PKCδ impaired vessel formation and angiogenic factor expression in diabetic ischemic limb.

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Abstract

Decreased collateral vessel formation in the diabetic peripheral limbs is characterised by abnormalities of the angiogenic response to ischemia. Hyperglycemia is known to activate protein kinase C (PKC) affecting the expression and activity of growth factors such as VEGF and PDGF. The present study investigates the role of PKCδ in diabetes-induced poor collateral vessel formation and inhibition of angiogenic factors expression and actions. Ischemic adductors muscles of diabetic Prkcd<sup>+/+</sup> mice exhibited reduced blood reperfusion, vascular density and number of small vessels as compared to non-diabetic Prkcd<sup>+/+</sup> mice. By contrast, diabetic Prkcd<sup>−/−</sup> mice showed significant increased blood flow, capillary density and number of capillaries. Although expression of various PKC isoforms were unchanged, activation of PKCδ was increased in diabetic Prkcd<sup>+/+</sup> mice. VEGF and PDGF mRNA and protein expression were decreased in muscles of diabetic Prkcd<sup>+/+</sup> mice and normalized in diabetic Prkcd<sup>−/−</sup> mice. Furthermore, phosphorylation of VEGFR2 and PDGFR-β were blunted in diabetic Prkcd<sup>+/+</sup> mice but elevated in diabetic Prkcd<sup>−/−</sup> mice. The inhibition of VEGFR2 and PDGFR-β activity was associated with increased SHP-1 expression. In conclusion, our data have uncovered the mechanisms by which PKCδ activation induced poor collateral vessel formation offering potential novel targets to regulate angiogenesis therapeutically in diabetic patients.
The main long-term complications from diabetes are vascular diseases, which are in turn the main causes of morbidity and mortality in diabetic patients (1). Diabetic vascular complications affect several important organs, including the retina, kidney, and arteries (2; 3). Peripheral vascular diseases are the major risk factor for non-traumatic lower-extremity amputation in patients with diabetes (4), characterized by insufficient collateral vessel development to support the loss of blood flow through occluded arteries in the ischemic limbs (5). Multiple abnormalities in the angiogenic response to ischemia have been documented in the diabetic state and are dependent on complex interactions of multiple growth factors and vascular cells. Experiments to improve angiogenesis and vascular cell survival by local infusion of the vascular endothelial growth factor (VEGF) or angiopoietin by increasing its expression have also been reported in non-diabetic animal models (6; 7). Moreover, animal studies using the platelet-derived growth factor (PDGF) have been done to improve collateral vessel formation and vascular healing in diabetic state (8). Clinical trials using recombinant of growth factors have noted transient improvement of myocardial and distal leg circulation (9-11). However, such favorable vascular effects appeared to produce limited clinical benefits (12). Local administration of growth factors such as VEGF by gene therapy in the setting of diabetes does not appear to have the beneficial long-term effects seen in the absence of diabetes nor improve quality of life (13; 14). One potential problem with normalizing VEGF or PDGF action alone is that a variety of growth factors may be needed to establish and maintain the capillary bed.

Various studies have clearly identified that the expression of growth factors such as VEGF, PDGF and stromal-derived factor-1 (SDF-1) are critically important in the formation of collateral vessels in response to ischemia (15-17). Previous studies suggested that hyperglycemia attenuates VEGF production and levels in myocardial tissue and animal models of wound repair
Furthermore, decreased VEGF and PDGF expression in the peripheral limbs and nerves of diabetic animals and rodents have been reported (19-21). Although the underlying mechanism of reduction of VEGF and PDGF expression in diabetes is not clear, it is well-known that the major inducers of VEGF and PDGF, i.e. hypoxia and oxidants, can both play a role in diabetes. We and others researchers have reported that variation in PDGF signaling, rather than expression, is linked to morphological abnormalities in the retina and in collateral capillary formation in ischemic limb model of diabetic animals(22; 23). Clearly, poor collateral vessel formation during ischemia induced by diabetes is attributable to the lack of production and/or action of critical growth factors such as VEGF and PDGF. Therefore, further studies of the basic mechanisms of hyperglycemia-induced activation of toxic metabolites such as activation of protein kinase C (PKC) are needed to identify how these proteins contribute to growth factor deregulation.

Protein kinase C, a member of a large family of serine/threonine kinases, plays a role in the pathophysiology of vascular complications. When activated, PKC phosphorylates specific serine or threonine residues on target proteins that vary depending on cell type. There are multiple isoforms of PKC that function in a wide variety of biological systems (24). PKC activation increases endothelial permeability, decreases blood flow and both the production and the response of angiogenic growth factors contributing to the loss of capillary pericytes, retinal permeability, ischemia, and neovascularisation (25-29). In smooth muscle cells, previous data have demonstrated that high glucose levels activate PKCα, β, δ and ε but not the atypical PKCζ (30; 31). In general, high levels of glucose-induced PKC activation cause vascular dysfunction by altering the expressions of growth factors such as the VEGF, PDGF, transforming growth factor-β, and others (32-34). PKCδ has been proposed to participate in smooth muscle cell
apoptosis and deletion of this PKC isoform led to increase arteriosclerosis (35). Moreover, we have previously demonstrated that diabetes-induced PKCδ activation generate PDGF unresponsiveness causing pericyte apoptosis, acellular capillaries and diabetic retinopathy (23). Therefore, we hypothesized that PKCδ activation could be involved in pro-angiogenic factor inhibition triggering poor collateral vessel formation in diabetes.
Research Design and Methods

Reagents and antibodies - Primary antibodies for immunoblotting were obtained from commercial sources including actin (HRP; I-19), SHP-1 (C19), VEGF (147), PKCα (C-20), PKCβ (C-18), PKCε (C-15), NOS3 (C-20), antibodies from Santa Cruz Biotechnology Inc., phospho-tyrosine, phospho-PKCδ (Thr 505), PKCδ, phospho-VEGFR2 (Y1175), VEGFR2, phospho-PDGFR-β (Tyr 1009), PDGFR-β antibodies from Cell Signaling, anti-α smooth muscle actin from Abcam, polyclonal antibody against PTP1B, CD31 from BD Bioscience, SHP-2, SHP-1 antibodies from Millipore, and rabbit and mouse peroxidase-conjugated secondary antibody from GE Healthcare Bio-Sciences. All other reagents employed including EDTA, leupeptin, phenylmethylsulfonyl fluoride, aprotinin, and Na3VO4 were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

Animal and experimental design – Six weeks-old C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our animal facility. Prkcd−/− mice, described previously and provided by Dr. Michael Leitges (35), were generated by the insertion of a LacZ/neo cassette into the first transcribed exon of the PKCδ gene. As a consequence of the insertion, the transcription of PKCδ was abolished leading to a null allele. Prkcd−/− mice with mixed background of 129SV and C57BL/6J strains were crossbred ten generations (F12) with wild-type C57BL/6J background from Jackson Laboratory. Animals were rendered diabetic for a two month period by streptozotocin (STZ) (Sigma) (50 mg/kg in 0.05M citrate buffer, pH. 4.5, i.p.) on five consecutive days after overnight fast; control mice were injected with citrate buffer. Blood glucose was measured by Glucometer (Contour, Bayer Inc). Throughout the period of study, animals were provided with free access to water and standard rodent chow (Harlan.
Teklad, Madison, WI). All experiments were conducted in accordance with the Canadian Council of Animal Care and University of Sherbrooke Guidelines.

**Hindlimb ischemia model** – We have assessed blood flow in non-diabetic and two months diabetic Prkcd\(^{+/+}\) and Prkcd\(^{-/-}\) mice. Animals were anesthetized and the entire lower extremity of each mouse was shaved. A small incision was made along the thigh all the way to inguinal ligament and extending superiorly towards the mouse abdomen. Femoral artery was isolated from the femoral nerve and vein, and ligated distally to the origin of the arteria profunda femoris. The incision was closed by interrupted 5-0 sutures (Syneture).

**Laser Doppler Perfusion Imaging and physical examination** – Hindlimb blood flow was measured using a laser Doppler perfusion imaging (PIMIII) system (Perimed Inc). Consecutive perfusion measurements were obtained by scanning the region of interest (hindlimb and foot) of anesthetized animals. Measurements were performed pre- and post-artery ligation, and additionally on postoperative days 7, 14, 21 and 28. To account for variables that affect blood flow temporally, the results at any given time were expressed as a ratio against simultaneously obtained perfusion measurements of the right (ligated) and left (non-ligated) limb. Tissue necrosis was scored to assess mice that had to be euthanized during the course of the experiment due to necrosis/loss of toes.

**Histopathology and Tunnel assay** – Right and left abductor muscles from Prkcd\(^{+/+}\) and Prkcd\(^{-/-}\) mice were harvested for pathological examination and sections were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 18 h and then transferred to 90% ethanol for light
microscopy and immunohistochemistry. Paraformaldehyde-fixed tissue was embedded in paraffin and 6 µm sections were stained with hematoxylin & eosin (Sigma). Apoptotic cells were detected using the TACS 2 Tdt-Fluor in situ apoptosis detection kit (Trevigen, Gaitherburg, CT, USA) according to the manufacturer’s instructions.

Immunofluorescence – Adductor muscles were blocked with 10% goat serum for 1 h and were exposed in sequence to primary antibodies (CD31 and α-smooth muscle actin, 1:100) over night following by incubation with secondary antibodies Alexa-647 conjugated anti-rabbit IgG and Alexa-594 conjugated anti-mouse (Jackson ImmunoResearch Laboratories; 1:500). Confocal images were captured on a Zeiss LSM 410 microscope; images of 1 experiment were taken at the same time under identical settings and handled in Adobe Photoshop similarly across all images.

Immunoblot analysis – Adductors muscles were lysed in Laemmli buffer (50 mM Tris [pH 6.8], 2% SDS, and 10% glycerol) containing protease inhibitors (1 mmol/l phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mmol/l Na3VO4; Sigma). Protein amount was measured with BCA kit (Bio-Rad). The lysates (10-20 µg protein) were separated by SDS-PAGE, transferred to PVDF membrane, and blocked with 5% skim milk. Antigens were detected using anti-rabbit horseradish peroxidase-conjugated antibody for other Western blotting, and detected with the ECL system (Pierce Thermo Fisher, Piscataway, NJ). Protein content quantification was performed using computer-assisted densitometry (Image J; NIH).

Real time PCR analysis – Real-time PCR was performed to evaluate mRNA expressions of PKC-α, PKC-β, PKC-δ, PKC-ε, VEGF, PDGF, KDR/Flk-1, PDGFR-β, eNOS, SDF-1, FGF2,
SHP-1, SHP-2 and PTP1B of non-ischemic and ischemic limbs. Total RNA was extracted from adductor muscles with TRI-REAGENT, as described by the manufacturer and RNeasy mini kit (Qiagen, Valencia, CA). The RNA was treated with deoxyribonuclease I (DNase I; Invitrogen) to remove any genomic DNA contamination. Approximately 1 µg RNA was used to generate cDNA using SuperScript III reverse transcriptase and random hexamers (Invitrogen) at 50 °C for 60 min. PCR primers and probes are listed in supplementary table 1. GAPDH and 18S ribosomal RNA expression was used for normalization. PCR products were gel purified, subcloned using QIA quick PCR Purification kit (Qiagen), and sequenced in both directions to confirm identity.

*Nuclear extract and non-radioactive transcription factor assay.* – Adductors muscles were lysed and nuclear-specific proteins were isolated using the NucBuster Protein Extraction Kit (Novagen, Madison, WI) according to the manufacturer’s instructions. Detection of HIF-1α in the nucleus was quantified using the non-radioactive transcription factor assay kit (Cayman, Ann Arbor, MI). Briefly, 20 µg of nuclear protein was incubated for 24 hours in 96-well plate containing immobilized specific double stranded DNA (dsDNA) consensus sequence of HIF-1α response element. HIF-1α contained in the nuclear extract was linked specifically to the HIF-1α response element. Wells were washed five times and the HIF transcription factor complex was detected by addition of a specific primary antibody directed against HIF-1α and incubated for an hour. Wells were washed five times and exposed with secondary antibody conjugated to HRP for another hour. Wells were then washed five times and developing agent was added to provide a sensitive colorimetric readout at 450 nm (*Infinite M200*, Tecan Group Ltd., Switzerland) to quantify nuclear HIF-1α levels.
Statistical analyses – The data were shown as mean ± SD for each group. Statistical analysis was performed by unpaired $t$ test or by one-way analysis of variance (ANOVA) followed by Tukey’s test correction for multiple comparisons. All results were considered statistically significant at $P < 0.05$. 
Results

Deletion of PKC delta improved reperfusion and vascular response ischemia on diabetic limbs. Non-diabetic and diabetic male Prkdc
t mice and control littermates (Prkcd
t+/+)
were subjected to unilateral right femoral artery ligation. Body weight and fasting glucose levels were measured at the sacrifice (Supplementary table 2). Blood flow reperfusion was assessed using the PIMIII laser Doppler imaging system (Fig 1A). Diabetic Prkcd
t+/+
exhibited reduced blood reperfusion of ischemic limb as compared to non-diabetic Prkcd
t+/+
(P=0.0046). In contrast, reperfusion of blood flow of diabetic Prkcd
t+/+
was significantly improved (P=0.0003) compared to diabetic Prkcd
t+/+
mice and similar to non-diabetic Prkcd
t+/+
and Prkcd
t+/+
mice 28 days after the ligation (Fig 1B). Since diabetic patients are at high risk of lower limb amputation, we have assessed limb necrosis and apoptosis. Impaired reperfusion in ischemic limbs of diabetic Prkcd
t+/+
mice was associated with elevated tissue necrosis, amputation (Fig. 1C) and apoptosis (Fig. 2) as compared to non-diabetic counterparts.

One main effect of hypoxia is to induce angiogenesis and to promote new capillary formation. To test whether activation of PKCδ is responsible for poor collateral vessel formation in diabetes, we have measured capillary density and capillary diameter in the ischemic adductor muscles. Figure 3 demonstrated that adductor muscles of diabetic Prkcd
t+/+
mice displayed a 31% vascular density reduction as compared to non-diabetic Prkcd
t+/+
mice. The decline of capillary density was accompanied with a 50% reduction in number of vessels with diameter of 50 µm or less. Interestingly, diabetic Prkcd
t−/−
mice showed a significant increase in capillary density and number of vessels with a diameter of < 50 µm as compared to diabetic Prkcd
t+/+
mice (Fig. 3D).
**PKCδ is activated in diabetic ischemic limb.** Hyperglycemia is known to activate multiple PKC isoforms, preferably the β and δ isoforms in vascular cells. Expression of various isoforms of PKC was assessed by quantitative PCR in muscle tissues (Fig. 4). Compared to non-diabetic *Prkcd*+/+ mice, mRNA expression of PKCβ and δ was modestly increased in adductor muscles of diabetic *Prkcd*+/+ mice and unchanged in *Prkcd*−/− mice (Fig. 4B and 4D). There was no significant difference in the mRNA expression of PKCα and ε (Fig. 4A and 4C). Although, diabetic *Prkcd*+/+ mice did not exhibit higher levels of protein expression of PKCα, β2, ε or δ isoforms, adductor muscles of *Prkcd*+/+ mice showed a significant and an impressive increase of PKCδ phosphorylation (Thr 505) (*P* = 0.0040), as a marker of PKCδ activation, 28 days after unilateral femoral artery ligation in comparison with non-diabetic *Prkcd*+/+ mice (Fig. 5).

**Inhibition of PKCδ promotes pro-angiogenic growth factor expression and activation.** To explain how the absence of PKCδ improved reperfusion in diabetic ischemia limb, we have performed a wide analysis of the gene and protein expression of angiogenic-related factors and their receptors. Quantitative gene expression analyses by real-time PCR indicated that VEGF-A, PDGF-B and PDGF receptor (PDGFR)-β mRNA expression were significantly decreased in adductor muscles of diabetic compared to non-diabetic *Prkcd*+/+ mice by 46%, 30% and 63%, respectively (Fig. 6A, 6C and 6D). The reduction of these genes in diabetic *Prkcd*+/+ mice was not observed in diabetic *Prkcd*−/− mice. Moreover, mRNA expression of VEGF receptor (VEGFR)2 (KDR/Flk-1), PDGF-B and PDGFR-β were significantly upregulated in diabetic *Prkcd*−/− compared to diabetic *Prkcd*+/+ mice (Fig. 6B, 6C, and 6D). These results suggest that impaired PDGF and VEGF expression by PKCδ activation might be the contributing factor for poor collateral vessel formation in diabetes. Expression of other angiogenic factors such as SDF-
1, FGF-2 and endothelial nitric oxide synthase (eNOS), as well as transcriptional factor activity of hypoxia-inducible factor-1 alpha (HIF-1α) were unchanged within all groups of mice (Fig. 6E, 6F, 6G, 6H and supplementary fig. 1). In contrast to four weeks after femoral artery ligation, transcriptional factor activity and mRNA levels of HIF-1α were significantly decreased in diabetic Prkcd<sup>+/+</sup> mice compared to non-diabetic Prkcd<sup>+/+</sup> and diabetic Prkcd<sup>−/−</sup> mice (Supplementary fig. 2 and 3).

**VEGFR2 and PDGFR-β activation is decreased in diabetic ischemic muscles.** To further investigate the mechanisms of impaired angiogenic response to restore blood flow in diabetes, the expression, activation and signaling pathway of VEGF-A, PDGF-B and their respective receptors (VEGFR2 and PDGFR-β) were examined. Protein expression of PDGF-B was significantly decreased in diabetic versus non-diabetic adductor muscles of wild-type animals. In contrast, VEGF-A and PDGF-B protein expression were elevated in ischemic limb of diabetic PKCδ null mice (Fig 7A and 7B). Phosphorylation of VEGFR2 and PDGFR-β were inhibited in ischemic adductor muscles of diabetic as compared to non-diabetic Prkcd<sup>+/+</sup> mice. On the contrary, activation of Src was elevated in adductor muscles of diabetic Prkcd<sup>+/+</sup> mice compared to non-diabetic Prkcd<sup>+/+</sup> and Prkcd<sup>−/−</sup> mice (Fig. 7B). Interestingly, tyrosine phosphorylation of VEGFR2 and PDGFRβ as well as PLC-γ1, Akt and ERK phosphorylation was greatly enhanced in Prkcd<sup>−/−</sup> mice compared to diabetic Prkcd<sup>+/+</sup> mice (Fig 7A and 7B). We did not observe any changes in the eNOS protein expression amongst experimental groups (Fig 7A).

**Expression of SHP-1 caused VEGFR2 and PDGFRβ inactivation.** We have previously shown that activation of PKCδ leads to increased expression of SHP-1 which inhibits the PDGF
signaling pathway and promotes retinal pericyte apoptosis in diabetic animals. To determine whether SHP-1 is implicated in PKCδ-induced VEGFR2 and PDGFR-β dephosphorylation in diabetic ischemic adductor muscles, we have measured SHP-1 expression by quantitative PCR and immunoblot analysis. Figure 8A and 8B indicates that mRNA expression of SHP-1, but not SHP-2 or PTP1B, was elevated in diabetic Prkcd+/+ mice, whereas in Prkcd−/− mice, SHP-1 is clearly downregulated. We have confirmed through immunoblot analysis that SHP-1 protein expression was elevated by 2.3-fold in ischemic adductor muscles of diabetic Prkcd+/+ mice as compared to non-diabetic Prkcd+/+ mice. The increase expression of SHP-1 was not observed in diabetic Prkcd−/− mice (Fig. 8C). No change was detected in protein expression of SHP-2 and PTP1B within all groups of mice (Fig. 8D).
Discussion

Diabetes is associated with the progression of vascular complications such as peripheral arterial diseases and a major risk factor for lower limb amputations (4). In the present study, we have demonstrated that activation of PKCδ diminishes the expression of VEGF and PDGF, two critical pro-angiogenic factors, contributing to poor capillary formation and blood flow reperfusion of the ischemic limbs. In addition to reducing expression of VEGF and PDGF, phosphorylation of VEGF and PDGF receptors was abrogated in diabetic ischemic muscles as compared to non-diabetic ischemic muscles. The inhibition of growth factor receptor phosphorylation was associated with the upregulation of SHP-1 expression, which has been reported to de-activate tyrosine kinase receptors such as VEGF and PDGF receptors. Overall, deletion of PKCδ prevents the reduction of VEGF and PDGF expression, and re-establishes KDR/Flk-1 and PDGFR-β phosphorylation, favoring new capillary formation and blood flow reperfusion.

Wound healing is a complex, well-orchestrated and dynamic process that involves a coordinated and precise interaction of various cell types and mediators. Given the fundamental contribution of VEGF and PDGF to the angiogenic process, the mechanism by which activation of PKCδ isoform prevents growth factors expression and signaling actions may provide a better understanding on how diabetes reduces collateral vessel formation in ischemic limb. In this study, we have demonstrated that PKCδ was activated in diabetic ischemic muscles and reduced blood flow reperfusion contributing to tissue necrosis, amputation and apoptosis. Previous studies have reported that PKCδ is involved in vascular cell apoptosis. PKCδ activates p-38 MAPK, p53 and caspase-3 cleavage to favor endothelial (36) and smooth muscle cell apoptosis (37; 38). Therefore, deletion of PKCδ may enhance vascular cell migration and proliferation, two
significant steps in the formation of new blood vessels. Total expression of PKC isoform in ischemic muscles was slightly affected by diabetes, probably due to fact that mRNA and protein analysis were performed 28 days after femoral artery ligation. However, phosphorylation of PKCδ on threonine 505, phosphorylation site within the activation loop, clearly suggests that PKCδ is activated in diabetic ischemic limbs compared to non-diabetic muscles. Previous data have shown that the inhibition of PKCδ, using an isozyme-specific peptide, improved number of microvessels and cerebral blood flow following acute focal ischemia in normotensive rats (39). Our data demonstrate that deletion of PKCδ restores blood flow perfusion in diabetic ischemic muscles by promoting the number of capillaries and reducing tissue apoptosis.

The reduction of VEGF and PDGF receptor expression and downstream signaling pathway is associated with impaired angiogenesis process in diabetic foot ulcer and ischemic diseases. Our results indicate that diabetes-induced PKCδ activation decreases VEGF, PDGF, KDR/Flk-1 and PDGF receptor-β mRNA expression in ischemic limb which is completely restored in PKCδ null mice. Interestingly, impaired angiogenic response in ischemic arterial diseases of type 1 and type 2 diabetes is associated with VEGF inhibition in endothelial cells and monocytes (13; 40). It is possible that the ablation of PKCδ may also affect VEGF signaling in monocytes, which may contribute to vessel formation abnormalities. However, this assumption will need further investigation. HIF-1α is a master regulator of angiogenic factors in response to tissue hypoxia. Previous study has shown that HIF-1α gene transfer increased recovery of limb perfusion increasing eNOS activation and vessel density (41). However, in our study, increase expression of VEGF in muscles of PKCδ deficient mice may not be entirely due to upregulation of HIF-1α. Since protein extraction was performed four weeks after the femoral artery ligation, it is possible that the expression of HIF-1α could have returned to basal levels. This hypothesis is supported
by results obtained two weeks after the surgery. Our data have demonstrated that HIF-1α transcriptional factor activity and mRNA expression were increased in non-diabetic and diabetic PKCδ null mice two weeks only after surgery (Supplementary fig. 2 and 3). Beside VEGF and PDGF expression, our data suggest that PKCδ activation disrupts the VEGF and PDGF signaling whereas in PKCδ deficient mice, the activity of VEGFR2, PDGFR-β, PLCγ1, Akt and ERK is enhanced. Surprisingly, Src phosphorylation was increased in ischemic muscles of diabetic wild-type mice even if PDGFR-β activity was reduced. However, previous study has reported that reactive oxygen species (ROS) production induced Src phosphorylation (42). Since ROS are massively produced in ischemic and hyperglycemic conditions, it is probable that ROS production is responsible for Src phosphorylation seen in diabetic wild-type mice.

There is strong evidence that progenitor cell recruitment and homing participate to angiogenesis and wound repair which are guided by SDF-1 (43). Although the number of progenitor cells is reduced in diabetic mice, inadequate progenitor cell mobilization has been proposed as one potential mechanism of impaired angiogenesis (44). However, our results did not observe any change in SDF-1 expression in PKCδ null mice suggesting that mobilization and local trafficking of progenitor cells to the ischemic site was not affected by PKCδ isoform.

Despite advances in revascularization techniques, limb salvage and relief of pain cannot be achieved in many diabetic patients with diffuse peripheral vascular diseases. VEGF-mediated gene therapy has shown promising results as an innovating method in the treatment of severe cardiovascular diseases. However, randomized gene therapy study failed to meet the primary objective of significant amputation reduction (45). During the 10-year follow-up period, no significant differences were detected in the number of amputations or causes of death with the use of transient VEGF-A-mediated gene therapy. One reason for this lack of improvement is
perhaps because the neovascularization requires the interaction of multiple growth factors that can promote, in a synergic manner, new and mature blood vessels. Enhancing the responsiveness of diabetic vascular cells to pro-angiogenic factors may offer a potential new approach to treat peripheral arterial diseases. Protein tyrosine phosphatase is a group of proteins that is critical in abating cell response to growth factors by inhibiting tyrosine kinase phosphorylation. Our results demonstrated that SHP-1 expression was increased in diabetic ischemic muscles and was responsible for VEGF and PDGR receptor dephosphorylation. Although not significant, a slight rise in SHP-2 (18%) and PTP1B (37%) expression was observed in diabetic PKCδ null mice. Previous studies have shown that PDGF activation enhanced SHP-2 and PTP1B activity (46; 47), which may explain our results. We have reported that activation of PKCδ induces the expression of SHP-1 in cultured pericytes exposed to high glucose concentrations and inhibits PDGF signaling pathway contributing to pericyte apoptosis (23). Others studies have also shown that SHP-1 is a negative regulator of VEGF signal transduction and inhibits endothelial cell proliferation (48; 49). Interestingly, silencing SHP-1 increased phosphorylation of KDR/Flk-1 and markedly enhanced capillary density in a non-diabetic hindlimb ischemia model (50). However, our current study does not provide direct link between SHP-1 expression and reduced angiogenesis, which will require further investigations. Nevertheless, our findings have identified PKCδ, and potentially SHP-1, as potential therapeutic targets for the treatment of diabetic peripheral arterial diseases and cardiovascular complications.

In summary, we have provided evidence that PKCδ is activated by diabetes in ischemic muscles and induced SHP-1 expression contributing to VEGF and PDGF unresponsiveness and poor angiogenesis response. Although various therapies are partly successful in restoring blood flow to the affected tissues, there is no effective strategy to specifically produce new functional
vessels to dismiss diabetic ischemic stress. Our data enhanced our understanding of the mechanisms underlying poor collateral vessel formation induced by PKC activation and may offer potential novel targets to regulate angiogenesis therapeutically in patients with diabetes.
Acknowledgements

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References


17. Semenza GL: Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology (Bethesda) 2009;24:97-106


Figure legends

Figure 1: Blood flow reperfusion and recovery limb from ischemia. (A) Laser Doppler imaging and reperfusion analysis of non-diabetic Prkcd+/+ and Prkcd−/− mice. (C) Morphological and observational analysis of necrosis and amputation of post-surgery. Results are shown as mean ± SD of 10-12 mice per group. * P = 0.0046 versus NDM Prkcd+/+, † P = 0.0003 versus DM Prkcd−/−.

Figure 2: Vascular cell apoptosis analysis in ischemic muscles. Immunofluorescence of apoptotic positive cells (red) and CD31 (blue) in the ischemic adductor muscles of non-diabetic (white bars) and diabetic (black bars) Prkcd+/+ and Prkcd−/− mice. Results are shown as mean ± SD of 3 sections of 6-7 mice per group. * P = 0.05 versus NDM Prkcd+/+, † P < 0.05 versus DM Prkcd+/+.

Figure 3: Histological and vascular density analysis. (A) Structural analysis of the ischemic muscles stained hematoxylin and eosin (H&E) and (B) immunofluorescence of endothelial cells (red) and α–smooth muscle actin (green) labelling in the ischemic adductor muscles of non-diabetic (white bars) and diabetic (black bars) Prkcd+/+ and Prkcd−/− mice. (C) Quantification of the vascular density and (D) the number of vessels smaller than 50 µm. Results are shown as mean ± SD of 3 sections of 6 mice per group.

Figure 4: Expression of PKC isoforms mRNA. Quantitative real-time PCR of PKCα, PKCβ, PKCδ and PKCe mRNA expression in ischemic adductors muscles of non-diabetic (white bars)
and diabetic (black bars) Prkcd$^{+/+}$ and Prkcd$^{-/-}$ mice. Results are shown as mean ± SD of 6-7 mice.

**Figure 5: Increased PKCδ activity in muscles of diabetic mice.** Expression of phospho-PKCδ (thr 505), PKCα, PCKβ, PKCδ, PKCε and actin were detected by immunoblot and densitometric quantitation was measured. Results are shown as mean ± SD of 4-6 independent experiments.

**Figure 6: mRNA expression of angiogenic factors.** Quantitative real-time PCR of VEGF, PDGF, KDR/Flk-1, PDGFR-β, eNOS, FGF-2 and SDF-1 mRNA expression as well as nuclear transcriptional factor activity of HIF-1α in ischemic adductors muscles of non-diabetic (white bars) and diabetic (black bars) Prkcd$^{+/+}$ and Prkcd$^{-/-}$ mice. Results are shown as mean ± SD of 6-7 mice. * $P = 0.05$ versus NDM Prkcd$^{+/+}$, † $P < 0.05$ versus DM Prkcd$^{+/+}$.

**Figure 7: Increased expression and activity of VEGF and PDGF signaling pathway in Prkcd$^{-/-}$.** Expression of (A) eNOS, PLC-γ1, phosphor-PLCγ1, VEGF-A, phospho-VEGFR2, VEGFR2, ERK1/2, phospho-ERK1/2 (B) PDGF-B, phospho-PDGFR-β, PDGFR-β, Src, phosphor-Src, Akt, phosphor-Akt and actin in ischemic adductors muscles of non-diabetic (white bars) and diabetic (black bars) Prkcd$^{+/+}$ and Prkcd$^{-/-}$ mice. Protein expression was detected by Western blot and densitometric quantitation was measured. Results are shown as mean ± SD of 4-6 independent experiments. * $P = 0.05$ versus NDM Prkcd$^{+/+}$, † $P < 0.05$ versus DM Prkcd$^{+/+}$.

**Figure 8: Increased expression of SHP-1 in ischemic adductor muscles of diabetic mice.** Quantitative real-time PCR of (A) SHP-1, (B) SHP-2 and PTP1B mRNA, and (C) protein
expression of SHP-1, (D) SHP-2, PTP1B and their corresponding loading control (actin) in ischemic adductors muscles of non-diabetic and diabetic Prkcd+/+ and Prkcd−/− mice. Protein expression was detected by immunoblot and densitometric quantitation was measured. Results are shown as mean ± SD of 4-6 independent experiments. * P = 0.05 versus NDM Prkcd+/+, † P < 0.05 versus DM Prkcd+/−.
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Figure 3: Histological and vascular density analysis. (A) Structural analysis of the ischemic muscles stained hematoxylin and eosin (H&E) and (B) immunofluorescence of endothelial cells (red) and α-smooth muscle actin (green) labelling in the ischemic adductor muscles of non-diabetic (white bars) and diabetic (black bars) Prkcd^+/+ and Prkcd^-/- mice. (C) Quantification of the vascular density and (D) the number of vessels smaller than 50 µm. Results are shown as mean ± SD of 3 sections of 6 mice per group.

195x154mm (300 x 300 DPI)
Figure 4: Expression of PKC isoforms mRNA. Quantitative real-time PCR of PKCα, PKCβ, PKCδ and PKCε mRNA expression in ischemic adductors muscles of non-diabetic (white bars) and diabetic (black bars) Prkcd\(^{+/+}\) and Prkcd\(^{-/-}\) mice. Results are shown as mean ± SD of 6-7 mice.

196x155mm (300 x 300 DPI)
Figure 5: Increased PKCd activity in muscles of diabetic mice. Expression of phospho-PKCδ (thr 505), PKCα, PKCβ, PKCδ, PKCε and actin were detected by Western blot and densitometric quantitation was measured. Results are shown as mean ± SD of 4-6 independent experiments.
**Figure 6: mRNA expression of angiogenic factors.** Quantitative real-time PCR of VEGF, PDGF, KDR/Flik-1, PDGFR-β, eNOS, FGF-1 and SDF-1 mRNA expression as well as nuclear transcriptional factor activity of HIF-1α in ischemic adductors muscles of non-diabetic (white bars) and diabetic (black bars) Prkcd\(^{+/+}\) and Prkcd\(^{-/-}\) mice. Results are shown as mean ± SD of 6-7 mice. * P = 0.05 versus NDM Prkcd\(^{+/+}\), † P < 0.05 versus DM Prkcd\(^{+/+}\)
Figure 7: Increased expression and activity of VEGF and PDGF signaling pathway in Prkcd<sup>−/−</sup>.
Expression of (A) eNOS, PLC-γ1, phospho-PLCγ1, VEGF-A, phospho-VEGFR2, VEGFR2, ERK1/2, phospho-ERK1/2 (B) PDGF-B, phospho-PDGFR-β, PDGFR-β, Src, phospho-Src, Akt, phospho-Akt and actin in ischemic adductors muscles of non-diabetic (white bars) and diabetic (black bars) Prkcd<sup>+/+</sup> and Prkcd<sup>−/−</sup> mice. Protein expression was detected by Western blot and densitometric quantitation was measured. Results are shown as mean ± SD of 4-6 independent experiments. * P = 0.05 versus NDM Prkcd<sup>+/+</sup>, † P < 0.05 versus DM Prkcd<sup>+/+</sup>.
Figure 8: Increased expression of SHP-1 in ischemic adductor muscles of diabetic mice.
Quantitative real-time PCR of (A) SHP-1, (B) SHP-2 and PTP1B mRNA, and (C) protein expression of SHP-1, (D) SHP-2, PTP1B and their corresponding loading control (actin) in ischemic adductors muscles of non-diabetic and diabetic Prkcd\(^{+/+}\) and Prkcd\(^{-/-}\) mice. Protein expression was detected by Western blot and densitometric quantitation was measured. Results are shown as mean ± SD of 4-6 independent experiments. * P = 0.05 versus NDM Prkcd\(^{+/+}\), † P < 0.05 versus DM Prkcd\(^{+/+}\).

226x197mm (300 x 300 DPI)
**Supplementary Table 1:** Sequence of primers.

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<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe</th>
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<td>GAT GTC ATA ATA CCT</td>
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**Supplementary Table 2:** Body weight and fasting glucose levels of non-diabetic and diabetic Prkcd<sup>+/+</sup> and Prkcd<sup>-/-</sup> mice at the sacrifice.

<table>
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<tr>
<th></th>
<th>NDM Prkcd&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>DM Prkcd&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>NDM Prkcd&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>DM Prkcd&lt;sup&gt;-/-&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>32.9 ± 4</td>
<td>25.3 ± 3.1</td>
<td>33.3 ± 2.2</td>
<td>24.7 ± 1.3</td>
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<tr>
<td>Blood glucose (mg dl&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>106 ± 9</td>
<td>443 ± 91</td>
<td>96 ± 31</td>
<td>477 ± 111</td>
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</tbody>
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Supplementary figure 1: mRNA expression of HIF-1α after 4 weeks of the femoral artery ligation. Quantitative real-time PCR of HIF-1α mRNA expression in ischemic adductors muscles of non-diabetic (white bars) and diabetic (black bars) Prkcd<sup>+/+</sup> and Prkcd<sup>−/−</sup> mice. Results are shown as mean ± SD of 6-7 mice.

Supplementary figure 2: HIF-1α nuclear binding assay after 2 weeks of the femoral artery ligation. Transcriptional factor assay of HIF-1α in nuclear extract of ischemic adductors muscles of non-diabetic (white bars) and diabetic (black bars) Prkcd<sup>+/+</sup> and Prkcd<sup>−/−</sup> mice. Results are shown as mean ± SD of 3-4 mice. * P = 0.05 versus NDM Prkcd<sup>+/+</sup>, † P < 0.05 versus DM Prkcd<sup>+/+</sup>. 
Supplementary figure 3: mRNA expression of HIF-1α after 2 weeks of the femoral artery ligation. Quantitative real-time PCR of HIF-1α mRNA expression in ischemic adductors muscles of non-diabetic (white bars) and diabetic (black bars) Prkcd<sup>+/+</sup> and Prkcd<sup>-/-</sup> mice. Results are shown as mean ± SD of 3-4 mice. * P = 0.05 versus NDM Prkcd<sup>+/+</sup>, † P < 0.05 versus DM Prkcd<sup>+/+</sup>.