

New Insights into the Molecular Mechanisms of Podocytes Injury in Diabetes

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Abstract

Pandemia of diabetes mellitus (DM) remains one of the biggest worldwide medical problems associated with dangerous complications, particularly diabetic nephropathy (DN), which is the leading cause of the end-stage renal disease. It was confirmed convincingly today that podocytes injury is implicated primarily or secondarily in various proteinuric glomerular diseases, including DN. The characteristic signs of podocytopathy are podocytes hypertrophy, foot process effacement, detachment from the glomerular basal membrane and apoptosis. Structural and functional alterations of podocytes evolve early, outpacing clinically significant albuminuria. The increasing loss of podocytes is associated with morphological and clinical signs of DN progression.

This review details the molecular and cellular events, mediators and signaling pathways that contribute to podocytes injury in DM, discusses the most important achievements, clinical and experimental data in these issues, describes the urinary biomarkers of podocytes injury.

Keywords: Diabetic nephropathy; Podocytes injury; Podocytes effacement; Proteinuria; Podocytopenia; Glomerulosclerosis; Urinary biomarkers

Abbreviations:

DM: Diabetes Mellitus, DN: Diabetic Nephropathy, AU: Albuminuria, PU: Proteinuria, MAU: Microalbuminuria, NAU: Normoalbuminuria, T1DM: Type1 Diabetes Mellitus, T2DM: Type2 Diabetes Mellitus, FP: Foot Process, GBM: Glomerular Basal Membrane, SD: Slit Diaphragm, GFR: Glomerular Filtration Rate, RAAS: Renin-Angiotensin-Aldosterone-System, AT-II: Angiotensin II, AT1R: AT-II type 1 receptors, PPR: Peceptor for prorenin, AGEs: Advanced Glycation End Products, RAGE: Receptor for AGE, EMT: Epithelial-mesenchymal transition, TGF-β1: Transforming Growth Factor β1, VEGF: Vascular Endothelial Growth Factor, VEGFR: Vascular Endothelial Growth Factor Receptor, ROS: Reactive Oxygen Species, MAPK: Mitogen-

activated Protein Kinase, TRPC: Transient Receptor Potential Canonical Channel, NADPH: Nicotinamide Adenine Dinucleotide Phosphate, NOX: NADPH Oxidases, mTOR: Mammalian Target of Rapamycin, MMP-9: Matrix Metalloproteinase 9, NFκB: Nuclear Factor Kappa-light-chain-enhancer of Activated B cells, WT1: Wilms Tumor Protein 1, ELISA: Enzymelinked Immunosorbent Assay

Introduction

Till today pandemia of diabetes mellitus (DM) remains one of the biggest worldwide medical problems. 425 million people in the World have DM, there will be 629 million in 2045 [1]. One of the greatest dangers of DM is diabetic nephropathy (DN) that develops in 30-40% of diabetic patients and often leads to the end-stage renal disease [2,3].

The various cell types are involved in the kidney damage including glomerular endotheliocytes [4], mesangiocytes [5], podocytes [6] and tubular epitheliocytes [7]-all of them are the targets of hyperglycemic injury. Several decades ago scientific interest was focused on the mesangium proliferation, mesangial matrix expansion as a cellular and molecular basis of DN ("mesangiocentric" DN concept) [8]. However, this DN theory did not explain clearly the origin of proteinuria (PU), especially massive. While a full understanding of the PU mechanisms in DN remains to be achieved, emerging studies have emphasized the crucial role of podocytes in these processes [9-15].

It was confirmed by the morphological data, that podocytes changes are primarily or secondarily implicated in various proteinuric glomerular diseases [16,17]. Moreover, there are evidences of podocytes depletion associated with the glomerulosclerosis in chronic progressive glomerulopathies, including DN [9]. Therefore, podocytes dysfunction and loss, their relationship with metabolic and hemodynamic disorders have attracted scientific attention today in order to find new methods of early DN diagnostic and prevention of disease progression.

In this article the molecular mechanisms of podocytes injury in DN are discussed, the most important achievements in these issues, clinical and experimental data are reviewed.

Podocytes Pathobiology in DN

The unique podocyte structure provides a wide range of its functions and the adaptive responses in physiological conditions, but at the same time makes this cell very sensitive to injury. Under the influence of various pathogenic factors (toxic, metabolic, hemodynamic) structural and functional changes occur in podocytes [9,10,15,18,19]. The characteristic features of podocytes injury (podocytopathy) are the foot process (FP) widening and effacement, podocytes hypertrophy, apoptosis, detachment from the glomerular basal membrane (GBM) and loss into the urinary space (podocyturia) and podocytes number reduction in the glomeruli (podocytopenia).

The diagnosis of podocytopathy can be carried out by morpho-pathological examination of kidney biopsy material (light and electron microscopy, immunohistochemical identification of podocytes specific proteins) or by the detection and quantification of urinary biomarkers (Figure 1).

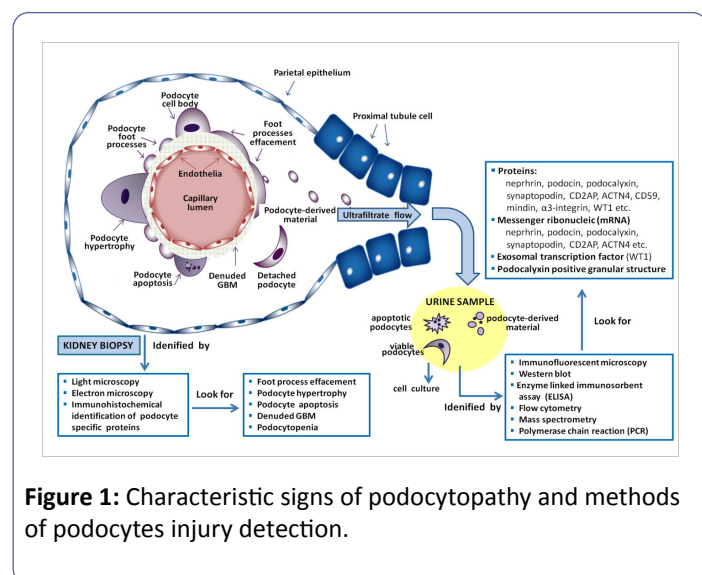


Figure 1: Characteristic signs of podocytopathy and methods of podocytes injury detection.

Podocytes effacement

Podocytes can undergo a process termed effacement, where the normal cellular architecture of the FP is altered and results in flattening and spreading of the FP along the GBM and disruption of the slit diaphragm (SD). The effacement can manifest whenever the following occurs: i) the disruption of SD and its related protein-protein interactions; ii) the interference in the normal association of the FP with the underlying GBM; iii) the reorganization of the actin cytoskeleton and its related protein-protein interactions; iv) the disruption of the normal negatively charged apical surface of the FP [9]. The change in the podocyte shape is not simply a passive process following injury; it is the result of complex molecular interactions of different protein domains of podocytes [17].

The FP widening and effacement in type 1 (T1DM) and 2 DM (T2DM) has been confirmed by a number of experimental and clinical studies, and a direct correlation has been established between the severity of these changes and the albuminuria (AU)/PU degree [6,10,20,21].

The mechanisms of podocytes actin cytoskeleton damage in DN are still being studied. The role of improper calcium handling due to abnormal activation of the calcium TRPC6 and 5 (transient receptor potential canonical) channels, dysregulation of Rho GTPases activity and violated RhoA signaling in podocytes is widely discussed [22-24].

The slit diaphragm, the role of nephrin

The unique barrier, that regulates passage of macromolecules from the blood to the urine, is the SD, which represents the contacts between podocytes. Several proteins (nephrin, CD2AP, ZO-1, FAT, nephrin-like1, podocin, P-cadherin) determine the SD structure [14,25,26].

Transmembrane protein nephrin functions not only as the core component of the extracellular SD filtration network but also as a signaling scaffold via interactions at its short intracellular region [26]. The extracellular nephrin domain connects with nephrin molecules from the neighboring FP. The cytoplasmic nephrin domain is phosphorylated on tyrosine residues, that is critical to the regulation of various intracellular podocytes pathways, including the actin cytoskeleton remodeling, the PI3 kinase/Akt signal pathway activation, the calcium signaling and nephrin endocytosis [22,27-29]. Nephrin colocalizes with adherence junction proteins such as P-cadherin or FAT, and anchors lipid rafts to the filtration slit. The interaction with CD2AP connects nephrin to the actin cytoskeleton. Nephrin binding to podocin provides the function in cell signaling [26,30].

As it was shown by previous morphological studies in animal models and in humans, the impairment of nephrin expression in the SD was observed not only in congenital nephrotic syndrome with a NPHS1 gene mutation, but also in various acquired nephropathies [31-34]. The different studies of kidney biopsies in patients with DN revealed the decreased glomerular expression of nephrin and the interrelation of these changes with the disorder of podocytes FP [35-37].

It is discussed that focal defects of the SD due to nephrin dissociation from podocytes actin cytoskeleton with following nephrin separation and its excretion into urine (nephrinuria), are some of the mechanisms of PU in various nephropathies [38]. Nephrinuria as a marker of podocytes damage was found in DM [34,37,39]. In a cross-sectional study Patari A, et al. assessed the presence of nephrin fragments in the urine of patients with T1DM by immunoblotting method [39]. Nephrinuria was revealed in 30% of DM patients with normoalbuminuria (NAU), in 17% of patients with microalbuminuria (MAU), and in 28% of patients with PU, whereas it was not detected in healthy individuals. Belinda Jim performed a quantitative estimation of nephrinuria by ELISA method in T2DM patients [37]. Nephrinuria was found in 100% of diabetic patients with MAU and PU, as well as in 54% of patients with NAU.

Relatedly, observation of the FVB/NJ Akita mouse DN model noted that urinary nephrin can be revealed before detectable AU develops [40]. It was shown that nephrinuria, evaluated by different methods, is closely correlated with the clinical manifestations of DN (MAU/PU, arterial hypertension, glomerular

filtration rate (GFR)) [34,37,41-43]. In the work of Ng D. nephrinuria was associated with lower GFR even among normoalbuminuric diabetic patients, who are traditionally perceived as having a low risk of chronic kidney disease [34]. The presented data pointed that nephrinuria is a sensitive indicator of glomerular injury in DM, therefore, it has a good perspectives of application for early diagnostic and monitoring of DN.

Podocyturia

Glomerular podocytes damage is accompanied by appearance of not only their structural-functional proteins in the urine, but also whole podocytes themselves due to their detachment from GBM [44]. The alterations in the level or activity of adhesive molecules are discussed as a possible mechanism of podocytes detachment. It was shown by the experimental models of DN and in the podocytes cell culture that even the short-term influence of hyperglycemia leads to the reduced expression of $\alpha3\beta1$ -integrins in podocytes and $\alpha3\beta1$ -integrin receptors in GBM, to the podocytes detachment from the GBM and their appearance in the urine (podocyturia) [45-47]. Disconnected from GBM podocytes usually die as a result of violations of cell-matrix relationships that are necessary to maintain the podocytes viability. Nevertheless, Vogelmann SU and other investigators were able to culture cells expressing typical podocytes markers from urine, indicating that some podocytes may remain viable [48].

Nakimura T, et al. [49] investigated in T2DM patients the urinary podocytes by immunofluorescence using antibodies against surface protein podocalyxin. The podocyturia was revealed in a half of patients with MAU and in 80% of patients with PU, furthermore, the number of labeled podocytes in the urine of patients with PU was significantly higher than in patients with MAU. Yu et al. [50] demonstrated that podocyturia was a more sensitive marker of glomerular damage than Hara et al. [51] using the ELISA method detected the podocyturia in a half of diabetic patients with normoalbuminuria, suggesting that the podocytes injury occurs early, prior to MAU appearance. Ye et al. [52] by a flow cytometry method also demonstrated the significantly increased number of podocalyxin-positive podocytes in DM patients with normal AU. The nonparametric ROC analysis confirmed that the sensitivity and specificity of the podocyturia are significantly greater than creatinine clearance, traditionally used for DN assessment.

Urinary biomarkers of podocytes injury, methods of detection

Whole podocytes themselves (viable as well as apoptotic), podocytes derived proteins, exosomes and granular structures may be detected in the urine of patients with glomerulopathy-all of them may be used as potential urinary tests for podocytes injury (**Figure 1**). Urinary biomarkers that have been found to be specific for podocytes detection include proteins (nephrin, podocin, synaptopodin, podocalyxin, mindin, Wilms tumor protein 1 (WT1) and etc.), specific messenger ribonucleic acids (mRNAs) (mRNAs of nephrin, podocin, synaptopodin, podocalyxin, CD2-associated protein-CD2AP, an exosomal transcription factor (WT1), and podocalyxin positive granular

structures. Different techniques can be used to identify the podocytes derivations, including the immunofluorescent staining, western blot, enzymelinked immunosorbent assay (ELISA), flow cytometry, mass spectrometry, polymerase chain reaction.

The main advantages of urinary diagnostic tests are high specificity, non-invasive detection and reproducibility, availability of measurement by relatively simple and sensitive methods (for instance, ELISA).

Over the past few decades, in addition to described above nephrin, other urinary podocytes biomarker were detected in DM, their associations with the clinical and morphological characteristics of DN were confirmed [41,42,51-56]. The utilization of the podocyte specific urinary biomarkers has not yet translated into common clinical practice, while the age-old quantification of PU, GFR and renal biopsy are the mainstays that clinicians have at hand. But these urinary biomarkers have good prospects for use in the near future as indicators of diabetic glomerulopathy presence/progression and therapeutic efficacy that in some cases may be more advantageous than the established parameters currently used in practice.

Podocytes apoptosis

Two mechanisms are proposed to explain the podocytes loss: detachment and apoptosis. The increased programmed cell death as a major cause of reduced podocyte number, leading to PU and glomerulosclerosis was showed in transforming growth factor $\beta1$ (TGF- $\beta1$) transgenic mice, CD2AP-/- mice and puromycin aminonucleoside (PAN)-treated rats [57-60]. It was discussed that ~90% of urinary podocytes are apoptotic [9]. The previous data also demonstrated that in DM podocytes are shed from the GBM not as a result of a defect in the podocytes themselves, but in response to some damaging stimulus in the glomerular compartment. Regulation of podocytes survival and death depends on the balance of pro- and antiapoptotic factors [9]. Apoptosis of podocytes is activated by the following factors: hyperglycemia, angiotensin II (AT-II), AT1-receptor, TGF- $\beta1$, Smad-7, reactive oxygen species (ROS), detachment from the GBM, mechanical tension, reduction of inhibitors of activated cyclic kinases p27 and p21, the basic fibroblast growth factor, apoptosis-inducing factor [9,57-59,61,62]. Antiapoptotic effects have been confirmed for a number of factors, including cyclin I, podocytes proteins (nephrin and CD2AP), intracellular apoptosis inhibitor Bcl-2, preserved cell-cell contacts, vascular endothelial growth factor (VEGF), hepatocytes growth factor, insulin-like factor, heat shock protein 27 and etc., [9,63].

A number of works *in vivo* and *in vitro* has already shed light on the delicate molecular mechanisms of podocyte apoptosis induced by leading damaging factors in DM, it will be detailed in the next section.

Autophagic process

Autophagy is the natural, multi-step process of lysosomal degradation, mediated by the autophagy-related genes and their associated enzymes. It plays a crucial role in removing protein aggregates and damaged or excess organelles, including

mitochondria, to maintain intracellular homeostasis. Adaptive autophagy may protect renal cells, including podocytes, against cellular stresses under diabetic conditions.

Impairment of podocytes autophagy by up regulation of mTORC1 pathways is implicated in the initiation and progression of DN [22,64,65]. Hyperglycaemia stimulates oxidative stress and ROS generation with activation of mTORC1, reduction of MAPK and Sirt1 that leads to autophagy impairment. Thus, improvement of autophagy by mTORC1 inhibitor, or MAPK and Sirt1 activators may be novel therapeutic options in DM, which in addition to RAAS blockers may slow down the DN progression.

Epithelial-mesenchymal transition (EMT)

The podocytes loss is facilitated by the activation of EMT [66]. It was shown in a cell culture that under the influence of the main inducer of EMT-TGF- β 1, podocytes lose ability to express proteins specific for podocytes (nephrine, podocin, P-cadherin, E-cadherin, ZO-1, etc.), they change epithelial phenotype and start to express the markers specific for mesenchymal cells (fibroblast-specific protein-1 (FSP1), desmin, matrix metalloproteinase 9 (MMP-9), α -Smooth muscle actin, etc.) [67,68]. As a result of these processes, the podocytes lose the normal cytoskeleton structure, cell polarity, intercellular contacts, and become mobile, resulting in enhanced detachment from GBM and the development of podocyturia. Like fibroblasts, the transdifferentiated podocytes acquire the ability to produce matrix proteins (fibronectin, collagen, etc.), thus speeding up the glomerulosclerosis formation.

In a retrospective cross-sectional analysis, greater amounts of FSP1-positive podocytes were observed in urinary sediments of diabetic patients with MAU than in those with normalbuminuria [69]. FSP1-positive podocytes selectively expressed Snail1, a known trigger for EMT. This study suggests that appearance of FSP1 in podocytes of diabetic patients is associated with more severe clinical and pathological findings of DN and correlates with podocytes detachment due to EMT process.

Podocytes hypertrophy

Glomerular hypertrophy is present in the early phase of DN. Podocytes in enlarged glomeruli have to cover a larger GBM area and are subjected to increased mechanical stress and injury [9,70,71]. Biochemically, hypertrophy is characterized by the entry of podocytes in G1-phase of the cell cycle, accompanied by an increase of protein content in the cells; however, under the certain circumstances, the podocytes stop in G1/S-phase without specific to the S-phase increase in DNA synthesis. These intracellular events determine the increase in size (but not numbers) of podocytes. The initial hypertrophy of the damaged podocytes is considered as adaptive. By this way the neighboring epithelial cells try to "patch up" the locuses of denuded GBM. However, after a certain time, the hypertrophy becomes maladaptive because the inducing mechanisms activate the apoptosis of hypertrophied and adjoining podocytes simultaneously. It was demonstrated *in vitro* and *in vivo* in diabetes that AT-II induces the podocytes hypertrophy and

regulates its intensity by increasing in the synthesis of inhibitors of cyclin-dependent kinases p21 and p27 [71,72].

Podocytopenia

Due to the limited proliferative capacity of podocytes, a massive podocytes loss leads to reduction in the glomerular podocytes number (podocytopenia). The consequences of different degrees of podocytes loss were demonstrated in rat models of regulated podocytes depletion [16,72,73]. Podocytes loss less than 20% causes the mesangial expansion only. Loss of more than 20% of podocytes leads to appearance of denuded areas of GBM and formation of synechia with Bowman's capsule. When a glomerulus losses between 20-40% of its podocytes, it renders itself to segmental sclerosis. The glomerulus becomes progressively sclerotic with more than 40% podocyte loss. Expressed depletion of more than 60% of podocytes leads to irreversible defect of the glomerular filter with the persistent high PU and the global glomerulosclerosis.

There are emerging experimental and clinical studies showing that the number of podocytes in the glomeruli is an important determinant of DN progression. Therefore, Steffes MW, et al. [74] in a longitudinal study of patients with T1DM have demonstrated that the decline in glomerular podocytes number is directly correlated with PU increase, and that podocytopenia can develop even in a short duration of DM. Meyer T, et al. [75] revealed in a longitudinal study of the Pima Indian population with T2DM that glomerular podocytes number was the most significant predictor of PU growth and GFR decline. Vestra M, et al. [21] showed in European cohort of patients with T2DM that glomerular podocytes pool decreases during progression NAU to PU. In the cross-sectional study of kidney biopsies of patients with T2DM White K, et al. [76] established a significant correlation between PU and the decrease of podocytes layer density and the reduction of podocytes number in the glomeruli.

Molecular events, mediating the podocytes injury in DM hyperglycemia

It was shown in murine T1DM and T2DM models that podocytes apoptosis increased sharply with onset of hyperglycemia and coincided with the onset of AU [61]. Elevated extracellular glucose concentration rapidly increased ROS generation *via* nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and mitochondrial pathways and induced podocytes apoptosis by activation of proapoptotic mitogen-activated protein kinase (MAPK) p38 and caspase-3 cascade [61]. Inhibition of NADPH oxidase activity prevented the podocytes apoptosis and AU [61]. Wang W, et al. showed that hyperglycemia induced mitochondria fission by promoting phosphorylation and lead to consequent generation of mitochondrial ROS [77]. Eid AA, et al. demonstrated that high glucose induced ROS by sequential upregulation of cytochrome p450 and its metabolite 20-hydroxyeicosatetraenoic acid, subsequent increase in the NADPH oxidases (Nox1 and Nox4) expression, and NADPH-dependent superoxide anion generation [78]. It was also shown that treatment of podocytes with named cytochrome p450 metabolite induced podocytes apoptosis similar to the effects of high glucose concentration. At the same

time inhibition of cytochrome P450A in OVE26 mice (T1DM model) prevented oxidative stress and reduced both FP effacement and apoptosis and significantly decreased AU [78]. It was demonstrated by Liu B.C. that high glucose induced ROS and activated TRPC6 resulting in intracellular Ca²⁺ overload and apoptosis [79]. It was reported in other studies that exposure of podocytes to high glucose increased the intracellular Ca²⁺ concentration and led to calcineurin activation and subsequent nuclear accumulation of nuclear factor of activated T-cells [80], whereas its inhibition ameliorated podocytes injury and DN in db/db mice [81].

Insulin signaling

Podocytes express the insulin receptors and are the main target of insulin action in the glomerulus, exhibiting insulin-dependent PI3K and MAPK signaling both *in vitro* and *in vivo* [30,82,83]. There is accumulating evidence that glomerular function is impaired when the podocytes are rendered insulin resistant. In animal models of type 1 and T2DM, glomerular insulin signaling was lost early in the DN progression of diabetic kidney disease [84]. Podocyte-specific deletion of insulin receptor causes AU, increased glomerular matrix accumulation, GBM thickening, changes of podocytes morphology and apoptosis [85].

The exposure of podocytes to high glucose abrogates insulin signaling by increasing the molecule Src homology-2 domain-containing phosphatase-1, which binds to the insulin receptors and prevents downstream signaling [86].

Impaired insulin signaling by altered levels of fatty acids in DM and related metabolic syndrome or proinflammatory cytokines that accumulate during insulin resistance is implicated in podocyte injury [30, 82, 83]. At the same time, the restoring insulin sensitivity and glucose uptake in glomerular podocytes can ameliorate the podocytes injury in DM [82,87].

The insulin pathway is closely associated with the mTOR (mammalian target of rapamycin) signaling in podocytes, which is important for cellular sensing of nutrient and growth factor levels as well as cellular stress [22,30]. Insulin via the insulin receptor and connection with insulin-like growth factor activates mTORC1 through Akt signaling; conversely, mTORC2 inhibits insulin signaling.

Under physiologic conditions, mTOR activity is essential for normal podocyte function. However, in DM elevated mTOR activity also mediates adverse effects, including podocytes hypertrophy, apoptosis, impairment of autophagy [22,30,64].

Renin-Angiotensin-Aldosterone System (RAAS)

Recent studies have shown that podocytes generate many components of the RAAS and express receptors of RAAS, including AT-II, mineralocorticoid and prorenin receptors [88-91]. High glucose concentrations induce AT-II synthesis by podocytes through activation of angiotensinogen expression [6]. Apart from hyperglycemia, the podocytes production of AT-II is activated by ROS, mechanical stretching, and PU components [91-93].

Crucial functions of podocytes, such as contraction, apoptosis, cytoskeletal organization and autophagocytosis, were shown to be regulated by the AT-II type 1 receptors (AT1R) [91,95-97]. Thus, it is believed that AT-II may promote PU via AT1R in podocytes without affecting blood pressure and glomerular hemodynamic changes. There is a large body of evidence that RAAS inhibitors reduce PU by inhibiting the effect of AT-II on podocytes directly [49,96,98,99]. So, it was demonstrated that podocytes responded to AT-II with an increase of the intracellular Ca²⁺ concentration and activation of Cl-conductance leading to podocytes depolarization. At the same time in rat glomerular *in situ* and in cell culture the effect of AT-II on Ca²⁺ and podocytes depolarization was completely inhibited by low concentrations of the selective AT1R antagonist losartan, suggesting that the effects were mediated by the AT1R [95]. Recently it was shown AT-II contributes to podocytes injury by increasing TRPC6 expression and this pathway is implicated in podocytes injury occurring in DN [23,24,100-102]. AT-II modulates the podocytes dysfunction by suppressing of the nephrin expression [103]. *In vitro* exposure to stretch induced a decrease of nephrin expression, which was mediated *via* AT1R [103]. Experimental studies in rat model with streptozotocin-induced DM and clinical studies in patients with T2DM showed that treatment with angiotensin-converting enzyme inhibitors or AT1R antagonists restored the nephrin expression and prevented the podocytes loss [49,98,99,102]. AT-II via AT1R stimulation increases nephrin- β -arrestin2 binding, nephrin endocytosis and glomerular permeability in mice [104]. This molecular mechanism of AT-II supports the use of AT1R blockers to prevent AU even in normotensives. AT-II increased VEGF expression in podocytes and AT-II mediated increase of 3 (IV) collagen expression were impaired by an inhibitor of VEGF signaling [106,107]. AT-II also decreased the expression of heparan sulfate proteoglycans on the cell surface and in the extracellular matrix of podocytes *in vitro*, suggesting that it might regulate the GBM permselectivity [108].

The expression of the prorenin receptors (PRR) by podocytes has been detected recently [88,90]. Under diabetic conditions, elevated plasma prorenin and rennin bind to the PRR in podocytes, resulting in an increased catalytic activity of these proteins and subsequently in local AT-II production [91, 108]. Moreover, prorenin and renin via PRR can activate ATII-independent intracellular MAPK p38 signaling, leading to podocytes apoptosis and PU [90,91,109]. The PRR were shown to be essential for podocytes autophagy and survival [110]. It has been revealed recently, that AT-II promotes autophagy in podocytes by ROS generation and enhanced podocytes expression of autophagic genes such as LC3-2 and beclin-1 [97]. As it was expected, the proautophagic effect of AT-II was inhibited by antioxidants [98]. The PRR have been assumed to be involved in the pathogenesis of DN by the release of cytokines [111]. The renin inhibitor aliskiren inhibited glucose-induced intracellular AT-II levels and profibrotic and apoptotic effects of high glucose concentrations in podocytes *in vitro*, although it did not inhibit PRR-dependent extracellular signaling [112,113].

Podocytes express mineralocorticoid receptors, and aldosterone may implement pleiotropic effects via this

communication [91,114]. Aldosterone is an important player of podocyte injury. Recently it has been shown that aldosterone induces the mitochondrial dysfunction, an increase of ROS and a reduction of the nephrin expression in podocytes [115]. So, the treatment with aldosterone antagonists and prevention of podocytes oxidative stress can be the other ways of podocytopathy correction [114,115].

Transforming growth factor β 1 (TGF- β 1)

Hyperglycemia, advanced glycation end-products (AGEs), VEGF, PU components (mainly albumin) activate the TGF- β 1 synthesis by podocytes [10,57,116]. Unlike mesangial cells, podocytes do not overexpress TGF- β 1 in response to AT-II. AT-II increases the podocytes expression of the TGF- β type II receptors [39]. Furthermore, AT-II may enhance the ROS generation and, subsequently, oxidative stress activates the latent TGF- β and the TGF- β signaling system [117,118].

TGF- β 1 suppresses the glomerular expression of α 3 β 1 integrin that leads to the impaired podocytes adhesiveness to GBM and their detachment [8,118,119].

The significant increase of TGF- β 1 induces podocytes apoptosis, which promotes podocyturia and podocytopenia. TGF- β 1 regulates the apoptosis *via* the Smad3- and Smad7-signalling pathways, which in turn inhibit NF- κ B, that encodes the synthesis of various cell survival factors [6,10,57,58]. It was revealed that TGF- β 1-induced mitochondrial Nox4 upregulation *via* the TGF- β 1receptor-Smad3 pathway is responsible for ROS production, mitochondrial dysfunction and apoptosis [120]. TGF- β 1 also mediates Smad-independent podocytes apoptosis *via* MAPK p38 and caspase-3 activation [82,118,119,121]. Recently an alternative theory has been put forward for TGF- β 1 mediated podocytes injury. TGF- β 1 increases mitochondrial membrane potential and oxygen consumption rate *via* mTOR pathway, resulting in increased ROS generation and podocytes apoptosis [122].

Several studies have shown an important role of TGF- β 1 as a key EMT inductor in various kidney diseases, including DN [66]. TGF- β 1 induces the desmosomes destruction, cell-matrix remodeling, stress fibers formation [57,64]. It was demonstrated that activated TGF- β 1 signaling in the podocytes may lead to the changes of podocytes phenotype, characterized by reduced expression of SD proteins, increased expression of mesenchymal markers (such as Snail, FSP1, desmin) and overproduction of α 3(IV) collagen and matrix-modifying enzyme MMP-9 [67,116-118,122]. Yamaguchi, et al. [69] revealed that FSP1-positive podocytes in the urine of diabetic patients selectively expressed Snail1 and integrin-linked kinase, a well-known trigger for EMT. The number of FSP1-positive podocytes correlated with the clinical and pathological findings of DN [69].

Furthermore, TGF- β 1 stimulates the VEGF expression by podocytes, involving in the mechanisms of increased glomerular permeability and glomerulosclerosis formation in DN [6,118,119].

To summarize, TGF- β 1 exerts multiple effects on podocytes in DM: (i) it reduces the podocytes adhesion to the GBM resulting in podocyturia; (ii) it induces the collagen synthesis by

podocytes resulting in GBM thickening (iii) it induces the podocytes EMT resulting in podocytes depletion; (iv) it impairs the architecture of SD and alters the podocytes permselectivity; (v) it increases of podocytes apoptosis resulting in podocytes depletion.

Vascular Endothelial Growth Factor A (VEGF-A)

VEGF-A is produced in great quantities by the glomerular podocytes. It is important for maintaining of the glomerular filtration barrier integrity and is a survival factor for podocytes, endothelial and mesangial cells [6, 124-126]. Activation of VEGF synthesis in podocytes was shown *in vitro* and *in vivo* by high glucose concentrations, AT-II (*via* ATR1 and ATR2), TGF- β 1, HIF-1 α (*via* AT-II and ATR2), AGEs, ROS, insulin) [6,124,127].

VEGF maintains glomerular filtration barrier integrity by signaling through VEGF-receptor 2 (VEGFR2), which is most abundantly expressed in the glomerular endothelium. There are clinical and experimental evidences that in DM glomerular VEGF-A expression and urinary levels can be elevated in the early stages of kidney disease [128-130] and significantly decreased in the foci of glomerulosclerosis that appears to be linked to the development of podocytopenia [6,30,131]. Interestingly, the induction of podocyte VEGF-A overexpression in adult mice causes glomerular disease with GBM thickening, glomerulomegaly, mesangial proliferation and FP effacement, a phenotype strikingly similar to early DN [132].

VEGF by binding the VEGFR2-nephrin-actin complex activates it and thus regulates the size and form of podocytes [6,124]. Experimental data showed that antiapoptotic action of nephrin is associated with its phosphorylation under the influence of VEGF *via* activation of Bcl-2 protein [124,133]. In contrast, reduce of nephrin phosphorylation contributes to podocytes apoptosis *via* nephrin linking with β -arrestin-2 [134]. DM violates the autoregulation of VEGF-signalling pathways. Thus, VEGF inhibits the nephrin formation in podocytes, promoting the FP violation. The decrease of VEGF expression impairs the podocytes survival, accelerating the podocytopenia [124]. The evidence has been obtained that VEGF-A stimulates the production of the α 3 chain of type IV collagen by podocytes, resulting GBM thickening and violation of its permeability [6,124]. It is believed that this synthesis is mediated by VEGF-A *via* TGF- β 1 and TGF- β 1receptor [6,124].

Reactive oxygen species (ROS)

Hyperglycemia and RAAS activation trigger the oxidative stress and ROS formation [13,15,135]. An important source of ROS production is NOX, which induces superoxide production *via* oxidation in the mitochondria, inflammation and stress, such ROS are implicated in the pathogenesis of DN [15,135-137]. Nox4 isoform, which is most abundantly expresses in the kidney and localizes within mesangial and tubular cells, podocytes, plays a critical role in oxidative stress in kidney disease [6,13,136,137]. Presented above data, mainly experimental, demonstrated ROS-induced actin polymerization resulting in cytoskeleton and FP violations, podocytes detachment, apoptosis and loss. So, in mice with streptozotocin-induced DM,

the kidney tissue expressed high concentration of NOX and intensively produced ROS [13]. Suppression of renal NOX is a promising strategy against renal damage [135-137]. Antioxidant drugs including apocynin, an inhibitor of the translocation of cytosolic components of NOX, p38 MAPK inhibitor, or tempol, a superoxide dismutase mimetic, also show renoprotective effect in DM, resulting in restoration of $\alpha 3$ -integrins expression by podocytes, reduction of podocyte apoptosis and detachment, mesangial matrix expansion and diminution of AU/PU [6,13].

Glycation end-products (AGEs)

AGEs generated from non-enzymatic glycation and oxidation of proteins or lipids. AGEs renal toxicity is mediated by three mechanisms: i) AGEs can deposit in renal tissue (mesangium, endothelial cells, GBM and podocytes) and modify its structure and function; ii) AGEs can be formed in situ, where they exert a profibrotic effect; iii) AGEs can bind to cell receptors [13,138]. The podocytes appear to be sensitive targets of AGEs actions as they express the receptor for AGEs (RAGE). So, the *in vitro* experiments demonstrated that glycated albumin inhibited nephrin expression in cultured podocytes through the RAGE engagement [139]. The harmful AGEs effect on podocytes is also realized through apoptosis activation by increasing of cell cycle inhibitor p27 synthesis [13,140]. There is accumulating evidence to suggest the pathophysiological crosstalk between the RAS and AGEs-RAGE axis in DN. AT-II through AT₂ receptors activates the expression of RAGE by podocytes [13,138,140]. The AGEs-RAGE-mediated ROS generation stimulates the production of TGF- β 1 via MAPK, and NF κ BAGEs induces TGF β -1 overexpression in podocytes.

AGEs inhibit the expression of neuropilin-1, a transmembrane receptor involved in cell adhesion, and reduces ZO expression, and tight junctions in the SD. AGEs also modify the expression of a cytoskeleton protein actin. All these changes result in increased AU [13,138,140].

AGEs-rich culture medium was found to induce podocytes apoptosis via induction of the transcription factor FOXO4 that contributes to podocytes loss. AGEs-RAGE binding in the podocytes upregulates the MCP-1 release leading to inflammation [13,138,140].

At the same time, the blockade of the AGE effects with anti-AGE agents prevented DN progression in experimental DM model [13,138,140]. Inhibition of the AGE formation and/or the blockade of RAGE-downstream pathways will be a promising therapeutic strategy for DN treatment.

Conclusion

Our understanding of podocytes biology has improved significantly in the past decade, we have more detailed knowledge of the mediators and signaling pathways that contribute to podocytes injury in diabetes. Despite the treatments with drugs that block the RAAS and combined antihyperglycaemic drugs, there is still a risk for the DN progression. Undoubtedly, a deeper insight into molecular mechanisms of podocytes injury in DM will lead to new therapeutic options for improving the survival of patients with

DN. In addition to the traditional RAAS blockade, the inhibition of AGEs formation and/or the blockade of RAGE-downstream pathways, the suppression of renal NADPH oxidase and MAPK p38, the impact on VEGF and TGF- β 1 signaling, improvement of autophagy by mTORC1 pathways regulation may be promising therapeutic approaches for DN. Furthermore, new non-invasive methods for detection of podocytopathy based on the evaluation of the specific podocytes biomarkers in the urine are available today and need to be put into common clinical practice. These urinary tests have good perspectives for early diagnostic and monitoring of DN.

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