Overview of Glucose Signaling in Mesangial Cells in Diabetic Nephropathy

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Clinical studies such as the Diabetes Control and Complications Trial (DCCT) in subjects with type 1 diabetes (1), the UK Prospective Diabetes Study (UKPDS) in subjects with type 2 diabetes (2), and the Kumamoto Study in Japanese subjects with type 2 diabetes (3) clearly link longstanding hyperglycemia to diabetic nephropathy. Furthermore, the regression of glomerular pathologic changes by the maintenance of normoglycemia for 10 yr was shown in eight subjects with type 1 diabetes who were treated with pancreas transplantation (4). Therefore, the understanding of the hyperglycemia-related molecular pathogenesis of diabetic nephropathy is truly needed to provide further insight into therapeutic strategies for diabetic nephropathy.

Diabetic nephropathy is characterized histologically by an accumulation of extracellular matrix (ECM) proteins in the glomerular mesangium (5). Functionally, an increase in GFR, glomerular hyperfiltration, found in the early phase of diabetes has been proposed to be related to the future development of diabetic nephropathy (6,7). These abnormalities could be caused by functional changes in diabetic glomeruli, particularly in glomerular mesangial cells because mesangial cells were found to be capable of producing ECM proteins (8,9) and regulating GFR through their contractility (10,11). An enhancement of the production of type IV collagen and fibronectin (12–14), a reduction of the contractile responsiveness to angiotensin II (15–17), and an overproduction of vasorelaxing eicosanoids (18–20) have been shown in diabetic glomeruli and mesangial cells cultured under high-glucose conditions. These functional changes in glomeruli and mesangial cells in diabetes are considered to be caused by the metabolic abnormalities in glomeruli and mesangial cells specific to diabetes. In this review, we would like to describe glucose-induced signaling abnormalities that lead to functional disturbances of mesangial cells in the diabetic milieu.

Glucose Transport into Mesangial Cells

The first step of glucose signaling is the transport of glucose into the cells through specific glucose transporters. Previous studies indicate that mesangial cells express only a small amount of insulin receptors or insulin-sensitive facilitative glucose transporters (GLUT-4). Alternatively, mesangial cells were found to express two types of glucose transporters, facilitative and sodium-coupled transporters (21), and brain type glucose transporter (GLUT-1) was shown to be a predominant isofrom (22,23). Therefore, excessive extracellular glucose in the diabetic milieu will easily enter the cells through GLUT-1 in an insulin-independent manner and induce various signaling pathways. The importance of GLUT-1 was suggested by the overexpression of GLUT-1 in mesangial cells, in which an excessive production of ECM proteins was observed even under normal glucose conditions (24,25). Furthermore, glucose-induced fibronectin production was found to be reduced by antisense GLUT-1 in mesangial cells (26). We have found that transforming growth factor-β1 (TGF-β1), one of the cytokines playing an important role in the development of diabetic nephropathy, is able to stimulate glucose uptake in mesangial cells by inducing the expression of mRNA and protein of GLUT1 (27). Our results also indicate that endogenous TGF-β1 produced by mesangial cells under high-glucose conditions stimulates glucose uptake and thus may accelerate glucose-induced metabolic abnormalities in mesangial cells.

Glucose-Induced Signaling Pathways: Metabolic Pathways

Glucose that enters the cells will be metabolized mainly by the glycolytic pathway. However, in the presence of excessive glucose, it will also be metabolized by various pathways and activate various signaling pathways indicated in Figure 1. An increase in the entry of glucose into the polyl pathway, the diacylglycerol (DAG) synthetic pathway, and the hexosamine pathway was found in mesangial cells cultured under high-glucose conditions.
been identified in glomerular cells including mesangial cells (28), and the accumulation of sorbitol and concomitant depletion of myo-inositol was found in mesangial cells under high-glucose conditions (29). Several mechanisms by which the activation of polyol pathway causes functional derangement of the cells are proposed. One of them is an increase in the ratio of NADH/NAD$^+$ resulting from the conversion of sorbitol to fructose. This increase in the ratio of NADH/NAD$^+$ is proposed to be linked to de novo synthesis of diacylglycerol (DAG), resulting in the activation of protein kinase C (PKC), which will be described below. The role of the polyol pathway in the development of diabetic nephropathy has been examined using specific inhibitors of polyol pathway, aldose reductase inhibitors. Although aldose reductase inhibitors were shown to correct glomerular hyperfiltration and albuminuria in diabetic rats as well as in diabetic humans (30–33) and the mesangial expansion in diabetic rats (34,35), other reports have not been able to confirm the effect of aldose reductase inhibitor on albuminuria, hyperfiltration, or mesangial expansion in diabetic animals (36,37). The study using transgenic mice over-expressing human aldose reductase revealed that only occlusion of renal vessels and deposits in Bowman’s capsule were seen in the kidney (38). However, another recent study in rats transgenic for aldose reductase showed that diabetes-induced albuminuria was completely prevented by the overexpression of aldose reductase, although the transgene was expressed in proximal tubular segments but not in glomeruli (39). Therefore, it remains unsolved whether the polyol pathway is in fact linked to the evolution of diabetic nephropathy.

**PKC-MAPK Pathway.** PKC, a family of serine-threonine kinases, has at least eleven isoforms that can be categorized into classical PKC (α, β1, β2, γ), novel PKC (δ, ε, η, θ, μ), and atypical PKC (ζ, λ) on the basis of their common structural features (40).

The classical PKC enzymes contain two cysteine-rich zinc finger-like motifs (C1 region), which are essential for interaction with phospholipid and diacylglycerol (DAG), and a Ca$^{2+}$-binding domain (C2 region) in their regulatory domain. In the novel PKC enzymes, Ca$^{2+}$ is not required because the C2 region is absent. Instead, they are activated by phosphatidyl-
serine and DAG or phorbol esters. The atypical PKC enzymes, which lack the C_2 region and one of the cysteine-rich zinc finger-like motifs in the C_1 region, are not activated by Ca^{2+}, DAG, or phorbol esters, but they are dependent on phosphatidylyserine and activated by cis-un-saturated fatty acids.

PKC activation is involved in regulating a number of vascular functions such as vascular permeability (41), contractility (42), cell proliferation (43), extracellular matrix protein synthesis (44), and signal transduction cascade for hormones (45) and growth factors (46). We and others have reported that high glucose increases DAG levels and subsequently activates PKC in vascular cells and tissues (47–49). The mechanism responsible for the activation of PKC by high glucose appears to be related to an elevation of *de novo* DAG levels from the glycolytic intermediates, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Figure 1). However, PKC activation by high glucose seems unlikely to be dependent on hydrolysis of phosphatidylinositol by phospholipase C and phosphatidylcholine by phospholipase D. This is supported by the data that inositol phosphate production, which is derived from hydrolysis of phosphatidylinositol, is unchanged in cultured aortic smooth muscle cells (50) and mesangial cells exposed to elevated glucose levels (51).

Other biochemical abnormalities by which high glucose and/or diabetes can produce diabetic nephropathy might be involved in PKC activation. The effect of the polyol pathway on high glucose-induced PKC activation was demonstrated by the fact that treatment with aldose reductase inhibitors prevented high glucose–induced PKC activation in mesangial cells (52,53). Similarly, aldose reductase has been implicated to be an obligatory mediator upstream of PKC activation by high glucose in human lens epithelial cells (54). Binding of advanced-glycation endproducts (AGE) to receptor for AGE (RAGE) produces a cascade of cellular signaling, such as PKC activation. Indeed, AGE-induced PKC activation has been shown to stimulate collagen mRNA expression in human mesangial cells (55). Moreover, Amadori-modified glycated albumin can also stimulate PKC activation, followed by increased production of TGF-β protein and type IV collagen in glomerular endothelial cells, even in physiologic glucose concentrations (56). Recent data by Ha et al. (57) have demonstrated that inhibition of high glucose-induced PKC activation effectively abrogated reactive oxygen species generation and nuclear factor-κB (NF-κB) activity, resulting in decreasing monocyte chemoattractant protein-1 secretion in mesangial cells. Alternatively, activation of PKC could also affect AGE formation, possibly through reactive oxygen species generation (58). Taken together, there is accumulating evidence that the different biochemical abnormalities produced by high glucose in *vivo* and/or hyperglycemia in *vivo* can influence one another, because many of the glucose metabolites are important sources for the different metabolic pathways.

Among eleven PKC isoforms, PKC α, β, β2, δ, and ε exhibit enhanced activation in the glomeruli of diabetic rats as well as mesangial cells exposed to high glucose by Western blot analysis and/or confocal microscopy (54,59,60). In addition, the activation of PKC-ζ isoforms has been shown in cultured mesangial cells exposed to high glucose (61), although the mechanism for activation of PKC-ζ, which is independent of DAG, is unclear. The differences in the findings obtained from many laboratories on the identity of the PKC isoform(s) activation by high glucose and/or diabetes might be due to differences in the methodology used to detect the presence/localization of the PKC isoforms as well as to measure their activity.

In the renal glomeruli, mesangial expansion and capillary basement membrane thickening, mainly composed of type IV collagen, fibronectin, and laminin, are classical manifestations of diabetic nephropathy. The effect of high glucose on the expression of fibronectin and type IV collagen can be prevented by general PKC inhibitors such as staurosporine or calphostin C (62,63), and treatment with PKC agonists stimulates type IV collagen expression (63) and fibronectin accumulation (64), suggesting that the effects of high glucose on increasing production of extracellular matrix proteins are mediated through PKC activation. PKC activation increases the expression of TGF-β1, a prototypical multifunctional cytokine, which is one of the most important growth factors in the regulation of extracellular matrix protein accumulation in diabetic nephropathy. One possible mechanism by which high glucose increases the expression and synthesis of TGF-β1 and extracellular matrix proteins is by PKC’s actions on inducing transcription factors c-fos and c-jun, which form complexes for activated protein-1 (AP-1) binding site, because increased mRNA expression of c-fos and c-jun protooncogenes has been demonstrated in cultured mesangial cells exposed to high glucose (65) and in rat glomeruli after induction of diabetes (66). Moreover, high glucose–induced TGF-β promoter activation with the use of luciferase reporter was inhibited by a PKC inhibitor through decreasing AP-1 activity (67).

Mitogen-activated protein kinases (MAPK), including the extracellular signal-regulated protein kinase-1/2 (ERK 1/2), stress-activated c-Jun N-terminal kinase (JNK), and p38 MAPK, play a key role in the intracellular signal transduction cascade to integrate the transcription of genes responsible for a variety of cellular responses relevant to diabetic nephropathy such as cell growth, differentiation, and extracellular matrix synthesis (68). We and others have shown that ERK as well as p38 MAPK are activated in mesangial cells exposed to high glucose and in rat glomeruli of early type 1 diabetes (69–73). Recently, Whiteside et al. (74) have found that mesangial cell p38 MAPK activation in response to endothelin-1, angiotensin II, and platelet-derived growth factor is significantly augmented in high glucose media. In a type 2 diabetic model, ERK activity was reported to be significantly activated in renal cortex of *db/db* mice as compared with nondiabetic mice (75). The co-activation of PKC and MAPK by high glucose reinforces the notion that PKC plays an important role in the process of ERK activation. For example, we found that a general PKC inhibitor, calphostin C, was able to prevent the activation of ERK under high glucose (69). Furthermore, the specific inhibitor for PKC-β isoform LY 335351 can also inhibit hyperglycemia-induced glomerular ERK activation in diabetic rats (unpublished data by Kitada M, Koya D, Haneda M). The mechanism of high glucose-induced p38 MAPK in mesangial cells was recently shown to be mediated possibly via reactive oxygen species or upstream signaling proteins rather.
than changes in the amount of p38 MAPK and PKC activation (73,74). In contrast, in vascular smooth muscle cells, PKC-δ dependent activation of p38 MAPK has been proposed (71).

One of the candidate targets of ERK is cytosolic phospholipase A₂ (cPLA₂), which was shown to be phosphorylated and activated by ERK (76). Indeed, the activities of cPLA₂ were found to be enhanced in mesangial cells exposed to high glucose, and this activation was prevented by PD98059, an inhibitor of an upstream kinase of ERK (MEK) (69). Recent reports also suggest that the activation of ERK in mesangial cells under high-glucose conditions is responsible for the overproduction of TGF-β and ECM proteins such as type IV collagen and fibronectin, possibly through AP-1 activation (67,77).

Moreover, ERK pathway was shown to be associated with mesangial cell hypertrophy through the induction of a newly identified protein, p8, which is induced in mesangial cells by endothelin as well as high glucose and in diabetic kidney (78). The functional role of p8, which is a helix-loop-helix protein with a nuclear localization signal with a modest sequence homology to high mobility group (HMG) protein, in mesangial cell hypertrophy was confirmed by employing p8 knock down using the recently developed method of RNA interference (78).

To understand the functional and pathophysiologic significance of PKC-MAPK activation, the effects of vitamin E (d-α tocopherol), thiazolidinediones, and a specific inhibitor for PKC-β isoform LY 333531 were tested on renal dysfunction and pathology in diabetic animals and in mesangial cells exposed to high glucose. Vitamin E and thiazolidinediones can prevent glomerular hyperfiltration as well as albuminuria in streptozotocin (STZ)-induced diabetic rats by inhibiting PKC-MAPK activation through decreasing DAG levels without changing plasma glucose levels (79). Oral administration of LY333531 to diabetic rats can also normalize GFR as well as albuminuria in parallel with inhibition of PKC activity without affecting DAG levels. Furthermore, LY333531 was able to prevent the diabetes-induced abnormalities in mRNA expression of TGF-β1, type IV collagen, and fibronectin in glomeruli of diabetic rats (79). In addition, an expansion of glomerular mesangium, one of the important histologic characters of diabetic nephropathy, was significantly ameliorated in db/db mice by the treatment with PKC-β inhibitor. The enhanced expression of TGF-β and ECM proteins in glomeruli of db/db mice was also shown to be prevented by the PKC-β inhibitor (80).

As may be suspected, PKC-β inhibition with LY333531 treatment has been shown to attenuate the progression of diabetic nephropathy functionally and structurally in STZ-induced mRen-2 rats, which develop hypertension dependent on activation of the renin-angiotensin system, in addition to hyperglycemia (81). However, it should be noted that the activation of other PKC isoforms by high glucose and/or diabetes may also contribute to diabetic nephropathy. Further studies are needed to clarify the relevance of each PKC isoform, especially by the development of inhibitors specific for other PKC isoforms, in addition to PKC-β, and by other ways using knock-in and/or knock-out of each PKC isoform in mesangial cells or mice.

Because activation of PKC-MAPK pathway could play a crucial role in mediating the development and progression of diabetic nephropathy, the use of inhibitors of PKC and MAPK would be valuable therapeutic strategies. However, PKC-MAPK inhibition with nonspecific inhibitors may increase the risk of adverse effects because PKC-MAPK acts as a general signaling system in cellular function. Therefore, an inhibitor such as PKC-β inhibitor, which has specificity for a single cellular signaling, could potentially obviate most of the adverse effects. The ongoing phase 3 clinical trials with a PKC-β inhibitor may hopefully reveal in the near future if there are benefits in patients with diabetic vascular complications.

**Hexoamine Pathway.** In the glycolytic pathway, glucose is converted to glucose-6-phosphate via hexokinase and subsequently to fructose-6-phosphate. Glucosamine-6-phosphate is then formed using glutamine as an amino donor under control of the rate-limiting enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT). Glucosamine-6-phosphate is further converted to uridine-5-diphosphate-N-acetylglucosamine (UDP-GlcNAc), resulting in proteoglycan synthesis and O-linked glycoproteins (82).

Inhibition of GFAT using either azaserine or antisense oligonucleotides has been shown to abrogate high glucose–induced TGF-β1 overexpression and subsequent effects on mesangial cell proliferation and matrix production through decreasing glucosamine metabolites in mesangial cells (83). Interestingly, this pathway is also associated with PKC activation because exposure to glucosamine resulted in the translocation of PKC α, β, and ε to plasma membrane and GFAT inhibitor azaserine attenuated the high glucose-induced translocation of PKC-β (84). Recently, it was also reported that azaserine suppressed high glucose–induced PKC activation and plasminogen activator inhibitor-1 (PAI-1) expression in mesangial cells, which is correlated with increased extracellular matrix accumulation in various kidney disease including diabetic nephropathy (85). The glycosylated modulation of the transcription factor Sp1 by N-acetylglucosamine is proposed to explain the link between increased hexoamine pathway and high glucose-induced PAI-1 overexpression in vascular smooth muscle cells (86). In addition, protein kinase A (PKA) activity through high glucose-induced hexoamine pathway is also involved in extracellular matrix protein laminin synthesis in mesangial cells (87).

**Glucose-Induced Signaling Pathways: The TGF-β System**

Various cytokines and growth factors are considered to be upregulated as a consequence of metabolic pathways described above and to play an important role in the development and progression of diabetic nephropathy. Among them, TGF-β, which promotes renal cell hypertrophy and stimulates extracellular matrix accumulation, has been shown to mediate virtually all of the pathologic changes of diabetic kidney disease (88). Several studies have concluded that high glucose stimulates the production of TGF-β and other intermediary growth factors (89). Transcriptional activation of the TGF-β1 gene by high ambient glucose has been demonstrated in mouse mesangial cells (90). The promoter of the mouse or human TGF-β1
gene has a consensus nucleotide sequence termed “glucose response element,” CACGTG, that seems to be the target for transcriptional activation by high glucose (90). In addition, the TGF-β1 promoter also has multiple AP-1-like consensus sites that respond to phorbol-ester/PKC stimulation (91). As described above, increased activities of PKC and the MAP kinase cascades in high-glucose conditions are essential for regulation of the promoter activity of TGF-β1 through the activation of the AP-1 complex (67,77,91).

Studies employing neutralizing anti-TGF-β antibodies have provided convincing evidence that the prostatic and hypertrophic effects of high ambient glucose in cultured renal cells are largely mediated by autocrine production and activation of TGF-β. In particular, such studies have involved glomerular mesangial cells (92), glomerular epithelial cells (93), proximal tubular cells (94), and renal cortical fibroblasts (95). Additionally, high ambient glucose upregulates TGF-β receptor mRNAs and proteins in cultured mesangial cells and tubular epithelial cells (96,97). Elevated renal TGF-β mRNA and protein levels have been found in various experimental animal models of diabetes mellitus such as STZ-diabetic rats or mice and db/db mice (98). This event coincides with the development of kidney hypertrophy, which is likely linked to the increased expression of TGF-β1 and type II TGF-β receptor (99) in the mouse kidney. Hyperglycemia is required for up-regulation of TGF-β and extracellular matrix production because restoration of normoglycemia by insulin attenuates the renal cortical (99) and glomerular (100) expression of these molecules.

The Smad family of proteins has been recently identified as a predominant signal transducer of TGF-β (101). Binding of TGF-β to its type II receptor and subsequent recruitment of the type I receptor results in the phosphorylation and activation of receptor-regulated Smads (R-Smads), Smad2 and Smad3. After associating with a common-Smad (Co-Smad), Smad4, Smad complex translocates into the nucleus, where it regulates the expression of target genes through the binding to Smad-binding element (SBE) (102). In the kidney of diabetic db/db and STZ mice, both the nuclear localization of Smad3 and the nuclear binding to SBE as well as the expressions of TGF-β ligand and its receptor were shown to be increased (103,104). Therefore, these observations indicated that the TGF-β system (consisting of TGF-β itself, its receptor, and the signaling pathway) was activated in the kidney of diabetes.


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