Protein Kinase C Activation Contributes to Microvascular Barrier Dysfunction in the Heart at Early Stages of Diabetes

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Abstract—The functional disturbance of microvasculature is recognized as an initiating mechanism that underlies the development of various diabetic complications. Although a causal relationship between microvascular leakage and tissue damage has been well documented in diabetic kidneys and eyes, there is a lack of information regarding the barrier function of coronary exchange vessels in the disease state. The aim of the present study was to evaluate the permeability property of coronary microvessels during the early development of experimental diabetes with a focus on the protein kinase C (PKC)-dependent signaling mechanism. The apparent permeability coefficient of albumin (Pa) was measured in isolated and perfused porcine coronary venules. The administration of high concentrations of D-glucose induced a dose-dependent increase in the Pa value, which was prevented by blockage of PKC with its selective inhibitors bisindolylmaleimide and Goe 6976. More importantly, an elevated basal permeability to albumin was observed in coronary venules at the early onset of streptozotocin-induced diabetes. The hyperpermeability was corrected with bisindolylmaleimide and the selective PKCβ inhibitor hispidin. Concomitantly, protein kinase assay showed a high PKC activity in isolated diabetic venules. Immunoblot analysis of the diabetic heart revealed a significant subcellular translocation of PKCβII and PKCe from the cytosol to the membrane, indicating that the specific activity of these isoforms was preferentially elevated. The results suggest that endothelial barrier dysfunction attributed to the activation of PKC occurs at the coronary exchange vessels in early diabetes. (Circ Res. 2000;87:412-417.)

Key Words: diabetes ■ microcirculation ■ permeability ■ protein kinases

Cardiovascular complications represent the major cause of morbidity and mortality in patients with diabetes mellitus. A functional disturbance in the microvasculature independent of large-vessel atherosclerosis has been implicated in the development of end-organ damage and clinical abnormalities manifested as nephropathy, retinopathy, neuropathy, and skin ulceration. Characteristic changes in the microcirculation during the early stages of diabetes include autoregulatory dysfunction and increased endothelial permeability. Capillary leakage has been documented in various tissues of experimentally diabetic animals, including the retina, aorta, skin, intestine, kidney, and heart. Endothelial barrier dysfunction, occurring before microvascular sclerosis and other structural alterations, is considered to be one of the initiating mechanisms that underlies the pathogenesis of microangiopathic complications.

The precise cause of vascular hyperpermeability in diabetes has not been established. Recent evidence suggests that hyperglycemia-induced de novo synthesis of diacylglycerol and the subsequent activation of protein kinase C (PKC) constitute an important signaling pathway that leads to endothelial dysfunction. The PKC activity has been found to be upregulated under the hyperglycemic or diabetic condition in microangiopathy-prone tissues, such as the retina, renal glomeruli, aorta, and heart. The in vivo administration of specific PKC inhibitors normalized microvascular blood flow and permeability in diabetic rats. In cultured endothelial cells, glucose caused a dose-dependent increase in macromolecular permeability that was abolished with the PKC inhibitors staurosporine and Goe 6976, which supports the role of PKC in the mediation of diabetes-induced endothelial barrier dysfunction.

In the coronary system, increased microvascular permeability may in large part contribute to myocardium insufficiency and ventricular dysfunction, as frequently seen in diabetic heart disease. Although the causal relationship between microvascular leakage and tissue damage has been well documented in diabetic kidney and retina, there is a paucity of information about the pathophysiological consequence of diabetes that extends particularly to the coronary exchange vessels. Therefore, the aim of the present study was to systematically examine the effect of diabetic hyperglycemia on coronary microvascular permeability, with a focus on the PKC-dependent signaling mechanism. An in situ model of...
intact perfused coronary venules was used to provide a direct assessment of the barrier property of venular endothelium. The results showed that albumin permeability was markedly elevated in coronary venules isolated from streptozotocin (STZ)-induced diabetic pig hearts and in venules treated with high concentrations of glucose. Upregulation of PKC activity was found in these vessels as well as in cultured coronary endothelial cells subjected to high concentrations of glucose. Correspondingly, the inhibition of PKC with pharmacological agents greatly attenuated the diabetes-induced venular hyperpermeability. We therefore suggest that the activation of PKC has an important role in the development of microvascular endothelial dysfunction during the early stages of diabetes.

Materials and Methods

The PKC inhibitors bisindolylmaleimide (BIM), Goe 6976, hispidin, and 2,2′,3′,3′4,4′-hexahydroxy-1,1′-biphenyl-6,6′-dimethanol di-methyl ether (HBDDE) were obtained from Calbiochem. STZ was ordered from Upjohn. Antibodies to PKCα, PKCβII, and PKCζII were obtained from Santa Cruz, and anti-PKCε was obtained from Transduction Laboratories.

Diabetes was induced in Yorkshire pigs that weighed 9 to 12 kg through the intravenous injection of STZ (150 mg/kg). Pigs were fed with a commercial diet that contains ammonium chloride (20 g/kg hog chow) the day before STZ injection. Only those that developed with a commercial diet that contains ammonium chloride (20 g/kg hog chow) the day before STZ injection. Only those that developed sustained hyperglycemia with a blood glucose level of >300 mg/dL, were included in this study.

The technique of isolation and cannulation of microvessels has been described in detail in our previous publications. Briefly, a coronary venule of 30 to 50 μm in diameter was dissected and cannulated with 4 micropipettes (Figure 1), of which each was connected to a reservoir to allow independent control of intraluminal pressure and flow. The vessel was interchangeably perfused with a physiological salt solution and the same solution containing fluorescein isothiocyanate-albumin. The changes in the fluorescence intensity in the venule and its adjacent area were measured with a video photometer that had an optical window positioned over the vessel. The apparent solute permeability coefficient of albumin (Pa) was calculated with the equation \( \frac{1}{P_a} = \frac{1}{D_e} \frac{dI_f}{dt} \frac{r}{2} \), where \( D_e \) is the initial step increase in fluorescent intensity, \( \frac{dI_f}{dt} \) is the initial rate of gradual increase in intensity as solutes diffuse out of the vessel into the extravascular space, and \( r \) is the venular radius.

PKC activity was measured in both freshly isolated coronary venules and cultured coronary venular endothelial cells (CVECs) with a MESACUP protein kinase assay kit (Medical and Biological Laboratories). To obtain vessel samples, 20 to 30 coronary venules were dissected from the heart of diabetic or control pigs and quickly homogenized, and the soluble fraction of the cells was then collected. This produced an initial step increase, followed by a gradual increase, in the intensity of fluorescence. There was a step decrease in intensity when the fluorescent-labeled molecules were washed out of the vessel lumen by switching the perfusion back to the outer inflow pipette. The Pa value was calculated with the equation \( P_a = \frac{1}{D_e} \frac{dI_f}{dt} \left( \frac{r}{2} \right) \), where \( \Delta I \) is the initial step increase in fluorescence intensity, \( \frac{dI_f}{dt} \), is the initial rate of gradual increase in intensity as solutes diffuse out of the vessel into the extravascular space, and \( r \) is the venular radius.

Figure 1. Schematic diagram showing the measurement of Pa in an isolated and cannulated coronary venule. The microvessel was cannulated with 4 micropipettes (pipette in pipette), with each pipette connected to a reservoir to allow independent control of intraluminal pressure and flow. The fluorescence intensity was measured with a video photometer with an optical window positioned over the venule and adjacent space. In each measurement, the isolated venule was first perfused with a physiological salt solution through the outer inflow pipette to establish the baseline intensity. The venular lumen was then quickly filled with fluorochemicals by switching the perfusion to the inner inflow pipette that contained fluorescein isothiocyanate (FITC)-albumin. This produced an initial step increase, followed by a gradual increase, in the intensity of fluorescence. There was a step decrease in intensity when the fluorescent-labeled molecules were washed out of the vessel lumen by switching the perfusion back to the outer inflow pipette. The Pa value was calculated with the equation \( P_a = \frac{1}{D_e} \frac{dI_f}{dt} \left( \frac{r}{2} \right) \), where \( \Delta I \) is the initial step increase in fluorescence intensity, \( \frac{dI_f}{dt} \), is the initial rate of gradual increase in intensity as solutes diffuse out of the vessel into the extravascular space, and \( r \) is the venular radius.

The hyperpermeability response to glucose was markedly suppressed during inhibition of PKC (Figure 3). Administration of the PKC inhibitors BIM (10−4 mol/L) (n=7) and Goe 6976 (10−7 mol/L) (n=6) did not significantly alter the

Results

Effect of High Glucose on Venular Permeability

Figure 2 demonstrates the effect of high concentrations of D-glucose on the permeability of isolated coronary venules. An increase in glucose concentration caused a rapid, dose-dependent increase in albumin permeability. A maximal response was observed at 60 minutes after the administration of glucose. The changes in Pa in response to various concentrations of glucose were as follows: from the basal value of 3.02±0.32×10−6 cm/s to 5.15±0.36×10−6 cm/s at 12.5 mmol/L (n=8, P<0.05), from 3.17±0.28×10−6 cm/s to 6.45±0.67×10−6 cm/s at 25 mmol/L, (n=10, P<0.05), from 2.86±0.43×10−6 cm/s to 6.79±0.75×10−6 cm/s at 50 mmol/L (n=6, P<0.05), and from 2.52±0.29×10−6 cm/s to 9.01±1.46×10−6 cm/s at 100 mmol/L (n=9, P<0.05).

The hyperpermeability response to glucose was markedly suppressed during inhibition of PKC (Figure 3). Administration of the PKC inhibitors BIM (10−4 mol/L) (n=7) and Goe 6976 (10−7 mol/L) (n=6) did not significantly alter the
baseline permeability, but they completely prevented the increase in permeability caused by the high concentration (50 mmol/L) of glucose.

Effect of Diabetes on Venular Permeability

STZ-induced diabetes significantly impaired the barrier function of coronary venules in pigs as early as 4 weeks after administration of the drug (Figure 4A). Specifically, the coronary venular permeability was $2.85 \pm 0.19 \times 10^{-6}$ cm/s in nondiabetic pigs at the basal condition ($n=27$), $2.62 \pm 0.62 \times 10^{-6}$ cm/s at 2 weeks of diabetes ($n=4, P>0.05$ versus nondiabetic), $4.11 \pm 0.33 \times 10^{-6}$ cm/s at 4 weeks of diabetes ($n=9, P<0.05$ versus nondiabetic), and $6.69 \pm 0.55 \times 10^{-6}$ cm/s at 6 to 8 weeks of diabetes ($n=15, P<0.05$ versus nondiabetic).

Although the PKC inhibitor BIM did not alter the basal permeability of normal, nondiabetic vessels, it dose-dependently attenuated diabetes-induced hyperpermeability (Figure 5). In venules from pigs at 8 weeks of diabetes, the $P_a$ value was $7.47 \pm 1.12 \times 10^{-6}$ cm/s ($n=7$). This value was reduced with BIM to $6.38 \pm 1.45 \times 10^{-6}$ cm/s at $10^{-2}$ mol/L ($n=5, P>0.05$), $4.42 \pm 1.45 \times 10^{-6}$ cm/s at $10^{-5}$ mol/L ($n=7, P<0.05$), and $3.67 \pm 0.81 \times 10^{-6}$ cm/s at $10^{-5}$ mol/L ($n=7, P<0.05$). Furthermore, as shown in Figure 6, although the selective PKCa inhibitor HBDDE ($8 \times 10^{-5}$ mol/L) did not affect the high $P_a$ in diabetic venules ($n=4$), the selective $\beta$-isoform inhibitor hispidin ($4 \times 10^{-6}$ mol/L) greatly attenuated diabetes-induced hyperpermeability in coronary venules ($6.69 \pm 0.55 \times 10^{-6}$ cm/s before and $3.06 \pm 0.29 \times 10^{-6}$ cm/s after hispidin, $n=7, P<0.05$).

PKC Activity

The protein kinase assay showed that the overall enzymatic activity of PKC was upregulated in diabetic venules (Figure 4B), with a time course that correlated with that of diabetes-induced venular hyperpermeability. In freshly isolated coronary venules, PKC activity at 2 weeks of diabetes was $100 \pm 12.02\%$ of the control value obtained from nondiabetic
pigs (n=3, P>0.05), 163.99±72.06% of control at 4 weeks (n=3, P>0.05), and 328.33±51.74% of control at 6 to 8 weeks (n=3, P<0.05). In cultured CVECs, high glucose (50 mmol/L) treatment induced a 73.71±10.78% increase in PKC activity. In Western blot analysis (Figure 7), diabetes did not alter the subcellular distribution of PKC\textsubscript{\textalpha} and PKC\textsubscript{\textbeta I} but induced a significant translocation of PKC\textsubscript{\textbeta II} and PKC\textsubscript{\textepsilon} from the cytosol to the membrane, indicating an upregulation in the activity of the \textbeta II- and \textepsilon-isoforms.

**Discussion**

The results of the present study demonstrated that an elevation of the glucose concentration in the perfusion media induced a rapid, dose-dependent increase in the permeability of coronary venules. The hyperpermeability response was prevented during pharmacological inhibition of PKC. More importantly, an elevated basal permeability was found in coronary venules at 4 to 8 weeks after STZ-induced diabetes, which was concomitant with an increase in PKC activity in the same tissue. Furthermore, Western blot analysis of the diabetic heart tissue revealed that the membranous distributions of PKC\textsubscript{\textbeta II} and PKC\textsubscript{\textepsilon} were preferentially increased, whereas PKC\textsubscript{\textalpha} and PKC\textsubscript{\textbeta I} did not significantly change. Correlatively, a wide-spectrum PKC inhibitor and a PKC\textsubscript{\textbeta} -specific blockade were able to restore the barrier function of the diabetic coronary venules. The results support a role for PKC in the mediation of microvascular endothelial dysfunction at the early stages of diabetes.

Microvascular endothelial dysfunction plays an important role in the development of diabetic cardiovascular complications.\textsuperscript{1} As an early pathophysiological alteration, microvascular leakage precedes structural abnormalities in the microvasculature.\textsuperscript{19} Increased extravasation of albumin from microvessels is seen in various tissues of diabetic subjects.\textsuperscript{3-6} In rats, vascular hyperpermeability is demonstrated as early as 4 weeks after the onset of STZ-induced diabetes.\textsuperscript{20} However, due to the technical limitations, diabetes-induced early functional changes in the coronary microvascular system remain largely unclarified, and even less is known about the permeability property of coronary exchange vessels under hyperglycemic conditions. In this regard, the present study is the first to show that the coronary venular barrier function is
damaged by short-term diabetes. The increase in the permeability of porcine coronary venules occurred 4 weeks after the onset of diabetes, and the hyperpermeability effect was comparably seen in the same types of vessels subjected to high glucose stimulation. This finding is in agreement with the hypothesis that hyperglycemia triggers microvascular endothelial dysfunction during the early period of diabetes.\(^{(19)}\)

The rapid permeability response of venules to diabetes and high glucose indicates that an impairment of endothelial barrier function may be an initial pathophysiological change in the development of diabetic heart disease. In support of our data, other experiments have demonstrated a dose-dependent increase in transendothelial flux of albumin in cultured cells incubated with high concentrations of glucose.\(^{(7,21,22)}\) Electron microscopy observation of intact perfused rat hearts\(^{(23)}\) revealed the formation of endothelial gaps upon hyperglycose stimulation.

A number of hypotheses have been proposed to explain the injurious effect of hyperglycemia, including the poloyl pathway, nonenzymatic glycation, oxidant generation, and PKC activation.\(^{(24,25)}\) Of these mechanisms, the PKC signaling has been increasingly recognized as an early and common pathway that leads to vascular complications.\(^{(18,26,27)}\) It is well accepted that elevated blood glucose levels increase the de novo synthesis of diacylglycerol, which in turn stimulates PKC.\(^{(28)}\)

The vascular effects of PKC activation are characterized by endothelial dysfunction and microcirculatory disturbance.\(^{(5,20)}\) Recent studies have revealed a direct relationship between the activity of PKC and vascular permeability in various organs.\(^{(26,30,31)}\) Oral administration of the selective PKC inhibitor LY333531 can normalize the microcirculation in retina and kidney of diabetic rats.\(^{(29)}\) Another PKC inhibitor, LY290181, prevents glucose-induced increases in endothelial permeability and corresponding vascular changes in vivo.\(^{(13)}\) Our experiments also support the central role of PKC activation in the pathophysiological regulation of microvascular permeability in diabetes.

PKC is a family of serine/threonine kinases that consists of >10 isoforms with distinct cellular function and different expression patterns.\(^{(26)}\) Previous investigations\(^{(11,32,33)}\) have revealed augmentation of PKCa, PKC\(\beta\), and PKCe activity in the myocardium of diabetic rats. Despite the fact that various combinations of PKC isoforms are activated in the diabetic heart, PKC\(\beta\)II activity seems to be predominant in all vascular tissues.\(^{(18)}\) Indeed, the \(\beta\)-isoenzyme was found to be upregulated in porcine hearts from the present study, and the PKC\(\beta\) inhibitor displayed a significant effect in blocking the hyperpermeability response to diabetes in coronary microvessels. Therefore, it is likely that the microvascular endothelial barrier dysfunction in the heart during early diabetes is, at least in part, attributed to the activation of PKC\(\beta\). In this regard, PKC\(\beta\) may be a potential therapeutic target for vascular complications and cardioangiopathy in diabetes. Regarding the other isoforms, although our experiment showed an elevated PKCe activity in the heart, we were unable to define its role in the regulation of venular permeability due to the lack of specific inhibitors.

It should be noted that the previous information regarding the exchange process in diabetes was obtained either through the measurement of tracer clearance, the result of which is complicated by hemodynamic effects and extrinsic factors, or through in vitro assays on cultured cells derived from large vessels, which renders difficulties in the identification of mechanisms distinct to particular microvascular beds. In contrast, our intact perfused venule model enabled a direct assessment of microvascular permeability under conditions in which various influencing factors were tightly controlled, producing results that are physiologically relevant and specific to the exchange vessels of the heart. Furthermore, the cumulative measurement of PKC activity in the diabetic venules directly supports the pharmacological approaches used in the evaluation of PKC-elicited coronary hyperpermeability.

In conclusion, the present study indicates that the permeability of coronary venules is increased in the early stages of experimental diabetes. We suggest that activation of PKC\(\beta\) in response to increased glucose levels is involved in the signaling mechanisms that underlie diabetes-induced coronary microvascular dysfunction.

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