Targeting Vascular Endothelial Cell Insulin Resistance in Type 2 Diabetes Mellitus: Is Protein Kinase Cβ the “Bulls-eye” for Reducing Vascular Risk in Diabetes?

Running title: Pierce; Endothelial cell insulin resistance and PKCβ in diabetes

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More than 25 million Americans or 8.3% of the population have diabetes mellitus of which approximately 95% of diagnosed cases are type 2 diabetes. Between 1990 and 2010, the number of new cases of diabetes has almost tripled in the U.S. and the Centers for Disease Control projects that 1 in 3 Americans will have diabetes by 2050 if this trend continues. The presence of type 2 diabetes increases the risk of microvascular complications such as retinopathy and nephropathy, and macrovascular disease most notably atherosclerotic coronary artery disease (CAD). Indeed, CAD-related death remains the primary cause of mortality in patients with type 2 diabetes even on standard glucose control. However, more intensive glucose control is not associated with reduced CAD risk and may even increase mortality, therefore there remains a critical need to determine novel adjunct therapy to standard glycemic control that will reduce vascular risk in patients with type 2 diabetes.

In 1996, a seminal paper by Ishii et al. demonstrated that selective inhibition of the β isoform of protein kinase C (PKCβ), a serine/threonine protein kinase that becomes activated in nutrient excess states such as hyperglycemia, resulted in significant improvement in retinal and glomerular microvascular function in diabetic rats in the absence of any alterations in glycemia or blood pressure. This study elucidated PKCβ as a potential novel target in diabetes-related vascular complications and identified increased tissue concentrations of diacylglycerol (DAG), likely as a result of excess de novo synthesis in hyperglycemia, as the primary activator of PKCβ in rats. Consistent with this, DAG concentration and PKCβ activity are increased in aortas of obese insulin resistant rats and are associated with reduced insulin-stimulated activation of protein kinase B (Akt). Akt is a serine/threonine kinase regulated by upstream phosphatidylinositol 3 kinase (PI3K) that directly phosphorylates nitric oxide synthase (eNOS) at serine 1177 (eNOSser1177), resulting in eNOS activation and nitric oxide (NO) synthesis.
Interestingly, the reduction in insulin-stimulated Akt phosphorylation and NO bioavailability in aortas of obese insulin resistant rats are partly reversed after 2 weeks of PKCβ inhibition. Additionally, 2 weeks of PKCβ blockade improves NO-mediated endothelium-dependent vasodilation (EDV) of mesenteric arteries in streptozotocin-induced diabetic rats. Consistent with the animal data, overexpression of PKCβ1 or β2 in cultured endothelial cells demonstrate a decrease in insulin-mediated Akt phosphorylation and eNOS expression, and exposure of bovine aortic endothelial cells to high glucose for 24 hours results in reductions in insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), phosphorylation of Akt and eNOS and NO production. Taken together, these studies provide strong in vivo and in vitro evidence that increased endothelial PKCβ activity in diabetes and/or hyperglycemia results in “endothelial insulin resistance” phenotype characterized by impairments in the insulin-mediated IRS-1/Akt/eNOS pathway and EDV. Thus, these data support the idea that PKCβ inhibition may be a novel clinical strategy to reverse endothelial insulin resistance and dysfunction in humans with diabetes.

However, results from several large randomized multicenter trials investigating the efficacy of the selective PKCβ inhibitor ruboxastaurin on diabetic retinopathy and nephropathy have been equivocal. Also, several small clinical studies in humans testing the efficacy of short-term PKCβ inhibition on peripheral vascular EDV in humans have been disappointing. Although Beckman et al. demonstrated that seven days of oral ruboxastaurin prevented a reduction in forearm resistance artery EDV after 6 hours of experimentally induced-hyperglycemia in healthy adults, two weeks of ruboxastaurin had no effect on forearm EDV in middle-aged adults with type 2 diabetes. In another study, six weeks of ruboxastaurin appeared to improve conduit artery EDV (brachial artery flow-mediated dilation) in middle-aged patients...
with Type 2 diabetes. However, the differences between groups were largely the result of a decline in EDV in the placebo group rather than a treatment effect of ruboxastaurin in the patients with diabetes. Collectively, the available evidence in humans to date suggest a lack of efficacy of PKCβ inhibition on microvascular disease outcomes and vascular EDV in patients with type 2 diabetes.

In the current issue of Circulation, Tabit et al. describe an interesting cross-sectional study where they compared basal and insulin-mediated alterations in eNOS activation before and after ex vivo PKCβ blockade in venous endothelial cells that were freshly isolated from patients with type 2 diabetes and healthy controls. They report a paradoxical higher basal expression of phosphorylated eNOS<sub>Ser1177</sub> in endothelial cells from patients with type 2 diabetes compared with healthy controls. The finding of increased phosphorylation of eNOS<sub>Ser1177</sub> in the absence of differences in total eNOS protein expression also has been observed in obese humans. This observation may represent a compensatory upregulation of eNOS phosphorylation in the setting of excessive superoxide production from increased eNOS uncoupling and/or NADPH oxidase activity in diabetes and obesity that results in reduced NO bioavailability and accumulation of nitrotyrosine in the vascular wall. In a novel translational approach, the authors exposed the isolated endothelial cells to insulin before fixation, and this resulted in a reduction in phosphorylated eNOS<sub>Ser1177</sub> in cells from the diabetic patients but an increase in phosphorylated eNOS<sub>Ser1177</sub> in the healthy control cells suggestive of insulin resistance in endothelial cells of diabetics. Moreover, as expected brachial artery EDV was lower in the patients with diabetes, but a new finding was that a greater increase in insulin-mediated phosphorylation of eNOS<sub>Ser1177</sub> was associated with higher brachial EDV, consistent with the idea that abnormal insulin-mediated eNOS regulation is associated with impaired endothelial function in type 2 diabetes.
Perhaps a more intriguing finding was that co-incubating the insulin-treated cells from diabetics with a selective PKCβ inhibitor reversed the abnormal decrease in insulin-stimulated phosphorylation of eNOS\textsuperscript{Ser177} but had no effect on phosphorylation of inhibitory eNOS\textsuperscript{Thr495} in diabetics. Consistent with animal models of insulin resistance and cultured endothelial cells exposed to experimental high-glucose,\textsuperscript{4} these data support the notion that enhanced PKCβ activity negatively modulates eNOS regulation in the human diabetic endothelium.

Interestingly, endothelial cells exposed to high glucose \textit{in vitro} also demonstrate enhanced activity of nuclear factor kappa B (NFκB), a key proinflammatory transcription factor that regulates hundreds of inflammatory genes, and is attenuated after PKCβ blockade.\textsuperscript{13} Consistent with these \textit{in vitro} findings, the study by Tabit et al.\textsuperscript{11} report that endothelial cells from diabetic patients compared with control subjects, demonstrate a trend for higher expression of NFκB p65 and intracellular adhesion molecule-1 (ICAM-1), and reduced expression of the inhibitor protein of NFκB (IκBα). This is significant because ICAM-1 is responsible for leukocyte adhesion to the endothelium during the early stages of atherosclerosis and endothelial dysfunction. Indeed, endothelial cells exposed to high glucose \textit{in vitro} demonstrate a rapid increase in NFκB activation\textsuperscript{6} and increased expression of a variety of proinflammatory molecules that can be attenuated by PKCβ or NFκB blockade.\textsuperscript{14} In the current study, acute \textit{ex vivo} PKCβ inhibition in endothelial cells from diabetic patients resulted in increased IκBα expression consistent with reduced NFκB activation. However, ICAM-1 was not measured again after treatment so it is unknown whether transcription NFκB-regulated genes was altered by PKCβ blockade. Taken together, these data indicate a clear link between elevated PKCβ activity and NFκB activation in endothelial cells exposed to chronic hyperglycemia, suggesting the intriguing possibility that endothelial PKCβ activity directly modulates NFκB-related...
inflammation in the diabetic endothelium.

The findings of this study should be interpreted in the context of several limitations. A significant limitation of the study is that patients were not treated with systemic PKCβ inhibition in a randomized controlled study, therefore it is unknown if brachial artery EDV would have improved following chronic PKCβ inhibition with ruboxistaurin. Also, because insulin-mediated phosphorylation of eNOS\textsuperscript{ser1177} was the primary readout of endothelial insulin resistance, it remains uncertain whether insulin-mediated NO bioavailability was truly increased after PKCβ blockade because eNOS enzyme activity and/or NO were not directly measured in the isolated cells. Although this would be technically difficult given the small number of cells obtained from the patients with this technique. Lastly, without quantifying nuclear localization or DNA binding activity of p65, and ICAM-1 expression after \textit{ex vivo} PKCβ inhibition, it remains unknown whether PKCβ blockade reverses NFκB-related inflammation in endothelial cells of diabetic humans.

The study has several strengths including novel assessments of protein expression from freshly isolated endothelial cells and assessments of vascular endothelial function in patients with type 2 diabetes and healthy adults. This allowed for unique molecular insight into the integrative roles of PKCβ, eNOS regulation and NFκB activity in the endothelium of diabetic vs. healthy humans. Importantly, results of insulin-stimulated eNOS regulation were not different among patients taking vs. not taking insulin and other medications. However, despite the unique findings of the current study it should be emphasized that chronic PKCβ blockade has not as of yet translated into improved vascular outcomes in patients with type 2 diabetes. Therefore, the need to develop new anti-diabetic drugs or investigate ‘old’ drugs that have pleiotropic effects on the vasculature beyond glucose lowering remains an important focus of investigation in diabetes.
One such “old” drug is metformin which is already widely used in patients with type 2 diabetes. In addition to its established effects on glycemic control, metformin also inhibits PKC-β and NFκB signaling in glucose- and cytokine-exposed endothelial cells suggesting a mechanism to explain metformin’s clinical benefits not attributable to glucose control. Non-acetylated salicylates such as salsalate have gained interest in recent years in type diabetes because they have been shown to inhibit NFκB activation in cytokine-stimulated endothelial cells, increase insulin sensitivity of muscle and liver in obese/insulin resistant rodents, improve glycemic control in humans with diabetes and restore endothelial function in older obese adults. Although salsalate’s effects on PKCβ are unknown, in vitro and in vivo studies strongly implicate NFκB in mediating endothelial insulin resistance in hyperglycemia. Moreover, a recent elegant study demonstrates that functional NFκB inhibition in transgenic rodents has a profound influence in the prevention of endothelial and systemic insulin resistance in diet- and genetic-induced obesity. Taken together, these studies support the general concept that targeted NFκB inhibition may be a new but logical approach to treat endothelial insulin resistance and dysfunction in humans with type 2 diabetes.

In summary, the study by Tabit et al. demonstrates for the first time in humans that PKCβ and NFκB are key mechanisms involved in endothelial cell insulin resistance in patients with type 2 diabetes. Given the disappointing results of PKCβ inhibitors, clinical trials targeting NFκB with salsalate or newly developed more selective NFκB inhibitors may be the new therapeutic “bulls-eye” for reducing vascular risk in patients with type 2 diabetes.
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Conflict of Interest Disclosures: None.

References:


**Figure Legend:**

**Figure 1.** Integrative scheme demonstrating increased protein kinase C beta activity in type 2 diabetes that mediates a decrease in insulin-mediated phosphorylation of endothelial nitric oxide synthase at serine 1177, nitric oxide bioavailability and endothelial function, as well as increased nuclear factor kappa B activation and expression of intercellular adhesion molecule-1.

Abbreviations: PIP$_2$, phosphatidylinositol biphosphate; PIP$_3$, phosphatidylinositol triphosphate; DAG, diacylglycerol; IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol 3 kinase; PDK-1, phosphoinositide-dependent kinase-1; Akt, protein kinase B; eNOS, endothelial nitric oxide synthase; Ser1177, serine 1177; NO$\cdot$, nitric oxide; PTEN, phosphatase and tensin homolog; PKC$\beta$, protein kinase C beta; IKK$\beta$, I kappa kinase beta; NF$\kappa$B, nuclear factor kappa B; I$\kappa$B$\alpha$, I kappa B alpha; ICAM-1, intercellular adhesion molecule-1; NADPH$,\,$ nicotinamide adenine dinucleotide phosphate; $O_2^\cdot$, superoxide anion radical; ONOO$^-$, peroxynitrite radical.
High circulating glucose

NADPH oxidase

DAG

PKCβ

IKKβ

IKBα

NFκB

NFκB (p65/p50)

nucleus

ICAM-1 + other proinflammatory proteins

Insulin Receptor

PI3K

PIKδ1

PTEN

Akt

Ser1177

eNOS

Uncoupling in diabetes?

O2→ NO•

Endothelial function

P = phosphorylation

→ = activates

= inhibits

OnOO → NO• + O2•

Nitrotyrosine

ONOO−

LY379196

SALSALATE

de novo synthesis

P = phosphorylation
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