Platelet-derived growth factor C promotes revascularization in ischemic limbs of diabetic mice

Junji Moriya, MD, PhD,* Xiumin Wu, PhD, Jose Zavala-Solorio, PhD, Jed Ross, MS, Xiao Huan Liang, MS, and **Napoleone Ferrara, MD,*** *South San Francisco, Calif*

Background: **Platelet-derived growth factor C (PDGF-C) has been reported to promote angiogenesis independently of vascular endothelial growth factor (VEGF), although its significance in postnatal angiogenesis in vivo remains poorly understood. VEGF has been employed as a major molecular tool to induce therapeutic angiogenesis. However, VEGF therapy is not very effective in models of cardiovascular diseases associated with diabetes, and the mechanisms of this phenomenon still remain to be elucidated.**

Methods: **We used a murine model of hind limb ischemia and of streptozotocin-induced diabetes.**

Results: **Expression of PDGF-C and its receptor PDGFR-α were markedly upregulated in ischemic limbs. Treatment with a neutralizing antibody against PDGF-C significantly impaired blood flow recovery and neovascularization after ischemia almost to the same extent as a VEGF-neutralizing antibody. Mice deficient in PDGF-C exhibited reduced blood flow recovery after ischemia compared with wild-type mice, confirming a strong proangiogenic activity of PDGF-C. Next, we injected an expression vector encoding PDGF-C into ischemic limbs. Blood flow recovery and neovascularization after ischemia were significantly improved in the groups treated with PDGF-C compared with controls. Attenuation of angiogenic responses to ischemia has been reported in patients with diabetes even after VEGF treatment, although a precise mechanism remains unknown. We hypothesized that PDGF-C might relate to the impaired angiogenesis of diabetes. We tested this hypothesis by inducing diabetes by intraperitoneal injection of streptozotocin. Expression levels of PDGF-C at baseline and after ischemia were significantly lower in limb tissues of diabetic mice than in those of control mice, whereas expression levels of other members of the PDGF family and VEGF were not changed or were even higher in diabetic mice. Introduction of VEGF complementary DNA expression plasmid vector into ischemic limbs did not improve blood flow recovery. However, these changes were effectively reversed by additional introduction of the PDGF-C complementary DNA plasmid vector.**

Conclusions: **These results indicate that downregulation of PDGF-C expression in limb tissues of diabetic mice contributes to impaired angiogenesis and suggest that introduction of PDGF-C might be a novel strategy for therapeutic angiogenesis, especially in the diabetic state. (J Vasc Surg 2014;59:1402-9.)**

Clinical Relevance: **Angiogenesis and arteriogenesis after ischemia are attenuated in most diabetic patients, although the precise mechanisms remain unclear. Platelet-derived growth factors (PDGFs) have a variety of functions on many cell types, and PDGF-C stimulates angiogenesis and revascularizes ischemic tissues. This study indicates the role for PDGF-C as a critical regulator of impaired angiogenesis of diabetes and suggests that PDGF-C might be a novel target for the treatment of ischemic cardiovascular diseases in diabetes.**

Therapeutic angiogenesis, which involves the use of proangiogenic factors or stem/progenitor cells for the treatment of ischemic cardiovascular diseases, is a promising concept. Vascular endothelial growth factor (VEGF) has long been recognized as the key regulator of physiologic and pathologic angiogenesis[1,2] and has been used as a major molecular tool to induce therapeutic angiogenesis.[3]

However, the limited successes of therapeutic angiogenesis, using VEGF or other angiogenic factors, emphasize the challenges in reconstructing a functional vascular network.[3-6] Indeed, several clinical studies have shown limited benefits of therapeutic angiogenesis in patients with ischemic cardiovascular disease.[7] This is presumably because these patients tend to have multiple risk factors for atherosclerosis, such as diabetes, that interfere with the response to treatment.[8,9] Therefore, it is important to develop novel strategies for therapeutic angiogenesis, and further research on the basic mechanisms of angiogenesis needs to be performed.

Platelet-derived growth factors (PDGFs) have a variety of effects on many cell types. They stimulate proliferation, migration, and differentiation of mesenchymal and other cell types in developing and adult tissues. The PDGF family comprises four genes: PDGF-A, PDGF-B, and the more recently discovered PDGF-C and PDGF-D.[10,11] PDGF receptor α (PDGFRα) and PDGF receptor β (PDGFRβ)

From Genentech Inc.

*Currently affiliated with Moores Cancer Center, University of California, San Diego, La Jolla, Calif.

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Reprint requests: Napoleone Ferrara, MD, Moores Cancer Center, University of California, San Diego, 3855 Health Sciences Dr, La Jolla, CA 92093 (e-mail: nferrara@ucsd.edu).

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are known to be the receptors for PDGFs. PDGF-C binds to and activates PDGFRα. PDGF-C does not bind to PDGFRβ, but when PDGFRβ is coexpressed with PDGFRα in the same cells, it can also be activated by PDGF-C.[12,13]

PDGF-C signaling through these receptors is important for the development of connective tissues and for wound healing.[10,14,15] Recent studies have shown that PDGF-C also stimulates angiogenesis in chick embryos and mouse corneas[12] and that it revascularizes ischemic tissues via effects on endothelial or bone marrow-derived cells.[16] Moreover, PDGF-C mediates the angiogenic properties of tumor-associated fibroblasts, and inhibition of PDGF-C signaling reduced angiogenesis in tumors refractory to anti-VEGF treatment.[17] Collectively, these studies support the notion that PDGF-C is a promising target for therapeutic angiogenesis and antiangiogenic therapy.[13,18] However, the precise roles of PDGF-C in pathologic and postnatal angiogenesis remain largely unclear.

Diabetes is a risk factor for the development of cardiovascular diseases associated with impaired angiogenesis.[19,20] Vascular complications of diabetes have been generally explained by disorganized expression of angiogenic factors such as VEGF.[21] However, diabetes is a paradoxical disease, associated with excessive angiogenesis in the retina and, conversely, with impaired collateral vessel formation in the ischemic limbs and hearts.[22-24] An explanation for this paradox still remains to be elucidated.

In this study, we investigated the role of PDGF-C in postnatal angiogenesis and found that PDGF-C expression is upregulated in ischemic tissues, along with neuropilin 1 (Nrp1) and Nrp2, which function as cell-surface receptors for axon guidance molecules and also as coreceptors for members of the VEGF family.[25] These upregulations were attenuated in a diabetic mouse model, possibly impairing revascularization after ischemia. Introduction of PDGF-C, but not VEGF, markedly improved revascularization after ischemia in diabetic mice. These findings suggest that PDGF-C might be a novel target for impaired angiogenesis of diabetes in which VEGF treatment is ineffective.

METHODS

The animal experiments in this study were approved by our Institutional Review Board.

Experimental animals. Male C57/BL6 mice (8 to 12 weeks old) were obtained from Charles River (Wilmington, Mass). The generation of PDGF-C-deficient mice (in C57/BL6 background) has been described previously.[26] For the type 1 diabetic model, mice were given daily intraperitoneal injections of streptozotocin (STZ) in 0.1 mol/L sodium citrate (pH 4.5) at the dose of 50 mg/kg body weight for 5 days.

Murine model of hind limb ischemia. After mice were anesthetized with a mixture of oxygen and isoflurane (3%-5% for induction and 2% for surgery), hind limb ischemia was generated as described previously.[27] Briefly, the proximal part of the femoral artery and the distal portion of the popliteal artery were ligated and removed after all side branches had been dissected free.

For immunoneutralization studies, we performed two intraperitoneal injections of antibodies (immediately and 5 days after ischemic surgery) at a dose of 5 mg/kg or 10 mg/kg body weight. The antibodies used for the study were anti-Ragweed antibody (control), anti-VEGF antibody (Clone G6-31 or B20-4.1),[28] anti-PDGF-C antibody (Clone 1E5.9, 2E7.1.6.11; Liang et al unpublished data), anti-PDGF-A antibody (Clone 1.6c12.12), anti-NRP1 antibody (Clone YW107.4.87), and anti-NRP2 antibody (Clone YW68.11.70; Genentech Inc). Human $VEGF_{165}$ or human PDGF-C complementary DNA (cDNA) was subcloned into a pCAGGS expression vector.[29] Empty pCAGGS vector was used as a control. The authenticity of the constructs was verified by sequencing.

For in vivo gene transfer, we exposed thigh muscles by incising the skin and injected the naked plasmid into the muscle, immediately and 5 days after surgery, at the dose of 100 μg in 100 μL phosphate-buffered saline. Buprenorphine was administered at a dose of 0.05 to 0.1 mg/kg body weight subcutaneously for 2 days after surgery or incision in the skin.

Ischemic limb samples were harvested for RNA analysis. Vastus and rectus femoris muscle tissues were removed from the ischemic limbs after systemic perfusion with phosphate-buffered saline and immediately soaked in RNA*later* RNA stabilization reagent (Qiagen, Valencia, Calif). according to the manufacturer's instructions.

Laser Doppler perfusion analysis. Laser Doppler perfusion imaging analyzer (Moor Instruments, Devon, United Kingdom) was used to record blood flow measurements on days 1, 3, 7, 10, 14, 21, and 28 after surgery. For quantification, ratios of readable units from the images in ischemic to nonischemic hind limb were determined. Mice showing adverse effects (severe fighting wounds, blackened toes, self-mutilation of the compromised limb) were euthanized immediately and excluded from the analysis.

RNA analysis. Limb muscle samples were homogenized by using the TissueLyser (Qiagen) according to the manufacturer's instructions. Total RNA was prepared by an RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was prepared using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, Calif). Quantitative real-time polymerase chain reaction (PCR) was performed by using the Applied Biosystems 7500 Real-Time PCR System with the Taqman Gene Expression Assays and the Taqman Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase messenger RNA was used as the endogenous control for all experiments. At least three biological replicates were included for each condition.

Statistical analysis. Data are shown as means ± standard error of the mean. In all experiments, comparisons between two groups were based on a two-sided Student *t*-test, and one-way analysis of variance was used to test for differences among more groups. P values of $<.05$ were considered statistically significant.

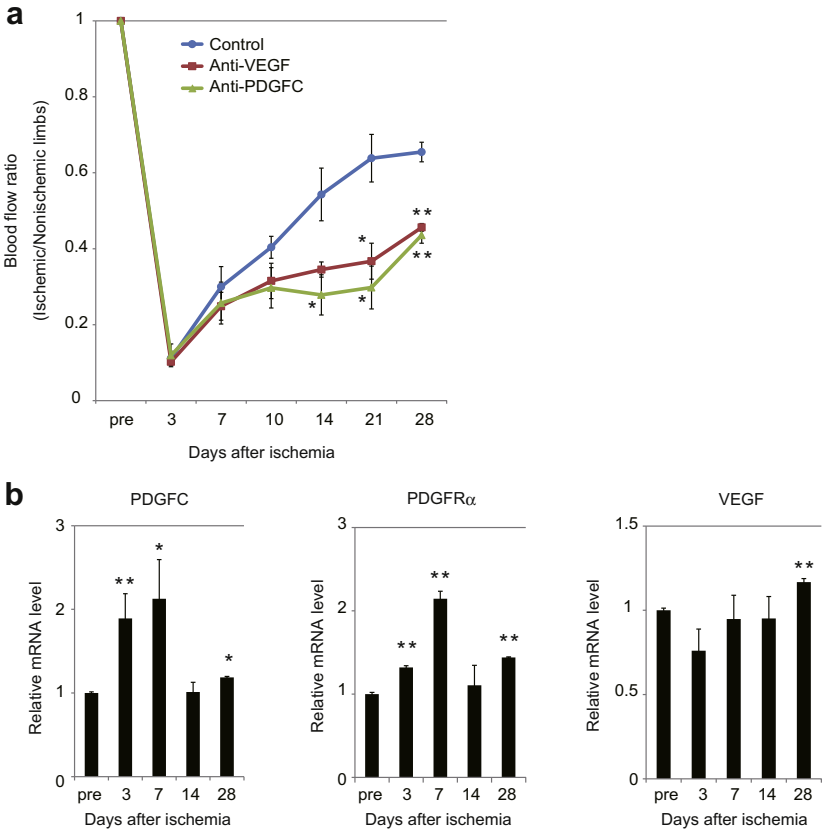

Fig 1. Platelet-derived growth factor (*PDGF*)-C contributes to revascularization in a murine model of hind limb ischemia. **a,** Mice were treated with anti-Ragweed antibody (*control*), antivascular endothelial growth factor (*VEGF*) antibody (*anti-VEGF*), or anti-PDGF-C antibody (*anti-PDGFC*) at the dose of 10 mg/kg body weight immediately and 5 days after ischemia. Blood flow recovery was analyzed by laser Doppler perfusion imaging as described in the Methods section. $*P < .05$, $**P < .01$ vs control (n = 5-8 for each group). Data represent means ± standard error of the mean. **b,** The messenger RNA (*mRNA*) levels of PDGF-C, PDGF receptor α (*PDGFRα*), and VEGF in ischemic limbs at several time points were assessed by real-time reverse transcription polymerase chain reaction analysis. $*P < .05$, $**P < .01$ vs pre (n = 3-5 for each group). Data represent means ± standard error of the mean.

RESULTS

Upregulation of PDGF-C expression contributes to revascularization in murine model of hind limb ischemia. We sought to investigate the factors that contribute to revascularization in hind limb ischemia. To this end, we used specific antibodies directed against several proangiogenic molecules. A murine model of hind limb ischemia was generated by unilateral femoral artery ligation, and each antibody was intraperitoneally administered after ischemia. Revascularization was measured by laser Doppler perfusion imaging. As expected, treatment with anti-VEGF antibodies strongly inhibited blood flow recovery for up to 28 days after ischemia (Fig 1, *a*). Among the antibody treatments tested, anti-PDGF-C markedly inhibited blood flow recovery, almost to the same extent as anti-VEGF (Fig 1, *a*). In addition, treatment with anti-Nrp1 or anti-Nrp2 significantly inhibited blood flow recovery (Supplementary Fig 1, *a*, online only), whereas anti-PDGF-A had no significant effect (Supplementary Fig 1, *b*, online only). We also confirmed that the effects of anti-PDGF-C on revascularization were dose-dependent (Supplementary Fig 1, *c*, online only).

Next, we performed quantitative real-time reverse transcription PCR analysis of ischemic tissues of mice to examine the expression of these angiogenic factors. Expression of PDGF-C and its receptor PDGFRα were markedly increased in ischemic limbs at 3 days after surgery, and this increase persisted until day 28 (Fig 1, *b*). Expression of Nrp1 and Nrp2 was also significantly increased up to 28 days after surgery (Supplementary Fig 2, *a*, online only). However, expression of VEGF in ischemic limbs significantly increased only at day 28 (Fig 1, *b*). Expression of other members of the PDGF family (PDGF-A, PDGF-B, PDGF-D) and PDGFRβ was increased only modestly at an early stage of ischemia and showed no significant increase, or even decreased at a later stage (Supplementary Fig 2, *a*,

online only). Upregulation of PDGF-C expression after ischemia was not altered by injection of control or anti-PDGF-C antibody (Supplementary Fig 2, *b*, online only). These results indicate that other than VEGF, upregulation of PDGF-C expression, along with Nrp1 and Nrp2, contributes to revascularization after ischemia.

PDGF-C positively regulates revascularization after ischemia. To further investigate the effects of PDGF-C on revascularization after ischemia, we introduced an expression vector encoding VEGF, a vector for PDGF-C cDNA, or the vectors for both angiogenic factors. Because intramuscular naked plasmid injection using pCAGGS vector allows long-term systemic delivery of target cDNA expression,[30] we used this vector to induce PDGF-C and VEGF expression. Injection of the VEGF vector into ischemic limbs significantly increased blood flow recovery compared with the control group (Fig 2, *a*). Intriguingly, injection of the PDGF-C vector also accelerated blood flow recovery almost to the same extent as that of the VEGF vector (Fig 2, *a*). However, blood flow recovery did not differ significantly between the VEGF group and the VEGF plus PDGF-C group (Supplementary Fig 3, online only). Next, we induced hind limb ischemia in PDGF-C-deficient mice to examine the consequences of loss of function of this molecule. Consistent with the results of our antibody injection or gene transfer experiments, PDGF-C-deficient mice showed less blood flow recovery than wild-type mice (Fig 2, *b*). These results suggest that PDGF-C positively regulates blood flow recovery after ischemia.

PDGF-C expression is downregulated in diabetic mice. PDGF signaling is reported to play a critical role in regulating pancreatic β-cell proliferation.[31] Angiogenic responses to ischemia were also shown to be attenuated in patients with diabetes,[32,33] although the precise mechanisms still remain elusive. We hypothesized that PDGF-C might relate to the impaired angiogenesis of diabetes. To test this hypothesis, we used a murine model of type 1 diabetes generated by an intraperitoneal injection of STZ (50 mg/kg daily for 5 days). We confirmed that body weights were significantly lower and that blood glucose levels were much higher in diabetic mice than in control mice (Supplementary Fig 4, *a* and *b*, online only).

We next assessed the baseline gene expression of limb tissues in diabetic mice by quantitative real-time reverse transcription PCR analysis 14 days after the first STZ injection. Among the genes tested, PDGF-C was markedly downregulated compared with control mice (Fig 3, *a*). Interestingly, expression of Nrp1 and Nrp2 was also significantly downregulated (Supplementary Fig 4, *c*, online only). There was also a slight decrease in PDGF-B expression, whereas expression of the other factors (PDGF-A, PDGF-D, PDGFRα, and VEGF) showed no significant difference (Fig 3, *a*).

At 14 days after the first STZ injection, mice underwent hind limb ischemia surgery. The decreased expression of the PDGF-C gene in diabetic mice persisted after ischemia, along with significantly lower induction of PDGFRα expression (Fig 3, *b*). Upregulation of Nrp1 and Nrp2 expression after ischemia was also diminished in diabetic mice (Supplementary Fig 4, *c*, online only). However, expression of VEGF did not significantly differ from control mice at day 7 after ischemia, although it showed a significant decrease at day 3 (Fig 3, *b*). Consistent

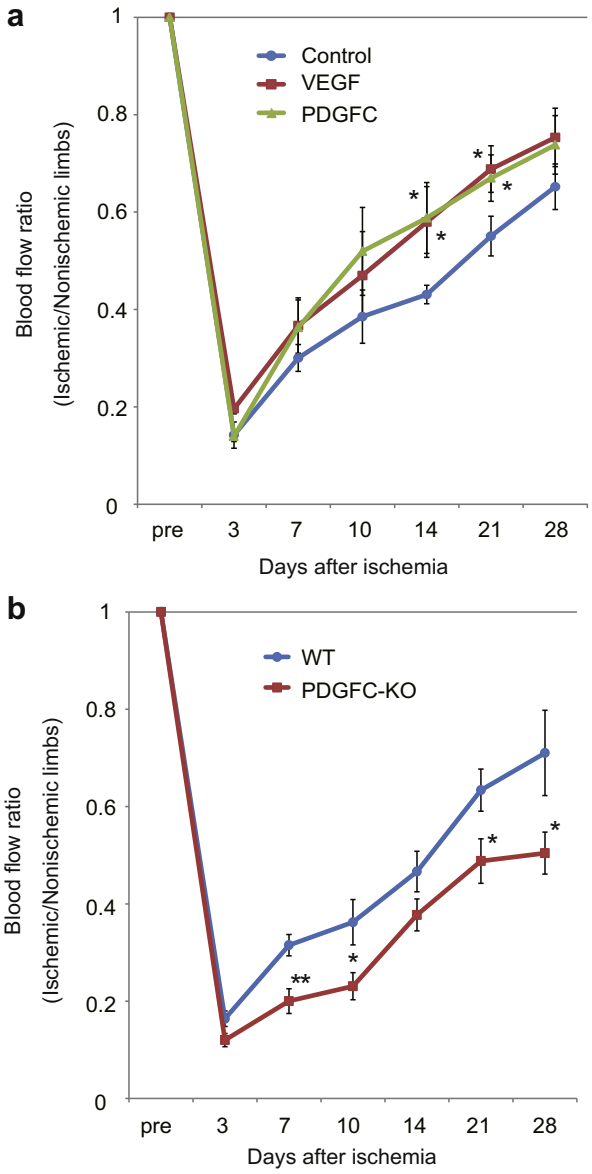

Fig 2. Platelet-derived growth factor (*PDGF*)-C positively regulates revascularization after ischemia. **a,** Ischemic limbs of mice were intramuscularly injected with empty vector (*control*), vascular endothelial growth factor expression vector (*VEGF*), or PDGF-C expression vector (*PDGF-C*). Blood flow recovery was analyzed. $^{*}P < .05$ vs control (n = 5-11 for each group). Data represent means ± standard error of the mean. **b,** Ischemic limbs of wild-type (*WT*) mice and PDGF-C knock-out (*KO*) mice were analyzed for blood flow recovery. $^{*}P < .05$, $^{**}P < .01$ vs WT (n = 9-10 for each group). Data represent means ± standard error of the mean.

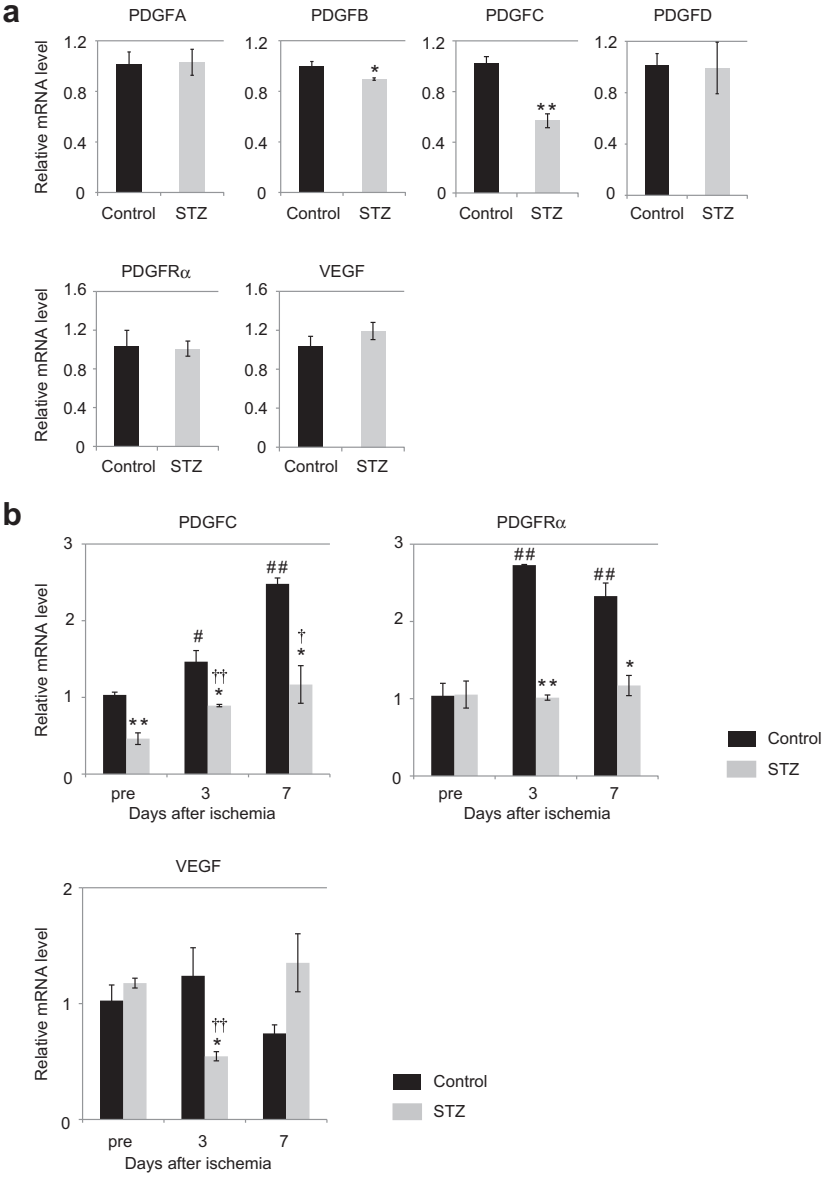

Fig 3. Platelet-derived growth factor (*PDGF*)-C expression is downregulated in diabetic mice. **a,** The baseline messenger RNA (*mRNA*) levels of PDGF-A, PDGF-B, PDGF-C, PDGF-D, PDGF receptor α (*PDGFRα*), and vascular endothelial growth factor (*VEGF*) in limb tissues of nondiabetic (*Control*) or streptozotocin (*STZ*)-treated diabetic mice were assessed by real-time reverse transcription-polymerase chain reaction analysis. $^{*}P < .05$, $^{**}P < .01$ vs control (n = 4-8 for each group). Data represent means ± standard error of the mean. **b,** Time course of relative PDGF-C, PDGFRα, and VEGF mRNA expression in ischemic limbs of nondiabetic (Control) or diabetic (STZ) mice. $^{*}P < .05$, $^{**}P < .01$ vs control; $^{\#}P < .05$, $^{\#\#}P < .01$ vs precontrol; $^{\dagger}P < .05$, $^{\dagger\dagger}P < .01$ vs pre-STZ (n = 4-8 for each group). Data represent means ± standard error of the mean.

with our hypothesis, these results raise the possibility that decreased expression of PDGF-C may be a contributor to impaired angiogenesis in diabetes.

Introduction of PDGF-C improves revascularization after ischemia in diabetic mice. We first induced hind limb ischemia in diabetic mice and analyzed blood flow recovery. Laser Doppler perfusion imaging revealed that blood flow recovery after ischemia was significantly impaired in diabetic mice compared with controls (Fig 4, *a*). Moreover, unlike control mice, injection of the VEGF vector into ischemic limbs of diabetic mice did not improve revascularization after ischemia (Fig 4, *a*; Fig 2, *a*).

Next, we injected an expression vector encoding PDGF-C. Laser Doppler perfusion imaging revealed that

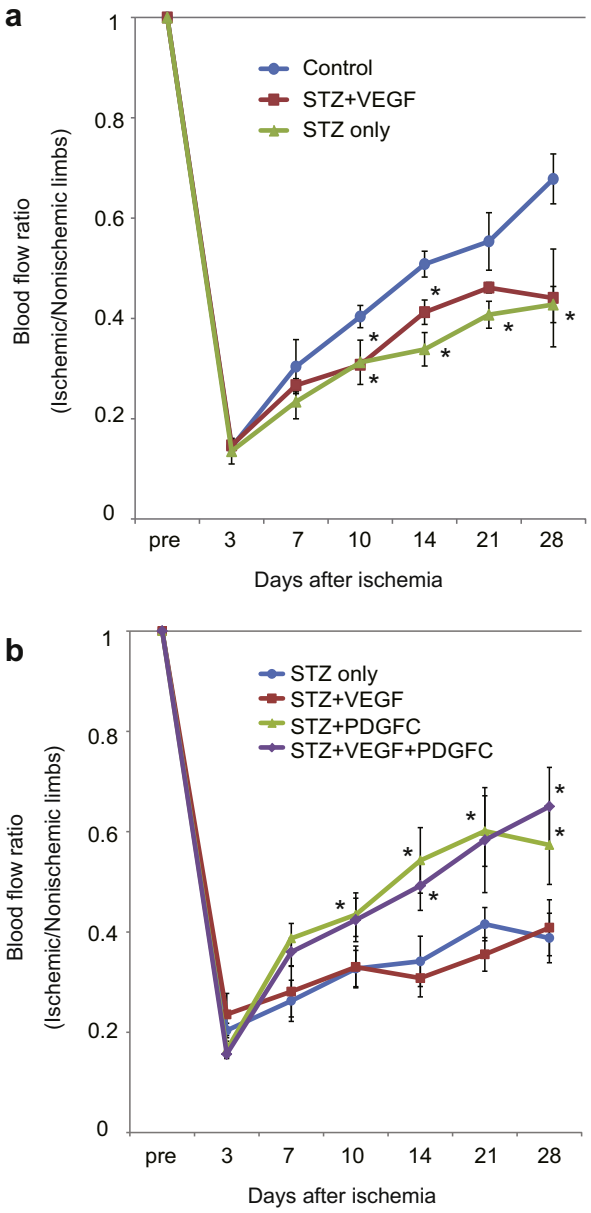

Fig 4. Platelet-derived growth factor (*PDGF*)-C introduction improves revascularization after ischemia in diabetic mice. **a,** Blood flow recovery in ischemic limbs of nondiabetic mice after treatment with empty vector (*Control*) or of diabetic mice after treatment with vascular endothelial growth factor (*VEGF*) vector (streptozotocin [*STZ*] + *VEGF*) or empty vector (*STZ only*). Blood flow was assessed as described in the Methods section. **P* < .05 vs control (n = 4-9 for each group). Data represent means ± standard error of the mean. **b,** Blood flow recovery in ischemic limbs of diabetic mice treated with an empty vector (STZ only), VEGF expression vector (STZ + VEGF), PDGF-C expression vector (*STZ + PDGFC*), or VEGF expression vector plus PDGF-C expression vector (*STZ + VEGF + PDGFC*) was analyzed. **P* < .05 vs STZ only (n = 5-7 for each group). Data represent means ± standard error of the mean.

the poor response of revascularization to VEGF treatment was effectively overcome by PDGF-C vector injection (Fig 4, *b*). However, just like in nondiabetic mice, introduction of the PDGF-C cDNA plus the VEGF cDNA did not have an additive effect compared with that of the PDGF-C cDNA alone (Fig 4, *b*; Supplementary Fig 3, online only). These results indicate that introduction of PDGF-C is effective for promoting revascularization after ischemia, especially when VEGF treatment is ineffective, such as in the diabetic state.

DISCUSSION

The present study demonstrates that PDGF-C accelerates revascularization in a murine model of hind limb ischemia. Cerebral vascular abnormalities with incomplete vascular smooth muscle cell coverage have been described in PDGF-C-deficient mice in C57/BL6 background, indicating that PDGF-C has an important role in vascular development.[26] Moreover, Li et al[16] reported that exogenous administration of PDGF-C stimulates vessel growth in the ischemic hind limb. We found in the present study that pharmacologic inhibition or genetic disruption of PDGF-C leads to impaired revascularization after ischemia, suggesting that endogenous PDGF-C also has a crucial role in postnatal angiogenesis.

Our results also suggest that expression of PDGF-C is downregulated in ischemic tissues of diabetic mice, thereby contributing to their impaired blood flow recovery after ischemia; however, expression of VEGF was not impaired. A very recent study reported that PDGF signaling controls pancreatic β-cell proliferation, suggesting a link between PDGF and pathogenesis of diabetes.[31] On the basis of our present findings, we suggest that PDGF plays a role in the development of diabetes-related vascular complications. Further studies are clearly needed to confirm these findings in additional models of diabetes.

Recent evidence suggests that PDGF is a potent neuroprotective factor and that its introduction can reduce neurodegeneration.[34,35] The vascular and nervous systems have several anatomic similarities, and the parallels between these systems extend to the molecular level.[36-38] Thus, it is possible that PDGF treatment not only augments blood flow but also rescues neurons from degeneration after ischemic insult.

That PDGF-C has a variety of cellular targets is well established. Previous reports have shown that PDGF-C promotes proliferation, survival, and migration of pericytes, endothelial cells, and fibroblasts.[39] Thus, PDGF-C very likely exerts its proangiogenic effect not only through direct effects on endothelial cells but also by acting on other vascular and perivascular cell types. Addressing these issues is the subject of future studies. How PDGF-C expression is downregulated in ischemic tissues of diabetic animals also remains to be determined. Because the angiogenic pathways induced by PDGF-C are known to be mostly VEGF-independent,[39] the downregulation of PDGF-C

expression in diabetes might be mediated by VEGF-independent mechanisms as well.

We found that expression levels of Nrp1 and Nrp2 were significantly downregulated in diabetic mice. This is consistent with the previous study by Schiekofer et al[21] in a model of type 2 diabetes. They concluded that impaired ischemia-induced neovascularization in type 2 diabetes is associated with the collapse of an "angiogenic network" in the ischemic limb. However, it still remains unknown how Nrp1 and Nrp2 are downregulated and contribute to the pathogenesis of angiogenic impairment of diabetes. Neuropilins are known to be the coreceptors for VEGF and class 3 semaphorins, potentiating signals of these molecules.[40] Although VEGF signals promote angiogenesis, semaphorin signals mainly inhibit angiogenesis. Therefore, together with reduced PDGF-C, lower expression of Nrp1 and Nrp2 might lead to impaired angiogenesis after ischemia in diabetes. Further studies are needed to determine the role of neuropilins under diabetic state.

CONCLUSIONS

Our results indicate that PDGF-C expression is downregulated in ischemic tissues in a mouse model of diabetes, resulting in angiogenic impairment. Delivery of the PDGF-C gene is sufficient to restore blood flow after ischemia. Therefore, PDGF-C might be a novel therapeutic option for ischemic cardiovascular diseases in diabetic patients.

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AUTHOR CONTRIBUTIONS

Conception and design: JM, XW, JR, XH, NF
Analysis and interpretation: JM, XW, JZ, JR
Data collection: JM, JZ, XH
Writing the article: JM
Critical revision of the article: JM, XW, JR
Final approval of the article: JM, NF
Statistical analysis: JM
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Overall responsibility: NF

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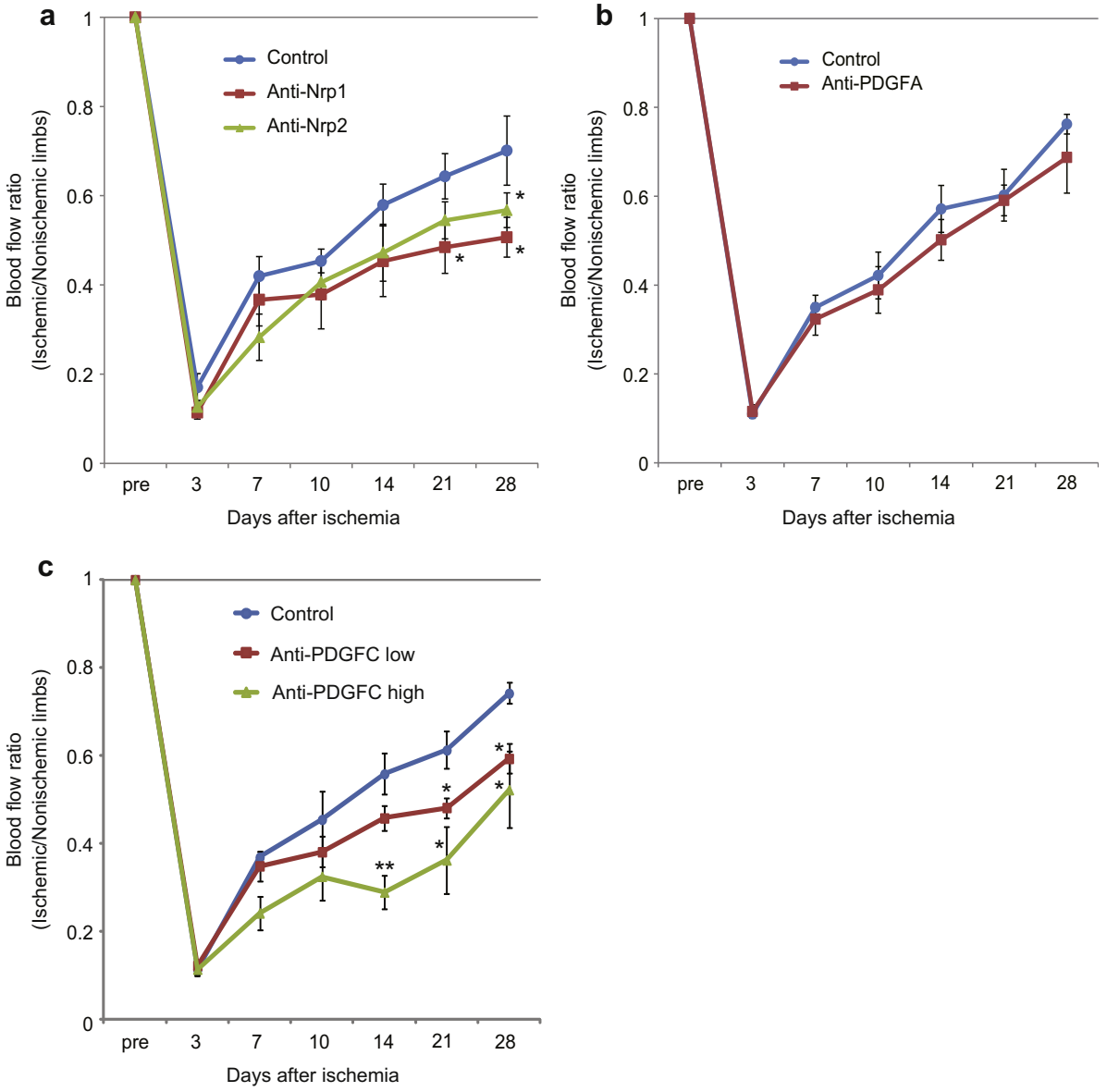
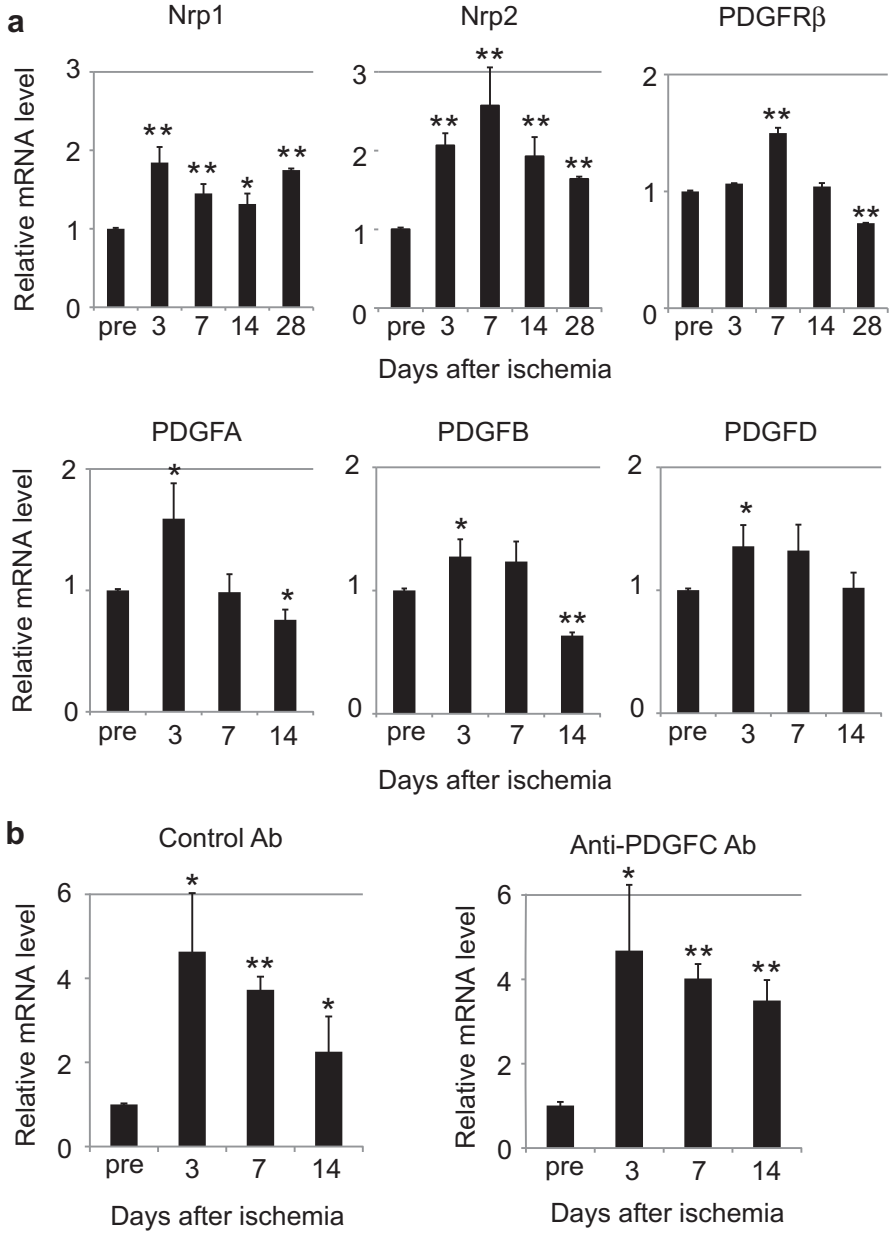
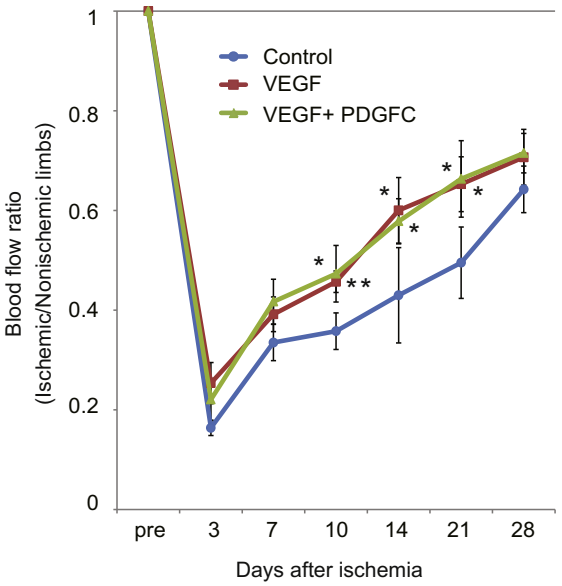


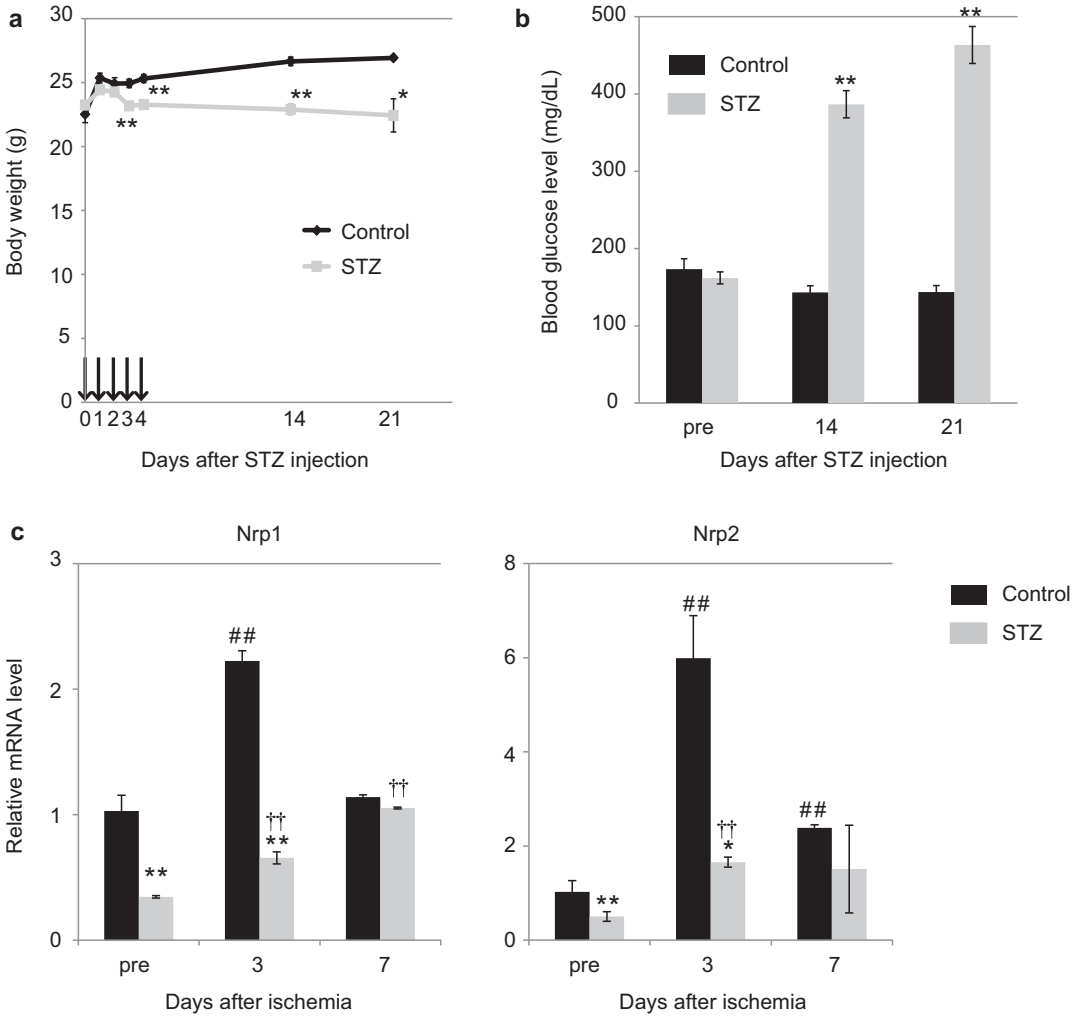
Supplementary Fig 1 (online only). Neuropilin (*Nrp*)1 and Nrp2, along with platelet-derived growth factor (*PDGF*)-C, contribute to revascularization after ischemia. **a,** Mice were treated with anti-ragweed antibody (*control*), anti-Nrp1 antibody (*anti-Nrp1*), or anti-Nrp2 antibody (*anti-Nrp2*) at the dose of 10 mg/kg body weight immediately and 5 days after induction of ischemia. Blood flow recovery was analyzed by laser Doppler perfusion imaging. $*P < .05$ vs control (n = 5 for each group). Data represent means ± standard error of the mean (SEM). **b,** Mice were treated with anti-ragweed antibody (*control*), or anti-PDGF-A antibody (*anti-PDGFA*) at the dose of 10 mg/kg body weight immediately and 5 days after ischemia induction, and blood flow recovery was analyzed. Data represent means ± SEM (n = 5 for each group). **c,** Mice were treated with anti-ragweed antibody (*control*), anti-PDGF-C antibody at the dose of 5 mg/kg body weight (*anti-PDGFC low*) or 10 mg/kg body weight (*anti-PDGFC high*) immediately and 5 days after ischemia, and blood flow recovery was analyzed as above described. $*P < .05$, $**P < .01$ vs control (n = 3-8 for each group). Data represent means ± SEM.



Supplementary Fig 2 (online only). Expression levels of neuropilin 1 (*Nrp1*), Nrp2, and platelet-derived growth factor (*PDGF*) family members after ischemia. **a,** The messenger RNA (*mRNA*) levels of Nrp1, Nrp2, platelet-derived growth factor receptor β (*PDGFRβ*), PDGF-A, PDGF-B, and PDGF-D in ischemic limbs at several time courses were assessed by real-time reverse transcription polymerase chain reaction analysis. $*P < .05$, $**P < .01$ vs pre (n = 4-8 for each group). Data represent means ± standard error of the mean. **b,** The PDGF-C mRNA levels in ischemic limbs of mice treated with an anti-ragweed antibody (*control Ab*) or anti-PDGF-C antibody (*anti-PDGFC Ab*) were assessed as described above. $*P < .05$, $**P < .01$ vs pre (n = 4-6 for each group). Data represent means ± standard error of the mean.



Supplementary Fig 3 (online only). Effect of combined introduction of the platelet-derived growth factor C (*PDGFC*) and the vascular endothelial growth factor (*VEGF*) expression vectors into ischemic limbs on blood flow recovery after ischemia. **a,** Ischemic limbs of mice were injected with empty vector (*control*), VEGF expression vector (*VEGF*), or VEGF expression vector plus PDGF-C expression vector (*VEGF + PDGFC*) as described in the Methods section. Blood flow recovery was analyzed. $*P < .05$, $**P < .01$ vs control (n = 6-13 for each group). Data represent means ± standard error of the mean.



Supplementary Fig 4 (online only). Expressions of neuropilin 1 (*Nrp1*) and Nrp2 are downregulated in diabetic mice. **a,** Mice treated with an intraperitoneal injection of sodium citrate buffer (*control*) or streptozotocin (*STZ*) were weighed. The *black arrows* indicate the timing of injection. $^{*}P < .05$, $^{**}P < .01$ vs control (n = 5 for each group). Data represent means ± standard error of the mean (SEM). **b,** Blood glucose levels of mice injected with sodium citrate buffer (*control*) or STZ were measured. $^{**}P < .01$ vs control (n = 5 for each group). Data represent means ± SEM. **c,** Time course of relative Nrp1 and Nrp2 messenger RNA (*mRNA*) expression in ischemic limbs of nondiabetic (*control*) or diabetic (*STZ*) mice. $^{*}P < .05$, $^{**}P < .01$ vs control; $^{\#\#}P < .01$ vs precontrol; $^{\dagger\dagger}P < .01$ vs pre-STZ (n = 4-8 for each group). Data represent means ± SEM.