Blockade of Bradykinin Receptor B1 but Not Bradykinin Receptor B2 Provides Protection From Cerebral Infarction and Brain Edema

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Background and Purpose—Brain edema is detrimental in ischemic stroke and its treatment options are limited. Kinins are proinflammatory peptides that are released during tissue injury. The effects of kinins are mediated by 2 different receptors (B1 and B2 receptor [B1R and B2R]) and comprise induction of edema formation and release of proinflammatory mediators.

Methods—Focal cerebral ischemia was induced in B1R knockout, B2R knockout, and wild-type mice by transient middle cerebral artery occlusion. Infarct volumes were measured by planimetry. Evan’s blue tracer was applied to determine the extent of brain edema. Postischemic inflammation was assessed by real-time reverse-transcriptase polymerase chain reaction and immunohistochemistry. To analyze the effect of a pharmacological kinin receptor blockade, B1R and B2R inhibitors were injected.

Results—B1R knockout mice developed significantly smaller brain infarctions and less neurological deficits compared to wild-type controls (16.8 ±4.7 mm³ vs 50.1 ±9.1 mm³, respectively; \(P<0.0001\)). This was accompanied by a dramatic reduction of brain edema and endothelin-1 expression, as well as less postischemic inflammation. Pharmacological blockade of B1R likewise salvaged ischemic tissue (15.0 ±9.5 mm³ vs 50.1 ±9.1 mm³, respectively; \(P<0.01\)) in a dose-dependent manner, even when B1R inhibitor was applied 1 hour after transient middle cerebral artery occlusion.

In contrast, B2R deficiency did not confer neuroprotection and had no effect on the development of tissue edema.

Conclusions—These data demonstrate that blocking of B1R can diminish brain infarction and edema formation in mice and may open new avenues for acute stroke treatment in humans. (Stroke. 2009;40:285-293.)

Key Words: bradykinin ■ edema ■ endothelin-1 ■ inflammation ■ stroke

Brain edema is a frequent cause of secondary infarct growth and subsequent deterioration of neurological symptoms.1,2 Recently, the life-saving effect of decompressive surgery has been established in patients with malignant middle cerebral artery (MCA) infarction leading to large brain edema.3 However, craniectomy is very invasive and so far no medication, eg, steroids or hyperosmolaric solutions (eg, mannitol, sorbitol) has proven to effectively reduce brain edema.4-6 The molecular mechanisms underlying edema formation in ischemic stroke are largely unknown. The kallikrein–kinin system plays an important role in the regulation of vascular permeability and has been invoked in edema formation.7,9 Kinins (eg, bradykinin, kallidin) are biologically highly active proinflammatory peptide hormones that are released by kallikreins from their precursors, kininogens, during various kinds of tissue injury, including focal and global brain ischemia.10-13 All components of the so-called kallikrein–kinin system have been identified in the brain.14-16 The cellular effects of kinins are mediated by 2 different bradykinin receptors. B1R is expressed at low levels under normal conditions but is induced selectively during inflammation by soluble mediators, eg, IL-1β or tumor necrosis factor-α. In contrast, B2R is constitutively expressed and mediates the majority of bradykinin physiological effects.8,17-20 Activation of B1R and B2R triggers inflammatory processes in the target organ such as the release of proinflammatory cytokines or the attraction of immune cells, as well as increased vascular permeability.8,21,22

In the present study, we analyzed in parallel the effect of B1R and B2R deficiency or blockade on infarct size, edema formation, and inflammatory processes in a mouse model of focal ischemic stroke. We could show that inhibition of B1R but not B2R protects from ischemic brain damage and is associated with less edema formation and attenuation of the local postischemic inflammatory response.

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vasculature was assessed by extravasation of Evan’s blue tracer (Sigma Aldrich; n=4/group; online).

**Invasive Hemodynamics**

For invasive hemodynamics mice were anesthetized with 2.5% isoflurane and catheterized via the right carotid artery with a high-fidelity 1.4-F Millar micro-tip catheter as described.23 Systolic and diastolic blood pressure and heart rate were measured 10 minutes after intravenous application of 0.9% NaCl (controls), R-715 (1 mg/kg body weight), and Hoe-140 (0.2 mg/kg body weight; n=5/group). Hemodynamic data were digitized via a MacLab system (AD Instruments) connected to an Apple G4 PowerPC computer (Apple Computer, Inc) and analyzed.

**PCR Studies**

Ischemic infarctions mostly spared the neuroect of B1R KO mice (Figures 2, 3). Therefore, basal ganglia (instead of total hemispheres), which were regularly included in the ischemic infarctions also in B1R KO mice (Figures 2,3), were dissected from the brains in WT and B1R KO mice at 4 hours (n=8 per group) and 24 hours (n=8 per group) after tMCAO. This mode of sampling excluded biased PCR results because of different infarct sizes between the groups. Real-time reverse-transcription polymerase chain reaction against murine B1R and B2R, IL-1β, transforming growth factor β-1, tumor necrosis factor-α, and endothelin-1 was performed using routine procedures (online).

**Histology and Immunohistochemistry**

Formalin-fixed brains embedded in paraffin from WT and B1R and B2R KO mice at day 1 after tMCAO (n=6/group) were cut into 4-μm-thick sections 0.5 mm anterior from bregma and stained with hematoxylin and eosin. Invading immune cells (T cells, macrophages, neutrophilic granulocytes) within the infarcted basal ganglia were detected by routine immunohistochemistry and quantified (online).

**Statistical Analysis**

Data are expressed as mean±SD. For statistical analysis PrismGraph 4.0 (GraphPad Software) software package was used. Brain edema formation in WT and B1R KO animals as measured by extravasation of Evan’s blue (Figure 4B) was compared using the nonparametric Mann-Whitney test. For comparison of survival curves (Figure 2B), the log-rank test was used. All other data were tested for Gaussian distribution with the D’Agostino and Pearson omnibus normality test and then analyzed by Bonferroni-corrected 1-way ANOVA or 2-way ANOVA (reverse-transcription polymerase chain reaction data). P<0.05 was considered statistically significant.

**Results**

**B1R Blockade Protects From Focal Cerebral Ischemia**

In a first set of experiments, we analyzed the mRNA expression pattern of B1R and B2R in ischemic brains from C57BL/6 mice over time (Figure 1). Both receptors were constitutively expressed at low levels in sham-treated animals. B1R and B2R mRNA expression significantly increased ~9-fold after 4 hours (P<0.0001), and in the case of B1R further increased until 24 hours (16.4±2.9-fold induction; P<0.01; Figure 1). Taken together, these data indicate that both kinin receptors are expressed in the murine brain and, just like their ligand bradykinin,12 undergo induction after focal cerebral ischemia, suggesting a functional role of the kalikrein–kinin system in ischemic stroke.

To investigate the functional role of B1R and B2R in experimental cerebral ischemia 60 minutes of tMCAO was induced in WT and B1R and B2R KO mice. Twenty-four
hours after reperfusion, the infarct volumes in B1R KO animals assessed by TTC staining were dramatically reduced to ~30% of the infarct volumes in WT mice (Figure 2A; 16.8±4.7 mm³ vs 50.1±9.1 mm³, respectively; P<0.0001). Importantly, infarct volumes in B1R-null mice did not further increase between day 1 and day 3 (16.8±4.7 mm³ vs 21.3±4.3 mm³, respectively; P>0.05) and remained significantly smaller compared to WT controls (21.3±4.3 mm³ vs 54.2±7.1 mm³, respectively; P<0.01; Figure 2A). The reduction in infarct size was functionally relevant, because the Bederson score assessing global neurological function (0.6±0.9 vs 2.9±0.8, respectively; P<0.0001) and the grip test that specifically measures motor function and coordination (2.7±0.9 vs 4.1±0.7, respectively; P<0.0001) were significantly better in B1R-deficient mice than in WT mice at day 1 after tMCAO (Figure 2B). In contrast, lack of B2R did not confer neuroprotection after focal cerebral ischemia because the infarct volumes and neurological deficits in B2R KO mice 24 hours after reperfusion were comparable to those in WT animals (Figure 2A,B). Moreover, B1R blockade significantly increased long-term survival in mice after tMCAO. Whereas 6 of 10 WT mice and 4 of 10 B2R KO died
5 days after infarct induction, 100% (10/10) of B1R-deficient mice survived (P<0.05; Figure 2B). Consistent with the TTC stains, histological analysis revealed massive ischemic infarction of the basal ganglia and neocortex in WT animals, but only limited infarctions that were mostly restricted to the basal ganglia in B1R KO mice (Figure 2C).

As congenital B1R deficiency protects mice from ischemic stroke, pharmacological targeting of B1R should provide similar protection. To test the protective potential of B1R inhibition, WT mice received 2 different doses of R-715 1 hour after the induction of tMCAO. Whereas 500 μg R-715/kg body weight had no significant impact on infarct size and neurological status, the higher dose of 1 mg R-715/kg body weight markedly reduced the infarcted brain volume at 24 hours compared to untreated controls (15.0±9.5 mm³ vs 50.1±9.1 mm³, respectively; P<0.01; Figure 3A). At this concentration, R-715–treated mice showed a significantly better Bederson (1.3±0.7 vs 2.9±0.8, respectively; P<0.01) and grip test score (2.7±0.9 vs 4.3±0.7, respectively; P<0.01). In contrast, the selective B2R inhibitor Hoe-140 did not confer neuroprotection when applied in the acute phase after ischemic stroke (Figure 3A and 3B).

It is known that the kallikrein–kinin system plays an important role in blood pressure regulation that could influence edema formation and infarct volume after ischemic stroke. We therefore analyzed the effect of the pharmacological B1R and B2R inhibitors R-715 and Hoe-140 on blood pressure and heart rate in C57BL/6 mice. Ten minutes after a single injection of R-715 (1 mg/kg body weight), a dosage that was neuroprotective when applied 1 hour after tMCAO (Figure 3A,B), or Hoe-140 (0.2 mg/kg body weight), no significant differences in systolic (86.4±8.0 mm Hg vs 83.8±12.3 mm Hg or 85.7±6.5 mm Hg; P>0.05) or diastolic blood pressure (55.6±5.6 mm Hg vs 55.2±14.6 mm Hg or 56.4±6.7 mm Hg; P>0.05) and heart rate (491.4±70.2 min⁻¹ vs 487.4±23.3 min⁻¹ or 469.4±62.7 min⁻¹; P>0.05) were found compared to vehicle-treated controls (Supplemental Figure II). This is in line with our previously published findings showing that B1R KO mice and animals deficient in both B1R and B2R are normotensive.24–26 These results exclude that blood pressure alterations caused the different infarct volumes and edema formation after B1R or B2R blockade in experimental stroke.
B1R Deficiency Reduces Brain Edema Formation After Focal Cerebral Ischemia

To further investigate the mechanism by which B1R deficiency protects from cerebral ischemia, the extent of brain edema formation as measured by the free water content of the ischemic hemisphere was assessed (Figure 4A). At day 1 after tMCAO, brain water content in the ischemic hemisphere of B1R KO mice was significantly reduced compared to WT mice, Bonferroni post hoc test. B, Representative corresponding coronal brain sections (left) of WT and B1R KO mice at day 1 after tMCAO after injection of Evan’s blue. Note that Evan’s blue extravasation was even absent in areas where infarction was present in B1R KO mice (basal ganglia; dotted line). B, Volume of Evan’s blue extravasation (right) determined by planimetry in the ischemic hemisphere of WT and B1R KO mice 24 hours after 60 minutes of tMCAO. **P<0.01, 1-way ANOVA compared to WT mice, Bonferroni post hoc test. B, Representative corresponding coronal brain sections (left) of WT and B1R KO mice 24 hours after tMCAO. C, Relative gene expression of endothelin-1 in the ischemic basal ganglia of WT and B1R KO mice 4 and 24 hours after tMCAO compared to sham-treated controls (n=4/group). **P<0.01, 2-way ANOVA, Bonferroni post hoc test.

B1R Deficiency Reduces Postischemic Inflammation in the Brain

Several studies have demonstrated that B1R deficiency modulates the inflammatory response under various pathophysiological conditions.34,35 We therefore analyzed the mRNA expression levels of several prototypic proinflammatory and antiinflammatory cytokines in the brains of WT and B1R KO mice after tMCAO over time (Figure 5). The amount of IL-1β mRNA in the infarcted basal ganglia of WT mice was elevated already 4 hours after tMCAO and massively increased until 24 hours in WT animals. In contrast, no significant endothelin-1 induction was observed in B1R KO mice at any time point after tMCAO, and endothelin-1 expression was significantly lower after 24 hours compared to WT controls (2.6±0.6-fold induction vs 5.2±6.3 induction, respectively; P<0.001).

Figure 5. Relative gene expression of IL-1β, transforming growth factor β-1 (TGFβ-1), and tumor necrosis factor-α (TNFα) in the ischemic basal ganglia of WT and B1R KO mice 4 and 24 hours after tMCAO compared to sham-treated controls (n=8/group). **P<0.01, *P<0.05, 2-way ANOVA, Bonferroni post hoc test.
animals (2.4±0.41-fold induction vs 0.9±0.2-fold induction, respectively; \( P<0.05 \)) and transforming growth factor β-1 expression sustained until day 1. Tumor necrosis factor-α levels did not differ between WT and B1R KO mice at any time point (Figure 5).

To further analyze the extent of the inflammatory response, we quantified the amount of immune cells invading the ischemic basal ganglia by immunohistochemistry (Figure 6A,B). Twenty-four hours after the induction of tMCAO, the majority of neutrophilic granulocytes were detected in the brain parenchyma of WT mice (Figure 6A, arrows), whereas some of them were still in the process of evading cerebral blood vessels (Figure 6A, arrowheads). In contrast, significantly less neutrophilic granulocytes (14.0±4.6 vs 29.0±11.3; \( P<0.05 \)), macrophages (14.0±5.3 vs 32.6±11.1; \( P<0.001 \)), and T cells (1.0±0.6 vs 4.0±1.0; \( P<0.0001 \)) invaded the ischemic basal ganglia of B1R KO mice compared to WT controls (Figure 6A,B).

**Discussion**

The main finding of the current study is that B1R blockade profoundly reduces infarct volumes and neurological deficits after experimental ischemic stroke in mice by salvaging the neocortex. Within the basal ganglia that were similarly affected by infarctions in WT and B1R KO mice, brain edema formation was less in B1R KO mice and the local inflammatory response was attenuated.

Although B1R and B2R have been detected in the rodent central nervous system, little is known about the expression of kinin receptors in the mouse brain after experimental stroke.8,18 Real-time reverse-transcription polymerase chain reaction revealed that both receptors are profoundly induced at very early stages after tMCAO. The time course of B1R and B2R induction in our study thus paralleled the formation of their ligand bradykinin after tMCAO in mice,12 suggesting a functional relevance for the kallikrein–kinin system in the acute phase of ischemic stroke. The cells expressing B1R and B2R in the (infarcted) brain remain to be further characterized but might be neurons, microglia, and endothelial cells.8

Previous studies could show that the blockade of the B1R protects from ischemic tissue damage, eg, in models of myocardial or renal infarction.34–37 This protective effect was accompanied by reduced ischemia-related inflammation. We further extend these findings by demonstrating that B1R is also critically involved in infarct development in the brain. Importantly, decreased stroke volumes in B1R-deficient mice were accompanied by a significant reduction in neurological deficits and mortality, and the pharmacological B1R blockade was still effective when performed 1 hour after the induction of stroke. This underlines the functional significance of this novel approach and indicates a potential suitability of selective B1R inhibitors13 for clinical application during the acute phase of ischemic stroke in humans.

The kallikrein–kinin system plays an important role in regulating blood pressure,8 which in turn could influence infarct volume after ischemic stroke. The pharmacological blockade of B1R and B2R had no effect on blood pressure and heart rate in our study. This is in line with our previous findings demonstrating that B1R KO mice and animals deficient in both kinin receptors (B1R/B2R double KO mice) are normotensive.24–26 Moreover, B1R antagonists did not change blood pressure in normotensive rats.38,39 These results indicate that blood pressure effects cannot account for the profound stroke protection in B1R-deficient mice or after pharmacological blockade of B1R.

Besides preventing infarct development within the neocortex, brain edema formation within areas of infarction in the basal ganglia was also reduced after blockade of B1R, as...
indicated by lack of Evan’s blue extravasation regularly seen in WT mice. Brain edema is considered an important secondary step in lesion development after stroke and reaches its maximum between day 1 and day 3.12 Interestingly, induction of endothelin-1 transcripts was nearly absent in the ischemic basal ganglia of B1R-null mice. In contrast, high levels of endothelin-1 were found at 4 hours and 24 hours after experimental stroke in WT animals, thus confirming previous observations.40,41 Endothelin-1 has been shown to be critically involved in regulating vascular integrity and edema formation under various pathophysiological conditions, including ischemic stroke.42,43 Mice overexpressing endothelin-1 developed more brain edema and larger cerebral infarctions after tMCAO.44 Moreover, pharmacological blockade of the endothelin type A receptor attenuated ischemic brain injury, edema formation, and blood–brain barrier disruption in rats.40,45 Most interestingly, high serum levels of endothelin-1 have very recently been shown to predict severe cerebral edema in patients with acute ischemic stroke after recombinant tissue plasminogen activator treatment.46 The molecular pathways that link the kallikrein–kinin system and endothelin-1–driven mechanism in ischemic stroke need to be further established.

The second mechanism by which B1R deficiency probably conveyed neuroprotection after focal cerebral ischemia was attenuation of the local inflammatory response.47 Twenty-four hours after tMCAO, the majority of invading neutrophilic granulocytes was already detected in the brain parenchyma of WT mice, whereas some cells were still evading the cerebral blood vessels, which confirms previous observations.47 In contrast, the number of neutrophils in the ischemic basal ganglia of B1R KO mice was significantly reduced. Leukocyte invasion has been shown to contribute to stroke development, probably by impairing reperfusion of the cerebral microvasculature.48,49 In addition, less T cells infiltrated the infarcted brain of B1R-deficient animals. Interestingly, several recent studies could demonstrate that 24 hours after 60 minutes of tMCAO, brain infarct volumes were significantly reduced in mice lacking T cells.50,51 The mechanism by which T-cell deficiency mediates neuroprotection in experimental stroke seems to be at least partly mediated by reduced interactions between lymphocytes and platelets in the cerebral microvasculature, leading to improved tissue reperfusion. Finally, the expression of various soluble immune mediators after tMCAO was altered. B1R mutant mice expressed less IL-1β within the ischemic brain, whereas the amount of transforming growth factor β-1 was increased compared to WT controls. IL-1β is a prototypic proinflammatory cytokine that has been attributed to aggravate ischemic brain damage.47 In contrast, transforming growth factor β-1 exerts pleiotropic immune functions and has been shown to mediate neuroprotection in different stroke models, eg, by augmenting antiapoptotic mechanisms.52 The cells producing these cytokines after tMCAO need to be further characterized but might include neurons, microglia, or invading immune cells.47

Previous investigations using B2R-deficient mice in experimental stroke produced contradictory results. Gröger et al 12 reported that B2R KO mice are protected from cerebral ischemia after tMCAO. The reasons for this discrepancy to our results are not clear at present but may be explained by different study designs, eg, the relatively small sample size.12 Differences in the time of brain ischemia (45 min vs 60 min) obviously did not account for the divergent results, because we also observed identical infarct volumes after 45 minutes of tMCAO at day 1 in WT and B2R KO mice (not shown). In obvious contrast to Gröger et al,12 Xia et al53 postulated that posts ischemic brain injury is exacerbated in mice lacking the B2R. Numerous studies did not find a detrimental effect of B2R deficiency or blockade on stroke outcome.12,54,55 Infarct size in WT mice after 90 minutes of MCAO was surprisingly small in the study by Xia et al,53 whereas the B2R KO mice had developed infarcts that were the size that one would usually expect in WT mice after 90 minutes of tMCAO. We and others27,28,55,56 could demonstrate that 60 minutes of occlusion of the MCA already causes infarct volumes between 50 and 80 mm³ at day 1 after MCAO, eg, in C57BL/6, SV129, or Swiss mice. In the article by Xia et al,53 90 minutes of occlusion only led to infarct volumes of 12.8±7.3 mm³ in the WT group. These very small infarctions in the control group are highly suggestive for insufficient vessel occlusion and additional reasons for those discrepant findings have been addressed elsewhere,57,58.

Taken together, our present study provides evidence that blocking of the B1R can diminish brain edema and cerebral infarction in mice and may open new avenues for acute stroke treatment in humans in the future. Whether this novel strategy is also applicable in severe central nervous system pathologies other than ischemic stroke, such as intracranial bleeding or head trauma, needs to be further established.

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Disclosures
None.

References


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