25 *Protein Kinase C Inhibitors and Diabetic Microvascular Disease*

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Abstract: Diabetes is characterized by major changes in the microvascular system. Multiple pathways exist that explain the occurrence of vascular and cellular changes in diabetes. These are abnormalities in oxidation pathways, polyol pathways, glycation end products and protein kinase C (PKC) pathways. Drugs that inhibit PKC-beta are being developed and some of the drugs such as riboxstaurin that are being tried in large randomized trials appear promising for diabetic retinal disease. The drug development is in its infancy and much more pharmacological effort and clinical trials are needed before such drugs are routinely prescribed.

INTRODUCTION

Diabetes mellitus is characterized by hyperglycemia, a relative or absolute deficiency of insulin and the development of diabetic-specific pathology in retina, renal glomerulus and peripheral nerves. It is also associated with accelerated atherosclerotic macrovascular disease affecting arteries that supply heart, brain and lower extremities. This condition is more extensive and progressive in diabetics as compared to non-diabetics. Due to the extensive nature of microvascular disease in diabetes, blindness and end stage renal disease occurs. More than 60% of the diabetes mellitus patients suffer from neuropathy which includes distal symmetric polyneuropathy, mononeuropathy, autonomic neuropathy causing erectile dysfunction, urinary incontinence, gastroparesis and nocturnal diarrhea. Diabetes leads to 50% of all nontraumatic amputations and 2-6 fold increase in cardiovascular complications. Overall micro vascular diabetic disease leads to 7-10 years of shorter life expectancy. Large clinical trials show a strong relationship between hyperglycemia and diabetic micro- and macrovascular complications in both type 1 and type 2 diabetes mellitus.

MECHANISMS OF HYPERGLYCEMIA INDUCED DAMAGE (FIG. 25.1)

There have been four major hypothesis which involve the hyperglycemia causing diabetic complications. These pathways are (i) increased polyol pathway flux; (ii) increased intracellular advanced glycated end-product (AGE) formation; (iii) increased hexosamine pathway flux; and (iv) activation of protein kinase C (PKC). This is schematically shown in Figure 25.1. However, there is no unifying hypothesis that links all these mechanisms together. One hypothesis considers that hyperglycemia is associated with accumulation of AGE and the production either denovo or by hydrolysis of diacylglycerol and oxidative stress. This increases or activates the PKC system, specifically PKC-β induced mechanisms. The PKC-β activation leads to micro vascular damage including retinopathy with visual loss, neuropathy with ulcers and amputations, and nephropathy with renal failure.

PROTEIN KINASE C

Protein kinase C (PKC), a ubiquitous, phospholipid-dependent enzyme, is involved in signal transduction associated with cell proliferation, differentiation, and apoptosis. At least eleven closely related PKC isozymes have been reported that differ in their structure, biochemical properties, tissue distribution, sub cellular localization, and substrate specificity. They are classified as conventional (a, b1, b2, g), novel (d, e, h, g, m), and atypical (z, l) isozymes. Conventional PKC isozymes are Ca^{++}dependent, while novel and atypical isozymes do not require Ca^{++} for their activation. All PKC isozymes, with the exception of z and l, are activated by diacylglycerol (DAG). PKC isozymes negatively or positively regulate critical cell cycle transitions, including cell cycle entry and exit and the G_1 and G_2 checkpoints. All PKC isoforms show different distribution among various cells. The a, d, and z isoforms are found in all cells. The g isoform is found only in neuronal cells. The b, e, and l isoforms are found in various tissues, whereas h and t isoforms are predominantly found in epithelial and immune cells.

ACTIVATION OF PROTEIN KINASE C (FIG. 25.2)

PKC activation is a critical step in the pathway to diabetic microvascular complications. Preclinical data suggest that the PKC-β isoform plays a major role in this process. In animal diabetes models specific inhibition of PKC-β normalizes many diabetic related changes in vascular functions, e.g. retinal blood flow, endoneural blood flow and sensory and motor nerve conduction velocity. PKCs are a family of at least 11 isoforms, 9 of which are activated by the lipid second-messenger diacylglycerol (DAG). Intracellular hyperglycemia increases DAG content in cultured microvascular cells and in the retina and renal glomeruli of diabetic animals. Intracellular hyperglycemia appears to increase DAG content primarily by increasing its de novo synthesis from the glycolytic intermediate glyceraldehyde-3-phosphate via reduction to glycerol-3-phosphate and stepwise acylation. Increased de novo synthesis of DAG activates PKC both in cultured vascular cells and in retina and glomeruli of diabetic animals. Increased DAG primarily activates the β and δ isoforms of PKC, but increases in other isoforms have also been found, such as *PKC*-α and PKC-ε isoforms in the retina and *PKC*-α and *PKC*-δ in the glomerulus of diabetic rats.

Another mechanism of PKC activation involves transient receptor-mediated stimulation of phospholipase C, which hydrolyzes inositol phospholipids of the plasma membrane to yield DAG, which again activates PKC after binding to an amino-terminal domain of the enzyme. In diabetes, however, intracellular DAG can also be increased in vascular tissue through *de novo* synthesis from glucose via glyceraldehyde 3-phosphate and phosphatidic acid or from nonesterified fatty acids. In particular, the β2 isoform of PKC is activated in the retina and renal glomeruli, and treatment with an inhibitor specific for the PKC β2 isoform partly normalizes retinal blood flow and albuminuria in rats with streptozotocin-induced diabetes. Likewise, the same inhibitor decreases mesangial expansion and albuminuria in diabetic db/db mice.

Theoretically, PKC may be activated in diabetes independently of DAG synthesis. Thus, *cis*-unsaturated fatty acids may synergistically increase the effect of DAG at basal levels of Ca^{++}. Furthermore, superoxide may directly activate PKC by releasing zinc from the zinc finger of the enzyme. Whether PKC is activated by such mechanisms in diabetes is unknown. It is interesting that treatment with vitamin E inhibits PKC by lowering vascular DAG concentrations, perhaps by increasing the enzymatic breakdown of DAG to phosphatidic acid or by activating protein phosphatase 2A, which will dephosphorylate PKC. This may be independent of the antioxidant effects of the vitamin.

Blood Flow Abnormalities

In early experimental diabetes, activation of PKC-β isoforms has been shown to mediate retinal and renal blood flow abnormalities, perhaps by depressing NO production and increasing

endothelin-l activity. Abnormal activation of PKC has been implicated in the decreased glomerular production of NO induced by experimental diabetes and in the decreased smooth muscle cell NO production induced by hyperglycemia. PKC activation also inhibits insulin-stimulated expression of endothelial nitric oxide synthase (eNOS) messenger RNA (mRNA) in cultured endothelial cells. Hyperglycemia increases endothelin 1-stimulated mitogel-activated protein kinase activity in glomerular mesangial cells by activating PKC isoforms. The increased endothelial cell permeability induced by high glucose in cultured cells is mediated by activation of PKC-α, however. Activation of PKC by elevated glucose levels also induces expression of the permeability-enhancing factor VEGF in smooth muscle cells.

Chemokine Activation

In addition to affecting hyperglycemia-induced abnormalities of blood flow and permeability, activation of PKC contributes to increased micro vascular matrix protein accumulation by inducing the expression of transforming growth factor (TGF)-β_1, fibronectin, and $\alpha_1$type-5 collagen in both cultured mesangial cells and in the glomeruli of diabetic rats. This effect appears to be mediated through PKC's inhibition of nitric oxide production. Hyperglycemia-induced expression of laminin-C1 in cultured mesangial cells is independent of PKC activation, however. Hyperglycemia-induced activation of PKC has also been implicated in the over expression of the fibrinolytic inhibitor (PAI-1) and in the activation of the pleiotrophic transcription factor nuclear factor (NF-κB) in cultured endothelial cells and vascular smooth muscle cells.

Endothelial Dysfunction

Vascular PKC activation causes endothelial dysfunction. Impaired endothelium-dependent relaxation during high glucose concentrations is prevented by a PKC inhibitor. Oral administration of a selective inhibitor of the PKC-β isoform prevents endothelial dysfunction in rats with streptozotocin induced diabetes or in healthy humans after 6 hours of hyperglycemic clamp. Activation of PKC in the vasculature by high glucose concentrations elicits a host of signaling responses in different vascular cell types. For example, increased concentrations of OAC and PKC activation result in increased expression of angiotensin-2 in proximal tubular cells. Induction of TGF-β in the kidney in diabetic rodents is dependent on PKC. Furthermore, increased permeability of endothelial cells cultured at high glucose concentrations is mediated by PKC.

Complex Vascular Pathways

The complexity of the role of PKC in signal transduction pathways important for pathogenesis of micro vascular complications is illustrated by the fact that PKC activity may be both upstream and downstream of other mediators such as vascular endothelial growth factor (VEGF) and endothelin-1 (ET-1). Thus, activation of PKC by high glucose may induce VEGF and ET-1, but VEGF-induced proliferation of endothelial cells and retinal neovascularization are dependent on PKC. Furthermore, the induction of ET-1 by POCP in retinal pericytes and stimulation of mitogen-activated protein kinase (MAPK) by ET-1 in mesangial cells cultured in high concentrations of glucose are dependent on PKC. Other roles for PKC in several signal transduction mechanisms are also reported.

Nervous system: Although the activity of protein kinase C (PCK) in nerve is uncertain, it is known that inhibition of PCK-β will reduce oxidative stress and will normalize the deficits in blood flow and nerve conduction. In endothelial cells, high glucose causes activation of NF-κB. Co-incubation with a selective PKC inhibitor, calphostin C, produced a concentration-dependent inhibition of glucose-induced activation of NF-κB, suggesting that PKC is important at the

endothelial cell level in the activation of adhesion molecules and the generation of reactive oxygen species particles.

Protein Kinase C Inhibitors

There is a long list of chemical substances found to inhibit the activity of PKC (Table 25.1). Two PKC inhibitors, LY333531 and PKC412, are being developed to reduce micro vascular complications in patients with diabetes.

Table 25.1: Protein kinase C inhibitors under evaluation

Drug classes	*Chemical compounds*
Macrocyclic bis(indolyl) maleimides	LY-333531 (riboxistaurin) LY-379196 LY-317615
Midostaurins	PKC 412 CGP 41251 N-benzoylstaurosporine
Others	7-hydroxystaurosporine (UCN-01) Bryostatin 1 Perifosine Ilmofosine RO 31-8220 RO 32-0432 GO 6976 ISIS-3521 (CGP 64128A)

Ruboxistaurin: One of these, ruboxistaurin (LY33353l), is a specific inhibitor of PKC-β (09) and has been found to block vascular complications of diabetes, including abnormalities in retinal blood flow, neovascularization, and VEGF-mediated effects on permeability in animal models. In a small trial (*n* = 29) of diabetic patients with no or minimal retinopathy, the drug was well tolerated with no adverse events noted and normalized mean circulation time and retinal blood flow abnormalities. The PKC-β Inhibitor Diabetic Macular Edema Study (PKC-DMES) was a multicentric, multinational, double masked, placebo-controlled trial with patients followed for up to 52 months. The trial included 686 patients with diabetic macular edema (DME) and mild-to-moderate non-proliferative diabetic retinopathy (NPDR). There was no statistically significant reduction in progression of retinopathy or incidence of macular edema found in this trial. However, ruboxistaurin, when given at 32 mg, displayed a trend (P = 0.04) toward a positive effect on the secondary outcome of occurrence of DME involving or imminently threatening the center of the macula. When patients with poor glycemic control at baseline were excluded (defined as $HbA_{1c} \geq 10\%$), ruboxistaurin displayed a borderline positive effect (P = 0.019) on the occurrence of DME involving or imminently threatening the center of the macula. Additional clinical trials are under way to assess whether this compound can delay or stop the progression of diabetic retinopathy and macular edema at their earliest stages.

The Protein Kinase C Diabetic Retinopathy Study (PKC-DRS) was recently completed. This study is a phase II/III randomized, multidose (three doses compared with placebo), multicentric trial. The aim was to slow the progression of nonproliferative diabetic retinopathy (NPDR) or vision loss, including cases of moderate to severe NPDR without prior treatment for proliferative retinopathy. The eyes were examined every 3 months, with retinal photographs taken every 6 months for a minimum follow-up of 36 months. Of the 617 patients recruited, 252 were allocated to placebo. Active treatment was with 8, 16, or 32 mg of ruboxistaurin. The primary end point was either progression of diabetic retinopathy or photocoagulation. Although the study failed to show any significant effect on these primary end points, there was a 32% risk reduction *(P* =

0.029) of moderate visual loss in patients treated with 32 mg of ruboxistaurin compared with placebo. There was also a trend of moderate visual loss sustained over 6 months being reduced in patients taking the highest dose of ruboxistaurin. Patients with higher levels of retinopathy at entry had more benefit from the highest dosage, compared with patients with less-severe retinopathy at baseline. Patients with higher levels of diabetic macular edema at baseline also had a greater benefit from the high-dose ruboxistaurin treatment with regard to sustained moderate visual loss. Although the study did not lead to a statistically significant effect on the primary end point of progression of retinopathy or application of focal photocoagulation, it displayed a trend toward a positive effect on moderate visual loss.

Thus, initial clinical trial data suggest that such PKC inhibitors are safe and can be well tolerated in humans and might have beneficial effects in preventing visual loss from diabetic retinopathy. However, the actual efficacy of the PKC inhibitor and the patient populations responsive to the treatment must be clarified in ongoing clinical trials.

PKC 412: The second PKC inhibitor, PKC412, is under development for treatment of diabetic retinopathy and other indications. This compound inhibits multiple PKC isoforms such as α, β, and γ, and at least two other receptor kinases. Orally administered PKC412 effectively inhibited retinal and choroidal neovascularization in a mouse model. The broader inhibitory potential of this drug, however, suggests that it may impede other physiologic processes. In a phase 1 trial of cancer patients, PKC412 was found to be generally tolerable at the doses tested, but further studies are needed to investigate the utility of PKC412 in preventing diabetic retinopathy/macular edema.

Others: The role of PKC isoforms in signal transduction pathways involved in regulation of the cell cycle, apoptosis, angiogenesis, differentiation, invasiveness, senescence and drug efflux are reviewed, along with the clinical results on the current crop of PKC inhibitors, including midostaurin (PKC-412, CGP 41251, N-benzoylstaurosporine), UCN-01 (7-hydroxystaurosporine), bryostatin 1, perifosine, ilmofosine, Ro 31-8220, Ro 32-0432, GO 6976, ISIS-3521 (CGP 64128A) and the macrocyclic bis (indolyl) maleimides (LY-333531, LY-379196, LY-317615).

An appreciation of the complex, often contradictory roles of PKC isoforms in signal transduction pathways involved in cancer is important for interpreting the clinical results observed with PKC inhibitors of varying selectivity. An antisense oligonucleotide, ISIS-3521 and two orally available small molecule inhibitors, LY 333531 and midostaurin, have now advanced to latter stage development for cancer and/or other indications. These compounds have varying levels of selectivity for the PKC isoforms and for the kinase and initial safety and early clinical efficacy have been encouraging. At this stage, the potential of PKC inhibition for the treatment of cancer has not been fully realized. The concurrent inhibition of multiple PKC isoforms may yet provide an improved clinical outcome in treating cancers in view of the complex interrelated roles of the PKC isoforms.

PKC inhibitors in peripheral neuropathy: Forty percent of people with diabetes have peripheral neuropathy. Till date the management includes intensive glycemic control, but no successful study on reversal or abrogation of underlying disease process. Diabetes with AGE, DAG, oxidative stress and PKC B activation leads to micro vascular dysfunction. It has impact on endothelial derived nitric oxide synthesis and the balancing factors – "the yin and the yang" of vasoconstriction and vasodilatation with endothelin 1 increase and angiotensin II increase. This leads to repeated assaults on the system and ultimately culminates in structural phase causing neuropathy, ulcers and amputation. In a phase 2, multicentric, placebo controlled, randomized, double blind study 250 patients with type 1 or type 2 diabetes with peripheral neuropathy, measured by vibration detection threshold (VDT), were included. The drug used was LY 333531 as 32 mg or 64 mg once daily for 58 weeks and end points assessed at 1, 3, 6 and 12 months. This drug was well tolerated. There was not deterioration of glycemic control and no impairment of

immune function. No worsening of VDT was noted. Larger studies are needed to clarify the role of PKC inhibitors in diabetic neuropathy.

FURTHER READING

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