ABSTRACT

Chronic hyperglycemia is the main cause of diabetic microvascular complications of the kidney and retina. Multiple theories have been proposed to explain the adverse effects of hyperglycemia, including increased flux through the polyol pathway, excessive formation of advanced glycation end-products, oxidative stress, and altered signaling pathways such as protein kinase C (PKC). This article will review the evidence that has been published in support of these theories for their role in causing the pathologies observed in the kidney and renal glomeruli of diabetic animals and humans. Special emphasis will be placed on the activation of the PKC pathway, a target with the potential of mediating the adverse effects of hyperglycemia and its resultant metabolites. At present, clinical trials are in progress to determine the efficacy of PKC-β isoform inhibitors for the treatment of diabetic microvascular complications. (Adv Stud Med. 2005;5(1A):S10-S19)

DIABETIC NEPHROPATHY

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease and one of the leading causes of morbidity and mortality in patients with diabetes. It is initially characterized by glomerular hemodynamic abnormalities that lead to glomerular hyperfiltration. Over time, these characteristics progress to glomerular structural damage as evidenced by microalbuminuria. With the continued decline in glomerular function, overt proteinuria, decreased glomerular filtration rate, and end-stage renal failure will result. Although the rate of glomerular function decline to end-stage renal failure varies according to the individual, the rate of decline is constant for that individual.

Type 1 and type 2 diabetes mellitus cause morphologic changes in the kidney. Initially, after the manifestation of diabetes, the kidney and glomeruli size increase as does the glomerular filtration rate. Chronically, the glomerular basement membrane (GBM) thickens, the mesangial extracellular matrix (ECM) progressively expands, and glomerular mesangial cells (GMCs) undergo minimal proliferation. ECM accumulation is likely the main pathology that causes decreased renal function because it alters filtration function and interactions among mesangial cells, endothelial cells, and podocytes, leading to mesangial expansion and glomerulosclerosis. The mesangial ECM progressively expands as a result of an increased synthesis and a decreased degradation of mesangial ECM proteins, including collagens IV, VI, and fibronectin. Along with development of glomerular abnormalities, tubulointerstitial fibrosis may also occur.

The earliest clinical indication of DN is persistent microalbuminuria resulting from abnormal glomerular filtration and possibly tubular uptake of albumin. The abnormal glomerular filtration is partly caused by a decrease in the synthesis of heparan sulfate proteo-
glycan, an anionic barrier of the GBM.\(^2,10\) It is also partly a result of the increased expression of cytokines, such as angiotensin, endothelin-1 (ET-1), and vascular endothelial growth factor (VEGF), that affect vascular flow and permeability.\(^2\)

Several factors affect the pathogenesis of DN, including hyperglycemia, intraglomerular and systemic hypertension, activation of the renin-angiotensin system (RAS), and several growth and inflammatory factors.\(^2\) Strict glycemic control can prevent or ameliorate the abnormalities in GMCs that result from persistent hyperglycemia\(^11,12\); these abnormalities play a key role in the onset and development of DN. Regression of early pathologic changes associated with DN has been reported in patients with type 1 diabetes mellitus who underwent pancreas transplantation that normalized blood glucose.\(^13\) This study underscores the role of hyperglycemia in the pathogenesis of DN.

This review addresses the potential mechanisms by which hyperglycemia adversely affects GMCs leading to DN. Theories explaining the adverse effects of hyperglycemia leading to diabetic complications will be described briefly, but the focus will be on the role of protein kinase-C (PKC) activation in the pathogenesis of DN.

**GLUCOSE ENTRY INTO GLOMERULAR MESANGIAL CELLS**

Chronic exposure to glucose alters the expression of glucose transporters in the GMCs,\(^14-16\) in turn, triggering some of the pathologic changes associated with DN.\(^16\)

Glamorous mesangial cells express primarily 2 types of glucose transporters: the facilitative-type glucose transporter (GLUT-1) and the sodium-coupled glucose transporter,\(^14,17\) but they also express a small amount of insulin-sensitive glucose transporter (GLUT-4). GLUT-1 is the predominant isoform in the mesangial cells, and excessive extracellular glucose increases glucose uptake in an insulin-independent manner. High glucose levels also appear to cause increased expression of GLUT-1 that further accelerates the entry of glucose into the cells.\(^14\)

Glamorous mesangial cells isolated from patients with diabetes mellitus demonstrate increased GLUT-1 expression and glucose uptake.\(^15\) Animal cell data show the overexpression of GLUT-1 increases glucose uptake and stimulates ECM protein production, even during euglycemia.\(^16\) Therefore, chronic exposure to hyperglycemia and increased expression of GLUT-1 contribute to the typical features of cellular hypertrophy (eg, the excessive production of cytokines, ECM, and the expansion of the mesangium) that are characteristic of DN.

**BIOCHEMICAL MECHANISMS OF ADVERSE EFFECTS OF HYPERGLYCEMIA**

Several theories have emerged regarding how hyperglycemia induces the pathologic changes leading to microvascular complications, including increased flux through the polyol pathway, excessive formation of advanced glycation end-products (AGEs), oxidative stress, and activation of PKC as a result of increases in glycolysis or lipid synthesis.\(^2\) Each of these pathways can generate toxic and reactive metabolites that then alter several key signaling intracellular pathways (Figure 1).

In addition to hyperglycemia, other factors have been recognized as accelerating the progression of DN, including hypertension, activation of RAS, increased expression of fibrotic factors, and increased expression of VEGF.\(^2\) These factors are targets for intervening in the pathogenesis of DN.

Loss of renal function in DN is prevented or minimized by controlling hypertension, and a variety of antihypertensive agents have proven effective in controlling hypertension.\(^18\) Blockade of RAS is considered as the first-line treatment of DN with hypertension.\(^19\) It also exerts a positive effect on the hemodynamic and nonhemodynamic RAS effects in the kidney. The RAS affects vasodilators (eg, nitric oxide) or vasoconstrictors (eg, ET-1) that influence intraglomerular blood pressure. When activated, it increases albumin permeability and induces expressions of several cytokines.\(^20\)

The increased expression of fibrotic factors, such as transforming growth factor β (TGF-β) and connective tissue growth factor (CTGF),\(^2,21\) are likely to play a role in the mesangial expansion in DN. VEGF expression is increased in the kidneys of patients with diabetes; with its potent vasodilator and permeability-inducing actions, VEGF expression may be implicated in the development of albuminuria.

Although all these factors likely interact in the pathogenesis of DN, the driving force behind the blood flow changes and cytokine expression is thought to be hyperglycemia, directly or indirectly.
When intracellular glucose becomes elevated, it causes an increased flux through the polyol pathway²,²² that may cause vascular and neuronal abnormalities.

The polyol pathway²³ converts glucose to fructose using 2 enzymes: aldose reductase (AR) and sorbitol dehydrogenase (SDH). These enzymes reduce glucose to sorbitol with the aid of the cofactor nicotinamide adenine dinucleotide phosphate (NADPH), and then convert sorbitol to fructose with the cofactor NAD⁺. AR has a low affinity for glucose when euglycemia is present,² thus when blood glucose is in the reference range, it accounts for only a small percentage of glucose being metabolized. Evidence suggests the polyol pathway is active in the kidney when hyperglycemia is present. AR and SDH have been identified in glomerular cells, including mesangial cells, and increased sorbitol levels are observed in mesangial cells that are exposed to high concentrations of glucose.²⁴-²⁶ In addition, the metabolic changes caused by increased flux of the polyol pathway are consistent with the renal changes that occur in the presence of hyperglycemia, including sorbitol accumulation, a substantial depletion of available NADPH caused by AR reaction that diminishes nitric oxide production by endothelial cells, increased prostaglandin production (increased prostaglandin E₂ [PGE₂] may contribute to abnormal filtration of glomeruli), and increased NADPH/NAD⁺ ratio that blocks the glucoytic pathway resulting in oxidative stress, increased de novo diacylglycerol (DAG) synthesis, and increased production of AGEs that activate PKC.²,²⁷-²⁹

Aldose reductase inhibitors have been investigated as potential therapeutic agents; however, in vivo studies in diabetic animals and clinical studies were largely disappointing.² In diabetic rats, AR inhibitors have inhibited mesangial expansion, but they did not affect.³⁰ AR inhibitors have been tested in humans for retinopathy and neuropathy, but there is no clinical evidence for their benefit in nephropathy.

**Advanced Glycation End-Products Pathway**

When hyperglycemia is present, proteins and lipids undergo nonenzymatic glycosylation generating AGEs, intracellularly and extracellularly.³¹ The concentration of AGEs is increased in many vascular and nonvascular tissues of patients with diabetes,³² and it is thought to be linked to diabetic complications.

The evidence that ties AGEs to the complications of diabetes mellitus is broad-based. Accumulation of AGEs in skin collagen correlates with the severity of
diabetic complications, including DN. Reports indicate that AGEs, including Nε-(carboxymethyl)lysine and pentosidine, accumulate in the renal cortex of diabetic rats and in sclerosing glomeruli of patients with diabetes. Accumulation of AGEs in the human glomerulus has also been correlated with the severity of DN. Administration of AGEs to nondiabetic rats has been reported to cause albuminuria and glomerulosclerosis.

The toxic effects of AGEs likely result from their binding with cell surface receptors and their accumulation in ECM structural proteins. Surface cell sites where binding occurs include the receptors for AGE (RAGE), the AGE-receptor complex (gelatin-3, OST-48, and 80K-H), macrophage scavenger receptors (type I and II), and CD-36. Among these receptors, RAGE appears to be the major receptor that alters cellular signaling pathways causing cellular dysregulation. Diabetic mice overexpressing RAGE exhibit accelerated features of DN, including albuminuria, glomerular hypertrophy, and glomerulosclerosis, compared to their other diabetic littermates.

AGE-RAGE interactions can initiate a range of cellular signaling cascades, including p28 (ras)-extracellular signal-regulated protein kinases (Erk1/2), Jak2/Stat3, and transcription nuclear factor-κB (NFκB), which mobilize multiple transcription genes. Erk1/2 activation has been associated with mesangial cell growth, enhanced expression of growth factors, and ECM proteins. In addition, AGE-RAGE interactions have been shown to activate NADPH oxidase resulting in increased reactive oxygen species (ROS) generation by unknown mechanisms. AGEs have also activated PKC, increased expression of growth factors, and ECM proteins. In addition, AGE-RAGE interactions have been shown to activate NADPH oxidase resulting in increased reactive oxygen species (ROS) generation by unknown mechanisms. AGEs have also activated PKC.

In an animal model of diabetes mellitus, AG ameliorates albuminuria and GBM thickening. Unfortunately, a clinical trial of AG has been limited because of its toxicity. In the animal model, AGE cross-link breakers, such as ALT-711, have been effective in preventing conditions associated with DN (ie, microalbuminuria, renal hypertrophy, and glomerulosclerosis). Additional studies are needed to determine the usefulness of AGE cross-link breakers in patients with DN.

**Reactive Oxygen Intermediate Pathway**

Oxidative stress results from the imbalance between the generation of ROS (superoxide [O2−], hydrogen peroxide [H2O2], and hydroxyl radical [OH−]) and the antioxidative mechanisms (eg, superoxide dismutase, catalase, and glutathione peroxidase). In physiologic conditions, ROS is continuously generated but effectively eliminated by the antioxidant system. However, when ROS production exceeds a system’s antioxidant defense capacity, the increased/unstable ROS will alter essential biologic cellular proteins or DNA, causing cellular damage. ROS is increased in diabetes mellitus and may be the cause of neuronal and vascular complications, including DN.

In diabetes mellitus, ROS generation may be increased through multiple mechanisms, including increased flux though the polyol pathway, increased formation of AGEs, mitochondrial superoxide overproduction, and activation of PKC. Furthermore, PKC activation and ROS generation are interrelated. The overexpression of PKC β2 stimulates NADPH oxidase activity that causes increased ROS generation in GMCs. ROS, in turn, activates PKC perhaps through tyrosine phosphorylation.

Recent studies suggest that NADPH oxidase (primarily found in phagocytic cells) is the main source of ROS in nonphagocytic cells, including mesangial cells, smooth muscle cells, and endothelial cells. NADPH oxidase consists of several subunits, including membrane-bound cytchrome b558 (composed of gp91-phox, p22-phox) and cytosolic subunits (Rac, p40-phox, p47-phox, and p67-phox). Activation of NADPH oxidase requires phosphorylation of its components by several kinases, including PKC. Upon activation, the cytosolic components translocate en bloc and bind to the membranous components.
Disassociation of p40-phox from p67-phox is necessary for NADPH oxidase activation. Expression of p22-phox, Rac, p47-phox, p67-phox, and gp91-phox has been confirmed in GMCs. Hyperglycemia associated with diabetes mellitus activates cellular NADPH oxidase, resulting in increased ROS generation. The markers of oxidative stress, including 8-hydroxydeoxyguanosin (8-OHdG) and superoxide synthesis, was demonstrated in the glomeruli of diabetic rats.

In addition to their cytotoxic effects, ROS can act as a second messenger (eg, in response to angiotensin II or PDGF) through redox-sensitive gene transcription factors and cell signaling pathways. ROS has been shown to cause increased expression of several cytokines, including TNF-β, tumor necrosis factor α, monocyte chemoattractant protein (MCP)-1 and MCP-3, vascular cell adhesion molecule 1, interleukin (IL)-1, IL-6, and IL-8, ECM proteins, such as type IV collagen. Activation of NF-kB and activator protein 1 by ROS are likely to play an important role in causing these cellular changes.

Results of antioxidant treatment for complications associated with diabetes mellitus, including DN, have had mixed results: positive in animal models, equivocal in humans. In diabetic rats, vitamin E has attenuated DN (glomerular expansion, albuminuria, and reduction of glomerular filtration rate). In patients with diabetes, high-dose vitamin E restored renal filtration and retinal blood flow. However, large studies using lower-dose vitamin E or other antioxidants have not shown any benefit in delaying the progression of diabetic microvascular or cardiovascular pathologies.

The lack of efficacy of antioxidants in human trials could be attributed to multiple factors. One reason often cited is the inability of currently available antioxidants to penetrate tissues and to neutralize oxidants induced by hyperglycemia. Alternatively, it is also possible that oxidative stress is not the major initiator of diabetic vascular complications (especially those complications involving microvessels). Oxidative stress may be an accelerator of DN and microvascular diseases similar to hypertension, and antioxidants may prove useful in combination with other treatments of diabetic vascular disease.

PROTEIN KINASE-C PATHWAY

Protein kinase C is a family of serine-threonine kinases that are multifunctional isoenzymes acting as an intracellular signal transduction system for many hormones and cytokines. There are 11 known isoenzymes that are classified into the following 3 subgroups: conventional PKCs (α, β1, β2, and γ), which are Ca2+-dependent and activated by phosphatidyserine (PS) and the second messenger DAG; novel PKCs (δ, ε, η, and ζ), which are Ca2+-independent and regulated by DAG and PS; and atypical PKCs (ζ and θ), which are Ca2+-independent and do not require DAG for activation (although PS regulates activity). In addition, all PKCs appear to require phosphorylation by phosphoinositide-dependent protein kinase 1, a downstream effector of PI3K, for full activity. PKC α, β1, β2, δ, ε, and ζ are likely to be ubiquitous isozymes found in most or all tissues. PKC γ is expressed mainly in the neuronal tissues and spinal cord, whereas PKC θ is expressed in skeletal muscle and hematopoietic cells.

The PKC isoenzymes reside in the cytosol in their inactive state. However, upon cellular activation they are targeted to distinct subcellular locations, including the plasma membrane, nucleus, and cytoskeleton suggesting isoform-specific biologic roles. Binding of activated PKC to receptors for activated C kinase (RACKs) is likely to be an important step for the signal transduction of PKC because the RACKs anchor multiple other enzymes and then provide a signal complex. DAG, a physiologic activator of PKC, is synthesized by pathways, including phospholipase C, de novo synthesis from the glycolytic intermediates and metabolism of phosphatidylcholine by phospholipase D, and glycerol 3-phosphate. DAG levels also can be reduced by phosphorylation (DAG kinases), hydrolysis (DAG lipase), and transacylation (sn-glycerol-3-phosphate acyltransferase).

In diabetes mellitus, PKC can be activated by several mechanisms, including increased DAG levels by de novo synthesis or inhibition of DAG kinase (Figure 2). PKC α, β1, β2, δ, ε, and ζ isoforms were observed in cultured mesangial cells, glomeruli, and tubules. In certain studies, all of these isoforms have been activated by high glucose levels or diabetes mellitus in mesangial cells or glomeruli.

Activation of PKC in diabetes mellitus has effects on various intracellular signal transduction systems, including the enhancement of Erk1/2 and p38 mitogen-activated protein (MAP) kinase cascades, and NADPH oxidase. PKC was shown to activate Erk1/2 by stimulating Raf-1, mitogen-activated protein kinases, and antioxidants may prove useful in combination with other treatments of diabetic vascular disease.
kinase kinase (MEK), and an intermediate downstream of mitogen-activated protein/extracellular signal-regulated kinase (MEK) or by inhibiting the Raf kinase inhibitory protein. Erk1/2 activation by high glucose was demonstrated to be PKC-dependent. The activation of PKC and increased activity of Erk1/2 have been observed in cultured mesangial cells at high glucose concentration and in glomeruli of streptozotocin-induced diabetic rats, whereas calphostin C (a general PKC inhibitor) or ruboxistaurin (RBX; a PKC-β isoform selective inhibitor) prevented the activation of Erk and p38 MAP kinase.

Increases in RAS expression or actions also are closely associated with PKC-β isoform activation. Angiotensin-converting enzyme inhibitors have been shown to reduce diabetes-induced PKC activity in the rat tissue glomeruli, and the PKC-β inhibitor (RBX) abolished the vasoconstrictive effect of angiotensin II in the arteriole of the glomerulus of the isolated rat kidney. Activated PKC inhibits Na+K+ATPase, an integral component of the sodium pump, and also regulates the maintenance of cellular integrity and functions, such as contractility, growth, and differentiation. Activation of PKC has been reported to increase the actions of several cytokines at signaling levels or at the transcription step (Figure 2). For example, activation of PKC can enhance the effects of angiotensin, VEGF, or ET-1 because these cytokines partly mediate their biologic actions by PKC activation. In addition, PKC activation has been shown to enhance the expression of VEGF, ET-1, PDGF-B, triple gene block β, and CTGF. Among PKC isoforms, PKC-β isoforms may be the most sensitive to changes in DAG levels. In diabetic rats, chronic hyperglycemia predominantly activated PKC-β2 isoform in the glomeruli, retinas, aorta, and heart.

Ruboxistaurin, a specific PKC-β isoform inhibitor, has been shown to ameliorate many functional and structural features of experimental diabetes. RBX inhibits PKC β1 and β2 at nanomole concentrations and shows 50-fold or greater affinity for PKC isoform compared to any other PKC isoforms or kinases tested. In diabetic rat GMCs exposed to high glucose concentrations, RBX inhibited PKC activity, arachidonic acid release and PGE2 production, and normalized Na+K+ATPase activity. In experimental diabetic animals, administration of RBX prevented elevated glomerular filtration rate, albuminuria, mesangial expansion, production of ECM proteins, including collagen IV and fibronectin, expression of TGF-β, and CTGF, and glomerulosclerosis (despite continued hyperglycemia). Kelly et al reported that RBX treatment in diabetic REN rats, which were hypertensive and overexpressed renin, inhibited mesangial expansion and proteinuria, suggesting that PKC inhibition could have additive effects with agents that inhibit angiotensin.

The safety and efficacy of RBX were evaluated in 29 patients with type 1 or type 2 diabetes mellitus in a 1-month clinical study. Results of the trial showed significant improvement in retinal blood flow and mean circulation time, suggesting safety and efficacy for RBX in treating vascular complications caused by diabetes mellitus. Clinical studies evaluating RBX in nephropathy, retinopathy, and neuropathy are ongoing.

**CONCLUSIONS**

Hyperglycemia may exert its negative effect on the kidney by the generation of toxic and reactive metabolites, leading to the activation of PKC. The development of PKC inhibitors may provide a new therapeutic approach for the prevention and treatment of diabetic kidney disease.

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**Figure 2. Protein Kinase-C Activation**

- **Hyperglycemia**
  - DAG
  - PKC activation (β + δ isoforms)
  - Vasotropic substances
  - Angiotensin
  - VEGF
  - ET-1
  - PDGF-B
  - IGF-1
  - Fibrotic factors
  - CTGF
  - TGF-β
  - Oxidants
  - Glomerulosclerosis
  - Tubular fibrosis
  - Renal failure

- **Renal pathologies**
  - Glomeruli
  - Tubules
  - Clinical results
  - GFR
  - Hypertrophy
  - Nephromegaly
  - Protein load
  - Albuminuria
  - Proteinuria
  - BUN + creatinine

BUN = blood urea nitrogen; DAG = diacylglycerol; CTGF = connective tissue growth factor; ET-1 = endothelin-1; GFR = glomerular filtration rate; IGF-1 = insulin-like growth factor-1; PGE2 = prostaglandin E(2); PKC = protein kinase C; TGF-β = transforming growth factor β; VEGF = vascular endothelial growth factor.
lites that alter intracellular signaling pathways. Understanding the interactions among these important signaling pathways is critical for unlocking the pathogenesis of DN. Neutralization of glucose and its toxic metabolites by innovative therapies, such as antioxidants or AGE inhibitors, or blocking the cellular signaling pathways involved in glucose toxicity by PKC-β isoform inhibitors, such as RBX, may offer a novel and potentially beneficial approach to the treatment and prevention of diabetic microvascular complications, including DN. The understanding of the interactions among these signaling pathways is critical for understanding the pathogenesis of DN and for designing innovative therapeutic interventions for complications from diabetes mellitus.

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