Kinin receptor agonism restores hindlimb postischemic neovascularization capacity in diabetic mice

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Abbreviations
ACE: angiotensin I-converting enzyme/kininase II
B1R: B1 bradykinin receptor
B1R-ag: B1 bradykinin receptor agonist
B2R : B2 bradykinin receptor

B2R-ag : B2 bradykinin receptor agonist

BM-MNCs : Bone marrow mononuclear cells

Diab : diabetic mice vehicle treated

EBM2 : endothelial basal medium

EPC : endothelial progenitor cell

KKS : kallikrein-kinin system

MCP-1 : Monocyte chemoattractant protein 1

NonDiab : non-diabetic mice

VEGF : Vascular Endothelial Growth Factor

**Recomended section:** cardiovascular
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 non-diabetic animals while ACE/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 neovascularisation in diabetic mice with limb ischemia and have therapeutic effect. Selective pseudopeptide kinin B1 or B2 receptor agonists resistant to peptidase action were administered by osmotic minipumps at non-hypotensive dosage for 14 days after unilateral femoral artery ligation in mice previously rendered diabetic by streptozotocin. Comparison was made with ligatured, non-agonist treated non-diabetic and diabetic mice. Diabetes reduced neovascularisation, assessed by microangiography and histological capillary density analysis, by roughly 40%. B1 receptor agonist or B2 receptor agonist similarly restored neovascularization in diabetic mice. Neovascularization in agonist-treated diabetic mice was undistinguishable from non-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 muscle during B1 receptor agonist or B2 receptor agonist treatment and VEGF level 2 fold. Both treatments increased, by 50 to 100%, circulating CD45/CD11b-positive monocytes and CD34+/VEGFR2+ progenitor cells. Thus, selective pharmacological activation of B1 or B2 kinin receptor overcomes diabetes effect on postischemic neovascularization and restores tissue perfusion, through monocyte/macrophage mobilisation. 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 (Johannesson et al., 2009). Several molecular and cellular defects are involved in impairment of neovascularization development in diabetes. These defects include reduced monocytes mobilisation and altered vascular endothelial growth factor (VEGF) synthesis and signalling (Rivard et al., 1999; Waltenberger et al., 2000). Also, the pro-angiogenic 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 suppress 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. We show here that kinin receptors can be targeted for this purpose.

Kinins are potent vascular endothelium activators, triggering release of endothelial mediators promoting smooth muscle 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 mainly inactivated in the circulation by the angiotensin I-converting enzyme (ACE/kininase II). Kinins exert their biological effects by stimulating two distinct G-protein
coupled receptors, the B1 bradykinin receptor (B1R) and the B2 bradykinin receptor (B2R). B2R are constitutively synthesized in tissues and mediate most biological effects of kinins. B1R are induced in pathological situations such as ischemia, inflammation or diabetes. The role of B1R in ischemic diseases and diabetes is however unclear (Couture et al., 2014).

Studies have suggested a role for the kallikrein-kinin system (KKS) in postischemic neovascularization in 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; Emanueli et al., 2001b; Emanueli et al., 2002; Emanueli and Madeddu, 2002). These experimental studies suggest 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 a large part, by kinin receptor signalling (Silvestre et al., 2001; Li et al., 2008). Although tissue kallikrein concentration was found 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 intervention 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. We show here 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 5 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 glycaemia ≥ 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; Waeckel et al., 2005). Immediately after inducing ischemia, mice were implanted with osmotic minipumps (Alzet, model 1002, Charles River Laboratories, l’Arbresle, France) delivering either the selective B1R agonist SarLys[Hyp3, Igl5, 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 non hypotensive dose of 720 nmol/kg.day⁻¹ or vehicle (saline). An additional, non-diabetic 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 below.

All mice were housed with a 12h 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 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. Briefly, four groups of ten week-old male C57BL/6J mice, diabetic or not, (n=10/group, Charles River Laboratories, l’Arbresle, France) 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; Waeckel et al., 2005).

**Microangiography** Blood vessel density was evaluated by high-definition microangiography (Trophy X-Ray system, France) 14 days after artery ligation. Mice were anesthetized (Ketamine 60mg/kg, Xylazine 15mg/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 microangiographic score: ratio of the percentage of pixels per image occupied by blood vessels in the quantification area in the ischemic and non-ischemic hindlimb.

**Capillary Density Analysis** Microvessel density was assessed by histological analysis at 14 days at sacrifice. Ischemic and non-ischemic gastrocnemius muscles were dissected and frozen in isopentane solution cooled in liquid nitrogen. Cryosections (7µm) were incubated
for 30 minutes in a solution of PBS containing 5% BSA at room temperature and then 30 minutes with a rabbit polyclonal antibody directed against total fibronectin (abcam, Paris, France, dilution 1:50) (Napoli et al., 2005). Capillaries were revealed with a fluorescent FITC anti-rabbit antibody (abcam, Paris, France, dilution 1:10) and capillary density (capillary object/field) was determined using Leica V03 software.

**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 day 7 and day 14 after artery ligation as previously described (Silvestre et al., 2001; Mallat et al., 2002). Briefly, 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 non-ischemic limb. Image was quantified using the associated software. Results are presented by using a colour scale: from low blood flow in blue to high blood flow in red.

For the three methods of quantification of neovascularisation, results are expressed as ratio of signal value for ischemic limb to signal value for non-ischemic 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**

Effect of agonist treatment on CD45/CD11b-positive cells and lin−/Sca1+/cKit+ progenitors cells mobilization in response to tissue ischemia, was assessed 7 days after artery ligation (Asahara et al., 1997; Dimmeler, 2010) in dedicated groups of mice treated as described above. 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, monocytes cells were incubated with
antibody directed against CD45 (1:100; eBioscience, Paris, France) and CD11b (0.25µg/million cells; Biolegend, Saint Quentin Yvelines, France) for 30 minutes at 4°C. Number of CD45/CD11b-positive cells was analysed using FlowJo software. For lin⁻/Sca1⁺/cKit⁺-progenitors, mononuclear cells were incubated with antibodies directed against mouse hematopoietic lineage (1:100; ebioscience, Paris, France), Sca1 (1:100; ebioscience, Paris, France) and cKit (1:100; ebioscience, Paris, France). 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 with antibody directed against CD34 (1:100; eBioscience, Paris, France) and VEGFR2 (1:100; eBioscience, Paris, France) and double positive cells were analysed using FlowJo software.

**Isolation of Bone Marrow Mononuclear Cells and Endothelial Progenitor cells differentiation assay**

Bone marrow cells were obtained by flushing tibias and femurs of non-diabetic and diabetic mice treated or not 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 (5x10⁶ BM-MNCs/well) coated with 0.1% rat plasma vitronectin-0.1% gelatine (Sigma-Aldrich, Lyon, France). The cells were maintained for 4 days in endothelial basal medium (EBM2, Gibco, LifeTechnologies, 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). Briefly, non-adherent cells were removed and adherent cells were stained with DiI-AcLDL and FITC-BS-1 lectin. Cells were incubated for 1 hour at 37°C with DiI-AcLDL (2.5µg/ml, Tebu-bio) in culture medium. Cells were then washed three times with...
PBS and fixed with 2% paraformaldehyde and incubated with FITC-labeled BS-1 lectin (10µg/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 non-diabetic mice and plated in absence or presence of B1R agonist (10^{-6} and 10^{-7} mol/l) or B2R agonist (10^{-6} and 10^{-7} mol/l) for 4 days. Number of EPCs was quantified as described above.

**Evaluation of macrophages infiltration in ischemic muscle**

Frozen tissue sections (7µm) of gastrocnemius muscle sampled at 14 days after arterial occlusion were incubated with rat monoclonal antibody directed against Mac-3 (1:50, BDpharmingen, Le Pont-de-Claix, France) to identify and quantify infiltrated macrophages. Immunostains were visualised using avidin-biotin horseradish peroxidase visualization systems (Vectastain ABC kit, Vector Laboratories, France) and Mac3-positive cells were counted using ImageJ software in 4 randomly chosen fields.

**Quantification of Monocyte chemoattractant protein 1 (MCP-1) mRNA and protein in muscle and blood**

Chemokine MCP-1 level is a critical, rate-limiting factor for monocytes/macrophages infiltration in ischemic tissue (Ito et al., 1997a; Hong et al., 2005). Total RNA was isolated from the gastrocnemius muscle using TRIzol (Invitrogen, 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 gene 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 $2^{\Delta\Delta CT}$ method.
comparative method for each sample (Livak and Schmittgen, 2001).

Plasma MCP-1 concentration was determined in blood sampled at 7 days using mouse MCP-1 ELISA kit (Invitrogen, France) according to manufacturer’s instructions.

**Measurement of Vascular endothelial growth factor (VEGF) protein level in muscle**

Fragments of gastrocnemius muscle were homogenized in 200µL of RIPA buffer containing protease inhibitors (cOmplete, Roche, Germany). Protein concentration was determined and 20µg of protein were separated by SDS-PAGE and then blotted onto a nitrocellulose sheet. Membranes were probed with antibodies directed against VEGF-A (1:1,000; Santa Cruz Biotechnology, Heidelberg, Germany) and beta-Actin (1:10,000; Sigma, St Louis, Mo). Stained proteins were detected by chemiluminescent reaction (ECL+ kit, Amersham Biosciences, Swiss). Signals were analysed in ImageQuant LAS 4000 (GE Healthcare, France) and quantified using Multi Gauge software 2.0 (FujiFilm, Japan). Results for VEGF were normalized to actin and expressed as ratio of ischemic to non-ischemic hindlimb.

**Statistical analysis**

Results were expressed as mean ± SEM. Statistical difference was analysed using one-way or two-way ANOVA for comparing effects of diabetes and treatment in mice. ANOVA was followed by ad hoc multiple comparison Tukey test. $P<0.05$ was considered as significant.
RESULTS

Physiological data

Body weight was not different among experimental groups. Glycaemia was significantly higher in diabetic mice when compared to non-diabetic mice (Table 1). Treatment with B1R or B2R agonist did not alter fasting glycaemia in diabetic mice.

Effect of B2R agonist treatment on blood pressure

Chronic administration of B2R agonist had no hypotensive effect in non-diabetic 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 influenced by diabetes or agonists treatment (Figure 1). In addition, six days administration of the B2R agonist at different doses to diabetic and non-diabetic 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 non-diabetic mice (ischemic/non ischemic hindlimb score ratio 0.60 ± 0.06 versus 0.99 ± 0.13, respectively, \(p<0.05\)). In diabetic mice, B1R or B2R agonist treatment increased vessel density in the ischemic hindlimb, (both \(p<0.01\), compared to vehicle-treated diabetic mice). Both treatments restored the angiographic score to values similar to non-diabetic animals (Figure 2A).
Microangiographic data were confirmed by capillary density analysis (Figure 2B). In diabetic mice, the ischemic/non-ischemic hindlimb capillary density ratio was decreased by roughly 50% by comparison with non-diabetic 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 to vehicle-treated diabetic mice) back to values observed in non-diabetic animals.

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

**Effects of diabetes and agonist treatment on progenitors cells mobilization and differentiation**

No significant effect of diabetes and B1R or B2R agonist treatment on circulating lin\(^-\)/Sca1\(^+\)/cKit\(^+\) was observed (supplemental figure). Furthermore, in bone marrow from treated and non treated mice, B1R or B2R agonist treatment had no effect on number of EPC derived from BM-MNCs when compared with vehicle-treated diabetic mice (supplemental figure). Additionally, in vitro, BM-MNCs differentiation into EPC was unaffected when BM-MNCs isolated from non-treated diabetic mice were cultured for 4 days in presence of B1R agonist \((10^{-6} \text{ and } 10^{-7} \text{ mol.L}^{-1})\) or B2R agonist \((10^{-6} \text{ and } 10^{-7} \text{ mol.L}^{-1})\) (data not shown).
In contrast, the number of circulating CD34+/VEGFR2+ cells was significantly decreased in diabetic mice when compared with non-diabetic mice (0.08 ± 0.01% vs 0.18 ± 0.01%, respectively, \( p < 0.05 \)). Three days of treatment with either B1R or B2R agonist increased circulating CD34+/VEGFR2+-mononuclear cells level in diabetic mice back to values observed in non-diabetic mice (B1R-ag: 0.20 ± 0.01%; B2R-ag: 0.17 ± 0.03%, both \( p < 0.05 \) versus vehicle-treated diabetic mice, Figure 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 to 50% by comparison with vehicle-treated diabetic mice (B1R-ag: 14.2 ± 0.6%, \( p < 0.05 \); B2R-ag: 15.9 ± 0.8%, \( p < 0.01 \) versus vehicle-treated: 10.7 ± 0.5%, Figure 4).

Effect of diabetes and agonist treatment on macrophage infiltration in ischemic tissue

Fourteen days after surgery the number of Mac3-positive cells was reduced by 62% in the gastrocnemius muscle of vehicle-treated diabetic mice, compared with non-diabetic mice (\( p < 0.001 \), Figure 5). B1R and B2R agonist treatments induced a more than 3-fold increase in macrophage infiltration in diabetic mice compared to vehicle-treated diabetic mice (\( p < 0.001 \)).

Effects 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 non-diabetic mice (\( p < 0.05 \), Figure 6). B1R or B2R agonist treatment restored VEGF protein levels in muscle to levels similar to non-diabetic animals.
**Effect of diabetes and B2R agonist treatment on MCP-1 mRNA and protein level**

In non-diabetic mice, ischemia was associated with a 2.8-fold increase in MCP-1 mRNA level in gastrocnemius muscle when compared with non-ischemic non-diabetic mice \( (p<0.05) \) but this effect was abrogated in vehicle-treated diabetic mice (Figure 7A). In diabetic mice, B1R agonist treatment had no effect on MCP-1 mRNA levels. By contrast, B2R agonist treatment increased MCP-1 mRNA level in ischemic muscle 6.6-fold \( (p<0.05) \), when compared with contralateral non-ischemic 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, Figure 7B).
DISCUSSION

The present study shows that selective kinin receptor activation by potent pharmacological agonists reverses the defect in postischemic neovascularization observed in diabetic mice. Pharmacological activation of either B1 or B2 receptor has therapeutic effect in hindlimb ischemia, restoring blood flow to ischemic tissues. These observations demonstrate by 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/macrophages mobilisation and activation is involved in restoration of postischemic neovascularisation 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 two weeks, in non-diabetic 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 non-diabetic 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 two-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 previous observation of 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 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, while diabetes suppresses B2R signalling in the heart it does not alter B2R function in proangiogenic cells.

We studied cellular pathways involved in neovascularisation development and known to be defective in diabetes to address mechanism of restoration of neovascularisation by kinin receptor agonists. Post ischemic 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 glycaemic control (Yue et al., 2011). Consistent with these human studies, we observed in diabetic mice a decrease in number of circulating CD34+/VEGFR2+ cells compared to non-diabetic mice. Treatment with either B1R or B2R agonist counteracted the effect of diabetes and increased CD34+/VEGFR2+ cell number. Given previous observation that stimulation of CD34+/VEGFR2+ cells mobilisation by IL-11 treatment results in enhanced collateral vessel growth in a model of femoral artery ligation (Aitsebaomo et al., 2011), the present observation suggest 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-MNCs differentiation into EPCs (supplemental figure).
There is increasing evidence that inflammation is involved in neovascularization development in the setting of ischemia. Inflammatory cells have been shown to accumulate in 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., 2002; Silvestre et al., 2003; Stabile et al., 2003; Hong et al., 2005; Waeckel et al., 2005; Stabile et al., 2006; Waeckel et al., 2006; Hillmeister et al., 2011). In diabetes, monocytes dysfunction and reduced VEGF production have been observed (Waltenberger et al., 2000; Tchaikovski et al., 2009; Waltenberger, 2009; Carr et al., 2011). Consistent with these studies, we observed a sharp reduction in number of infiltrated macrophages and decreased VEGF protein level in ischemic muscle of diabetic mice compared to non-diabetic mice. B1R or B2R agonist treatment fully restored the inflammatory response. Both treatments increased circulating monocytes CD45/CD11b-positive cells in blood and monocyte/macrophages infiltration in ischemic tissue in diabetic mice. These results suggest that the proangiogenic effect of the agonists is due, at least partly, to restoration of the cellular inflammatory response to ischemia.

We observed that B2R activation stimulates MCP-1 synthesis, consistent with its effect on monocytes mobilisation, MCP-1 upregulates hypoxia-inducible factor 1 alpha gene expression and subsequently vascular endothelial growth factor-A synthesis (Hong et al., 2005). This chemokine is a major regulator of monocytes/macrophages trafficking in ischemic area and is implicated in postischemic neovascularization. After vessel occlusion, administration of MCP-1 stimulates monocyte trafficking in the adventitia of growing arteries while blockade of MCP-1 receptor, CCR2, inhibits this process (Ito 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 absence of leukocytes infiltrates
(Salcedo et al., 2000). However, although triggering monocyte mobilization and increasing macrophage infiltration in ischemic tissue similarly to the B2R agonist, the B1R agonist had no effect on MCP-1. Yet, monocytes, a major source of MCP1, display 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 (Ahluwalia and Perretti, 1996; Duchene et al., 2007).

In summary, the present study demonstrates that kinin receptor signalling has strong proangiogenic potential in hindlimb ischemia in diabetes in mice. It shows that, in this experimental setting, B1R and B2R signalling are equally effective. The proangiogenic effect of B1R and B2R activation in diabetes results, at least in part, from monocyte mobilisation and macrophage infiltration and from mobilisation 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, like hypotension or angioedema, is a concern (Alhenc-Gelas et al., 2011), albeit no hypotension or lethality was observed in chronically agonist treated diabetic or non-diabetic mice.
Authorship contributions

Participated in research design: Desposito, Waeckel, Alhenc-Gelas, Roussel and Bouby

Conducted experiments: Desposito, Waeckel, Potier and Chollet

Contributed new reagents or analytic tools: Gobeil

Wrote and contributing to the writing of the manuscript: Desposito, Waeckel, Alhenc-Gelas, Roussel, Bouby and Gobeil
References


Footnotes:

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Disclosure:

Roussel : consultant for / recipient of research grants from: sanofi-aventis, MSD Chibret, Servier, Roche, Eli Lilly, Astra Zeneca, BMS, Novartis, Novo Nordisk, and Lifescan;

Alhenc-Gelas : recipient of research grant from Bristol/Myers/Squibb
Figures legends:

Figure 1. Systolic blood pressure in non-diabetic (NonDiab) and diabetic (Diab) mice after 3 weeks (A) or 5 weeks (B) of treatment with 720 nmol/kg.day^{-1} of B2 receptor agonist or saline. Values are mean±SEM, n=10/group, effect of diabetes or treatment=NS.

Figure 2. Representative illustrations and quantitative evaluation of data for microangiography (A) and capillary density analysis (B) at day 14 and for foot perfusion measurement (C) at day 7 and day 14 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). Graphs show means ± SEM of ratio of ischemic to non-ischemic hindlimb values (Isch/Nonisch ratio) for angiographic score (A), capillary density (B) and blood flow (C). n=10/group; $p<0.05, $$p<0.01$ vs NonDiab mice; **$p<0.01$, ***$p<0.001$ vs Diab mice. Scale bar represents 100µm. Color scale for blood flow: blue low, yellow intermediate, red high.

Figure 3. Circulating CD34/VEGFR2-positive cells quantified by flow cytometry 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 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 mean±SEM, n= 6 mice/group; $\Psi p<0.05$ vs NonIsch; $\Psi p<0.05$ vs NonDiab mice; *$p<0.05$ vs Diab mice."
Figure 4. Circulating CD45/CD11b-positive monocyte cells quantified by flow cytometry 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 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$^3$ on the vertical axis and FITC-A is 10$^3$ on the horizontal axis in each panel. The bar graph shows mean±SEM, n=6 mice/group; $p<0.05$ vs NonDiab mice; *p<0.05 **p<0.01 vs Diab mice.

Figure 5. Representative photomicrographs and quantitative evaluation of Mac3-positive cells in ischemic gastrocnemius muscle of 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 14 days. Arrows represent positive staining for Mac3. Values are mean±SEM; n=5/group; $p<0.05$, $$$p<0.001$ vs NonDiab mice; ***p<0.001 vs Diab mice. Scale bar represents 100µm.

Figure 6. Representative blots and quantitative evaluation of VEGF protein level in ischemic gastrocnemius muscle of 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 14 days. Values are mean±SEM of ischemic to non-ischemic muscle ratio. VEGF level is normalized to actin level. n=7/group; $p<0.05$ vs NonDiab mice; *p<0.05 vs Diab mice.
Figure 7. MCP-1 mRNA level in ischemic and non-ischemic gastrocnemius muscle (A) and MCP-1 protein level in blood (B) of 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 14 days. MCP-1 mRNA is normalized to 18S. Values are mean±SEM; n=5/group; ψp<0.05 vs Non-ischemic muscle of NonDiab mice; $$$p<0.001 vs ischemic muscle of NonDiab mice; **p<0.01 vs all other groups; ***p<0.001 vs ischemic muscle of Diab mice.
Table 1. Baseline body weight and fasting plasma glucose of experimental groups

Baseline body weight and fasting plasma glucose in non-diabetic mice (NonDiab), diabetic mice (Diab) and diabetic mice treated with B1R agonist (Diab + B1R-ag) or B2R agonist (Diab + B2R-ag). Values are mean±SEM, n=20/group; **p<0.01 vs NonDiab mice.

<table>
<thead>
<tr>
<th></th>
<th>NonDiab</th>
<th>Diab</th>
<th>Diab + B1R-ag</th>
<th>Diab + B2R-ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>27 ± 0.4</td>
<td>26 ± 0.8</td>
<td>25 ± 0.8</td>
<td>25 ± 1.1</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>165 ± 27</td>
<td>388 ± 26**</td>
<td>385 ± 25**</td>
<td>387 ± 24**</td>
</tr>
</tbody>
</table>
Figure 3

![Diagram showing VEGFR2+ and CD34 expression levels across different groups, including NonDiab, Diab, Diab + B1R-ag, and Diab + B2R-ag.](image-url)
Figure 4

[Image of a bar chart and flow cytometry plots showing the percentage of circulating CD5/CD11b-positive cells across different groups (NonDiab, Diab, Diab + B1R-ao, Diab + B2R-ao), with statistical significance indicated by asterisks and a dollar symbol.]
Figure 5

NonDiab  Diab  Diab + B1R-ag  Diab + B2R-ag

Number of Mac3-positive cells (Cells/area)

NonDiab  Diab  Diab + B1R-ag  Diab + B2R-ag

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Kinin receptor agonism restores hindlimb postischemic neovascularization capacity in diabetic mice

Dorinne Desposito, Louis Potier, Catherine Chollet, Fernand Gobeil Jr, Ronan Roussel, Francois Alhenc-Gelas, Nadine Bouby, Ludovic Waeckel

Journal of Pharmacology and Experimental Therapeutics

Supplemental figures

(A)

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, 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.