PKCδ Impaired Vessel Formation and Angiogenic Factor Expression in Diabetic Ischemic Limbs

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Decreased collateral vessel formation in diabetic peripheral limbs is characterized 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 vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). The current study investigates the role of PKCδ in diabetes-induced poor collateral vessel formation and inhibition of angiogenic factors expression and actions. Ischemic adductor muscles of diabetic *Prkcd*$^{+/+}$ mice exhibited reduced blood reperfusion, vascular density, and number of small vessels compared with nondiabetic *Prkcd*$^{+/+}$ mice. By contrast, diabetic *Prkcd*$^{-/-}$ mice showed significant increased blood flow, capillary density, and number of capillaries. Although expression of various PKC isoforms was unchanged, activation of PKCδ was increased in diabetic *Prkcd*$^{+/+}$ mice. VEGF and PDGF mRNA and protein expression were decreased in the muscles of diabetic *Prkcd*$^{+/+}$ mice and were normalized in diabetic *Prkcd*$^{-/-}$ mice. Furthermore, phosphorylation of VEGF receptor 2 (VEGFR2) and PDGF receptor-β (PDGFR-β) were blunted in diabetic *Prkcd*$^{+/+}$ mice but elevated in diabetic *Prkcd*$^{-/-}$ 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. ***Diabetes*** **62:2948–2957, 2013**

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 nontraumatic lower limb amputation in patients with diabetes (4), characterized by collateral vessel development insufficient 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 depend on complex interactions of multiple growth factors and vascular cells.

Experiments to improve angiogenesis and vascular cell survival by local infusion of vascular endothelial growth factor (VEGF) or angiopoietin by increasing its expression have also been reported in nondiabetic animal models (6,7). Moreover, animal studies have used platelet-derived growth factor (PDGF) to improve collateral vessel formation and vascular healing in the diabetic state (8). Clinical trials using recombinant growth factors have noted transient improvement of myocardial and distal leg circulation (9–11). However, these 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 or to 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 in animal models of wound repair (5,18). Furthermore, decreased VEGF and PDGF expression in the peripheral limbs and nerves of diabetic animals and rodents has 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 other 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 an ischemic limb model of diabetic animals (22,23). Clearly, poor collateral vessel formation during diabetes-induced ischemia 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.

PKC, a member of a large family of serine/threonine kinases, is involved in the pathophysiology of vascular complications. When activated, PKC phosphorylates specific serine or threonine residues on target proteins that vary, depending on cell type. PKC has multiple isoforms that function in a wide variety of biological systems (24). PKC activation increases endothelial permeability and decreases blood flow and the production and response of angiogenic growth factors that contribute to the loss of capillary pericytes, retinal permeability, ischemia, and neovascularization (25–29).

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Previous data have demonstrated that high glucose levels in smooth muscle cells 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 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 increased arteriosclerosis (35). Moreover, we previously demonstrated that diabetes-induced PKCδ activation generates PDGF unresponsiveness, causing pericyte apoptosis, acellular capillaries, and diabetic retinopathy (23). We therefore hypothesized that PKCδ activation could be involved in proangiogenic factor inhibition that triggers poor collateral vessel formation in diabetes.

RESEARCH DESIGN AND METHODS

Reagents and antibodies. Primary antibodies for immunoblotting were obtained from commercial sources, including actin (horseradish peroxidase [HRP]; I-19), SHP-1 (C19), VEGF (147), PKCα (C-20), PKCβ (C-18), PKCε (C-15), and nitric oxide synthase (NOS) 3 (C-20) antibodies from Santa Cruz Biotechnology Inc.; phospho (p)-tyrosine, p-PKCδ (Thr 505), PKCδ, p-VEGF receptor 2 (VEGFR2; Y1175), VEGFR2, p-PDGF receptor-β (PDGFR-β; Tyr 1009), and PDGFR-β antibodies from Cell Signaling; anti-α smooth muscle actin from Abcam; polyclonal antibody against protein-tyrosine phosphatase 1B (PTP1B) and CD31 from BD Bioscience; SHP-2 and SHP-1 antibodies from Millipore; and rabbit and mouse peroxidase-conjugated secondary antibody from GE Healthcare Bio-Sciences. All other reagents used, including EDTA, leupeptin, phenylmethylsulfonyl fluoride, aprotinin, and Na_3VO_4, were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Animal and experimental design. C57BL/6J mice (6 weeks old) 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. This insertion abolished the transcription of PKCδ, leading to a null allele. *Prkcd*$^{-/-}$ mice with mixed background of 129SV and C57BL/6J strains were crossbred for 10 generations (F12) with wild-type C57BL/6J background from The Jackson Laboratory. Animals were rendered diabetic for a 2-month period by intraperitoneal streptozotocin injection (50 mg/kg in 0.05 mol/L citrate buffer, pH 4.5; Sigma) on 5 consecutive days after an overnight fast; control mice were injected with citrate buffer. Blood glucose was measured by Glucometer (Contour, Bayer Inc.). Throughout the study period, 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.

Hind limb ischemia model. We assessed blood flow in nondiabetic and *Prkcd*$^{+/+}$ and *Prkcd*$^{-/-}$ mice diabetic for 2 months. Animals were anesthetized,

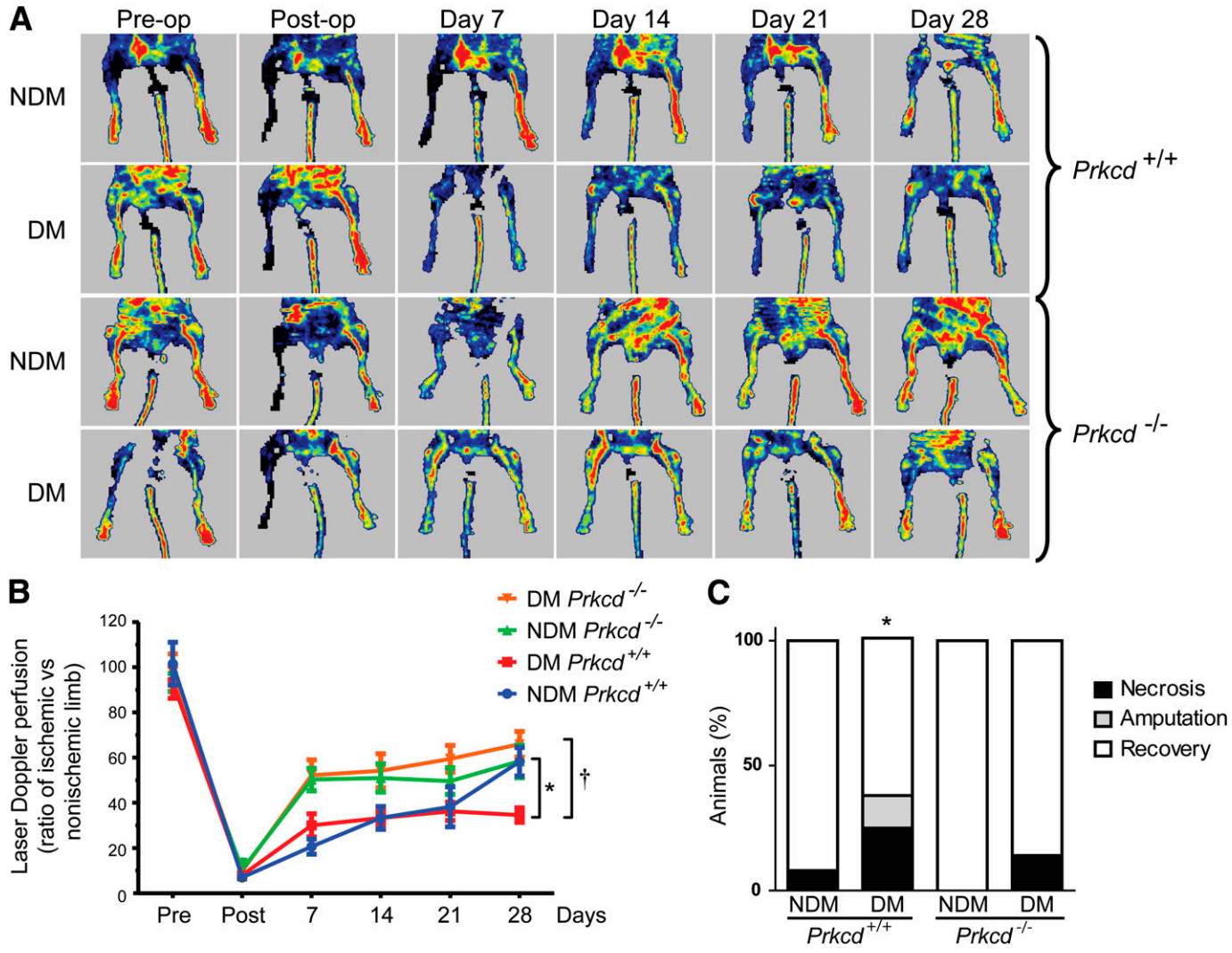

FIG. 1. Blood flow reperfusion and recovery limb from ischemia. Laser Doppler imaging and reperfusion analysis of diabetic (DM) and nondiabetic (NDM) *Prkcd*$^{+/+}$ and *Prkcd*$^{-/-}$ mice. *A* and *B*: Morphological and observational analysis of necrosis and amputation of ischemic limbs after surgery. *C*: Results are shown as mean ± SD of 10–12 mice per group. **P* = 0.0046 vs. NDM *Prkcd*$^{+/+}$, †*P* = 0.0003 vs. DM *Prkcd*$^{-/-}$.

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 toward the mouse abdomen. The 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. Hind limb blood flow was measured using PIM3 laser Doppler perfusion imaging (Perimed Inc.). Consecutive perfusion measurements were obtained by scanning the region of interest (hind limb and foot) of anesthetized animals. Measurements were performed before and after artery ligation and 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 (nonligated) limb. Tissue necrosis was scored to assess mice that had to be killed during the course of the experiment due to necrosis/loss of toes.

Histopathology and TUNEL assay. Right and left abductor muscles from *Prkcd*$^{+/+}$ and *Prkcd*$^{-/-}$ mice were harvested for pathological examination. 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 and eosin (Sigma). Apoptotic cells were detected using the TACS 2 Tdt-Fluor in situ apoptosis detection kit (Trevigen, Gaithersburg, MD) according to the manufacturer's instructions.

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

Immunoblot analysis. Adductor muscles were lysed in Laemmli buffer (50 mmol/L 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 Na_3VO_4; Sigma). Protein amount was measured with a BCA kit (Bio-Rad). The lysates (10–20 μg protein) were separated by SDS-PAGE, transferred to polyvinylidene fluoride membrane, and blocked with 5% skim milk. Antigens were detected using anti-rabbit HRP-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 with Image J software (National Institutes of Health).

Real-time PCR analysis. Real-time PCR was performed to evaluate mRNA expressions of PKCα, PKCβ, PKCδ, PKCε, VEGF, PDGF, KDR/Flk-1, PDGFR-β, endothelial NOS (eNOS), SDF-1, FGF2, SHP-1, SHP-2, and PTP1B of nonischemic 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 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. Glyceraldehyde-3-phosphate dehydrogenase and 18S ribosomal RNA expression were used for normalization. PCR products were gel purified, subcloned using a QIA quick PCR Purification kit (Qiagen), and sequenced in both directions to confirm identity.

Nuclear extract and nonradioactive transcription factor assay. Adductor muscles were lysed and nuclear-specific proteins isolated using the NucBuster Protein Extraction Kit (Novagen, Madison, WI) according to the

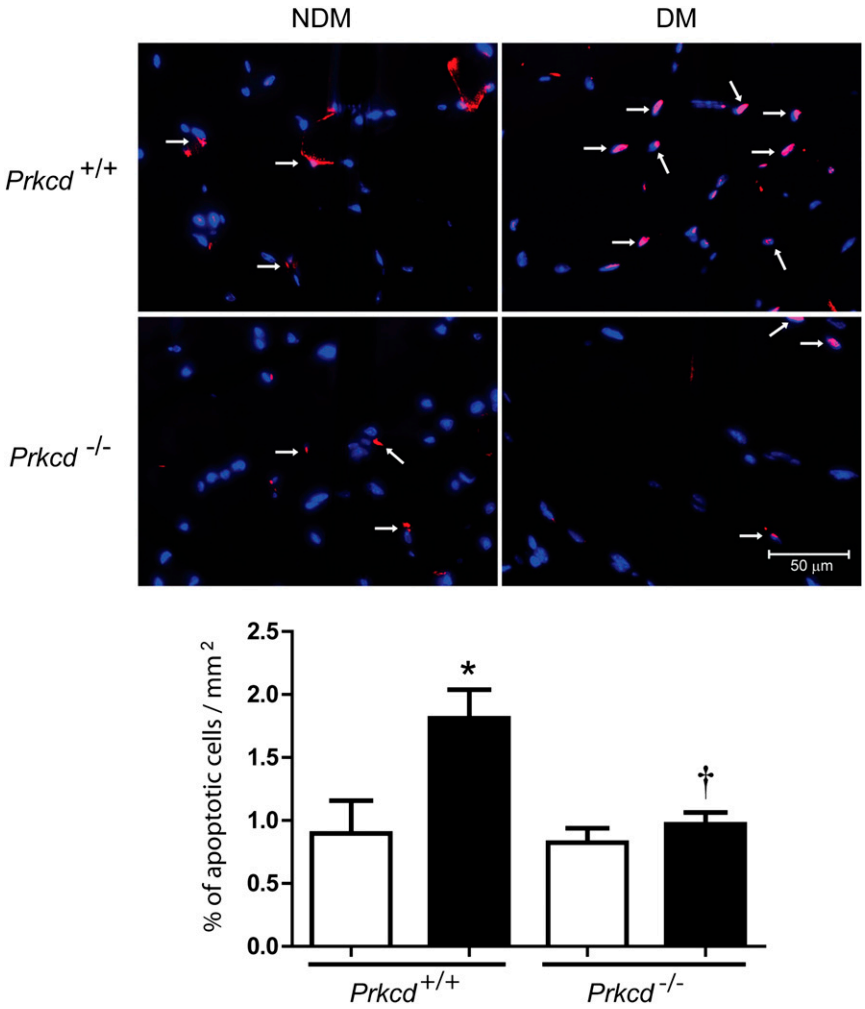

FIG. 2. Vascular cell apoptosis analysis in ischemic muscles. Immunofluorescence of apoptotic-positive cells (red) and CD31 (blue) (*top panels*) and percentage of apoptotic cells (*bottom panel*) in the ischemic adductor muscles of nondiabetic (NDM, □) and diabetic (DM, ■) *Prkcd*$^{+/+}$ and *Prkcd*$^{-/-}$ mice. Results are shown as mean ± SD of three sections of six to seven mice per group. White arrows represent colocalization of CD31 and the apoptotic-positive marker. **P* = 0.05 vs. NDM *Prkcd*$^{+/+}$, †*P* < 0.05 vs. DM *Prkcd*$^{+/+}$.

manufacturer's instructions. Detection of hypoxia-inducible factor-1α (HIF-1α) in the nucleus was quantified using the nonradioactive transcription factor assay kit (Cayman, Ann Arbor, MI). Briefly, nuclear protein (20 μg) was incubated for 24 h in a 96-well plate containing immobilized specific double-stranded DNA consensus sequence of the 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 1 h. Wells were washed five times and exposed with secondary antibody conjugated to HRP for 1 h. 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., Männedorf, Switzerland) to quantify nuclear HIF-1α levels.

Statistical analyses. The data are shown as mean ± SD for each group. Statistical analysis was performed by unpaired *t* test or by one-way ANOVA, followed by the Tukey test correction for multiple comparisons. All results were considered statistically significant at $P < 0.05$.

RESULTS

Deletion of PKCδ improved reperfusion and vascular response ischemia on diabetic limbs. Nondiabetic and diabetic male *Prkcd*$^{-/-}$ mice and control littermates (*Prkcd*$^{+/+}$) underwent unilateral right femoral artery ligation. Body weight and fasting glucose levels were measured when mice were killed (Supplementary Table 2). Blood flow reperfusion was assessed using the PIM 3 laser Doppler imaging system (Fig. 1*A*). Diabetic *Prkcd*$^{+/+}$ mice exhibited reduced blood reperfusion of the ischemic limb compared with nondiabetic *Prkcd*$^{+/+}$ mice ($P = 0.0046$). In contrast, reperfusion of blood flow of diabetic *Prkcd*$^{-/-}$ mice was significantly improved ($P = 0.0003$) compared with diabetic *Prkcd*$^{+/+}$ mice and similar to nondiabetic *Prkcd*$^{+/+}$ and *Prkcd*$^{-/-}$ mice 28 days after the ligation (Fig. 1*B*). Because diabetic patients are at high risk of lower limb amputation, we assessed limb necrosis and apoptosis. Impaired reperfusion in ischemic limbs of diabetic *Prkcd*$^{+/+}$ mice was associated with elevated tissue necrosis, amputation (Fig. 1*C*), and apoptosis (Fig. 2) compared with nondiabetic 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 measured capillary density and capillary diameter in the ischemic adductor muscles. Figure 3 demonstrated that the adductor muscles of diabetic *Prkcd*$^{+/+}$ mice displayed a 31% vascular density reduction compared with nondiabetic *Prkcd*$^{+/+}$ mice. The decline of capillary density was accompanied with a 50% reduction in number of vessels with a diameter of 50 μm or less. Interestingly, diabetic *Prkcd*$^{-/-}$ mice showed a significant increase in capillary density and number of vessels with a diameter of less than 50 μm compared with diabetic *Prkcd*$^{+/+}$ mice (Fig. 3*D*).

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 with nondiabetic *Prkcd*$^{+/+}$ mice, mRNA expression of PKCβ and δ was modestly increased in adductor muscles of diabetic *Prkcd*$^{+/+}$ mice and unchanged in *Prkcd*$^{-/-}$ mice (Fig. 4*B* and *D*). There was no significant difference in the mRNA

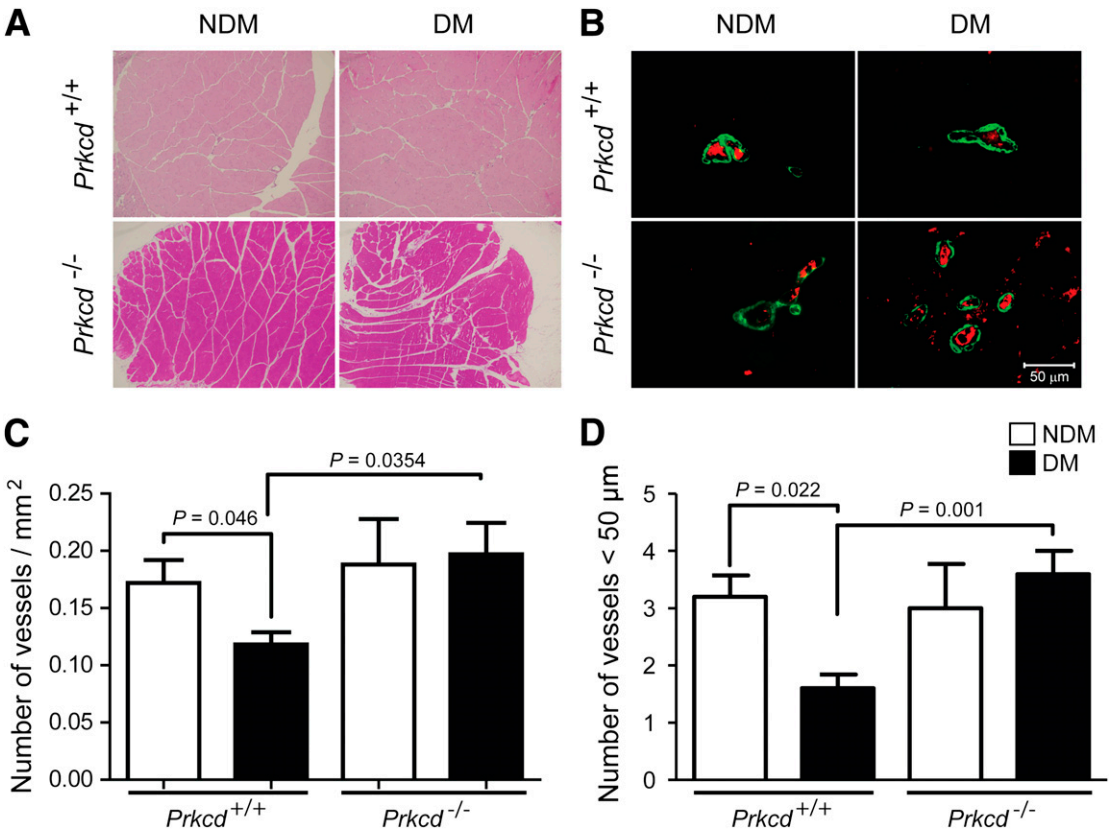

FIG. 3. Histological and vascular density analysis. Structural analysis of the ischemic muscles stained with hematoxylin and eosin (*A*) and immunofluorescence of endothelial cells (red) and α-smooth muscle actin (green) labeling (*B*) in the ischemic adductor muscles of nondiabetic (NDM) and diabetic (DM) *Prkcd*$^{+/+}$ and *Prkcd*$^{-/-}$ mice. Quantification of the vascular density (*C*) and the number of vessels smaller than 50 μm (*D*). Results are shown as mean ± SD of three sections of six mice per group.

expression of PKCα and -ε (Fig. 4*A* and *C*). 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 impressive increase of PKCδ phosphorylation (Thr 505; P = 0.0040), as a marker of PKCδ activation, 28 days after unilateral femoral artery ligation compared with nondiabetic *Prkcd*$^{+/+}$ mice (Fig. 5).

Inhibition of PKCδ promotes proangiogenic growth factor expression and activation. To explain how the absence of PKCδ improved reperfusion in diabetic ischemic limbs, we 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 PDGFR-β mRNA expression was significantly decreased in the adductor muscles of diabetic mice by 46, 30, and 63%, respectively, compared with nondiabetic *Prkcd*$^{+/+}$ mice (Fig. 6*A*, *C*, and *D*). The reduction of these genes in diabetic *Prkcd*$^{+/+}$ mice was not observed in diabetic *Prkcd*$^{-/-}$ mice. Moreover, mRNA expression of VEGFR2 (KDR/Flk-1), PDGF-B, and PDGFR-β was significantly upregulated in diabetic *Prkcd*$^{-/-}$ compared with diabetic *Prkcd*$^{+/+}$ mice (Fig. 6*B–D*). 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 eNOS, as well as transcriptional factor activity of HIF-1α, was unchanged within all groups of mice (Fig. 6*E–H* and Supplementary Fig. 1). In contrast to 4 weeks after femoral artery ligation, transcriptional factor activity and mRNA levels of HIF-1α were significantly decreased in diabetic *Prkcd*$^{+/+}$ mice compared with nondiabetic *Prkcd*$^{+/+}$ and diabetic *Prkcd*$^{-/-}$ mice (Supplementary Figs. 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 and PDGF-B and their respective receptors (VEGFR2 and PDGFR-β) were examined. Protein expression of PDGF-B was significantly decreased in diabetic versus nondiabetic adductor muscles of wild-type animals. In contrast, VEGF-A and PDGF-B protein expression was elevated in the ischemic limb of the diabetic PKCδ null mice (Fig. 7*A* and *B*). Phosphorylation of VEGFR2 and PDGFR-β was inhibited in ischemic adductor muscles of diabetic mice compared with nondiabetic *Prkcd*$^{+/+}$ mice. However, activation of Src was elevated in adductor muscles of diabetic *Prkcd*$^{+/+}$ mice compared with nondiabetc *Prkcd*$^{+/+}$ and *Prkcd*$^{-/-}$ mice (Fig. 7*B*). Interestingly, tyrosine phosphorylation of VEGFR2 and PDGFRβ, as well as PLCγ1, Akt, and ERK phosphorylation, was greatly enhanced in *Prkcd*$^{-/-}$ mice compared with diabetic *Prkcd*$^{+/+}$ mice (Fig. 7*A* and *B*). We did not observe any changes in the eNOS protein expression among experimental groups (Fig. 7*A*).

Expression of SHP-1 caused VEGFR2 and PDGFR-β 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

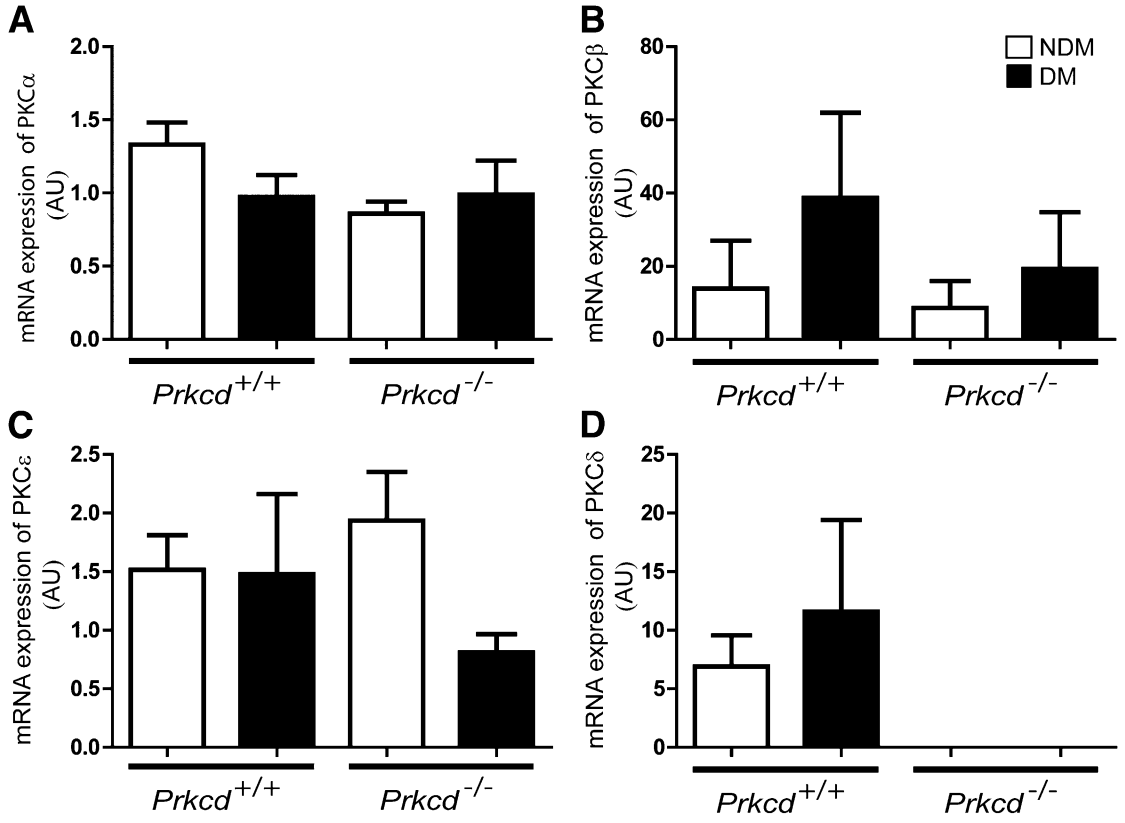

FIG. 4. Quantitative real-time PCR expression of mRNA isoforms of PKCα (*A*), PKCβ (*B*), PKCε (*C*), and PKCδ (*D*) in ischemic adductor muscles of nondiabetic (NDM) and diabetic (DM) *Prkcd*$^{+/+}$ and *Prkcd*$^{-/-}$ mice. Results are shown as mean ± SD of six to seven mice.

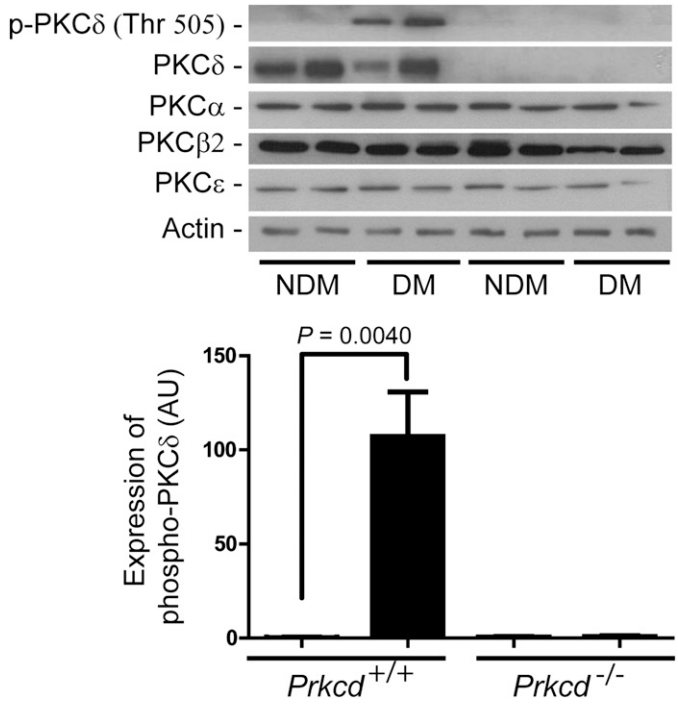

FIG. 5. Increased PKCδ activity in muscles of diabetic mice. Expression of p-PKCδ (Thr 505), PKCα, PKCβ, PKCδ, PKCε, and actin was detected by immunoblot in nondiabetic (NDM) and diabetic (DM) mice (*top panel*), and densitometric quantitation was measured in NDM (□) and DM (■) mice (*bottom panel*). Results are shown as mean ± SD of four to six independent experiments.

muscles, we measured SHP-1 expression by quantitative PCR and immunoblot analysis. Figure 8*A* and *B* indicates that mRNA expression of SHP-1, but not SHP-2 or PTP1B, is elevated in diabetic $Prkcd^{+/+}$ mice, whereas SHP-1 is clearly downregulated in $Prkcd^{-/-}$ mice. We confirmed through immunoblot analysis that SHP-1 protein expression was elevated by 2.3-fold in ischemic adductor muscles of diabetic $Prkcd^{+/+}$ mice compared with nondiabetic $Prkcd^{+/+}$ mice. The increase expression of SHP-1 was not observed in diabetic $Prkcd^{-/-}$ mice (Fig. 8*C*). No change was detected in the protein expression of SHP-2 and PTP1B within all groups of mice (Fig. 8*D*).

DISCUSSION

Diabetes is associated with the progression of vascular complications, such as peripheral arterial disease, and is a major risk factor for lower limb amputations (4). In the current study, we have demonstrated that activation of PKCδ diminishes the expression of VEGF and PDGF, two critical proangiogenic 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 compared with nondiabetic ischemic muscles. The inhibition of growth factor receptor phosphorylation was associated with the upregulation of SHP-1 expression, which has been reported to deactivate 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 of how diabetes reduces collateral vessel formation in the ischemic limb. In this study, we demonstrated that PKCδ is 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, mitogen-activated protein kinase, 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 because mRNA and protein analyses were performed 28 days after femoral artery ligation. However, phosphorylation of PKCδ on threonine 505, a phosphorylation site within the activation loop, clearly suggests that PKCδ is activated in the muscles of diabetic ischemic limbs compared with nondiabetic muscles. Previous data showed that the inhibition of PKCδ, using an isozyme-specific peptide, improved the number of microvessels and cerebral blood flow after 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 the 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 PDGFR-β mRNA expression in the 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 showed that HIF-1α gene transfer increased recovery of limb perfusion, increasing eNOS activation and vessel density (41). In our study, however, the increase in the expression of VEGF in muscles of PKCδ-deficient mice may not have been entirely due to upregulation of HIF-1α. Because protein extraction was performed 4 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 2 weeks after the surgery. Our data demonstrated that HIF-1α transcriptional factor activity and mRNA expression were increased in nondiabetic and diabetic PKCδ-null mice 2 weeks only after surgery (Supplementary Figs. 2 and 3). Besides VEGF and PDGF expression, our data suggest that PKCδ activation disrupts 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 the ischemic muscles of diabetic wild-type mice even if PDGFR-β

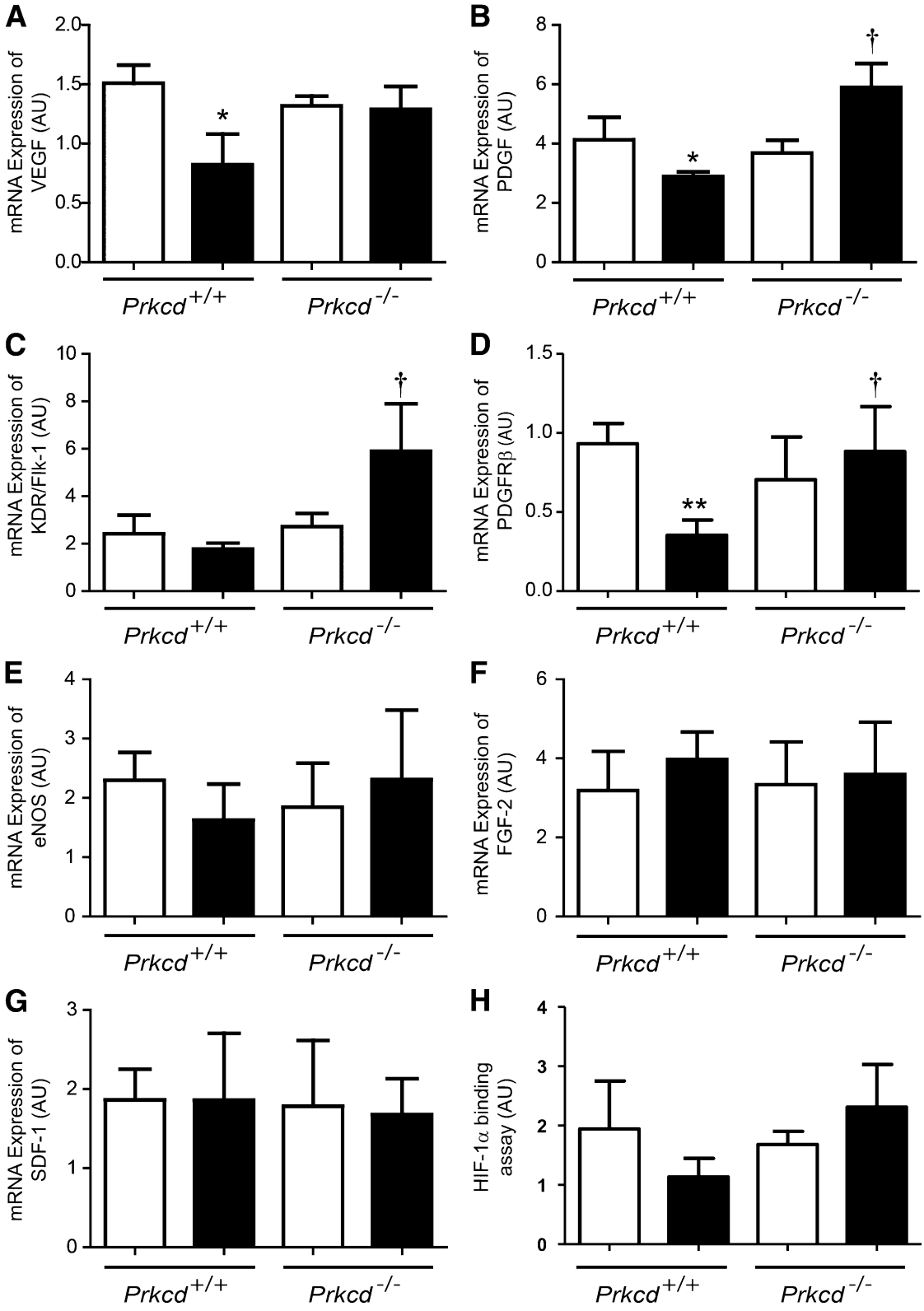

FIG. 6. mRNA expression of angiogenic factors. Quantitative real-time PCR of VEGF (*A*), PDGF (*B*), KDR/Flk-1 (*C*), PDGFR-β (*D*), eNOS (*E*), FGF-2 (*F*), and SDF-1 (*G*) mRNA expression and nuclear transcriptional factor activity of HIF-1α (*H*) in ischemic adductor muscles of nondiabetic (NDM, □) and diabetic (DM, ■) *Prkcd*$^{+/+}$ and *Prkcd*$^{-/-}$ mice. Results are shown as mean ± SD of six to seven mice. **P* = 0.05 vs. NDM *Prkcd*$^{+/+}$, ***P* < 0.01 vs. NDM *Prkcd*$^{+/+}$, †*P* < 0.05 vs. DM *Prkcd*$^{+/+}$.

activity was reduced. However, a previous study reported that reactive oxygen species (ROS) production induced Src phosphorylation (42). Because ROS are massively produced in ischemic and hyperglycemic conditions, it is probable that ROS production is responsible for the Src phosphorylation seen in diabetic wild-type mice.

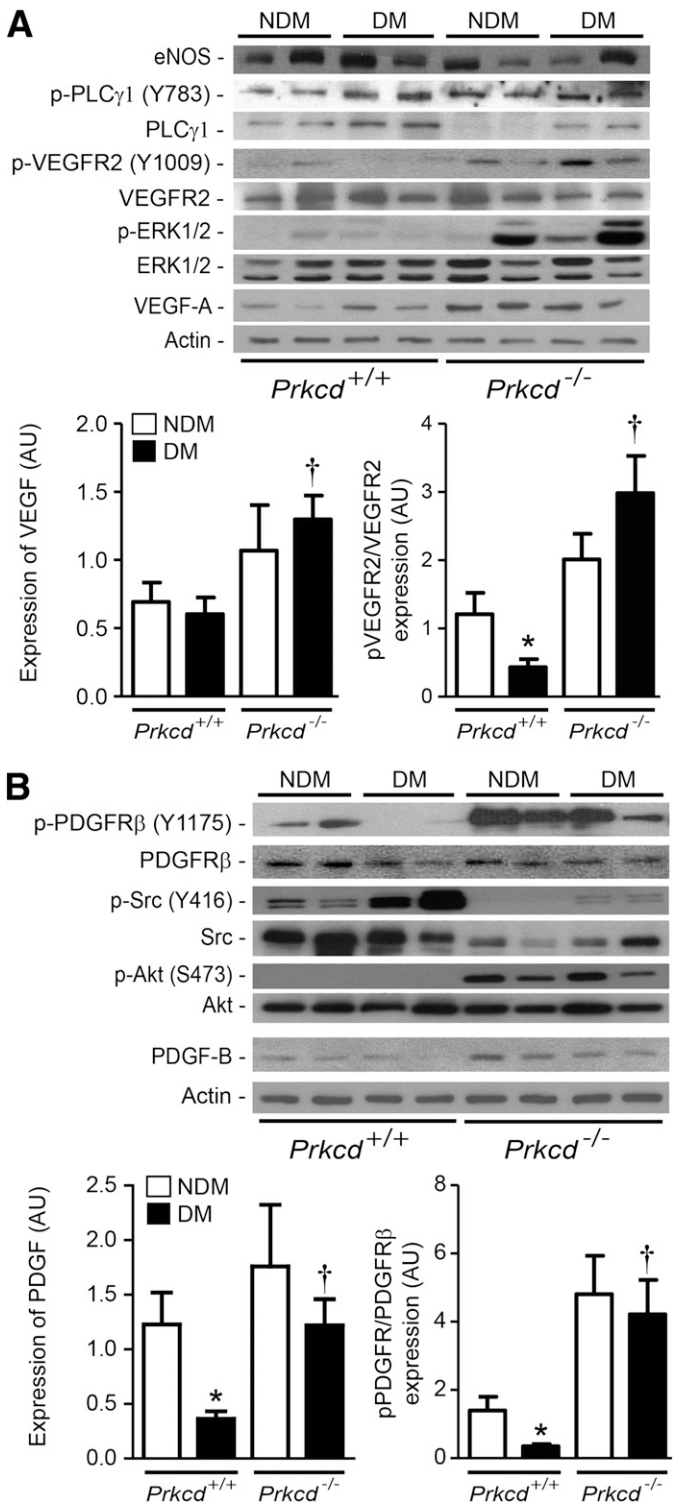

FIG. 7. Increased expression and activity of the VEGF and PDGF signaling pathway in diabetic (DM) and nondiabetic (NDM) *Prkcd*$^{-/-}$ mice. Expression of eNOS, PLCγ1, p-PLCγ1, VEGF-A, p-VEGFR2, VEGFR2, ERK1/2, p-ERK1/2 (*A*), and PDGF-B, p-PDGFR-β, PDGFR-β, Src, p-Src, Akt, p-Akt, and actin (*B*) in ischemic adductor muscles of *Prkcd*$^{+/+}$ and *Prkcd*$^{-/-}$ mice. Protein expression was detected by Western blot, and densitometric quantitation was measured. Results are shown as mean ± SD of four to six independent experiments. **P* = 0.05 vs. NDM *Prkcd*$^{+/+}$, †*P* < 0.05 vs. DM *Prkcd*$^{+/+}$.

There is strong evidence that progenitor cell recruitment and homing participate in 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 the PKCδ isoform.

Despite advances in revascularization techniques, limb salvage and pain relief cannot be achieved in many diabetic patients with diffuse peripheral vascular disease. VEGF-mediated gene therapy has shown promising results as an innovative method in the treatment of severe cardiovascular diseases. However, a randomized study of gene therapy 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 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 proangiogenic 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 the 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 nondiabetic hind limb ischemia model (50). However, our current study does not provide a 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 enhance 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.

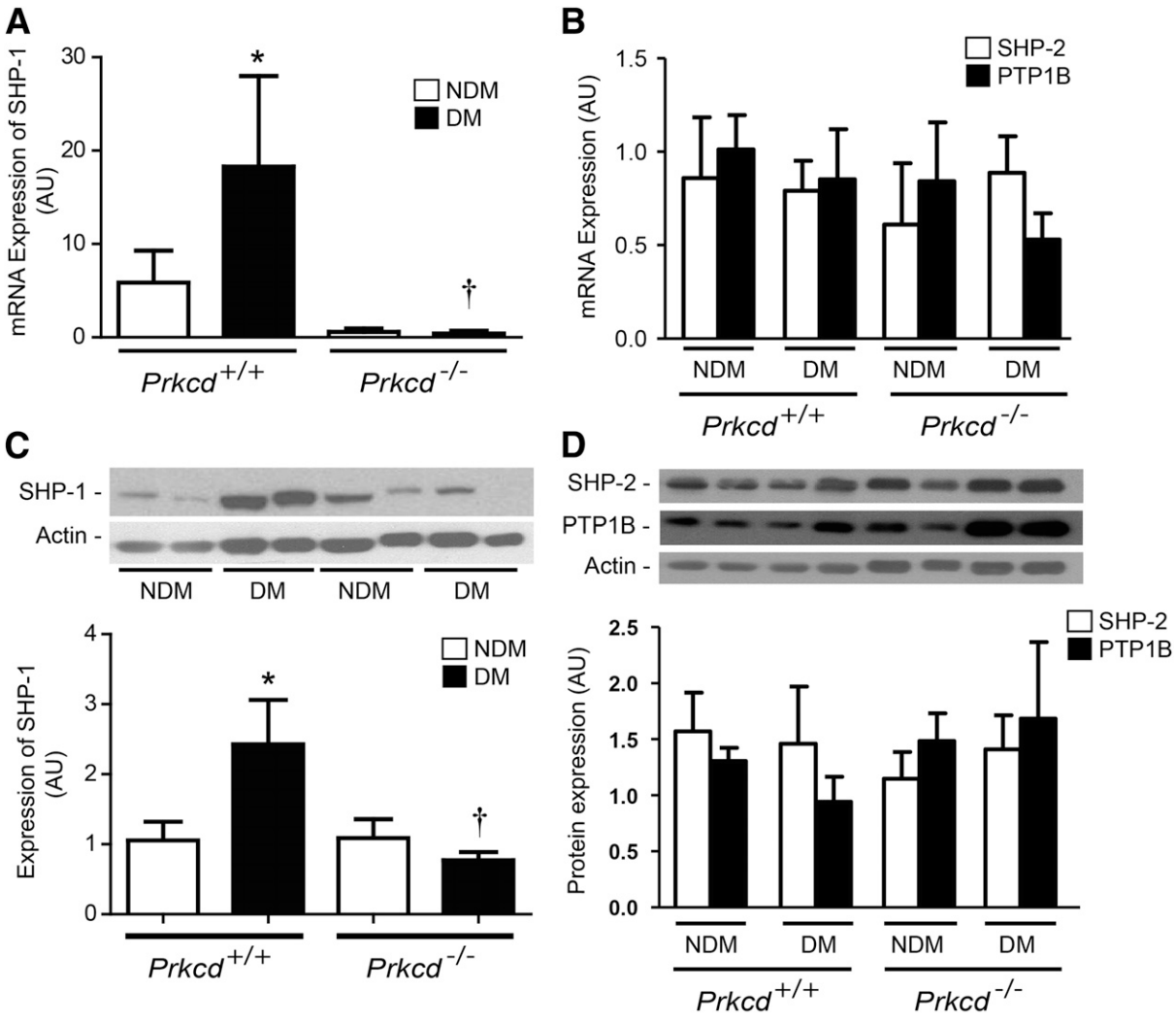

FIG. 8. Increased expression of SHP-1 in ischemic adductor muscles of diabetic (DM) and nondiabetic (NDM) mice. Quantitative real-time PCR of SHP-1 (*A*), SHP-2, and PTP1B mRNA (*B*), and protein expression of SHP-1 (*C*), SHP-2, PTP1B, and their corresponding loading control (actin) (*D*) in ischemic adductor muscles of NDM and DM *Prkcd*$^{+/+}$ and *Prkcd*$^{-/-}$ mice. Protein expression was detected by immunoblot, and densitometric quantitation was measured. Results are shown as mean ± SD of four to six independent experiments. **P* = 0.05 vs. NDM *Prkcd*$^{+/+}$, †*P* < 0.05 vs. DM *Prkcd*$^{+/+}$.

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No potential conflicts of interest relevant to this article were reported.

F.L., M.P., B.D., and P.G. performed experiments and analyzed the data. M.L. provided the *Prkcd*-deficient mice. A.G. performed animal care and researched data. F.L. and P.G. wrote the manuscript. P.G. is the guarantor of this work, and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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