Kinin Receptor Agonism Restores Hindlimb Postischemic Neovascularization Capacity in Diabetic Mice

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ABSTRACT

Limb ischemia is a major complication of thromboembolic diseases. Diabetes worsens prognosis by impairing neovascularization. Genetic or pharmacological inactivation of the kallikrein-kinin system aggravates limb ischemia in nondiabetic animals, whereas angiotensin I-converting enzyme/kininase II inhibition improves outcome. The role of kinins in limb ischemia in the setting of diabetes is not documented. We assessed whether selective activation of kinin receptors by pharmacological agonists can influence neovascularization in diabetic mice with limb ischemia and have a therapeutic effect. Selective pseudo-peptide kinin B1 or B2 receptor agonists resistant to peptidase action were administered by osmotic minipumps at a nonhypo-osmotic dosage for 14 days after unilateral femoral artery ligation in mice previously rendered diabetic by streptozotocin. Comparison was made with ligatured, nonagonist-treated nondiabetic and diabetic mice. Both treatments restored blood flow in the ischemic hindfoot, measured by laser-Doppler perfusion imaging. Macrophage infiltration increased 3-fold in the ischemic gastrocnemius hindfoot, measured by laser-Doppler perfusion imaging. Macrophage infiltration increased 3-fold in the ischemic gastrocnemius muscle during B1 receptor agonist or B2 receptor agonist treatment, and vascular endothelial growth factor (VEGF) level increased 2-fold. Both treatments increased, by 50–100%, circulating CD45/CD11b-positive monocytes and CD34+/VEGFR2+ progenitor cells. Thus, selective pharmacological activation of B1 or B2 kinin receptor overcomes the effect of diabetes on postischemic neovascularization and restores tissue perfusion through monocyte/macrophage mobilization. Kinin receptors are potential therapeutic targets in limb ischemia in diabetes.

Introduction

Limb ischemia secondary to arterial obstruction is a major cause of morbidity and premature mortality, especially in subjects with diabetes mellitus. Development of collateral blood vessels restores, to some extent, distal blood flow, limits ischemic damage, and is a critical factor for recovery. Reduced ability to develop new vessels contributes to enhanced severity of limb ischemia in diabetes (Johanssone et al., 2009). Several molecular and cellular defects are involved in impairment of neovascularization development in diabetes. These defects include reduced monocyte mobilization and altered vascular endothelial growth factor (VEGF) synthesis and signaling (Rivard et al., 1999; Waltenberger et al., 2000). Also, the proangiogenic capacity of bone marrow–derived progenitor cells has been shown to be impaired in diabetes, contributing to the defect in neovascularization (Tepper et al., 2002; Tamarat et al., 2004). Arterial desobstruction, the most straightforward treatment for restoring limb perfusion and suppressing ischemia, is not always feasible, especially in the presence of multiple and/or distal obstructive lesions. Pharmacological interventions promoting neovascularization and restoring distal blood flow are needed for improving prognosis of limb ischemia, especially in diabetes. Here, we show that kinin receptors can be targeted for this purpose.

Kinins are potent vascular endothelium activators, triggering release of endothelial mediators promoting smooth muscle

ABBREVIATIONS: ACE, angiotensin I-converting enzyme/kininase II; BM-MNC, bone marrow mononuclear cell; B1R, B1 bradykinin receptor; B2R, B2 bradykinin receptor; EPC, endothelial progenitor cell; FITC, fluorescein isothiocyanate; KKS, kallikrein-kinin system; MCP-1, monocyte chemoattractant protein 1; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.
relaxation, fibrinolysis, and inhibition of platelet aggregation (Furchgott and Vanhoutte, 1989; Brown et al., 2000). Kinins also activate progenitor cells with neovessel development capacity (Krankel et al., 2008; Spinetti et al., 2011). Kinins are synthesized in several organs, including arteries (Bergaya et al., 2001; Meneton et al., 2001). The peptides are released from precursors, kininogens, by kallikreins and are mainly inactivated in circulation by angiotensin I–converting enzyme (ACE/kininase II). Kinins exert their biologic effects by stimulating two distinct G protein–coupled receptors, the B1 bradykinin receptor (B1R) and the B2 bradykinin receptor (B2R). B2Rs are constitutively synthesized in tissues and mediate most biologic effects of kinins. B1Rs are induced in pathologic situations, such as ischemia, inflammation, or diabetes. The role of B1R in ischemic diseases and diabetes, however, is unclear (Couture et al., 2014).

Studies have suggested a role for the kallikrein-kinin system (KKS) in postischemic neovascularization in an experimental model in non-diabetic animals. Indeed, neovascularization development after femoral artery occlusion was severely reduced in tissue kallikrein-deficient mice (Stone et al., 2009; Spinetti et al., 2011). Conversely, local delivery of human tissue kallikrein gene was reported to accelerate angiogenesis in a model of hindlimb ischemia, an effect that was prevented by pharmacological or genetic inactivation of B1R or B2R (Emanueli et al., 2001a,b, 2002; Emanueli and Madeddu, 2002). These experimental studies suggest a potential therapeutic value of KKS activation in hindlimb ischemia. Interestingly, it has been shown that the proangiogenic effect of ACE inhibitors in hindlimb ischemia was mediated, in large part, by kinin receptor signaling (Silvestre et al., 2001; Li et al., 2008). Although tissue kallikrein concentration was increased in femoral venous effluent of patients with arterial obstruction (Porcu et al., 2002), kallikrein activity level is low in arteries and kinin production and accumulation are slow, even after ACE inhibition (Bergaya et al., 2001; Alhenc-Gelas et al., 2011). Developing new pharmacological interventions for KKS activation has potential therapeutic interest. Kallikrein cannot be easily targeted for pharmacological activation, but recently, potent selective and long-acting B1R and B2R agonists have been synthesized and their therapeutic efficacy has been documented in experimental cardiac ischemia (Belanger et al., 2009; Cote et al., 2009; Potier et al., 2013).

The aim of the present study was to evaluate the effects of B1R and B2R agonists in peripheral ischemia in diabetic mice. Here, we show that selective activation of kinin receptors by pharmacological agonists enhances neovascularization and restores blood flow after femoral artery occlusion in diabetic mice.

Materials and Methods

Animals and Treatments

Ten-week-old male C57BL/6J mice (Charles River Laboratories, l’Arbresle, France) were used. Diabetes was induced by five daily intraperitoneal injections of streptozotocin (50 mg/kg body weight in 0.05 mol/l sodium citrate, pH 4.5) (Bodin et al., 2009). Five weeks after the first injection, mice with established diabetes (fasting glycemia >250 mg/dl) underwent surgery for inducing unilateral hindlimb ischemia. Animals were anesthetized by isoflurane inhalation, and ischemia was induced by ligation of the right femoral artery as previously described (Silvestre et al., 2000; Waechel et al., 2005). Immediately after inducing ischemia, mice were implanted with osmotic minipumps (Alzet, model 1002; Charles River Laboratories) delivering either the selective B1R agonist SarLys[Hyp3, Ig5, DPhe8]desArg9-bradykinin (Cote et al., 2009) or the selective B2R agonist (Hyp[3],Thi[5],N(Chg)[7],Thi[8])-bradykinin (Belanger et al., 2009; Savard et al., 2013) at a nonhypotensive dose of 720 nmol/kg day−1 or vehicle (saline). An additional, nondiabetic group (n = 10) underwent arterial femoral ligation and received saline infusion via Alzet minipump.

A first series of mice (n = 10/group) was studied for 14 days after femoral ligation for neovascularization development. A second series (n = 10/group) submitted to ischemia with or without treatment was studied at 3 and 7 days for blood and bone marrow phenotypes (n = 5–7/group), as described later.

All mice were housed with a 12-hour light/dark cycle, and had free access to food and water. All experimental procedures were approved by the Charles Darwin Ethics Committee for Animal Experimentation (Pierre and Marie Curie University, Paris, France) and were performed in accordance with European legislation for the care and use of laboratory animals (L358-86/609/EEC).

Blood Pressure Measurement

As the B2R agonist dose dependently decreases blood pressure during acute administration in mice (Potier et al., 2013), we assessed the blood pressure effect of chronic B2R agonist treatment in dedicated groups of mice. In brief, four groups of 10-week-old male C57BL/6J mice, diabetic or not (n = 10/group; Charles River Laboratories), were treated with 720 nmol/kg day−1 or vehicle (saline) for 5 weeks via Alzet minipump. Systolic blood pressure was measured at 3 and 5 weeks by tail-cuff plethysmography in trained animals, as previously described (Meneton et al., 2001).

Quantification of Neovascularization

Postischemic neovascularization was evaluated by three different and complementary methods, as previously described (Silvestre et al., 2000; Waechel et al., 2005).

Microangiography. Blood vessel density was evaluated by high-definition microangiography (Trophy X-Ray system; Trophy Radiologie, Vincennes, France) 14 days after artery ligation. Mice were anesthetized (ketamine 60 mg/kg, xylazine 15 mg/kg) and a polyethylene catheter was inserted through laparotomy into the abdominal aorta for injecting contrast medium (barium sulfate, 1 g/ml). Images (two per animal) were acquired by a high-definition digital X-ray transducer and assembled to obtain a complete view of hindlimbs. Blood vessel density was expressed as a microangiographic score: ratio of the percentage of pixels per image occupied by blood vessels in the quantification area in the ischemic and nonischemic hindlimb.

Capillary Density Analysis. Microvessel density was assessed by histologic analysis at 14 days after sacrifice. Ischemic and nonischemic gastrocnemius muscles were dissected and frozen in isopentane solution cooled in liquid nitrogen. Cryosections (7 μm) were incubated for 30 minutes in a solution of phosphate-buffered saline containing 5% bovine serum albumin at room temperature, and then 30 minutes with a rabbit polyclonal antibody directed against total fibronectin (dilution 1:50; Abcam, Paris, France) (Napoli et al., 2005). Capillaries were revealed with a fluorescent fluorescein isothiocyanate (FITC) antirabbit antibody (dilution 1:10; Abcam), and capillary density (capillary object/field) was determined using Leica V10 software (Leica Microsystems, Wetzlar, Germany).

Laser Doppler Perfusion Imaging. Cutaneous foot perfusion was measured in anesthetized (1.5% isoflurane inhaling) mice by laser Doppler perfusion imaging (Moor Instruments, Millwey, UK) before (day 0) and at days 7 and 14 after artery ligation, as previously described (Silvestre et al., 2001; Mallat et al., 2002). In brief, mice were anesthetized by isoflurane inhalation and limbs were depilated. Mice were then placed on a heating plate at 37°C. Blood flow was measured in the foot of ischemic and nonischemic limbs. Image was quantified using the associated software. Results are presented using a color scale: from low blood flow in blue to high blood flow in red.
For the three methods of quantification of neovascularization, results are expressed as a ratio of signal value for ischemic limb to signal value for nonischemic limb. This allows each animal to be its own control and minimizes experimental variability.

**Analysis of Circulating Monocytes and Proangiogenic Progenitor Cells by Flow Cytometry**

The effect of agonist treatment on CD45/CD11b-positive cells and lin−Sca1+/cKit+ progenitor cell mobilization in response to tissue ischemia was assessed 7 days after artery ligation (Ashara et al., 1997; Dimmeler, 2010) in dedicated groups of mice treated as described earlier. Briefly, blood was withdrawn from the tail, and low-density mononuclear cells were studied by flow cytometry after immunostaining, as previously described (Tamarat et al., 2004; Waeckel et al., 2006). For CD45/CD11b, monocyte cells were incubated with antibody directed against CD45 (1:100; eBioscience, Paris, France) and CD11b (0.25 μg/million cells; BioLegend, Saint Quentin Yvelines, France) for 30 minutes at 4°C. Number of CD45/CD11b-positive cells was analyzed using FlowJo software (FlowJo, LLC, Ashland, OR). For lin−/Sca1+/cKit+ progenitors, mononuclear cells were incubated with antibodies directed against mouse hematopoietic lineage (1:100; eBioscience), Ska1 (1:100; eBioscience), and cKit (1:100; eBioscience). Density of double-positive cells among lineage-negative cells was evaluated by flow cytometry using FlowJo software.

In these mice, circulating CD34+/VEGFR2+ cells were also studied at 3 days after artery ligation. Low-density mononuclear cells were incubated against CD34 (1:100; eBioscience) and VEGFR2 (1:100; eBioscience), and double-positive cells were analyzed using FlowJo software.

**Isolation of Bone Marrow Mononuclear Cells and Endothelial Progenitor Cell Differentiation Assay**

Bone marrow cells were obtained by flushing tibias and femurs of nondiabetic and diabetic mice treated or not treated with agonists for 7 days. Bone marrow mononuclear cells (BM-MNCs) were isolated by density gradient centrifugation with Ficoll and immediately plated on 35-mm cell culture dishes (5 × 10^6 BM-MNCs/well) coated with 0.1% rat plasma vitronectin–0.1% gelatin (Sigma-Aldrich, Lyon, France). The cells were maintained for 4 days in endothelial basal medium (Gibco, Life Technologies, Paisley, UK), and endothelial progenitor cell (EPC) differentiation was evaluated as previously described (Tamarat et al., 2004; Waeckel et al., 2006). This assay measures accumulation of endothelial marker–bearing cells (Fadini et al., 2012). In brief, nonadherent cells were removed and adherent cells were stained with Dil-AcLDL and FITC–BS-1 lectin. Cells were incubated for 1 hour at 37°C with Dil-AcLDL (2.5 μg/ml; Tebu-bio, Le Perray en Yvelines, France) in culture medium. Cells were then washed three times with phosphate-buffered saline and fixed with 2% paraformaldehyde and inoculated with FITC–labeled BS-1 lectin (10 μg/ml; Sigma-Aldrich). Double-positive cells were considered as EPCs and counted per well. The ex vivo effect of agonists on BM-MNC differentiation was also studied. BM-MNCs were isolated from nondiabetic mice and plated in the absence or presence of B1R agonist (10−8 and 10−7 mol/l) or B2R agonist (10−8 and 10−7 mol/l) for 4 days. The number of EPCs was quantified as described earlier.

**Evaluation of Macrophage Infiltration in Ischemic Muscle**

Frozen tissue sections (7 μm) of gastrocnemius muscle sampled at 14 days after arterial occlusion were incubated with rat monoclonal antibody directed against Mac-3 (1:50; BD Pharmingen, Le Pont-de-Clair, France) to identify and quantify infiltrated macrophages. Immunostains were visualized using avidin-biotin peroxidase visualization systems (Vectastain ABC kit; Vector Laboratories, Les Ulis Cedex B, France), and Mac-3–positive cells were counted using ImageJ software (NIH, Bethesda, MD) in four randomly chosen fields.

**Quantification of Monocyte Chemoattractant Protein 1 mRNA and Protein in Muscle and Blood**

Chemokine monocyte chemoattractant protein 1 (MCP-1) level is a critical, route-limiting factor for monocyte/macrophage infiltration in ischemic tissue (Ito et al., 1997a; Hong et al., 2005). Total RNA was isolated from the gastrocnemius muscle using TRIZol (Invitrogen, Cergy Pontoise Cedex, France) and reverse-transcribed with superscript II reverse transcriptase as previously described (Bodin et al., 2009). The cDNAs were amplified and quantified using TaqMan Universal Master Mix and Assays-on-Demand Gene Expression Probes for genes of MCP-1 in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, France). Each sample was tested in triplicate. Data were normalized to 18S mRNA. Changes in the target gene were calculated by the 2−ΔΔCT comparative method for each sample (Livak and Schmittgen, 2001).

Plasma MCP-1 concentration was determined in blood sampled at 7 days using a mouse MCP-1 enzyme-linked immunosorbent assay kit (Invitrogen) according to the manufacturer’s instructions.

**Measurement of VEGF Protein Level in Muscle**

Fragments of gastrocnemius muscle were homogenized in 200 μl of radioimmunoprecipitation assay buffer containing protease inhibitors (Complete; Roche, Mannheim, Germany). Protein concentration was determined, and 20 μg of protein was separated by SDS-PAGE and then blotted onto a nitrocellulose sheet. Membranes were probed with antibodies directed against VEGF-A (1:1000; Santa Cruz Biotechnology, Heidelberg, Germany) and beta-actin (1:10,000; Sigma-Aldrich, St. Louis, MO). Stained proteins were detected by chemiluminescent reaction (ECL+ kit; Amersham Biosciences, Glattbrugg, Switzerland). Signals were analyzed in ImageQuant LAS 4000 (GE Healthcare, Vélizy-Villacoublay, France) and quantified using Multi Gauge software 2.0 (Fujifilm, Tokyo, Japan). Results for VEGF were normalized to actin and expressed as the ratio of ischemic to nonischemic hindlimb.

**Statistical Analysis**

Results were expressed as the mean ± S.E.M. Statistical difference was analyzed using one-way or two-way analysis of variance for comparing effects of diabetes and treatment in mice. Analysis of variance was followed by an ad-hoc multiple comparison Tukey test. P < 0.05 was considered as significant.

**Results**

**Physiologic Data.** Body weight was not different among experimental groups. Glycemia was significantly higher in diabetic mice when compared with nondiabetic mice (Table 1). Treatment with B1R or B2R agonist did not alter fasting glycemia in diabetic mice.

**Effect of B2R Agonist Treatment on Blood Pressure.** Chronic administration of B2R agonist had no hypertensive effect in nondiabetic or diabetic mice. Systolic blood pressure measured by tail-cuff plethysmography after 3 and 5 weeks of treatment with 720 nmol/kg day−1 of B2R agonist was not

**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Blood glucose (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NonDiab</td>
<td>27 ± 0.4</td>
<td>165 ± 27</td>
</tr>
<tr>
<td>Diab</td>
<td>26 ± 0.8</td>
<td>388 ± 26**</td>
</tr>
<tr>
<td>Diab + B1R-ag</td>
<td>25 ± 0.8</td>
<td>385 ± 25**</td>
</tr>
<tr>
<td>Diab + B2R-ag</td>
<td>25 ± 1.1</td>
<td>387 ± 24**</td>
</tr>
</tbody>
</table>

**P < 0.01 versus NonDiab mice.**

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influenced by diabetes or agonist treatment (Fig. 1). In addition, 6 days of administration of the B2R agonist at different doses to diabetic and nondiabetic mice had no effect on either systolic or diastolic blood pressure, measured through a catheter inserted into the carotid artery in anesthetized mice (data not shown).

**Effect of Diabetes and B1R or B2R Agonist Treatment on Postischemic Neovascularization and Blood Flow.** Hindlimb vessel density assessed by microangiography was reduced by roughly 40% in diabetic mice treated with vehicle when compared with nondiabetic mice (ischemic/nonischemic hindlimb score ratio 0.60 ± 0.06 versus 0.99 ± 0.13, \( P < 0.05 \)). In diabetic mice, B1R or B2R agonist treatment increased vessel density in the ischemic hindlimb (both \( P < 0.01 \) compared with vehicle-treated diabetic mice). Both treatments restored the angiographic score to values similar to nondiabetic animals (Fig. 2A).

Microangiographic data were confirmed by capillary density analysis (Fig. 2B). In diabetic mice, the ischemic/nonischemic hindlimb capillary density ratio was decreased by roughly 50% in comparison with nondiabetic mice (\( P < 0.05 \)). In diabetic mice, B1R or B2R agonist treatment increased capillary density in the ischemic hindlimb 2.5- and 2.6-fold, respectively (\( P < 0.01 \) compared with vehicle-treated diabetic mice), back to values observed in nondiabetic animals.

Effect on vessel density was associated with changes in tissue perfusion (Fig. 2C). At day 7, blood flow was reduced in all occluded groups by comparison with day 0, as expected, but flow remained higher in B1R agonist–treated diabetic mice than in other diabetic groups (\( P < 0.05 \) versus vehicle-treated diabetic mice). At day 14, foot perfusion remained low in vehicle-treated diabetic mice (\( P < 0.05 \) compared with nondiabetic mice). Treatment with either B1R or B2R agonist increased blood flow (\( P < 0.001 \) compared with vehicle-treated diabetic mice). At day 14, foot perfusion remained low in vehicle-treated diabetic mice (\( P < 0.05 \) compared with nondiabetic mice). Treatment with either B1R or B2R agonist increased blood flow (\( P < 0.001 \) compared with vehicle-treated diabetes).
diabetic mice), restoring foot perfusion to values similar to or slightly higher than nondiabetic animals. There was no significant difference between effect of B1R and B2R agonist treatment on neovascularization.

**Effects of Diabetes and Agonist Treatment on Progenitor Cell Mobilization and Differentiation.** No significant effect of diabetes and B1R or B2R agonist treatment on circulating lin−/Sca1+/cKit+ was observed (Supplemental Fig. 1). Furthermore, in bone marrow from treated and nontreated mice, B1R or B2R agonist treatment had no effect on number of EPCs derived from BM-MNCs when compared with vehicle-treated diabetic mice (Supplemental Fig. 1). Additionally, in vitro, BM-MNC differentiation into EPC was unaffected when BM-MNCs isolated from nontreated diabetic mice were cultured for 4 days in the presence of B1R agonist (10^{-6} and 10^{-7} mol·l^{-1}) or B2R agonist (10^{-6} and 10^{-7} mol·l^{-1}) (data not shown).

In contrast, the number of circulating CD34+/VEGFR2+ cells was significantly decreased in diabetic mice when compared with nondiabetic mice (0.08 ± 0.01% versus 0.18 ± 0.01%, \( P < 0.05 \)). Three days of treatment with either B1R or B2R agonist increased circulating CD34+/VEGFR2+ mononuclear cell levels in diabetic mice back to values observed in nondiabetic mice (B1R agonist: 0.20 ± 0.01%; B2R: 0.17 ± 0.03%; both \( P < 0.05 \) versus vehicle-treated diabetic mice) (Fig. 3).

**Effects of Diabetes and Agonist Treatment on Circulating Monocyte CD45/CD11b-Positive Cells.** Diabetes had no detectable effect on circulating CD45/CD11b-positive cells evaluated 7 days after femoral ligation. Treatment with either B1R or B2R agonist increased circulating CD45/CD11b-positive cells by 30–50% by comparison with vehicle-treated diabetic mice (B1R agonist: 14.2 ± 0.6%, \( P < 0.05 \); B2R agonist: 15.9 ± 0.8%, \( P < 0.01 \) versus vehicle-treated: 10.7 ± 0.5%) (Fig. 4).

**Effect of Diabetes and Agonist Treatment on Macrophage Infiltration in Ischemic Tissue.** Fourteen days after surgery, the number of Mac-3-positive cells was reduced by 62% in the gastrocnemius muscle of vehicle-treated diabetic mice, compared with nondiabetic mice (\( P < 0.001 \); Fig. 5). B1R and B2R agonist treatments induced a more than 3-fold increase in macrophage infiltration in diabetic mice compared with vehicle-treated diabetic mice (\( P < 0.001 \)).

**Effect of Diabetes and Agonist Treatment on Tissue VEGF Protein Level.** VEGF protein level was markedly reduced (−62%) in ischemic muscle of vehicle-treated diabetic mice compared with nondiabetic mice (\( P < 0.05 \); Fig. 6). B1R or B2R agonist treatment restored VEGF protein levels in muscle to levels similar to nondiabetic animals.

**Effect of Diabetes and B2R Agonist Treatment on MCP-1 mRNA and Protein Level.** In nondiabetic mice, ischemia was associated with a 2.8-fold increase in MCP-1 mRNA level in gastrocnemius muscle when compared with nonischemic nondiabetic mice (\( P < 0.05 \)), but this effect was abrogated in vehicle-treated diabetic mice (Fig. 7A). In diabetic mice, B1R agonist treatment had no effect on MCP-1 mRNA levels. By contrast, B2R agonist treatment increased MCP-1 mRNA levels in ischemic muscle 6.6-fold (\( P < 0.05 \)) when compared with contralateral nonischemic muscle, and 4.4-fold (\( P < 0.05 \)) when compared with ischemic muscle from vehicle-treated diabetic mice. B2R agonist, but not B1R agonist, moderately but significantly increased plasma MCP-1 protein levels (\( P < 0.01 \), compared with vehicle-treated diabetic mice; Fig. 7B).

**Discussion**

The present study shows that selective kinin receptor activation by potent pharmacological agonists reverses the defect

![Fig. 3. Circulating CD34/VEGFR2-positive cells quantified by flow cytometry in ligatured nondiabetic mice (NonDiab), vehicle-treated diabetic mice (Diab), and diabetic mice treated with 720 nmol/kg·day of B1R agonist (Diab + B1R-ag) or 720 nmol/kg·day of B2R agonist (Diab + B2R-ag) for 3 days. The top row shows flow cytometry results for FITC-A and PE-A staining of cells in representative experiments. The percentage of cells expressing both markers is displayed in the boxes drawn in the upper-right quadrant, where threshold PE-A is 10 on the vertical axis and FITC-A is 10 on the horizontal axis in each panel. The bar graph shows the mean ± S.E.M. (n = 6 mice/group); \(* P < 0.05 \) versus nonischemic (NonIsch); \( ^{*} P < 0.05 \) versus NonDiab mice; \( ^{*} P < 0.05 \) versus Diab mice. PE, phycoerythin.](https://jpet.aspetjournals.org/content/100/1/222/F3)
in postischemic neovascularization observed in diabetic mice. Pharmacological activation of either the B1 or B2 receptor has a therapeutic effect on hindlimb ischemia, restoring blood flow to ischemic tissues. These observations demonstrate, using a gain-of-function approach and new, specific pharmacological tools, the strong proangiogenic effect of KKS activation in hindlimb ischemia and the role of the two kinin receptor subtypes. The data further suggest that monocyte/macrophage mobilization and activation is involved in restoration of postischemic neovascularization capacity by kinin receptor agonists in diabetic mice.

Diabetes impairs postischemic neovascularization development after arterial obstruction, resulting in enhanced severity of leg ischemia in diabetic patients. Similarly, in the experimental setting, recovery from hindlimb ischemia induced by femoral artery ligation in mice or rats occurs quickly, in less than 2 weeks, in nondiabetic animals through neovessel formation, but in diabetic animals the neovascularization process is severely hampered, and recovery is delayed. A number of studies, focused on kallikrein or B2R, have suggested a role for KKS in postischemic neovascularization in nondiabetic mice or rats (Silvestre et al., 2001; Emanueli et al., 2002; Li et al., 2008; Smith et al., 2008; Stone et al., 2009). However, whether KKS activation can influence the defective postischemic neovascularization process in diabetic animals needed to be documented. Prophylactic gene therapy with human tissue kallikrein has been reported to ameliorate postischemic recovery in diabetic mice (Emanueli et al., 2004). In the present study, we show that 2-week treatment with selective, pharmacological B1R or B2R agonists started after femoral artery occlusion is sufficient for restoring normal postischemic neovascularization in mice with established diabetes. The effect of B2R agonist is consistent with a previous observation of a lack of proangiogenic effect of ACE inhibition in B2R-deficient diabetic mice (Ebrahimian et al., 2005). The role of B1R in postischemic neovascularization in diabetic animals has not been documented before, despite induction of this receptor by ischemia and hyperglycemia.

We found that B1R and B2R activation have a similar beneficial effect in hindlimb ischemia in diabetic mice. This is in contrast with cardiac ischemia where B1R, but not B2R, activation reduces ischemia-reperfusion damage in diabetic mice (Potier et al., 2013). These findings indicate that, although diabetes suppresses B2R signaling in the heart, it does not alter B2R function in proangiogenic cells.

We studied cellular pathways involved in neovascularization development and known to be defective in diabetes to address mechanism of restoration of neovascularization by kinin receptor agonists. Postischemic neovascularization is
not solely the result of proliferation of endothelial cells, but also involves mobilization of progenitor cells and vasculogenesis. Decreased number of circulating progenitor cells is observed in human diabetes, and circulating progenitor level is negatively related to glycemic control (Yue et al., 2011). Consistent with these human studies, we observed a decrease in number of circulating CD34+/VEGFR2+ cells in diabetic mice compared with nondiabetic mice. Treatment with either B1R or B2R agonist counteracted the effect of diabetes and increased CD34+/VEGFR2+ cell number. Given the previous observation that stimulation of CD34+/VEGFR2+ cell mobilization by interleukin-11 treatment results in enhanced collateral vessel growth in a model of femoral artery ligation (Aitsebaomo et al., 2011), the present observation suggests a role for these cells in the proangiogenic effect of kinin receptor agonists. On the other hand, our results do not support the hypothesis that this proangiogenic effect is mediated by lin/Sca1+/cKit+ mobilization or BM-MNC differentiation into EPCs (Supplemental Fig. 1).

There is increasing evidence that inflammation is involved in neovascularization development in the setting of ischemia. Inflammatory cells have been shown to accumulate in the ischemic area and positively modulate neovascularization and arteriogenesis through various mechanisms, including production of angiogenic factors, secretion of proinflammatory cytokines, and matrix degradation (Arras et al., 1998; Tamarat et al., 1997b; Arras et al., 1998; Heil et al., 2002; Waeckel et al., 2006). In addition, MCP-1 induces chemotaxis of human endothelial cells at nanomolar concentrations in the absence of leukocyte infiltrates (Salcedo et al., 2000). However, although triggering monocyte mobilization and increasing macrophage infiltration in ischemic tissue in a manner similar to the B2R agonist, the B1R agonist had no effect on MCP-1. However, monocytes, a major source of MCP-1, display a relatively high level of B1 receptor mRNA in mice with femoral artery occlusion (data not shown). The reason for the difference between the two receptors in coupling to MCP-1 gene expression remains unclear. Other chemokines beside MCP-1 can promote angiogenesis and

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There is increasing evidence that inflammation is involved in neovascularization development in the setting of ischemia. Inflammatory cells have been shown to accumulate in the ischemic area and positively modulate neovascularization and arteriogenesis through various mechanisms, including production of angiogenic factors, secretion of proinflammatory cytokines, and matrix degradation (Arras et al., 1998; Tamarat et al., 1997b; Arras et al., 1998; Heil et al., 2002; Waeckel et al., 2006). In addition, MCP-1 induces chemotaxis of human endothelial cells at nanomolar concentrations in the absence of leukocyte infiltrates (Salcedo et al., 2000). However, although triggering monocyte mobilization and increasing macrophage infiltration in ischemic tissue in a manner similar to the B2R agonist, the B1R agonist had no effect on MCP-1. However, monocytes, a major source of MCP-1, display a relatively high level of B1 receptor mRNA in mice with femoral artery occlusion (data not shown). The reason for the difference between the two receptors in coupling to MCP-1 gene expression remains unclear. Other chemokines beside MCP-1 can promote angiogenesis and
may be involved in B1R action (Ahuwalia and Perretti, 1996; Duchene et al., 2007).

In summary, the present study demonstrates that kinin receptor signaling has strong proangiogenic potential in hindlimb ischemia in diabetes in mice. It shows that, in this experimental setting, B1R and B2R signaling are equally effective. The proangiogenic effect of B1R and B2R activation in diabetes results, at least in part, from monocyte mobilization and macrophage infiltration, and from mobilization of proangiogenic CD34+/VEGFR2+ mononuclear cells. Treatment with B1 or B2 receptor agonist restores the impaired neovascularization capacity of diabetic mice. Development of kinin receptor agonists for therapeutic use in diabetic patients may thus be considered. Potential occurrence of side effects, such as hypotension or angioedema, is a concern (Alhenc-Gelas et al., 2011), albeit no hypotension or lethargy was observed in diabetic or nondiabetic mice chronically treated with agonists.

Authorship Contributions

Participated in research design: Despois, Waecelk, Alhelgas, Roussel, Boughy.

Conducted experiments: Despois, Waecelk, Potetier, Chollet.

Contributed new reagents or analytic tools: Gobeil.

Wrote or contributed to the writing of the manuscript: Despois, Waecelk, Alhelgas, Roussel, Boughy, Gobeil.

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Supplemental figures. (A) Circulating lin-/Sca-1-/c-Kit-positive cells in ligatured non-diabetic mice (NonDiab), diabetic mice vehicle treated (Diab) and diabetic mice treated with 720 nmol/kg.day\(^{-1}\) of B1R agonist (Diab + B1R-ag) or 720 nmol/kg.day\(^{-1}\) of B2R agonist (Diab + B2R-ag) for 7 days. (B) Quantification of AcLDL-Dil/BS-1 lectin-positive cells in bone marrow. BM-MNC were isolated from NonDiab mice mice, Diab mice and Diab + B1R-ag or Diab + B2R-ag mice, 7 days after femoral ligation. BM-MNC were cultured for 4 days and EPC were characterised as adherent cells with double positive staining for AcLDL-Dil and BS-1 lectin. Values are mean ±SEM; n=5-7/group; ***p<0.001 vs NonDiab mice.