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Activation of Protein Kinase C Isoforms and Its Impact on Diabetic Complications

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This Review is part of a thematic series on **Cardiovascular Complications of Diabetes and Obesity**, which includes the following articles:

The Impact of Macrophage Insulin Resistance on Advanced Atherosclerotic Plaque Progression [2010;106:58–67]

The RAGE Axis: A Fundamental Mechanism Signaling Danger to the Vulnerable Vasculature [2010;106:842–853]

The Promise of Cell-Based Therapies for Diabetic Complications: Challenges and Solutions [2010;106:854–869]

Activation of Protein Kinase C Isoforms and Its Impact on Diabetic Complications

Oxidative Stress and Diabetic Complications

The Polyol Pathway: Implications for Atherosclerosis and Cardiac Dysfunction in Diabetes

ER Stress, Inflammation, Obesity, and Diabetes

Epigenetics: Mechanisms and Implications for Diabetic Complications

Ann Marie Schmidt, Guest Editor

Activation of Protein Kinase C Isoforms and Its Impact on Diabetic Complications

Pedro Geraldes, George L. King

Abstract: Both cardio- and microvascular complications adversely affect the life quality of patients with diabetes and have been the leading cause of mortality and morbidity in this population. Cardiovascular pathologies of diabetes have an effect on microvessels, arteries, and myocardium. It is believed that hyperglycemia is one of the most important metabolic factors in the development of both micro- and macrovascular complications in diabetic patients. Several prominent hypotheses exist to explain the adverse effect of hyperglycemia. One of them is the chronic activation by hyperglycemia of protein kinase (PK)C, a family of enzymes that are involved in controlling the function of other proteins. PKC has been associated with vascular alterations such as increases in permeability, contractility, extracellular matrix synthesis, cell growth and apoptosis, angiogenesis, leukocyte adhesion, and cytokine activation and inhibition. These perturbations in vascular cell homeostasis caused by different PKC isoforms (PKC- α , - β 1/2, and PKC- δ) are linked to the development of pathologies affecting large vessel (atherosclerosis, cardiomyopathy) and small vessel (retinopathy, nephropathy and neuropathy) complications. Clinical trials using a PKC- β isoform inhibitor have been conducted, with some positive results for diabetic nonproliferative retinopathy, nephropathy, and endothelial dysfunction. This article reviews present understanding of how PKC isoforms cause vascular dysfunctions and pathologies in diabetes. (*Circ Res.* 2010;106:1319-1331.)

Key Words: protein kinase C ■ vascular cell biology ■ diabetes ■ cardiovascular diseases

Diabetes currently affects nearly 170 million people worldwide and this number will double by 2030, affecting both developed and developing countries.¹ The major cause complications in diabetic patients is complications, which are the result of interactions among systemic metabolic abnormalities such as hyperglycemia, dyslipidemia, genetic and epigenetic modulators and local tissue responses to toxic

metabolites. Complications involve large vessel obstructions, such as coronary artery diseases, atherosclerosis, and peripheral vascular diseases, and microvascular pathologies, including retinopathy, nephropathy, and neuropathy. Hyperglycemia is one of the major systemic risk factors for diabetic complications. The Diabetes Control and Complications Trial (DCCT) in type 1 diabetes² and the United Kingdom Pro-

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Non-standard Abbreviations and Acronyms

ACE	angiotensin-converting enzyme
AGE	advanced glycation end product
CTGF	connective tissue growth factor
DAG	diacylglycerol
EC	endothelial cell
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
ET-1	endothelin-1
MAPK	mitogen-activated protein kinase
MI	myocardial infarction
NTSS-6	neuropathy total symptoms score-6
PDGF	platelet-derived growth factor
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
RBF	retinal blood flow
RBX	ruboxistaurin
SMC	smooth muscle cells
TGF	transforming growth factor
VEGF	vascular endothelial growth factor

spective Diabetes Study in type 2 diabetes³ demonstrated that intensive blood glucose control delays the onset and retards the progression of diabetic microvascular complications. In contrast, reducing cardiovascular pathologies in type 2 diabetes requires the control of multiple metabolic factors, including hyperglycemia, free fatty acids, lipids, insulin resistance, and others. Hyperglycemia alone may not be sufficient to trigger diabetic alterations because only 30% to 40% of type 1 diabetic patients will ultimately develop chronic renal failure, suggesting that genetic and protective tissue factors may also play an important role.⁴ For example, the 50-Year Medalist Study at Joslin Diabetes Center, which recognizes patients who survive more than 50 years with type 1 diabetes, showed that significant numbers of diabetic patients live without severe complications regardless of their HbA_{1c} levels and other classical markers thought to be predictive for diabetic vascular complications.⁴ These data suggest that some patients possess genetic factors that can neutralize the adverse effects of hyperglycemia.

Multiple biochemical pathways have been proposed to link the adverse effects of hyperglycemia with vascular complications. Cellular mechanisms include increased activation of the polyol pathway,⁵ nonenzymatic glycation, and AGEs (advanced glycation end products) pathway,⁶ enhanced reactive oxygen production and actions,⁷ and activation of the diacylglycerol (DAG)–protein kinase (PK)C pathway.⁸ It is likely that hyperglycemia-induced intra- and extracellular changes lead to alterations of signal transduction pathways, affecting gene expression and protein function to cause cellular dysfunction and damage. The DAG-PKC pathway is one of the most studied pathways in cellular signaling induced by diabetes.⁹ In this review, we focus on the role of

PKC actions and related signaling changes in the development of diabetic vascular complications.

Activation of DAG-PKC Pathway in Diabetes

DAG levels are elevated chronically in the hyperglycemic or diabetic environment because of an increase in the glycolytic intermediate dihydroxyacetone phosphate. This intermediate is reduced to glycerol-3-phosphate, which subsequently increases de novo synthesis of DAG.¹⁰ In diabetes, total DAG levels are elevated in vascular tissues, such as the retina,¹¹ aorta, heart,¹² and renal glomeruli,^{13,14} and in nonvascular tissues, such as liver and skeletal muscles.¹⁵ However, there is no consistent change in DAG levels in the central nervous system and peripheral nerves.¹⁶ Various cell culture studies have shown that DAG levels increase in a time-dependent manner as glucose levels elevate from 5.5 to 22 mmol/L in aortic endothelial cells,¹² retinal pericytes,¹⁷ smooth muscle cells¹⁰ and renal mesangial cells.¹⁸

PKC, a group of enzyme members of the AGC (cAMP-dependent protein kinase/PKG/PKC) family, is a serine/threonine-related protein kinase that plays a key role in many cellular functions and affects many signal transduction pathways.¹⁹ There are multiple isoforms of PKC that function in a wide variety of biological systems.²⁰ The conventional PKC (cPKC) isoforms (PKC- α , - β 1, - β 2, and - γ) are activated by phosphatidylserine, calcium, and DAG or phorbol esters such as phorbol 12-myristate 13-acetate (PMA), whereas novel PKCs (nPKCs) (PKC- δ , - ϵ , - θ , and - η) are activated by phosphatidylserine, DAG or PMA, but not by calcium. The atypical PKCs (aPKCs) (PKC- ζ and - ι/λ) are not activated by calcium, DAG or PMA (Figure 1). Extensive and excellent reviews concerning PKC structural basic activation have been published.^{19–22} Given the present breadth of knowledge in this area, we focus our attention on how hyperglycemia modulates PKC activation. PKCs can also be activated by oxidants such as H₂O₂ in a manner unrelated to lipid second messengers²³ and by mitochondrial superoxide induced by elevated glucose levels.²⁴ Many abnormal vascular and cellular processes and deregulations, including endothelial dysfunction, vascular permeability, angiogenesis, cell growth and apoptosis, changes in vessel dilation, basement membrane thickening, and extracellular matrix (ECM) expansion; enzymatic activity alterations, such as in mitogen-activated protein kinase (MAPK), cytosolic phospholipase A₂, Na⁺–K⁺–ATPase; and alterations in several transcription factors (Figure 2) are attributed to multiple PKC isoforms that are changed by diabetes (Table 1). These PKC-induced vascular and tissue pathologies are discussed in detail in the following paragraphs.

PKC Activation: Cell Culture Studies

Endothelial Cells

Endothelial cells (ECs) regulate both vasodilator and vasoconstrictor substances mediating coagulation, platelet adhesion and immune function and control volume and electrolyte content of the intravascular and extravascular spaces. Tight junctions between ECs form a vascular barrier, which in the diabetic state becomes more vulnerable and permeable as a

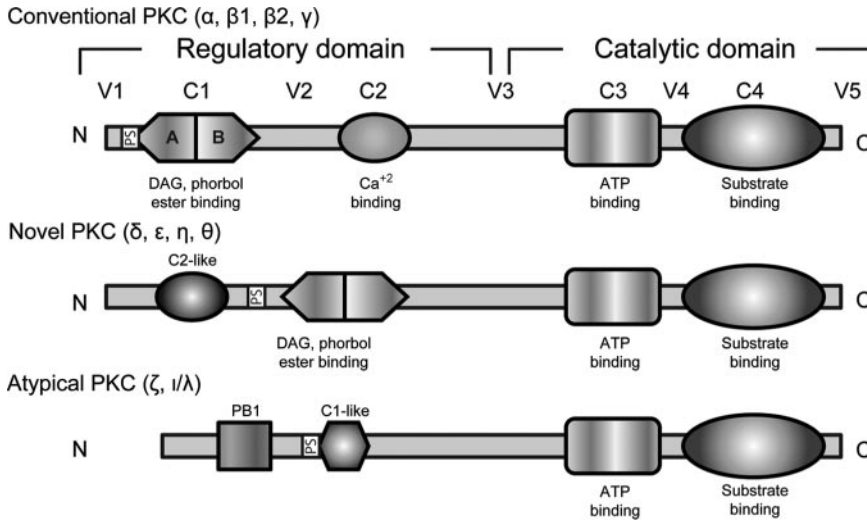


Figure 1. Schematic representation of the domain structure of PKC isoforms. Adapted from Newton^{19,20} and Steinberg.^{19,20}

result of EC abnormalities. PKC activation directly increases the permeability of albumin and other macromolecules through barriers formed by ECs.^{25,26} Moreover, Inoguchi et al reported that hyperglycemia or PMA inhibits gap junction intercellular communication in bovine aortic ECs. Staurosporine, a serine/threonine kinase inhibitor, prevented these effects of high glucose.²⁷ High glucose exposure induced translocation of PKC- α , - β 1, - β 2, and PKC- δ but not PKC- ϵ or PKC- ζ in retinal ECs.²⁸ A previous study showed that overexpression of PKC- β 1 in human dermal microvascular ECs enhances phorbol ester-induced increased permeability to albumin.²⁹ Hempel et al also showed that PMA increases permeability via translocation of PKC- α similar to that caused by high glucose levels. This permeability is prevented by staurosporine.³⁰ PKC activation also results in endothelium-dependent vasodilator dysfunction by altering the bioavailability of nitric oxide (NO), affecting vascular endothelial growth factor (VEGF) expression and actions and decreasing production of prostacyclin, as well as increasing the production of thromboxane, other cyclooxygenase-dependent vasoconstrictors and endothelin (ET)-1 (Figure

3).^{31–33} A previous study demonstrated that endothelial nitric oxide synthase (eNOS) expression is decreased in aortic ECs cultured in high glucose concentrations, resulting in a reduction of NO.³⁴ Treatment with calphostin C, an inhibitor of c- and nPKC (novel PKC), prevented high glucose levels from reducing NO production.³³ Another potential pathway by which high concentrations of glucose reduce NO bioavailability is by increasing superoxide production from NADPH oxidase in aortic ECs.³⁵ The induction of several subunits of NADPH oxidase by constant or intermittent high glucose concentrations is decreased with treatment of a PKC- β inhibitor (LY379196).³⁶ PKC activation also modulates vascular endothelial permeability and neovascularization via the expression of growth factors, such as VEGF/vascular permeability factor. One study showed that both VEGF/vascular permeability factor mitogenic and permeability actions are, in part, the result of the membrane expression of PKC- α and - β through tyrosine phosphorylation of phospholipase-C γ , which is reduced by a PKC- β inhibitor, LY333531.³⁷ Moreover, PKC- β is required for VEGF-induced retinal EC permeability by altering occluding phosphorylation.³⁸ One

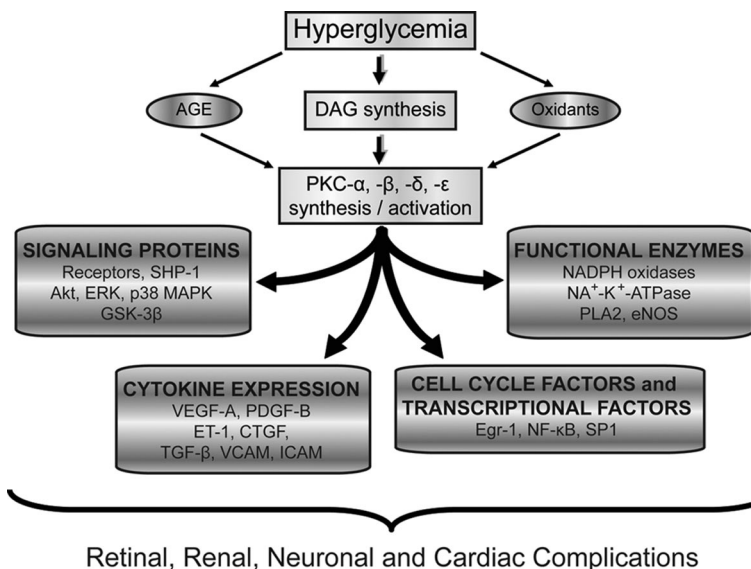


Figure 2. Schematic representation of biological targets of PKC isoform activation and synthesis.

Table 1. PKC Isoforms Detected in Vascular Tissues and Cell Types by Immunoblot Under Normal Conditions or Following Exposure to Hyperglycemia or Diabetic State

Tissue and Cultured Cell Type	PKC Isoforms Detected	PKC Isoforms Activated (Translocation and/or Abundance) by Hyperglycemia or Diabetes
Mouse retina	α , $\beta 1$, $\beta 2$, δ , ϵ , ζ	$\beta 2$, δ
Rat retina	α , $\beta 1$, $\beta 2$, δ , ϵ	α , $\beta 1$, $\beta 2$, δ , ϵ
Bovine pericytes	α , $\beta 1$, $\beta 2$, δ , ϵ , θ , ζ	δ
Bovine endothelial cells	α , $\beta 1$, $\beta 2$, δ , ϵ , γ , ζ	α , $\beta 2$, δ
Rat kidney	α , $\beta 1$, $\beta 2$, δ , ϵ , ζ	α , ϵ
Rat glomeruli	α , $\beta 1$, $\beta 2$, δ , ϵ	α , $\beta 1$, $\beta 2$, δ , ϵ
Rat mesangial cell	α , δ , ϵ , ζ	α , ζ
Rat corpus cavernosum	α , $\beta 1$, $\beta 2$, δ , ϵ	$\beta 2$
Rat sciatic nerve	α , $\beta 1$, $\beta 2$, δ , ϵ	α or no change
Rat aorta	α , $\beta 2$, δ , ϵ	$\beta 2$
Rat aortic smooth muscle cells	α , $\beta 2$, δ	$\beta 2$, δ
Rat heart	α , $\beta 2$, δ , ϵ , γ , ζ	α , $\beta 2$, δ , ζ
Rat cardiac myocytes	$\beta 2$, δ , ϵ	$\beta 2$, ϵ

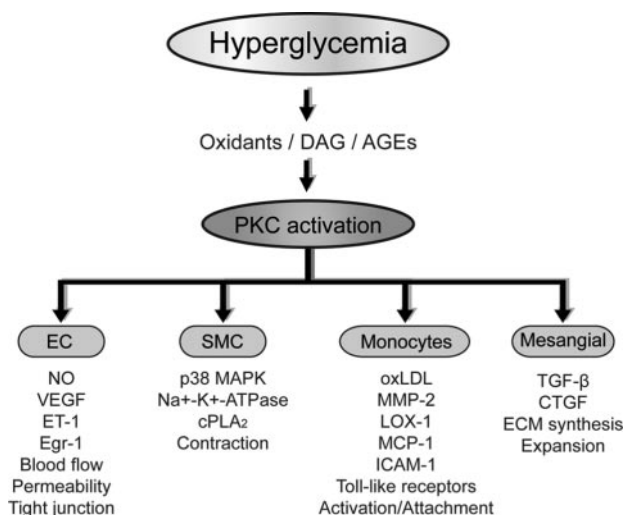
possible mechanism for vasoconstriction in retinal ECs caused by PKC action is probably increased expression of ET-1, a potent vasoconstrictor. Indeed, studies reported that ET-1 mRNA expression elevates in cultured retinal ECs exposed to high glucose levels.²⁸

Smooth Muscle Cells

Vascular smooth muscle cells (SMCs) are involved in regulation of vascular tone and could be an important target for injurious effects of hyperglycemia. An emerging body of evidence suggests that hyperglycemia-induced SMC pathogenesis, similar to EC pathogenesis, results from an imbalance between cell growth and cell death. In the presence of high glucose levels, SMCs have elevated DNA synthesis and contraction by PMA stimulation.^{39–41} Another group reported that high glucose levels reduce SMC apoptosis, which is blocked by PKC inhibitor (calphostin C).⁴² Moreover, rat SMCs exposed to high glucose concentrations exhibited increases membrane fraction expression of cPKC- β (conventional PKC- β) and nPKC- δ (novel PKC- δ) p38 MAPK phosphorylation and arachidonic acid release (Figure 3).⁴¹

Monocytes: Macrophages

Monocyte activation and transformation into macrophages are key steps in the atherosclerotic and inflammatory process. One of the earliest events in the pathogenesis of atherosclerosis is lipid accumulation in the arterial wall and formation of foam cells through uptake of modified or oxidized low-density lipoprotein by monocyte-derived macrophages.⁴³ Little is known about how hyperglycemia or diabetes affects PKC activity in circulating monocytes. However, activation of PKC in monocytes may participate in their initial adhesion to the vasculature and differentiation into macrophages. Hyperglycemia regulates the LOX-1 (leptin-like oxidized

**Figure 3.** Schematic representation of hyperglycemia-induced PKC activation affecting multiple cellular functions.

low-density lipoprotein receptor-1) and enhanced membrane PKC- $\beta 2$ expression in human monocyte-derived macrophages.⁴⁴ These effects of high glucose levels in LOX-1 expression are prevented by treatment with calphostin C or PKC- β inhibitor (LY379196). Ceolotto et al reported that monocytes isolated from diabetic patients showed higher membrane PKC activity compared to those from control subjects.⁴⁵ This study also demonstrated that membrane PKC- β activity, but not - α , isoform, is increased in diabetic monocytes.⁴⁵ Furthermore, Osto et al reported that inhibition of PKC- β reduced low-density lipoprotein uptake by macrophages.⁴⁶ This study also showed that treatment with LY333531 significantly attenuates macrophage activation by reducing intercellular adhesion molecules-1 and monocyte chemotactic protein-1 protein expression.⁴⁷ A recent study from Dasu et al reported that elevation of glucose levels in the media increases the expression of toll-like receptors 2/4 in the human acute monocytic leukemia cell line, whereas inhibition of PKC- α and PKC- δ prevents this effect.⁴⁸ Overall, these data suggest that PKC signaling could be in part responsible for macrophage activation/attachment and foam cell formation induced in the hyperglycemic state (Figure 3).

Mesangial Cells

The hallmark of diabetic kidney disease is the thickening of glomerular basement membrane and accumulation of ECM in glomerular mesangium and tubulointerstitium.⁴⁹ Histological analysis showed increases in type IV and VI collagen, fibronectin, and laminin and decreases in proteoglycans in the mesangium of diabetic patients with nephropathy, and probably in the vascular endothelium in general.⁵⁰ In vitro experiments reported that hyperglycemia or PMA increases type IV collagen and fibronectin expression in mesangial cells, which is prevented by calphostin C.^{51,52} Kapur-Drezgic et al reported that total PKC activity measured by in situ ³²P-phosphorylation of epidermal growth factor substrate is increased by hyperglycemia in rat mesangial cells.⁵³ Length of exposure to high glucose also influences PKC isoform translocation in these cells.⁵⁴ Previous studies reported that

exposure to high glucose levels or PMA stimulation increases PKC- α , - β 1, - β 2, PKC- δ , and - ϵ membrane phosphorylated fraction in mesangial cells.^{53–57} AGEs also play a significant role in the pathogenesis of vascular and renal complications associated with diabetes. Two groups indicated that AGEs modulate, directly or indirectly via oxidative stress, particulate and membrane localization of PKC- β in mesangial cells.^{58,59} In addition, the development of mesangial expansion and basement membrane thickening in diabetes correlates with increased expression of transforming growth factor (TGF)- β . The implication of PKC causing elevated production of ECM and TGF- β is further supported by several reports showing that LY333531 prevents hyperglycemia-increased ECM production and TGF- β expression in mesangial cells.⁶⁰ Another group demonstrated that high glucose concentrations stimulate PKC- ζ activity, as measured by immune complex kinase assay and immunofluorescence confocal microscopy. This activity is suppressed by a TGF- β receptor inhibitor (LY364947).⁶¹

PKC Activation: Animal Model Studies

Atherosclerosis

Diabetes macrovasculopathy is associated with structural and functional changes in large vessels that lead to blood flow obstruction, hypertension, myocardial infarction (MI) and possibly death. Atherosclerosis, characterized by endothelial dysfunction, cytokine expression, monocyte infiltration, SMC proliferation, impaired fibrinolysis combined with increased thrombosis, and chronic inflammation, causes ischemic heart diseases, MI, stroke, and peripheral arterial diseases.^{62–64} Hyperglycemia is believed to participate in atherosclerotic plaque formation. Intensive therapy treatment (Diabetes Control and Complications Trial study) showed a significant decrease in carotid artery intima/media thickness.⁶⁵ However, the disease state associated with insulin resistance can also accelerate atherosclerosis which suggests that metabolic abnormalities associated with diabetes, and not hyperglycemia per se, can increase the risk for macrovascular complications. Nevertheless, activation of PKC isoforms, especially the β isoform, has been the subject of recent investigation. Schmidt, Yan, and colleagues recently published supportive data on the potential role of PKC- β in atherosclerotic plaque formation. Depletion of PKC- β gene or treatment with LY333531 in apolipoprotein e-deficient mice decreased atherosclerosis by inhibiting the Erg-1 (early growth response-1) protein, which regulates vascular cell adhesion molecule expression and matrix metalloproteinase-2 activity preferentially in ECs.⁶⁶ However, additional isoforms may be involved in other steps of atherosclerosis. Novel PKC- δ participates in SMC apoptosis and deletion of this isoform, leading to formation of arteriosclerosis.⁶⁷ Therefore, whether it will be beneficial to inhibit multiple PKC isoforms to halt or prevent complex mechanisms related to atherosclerosis remains to be resolved.

Cardiomyopathy

Myocardial pathologies in diabetic patients include diastolic dysfunction, microvascular diseases, and interstitial fibrosis, the last of which could be the result of active tissue remodel-

ing contributing to reduction of cardiac contractility.⁶⁸ Independent of the severity of coronary artery disease, hypertension and other risk factors, diabetic patients have elevated risk of congestive heart failure, inadequate collateral vascular formation in response to ischemia⁶⁹ and greater likelihood of developing heart failure and reinfarction after MI.⁷⁰ Quantitative immunoblotting revealed a significant increase in membrane fraction expression of PKC- β 1 and - β 2 in human failed hearts compared to nonfailed.⁷¹ This study also showed that treatment with LY333531 decreases PKC activation in human heart.⁷¹ Previously, animal studies reported that PKC- α , - β 1, and - β 2 isoform mRNA and protein expression in the membrane fraction are increased in the heart and aorta in the hyperglycemic condition.^{12,72} Genetically modified mice have been developed to better examine the role of PKC in heart diseases. Transgenic mice overexpressing PKC- β 2 develop cardiomyopathy, supporting the role of PKC- β 2 in myocardial hypertrophy and fibrosis.⁷³ Both angiotensin-converting enzyme (ACE) and PKC- β inhibitors ameliorate the metabolic gene profile and affect PKC activity in diabetic hearts without altering circulating metabolites.⁷⁴ In cardiomyocytes, free fatty acids increased metabolic genes such as PDK4 and UCP3 and mediated the inhibition of basal and insulin-stimulated glucose oxidation, all of which are prevented by treatment with LY333531. Furthermore, treatment with islet cell transplantation, LY333531 and ACE inhibitor (captopril) improved diastolic function, increased glucose utilization by 36%⁷⁴ and attenuated myocyte hypertrophy and collagen deposition⁷⁵ in diabetic rat hearts. Together, these results suggest that both ACE and PKC- β inhibitors regulate fuel metabolic gene expression directly in the myocardium and consequently improve cardiac function and metabolism in diabetes. Other PKC isoforms may also play a role in cardiomyopathy. A recent study reported that PKC- α -deficient mice exhibit increased cardiac contractility and are less susceptible to heart failure following long-term pressure-overload stimulation.⁷⁶

Connective tissue growth factor (CTGF) expression may contribute to cardiac fibrosis. Interestingly, transgenic PKC- β 2 mice showed increased CTGF expression in the myocardium, suggesting that CTGF may act directly or indirectly with other cytokines to induce cardiac fibrosis.⁷³ By opposition, inhibition of PKC- δ exacerbated CTGF expression and potentially cardiac fibrosis in the presence of angiotensin II.⁷⁷ However, careful attention should be given to PKC- δ activation because this isoform regulates proapoptotic signaling in cardiomyocytes during myocardial ischemia/reperfusion injury.⁷⁸ Furthermore, cardiac-specific activation of PKC- ϵ prevented diabetes-induced pathogenetic changes in the heart, including ventricular function⁷⁹ (Figure 4).

Among the processes induced by hyperglycemia, activation of PKC may contribute to cardiomyopathy by inhibiting insulin's metabolic actions, perhaps by phosphorylation of serine/threonine residues on the insulin receptors or their substrates.⁸⁰ Cardiomyocytes isolated from nondiabetic animals and cultured in high glucose concentrations exhibited impaired insulin-stimulated glucose uptake compared to myocytes in normal glucose levels.⁸¹ Loss of insulin action in myocardium is associated with lower basal expression of

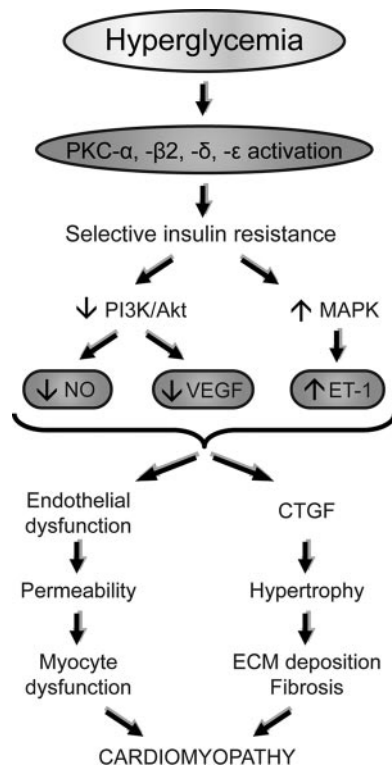


Figure 4. Schematic representation of various biological targets of PKC activation leading to selective insulin resistance and diabetic cardiomyopathy.

hypoxia-inducible factor-1 α , which affects VEGF expression in the myocardium.⁸² In contrast to nondiabetic patients, insulin-resistant and diabetic patients showed a downregulation of VEGF and its receptors.⁸³ Moreover, several studies of diabetic animals confirmed this finding, showing a reduction of mRNA and protein expression of VEGF and its receptors, cardiac VEGF signaling, and coronary capillary density in the myocardium.^{84,85} Interestingly, another group reported that diabetic patients with chronic coronary heart diseases show an elevation of myocardial VEGF expression but a reduction of its receptor expression and subsequently its downstream signal transduction.⁸⁶ Nevertheless, VEGF signaling impairment results in decreased formation of collateral circulation, which explains bigger infarct size and congestive heart failure after MI in patients with diabetes.

Retinal Tissues

The pathogenesis of diabetic retinopathy is a complex process involving multiple factors.⁸⁷ The early stage of diabetic retinopathy is characterized by loss of pericytes around capillaries in the retina. Indeed, pericyte loss occurs before clinically discernible retinopathy. This is followed by development of weakness in the capillary wall, leading to capillary aneurysm formation (microaneurysms) and fluid leakage from capillaries as their walls become more permeable, with increased adhesion of leukocytes and monocytes to the endothelium.⁸⁷ Hyperglycemia activates several PKC isoforms in retinal tissues, including PKC- α , - β , - δ , and - ϵ .^{12,88} PKC activation causes retinal vascular dysfunction by altering enzyme activities in ECs (NO, ET-1, VEGF) and peri-

cytes (platelet-derived growth factor [PDGF], reactive oxygen species, nuclear factor κ B, and MAPK).⁸⁹ In diabetic animals, blood flow is decreased in the retina but elevated in glomeruli.^{90–92} In streptozotocin-induced diabetic rats, treatment with LY333531 (0.1 to 10 mg/kg) improved retinal hemodynamic abnormalities.¹³ Interestingly, systemic oral administration for 2 weeks of LY333531 to diabetic rats from the onset of the disease normalized the retinal blood flow (RBF)¹³ and prevented the induction of ET-1 mRNA expression.⁹³ Furthermore, local intravitreal injection of LY333531 (5 nmol/L) reduced retinal PKC activation and retinal circulation time and restored RBF in diabetic rats.⁹⁴ Alterations in NO production and eNOS expression directly influence vascular hemodynamics such as contraction and relaxation, which may affect RBF. In vessels isolated from diabetic patients and animals, acetylcholine stimulation, which induces vessel relaxation, appears to be delayed.^{95,96} PKC agonist PMA provokes vascular relaxation impairment in normal arteries.⁹⁷

How diabetes regulates and increases proapoptotic factors has been researched for many years. However, the impact of hyperglycemia on reducing survival factors has been much less studied. We recently reported that both PKC- β and PKC- δ translocate to the membrane fraction in total retinal lysates of diabetic mice, but the consequences of these 2 PKC isoform activations were very different: PKC- δ induced cellular apoptosis,¹⁷ whereas PKC- β enhanced cellular growth.⁹⁸ Accordingly, an increase in membrane PKC- δ levels for several months of diabetes correlated with the appearance of retinal pericyte apoptosis in vitro and acellular capillaries in vivo. In vivo studies showed that induction of retinal PKC- δ of diabetic mice leads to PDGF resistance, which is not observed in *Prkcd*^{-/-} mice. Furthermore, specific inhibition of PKC- δ in pericytes in vitro or disruption of *Prkcd*^{-/-} mice prevented NF- κ B activation and restored signal transduction of PDGF. Our study found that hyperglycemia through PKC- δ actions promotes 2 distinct and equally important pathways by (1) increasing reactive oxygen species production and NF- κ B activity and (2) decreasing the important survival signaling pathway of PDGF by upregulating the expression of a protein tyrosine phosphatase, SHP-1. These findings identify a pivotal role for PKC- δ in causing retinal cell apoptosis and the formation of acellular capillaries (Figure 5).¹⁷

Renal Tissues

Many studies have correlated changes in hyperglycemia with severity and progression of nephropathy affecting both the glomeruli and renal proximal tubules.^{2,3,99,100} The specific effects of PKC isoforms on diabetic complications in the kidneys are just beginning to be determined using pharmacological approaches and genetically altered mice. Menne et al reported that diabetic *Prkca*^{-/-} mice are resistant to the development of albuminuria and glomerular hyperfiltration stimulated by VEGF.¹⁰¹ The translocation of PKC- α to renal cortical membranes by diabetes is associated with NADPH-dependent superoxide production and elevated renal serum and urinary VEGF.¹⁰² Furthermore, previous animal studies reported that deletion of the PKC- β gene or treatment with

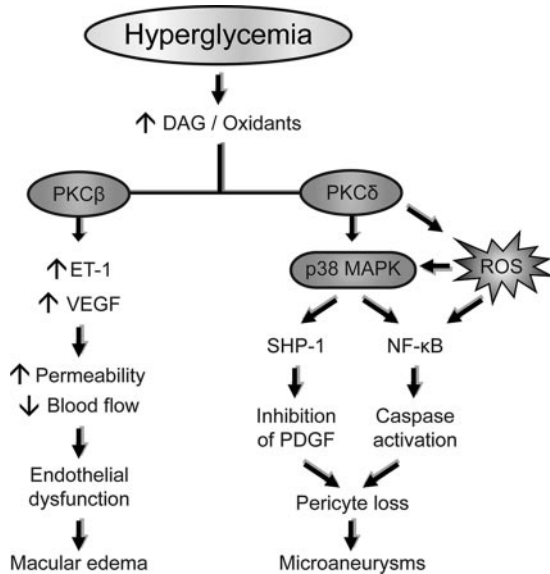


Figure 5. Schematic representation of potential biological targets of PKC activation causing diabetic retinopathy.

LY333531 protects against diabetes by causing increases in renal hypertrophy, glomerular hyperfiltration, ECM production, expression of CTGF, and production of TGF- β 1 and reactive oxygen species (Figure 6).^{13,60,103–105} Even in nondiabetic kidney disease, LY333531 treatment attenuated the impairment in glomerular filtration rate and reduced the extent of both glomerulosclerosis and tubulointerstitial fibrosis in subtotaly nephrectomized rats.¹⁰⁶ Interestingly, analysis of the renal phenotype in *Prkce*^{-/-} mice showed elevated albuminuria at 6 and 16 weeks of age, with increased tubulointerstitial fibrosis and mesangial cell expansion.¹⁰⁷ These data suggest that not all PKC isoforms have adverse effects on kidney pathologies.

Neuronal Tissues

The contribution of PKC activation to diabetic neuropathy still requires clarification. PKC contributes to diabetic neuropathy by a neurovascular mechanism such as blood flow and conduction velocity. Immunohistochemical analysis demonstrated the presence of PKC- α , - β 1, - β 2, - γ , PKC- δ , and - ϵ isoforms in nerve.^{108,109} A previous report demonstrated a reduction of PKC activity by direct measurement of sciatic nerve tissues in streptozotocin diabetic rats.¹¹⁰ These results contrast with more recent studies showing that treatment with nonselective PKC isoform inhibitor as well as selective PKC- β inhibitor improve neural function in diabetic animals.^{111,112} Initial evidence suggested that PKC is involved in the mechanism leading to reduced Na⁺-K⁺-ATPase activity, resulting in decreased nerve conduction and nerve regeneration.¹¹³ Membrane-associated PKC activity was reduced in diabetic mice treated with nonselective PKC inhibitors that restored or maintained Na⁺-K⁺-ATPase activity.^{111,114} Furthermore, previous studies examined the effect of diabetes in rat sciatic nerve and demonstrated that LY333531 treatment prevents the development of diabetic nerve dysfunction.¹¹⁵ Indeed, Cameron et al showed that treatment with LY333531 at low dose improves motor nerve conduction velocity,

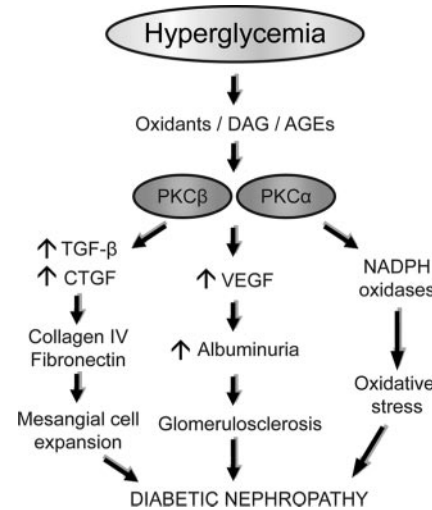


Figure 6. Schematic representation of various biological targets of PKC activation leading to diabetic nephropathy.

normalizes nerve blood flow and restores Na⁺-K⁺-ATPase activity in diabetic rats.¹¹⁶ However, in this study, diabetes did not affect nerve PKC activity or DAG levels. Overall, these studies suggest that PKC inhibition on nerve conduction improves nerve blood flow rather than nerve Na⁺-K⁺-ATPase activity.

Insulin Resistance

Although the principal function of insulin is to regulate and maintain glycemic control, this hormone has many vasotropic actions. Insulin normally stimulates vasorelaxation through a direct effect on blood vessels mediated by endothelium-derived NO.¹¹⁷ Insulin also promotes endothelial NO production by rapid posttranslational mechanisms,¹¹⁸ as well as eNOS gene expression.¹¹⁹ Therefore, insulin resistance, diabetes or reduction of insulin-stimulated regulation of endothelium-derived NO may be an important factor for vascular homeostasis.¹²⁰ Insulin signaling in vasculature of obese Zucker rats showed inhibition of the phosphoinositide 3-kinases (PI3Ks) pathway but no change in the extracellular signal-regulated kinases (ERK)1/2 cascade, suggesting a selective insulin resistance phenomenon.¹²¹ This observation was subsequently reported in skeletal muscle from obese people and patients with type 2 diabetes¹²² and in the myocardium of obese Zucker rats.¹²³ Vasculature of obese Zucker rats also exhibited elevated PKC activity¹²⁴ and reduced insulin-stimulated insulin receptor substrate tyrosine phosphorylation.¹²¹ The question of which PKC isoform's elevation is involved in this process has generated much interest. Treatment with LY333531 normalized the reduction of insulin-stimulated NO production in the aorta of obese Zucker rats.¹²⁴ Transgenic mice overexpressing PKC- β 2 exhibited decrease Akt activation in vascular cells after insulin stimulation.¹²⁴ Although strong evidence corroborates PKC-induced insulin signaling inhibition, the complex mechanisms involved are not fully understood. PKC activation prevents PI3K pathway at the insulin receptor substrate level, which affects several signaling molecules related to this pathway (see elegant review by Sampson and Cooper).¹²⁵

Table 2. Clinical Trials Using a PKC Inhibitor

Clinical Trials	Patients (N)	Treatment	Clinical Outcomes
Aiello et al (2006) ¹³¹	29	RBX 4, 16, 32 mg/day (1 month, dose- dependent)	Normalization of RBF at 32 mg/day
PKC-DRS (2005) ¹³³	252	RBX 8, 16, 32 mg/day (36–46 months) vs placebo	Significantly delayed occurrence of moderate visual impairment; no reduction in progression of proliferative DR
PKC-DMES (2007) ¹³⁴	686	RBX 4, 16, 32 mg/day (minimum 30 months) vs placebo	No significant effect of RBX to reduce progression of diabetic macular edema
PKC-DRS2 (2006) ¹³⁵	685	RBX 32 mg/day (36 months) vs placebo	40% risk reduction in vision loss
Tuttle et al (2005) ¹³⁷	123	RBX 32 mg/day (12 months) vs placebo	Decrease in urinary ACR
Vinik et al (2005) ¹³⁸	205	RBX 32, 64 mg/day (1, 3, 6, 12 months) vs placebo	Improvement with RBX 64 mg/day in patients with NTSS-6>6 at baseline
Beckman et al (2002) ¹⁴³	15	RBX 32 mg/day (7 days) vs placebo	Significant inhibition of endothelium-dependent vasodilatation after hyperglycemic clamp
Mehta et al (2009) ¹⁴⁴	108	RBX 32 mg/day (6–8 weeks) vs placebo	Trend but not significant improvement in flow-mediated dilatation

PKC-DMES indicates PKC-Diabetic Macular Edema Study; PKC-DRS, PKC-Diabetic Retinopathy Study.

Insulin actions mediating the ERK1/2 pathway are accentuated by PKC actions.¹²⁶ Thus, selective insulin resistance, caused by PKC activation, enhances insulin's proatherosclerotic mechanisms via ERK1/2 signaling or inhibits its anti-atherosclerotic mechanisms by inhibiting the PI3K/Akt pathway.¹²⁷

PKC Inhibitors and Human Clinical Trials

General PKC isoform inhibitors exist. However, these non-isoform-specific inhibitors interact with other ATP binding kinases and therefore display toxic and severe side effects in vivo. Suitable specific PKC isoform inhibitors for therapeutic or clinical studies should target the phospholipid or phorbol ester binding site of the PKC structure, called regulatory domain, or bind to the substrate or ATP binding site of the catalytic domain. For example, indolcarbazole or bisindolylmaleimide are selective inhibitors that target the ATP-binding site of catalytic domain. The PKC- β inhibitor ruboxistaurin (RBX) (also known as LY333531 or Arxxant, Eli Lilly, Indianapolis, Ind) is a class of bisindolylmaleimide.^{13,104} Rottlerin (mallotoxin), a natural product derived from *Malolotus philippensis*, has higher affinity for PKC- δ (IC₅₀, 3 to 6 μ mol/L) but also inhibits other isoforms of PKC (IC₅₀, >30 μ mol/L)¹²⁸ and other non-PKC kinases, such as MAPK, PKA, and glycogen synthase kinase-3.¹²⁹ In contrast, RBX shows selective inhibition for PKC- β 1 and PKC- β 2 with IC₅₀ values at 4.5 and 5.9 nmol/L, respectively.¹³⁰

RBX is the most studied PKC inhibitor in cellular, animal and especially human studies (Table 2). Phase I studies using RBX included patients who had diabetes for less than 10 years and no evidence of clinical retinopathy. This study determined the dose needed to normalize RBF, an early marker of diabetic retinopathy.¹³¹ The dose-response curve showed that a minimum dosage of 32 mg/d orally is required to prevent decreases in RBF in diabetic patients.¹³¹ Low doses did not completely normalize RBF. Few side effects were found during clinical trials that lasted up to 4 years.¹³² Phase II and phase III clinical trials were conducted in late stages of nonproliferative diabetic retinopathy, with the loss of visual acuity as the primary end point. The first 2 clinical

trials, PKC-Diabetic Retinopathy Study (PKC-DRS) and PKC-Diabetic Macular Edema Study (PKC-DMES),^{133,134} failed to reach primary outcomes because of multiple factors (unpowered, 3 treatment arms of differing dosages, high dropout rate of patients). However, there was a significant reduction in the secondary end point of the progression of diabetic macular edema to a certain stage. A much larger clinical trial, PKC-DRS2, was undertaken using a single oral dose (32 mg versus placebo), with the primary end point of visual acuity rather than diabetic retinopathy progression.¹³⁵ The results showed that RBX significantly prevents reduction of visual acuity in diabetic patients with moderate visual loss and decreases the onset of diabetic macular edema.¹³³ These clinical results suggest that PKC activation, especially of the β isoform, participates in the development of diabetic retinopathy. However, because treatment with RBX preserves visual acuity by decreasing capillary permeability or targeting the neural retina but cannot significantly delay the progression of diabetic retinopathy, these data suggest that inhibition of the PKC- β isoform alone is not enough to stop the early metabolic changes that are likely driving the progression of preproliferative diabetic retinopathy.

The role of PKC- β was also evaluated in patients with diabetic nephropathy. In kidney biopsies of diabetic patients, quantitative real-time PCR analysis showed a 9.9-fold increase in PKC- β mRNA expression as compared to control subjects.¹³⁶ A phase II clinical trial was conducted using RBX (32 mg/d) to determine whether PKC- β inhibition can be effective in type 1 and 2 diabetic patients with high proteinuria (>300 mg/d) treated with ACE inhibitors or angiotensin-receptor blockers. Results suggested that 1 year of treatment with RBX in addition to angiotensin II inhibitors or receptor blockers decreases the loss of glomerular filtration rate and proteinuria in diabetic patients.¹³⁷ However, more data and phase III clinical trials are needed to confirm the role of PKC and its subsequent signaling pathways in diabetic nephropathy, especially in very early histopathologic manifestations.

Vinik et al conducted a 1-year trial of RBX treatment, using a standardized clinical neurological examination to

measure changes in neuropathy sensory symptoms by the neuropathy total symptoms score-6 (NTSS-6) and quantitative sensory testing by the vibration detection threshold.¹³⁸ Patients with diabetic peripheral neuropathy taking placebo versus either 32 or 64 mg of RBX were evaluated at baseline and at 1, 3, 6, and 12 months in each group.¹³⁸ RBX had no significant effect on the overall patient population. However, results showed a significant change in the NTSS-6 at 6 months and 12 months in patients who had clinically significant neuropathy sensory symptoms (NTSS-6 > 6) at baseline when treated with RBX 64 mg compared with placebo. There were no treatment-related differences in change from baseline to end point for vibration detection threshold among all symptomatic patients. However, in a subset of patients with less severe symptomatic diabetic peripheral neuropathy, as defined by a measurable sural nerve action potential, RBX (32 mg and 64 mg) statistically improved vibration detection threshold compared with placebo.¹³⁸

Abnormal endothelial function has been demonstrated in type 1 and type 2 diabetes, as well as in obese, insulin-resistant patients whose insulin sensitivity correlated with the magnitude of endothelium-dependent vasodilation.^{139–141} Vascular permeability is increased as early as 4 to 6 weeks' duration of diabetes in patients, supporting the concept of EC dysfunction.¹⁴² A previous study also reported that infusion of glucose for 6 hours decreases endothelium-dependent vasodilation in nondiabetic and healthy people.¹⁴³ In the natural history of type 2 diabetes, the onset of hyperglycemia precedes endothelial dysfunction,¹⁴¹ whereas onset of endothelial dysfunction coincides with the presence of hyperglycemia in type 1 diabetes. Treatment with RBX (32 mg/d for 7 days) prevented endothelium-dependent vasodilatation abnormalities induced by hyperglycemia.¹⁴³ Moreover, a recent small, double-masked, placebo-controlled study in type 2 diabetes showed that RBX (32 mg/d) after 6 weeks improves femoral-mediated dilatation at 1 and 5 minutes after cuff deflation as compared to placebo.¹⁴⁴ These data suggest that PKC activation, especially the β isoform induced by hyperglycemia, may be responsible for the endothelial dysfunction observed in diabetic patients. Clearly, further studies are needed to determine whether RBX can effectively improve endothelial function in type 1 and 2 diabetic patients.

Summary

In summary, many years of human and animal data confirm the long-held belief that hyperglycemia perturbs arteries and vascular cell function. The source of such complications in diabetes is certainly multifactorial, with key roles identified for oxidants and glycation metabolites. However, a large body of literature supports hypothesis that hyperglycemia or diabetes leads to vascular DAG accumulation and ensuing PKC activation, causing a variety of cardiovascular defects. Indeed, modulation of PKC signaling transduction pathways affects the pathogenesis of various diabetic vascular complications. Activation of specific PKC isoforms by hyperglycemia and lipid metabolites is likely to be responsible for specific vascular pathologies such as EC and SMC dysfunction, ECM synthesis and fibrosis, monocyte activation, de-regulation of cytokines, and vascular insulin dysfunction.

These intermediate cellular alterations contribute significantly to the development of macrovascular complications such as cardiomyopathy and acceleration of atherosclerosis, and microvascular complications such as retinopathy and nephropathy. Clinical trials have shown potential for PKC- β inhibitor as a therapy for diabetic vascular complications. However, not all clinical trials demonstrated positive effects of PKC- β inhibition. For example, RBX trials did not exhibit robust effects on the improvement of painful neuropathy or sexual dysfunction in diabetic patients.¹⁴⁵ Therefore, more clinical trials targeting multiple PKC isoforms are urgently needed to test the effectiveness in delaying, stopping and even reversing diabetic vascular complications.

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Disclosures

None.

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