and downstream 5′-GTGCACTTGCAGTTCTTGCAG-3′) were purchased from Takara Bio Inc. Each sample was analyzed in triplicate and normalized for GAPDH mRNA expression. Immunofluorescent staining of MT-1/-2 protein was performed as described above. The immunofluorescence intensity in cultured mProx cells was calculated using the formula, x (density) $\times$ positive area (μm^2), using Lumina Vision software (Mitani Corporation).

2.5. Statistical Analysis. All values are means ± SEM. Statistically significant differences between groups were examined using one-way ANOVA followed by Scheffé's test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. MT-1/-2 Expression Was Increased in Diabetic Kidney. MT-1/-2 expression was observed in the renal cortex from 1 week after the induction of diabetes. Its expression increased gradually and was strongly upregulated at week 8 (Figure 1,(d),(e),(f)). In contrast, MT-1/-2 was hardly detected in the kidney of control rats (Figure 1, (a),(b),(c)). Renal sections counterstained with antiaquaporin 1 and antinephrin antibodies revealed that MT-1/-2 expression was predominantly localized in the proximal tubular epithelial cells (Figure 2(b)), and to a lesser extent in the podocytes of the diabetic kidneys (Figure 2(d)). In control rats, MT-1/-2 was weakly expressed in the proximal tubular epithelial cells (Figure 2(a)), but not in the podocytes (Figure 2(c)). Body weight, kidney weight, UAE, and HbA1c are shown in Table 1. Diabetic rats had a significantly lower body weight and higher kidney weight per body weight at 8 weeks, but not at 1 and 2 weeks after the induction of diabetes. Similarly, The UAE and HbA1c level in the diabetic rats was significantly higher than in the control rats at 8 weeks, but not at 1 and 2 weeks. Glomerular hypertrophy and mesangial matrix expansion, but not interstitial changes and tubular atrophy

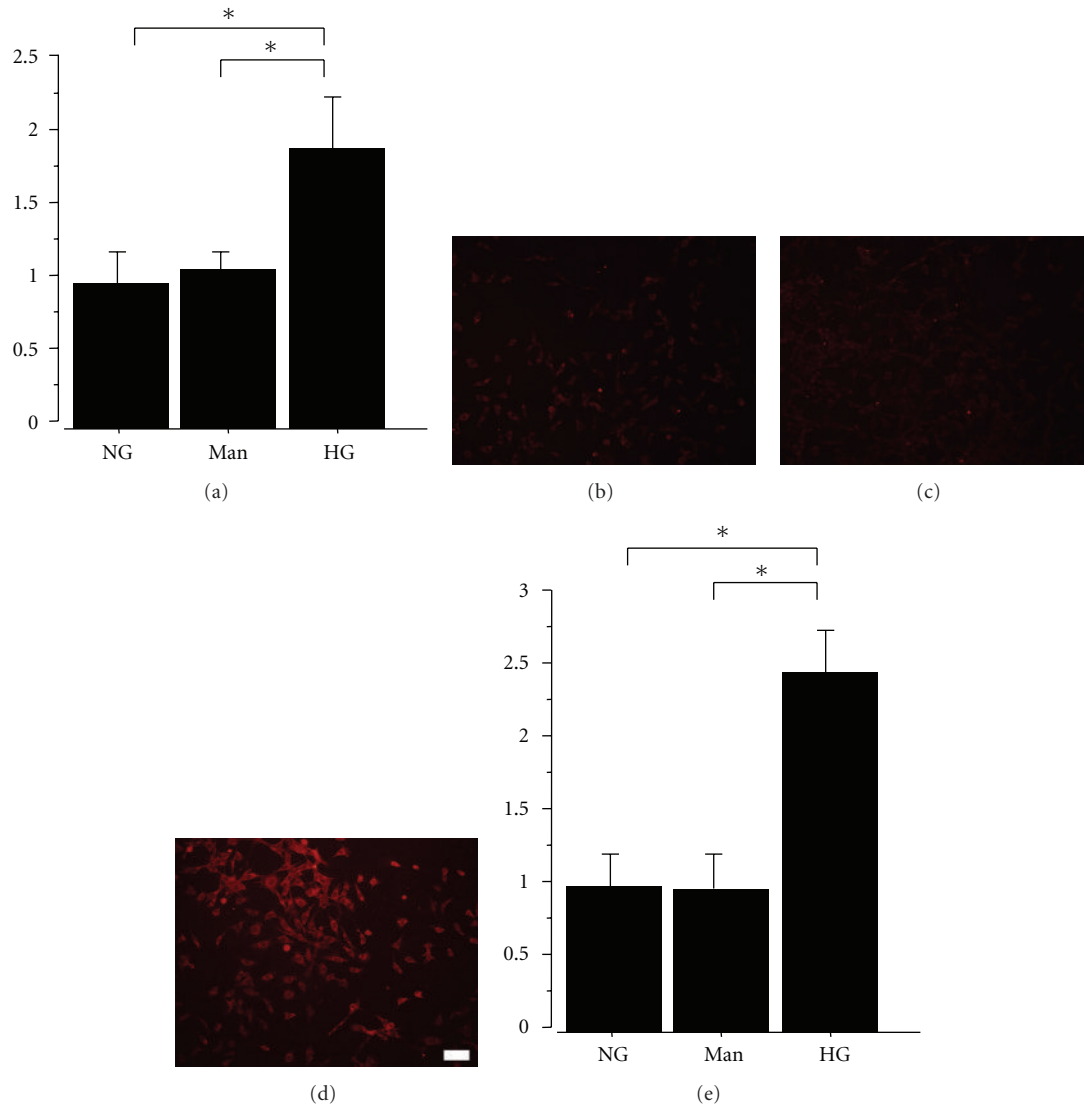

Figure 3: High glucose increases MT-1 mRNA and MT-1/-2 protein expression. mProx24 cells were serum-starved for 24 h before stimulation with high glucose or mannitol. (a) Cells were harvested after 24 h, and MT-1 mRNA expression was analyzed by qRT-PCR in three independent experiments and normalized for GAPDH. (b–e) MT-1/-2 protein expression was determined by immunofluorescent staining with anti-MT-1/-2 antibody 24 h after stimulation followed by densitometric analysis. Results are means ± SEM of three independent experiments. $^*P < 0.05$ versus high glucose; NG: normal glucose; Man: mannitol; HG: high glucose. Scale bar: 100 μm.

were observed in the diabetic rats as compared with control rats at 8 weeks (data not shown).

3.2. High Glucose Increased MT-1/-2 Expression in mProx24 Cells. qRT-PCR analyses revealed that exposure to the high glucose medium significantly increased MT-1 mRNA expression in mProx24 cells compared with normal glucose medium (Figure 3(a)). Similarly, high glucose, but not mannitol, significantly increased MT-1/-2 protein expression in mProx24 cells (Figures 3(b)–3(e)). These data indicate that high glucose increases the mRNA and protein expression of MT-1/-2 in mProx24 cells.

3.3. MT-1/-2 Expression Was Suppressed by Vitamin E. It is well known that high glucose increases the generation of ROS in various cells. To investigate the mechanism by which MT is induced by ROS in the high glucose condition, we examined the effects of an antioxidant, vitamin E, on MT-1/-2 expression in mProx24 cells. As shown in Figure 4, high-glucose-stimulated MT-1/-2 expression was significantly attenuated by vitamin E in a dose-dependent manner (Figure 4). Accordingly, these findings suggest that ROS generated by high glucose induces MT-1/-2 expression in the proximal tubular epithelial cells of the kidney.

4. Discussion

There is increasing evidence from experimental and clinical studies to suggest that oxidative stress plays a critical role in the pathogenesis and progression of diabetic

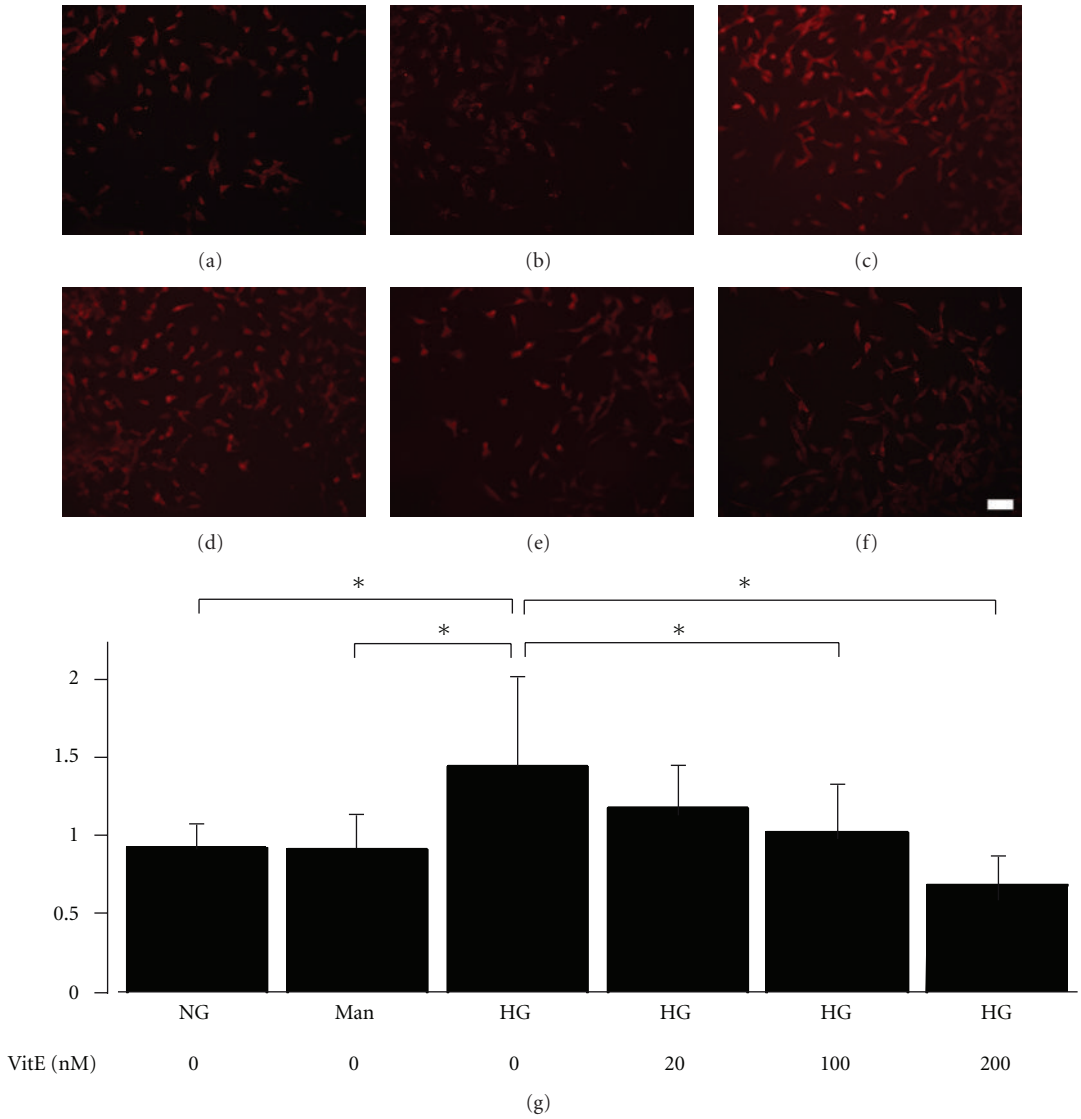

Figure 4: Vitamin E suppresses high glucose-induced MT-1/-2 expression. mProx24 cells were serum-starved and pretreated with vehicle or vitamin E for 24 h before stimulation with high glucose or mannitol. MT-1/-2 expression was determined by immunofluorescent staining. MT-1/-2 expression was not increased by mannitol (b) compared with normal glucose (a), but was increased by high glucose (c). High glucose-induced MT-1/-2 expression was attenuated by vitamin E pretreatment in a dose-dependent manner (d: 20 nM; E: 100 nM; F: 200 nM). The cells depicted are representative of three independent experiments. (g) Densitometric quantification of MT-1/-2 immunofluorescence. Results are means ± SEM of three independent experiments. $^*P < 0.05$ versus high glucose; NG: normal glucose; Man: mannitol; HG: high glucose; Vit E: vitamin E. Scale bar: 100 μm.

complications [21]. Since MT is a potent, endogenous and inducible antioxidant in various tissues [11, 12], we hypothesized that MT may be induced and act as an antioxidant in STZ-induced diabetic kidneys. Here, we found that high glucose induces the expression of MT-1/-2 mainly in proximal tubular epithelial cells and, to a lesser extent, in podocytes in rat kidneys. MT-1/-2 was dramatically expressed in renal proximal tubular epithelial cells within 1 week after inducing diabetes and gradually increased to week 8. MT-1/-2 expression seems to correlate with glucose level, but not with UAE, HbA1c, interstitial abnormalities. To our knowledge, this is the first report describing the localization and expression of MT-1/-2 in the diabetic kidney.

To elucidate the mechanism by which diabetes induces MT-1/-2 expression in proximal tubular epithelial cells, we investigated the effects of high glucose stimulation on mProx24, a murine renal proximal tubular epithelial cell line. We detected increased MT-1 mRNA and MT-1/-2 protein expression in the high glucose condition and found that high glucose-induced MT-1/-2 expression was suppressed by pretreatment with the antioxidant vitamin E. Vitamin E is well known to have high biological activity to protect cells from the propagation of free radical reactions [22, 23], thus we chose vitamin E in this study. These data suggest that ROS and oxidative stress, which are induced by high glucose, may be involved in the induction of MT-1/-2. Although

Table 1: Metabolic data at 1, 2, and 8 weeks after inducing diabetes.

	1 week	2 week	8 week
Body weight (g)			
Control	204 ± 6.3	241 ± 10.4	380 ± 13.3
Diabetic	198 ± 4.7	225 ± 11.5	248 ± 16.6*
Kidney weight (mg/g BW)			
Control	5.8 ± 0.4	5.6 ± 0.8	4.5 ± 0.7
Diabetic	5.9 ± 0.6	6.1 ± 1.0	6.7 ± 0.9*
UAE (μg/day)			
Control	110 ± 7.3	121 ± 8.1	137 ± 14.7
Diabetic	116 ± 5.7	125 ± 9.4	458 ± 24.5*
HbA1c (%)			
Control	3.7 ± 0.4	3.8 ± 0.6	3.8 ± 0.5
Diabetic	3.8 ± 0.3	4.3 ± 0.7	7.8 ± 0.9*

Data are means ± SEM; $^*P < 0.05$ versus the control group. BW: body weight; UAE: urinary albumin excretion; HbA1c: hemoglobin A1c.

several studies have shown that MT protein expression is increased in the kidney of diabetic animals [16–18], the cellular distribution of MTs has not been addressed. Our data provide the first evidence for the expression profile of MT-1/-2 in the diabetic kidney. We speculate that MT-1/-2 is highly induced in proximal tubular epithelial cells in compensation for oxidative stress induced by high glucose.

Our study has potential limitations. First, we speculated that MT-1/-2 expression was upregulated by ROS, but further studies are needed to elucidate the underlying mechanisms. Although Zn is known to induce the gene and protein expression of MTs [24], this essential trace element is unlikely to be involved in our findings because the same chow was provided to the control and diabetic rats. In this study, we showed that high-glucose-stimulated MT-1/-2 expression was attenuated by vitamin E *in vitro*, but we have no data about diabetic rats treated by vitamin E. MT-1/-2 expression in the diabetic state may differ between cells and tissues, and the mechanisms by which other antioxidants regulate the expression of MT remain unclear. Further studies are needed to elucidate these issues. Second, it is still controversial whether site-specific induction of MT plays an important role in diabetic nephropathy. Podocyte-specific overexpression of MT reduced diabetic nephropathy in transgenic mice [25]. However, no studies have investigated whether MT expression in proximal tubular epithelial cells has a protective effect in diabetic animal models. Therefore, diabetes models using MT-knockout mice are needed to answer this question.

In conclusion, renal ROS, which are induced by diabetes, upregulate MT-1/-2 expression in proximal tubular epithelial cells of the kidney. Our results suggest that MT-1/-2 might be a novel therapeutic target to treat diabetic nephropathy.

Acknowledgments

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Research Article

Dietary Restriction Ameliorates Diabetic Nephropathy through Anti-Inflammatory Effects and Regulation of the Autophagy via Restoration of Sirt1 in Diabetic Wistar Fatty (*fa/fa*) Rats: A Model of Type 2 Diabetes

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Aim. Despite the beneficial effects of dietary restriction (DR) on lifespan, age-related diseases, including diabetes and cardiovascular diseases, its effects on type 2 diabetic nephropathy remain unknown. This study examined the renoprotective effects of DR in Wistar fatty (*fa/fa*) rats (WFRs). *Methods*. WFRs were treated with DR (40% restriction) for 24 weeks. Urinary albumin excretion, creatinine clearance, renal histologies, acetylated-NF-κB (p65), Sirt1 protein expression, and p62/Sqstm 1 accumulation in the renal cortex, as well as electron microscopic observation of mitochondrial morphology and autophagosomes in proximal tubular cells were estimated. *Results*. DR ameliorated renal abnormalities including inflammation in WFRs. The decrease in Sirt1 levels, increase in acetylated-NF-κB, and impaired autophagy in WFRs were improved by DR. *Conclusions*. DR exerted anti-inflammatory effects and improved the dysregulation of autophagy through the restoration of Sirt1 in the kidneys of WFRs, which resulted in the amelioration of renal injuries in type 2 diabetes.

1. Introduction

According to the International Diabetes Federation (IDF) atlas in 2009, the estimated diabetes prevalence for 2010 has risen to 285 million, representing 6.6% of the world's adult population, with a prediction that by 2030 the number of people with diabetes will have risen to 438 million [1]. Diabetes results in vascular changes and dysfunction, and diabetic complications are the major cause of morbidity and mortality in diabetic patients. Among diabetic vascular complications, nephropathy is a leading cause of end-stage renal disease and an independent risk factor for cardiovascular diseases. Renal inflammation is recognized as one of the important pathophysiological mechanisms and therapeutic targets for the prevention of diabetic nephropathy and atherosclerosis [2].

Dietary restriction (DR) has a variety of effects on lifespan extension and the delayed onset of age-related diseases, including cardiovascular diseases and diabetes, and is accepted as the only established antiaging experimental paradigm [3, 4]. The beneficial effects of DR involve the function of the NAD^+-dependent deacetylase, Sirt1, the expression of which is induced by DR. Sirt1 has cytoprotective effects, including anti-inflammatory effects [5–7].

In addition, autophagy is a major intracellular process in which lysises in sthe damaged cytoplasmic organelles, including mitochondria, degrade protein aggregates and aged proteins [8, 9]. Although the renoprotective effects of autophagy have been shown in several animal experimental models such as aging [10] and acute kidney injury [11–14], the role of autophagy in diabetic nephropathy remains unknown. Moreover, Sirt1 is one of the positive regulators

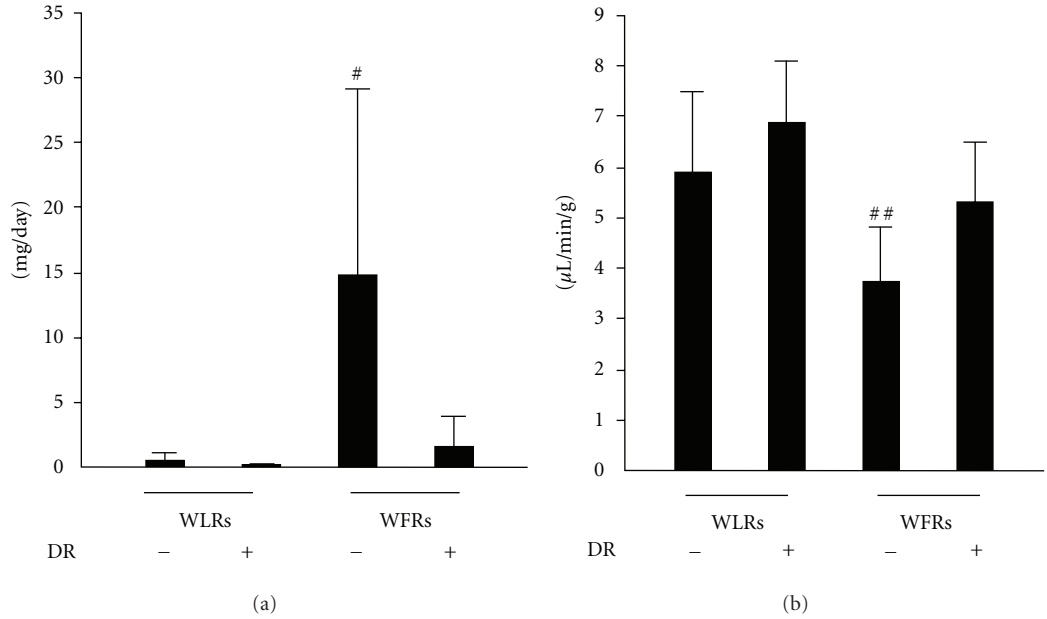

Figure 1: DR ameliorates urinary albumin excretion (a) and creatinine clearance (Ccr) (b) in Wistar fatty rats (WFRs). Treatment with DR reduced urinary albumin excretion and improved Ccr in WFRs. Data are means ± SD ($n = 9 - 11$, $^{\#}P < 0.01$ versus other groups, $^{\#\#}P < 0.05$ versus other groups).

of autophagy [15]. However, there is little information on whether DR ameliorates nephropathy in type 2 diabetes, and if so, whether the effects of DR are associated with Sirt1 through anti-inflammatory function or through the regulation of autophagy.

Therefore, the aim of this study was to investigate the potential effects of DR on inflammation and the regulation of autophagy in the diabetic kidney. We demonstrated that DR attenuated inflammation related to the increase in acetylated-NF-κB and the dysregulation of autophagy as a result of normalized mitochondrial morphologies through restoration of Sirt1 expression in the kidneys of WFRs.

2. Research Design and Methods

2.1. Materials and Antibodies. The anti-Sirt1 antibody was purchased from Millipore (Billerica, Mass). The anti-phospho-AMPKα (Thr172) and anti-AMPKα (23A3) antibodies were obtained from Cell Signaling Technology (Beverly, MA). The acetylated-NF-κB (p65, K310) and NF-κB (p65) antibodies were purchased from Abcam (Cambridge, MA). The anti-ED-1 antibody was purchased from Serotec (Oxford, UK). The anti-p62/Sqstm1 antibody was obtained from Medical & Biological Laboratories (Nagoya, Japan). The Rat Microalbuminuria ELISA kit (NEPHRAT II) was purchased from Exocell (Philadelphia, P).

2.2. Animals. Male diabetic Wistar fatty (*fa/fa*) rats (WFRs), a model of type 2 diabetes, and age-matched nondiabetic Wistar lean rats (WLRs) were obtained from Takeda Chemical Industries (Osaka, Japan) [16]. At 6 weeks of age, rats were divided into four groups: WLRs, WFRs and WLRs treated with DR, and WFRs treated with DR. The DR group was given a daily 40% restriction of the food consumption of WLRs or WFRs control rats. Food consumption was measured twice a week. Body weight and blood glucose levels were measured every two weeks in all animals. The blood pressure of conscious rats was measured at steady state using a programmable tail-cuff sphygmomanometer (BP98-A; Softron, Tokyo, Japan) once a month. After 24 weeks, individual rats were placed in metabolic cages for 24 h urine collection. The urine samples were stored at -80°C until analysis. Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital, and the right kidneys were removed and stored at -80°C for the experiments described below. After the collection of blood samples from the left cardiac ventricle, the left kidney was perfused with ice-cold phosphate-buffered saline (PBS) and 10% neutral-buffered formalin and removed. The Research Center for Animal Life Science of Kanazawa Medical University approved all experiments.

2.3. Blood and Urinary Analysis. Glycated-Hb levels were measured using a DCA 2000 analyzer (Siemens Medical Solutions Diagnostics, Tokyo, Japan). Triglycerides (TG) and total cholesterol (T-CHO) were measured using a Pure-Auto S TG-N kit (Sekisui Medical, Tokyo, Japan) and an L-type cholesterol *H*-test kit (Wako Pure Chemical Industries, Osaka, Japan), respectively. Serum creatinine was measured using a Cica liquid-S CRE kit (Kanto Chemical Co., Inc, Tokyo, Japan), and urinary creatinine concentration was measured with a BioMajesty JCA-BM12 (Hitachi, Tokyo, Japan). Urinary albumin excretion (UAE) was measured using an enzyme-linked immunosorbent assay (ELISA) kit,

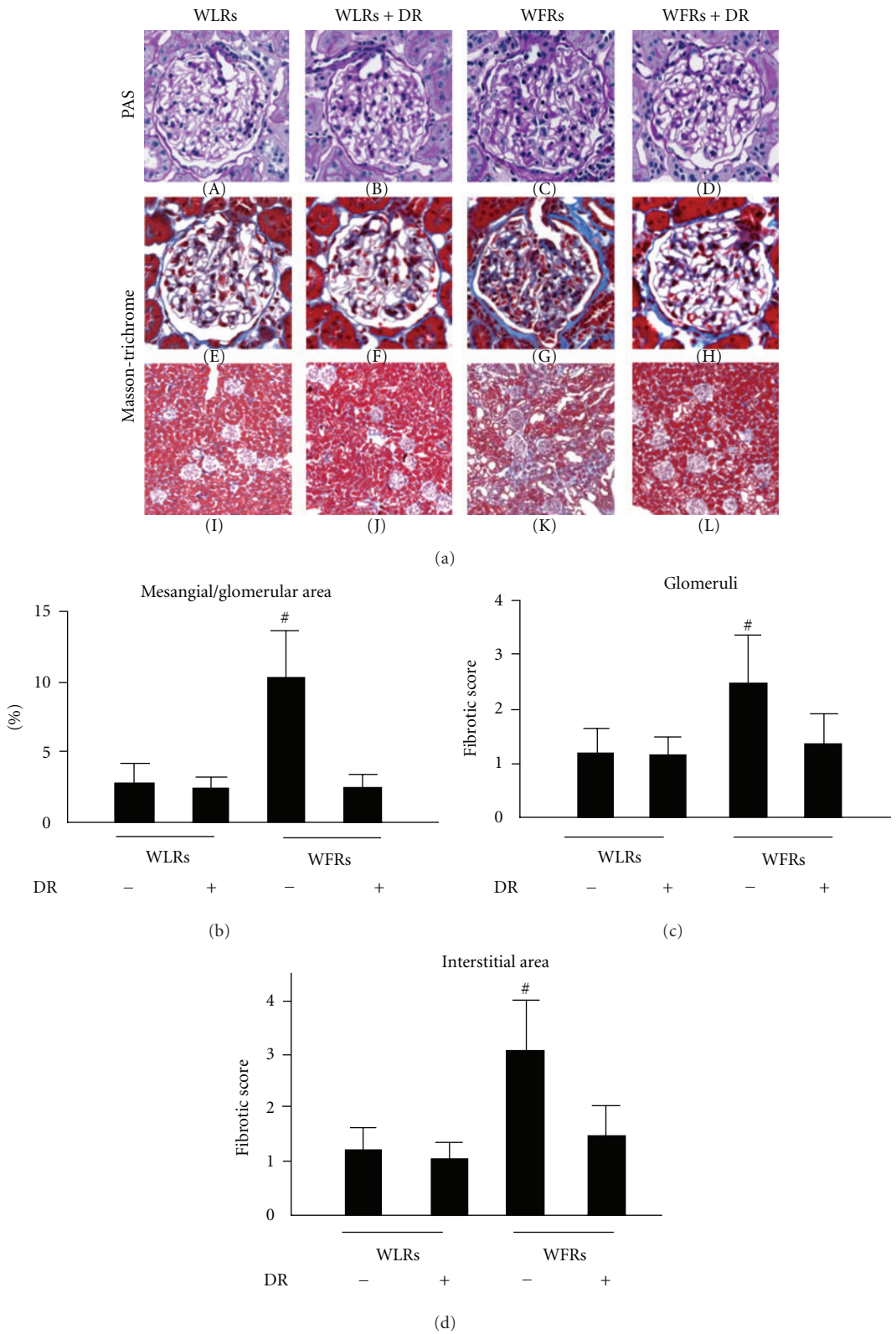

FIGURE 2: DR ameliorates mesangial expansion and renal fibrosis in WFRs. ((a)-(A) through (D)) Representative photomicrographs of PAS-stained kidney sections from four groups of rats. Data are the results of independent experiments in each group with six rats per group. Original magnification, ×400. (b) Quantitative assessment of the mesangial matrix area in the four groups of rats. Data are means ± SD. ($n = 6$, $^{\#}P < 0.01$ versus other groups). Treatment with DR reduced glomerular and interstitial fibrosis in WFRs. ((a)-(E) through (H), (I) through (L)) Representative photomicrographs of Masson-Trichrome staining in the four groups of rats. Data are the results of independent experiments in each group with six rats per group. Original magnification, ×400 for glomerular fibrosis and ×100 for interstitial fibrosis. (c) and (d) Quantitative assessment of fibrosis in the four groups of rats. Data are means ± SD ($n = 6$, $^{\#}P < 0.01$ versus other groups).

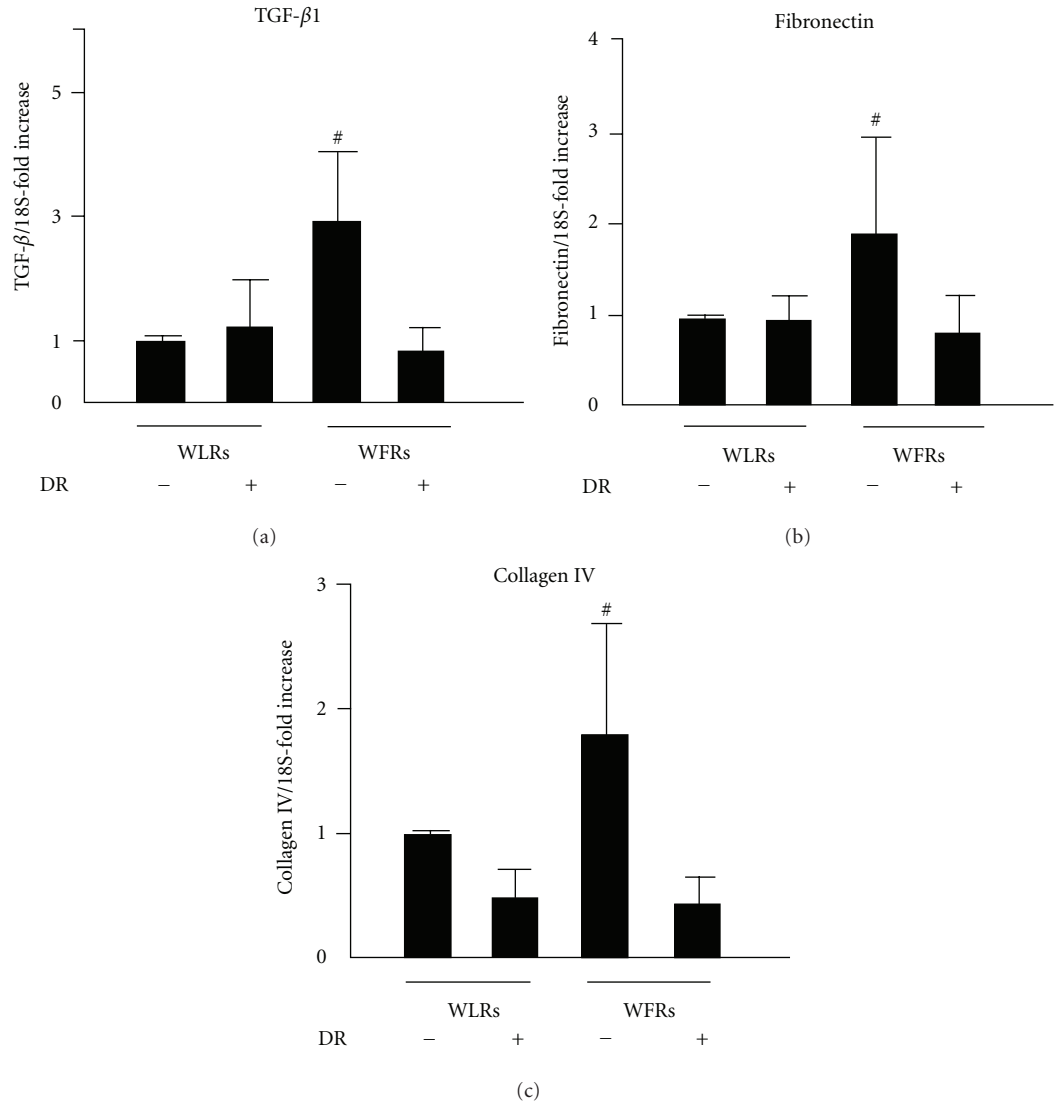

Figure 3: TGF-β, fibronectin, and collagen IV mRNA expression levels in the kidney. The mRNA expression levels of TGF-β (a), fibronectin (b), and collagen IV (c) were quantified using real-time PCR and expressed as fold increases from Wistar lean rats (WLRs). Data are means $\pm$ SD ($n = 9 - 11$, $^{\#}P < 0.05$ versus other groups).

and the results are expressed as the total amount of albumin excreted in 24 h urine collection. Body weight-adjusted creatinine clearance (Ccr) was calculated with the following equation: Ccr = urine creatinine (mg/dL) $\times$ urine volume (μL/min)/serum creatinine (mg/dL)/body wt (g) [17].

2.4. Morphological Analysis and Immunohistochemistry. Paraffin sections (3 μm thick) of the fixed and processed kidneys were stained with periodic acid/Schiff (PAS) reagent or Masson-Trichrome. To assess the mesangial expansion, 20 glomeruli, that were randomly selected from each rat were cut at the vascular pole, and the periodic acid/Schiff-(PAS-) positive material in the mesangial area and glomerular tuft area was measured using computer-assisted color image analysis (Micro Analyzer; Japan Poladigital, Tokyo, Japan) as described previously [18]. For the semiquantitative evaluation of fibrosis by Masson-Trichrome staining in the kidney, 20 randomly selected glomerulus or tubulointerstitial areas per rat were graded in a double-blind manner, as reported previously, with minor modifications [10].

Immunohistochemical staining of 3 μm paraffin sections was performed as described previously [18]. In brief, the sections were deparaffinized and rehydrated by incubating at room temperature in xylene three times for 3 min, in 100% ethanol twice for 3 min, in 95% ethanol twice for 3 min, and once in Tris-buffered saline (TBS; 0.1 mol/L Tris-HCl, pH 7.4, 0.15 mol/L NaCl) for 5 min. The sections were immersed in 3% H_2O_2 absolute methanol solution at room temperature for 15 min. After washing with TBS, sections were incubated overnight at 4°C with primary antibodies raised against ED-1 (1 : 50), followed by amino acid polymers that are conjugated to multiple molecules of peroxidase and antimouse IgG and washed with TBS. The sections were stained with ACE solution for 20 min at room

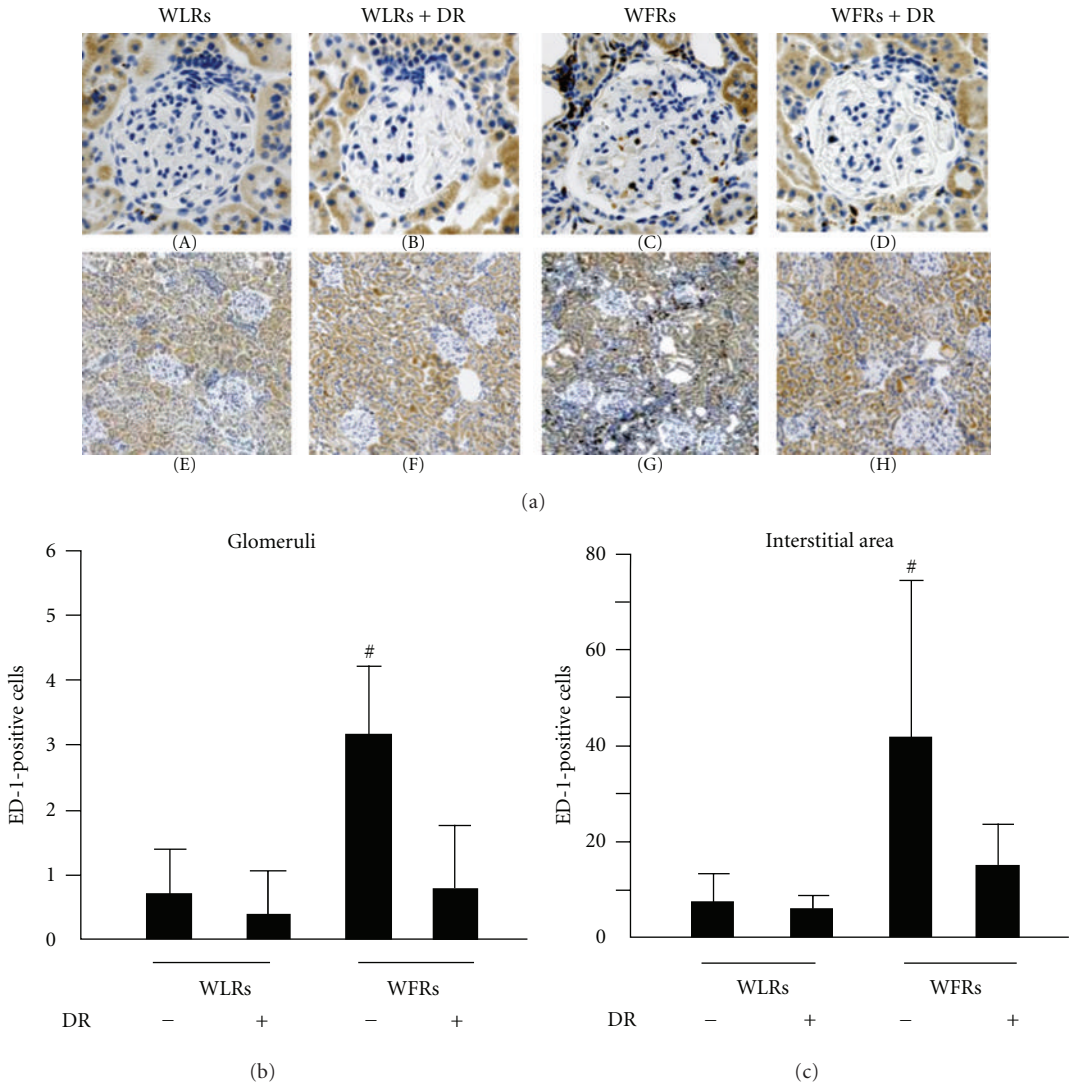

Figure 4: Treatment with DR suppresses the number of ED-1-positive cells in the kidney of WFRs. ((a)-(A) through (H)) Representative photomicrographs of renal ED-1-positive cells in the four groups of rats. Data are the results of independent experiments in each group with six mice per group. Original magnification, ×400 for glomerular ED-1 staining and ×100 for tubule-interstitial ED-1 staining. (b) and (c) ED-1-positive cells in glomerular and tubulointerstitial lesions. Data are means ± SD ($n = 6$, $^{\#}P < 0.05$ versus other groups, $^{\#\#}P < 0.01$ versus other groups).

temperature and washed with distilled water, followed by counterstaining with hematoxyline. For quantitative analysis of ED-1 staining, ED-1-labeled cells in the 20 randomly selected glomeruli and interstitial areas of the renal cortex were counted per each animal and analyzed individually as described previously [18, 19]. An investigator who was blinded to the sample identity performed the image analysis.

2.5. Western Blot Analysis. The renal cortex sample was homogenized in ice-cold RIPA buffer. Samples of protein solutions from the kidney were used for Western blotting. These samples were separated on 15% SDS-PAGE gels and transferred to a polyvinylidene difluoride filter (Immobilon; Millipore, Bedford, MA). After blocking with 5% milk, the filter was incubated overnight with an anti-acetylated-NF-κB (p65) (1 : 1000), NF-κB (1 : 1000), p62/Sqstm1 (1 : 1000), or Sirt1 antibody (1 : 1000) at 4°C. The filter was then incubated with the appropriate HRP-conjugated secondary antibodies (Amersham, Buckinghamshire, UK), and the bands were detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

2.6. Quantitative RT-PCR. The isolation of total RNA from the renal cortex and the determination of cDNA synthesis by reverse transcription and quantitative real-time PCR were performed as described previously [14]. The PCR primer sets are listed below. TGF-β: sense (5′-3′) TGCGCCTGCAGAGATTCAAG, antisense (5′-3′) AGGTAACGCCAGGAATTGTTGCTA, fibronectin: sense (5′-3′) GCACATGTCTCGGGAATGGA, antisense

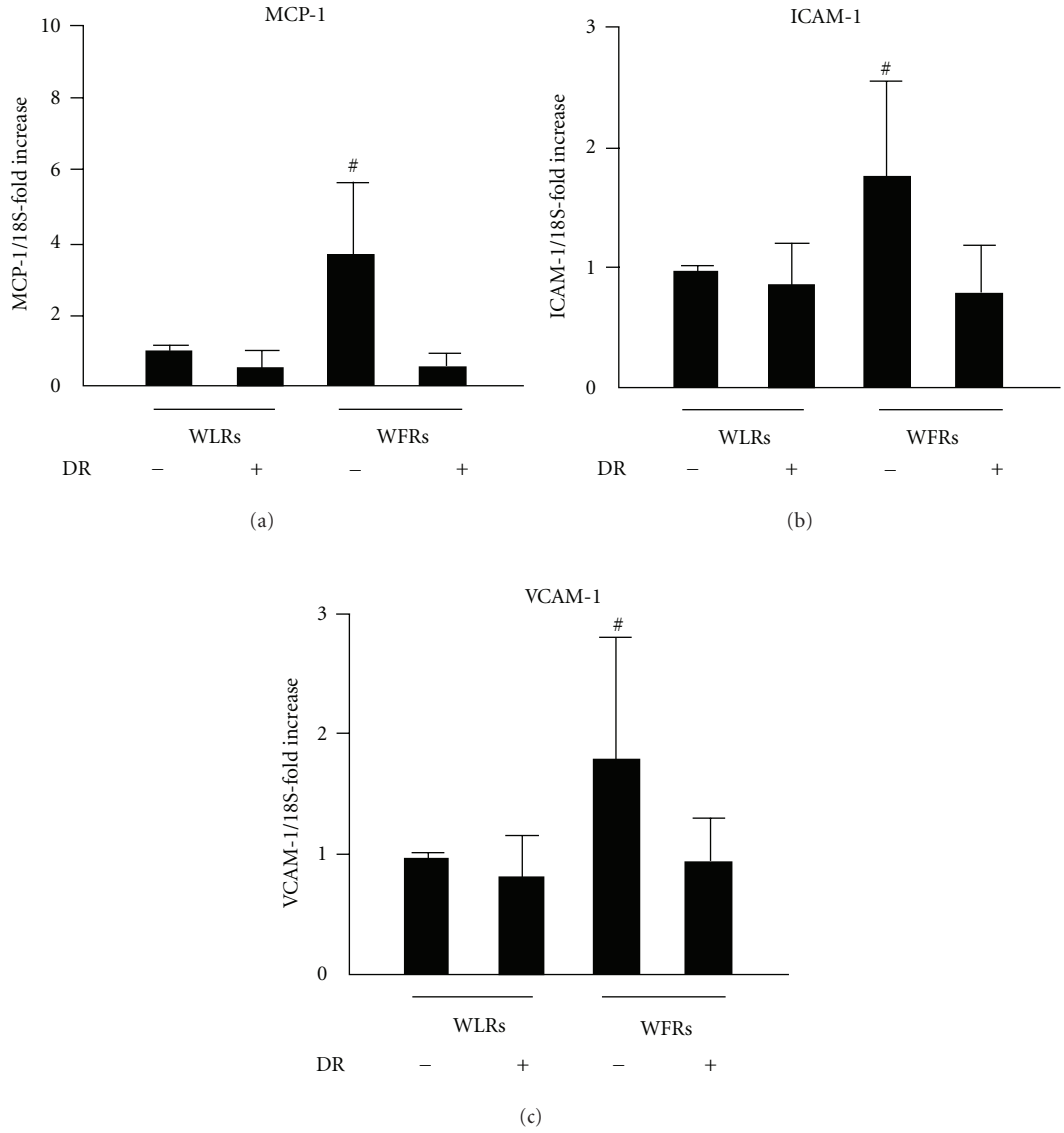

Figure 5: MCP-1, ICAM-1, and VCAM-1 mRNA expression levels in the kidney. The mRNA expression levels of MCP-1 (a), ICAM-1 (b), and VCAM-1 (c) were quantified using real-time PCR and expressed as fold increases from Wistar fatty rats. Data are means ± SD ($n = 9-11$, $^{\#}P < 0.05$ versus other groups).

(5′-3′) ACACGTGCAGGAGCAAATGG, collagen IV: sense (5′-3′) GTGTCAGCAATTAGGCAGGTCAAG, antisense (5′-3) CTGGTGTTGGAAACCCTGTGAA, MCP-1: Sense (5′-3′) CTATGCAGGTCTCTGTCACGCTTC, antisense (5′-3′) CAGCCGACTCATTGGGATCA, ICAM-1: sense (5′-3′) ACAAGTGCCGTGCCTTTAGCTC, Anti-sense (5′-3′) GATCACGAAGCCCGCAATG, VCAM-1: sense (5′-3′) GGA-TGCCGGAGTATACGAGTGTG, antisense (5′-3′) CAATGGCGGGTATTACCAAGGA,18S: Sense (5′-3′) TTCCGATAACGAACGAGACTCT, Anti-sense (5′-3′) TGGCTGAACGCCACTTGTC.

2.7. Electron Microscopy. Part of the harvested kidney was cut into small tissues blocks (1 mm^3) and fixed in 2% glutaraldehyde in 0.1 M potassium phosphate sodium buffer at 4°C for examination by electron microscopy. After postfixation with 2% osmium tetroxide, tissues were dehydrated in a series of graded ethanol solutions. Ethanol was then substituted for propylene oxide, and the samples were embedded in epoxy resin. Ultrathin sections were double stained with uranyl acetate and lead citrate. Sections were examined using a JEM1200EX electron microscope (JEOL, Tokyo) at 80 keV. The mitochondria morphology and autophagosomes in the proximal tubular cells were observed by electron microscopy as described previously [10, 14].

2.8. Statistical Analysis. Data are expressed as means ± SD. The Tukey multiple-comparison test was used to determine the significance of pairwise differences among three or more groups. $P < 0.05$ was considered significant.

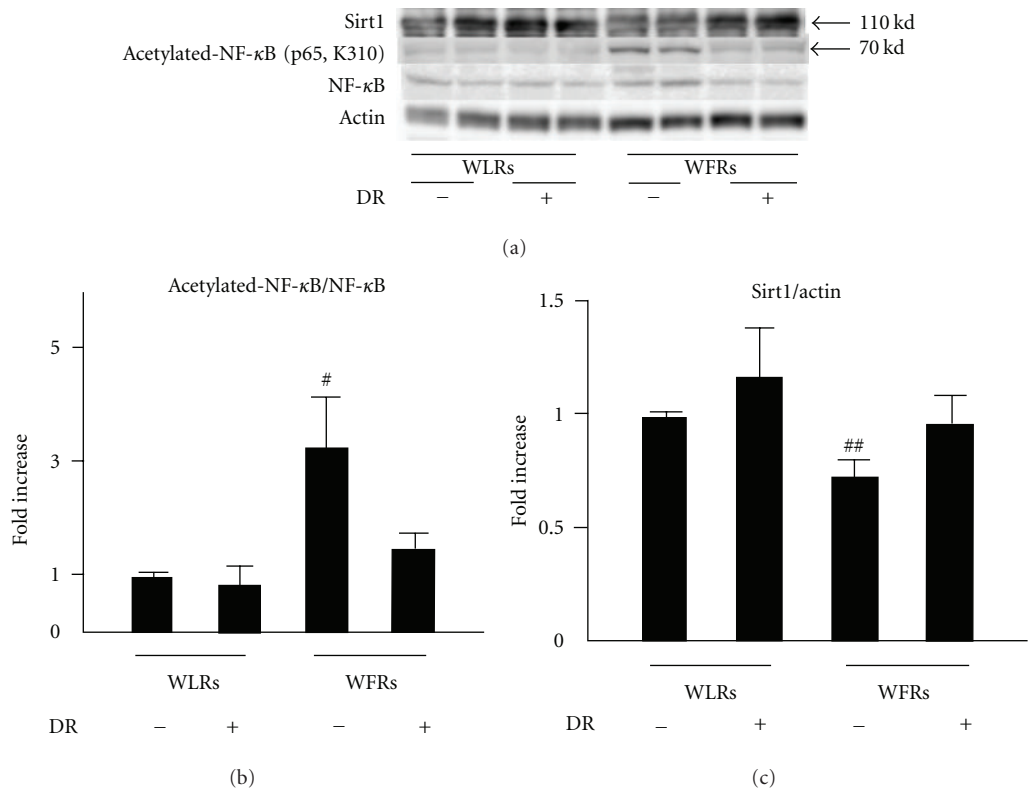

Figure 6: Acetylated-NF-κB (p65) and Sirt1 expression in the kidney. (a) Representative immunoblots of Sirt1, acetylated-NF-κB (p65), and NF-κB in protein extracts from the kidneys of rats of each group. Actin was loaded as an internal control. (b) Quantitative analysis of acetylated-NF-κB (p65) protein expression. (c) Quantitative analysis of Sirt1 protein expression. Data are means ± SD ($n = 6$, $^{\#}P < 0.01$ versus other groups, $^{\#\#}P < 0.05$ versus other groups).

3. Results

3.1. Characteristics of Experimental Rats. The characteristics of the four groups of rats at the end of the experimental period were shown in Table 1. The whole body and kidney weights were significantly higher in WFRs than in the other group. The systolic blood pressure (SBP) was not significantly changed in any groups. The WFRs exhibited elevated fasting blood glucose levels and glycated-Hb compared to WLRs, and DR induced a partial improvement in glycated-Hb levels by the end of the experimental period. Serum lipid profiles including T-CHO and TG levels were also significantly elevated in WFRs compared to WLRs; the increases in T-CHO and TG levels were partially rescued by DR (Table 1).

3.2. Changes in Urinary Albumin Excretion and Creatinine Clearance (Ccr). To evaluate the effects of DR on renal dysfunction in WFRs, we measured the urinary albumin excretion and Ccr. The values for urinary albumin excretion were higher, and the Ccrs were lower in WFRs than those in WLRs. Treatment with DR significantly reduced the urinary albumin excretion and restored Ccr, which indicated that DR ameliorated the functional abnormalities of nephropathy in WFRs (Figures 1(a) and 1(b)).

3.3. Changes in Kidney Morphology. Representative photomicrographs of mesangial matrix accumulation in the PAS-stained kidneys of the four groups were shown in Figure 2(a)—(A)–(D). The results of the quantitative analysis of mesangial matrix expansion in all groups were shown in Figure 2(b). Although the ratios of mesangial matrix/glomerular area were significantly larger in WFRs than that in WLRs, DR significantly ameliorated the mesangial expansion in WFRs.

The renal fibrosis determined by Masson-Trichrome staining (Figure 2(a)—(E) through (H) and (I) through (L)) also revealed a significantly higher score for renal glomerular and tubulointerstitial lesions in WFRs than those in WLRs (Figures 2(c) and 2(d)). DR reduced the increase in score for Masson-Trichrome staining in WFRs (Figures 2(c) and 2(d)).

3.4. mRNA Levels of TGF-β1, Fibronectin, and Collagen IV in the Kidney. We also assessed TGF-β1, fibronectin, and collagen IV mRNA expression levels in the kidney. The mRNA expression of these fibrosis-related genes was significantly higher in the kidneys of WFRs than that in the kidneys of WLRs (Figures 3(a)–3(c)). These alterations in WFRs were improved by DR.

Table 1: Effects of DR on body weight, kidney weight, blood pressure, blood glucose, glycated-Hb, and lipid profiles in the four groups of rats.

	WLRs ($n = 10$)	WLRs + DR ($n = 11$)	WFRs ($n = 10$)	WFRs + DR ($n = 9$)
Body weight (g)	426.3 ± 31.4	264.2 ± 32.7[a]	671.3 ± 70.3[a]	489.8 ± 34.8[b]
Kidney weight (g)	2.8 ± 0.17	1.8 ± 0.06[a]	3.0 ± 0.35[a]	2.23 ± 0.91[a]
Systolic blood pressure (mmHg)	121.9 ± 6.8	113.0 ± 11.0	126.3 ± 15.4	120.3 ± 5.1
Fasting blood glucose (mg/dL)	96.7 ± 8.8	95.7 ± 5.6	111.1 ± 11.9[c]	115.4 ± 6.1[c]
Glycated-Hb (%)	3.1 ± 0.25	3.1 ± 0.17	6.1 ± 0.56[c]	4.9 ± 0.9[c,d]
Total cholesterol (mg/dL)	132.2 ± 14.6	88.4 ± 6.7[a]	207 ± 32.3[c]	164.0 ± 26.5[b]
Triglyceride (mg/dL)	25.3 ± 4.9	20.5 ± 10.0	179.4 ± 93.3[c]	77.4 ± 25.5[c,d]

Data are means ± SD.
Data are means ± SD [a]$P < 0.01$ versus other groups, [b]$P < 0.05$ versus other groups, [c]$P < 0.01$ versus WLRs, WLRs + CR, [d]$P < 0.05$ versus WFRs.

3.5. Changes in Macrophage Infiltration in the Kidney. The number of ED-1 (a macrophage marker)-positive cells in the renal glomeruli and interstitial lesions was significantly higher in WFRs than that in WLRs (Figure 4(a)—(A) through (H)). DR reduced the number of ED-1-positive cells in the renal glomeruli and interstitial lesions of WFRs.

3.6. mRNA Levels of MCP-1, ICAM-1, and VCAM-1 in the Kidney. We next determined the mRNA expression levels of inflammation-related genes such as MCP-1, ICAM-1, and VCAM-1 in the kidney. The mRNA expression of these genes was significantly higher in the kidneys of WFRs than those of WLRs (Figures 5(a)–5(c)). These changes in WFRs were almost completely abrogated after DR. The results in Figures 2–5 indicate that DR reduces glomerular and interstitial histological abnormalities, including mesangial expansion, renal fibrosis, and macrophage infiltration, in the kidney of WFRs.

3.7. Changes in the Acetylation of NF-κB and Sirt 1 Protein Expression in the Kidney. We assessed the changes in acetylation of NF-κB and Sirt 1 protein expression in the kidney (Figures 6(a), 6(b), and 6(c)). The level of acetylated-NF-κB (p65) was significantly increased in the kidney of WFRs compared to that of WLRs. However, the protein expression of Sirt1 was decreased in the kidney of WFRs. This increase in acetylated-NF-κB in the kidney of WFRs was almost completely reversed, with the level being similar to the basal levels of WLRs, after treatment with DR, which was consistent with the restoration of Sirt1 expression.

3.8. Changes in Autophagy in the Kidney. The accumulation of p62/Sqstm1, which is degraded through an autophagy-lysosome pathway [8], was significantly enhanced in the kidneys of WFRs (Figures 7(a) and 7(b)). Mitochondrial morphology was also altered, resulting in marked swelling and the disintegration of cristae in the renal cortex of WFRs. Under normal circumstances, damaged mitochondria are degraded by the intracellular autophagy pathway [8]. In contrast to the proximal tubular cells of WFRs, DR resulted in a restoration of abnormal mitochondrial morphology with numerous autophagosomes in WFRs (Figure 7(c)).

4. Discussion

In this study, we demonstrated the potential benefits of DR in ameliorating renal injuries of type 2 diabetes. DR exerted anti-inflammatory effects and improved the dysregulation of autophagy through the restoration of Sirt1 protein expression in the kidney of WFRs.

Numerous reports have shown that DR extends the lifespan of yeast, worms, flies, and mammals [3]. Recently, Colman et al. also reported that DR delayed the onset of age-associated pathologies, including diabetes and cardiovascular disease, in rhesus monkeys [4]. Moreover, Fontana et al. showed that DR in humans improved metabolism and decreased serum C-reactive protein, TNF-α, and carotid IMT thickening [20]. These findings suggest that the various protective effects of DR against vascular impairment are exerted through the reduction of vascular inflammation, which is strongly involved in the molecular alterations that occur in aging or age-related diseases [21]. The inflammatory process is one of the pivotal mechanisms for the initiation and progression of diabetic nephropathy and atherosclerosis [2]. However, there is little information about whether DR improves diabetic nephropathy in type 2 diabetes, especially diabetes-induced inflammation in the kidney.

First, we investigated the effects of DR on renal functional and histological abnormalities in a model of type 2 diabetes. Diabetic WFRs clearly showed increased albuminuria, a reduction in Ccr, and increased mesangial expansion and renal fibrosis. Moreover, an increase in macrophage infiltration accompanied by the overexpression of inflammation-related genes, including MCP-1, ICAM-1, and VCAM-1, was observed in the kidney of WFRs. Treatment with DR resulted in complete improvement of diabetes-induced renal injuries, including inflammation, and a partial reduction of glycated-Hb levels by approximately 20% compared to WFRs. These results indicate that the effects of DR on diabetic nephropathy may be exerted through other factors rather than its effects of an improvement of systemic metabolism such as hyperglycemia and dyslipidemia. What is the other factor? We here focused on Sirt1 as the other factor induced by DR because DR is known to significantly increase the levels of Sirt1 protein expression in most tissues, including the kidney, and the beneficial effects of DR are linked to Sirt1 activation [5, 6, 22].

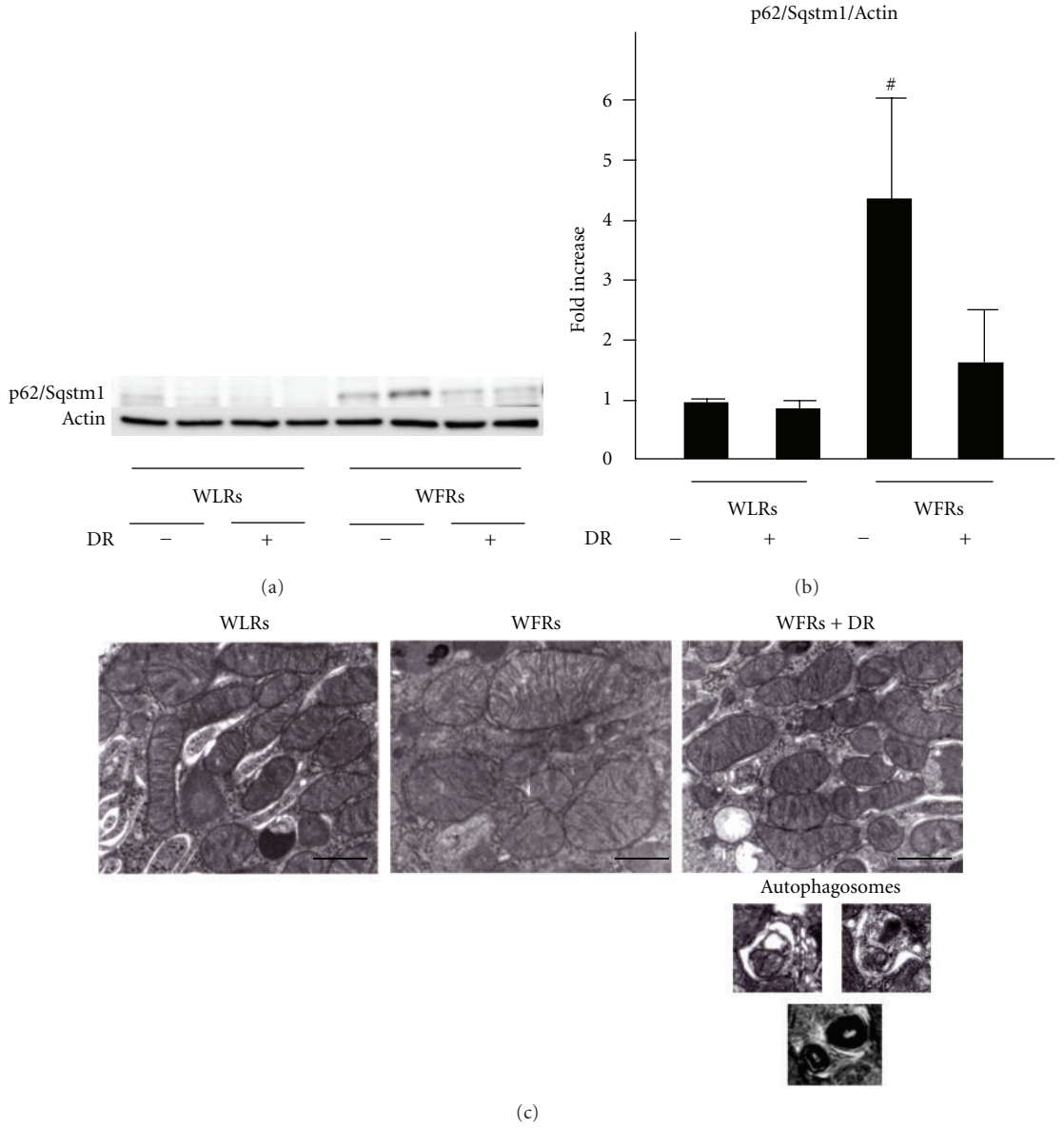

Figure 7: p62/Sqptm1 accumulation and electron microscopy in the kidney. (a) Representative immunoblots of p62/sequestosome 1 (Sqptm1) in protein extracts from the kidneys from rats of each group. Actin was loaded as an internal control. Data are means ± SD ($n = 6$, $^{\#}P < 0.01$ versus other groups). (b) Quantitative analysis of p62/Sqptm1expression. Data are means ± SD ($n = 6$, $^{\#}P < 0.05$ versus other groups). (c) Representative micrographs of proximal tubular cells from the, four groups of rats. Scale bar = 1 μm ($n = 3$).

Therefore, we asked whether Sirt1 protein expression was altered in the kidney of WFRs. Sirt1 protein expression was significantly decreased in the kidney of WFRs as compared to WLRs, and this alteration of Sirt1 expression was restored by treatment with DR. Sirt1 has anti-inflammatory properties through the deacetylation of NF-κB (p65) [23, 24], which plays a central role in the regulation of the expression of inflammation-related genes, such as MCP-1, ICAM-1, and VCAM-1. Several reports have also shown that reduced levels of Sirt1 lead to the upregulation of acetylated-NF-κB (p65), resulting in an increase in inflammation in the adipose tissue of high-fat diet-induced obese mice [25] or the monocytes of patients with chronic obstructive pulmonary disease (COPD) related to smoking [26]. In the present study, we showed that acetylated-NF-κB (p65) was clearly increased in the kidney of WFRs compared to WLRs, and this alteration of acetylated-NF-κB (p65) in WFRs was lessened by DR. This result is consistent with the restoration of Sirt1 protein expression. The p65 subunit of NF-κB selectively interacts with Sirt1, which promotes the deacetylation of the p65 subunit and leads to the attenuation of the transcriptional activity of NF-κB and inflammation [23]. Therefore, our results indicate that renal inflammation was induced by increased levels of acetylated-NF-κB (p65) owing to the

reduced levels of Sirt1 protein expression, and DR exerted anti-inflammatory effects through the restoration of Sirt1 expression in the kidney of WFRs.

Autophagy is a lysosomal degradation pathway in cellss and plays a crucial role in removing protein aggregates and damaged or excess organelles, such as mitochondria, to maintain intracellular homeostasis and maintain the cell health under various stress conditions [8, 9]. The renoprotective role of autophagy has been shown in several animal experimental models, such as aging [10] and acute kidney injuries [11–14]. In the present study, we found that mitochondrial morphological damages in the proximal tubular cells and p62/Sqstm1 accumulation in diabetic kidneys of WFRs occurred, which suggested that an impairment of autophagy system induced mitochondrial damage. Because Sirt1 is one of the positive regulators of autophagy, the decrease in Sirt1 expression observed in the diabetic kidney may lead to the dysregulation of autophagy. In addition, the phosphorylation of AMPK, which positively regulates Sirt1 activity [27, 28] and autophagy [8], was also decreased in the diabetic kidney (data not shown). DR improved the function of autophagy system, which resulted in normalization of mitochondrial morphological changes and p62/Sqstm1 accumulation accompanied by the restoration of Sirt1 expression and AMPK activation in the kidney of WFRs. We also confirmed the existence of numerous autophagosomes in the proximal tubular cells of the kidney in WFRs treated with DR, consistent with the existence of normal morphological mitochondria. In addition, when autophagy is inhibited, p62/Sqstm1 accumulation alters the NF-κB pathway and leads to inflammation [29, 30]. These data from previous reports suggest that p62/Sqstm1 accumulation associated with the dysregulation of autophagy is involved in increased inflammation. However, the role of autophagy in the pathogenesis of diabetic nephropathy and the relationship between inflammation and dysregulation of autophagy are still unclear. Therefore, further study will be needed to clarify these mechanisms in detail.

5. Conclusions

The current study demonstrated the beneficial effects of DR on renal injuries, including inflammation, in type 2 diabetic nephropathy model. The decrease in Sirt1 protein expression in the diabetic kidney may lead to inflammation through increased levels of acetylated-NF-κB (p65) and through the dysregulation of autophagy. DR may ameliorate the inflammation and the dysregulation of autophagy via the restoration of Sirt1 protein expression in the diabetic kidney. Therefore, Sirt1 may be a significant therapeutic target for the prevention of nephropathy in type 2 diabetes.

Conflict of Interests

The authors declare no conflict of interests.

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