Arteriogenesis Is Modulated By Bradykinin Receptor Signaling

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Rationale: Positive outward remodeling of pre-existing collateral arteries into functional conductance arteries, arteriogenesis, is a major endogenous rescue mechanism to prevent cardiovascular ischemia. Collateral arterial growth is accompanied by expression of kinin precursor. However, the role of kinin signaling via the kinin receptors (B1R and B2R) in arteriogenesis is unclear.

Objective: The purpose of this study was to elucidate the functional role and mechanism of bradykinin receptor signaling in arteriogenesis.

Methods and Results: Bradykinin receptors positively affected arteriogenesis, with the contribution of B1R being more pronounced than B2R. In mice, arteriogenesis upon femoral artery occlusion was significantly reduced in B1R mutant mice as evidenced by reduced microspheres and laser Doppler flow perfusion measurements. Transplantation of wild-type bone marrow cells into irradiated B1R mutant mice restored arteriogenesis, whereas bone marrow chimeric mice generated by reconstituting wild-type mice with B1R mutant bone marrow showed reduced arteriogenesis after femoral artery occlusion. In the rat brain 3-vessel occlusion arteriogenesis model, pharmacological blockade of B1R inhibited arteriogenesis and stimulation of B1R enhanced arteriogenesis. In the rat, femoral artery ligation combined with arterial venous shunt model resulted in flow-driven arteriogenesis, and treatment with B1R antagonist R715 decreased vascular remodeling and leukocyte invasion (monocytes) into the perivascular tissue. In monocyte migration assays, in vitro B1R agonists enhanced migration of monocytes. In chimeric mice generated by reconstituting wild-type mice with B1R mutant bone marrow showed reduced arteriogenesis, whereas bone marrow mutant mice as evidenced by reduced microspheres and laser Doppler flow perfusion measurements. Transplantation of wild-type bone marrow cells into irradiated B1R mutant mice restored arteriogenesis, whereas bone marrow chimeric mice generated by reconstituting wild-type mice with B1R mutant bone marrow showed reduced arteriogenesis after femoral artery occlusion. In the rat brain 3-vessel occlusion arteriogenesis model, pharmacological blockade of B1R inhibited arteriogenesis and stimulation of B1R enhanced arteriogenesis. In the rat, femoral artery ligation combined with arterial venous shunt model resulted in flow-driven arteriogenesis, and treatment with B1R antagonist R715 decreased vascular remodeling and leukocyte invasion (monocytes) into the perivascular tissue. In monocyte migration assays, in vitro B1R agonists enhanced migration of monocytes.

Conclusions: Kinin receptors act as positive modulators of arteriogenesis in mice and rats. B1R can be blocked or therapeutically stimulated by B1R antagonists or agonists, respectively, involving a contribution of peripheral immune cells (monocytes) linking hemodynamic conditions with inflammatory pathways. (Circ Res. 2011;109:524-533.)

Key Words: bone marrow transplantation ■ bradykinin receptors ■ collateral growth ■ leukocytes

Arteriogenesis is the process that involves the flow-induced outward remodeling of preexisting collateral arterial pathways into functional conductance arteries (biological bypass). As a result of the arteriogenesis process, blood perfusion to the compromised region is restored; therefore, it is regarded as a clinically highly relevant target. It is established that arteriogenesis is triggered by changes in local hemodynamic conditions and subsequent activation of inflammatory pathways. We previously showed that expression of kininogen, a precursor of the vasoactive kinin peptides, was selectively expressed in growing collaterals of the rat brain. Here we investigated the role of kinin signaling in bradykinin receptor-deficient mice for collateral growth and evaluated whether stimulation with bradykinin receptor antagonists/agonists may modulate arteriogenesis in mice and rats. Our data suggest that the kinin–receptor signaling pathway may act as a molecular link between changes in hemodynamic forces (artery occlusion) and the activation of inflammatory pathways, including attraction of bone marrow...
Arteriogenesis differs considerably from sprouting angiogenesis, with respect to the driving forces and underlying molecular mechanisms. Angiogenesis is the process in which endothelial cells sprout from vessels; it is driven by hypoxia and gradients of vascular endothelial growth factor and results in the formation of new capillaries within the ischemic tissue. In contrast, arteriogenesis occurs in response to complete stenosis of the feed artery and results in redistribution of flow by recruitment of collateral arterial pathways, resulting in positive outward remodeling of the vessel diameter. Local inflammatory processes in the vessel wall of growing collateral arteries lead to invasion of different leukocyte populations that accumulate in the perivascular space (monocytes, neutrophils), and drive collateral growth via the local production of paracrine cytokines. Application of proinflammatory mediators, such as GM-CSF and MCP-1, stimulate arteriogenesis therapeutically by enhancing, in particular, monocyte migration and increasing their adhesiveness to the activated endothelium. Therapeutic increase in arteriogenesis was shown to correlate significantly with reduction in experimentally induced stroke volume, and identification of novel therapeutic targets to treat arterial occlusive diseases is of major concern.

Biological effects of bradykinin receptor signaling share common features with processes involved in arteriogenesis, such as leukocyte recruitment and production of cytokines, and were shown to mediate cell proliferation and capillary sprouting. Vascular kininogen is cleaved by tissue kallikrein, resulting in the production of different kinin peptides, which constitute a family of major proinflammatory peptides and are produced locally at sites of inflammation. Kinins mediate their biological function by specific interaction with the two G-protein–coupled bradykinin receptors (B1R and B2R). The B2R is short-term, activated by the kinins bradykinin or Lys-bradykinin, is constitutively expressed in many different organs, tissues, and cells, and is involved in many vascular actions, such as vasodilation. B1R is activated by the kinins des-Arg9-bradykinin or Lys-bradykinin; once activated, it does not undergo rapid desensitization.

Here, we hypothesize that bradykinin receptors are required for collateral arterial growth and may be targeted for therapeutic arteriogenesis. We provide genetic and pharmacological evidence showing that B1R acts as a positive modulator of outward remodeling of collateral arteries. In bradykinin mutant mice, loss of bradykinin receptors led to a significant reduction in arteriogenesis. In rats, arteriogenesis can be therapeutically inhibited or stimulated by B1R antagonists/agonists. Furthermore, using B1R−/−/wild-type (WT) mixed BM chimeric mice, we demonstrate the pivotal role of B1R expression on peripheral immune cells in arteriogenesis. Finally, immune histology on collateral sections and human acute monocytic leukemia cell line (THP-1) migration assays specify the immanent role of B1R-driven monocyte migration.

Methods

Animal Experiments

For detailed method description see within the expanded Methods section available in the Online Supplement at http://circres.ahajournals.org.
mice showed a minor reduction in perfusion recovery 7 days after FAO as well (41.3%; Figure 1D), but they exhibited a complete flow recovery from FAO within 21 days. LDF measurement (total blood flow) identified no difference in hind limb perfusion between B1R<sup>−/−</sup>/H11002<sup>−/−</sup>/H11002<sup>−/−</sup>, B2R<sup>−/−</sup>/H11002<sup>−/−</sup>/H11002<sup>−/−</sup>, and WT mice before ligation. However, after FAO, perfusion recovery remained significantly reduced only in B1R<sup>−/−</sup> mice starting from day 3 to day 21 (Figure 1G, H). After perfusion, we examined collateral diameters and collateral numbers in the adductor and quadriceps region of the upper thigh (Figure 2). At baseline, lumen diameter and collateral number were not different; however, positive outward remodeling at day 7 after FAO showed markedly reduced blood flow recovery in B1R<sup>−/−</sup> mice compared to WT mice. Flow recovery in B2R<sup>−/−</sup> mice did not differ from that of WT mice.

**Figure 1. Hindlimb perfusion recovery 7 days after femoral artery ligation.** A, B, Schematic representation of the mice hindlimb collateral network. LCFA indicates lateral caudal femoral artery; SA, saphenous artery. Collateral network before (A) and after occlusion (B) of the femoral artery (FA). Occlusion results in a positive outward remodeling of collateral arteries and recovery of blood flow within 7 days. C, D, Hindlimb perfusion as measured by microspheres in arbitrary units. Perfusion is significantly reduced in B1R<sup>−/−</sup> and B2R<sup>−/−</sup> mice compared to wild-type (WT) mice at 7 days after ligation. E, F, At 21 days after FAO, collateral grown in B1R<sup>−/−</sup> remains significantly reduced but recovers in B2R<sup>−/−</sup> mice to levels of WT mice. G, H, Peripheral perfusion measured with laser Doppler flow (LDF) device over a time period of 21 days after FAO shows markedly reduced blood flow recovery in B1R<sup>−/−</sup> mice compared to WT mice. Flow recovery in B2R<sup>−/−</sup> mice does not differ from that of WT mice.

**Figure 2. Femoral artery ligation.** Collateral remodeling and monocyte migration. Reduced collateral remodeling (lumen expansion) in B1R<sup>−/−</sup> mice. Staining for smooth muscle actin (red) to analyze collateral lumen diameter (A) and collateral density (B) at baseline and 7 days after femoral artery ligation. Collateral lumen diameter and number in B2R<sup>−/−</sup> mice (C, D) and B1R<sup>−/−</sup> mice (E, F). Staining for ED1-positive macrophages (violet) 3 days after FAO around collateral vessels of the collateral area (quadriceps/adductor) in wild-type (G) and B1R<sup>−/−</sup> mice (H). Note reduced ED1-positive cell migration in B1R<sup>−/−</sup> mice.
icatinib), and collateral perfusion was measured again 7 days after FAO (Online Figure I), showing that reduction in perfusion recovery is similar to that measured in B1R−/− mice.

Perfusion Restoration After BM Transplantation
Leukocytes contribute importantly to arteriogenesis, and pericollateral ED1-positive cells (macrophages) were found to be reduced in B1R−/− mice (Figure 2G, H). To assess whether the reduced perfusion recovery observed in B1R−/− mice is the consequence of a deficient B1R expression of immune cells, we generated BM chimeric mice in a crossover design (Figure 3A). Recipient mice were subjected to FAO and collateral perfusion recovery was measured 7 days later by microsphere perfusion. Transplantation of BM from WT mice in WT mice showed a slightly reduced perfusion restoration in chimeras (WT BM into WT C57BL/6J; n=7; 40.1%) in comparison to nonirradiated WT mice, indicating that the radiation and transplantation effect collateral artery growth (Figure 3B). Transplantation of B1R−/− BM in B1R−/− mice revealed that perfusion restoration was strongly reduced (B1R−/− BM into B1R−/− chimeric mice; n=8; 24.5%). Likewise, B1R−/− BM to WT chimeric mice showed a significant decrease in hind limb perfusion (B1R−/− BM into WT mice; n=8; 27.6%) to levels that did not differ from nonirradiated B1R−/− mice (compare to Figure 1). Injection of WT BM into B1R−/− mice almost completely restored the arteriogenic deficit to levels comparable to those of WT animals (WT BM into B1R−/− chimeric mice; n=8; 44.8%). Next, we aimed at analyzing the fate of the BM-derived cells by transplanting BM of GFP reporter mice into B1R−/− and WT mice. Here, immunohistochemical evaluation of BM-derived leukocytes using a GFP antibody showed that GFP-positive cells invade the perivascular space of collateral arteries 3 days after FAO (Figure 3, D through G). GFP-positive immune cells basically gather around the collateral arteries. The number of invading GFP-positive cells in (GFP reporter mice BM into WT mice and GFP reporter mice BM into B1R−/− chimeras (E, G) gather around collateral arteries.

Figure 3. Hind limb perfusion recovery. Femoral artery occlusion (FAO) in bone marrow (BM)-grafted chimeric mice. A.Recipient mice were irradiated and then BM was isolated from wild-type (WT) or B1R−/− donor mice and injected crosswise into WT or B1R−/− recipient mice. Six weeks after generation of chimeric mice, hind limb perfusion restoration was measured 7 days after FAO by microsphere perfusion. B. Note that transplantation of B1R−/− BM into WT mice significantly reduced hind limb perfusion after FAO. Transplantation of WT BM into B1R−/− mice augmented hind limb perfusion after FAO. *P<0.05. C. BM from green fluorescent protein (GFP) reporter donor was transplanted into WT mice or B1R−/− recipient mice and FAO was performed for 3 days. Adductor/quadriceps muscles were taken for immunohistological stainings (collateral area). D and E. Staining for GFP-positive cells (chromogen red dye) and nucleus (blue). F and G. Staining for GFP-positive cells using fluorescent antibodies, GFP (green), smooth muscle actin (red), and nucleus (blue). GFP BM-derived cell in WT chimeras (D, F) and GFP BM-derived cell in B1R−/− chimeras (E, G) gather around collateral arteries.

Pharmacological Inhibition of Cerebral Arteriogenesis by Bradykinin Receptor Antagonist Treatment
Pharmacological modulation of the bradykinin receptor pathways was performed in the well-established nonischemic hypoperfusion 3-VO rat brain model. Here, induction of cerebral arteriogenesis by 3-VO results in recruitment of the posterior cerebral artery (PCA) and diameter changes of the growing collateral pathway can be measured (Figure 4A, 3B) directly. Three weeks after 3-VO induction of arteriogenesis, results in significant diameter increase of the PCA from 143 µm to 247 µm ipsilateral to the occluded carotid artery (Figure 4C). The pharmacological inhibition of cerebral arteriogenesis on B1R antagonist Des-(Arg9,Leu8) bradykinin treatment showed...
that 3 weeks after 3-VO, PCA diameter growth was significantly smaller (up to 198 μm) in comparison to PCA diameters of untreated control rats (Figure 4C).

Next, recovery of blood flow after 3-VO was measured using LDF by concurrent increased blood PCO2. Increase in PCO2 results in maximal vasodilation of brain vessels and, therefore, subsequent increase in blood flow. Therefore, this method shows cerebrovascular perfusion reserve capacity (CVRC), which correlates with cerebral collateralization (Figure 4D). CVRC, in response to CO2, is given in percent of total blood flow under normal conditions. Whereas in nontreated nonoperated rats the CVRC is ∼20% of total blood flow, CVRC immediately after 3-VO is completely abolished. Within 3 weeks after 3-VO, perfusion recovers to 10%. However, in B1R antagonist-treated rats, CVRC remained completely abolished and does not recover (Figure 4E). Negative values resulted because of the steal phenomenon.19 Application of a B2R antagonist icatibant inhibits cerebral arteriogenesis as well (Online Figure II), but inhibition was less as compared to B1R.

Therapeutic Stimulation of Cerebral Arteriogenesis by B1R Agonist Treatment

To evaluate the potential of stimulating cerebral arteriogenesis therapeutically, rats were treated with the specific B1R agonist Sar-[D-Phe8]-des-Arg9 bradykinin. As shown, therapeutic stimulation of cerebral arteriogenesis is measured best 1 week after 3-VO, when differences to adaptive arteriogenesis are most obvious.6 Seven days after 3-VO, PCA diameters of untreated rats increase from 143 μm to 213 μm (Figure 5B). In comparison, rats treated with a B1R agonist showed a significantly stronger collateral growth, and PCA diameters increase up to 251 μm. Likewise, measurement of perfusion recovery showed that stimulation with a B1R agonist significantly improves the CVRC (Figure 5C). CVRC reached 9% already within 1 week, thereby demonstrating a rapid recovery, which was comparable with the CVRC seen in untreated rats 3 weeks after 3-VO.

Inhibition of Shear Stress-Induced Arteriogenesis by B1R Antagonist Is Associated With Reduced Leukocyte–Monocyte Transmigration

B1R is expressed on leukocytes such as neutrophils and monocytes/macrophages,20 and here we performed immune histology to determine the effect of pharmacological inhibition of B1R signaling on leukocyte migration into the perivascular tissue of growing collaterals. Therefore, we used the femoral artery ligation model in rats, and surgery was combined with creation of an AV shunt between the artery
C P B1R agonist treatment increases CVR significantly (∗/H11021). *B1R agonist Sar[D-phe8]-des-Arg9 bradykinin. Furthermore, days after 3-VO, which can be enhanced by the stimulation with saline-treated control rats with B1R agonist Sar[D-phe8]-des-Arg9 bradykinin. Moreover, days after 3-vessel occlusion (3-VO). Comparison of saline-treated control to B1R agonist Sar[D-phe8]-des-Arg9 bradykinin, as well as by pretreatment with the B1R antagonists R715 (Figure 7). Migration assays demonstrated that monocyte migration is strongly increased in response to both B1R agonists and actually comparable with the MCP-1-positive control. However, pretreatment with the selective B1R antagonist R715 completely inhibited B1R agonist-induced monocyte migration.

**Discussion**

This study, for the first time to our knowledge, demonstrates the functional relevance of bradykinin receptors in arteriogenesis. The B1R especially acts as an important positive modulator of arteriogenesis involving the contribution of BM-derived cells.

The present study combines two established flow-driven arteriogenesis models: the hind limb FAO model, in which arteriogenesis is measured via microsphere perfusion and LDF in mice, and the previously well-documented 3-VO nonischemic hypoperfusion model for induction of arteriogenesis in the brain of the rat. Collateral growth was analyzed in a bradykinin receptor loss of function model, as well as by pharmacological modulation of the bradykinin receptor pathway. Analysis of bradykinin receptor-dependent collateral growth provides novel findings.

In particular, B1R is relevant for hind limb collateral artery growth in mice, and transplanting BM from WT to B1R-deficient mice showed the crucial role of B1R expression on BM-derived cells for collateral artery growth. Immunohistological staining demonstrated that BM-derived cells gather around collateral vessels, presumably to facilitate arteriogenesis. In cerebral arteriogenesis, B1R antagonist treatment significantly inhibits collateral growth, and application of B1R agonist can stimulate cerebral arteriogenesis therapeutically. The creation of an AV shunt to therapeutically enhance collateral flow in the rat hind limb after FAO demonstrated how B1R inhibition dramatically blocks macrophage/neutrophil migration into the perivascular tissue as well as the collateral diameter increase. Finally, an in vitro migration assay showed a strong enhancement of monocyte migration on stimulation with different B1R agonists.

Distal to the ligation with a vein. This shunt intervention increases shear stress, thereby promoting collateral artery growth and influx of leukocytes considerably, and thus making it highly suitable to quantify leukocyte transmigration into the collateral area. Staining for CD11b-positive and ED1-positive cells was performed on sections taken from the collateral region of rats 3, 7, and 14 days after FAO and AV shunt formation (Figure 6A–F). Staining for CD11b and ED1 showed clearly a marked invasion of leukocytes and macrophages into the perivascular tissue around collateral arteries. Rats treated with FAO over time with the B1R antagonist R715 demonstrated considerably lower numbers of leukocytes in the perivascular region, particularly the number of CD11b+/ED1+ cells. Angiographies showing the collateral area of the rat hind limb 7 days after FAO and AV shunt intervention demonstrated the strong promotion of collateral artery growth (Figure 6G–K). Treatment over time with B1R antagonist R715 markedly inhibited arteriogenesis, which corresponded with the reduced number of migrated leukocytes.

**B1R Agonist Treatment Increase Human Acute Monocytic Leukemia Cell Line Monocyte Migration**

Taking into account the pivotal role of BM-derived cells and monocyte vascular transmigration for arteriogenesis, we tried to answer the question whether stimulation of B1R expressed on monocytes may result in increased cell migration. Therefore, human acute monocytic leukemia cell line monocyte migration assays were performed by the application of 2 B1R agonists (Lys-[Des-Arg9] bradykinin and Sar-[D-Phe8]-des-Arg9 bradykinin), as well as by pretreatment with the B1R antagonists R715 (Figure 7). Migration assays demonstrated that monocyte migration is strongly increased in response to both B1R agonists and actually comparable with the MCP-1-positive control. However, pretreatment with the selective B1R antagonist R715 completely inhibited B1R agonist-induced monocyte migration.
reduced collateral vessel diameters 7 days after FAO (Figure 2). Perfusion measurements identified reduced arteriogenesis in B2R−/− mice 7 days after FAO; however, B2R−/− mice recovered from FAO within 3 weeks and showed similar blood flow as for WT mice. Presumably, B2R deficiency has only a minor effect on arteriogenesis, because another system may counterbalance the B2R deficiency and the B1R or B2R deficiency delays the subsequent B1R activation. Treatment with B1R antagonists leads to reduced collateral remodeling, as observed in bradykinin receptor-deficient mice, and proved that arteriogenesis can be modulated by pharmacological intervention into the bradykinin receptor signaling pathway.

Therapeutic modulation of cerebral arteriogenesis in the rat brain after 3-VO was determined by measuring diameters of the PCA and the change of blood flow on elevation in CO₂ level. Growth of cerebral collateral circulation (the posterior cerebral artery) 3 weeks after 3-VO is significantly inhibited by a B1R antagonist (Figure 4). Consequently, CVRC declines dramatically in rats treated with a B1R antagonist, and blood perfusion reserve does not recover within 3 weeks after 3-VO. Furthermore, cerebral arteriogenesis can be therapeutically stimulated by B1R agonist treatment. We previously demonstrated that stimulation with GM-CSF strongly enhances arteriogenesis and collateral flow in the rat brain 3-VO model. Here, we demonstrate for the first time to our knowledge a positive therapeutic effect on cerebral collateral artery growth by stimulation with a B1R agonist (Figure 5). In conclusion, a considerable impact on collateral perfusion was demonstrated for the B1R, which is weakly expressed in physiological conditions but is induced under inflammatory conditions in a variety of tissues.

In this context, results obtained in the hind limb and brain arteriogenesis models for the B2R showed that B2R is involved in active collateral remodeling, but only to a minor degree. Despite B2R being ubiquitously and constitutively expressed, ligand binding results in rapid desensitization and receptor degradation; therefore, B2R may be considered a less attractive target for therapeutic intervention.

Previously, modulation of angiogenesis in the context of the kallikrein kinin system was reported. However, arteriogenesis differs considerably from angiogenesis; we demonstrate that during arteriogenesis, preexisting collateral arteries grow in diameter (3-VO model), rather than capillaries, and are formed de novo and increase in number. Whereas angiogenesis depends on local ischemia, arteriogenesis is induced by increased hemodynamic shear forces.
growth. Grafting of BM from GFP reporter mice into B1R mice. Therefore, the expression of B1R on immune cells noticed for collateral blood flow in untreated B1R mutant mice, whereas transplantation of WT BM into B1R mice nearly rescued the phenotype noticed for collateral blood flow in untreated B1R mutant mice. Therefore, the expression of B1R on immune cells seems to be the pivotal determinant during collateral artery growth. Grafting of BM from GFP reporter mice into B1R mice and WT mice demonstrated that GFP-positive cells do not incorporate into the smooth muscle cell layer in the endothelial cell layer and seem to gather near the collateral area. Different studies clarified that monocytes or neutrophils do not incorporate into the vascular wall and their effect relies on paracrine mechanisms, namely the secretion of growth factors and cytokines that promote vascular growth.5,27 Leukocyte numbers around collateral vessels were not found to be significantly different between GFP/B1R−/− and GFP/WT chimeras. This demonstrated that transmigration was independent of the donor phenotype, because GFP-positive BM-derived cells still carried functional B1R.

To determine the functional role of B1R for leukocyte migration, rats were subjected to FAO, followed by surgical AV shunt formation. Eitenmüller et al21 demonstrated how collateral growth can be improved by connecting the femoral artery distal to the ligation with the femoral vein. AV shunt formation leads to a steep pressure gradient, which maximizes blood flow and shear stress within the collateral region. As a result, typical features of arteriogenesis such as leukocyte migration are augmented and can be studied in detail. Experiments using AV shunts such as performed with inhibitors of endothelial nitric oxide synthase efficiently showed mechanisms for modulating arteriogenesis.28 Likewise, our results obtained from the angiographies showed that under high shear conditions, arteriogenesis is reduced with B1R antagonist treatment (Figure 6I) and correlated with reduced leukocyte transmigration as presented in the histology. Immunostaining for CD11b (pan-leukocyte marker) showed a strong reduction in a number of positive cells in the collateral region of B1R antagonist-treated rats. Predominantly, staining for ED1 (monocyte marker) colocalized with CD11b, indicating that most cells belong to the monocyte/macrophage subtype (Figure 6). However, CD11b+ and ED1− cell staining indicated invasion of further subtypes of leukocytes into the perivascular space, presumably neutrophils.

Different studies demonstrated the important role of B1R or B1R antagonists for leukocyte recruitment and show partial or complete inhibition of recruitment in B1R−/− mice.29,30 Recent in vivo and in vitro studies suggested that B1R activation on neutrophils in inflammatory situations directly promotes neutrophil adhesion and migration into the perivascular tissue.31,32 B1R expression has been reported on macrophages as well; however, their role for recruitment of mononuclear cells seem to be controversial. By performing monocyte migration assay, we showed clearly that a B1R agonist promotes monocyte chemotaxis, which equals MCP-1 effects (Figure 7). Circulating monocytes have been shown to be the pivotal cellular factor during adaptive arteriogenesis, accelerating the remodeling process of collateral arterial tissue.33 Relevance was tested by MCP-1 infusion into the rabbit hind limb and it was proven in osteopetrotic mice that a genetic lack of monocytes results in almost no ability to increase collateral diameters.18,34

To the best of our knowledge, our study is the first showing direct evidence for the role of bradykinin receptor signaling in arteriogenesis. In the brain and the periphery, inhibition with bradykinin receptor antagonists lead to considerable modulation of arteriogenesis and provide direct evidence for therapeutic stimulation of cerebral collateral artery growth by application of B1R agonists. Recently, a predisposition to atherosclerosis in mice defi-
cient in kinin B1 receptor and apolipoprotein E was reported, indicating that B1R might be beneficial to treat atherosclerosis. This is a promising finding and might open an avenue for therapies designed to enhance collateral growth and to inhibit the development and progression of atherosclerosis. Furthermore, we demonstrate that functional B1R signaling on BM-derived cells is of major importance for collateral artery growth and hind limb arteriogenesis, and that B1R signaling governs monocyte recruitment in vitro. Data suggest that the structural adaptation of collateral arteries is modulated by B1R signaling by a molecular linkage of changes in hemodynamic forces (artery occlusion) and the activation of inflammatory processes.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Arteriogenesis, the growth of preexisting collateral anastomoses, is regulated by changes in hemodynamic forces (eg, increase in pulsatile shear stress on artery occlusion) and inflammatory processes (eg, monocyte invasion).
- The bradykinin receptor system is important for vascular biology (eg, increasing the level of tissue kallikrein and kinins protects from vascular ischemia). All components of the kallikrein kinin system can be produced locally in the vasculature.
- Bradykinin 1 receptor (B1R) is expressed upon inflammation of vascular cells (endothelium) and bone marrow (BM)-derived cells such as monocytes, dendritic cells, and neutrophils. B1R expression is relevant for neutrophil transmigration into the perivascular tissue.

What New Information Does This Article Contribute?

- Bradykinin receptors are relevant for flow-induced collateral artery growth (arteriogenesis) in the periphery as well as the brain. B1R in particular has a significant long-term impact on arteriogenesis.
- Collateral artery growth is critically dependent on the expression of B1R on BM-derived cells. Inhibition of B1R signaling (either in a molecular loss of function model or by pharmacological inhibition) significantly reduces monocyte transmigration during arteriogenesis.

- Collateral artery growth can be pharmacologically modulated by application of B1R agonists, which significantly stimulates arteriogenesis. In contrast, application of B1R antagonists significantly inhibits arteriogenesis in the brain and periphery, respectively.

Collateral artery growth (arteriogenesis) constitutes an effective biological rescue mechanism against detrimental effects of arterial stenosis. Whereas angiogenesis results in the de novo formation of capillaries locally at sites of ischemia, arteriogenesis refers to the pulsatile flow-induced growth of small preexisting collateral arteries into large functional conductance arteries, which circumvent sites of occlusion as biological bypasses. Here, we report that collateral artery growth is modulated by bradykinin receptor signaling and is particularly dependent on B1R expression on BM-derived cells. We demonstrate for the first time to our knowledge that B1R provides a molecular link between hemodynamic changes that precede adaptive arteriogenesis and the inflammatory processes that lead to perivascular monocyte invasion. Finally, we show that cerebral arteriogenesis can be therapeutically stimulated or inhibited by B1R agonist/antagonist treatment in mice. Collateral circulation can significantly influence the occurrence and size of cerebral infarction. Stimulating arteriogenesis constitutes a potential novel therapeutic option for cardiovascular disease.
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Arteriogenesis is modulated on bradykinin receptor signaling

Online supplement

1. Detailed Material and Methods section

Animal Experiments

Experiments were carried out in accordance with the German Law for the Protection of Animals and the National Institute of Health Guidelines for Care and Use of Laboratory Animals.

Femoral artery occlusion model and assessment of blood perfusion using microspheres-based technique

In B1R-/−, B2R-/- mice and wild-type (C57BL/6J) mice, the right femoral artery was ligated proximal and distal to the lateral caudal femoral artery as previously described. Anesthesia was induced by 100 mg/kg ketamine and 10 mg/kg xylazine. 7 days (n=10 per group) and 21 days (n=8 per group) post femoral artery occlusion collateral occlusion was measured to determine collateral artery growth using a microsphere-based perfusion technique as previously. In brief, the aorta was cannulated with a catheter proximal to the aortic bifurcation, and maximal vasodilatation of the collateral arteries was adjusted by adenosine perfusion (0.03 mg/mL/min per each microsphere solution). Subsequently, hind limb collateral networks were perfused with fluorescent microspheres of different colours at different pressure levels. Fluorescent microspheres were isolated, counted by FACS analysis, and normalized against a determined number of blue microspheres. Perfusion restoration is expressed as percentage of the perfusion in the occluded right hind limb relative to the non-occluded left hind limb. For pharmacological inhibition of peripheral arteriogenesis mice were subjected to FAO and treated as following: Control: 0.25ml/d saline; B2R antagonist Icatibant: 1mg/kg/d s.c.; B1R antagonist: R715 (50nmol/kg/d) s.c. by osmotic minipumps (Alzet, Model 1007D).

Femoral artery occlusion model and assessment of blood flow with LDF

FAO was performed in 12-week-old BR1 KO and BR2 KO mice as described. Non-invasive laser Doppler (LDF) imaging technique was used for repetitive quantification of hindlimb blood flow before FAO, immediately post FAO, at day 1, day 3, day 7, day 14 and day 21 post FAO. LDF measurement depends on the low-power light from a monochromatic stable laser beam directed on the mouse hindlimb (830 nm, laser diode, model LD12-HR, Moor Instruments, Millwey, Axminster, UK). Light is scattered by red blood cells and is photo-detected, depending on the Doppler principle. Data were processed to provide a blood flow measurement, and were describe in ‘flux’, a quantity that is proportional to the product of the average speed of the blood cells and the blood volume. Flux is expressed in arbitrary ‘perfusion units’ (indicated in supplemtal figure 1) and is calculated using the first moment of the power spectral density.

Femoral artery occlusion model and histology

Vessel diameters and collateral vessel density were determined 7 days post FAO. Adductor muscles (upper thigh) and the quadriceps muscle (muscles below/laterally of the femoral artery) were excised and embedded in paraffin for histology. Muscles were cut starting up to 2 mm proximal to the femoral artery occlusion into 5 µm slides and sections were stained using antibodies against SM-Actin (SM-Actin conjugated to Cy3 or FITC, Sigma). SM-actin positive vessels from three sections separated by 100 µm from 3 animals each were analysed. All SM-actin positive vessels within the area of interest were considered as putative collaterals (no capillaries), and numbers and diameter were averaged and determined from digitized images using ImageJ as software analysis tool. 3 days post FAO Mm. quadriceps and Mm. adductores of B1R -/- mice and mice chimeras (GFP- BL/6J, into WT and B1 KO C57BL/6J) were harvested for monocyte and BM-derived cell-staining. The following antibodies were used: Goat Anti-Mouse GFP (Abcam), Rabbit aGoat-Biotin (Vector Laboratories), Donkey aGoat-Cy2 (Jackson Lab), Mouse anti aSMC-Cy3 (Sigma), Rat aMouse-CD68-
Alexa 647 (AbSerotec). Nuclei were stained either with haematoxylin (Roth) or with Hoechst (Molecular Probes). Sections were viewed with a fluorescence and brightfield–microscope (Olympus FSX 100).

Measurement of hemodynamic perfusion reserve and diameters in the 3-VO rat brain model
The non-ischemic brain hypoperfusion 3-vessel occlusion model (3-VO) was applied to induce arteriogenesis in the brain by occlusion of both vertebral arteries, and the left carotid artery (CA) was carried out as previously described in detail. Ipsilateral blood flow measured by laser Doppler flowmetry (Perimed, Sweden) decreased to 50 per cent and persists. Successful 3-VO surgery without detectable neurological damage (immunohistological evaluation) has been described in detail by Busch et al. and Schneeloch et al. In brief, anesthesia was induced by injection intraperitoneal of 50 mg/kg ketamine and 4 mg/kg xylazine and maintained by inhalation of 2% to 4% isoflurane in oxygen. Local analgesia was achieved by ropivacaine. Cerebral blood flow (CBF) was measured to ensure cerebral hypoperfusion, by placing a laser probe directly on the skull bone above the frontoparietal cortex. Cerebrovascular anatomy was studied after maximal vasodilatation (50 mg/kg papaverine) by a modification of the post-mortem latex perfusion method of Maeda et al. and external PCA diameter was measured with a stereozoom microscope (Leica MZ6). The cerebrovascular hemodynamic reserve capacity (CVRC) was measured by assessing cerebrovascular reactivity. Rats were anesthetized seven versus 21 days after 3-VO, and veins were catheterized for application of acetazolamide (30 mg/kg Diamox, Sanofi Aventis). CVR was measured by LDF, and blood samples were taken before and after CVR for blood gas analysis. For pharmacological inhibition of cerebral arteriogenesis BR2 antagonist Icatibant (Jerini, 0.5 mg/kg/d) and BR1 antagonist Des-(Arg9,Leu8)-Bradykinin (Bachem, 50nmol/kg/d BR1) were injected s.c using osmotic minipumps (Alzet, Model 2004). Collateral growth in the brain was evaluated in rats by measuring diameters and CVRC 21 days post 3-VO. To evaluate the effects on therapeutically induced arteriogenesis via BR1, rats were treated subcutaneously with BR1 agonist Sar[D-phe8]-des-Arg9-Bradykinin (Tocris Bioscience 1mg/kg/d), and collateral growth was measured seven days post 3-VO.

Bone Marrow transplantation
Bone marrow transplantation was performed as described. First recipient mice (n=8) were lethally irradiated at a dose of 1,100 cGray (split dose) at the age of 8 months: mice were lethally irradiated at a dose of 700 cGray (1030 monitor units). Second, mice were reconstituted with 12 x10⁶ donor bone marrow cells via the tail vein. Bone marrow transplantation from donor mice (C57BL/6J) to recipient mice (C57BL/6J) was performed as follows: 1. B1R -/- (donor) in B1R -/- (recipient), WT (donor) in WT (recipient), B1R -/- (donor) in WT (recipient), WT (donor) in B1R -/- (recipient). Third, animals were allowed to recover for 6 weeks and femoral artery ligation was performed with measurement of collateral perfusion seven days post FAO.

Rat hind-limb shunt experiment and histology
Sprague Dawley Rats (weight 330g) (Harlan Winkelmann, Borchen, Germany) were subjected to a combined shunt treatment and i.p. implantation of osmotic minipumps (Charles River Laboratories, Sulzfeld, Germany ) containing R-715 (tocris bioscience, 70μg/kg bodyweight/day) for 3d, 7d or 14d (n=3). Phosphate buffered saline served as control. Anaesthesia was accomplished by intraperitoneal injection of Ketaminhydrochloride (100mg/Kg bodyweight) and Xylazine (4mg/Kg bodyweight). Postoperative analgesia was assured by subcutaneous injection of 0,03 mg/kg Kg Buprenorphin during the operation and again after 12h. At each time point Mm. quadriceps and Mm. Adductores were harvested for immuno-staining. The following antibodies were used: mouse anti-rat CD68-Alexa Fluor 647 (AbD serotec), mouse anti rat CD11b (AbD serotec), Cy2-conjugated anti-smooth muscle actin (Actg2) (Sigma), Cy3-conjugated donkey anti-mouse (Jackson ImmunoResearch). Nuclei were stained with DAPI (Molecular Probes). Sections were viewed with a confocal microscope (Leica SP5). For angiographic collateral growth was visualized seven days after treatment by post mortem angiographies via infusion of gelatine based barium sulphate contrast medium as described.
Monocyte migration assay

The human monocytic THP-1 cell line was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). Human monocytic THP-1 cell line (DSMZ) were cultivated in RPMI 1640 (10% FCS, 2mmol/L of L-glutamine, 100U/mL of penicillin, and 100μg/mL of streptomycin) at 95% relative humidity and 5% CO2 at 37°C. Assays were performed using transwell chambers (Becton Dickinson) with a gelatin-coated (0.2%) polycarbonate membrane with 8μm pores. Cells were pre-stimulated with 1ng/ml IL-1β (for activation of B1R expression) with or without 10nM antagonist R715 (Tocris Biosciences) for 30min at 37°C. Migration experiment was performed toward a gradient of B1R agonists Lys-[Des-Arg9]-Bradykinin (10nM, Bachem), Sar-[D-Phe8]-des-Arg9-Bradykinin (10nM, Tocris Bioscience) and MCP-1 control gradient (10ng/mL, Chemicon) for 2.5h. THP-1 monocyte migration to the lower surface of the filter was determined microscopically by counting numbers per high power field (HPF, magnification 320×). Three randomly chosen HPFs were counted per filter, and each experiment was performed three times.

Data Analysis

Student’s t-test or ANOVA followed by t tests using the Bonferroni was used for statistical analysis. P-values were adjusted using a false discovery rate procedure to achieve experiment-wide significance of 0.05. All results are presented as mean and standard error of the mean (SEM). Data were analyzed with PASW (SPSS 18) and graphs were made using Prism 4.
Supplemental Figure I

(A) Schematic representation of the mice hindlimb collateral network. (FA: femoral artery, LCFA: lateral caudal femoral artery, SA: saphenous artery). (B) Occlusion of the femoral artery results in a positive outward remodeling of collateral arteries and recovery of blood flow within 7 days. (C, D) Administration of the B1R antagonist R715 (B1Ri) and B2R antagonist Icatibant reduces hindlimb perfusion. *, p<0.05; **, p<0.01.
Supplemental Figure II
(A) Schematic representation of arterial adaptations in the 3-VO model. (B) Angiography of the circle of Willis in controls, and B2R antagonist treated rats. Rats treated with B2R antagonist icatibant demonstrated 3 weeks post 3-VO a smaller PCA diameter increase as compared to untreated rats (207 µm), furthermore CVRC in B2R antagonist treated rats is significantly reduced.
Supplemental References


