ABSTRACT

Although the molecular mechanisms involved in the pathogenesis of diabetic microvascular complications are complex, they are potential therapeutic targets.

Of the 4 mechanisms implicated in the development of glycemic injury in vascular tissue, the diacylglycerol (DAG)-mediated activation of protein kinase C (PKC) appears to be the most important and presently is the focus of intense investigation.

When activated by DAG, PKC has important effects on the endothelium. It causes cytoskeletal proteins to contract, leading to morphologic shape changes in the endothelial cell that weaken the intercellular junction and allow macromolecules to leak across the retinal or glomerular barrier in the eye and kidney. PKC also facilitates responses to the vascular endothelial growth factor (VEGF), such as new vessel formation, increased endothelial permeability, and leakage.

Of the 12 PKC isoenzymes that have been cloned and characterized thus far, 2 appear to be particularly involved in hyperglycemia-induced vascular injury—PKCβ1 and PKCβ2. Ruboxistaurin, an orally active molecule that is highly specific for these isoenzymes, has been shown to inhibit PKC activation and interfere with the progression of hyperglycemia to microvascular injury without interfering with any of the other fundamental kinases that are important to the function of many organs.

Experimental data have demonstrated that ruboxistaurin attenuates increased retinal PKC activity and retinal vessel permeability induced by exogenous VEGF. Animal studies also have shown that selective inhibition of the PKCβ2 pathway with the drug can restore endothelial barrier function, improve endothelial contractile and relaxant responses, and prevent the replication, migration, and proliferation of endothelial cells that are involved in new vessel formation.

Clinical trials of ruboxistaurin have shown the drug to be safe for patients, well tolerated, and capable of ameliorating diabetes-associated retinal abnormalities. Continuing trials involve patients with diabetic retinopathy, neuropathy, and nephropathy.

particularly involved in the pathogenesis of microangiopathy, as opposed to macroangiopathy.

Proteinuria and leakage of macromolecules across the endothelial barrier are especially significant in the pathogenesis and progression of diabetic vascular disease. The close correlation between the urinary albumin excretion rate and mortality indicates that leakage of macromolecules across the endothelial barrier in the kidney and elsewhere in the circulation is a surrogate marker of vascular health.

Long regarded as the inert inner lining of blood vessels, the endothelium has now been shown to be an active organ in its own right. It plays an important role in various aspects of vascular function, including thrombosis and hemostasis, vascular tone and structure, lipid transport, permeability, metabolic activity, angiogenesis, inflammation, tumor growth and metastasis, and immune response.

Many of these functions are highly relevant in diabetes. Angiogenesis, for example, is important both in diabetic retinopathy and the development of collateral blood vessels in the peripheral circulation of patients with lower limb ischemia and ulceration. Inflammation within atherosclerotic plaques and the microvasculature of skeletal muscle also is important in vascular complications and possibly in the development of insulin resistance and other metabolic abnormalities of diabetes.

The vasculature is regulated by the effects of various circulating components in the blood on the endothelium and the effects of the endothelium on vascular smooth muscle. Blood components include blood cells, physical forces such as shear stress and blood pressure, and vasoactive substances such as peptides, kinins, amines, and nucleotides. In response to the effects of these various blood components, the endothelium releases various endothelium-derived factors that lead to relaxation, contraction, or proliferation of the underlying vascular smooth muscle.

**MECHANISMS OF GLYCEMIA-INDUCED VASCULAR INJURY**

Four mechanisms have been implicated in the development of glycemic injury in vascular tissue: nonenzymatic glycation forming advanced glycosylation end-products, oxidative-reductive stress, aldose reductase activation, and diacylglycerol (DAG)-mediated activation of protein kinase C (PKC).

Nonenzymatic glycation of tissue proteins causes the structural and functional abnormalities associated with microangiopathy. An increase in reactive oxygen species is involved in the development of the characteristic features of vascular disease. Activation of the aldose reductase pathway also results in vascular tissue injury, but drugs developed to inhibit activation of this pathway have, for the most part, been withdrawn from the market because of lack of efficacy or serious adverse effects. Only one such agent is still on the market in Japan, and another is under investigation.

Diacylglycerol-mediated activation of the PKC pathway, however, is presently the focus of intense investigation, with various PKC isoenzymes cloned and characterized thus far and an inhibitor of PKC β now in ongoing and recently completed clinical trials.

**PROTEIN KINASE C PROPERTIES AND ACTIVATION**

Adding and removing phosphate groups is one of the main mechanisms by which the body regulates the activity of tissue proteins—whether they are receptors, enzymes, signal proteins, or transcription factors. In these reversible processes, kinases add phosphate groups to tissue proteins at serine and threonine residues, and phosphatases remove the phosphate groups.

Protein kinase C adds phosphate groups to a host of protein substrates within vascular tissues at serine and threonine residues and, thus, is considered one of the major serine/threonine-specific protein kinases. By adding phosphate groups, PKC modifies the receptor status of the phosphorylated substrate. PKC also plays an important role in a number of signal transduction pathways, is a calcium-dependent and phospholipid-dependent enzyme and, when activated by DAG (its main endogenous activator) translocates from the cytosol to the plasma membrane where many protein substrates exist, either as receptors or intracellular enzymes. Perhaps the most important feature of PKC is that it is not a single enzyme, but a family of multifunctional enzymes with different patterns of tissue distribution, different cofactor requirements, and different protein substrates.

Glucose enters endothelial and smooth muscle cells through glucose transporter-1 in the vascular cell membrane and then goes through the glycolytic pathway. When hyperglycemia is present, glycerol phosphate, which in turn leads to the de novo
synthesis of specific species of DAG. These DAG species, which vary in fatty acid composition, in turn activate one or more of the isoenzymes of PKC. Fatty acids, which are elevated in patients with diabetes mellitus (particularly type 2), augment PKC activation.

Experimental data show that DAG levels and PKC activity are consistently increased in the presence of hyperglycemia (Table). Studies of human cells yield similar findings. Williams et al demonstrated a dose-response relationship between glucose levels and PKC activity in human vascular smooth muscle,1 and Ceolotto et al demonstrated the same relationship in circulatory monocytes harvested from the blood of human subjects with and without diabetes.2 These findings support the hypothesis that high glucose levels cause widespread activation and translocation of PKC.

PROTEIN KINASE C ISOENZYMES, STRUCTURE, AND FUNCTION

Twelve PKC isoenzymes have been cloned and characterized thus far. They are divided into 3 groups: 4 conventional isoenzymes (cPKC α, β1, β2, γ) that require calcium or phospholipid as cofactors for activation; 5 novel isoenzymes that do not require calcium for activation; and 3 atypical isoenzymes that can be activated independently of calcium or phospholipid. The isoenzymes that seem to be particularly involved in hyperglycemia-induced vascular injury are β1 and β2, although γ and δ (in the novel group) are also involved to some extent.

Protein kinase C is a simple protein molecule that is divided into a regulatory domain containing calcium and DAG binding sites, and a catalytic domain, where the substrate binds when PKC is adding a phosphate group to a protein or a receptor within the cell. Novel isoforms lack a calcium-binding region, and atypical isoforms lack one motif in the phosphate/DAG region.

When hyperglycemia is present, there is an increased uptake of glucose into a variety of vascular cells, particularly endothelial and smooth muscle cells. This leads to an increase in de novo synthesis of DAG, which then activates one or more PKC isoenzymes, especially—but not exclusively—PKC β. The PKC isoenzymes then phosphorylate a host of tissue proteins at serine and threonine residues, leading to changes in the expression of certain gene and membrane transporters, all of which contribute to some of the structural and functional abnormalities associated with diabetic microangiopathy.

ENDOTHELIAL BARRIER FUNCTION AND PERMEABILITY

The endothelium is one of the most important target tissues affected by PKC activation. Under normal glycemic conditions, endothelial cells have tight intercellular junctions that prevent the movement of macromolecules such as albumin across the endothelial barrier. These tight junctions are maintained in part by the protein connexin-43.3 However, in diabetes mellitus increased levels of glucose, circulating hormones, and blood pressure, in addition to hypoxia, stimulate the activation of PKC, which causes cytoskeletal proteins to contract. This contraction leads to morphologic shape changes in the endothelial cell that weaken the intercellular junction and allow leakage of macromolecules,4 resulting in macular edema in the eye or proteinuria in the kidney, for example. Phosphorylation of connexin-43 by PKC also leads to a weakening of the intercellular junction.

Importantly, even when glucose levels are elevated, the weakening of GAP junction intercellular communication associated with hyperglycemia can be reversed.

Table. Relationship Between DAG Level and PKC Activity in Cultured Cells Exposed to High Glucose and in Tissues Isolated from Diabetic Animals

<table>
<thead>
<tr>
<th>DAG Level</th>
<th>PKC Activity</th>
</tr>
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<tbody>
<tr>
<td>Cultured cells</td>
<td></td>
</tr>
<tr>
<td>Retinal endothelial cells</td>
<td>↑</td>
</tr>
<tr>
<td>Aortic endothelial cells</td>
<td>↑</td>
</tr>
<tr>
<td>Aortic smooth muscle cells</td>
<td>↑</td>
</tr>
<tr>
<td>Renal mesangial cells</td>
<td>↑</td>
</tr>
<tr>
<td>Pericytes</td>
<td>→</td>
</tr>
<tr>
<td>Tissues</td>
<td></td>
</tr>
<tr>
<td>Retinal (diabetic rats and dogs)</td>
<td>↑</td>
</tr>
<tr>
<td>Heart (diabetic rats)</td>
<td>↑</td>
</tr>
<tr>
<td>Aorta (diabetic rats and dogs)</td>
<td>↑</td>
</tr>
<tr>
<td>Renal glomeruli (diabetic rats)</td>
<td>↑</td>
</tr>
<tr>
<td>Brain (diabetic rats)</td>
<td>→↑</td>
</tr>
<tr>
<td>Peripheral nerve</td>
<td>ND</td>
</tr>
</tbody>
</table>

↓ = decrease; ↑ = increase; → = no change; DAG = diacylglycerol; ND = not determined; PKC = protein kinase C.
or attenuated if a nonspecific PKC inhibitor known as calphostin C is also present.\(^5\)

Vascular endothelial growth factor (VEGF) plays an important role in inducing endothelial permeability and stimulating new vessel formation. VEGF is an important molecule in diabetic retinopathy, where increased intraocular levels of VEGF have been implicated in the development of macular edema and new vessels,\(^6\) and to a lesser extent in diabetic nephropathy.

Protein kinase C is involved in the activation of the VEGF gene and in VEGF actions on endothelial cells. As demonstrated in an in vitro study, the transcription of VEGF, also known as vascular permeability factor (VPF), was significantly higher for high glucose levels versus normal glucose and for high glucose levels versus high glucose combined with either of 2 nonspecific PKC inhibitors, calphostin C and H7 (\(P < .05\) for all comparisons).\(^7\) In other words, PKC activation increased hyperglycemia-induced VPF gene expression, and PKC inhibition blocked it.

Similarly, the permeability-inducing effects of VEGF are dependent on PKC activation as a downstream signaling molecule. As shown in one study, increasing concentrations of VEGF in bovine aortic endothelial cells were associated with increased PKC activity.\(^8\)

**Protein Kinase C-β Properties and Inhibition**

Protein kinase C plays a crucial role on either side of the VEGF pathway. PKC activation is necessary for high glucose levels to activate VEGF gene transcription and to signal responses to VEGF, such as new vessel formation, increased permeability, and leakage. As shown in Figure 1, the potential role of the PKC-β isoform in diabetic retinopathy recounts this sequence of events.

Ruboxistaurin, an orally active molecule that is exquisitely specific for PKC \(\beta_1\) and PKC \(\beta_2\), inhibits PKC activation and interferes with the progression of hyperglycemia to microvascular injury. In addition to its specificity for the \(\beta\) isoforms, ruboxistaurin does not interfere with any of the other fundamental kinases (eg, cAMP-dependent kinase) that are important to many organs and their function.\(^9\)

Early experimental data demonstrated that ruboxistaurin markedly attenuates increased retinal PKC activity and retinal vessel permeability induced by intraocular administration of exogenous VEGF.\(^10\) As shown in Figure 2, increased retinal vascular permeability following an injection of VEGF into the eye

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**Figure 1. Potential Role of PKC β in Diabetic Retinopathy**

- Hyperglycemia
- PKC-β activation
- VEGF/VPF production
- PKC-β activation
- Neovascularization

**Figure 2. Effect of PKC-β Inhibition with Ruboxistaurin on VEGF-Induced Retinal Vascular Permeability**

Increased retinal vascular permeability induced by an injection of VEGF into the eye (left panel). Note the leakage of edematous fluid into the macular zone. Pretreatment with ruboxistaurin almost completely abolishes the retinal permeability response to VEGF (right panel).

PKC \(\beta\) = protein kinase C beta; VEGF = vascular endothelial growth factor; VPF = vascular permeability factor.

Reproduced with permission from Aiello et al. *Diabetes*. 1997;46:1473-1480.\(^10\)
and subsequent leakage of edema into the macular zone are almost completely abolished by pretreatment with the PKC-β inhibitor.

The crucial role of PKC β in hyperglycemia-induced retinal disease was demonstrated in a study of retinal blood flow in rats with streptozotocin-induced diabetes. Similarly, ruboxistaurin dramatically attenuated the effects of VEGF on endothelial replication and migration in bovine aortic endothelial cells. Because the endothelial proliferation response to VEGF is involved in new vessel formation, particularly in the eye, the findings of this study and others suggest that selective inhibition of the PKC-β pathway may have an important beneficial effect on diabetic proliferative retinopathy.

Experimental data also demonstrate that ruboxistaurin has a favorable effect on diabetic microalbuminuria by restoring endothelial barrier function and reducing the leakage of protein across the endothelial barrier within the glomerulus. In addition, there are encouraging animal data showing that pretreatment with higher doses of ruboxistaurin restores sciatic nerve conduction velocity to control levels.

By impairing vascular responses to acetylcholine, which are mediated by nitric oxide, hyperglycemia impairs endothelium-dependent vasodilation. However, as shown in another experimental study, ruboxistaurin can improve the vasodilator response to acetylcholine and restore endothelial function.

As the studies described above indicate, there is strong evidence in a range of experimental settings that selective inhibition of the PKC-β pathway with ruboxistaurin can restore endothelial barrier function, improve endothelial contractile and relaxant responses, and prevent the replication, migration, and proliferation of endothelial cells that are involved in new vessel formation.

**Clinical Trials of Ruboxistaurin**

Against the backdrop of encouraging experimental data, several small clinical studies and several large clinical trials were designed to evaluate the efficacy, safety, and tolerability of ruboxistaurin in patients with diabetic microangiopathy, particularly those patients with sight-threatening macular edema.

An early phase IB study assessing the safety, pharmacokinetics, and pharmacodynamics of oral ruboxistaurin for 30 days in patients with type 1 or type 2 diabetes mellitus or mild nonproliferative diabetic retinopathy and diabetes mellitus for less than 10 years found that the PKC-β inhibitor was well tolerated by patients taking doses of 16 mg or less twice daily and had favorable pharmacodynamic effects on retinal blood flow (Figure 3). Preliminary results suggested that, as compared with a placebo, ruboxistaurin reduced mean circulation time in the retina in patients with type 1 or type 2 diabetes mellitus—evidence that this drug may have beneficial effects on impaired retinal blood flow.

The finding that ruboxistaurin was well tolerated by patients is reassuring because PKC plays an important role in liver function, neurologic function, and in many other organs in the body. Researchers presume that the drug’s specificity for only 2 of the 12 PKC isoenzymes accounts for its safety and tolerability.

The study also found that ruboxistaurin was not associated with any significant increase in adverse events, nor did it have any adverse effect on immune function—another reassuring finding because PKC plays an important role in white cell function and antibody formation. As expected, ruboxistaurin had no effect on glycemic control.

The encouraging findings regarding tolerability and amelioration of diabetes-associated abnormalities in retinal vascular function led to a series of large multicenter phase II and phase III clinical trials that were con-
ducted over the past 3 years in North America, the United Kingdom, and Europe. Some of these trials are continuing as extension studies, and other trials are under way, not only in patients with diabetic retinopathy but also in patients with neuropathy and nephropathy.

**CONCLUSIONS**

Activation of the DAG-PKC pathway has several important unwanted effects, especially ischemia and leakage, on microangiopathy in retinal, renal, and neural tissues.

Protein kinase C activation by high glucose seems to be strongly linked to the PKC-β_1_ and PKC-β_2_ isoenzymes. PKC-mediated phosphorylation of connexin-43 decreases tight junction intercellular communication, which in turn contributes to macular edema and proteinuria.

Encouraging data from experimental studies suggest that the inhibition of the PKC-β pathway with orally active ruboxistaurin is feasible and probably safe for long-term administration in humans. The studies also suggest that ruboxistaurin, by providing an effective blockade of hyperglycemia-induced vascular injury, may be useful as an adjunct to all currently available therapeutic approaches that are used to control glucose and blood pressure levels and to improve outcomes in patients with diabetes mellitus.

**REFERENCES**

2. Ceelotto G, Gallo A, Miola M, et al. Protein kinase C activity is acutely regulated by plasma glucose concentra