Inhibition of Protein Kinase C β Ameliorates Impaired Angiogenesis in Type I Diabetic Mice Complicating Myocardial Infarction

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Background: In recent studies, the inhibition of protein kinase C (PKC) β has been shown to improve diabetic vascular complications. However, the effect on angiogenesis in myocardial ischemia with diabetes mellitus (DM) is still unknown.

Methods and Results: Mice were divided into 3 groups: control, DM and DM+PKC-I groups (n=8, respectively). In the DM and DM+PKC-I groups, diabetes was induced by streptozotocin (STZ) (1.5 mg/body i.p.) for 5 days. Next, left anterior descending artery (LAD) ligation was performed in all groups. In the DM+PKC-I group, PKC β inhibitor (Cat. No. 539654; 10 nmol/L) was administered from days 1 to 10. After 4 weeks of LAD ligation, the animals were killed. Microvascular density was significantly improved by PKC β inhibitor (control: 87.9 ± 5.2/high-power field (HPF); DM: 51.4 ± 6.9/HPF; PKC-I: 80.3 ± 4.9/HPF; P<0.05). Expression of both vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS), which was decreased in the DM group, were significantly improved by inhibition of PKC β [VEGF (DM: 0.36 ± 0.11-fold and DM+PKC-I: 0.77 ± 0.07-fold vs. control); eNOS (DM: 0.35 ± 0.06-fold and DM+PKC-I: 0.73 ± 0.08-fold vs. control); both P<0.05].

Conclusions: Inhibition of PKC β ameliorated impaired angiogenesis by hyperglycemia in STZ-induced DM mice complicated by myocardial infarction. These results suggest a new possible indication of PKC β inhibitor for myocardial ischemia with DM. (Circ J 2012; 76: 943–949)

Key Words: Angiogenesis; Diabetes; Myocardial ischemia; PKC β inhibitor

The number of patients with diabetes mellitus (DM) in 2010 was estimated to be 285 million worldwide, equivalent to approximately 7% of the adult world population. This disease, which is characterized by a state of chronic hyperglycemia, is accompanied by macro- and microangiopathy in multiple organs, including the eyes, kidneys, nerves, peripheral arteries and heart. The development of coronary artery disease (CAD) is a major cause of mortality in patients with DM. Compared with patients without DM, those with the disease have a higher incidence of extensive CAD that is caused by an increased propensity for accelerated atherosclerosis and a significant impairment of coronary collateral vessel formation during myocardial ischemia. In addition, the angiogenic response to chronic myocardial ischemia is also impaired in patients with DM.

Although multiple molecular mechanisms are involved in the impairment of angiogenesis in the hyperglycemic state, recent studies have revealed that activation of protein kinase C (PKC) plays an important role in the development of diabetic vascular complications. PKC, which is a serine/threonine-related protein kinase, has multiple isoforms. In particular, the β isoform (PKC β 1 and 2) is expressed in cardiovascular tissues and activated by high levels of glucose and free fatty acids. In numerous experimental and clinical studies, the inhibition of PKC β has been shown to improve diabetic complications such as retinopathy and nephropathy. However, the effect on angiogenesis in myocardial ischemia with DM is still unknown. Our present study aimed to examine the effect of PKC β inhibitor on myocardial ischemia in the hyperglycemic state. We hypothesized that PKC β inhibitor would improve the impaired angiogenic response and reduces the myocardial infarction (MI) area in a streptozotocin (STZ)-induced DM mice model in which the animals underwent left anterior descending artery (LAD) ligation.

Methods

Animals

The committee on Animal Research at the University of Tsukuba approved the experimental protocols. The mice were cared for in accordance with the Guiding Principles for the Care and Use of Animals based on the Helsinki Declaration of
The C57BL/6J mice (Charles River, Yokohama, Japan) used were 8–10 weeks old and weighed 22–28 g at the time of the study. The mice were kept in standard mouse cages filled with woodchips, in groups of a maximum of 7 animals. The animals were subjected to a 14-h light/10-h dark cycle with a light intensity of 200 lux. Tap water and pelleted feed were given ad libitum in a room at 21–26°C and 40–65% relative humidity.

General Experimental Sequence and DM Induction
Mice were divided into 3 groups: control, DM, and DM plus PKC β inhibitor (DM+PKC-I) (n=8, respectively). In the DM and DM+PKC-I groups, diabetes was induced by intraperitoneal injection of STZ (Wako, Osaka, Japan; 1.5 mg/body) in normal saline for 5 days (days −5 to −1). In the control group, vehicle was administered. Fasting blood glucose levels were measured before STZ or vehicle administration, at operation and 4 weeks postoperatively. After the injection of STZ or vehicle, LAD ligation was performed in all groups. In the DM+PKC-I group, PKC β inhibitor (Cat. No. 539654, CAL-BIOCHEM, Darmstadt, Germany; 10 mmol/L) in normal saline was injected intraperitoneally from days 1 to 10. The dose of the PKC β inhibitor was decided after referring to previous reports using the PKC inhibitor, which had almost the same molecular weight and IC₅₀.14,15 In the control and DM groups, vehicle was administered instead of PKC β inhibitor. After 4 weeks of LAD ligation, the mice were killed and their hearts excised. The hearts were then transected at 2 mm distal to the ligation and divided into 2 sections: the basal and apex sections. The basal part was fixed in formalin and embedded in paraffin for histological analysis, while the apex part was frozen using liquid nitrogen and stored at −80°C for western blotting.

MI Model
Mice were anesthetized with a mixture of ketamine (500 mg/ml; Sankyo, Tokyo, Japan, 100 mg/kg) and xylazine hydrochloride (Sigma-Aldrich, Tokyo, Japan, 10 mg/kg). The intubation and extubation procedures used have been previously described.16 During mechanical ventilation, LAD ligation was performed. The surgical approach was made through a left lateral thoracotomy (entering between the 3rd and 4th ribs). After opening the pericardium, the LAD was ligated 1–2 mm distal to the left atrial appendage, using 7-0 polypropylene suture. The thoracic wall was closed with 1 or 2 interrupted stitches and the skin incision was closed by a running stitch.

Measurement of Infarct Size
From the basal part of the harvested heart, which was embedded in paraffin, 6-μm sections were cut and stained with Masson’s trichrome. The stained slides were examined under light microscopy and digitized for analysis with Motic Images Plus 2.1 U (Motic, Xiamen, China). Infarct size was assessed morphologically and calculated as the ratio of the average scar circumference of the endocardium and the epicardium.17

Immunohistochemistry
In this study, the Universal Immuno-enzyme Polymer (UIP) method, which was developed for immunohistochemical staining by Nichirei Bioscience Inc (Tokyo, Japan), was used. Paraffin-embedded myocardial sections, which were sliced at a thickness of 6 μm, were immersed in a 10 mmol/L sodium citrate buffer (pH 6.0) and heated by thermal microwave radiation. The sections were then incubated in 3% hydrogen peroxide for 15 min to inhibit endogenous peroxidise activity. After deparaffinization and rehydration, the sections were incubated overnight at 4°C with anti-platelet-endothelial cell adhesion molecule-1 (CD31) antibody (1:400) (AnaSpec, CA, USA). The sections were counterstained with hematoxylin and examined for microvascular density (MVD),18 which was measured in ×400 cross-sectional fields randomly selected from the peri-infarcted area and results are expressed as an average of 5 fields.

Western Blotting
Whole-cell lysates were isolated from the homogenized myocardial samples with a radio-immunoprecipitation assay buffer and centrifuged at 13,000 rpm for 30 min to separate the soluble and insoluble fractions. The protein concentration was measured spectrophotometrically at a 595 nm wavelength with a DC protein kit (Bio-Rad, CA, USA) and adjusted to 1 mg/ml among each sample. Equal amounts of proteins (20 μg) were loaded and separated by electrophoresis on sodium dodecyl-sulfate polyacrylamide gel (Wako, Osaka, Japan) and transferred to polyvinylidene fluoride membranes (Wako, Osaka, Japan). Each membrane was incubated overnight at 4°C with the following specific primary antibodies: anti-vascular endothelial growth factor (VEGF) antibody (1:1,000) (Santa Cruz, CA, USA), anti-VEGF receptor 1 (VEGFR1) antibody (1:500) (AnaSpec, CA, USA), anti-VEGF2 antibody (1:1,000) (EndoGene, Nanjing, China), anti-endothelial nitric oxide synthase (eNOS) antibody (1:500) (AnaSpec), and anti-β-actin antibody (1:1,000) (AnaSpec). The membranes were then incubated for 60 min at room temperature with secondary antibody (1:6,000) (Amersham, NJ, USA). Immune complexes were visualized with the Enhanced Chemiluminescence Detection System (Amersham, NJ, USA). Bands were quantified by densitometry of radioautographs films, which was performed with Photoshop 7.0 (Adobe, CA, USA). Band intensity was expressed as a ratio of the control group.

Statistical Analysis
Data are expressed as mean±SEM. Comparisons of variables among the 3 groups were performed by 1-way analysis of variance. A probability value less than 0.05 was considered significant. If a significant difference was recognized, a post-hoc test using the Games-Howell method was performed. All analyses were carried out with Stat View 5.0 (SAS, NC, USA).

Results
Change in Fasting Blood Glucose Levels
Before administration of STZ or normal saline, fasting blood glucose levels were not significantly different among the 3 groups (control: 121.4±3.1 mg/dl; DM: 135.6±10.0 mg/dl; DM+PKC-I: 134.7±3.4 mg/dl; P=0.18). Mice in the DM and DM+PKC-I groups had DM induced by administration of STZ (1.5 mg/body) for 5 days. Fasting blood glucose levels in the mice of both these groups were significantly higher at operation (control: 127.8±7.0 mg/dl; DM: 182.0±13.0 mg/dl; DM+PKC-I: 168.3±12.0 mg/dl; P<0.05) and 4 weeks postoperatively (control: 131.5±16.1 mg/dl; DM: 478.0±34.4 mg/dl; DM+PKC-I: 440.7±47.5 mg/dl; P<0.05) than in those of control group (Figure 1).

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Infernt Size and MVD
Myocardial infarct size was increased in the mice of the DM group compared with the control and DM+PKC-I groups; however, there was no significant difference among the 3 groups (control: 40.0±3.1%; DM: 48.0±2.7%; DM+PKC-I: 41.7±3.6%; P=0.19) (Figure 2). Meanwhile MVD of the peri-infarct area was significantly decreased in mice of the DM group compared with those of the control and DM+PKC-I groups (control: 87.9±5.2/HPF; DM: 51.4±6.9/HPF; DM+PKC-I: 80.3±4.9/HPF; P<0.05) (Figure 3).

Western Blotting
Figure 4 shows the results from the western blot analysis. VEGF is one of the important pro-angiogenic factors that mediates its effects through binding to VEGFR1 and 2. The DM group had significantly lower expression of VEGF, though the inhibition of PKC β led to significant improvement in VEGF expression that was impaired by hyperglycemia (DM: 0.36±0.11-fold and DM+PKC-I: 0.77±0.07-fold vs. control; P<0.05). Expression of eNOS, which is a downstream mediator in angiogenesis, showed the same pattern as VEGF (DM: 0.35±0.06-fold and DM+PKC-I: 0.73±0.08-fold vs. control; P<0.05). However, expression of VEGFR1 and 2 was similar among the groups [VEGFR1 (DM: 0.87±0.11-fold and DM+PKC-I: 0.78±0.08-fold vs. control; P=0.30), VEGFR2 (DM: 0.91±0.22-fold and DM+PKC-I: 0.85±0.24-fold vs. control; P=0.89)]. In addition, expression of β-actin, as an internal standard, was not significantly changed among the groups (DM: 0.97±0.11-fold and DM+PKC-I: 1.02±0.15-fold vs. control; P=0.96).

Discussion
Diabetes is a systemic disease that is accompanied by macro- and microangiopathy in multiple organs. In the patients with DM, myocardial pathologies include diastolic dysfunction, impaired angiogenesis, interstitial fibrosis, and severe CAD.
PKC, which is a serine/threonine-related protein kinase, mediates many intracellular signal transduction pathways and is activated by diacylglycerol (DAG), phosphatidylserine and calcium. The de novo synthesis of DAG is elevated in a hyperglycemic state because of an increase of dihydroxyacetone phosphate, which is an intermediate of the glycolytic pathway. PKC is activated mainly by DAG under such conditions. Furthermore, although various PKC isoforms have been reported to be activated by DM or hyperglycemia, PKC β isoforms play a more important role than other PKC isoforms in the organs in which diabetic vascular complications can occur. In the heart, it has been previously reported that mRNA and protein expression of PKC β isoforms are increased under hyperglycemic conditions, although MI causes a significant increase in the protein expression of PKC β isoforms in rat hearts. Therefore, we consider that activation of PKC β isoforms is an important cellular mechanism in the

Figure 3. MVD in the peri-infarct area. (A) Representative examples of CD31-staining in the peri-infarct area. (B) MVD in the mice of the DM group was significantly decreased compared with the control group (*P<0.05 vs. control). Inhibition of PKCβ improved the angiogenic response impaired by diabetes (**P<0.05 vs. DM) (n=8, each group). DM, diabetes mellitus; MVD, microvascular density; PKC, protein kinase C.
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Our present study showed that inhibition of PKC \( \beta \) isoforms significantly improved the MVD of the peri-infarct area, which was impaired by hyperglycemia in STZ-induced diabetic mice with MI. In addition, we confirmed that the expression of VEGF and eNOS were also improved by administration of PKC \( \beta \) inhibitor, although there was no significant change in the expression of VEGFR1 or 2. The phenomenon of angiogenesis, which is regulated by various angiogenic and anti-angiogenic factors, is the process of sprouting new capillaries from}

**Figure 4.** Expression of angiogenic mediators. (A) The DM group had significantly lower expression of VEGF, though the inhibition of PKC \( \beta \) led to significant improvement in VEGF expression that was impaired by hyperglycemia (*) \( P<0.05 \) vs. control, **(*) \( P<0.05 \) vs. DM) \((n=8, \text{each group})\). (B) Expression of eNOS showed the same pattern as VEGF (* \( P<0.05 \) vs. control, **(*) \( P<0.05 \) vs. DM) \((n=8, \text{each group})\). (C) Expression of VEGFR1 was not significantly different among the groups \( (P=0.30) \))\((n=6, \text{each group})\). (D) As in VEGFR2, there was no significant difference in expression of VEGFR2 \( (P=0.89) \))\((n=6-8/\text{group})\). (E) Expression of \( \beta \)-actin was measured as an internal control. There was no significant difference among the groups \( (P=0.96) \))\((n=5, \text{each group})\). DM, diabetes mellitus; eNOS, endothelial nitric oxide synthase; PKC, protein kinase C; VEGF, vascular endothelial growth factor.
In ischemic conditions, including MI, angiogenesis triggered by hypoxia occurs in order to compensate for the insufficient blood supply and maintain tissue functions. VEGF is one of the most important angiogenic factors and its expression in the myocardium is decreased in patients with DM compared with non-DM patients. This fact has been supported by several animal experiments, including our present study. Activation of PKC inhibits the action of insulin, presumably by phosphorylation of serine/threonine residues on the insulin receptor or their substrate. The insulin receptor, which is a receptor tyrosine kinase, shares many downstream mediators, such as phosphoinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAP-kinase), with angiogenic factors, including VEGF and eNOS. In addition, impairment of insulin’s action in the myocardium is associated with lower expression of hypoxia-inducible factor-1α (HIF-1α), which affects the expression of VEGF in the myocardium. HIF-1α also enhances angiogenesis in the heart via stromal cell-derived factor-1.

Nitric oxide (NO) is also important in ischemia-induced angiogenesis and its synthesis is regulated by eNOS. The generation of NO is regulated by both the absolute level of eNOS protein and protein kinase B (Akt)-mediated phosphorylation of the eNOS level. As mentioned earlier, the activation of PKCβ isoforms inhibits phosphorylation of eNOS via a PI3K/Akt pathway that is stimulated by insulin. Furthermore, VEGF itself has effects on the phosphorylation of eNOS through this same pathway. However, the level of expression of eNOS protein under diabetic conditions is still controversial. As it was in our study, it has been reported that eNOS protein expression is decreased in human coronary artery endothelial cells under hyperglycemic conditions, although other studies have reported an increase or no change in eNOS protein expression. To elucidate this inconsistency, further investigations are needed.

In summary, our present study suggests that in STZ-induced diabetic mice complicated with MI, inhibition of PKCβ isoforms ameliorates the angiogenic response that is impaired by hyperglycemia through improvements of VEGF and eNOS expression. Although we examined limited angiogenic factors in this study, previous studies implicated other mediators in the improvement of impaired angiogenesis by hyperglycemia because of the inhibition of PKCβ isoforms. For example, matrix metalloproteinase-2 (MMP-2), which is one of the extracellular endopeptides and upregulated by HIF-1α/VEGF, participates in angiogenesis through facilitating extracellular matrix degeneration to allow new vessel expansion. On the other hand, MMP-2 also has an anti-angiogenic effect because of production of angiotatin, which antagonizes the angiogenic effect of VEGF. In the diabetic condition, increased expression of MMP-2 contributes to impaired angiogenesis. In addition, it has been reported that inhibition of PKCβ reduces expression of MMP-2 in vitro. Therefore, we will investigate these points in the further studies.

**Clinical Implications**

Based on positive results in experimental animal models with induced diabetic vascular complications, clinical trials using PKCβ inhibitor have been performed and the effects on diabetic retinopathy and nephropathy have been reported. Ruboxistaurin (Eli Lilly, Indianapolis, IN, USA), which is a PKCβ inhibitor, has been deemed “approvable” by the United States Food and Drug Administration and is now undergoing additional clinical trials for diabetic retinopathy.

There have not been any previous reports on the effects of PKCβ inhibitor for impaired angiogenesis of the ischemic heart with diabetes, in either animal experimental or clinical studies. Therefore, there is a possibility that the results of our present study will create a new indication of PKCβ inhibitor.

In our present study, PKCβ inhibitor improved impaired angiogenic response by hyperglycemia, though the size of the myocardial infarct did not significantly change. We think that this is the limitation of therapeutic angiogenesis. Actually, although various types of therapeutic angiogenesis for CAD have been previously reported, including medications, recombining protein of angiogenic growth factors, gene transfer and stem cell therapy, none of these treatments has dramatically decreased the myocardial infarct without conventional direct revascularization, either coronary artery bypass grafting or percutaneous coronary intervention. These findings suggest the therapeutic angiogenesis for acute coronary ischemia is insufficient for the prevention of myocardial remodelling. However, for complete evaluation of therapeutic angiogenesis in acute coronary ischemia, it is necessary to evaluate not only morphological parameters, but also physiological parameters, such as cardiac function and exercise tolerance in clinical settings.

**Study Limitations**

Our present study used STZ-induced diabetic mice to mimic type I DM. Although hyperglycemia is common to models of types I and II DM, insulin resistance, which accompanies type II DM, affects endothelial function, oxidative stress, inflammation, atherosclerosis and angiogenesis. Further experiments with animal models of type II DM are needed. In addition, STZ-induced diabetic mice are too weak to survive surgery for LAD ligation at the time when remarkable hyperglycemia is caused by complete insulinemia. Therefore, we performed the surgery for LAD ligation at a fasting glucose level of approximately 180 mg/dl, which is about half the maximal hyperglycemia in this study, although there was a significant difference between the control and STZ-administered groups. Similar experimental models have been previously reported and it is especially important to pay attention to the glucose levels at the time of surgery creating MI.

**Conclusions**

Inhibition of PKCβ ameliorated the impaired angiogenesis brought on by hyperglycemia in STZ-induced diabetic mice complicated by MI, which is associated with improvement in VEGF and eNOS expression. Although the size of the myocardial infarct was not significantly improved, our results suggest a new potential indication of PKCβ inhibitor for MI in DM patients.

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**References**

9. Evcimen ND, King GL. The role of protein kinase C activation and angiogenesis in MI With DM


