Abstract—The bioavailability of nitric oxide is decreased in animal models and humans with diabetes mellitus. Hyperglycemia, in particular, attenuates endothelium-dependent vasodilation in healthy subjects. In vitro and in vivo animal studies implicate activation of protein kinase Cβ as an important mechanism whereby hyperglycemia decreases endothelium-derived nitric oxide. Accordingly, this study tested the hypothesis that inhibition of protein kinase Cβ would prevent impairment of endothelium-dependent vasodilation in healthy humans exposed to hyperglycemia. This study was a randomized, double-blind, placebo-controlled, crossover trial. Healthy subjects were treated with an orally active, selective, protein kinase Cβ inhibitor, LY333531, or matching placebo once a day for 7 days before vascular function testing. Forearm blood flow was measured using venous-occlusion, strain-gauge plethysmography. Endothelium-dependent vasodilation was measured via incremental brachial artery administration of methacholine chloride (0.3 to 10 μg/min) during euglycemia and after 6 hours of hyperglycemic clamp. The forearm blood flow dose-response curve to methacholine was significantly attenuated by hyperglycemia after placebo treatment (P=0.009 by ANOVA, euglycemia versus hyperglycemia) but not after treatment with LY333531. Inhibition of protein kinase Cβ prevents the reduction in endothelium-dependent vasodilation induced by acute hyperglycemia in healthy humans in vivo. These findings suggest that hyperglycemia impairs endothelial function, in part, via protein kinase Cβ activation. (Circ Res. 2002;90:107-111.)

Key Words: protein kinase C β nitric oxide hyperglycemia endothelium diabetes

Vascular disease is the principal cause of morbidity and mortality in patients with diabetes mellitus.1 Diabetes mellitus is associated with changes in endothelial cell function that augment the development of atherosclerosis. An important early change, decreased bioavailability of endothelium-derived nitric oxide, is linked to many of the pathological features of atherosclerosis including upregulation of leukocyte adhesion molecules, platelet activation, and an increased propensity for vasoconstriction.2–5 Previous studies have demonstrated decreased endothelium-dependent vasodilation, a physiological marker of decreased bioavailability of nitric oxide in both conduit arteries and resistance vessels in experimental models of diabetes and humans with type 1 and type 2 diabetes.4–6

The central importance of hyperglycemia to the development of cardiovascular disease in diabetes mellitus is becoming increasingly evident. Population studies have revealed that an incremental risk of cardiovascular disease is associated with higher levels of blood glucose, beginning in the upper normal range.7 Hyperglycemia, per se, impairs vasodilator function in animals and healthy humans, similar to that which occurs in patients with diabetes.3,9 This cannot be attributed to downregulation of endothelial nitric oxide synthase (eNOS), since glucose increases eNOS mRNA expression and protein levels in cultured endothelial cells and vessels from diabetic animals.10–11 Despite this upregulation, there is reduced endothelium-derived nitric oxide in hyperglycemic states. Of potential mechanisms by which hyperglycemia may decrease the bioavailability of nitric oxide, recent evidence implicates a prominent role for activation of protein kinase C.12–14

Protein kinase C is a cytoplasmic family of enzymes with a wide variety of actions in intracellular signal transduction.15 The activation of protein kinase C decreases endothelium-derived nitric oxide synthesis, whereas its inhibition augments nitric oxide release.16–18 Of the many types of protein kinase C in vascular tissue, the β isoforms are activated to a greater magnitude than other isoforms in response to hyperglycemia.19–20 Thus, this subtype may be central to the vascular dysfunction seen with hyperglycemia.

Recently, a selective inhibitor of protein kinase Cβ, LY333531, has been described.21 Investigations using this
compound in experimental models of diabetes indicate that inactivation of protein kinase Cβ abrogates many of the pathophysiological vascular changes seen in hyperglycemia. Accordingly, the purpose of this investigation was to test the hypothesis that hyperglycemia-induced activation of protein kinase Cβ decreases the bioavailability of endothelium-derived nitric oxide and impairs vasodilator function in healthy humans. Specifically, we sought to determine whether pretreatment with an inhibitor of protein kinase Cβ, LY333531, would prevent decreases in endothelium-dependent vasodilation caused by experimental hyperglycemia in intact, healthy humans.

Materials and Methods

Subjects
Fifteen healthy volunteers were recruited via newspaper advertisement and provided written, informed consent. All subjects underwent screening, consisting of a medical history, physical examination, and laboratory studies to obtain values for complete blood cell count, serum electrolytes, fasting glucose, blood urea nitrogen, creatinine, transaminases, alkaline phosphatase, and lipid profile. Subjects with hypertension, history of tobacco use, LDL or total cholesterol greater than the 75th percentile for age and gender, cardiovascular disease, or other disease were excluded. The protocol was approved by the Human Research Committee of Brigham and Women’s Hospital.

Study Design
The effect of protein kinase Cβ inhibition on endothelium-dependent vasodilation during hyperglycemia was studied in a randomized, double-blind, placebo-controlled, crossover design. All subjects were studied in the morning in the postabsorptive state, fasting after the previous midnight. Subjects were randomized to receive either LY333531 (Eli Lilly and Company), 32 mg orally once daily, or matching placebo for 7 days before and on the morning of each vascular function study. After a minimum 2-week washout period, subjects then crossed over and received the other medication for 7 days before the second study day. Female participants underwent vascular testing during the same menstrual phase each visit. Cyclooxygenase inhibitors, alcohol, and caffeine were prohibited for 12 hours before the study morning.

On the morning of each study, an indwelling antecubital venous catheter and brachial artery catheter were inserted. After a minimum of 30 minutes following catheter insertion, LY333531 levels and basal forearm blood flow were measured. Thereafter, endothelium-dependent vasodilation was assessed by measuring the forearm blood flow response to incremental intra-arterial doses of methacholine chloride (0.3, 1.0, 3.0, and 10.0 μg/min) infused at a flow rate of 0.388 mL/min. Each dose was administered for 6 minutes, and forearm blood flow measurements were made during the last 2 minutes of the infusion. After completion of euglycemic measurements, infusion of dextrose was initiated to maintain forearm hyperglycemia. After 6 hours of hyperglycemic clamp (see next section), basal forearm blood flow and the blood flow responses to methacholine were measured again. Endothelium-independent vasodilation was not tested because it has been demonstrated to be unimpaired in vitro, in animal models of hyperglycemia, and in intact humans with experimentally induced hyperglycemia.8,9,12,22,23 The vascular research laboratory was quiet, dimly lit, and temperature-controlled at 23°C.

Forearm Hyperglycemic Clamp
A forearm hyperglycemic clamp was used to raise and maintain forearm glucose concentration at 300 mg/dL (16.7 mmol/L) as previously described.9 Dextrose (50% solution) was infused via the brachial artery catheter into the forearm. Fifteen minutes after the infusion was started, the blood glucose level was determined from antecubital venous blood and the infusion rate adjusted to maintain the hyperglycemic clamp at 300 mg/dL. The infusion rate was adjusted every 10 to 15 minutes for the duration of the study, usually ranging between 0.1 and 0.3 mL/min. In addition, the somatostatin analogue, octreotide, was infused at 30 ng·kg⁻¹·min⁻¹, to suppress pancreatic insulin release, since insulin is a known vasodilator whose vascular effects are mediated, at least in part, by endothelium-derived nitric oxide.24,25 The octreotide infusion was initiated at least 15 minutes before the first hemodynamic measurement and maintained throughout the protocol. No vasoactive effects have been identified in studies using a similar dose of octreotide.9,26,27

Hemodynamic Measurements
Bilateral forearm blood flow was measured by venous-occlusion, mercury-in-silastic, strain-gauge plethysmography (D.E. Hokanson, Issaquah, Wash) using established methods. The hand circulation was excluded during data acquisition using wrist cuffs inflated to 200 mm Hg. A venous occlusion pressure of 40 mm Hg was generated by cuffs placed on each arm above the elbow for each measurement of forearm blood flow. Forearm blood flow was reported as milliliters per 100 mL of tissue/min. Arterial blood pressure was measured via the brachial artery cannula. The cannula was attached to a pressure transducer contiguous with an amplifier on a Gould physiological recorder. Heart rate was determined by the RR interval of a continuous ECG monitor.

Laboratory Analyses
Whole-blood glucose concentration was measured at the bedside by the glucose oxidase method using a glucose reflectometer (Lifescan, Inc). Concentrations of LY333531 were determined by liquid chromatography with tandem mass spectrometry (Advanced BioAnalytical Services, Inc).

Statistical Analyses
Descriptive measures are reported as mean±SD. Experimental measures are reported as mean±SE. Basal forearm blood flows and glucose concentrations were compared by paired two-tailed t tests. Two-way, repeated-measures ANOVA was performed to compare the dose-response curves during euglycemia and after 6 hours of hyperglycemic clamp, using the absolute increase in blood flow from the basal flow rate. Statistical significance was accepted at the 95% confidence level (P<0.05). Before the randomization code was broken, one subject was excluded from the analysis because of technical problems in acquiring forearm hemodynamic measurements. Data reported in the Results section do not include this subject. Additional statistical analysis was performed that included this subject’s data, and it did not change the significance of the interventions.

Results

Baseline Characteristics
The 14 evaluable subjects, aged 26±6 years, included seven men and seven women. At the screening visit, mean arterial pressure was 86±10 mm Hg, blood glucose was 82±15 mg/dL, and total cholesterol was 141±19 mg/dL. Values obtained for fasting glucose, total and HDL cholesterol, triglycerides, and systolic and diastolic blood pressure were within normal limits in every subject.

Effect of Hyperglycemia and Protein Kinase Cβ Inhibition
Forearm glucose concentrations averaged 317±45 mg/dL during 6 hours of hyperglycemia with placebo treatment and 336±83 mg/dL with LY333531 treatment (P=NS). LY333531 treatment resulted in plasma LY333531 concentrations of 16.3±4.3 nmol/L before euglycemia forearm
blood flow measurements and 3.5±2.8 nmol/L after 6 hours of hyperglycemia just before data acquisition.

Baseline forearm blood flow was measured before the methacholine chloride infusion in each condition. Basal euglycemic forearm blood flow did not differ between placebo or LY333531 treatment periods, 1.7±0.1 versus 1.6±0.1 mL per 100 mL/min, respectively, P=NS (Figure 1). Six hours of hyperglycemic clamp increased basal forearm blood flow compared with the respective euglycemic baseline in both settings: to 2.4±0.2 mL per 100 mL/min with placebo treatment (P=0.008) and to 2.9±0.2 mL per 100 mL/min with LY333531 treatment (P=0.001) (Figure 1). The increase in basal forearm blood flow from euglycemia to hyperglycemia was significantly greater with LY333531 pretreatment than placebo (P=0.037).

Incremental doses of methacholine chloride increased forearm blood flow during euglycemia and hyperglycemia. The forearm blood flow response to methacholine chloride during euglycemia was not significantly different with or without inhibition of protein kinase Cβ (P=NS). During placebo treatment, 6 hours of hyperglycemic clamp significantly diminished the forearm blood flow response to methacholine compared with euglycemia (P=0.009) (Figure 2). In contrast, with protein kinase Cβ inhibition, there was no significant difference in the forearm blood flow response to methacholine chloride between euglycemia and hyperglycemia (P=NS) (Figure 3).

Figure 1. Basal forearm blood flow at baseline euglycemia and during clamp, with and without PKCβ inhibition. Resting forearm blood flow significantly increased from euglycemic baseline to 6-hour hyperglycemic clamp with and without LY333531 (P=0.008 and 0.001, respectively). The increase in resting flow was greater in the setting of LY333531 (P=0.037). Throughout the vascular function studies, there was no change in forearm blood flow in the contralateral forearm. Blood pressure and heart rate also remained stable and without significant variation during the course of the study.

Discussion

The novel finding of this investigation is that selective inhibition of protein kinase Cβ prevents hyperglycemia-induced impairment of endothelium-dependent vasodilation in healthy, nondiabetic humans in vivo. These results suggest that activation of protein kinase Cβ by hyperglycemia occurs within hours and importantly contributes to endothelial dysfunction. To our knowledge, this is the first report in intact humans that specific inhibition of protein kinase Cβ preserves endothelium-dependent vasodilator function in the presence of hyperglycemia.

Hyperglycemia and Protein Kinase C Activation

Diabetes-related protein kinase C activation results in endothelial dysfunction manifested by decreased nitric oxide bioavailability, increased production of oxygen-derived free radicals, increased leukocyte adhesion molecule expression and leukocyte adhesion, increased albumin permeability, and impaired fibrinolysis.28–32 Experimentally, hyperglycemia activates protein kinase C in endothelial and vascular smooth muscle cells and consistently produces similar findings of endothelial dysfunction including decreased endothelium-dependent vasodilation.9,31,33,34 The effect of hyperglycemia on vascular smooth muscle cell function is less clear. Experimental evidence demonstrates both increased and decreased vasoconstriction in response to hyperglycemia.35,36 Hypersensitivity to vasoconstriction does not appear to contribute importantly to the impairment of endothelium-dependent vasodilation in humans with diabetes or healthy humans exposed to hyperglycemia.37–39

Of the many isoforms of protein kinase C activated by hyperglycemia, the β isoforms play a prominent role in vivo in vascular dysfunction. Protein kinase Cβ is preferentially activated in vascular endothelium in both diabetic rat and hyperglycemic dog models.19,20 Further, protein kinase Cβ causes physiological abnormalities in vivo including increased retinal mean circulation time, glomerular filtration rate, and albumin excretion time in an in vivo rat model.14
Thus, protein kinase Cβ contributes importantly to hyperglycemia-mediated vascular dysfunction.

Hyperglycemia-mediated protein kinase C activation may be caused by a number of mechanisms including elevated diacylglycerol concentration and mitochondrial superoxide anion overproduction. Diacylglycerol concentrations increase as a result of de novo synthesis from augmented glucose metabolism. Increased diacylglycerol concentrations cause membrane translocation and activation of protein kinase C. A recent study indicates that protein kinase C activation also may result from glucose-induced superoxide anion production. Increases in cytosolic glucose concentrations enhance intracellular superoxide anion production by generating an electrochemical gradient in the mitochondrial electron transport chain of the tricarboxylic acid pathway. Inhibitors of this pathway abrogate increases in protein kinase C activity. Mitochondrial superoxide overproduction may cause the activation of protein kinase C by inducing the de novo synthesis of diacylglycerol or phosphatidylcholine synthesis.

**Protein Kinase C and Endothelium-Derived Nitric Oxide**

Our study supports an important role for protein kinase Cβ activation as a cause of hyperglycemia-induced, impaired endothelium-dependent vasodilation in human arterial resistance vessels. Indeed, we observed that LY333531, a specific inhibitor of protein kinase Cβ, prevented the expected decrease in endothelium-dependent vasodilation during acute hyperglycemia. These findings cannot be explained by constitutive activity of protein kinase Cβ or by effects of LY333531 in euglycemic conditions, because the euglycemic responses to methacholine chloride, with and without LY333531, were not different. Because the change in response to methacholine becomes evident with hyperglycemia, it is likely that in healthy humans, significant protein kinase Cβ activation occurs as a consequence of hyperglycemia and decreases the bioavailability of endothelium-derived nitric oxide.

Experimental evidence suggests several mechanisms by which protein kinase C may decrease the bioavailability of nitric oxide. Activation of protein kinase C antagonizes phosphatidylinositol-3 kinase–mediated activation of endothelial nitric oxide synthase and decreases endothelial-derived perivascular nitric oxide concentration. In addition, activation of protein kinase C increases superoxide anion formation from several sources. Protein kinase C induces NAD(P)H oxidase to produce superoxide anion, which subsequently uncouples endothelial nitric oxide synthase and augments production of superoxide anion preferentially over nitric oxide. Superoxide anion further decreases the bioavailability of nitric oxide by reacting with it to form peroxynitrite. We have previously demonstrated that hyperglycemia-induced impairment of endothelium-dependent vasodilation can be reversed via infusion of an antioxidant in humans in vivo. Thus, our present observations lend support to the notion that hyperglycemic activation of protein kinase C in humans yields an oxidative stress that further decreases the bioavailability of nitric oxide.

It is conceivable that protein kinase C also may impair endothelium-independent vasodilation. Hyperglycemia activates protein kinase C in vascular smooth muscle cells and the contractile response in vascular smooth muscle is abnormal in animal models of diabetes mellitus and in humans with type 2 but not type 1 diabetes. However, in experimental and human models of hyperglycemia, no abnormality in endothelium-independent vasodilation has been demonstrated. Our observations do not exclude the possibility that hyperglycemia-mediated protein kinase C activation alters vascular smooth muscle vasodilator function, but these previous investigations make this possibility less likely.

**Basal Forearm Blood Flow**

As we and others have demonstrated previously, hyperglycemia significantly increases resting forearm blood flow from baseline euglycemia to the 6-hour hyperglycemic clamp. This effect has been mimicked by infusion of mannitol and hypertonic saline, implicating increased osmolality as a cause for this phenomenon. Interestingly, in our subjects, the increase in resting forearm blood flow in response to hyperglycemia was greater with inhibition of protein kinase Cβ. Our findings do not provide a precise mechanism for the change in resting forearm flow; however, unimpaired constitutive nitric oxide production, despite hyperglycemia, resulting from protein kinase Cβ inhibition, may contribute to this finding.

**Conclusion**

Hyperglycemia increases the activity of protein kinase Cβ, which decreases endothelium-dependent relaxation. This mechanism may contribute to vascular dysfunction in patients with hyperglycemia. Inhibition of protein kinase Cβ by LY333531 may improve vascular function in patients with diabetes mellitus. Protein kinase Cβ may therefore be an appropriate therapeutic target for patients with diabetes and vascular dysfunction.

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