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
Materials and Methods

Induction of Cerebral Ischemia

A total of 276 mice were used in this study. Animal experiments were approved by the Regierung von Unterfranken and conducted according to the recently published recommendations for research in mechanism-driven basic stroke studies. B1R and B2R knockout (KO) mice used in this study were described previously. B1R KO mice and animals deficient in both kinin receptors are normotensive. C57BL/6 wild-type (WT) mice (Charles River; Sulzfeld, Germany) served as controls. Focal cerebral ischemia was induced in 6- to 8-week-old mice by 60 minutes of transient middle cerebral artery occlusion (tMCAO), as described (Expanded Materials and Methods, available online at http://stroke.ahajournals.org). For polymerase chain reaction (PCR) studies, sham-treated C57BL/6 mice (n = 4) were used as controls. To determine the effect of pharmacological B1R inhibition in WT mice, the selective B1R inhibitor R-715 (Ac-Lys-[D-Arg9-Hyp3-Thi5-D-Tic7-Oci8-BK; 0.2 mg/kg body weight; Sigma Corporation) was administered intravenously 1 hour after tMCAO at a dosage of 500 μg/kg body weight (n = 10), and 1 mg/kg body weight (n = 10), respectively. For the selective blockade of B2R, Hoe-140 (D-Arg9-Hyp1-Thi15-d-Tic3-Oci6-BK; 0.2 mg/kg body weight; Sigma Aldrich) was injected (n = 10).

Laser Doppler flowmetry (Moor Instruments) was used in some animals (n = 6/group) to monitor regional cerebral blood flow in the MCA territory (supplemental Figure IA, available online at http://stroke.ahajournals.org) and the cerebral vasculature was assessed by perfusion with black ink (n = 3/group; supplemental Figure IB). Long-term survival of WT, B1R KO, and B2R KO mice after tMCAO was investigated over a period of 5 days (n = 10/group).

Assessment of Functional Outcome

Twenty-four hours after tMCAO, the modified Bederson score was used to determine global neurological function according to the following scoring system: 0, no deficit; 1, forelimb flexion; 2, decreased resistance to lateral push; 3, unidirectional circling; 4, escape (to the supports). String by forepaws and hind paws plus tail wrapped around string; 2, same as for 1, and attempts to climb onto string; 3, hangs onto string by 1 or both forepaws; 4, hangs onto string by forepaws and hind paws plus tail wrapped around string; and 5, escape (to the supports).

Determination of Infarct Size

Infarct volumes were quantified by planimetry from 2,3,5-triphenyl-tetrazolium chloride-stained (TTC; Sigma Aldrich) mouse brains, as described (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, undergo induction after focal cerebral ischemia, suggesting a functional role of the kallikrein–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

Figure 2. Infarct volumes, functional outcomes, and survival rates 24 hours and 3 days after tMCAO in WT, B2R KO, and B1R KO mice. A, Representative TTC stains (upper panel) of 3 corresponding coronal brain sections of WT and B2R and B1R KO mice at day 1 (left) and WT and B1R KO mice at day 3 (right) after tMCAO. A, Brain infarct volumes (lower panel) as measured by planimetry in WT and B2R and B1R KO mice at day 1 (left, n=10/group) and WT and B1R KO at day 3 (right, n=5/group) after tMCAO. B, Neurological Bederson score and grip test score (upper panel) as assessed at day 1 after tMCAO in WT and B2R and B1R KO mice (n=10/group). B, Survival rates of WT and B2R and B1R KO mice (n=10/group) (lower panel) until day 5 after tMCAO. C, Hematoxylin and eosin-stained sections of corresponding territories in the ischemic hemispheres of WT and B1R KO mice. Infarcts are restricted to the basal ganglia in B1R null mice but consistently include the neocortex in WT controls. Bar represents 100 μm. ***P<0.0001, **P<0.01, 1-way ANOVA compared to WT mice, Bonferroni post hoc test (infarct volumes and functional scores). *P<0.05, log-rank test (survival rates) compared to WT mice.
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/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 score (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. These results exclude that blood pressure alterations caused the different infarct volumes and edema formation after B1R or B2R blockade in experimental stroke.

![Figure 3](http://stroke.ahajournals.org/)

Figure 3. A, Representative TTC stains (upper panel) 24 hours after tMCAO of 3 corresponding coronal brain sections of mice treated with the B2R inhibitor Hoe-140 (0.2 mg/kg body weight) and the B1R inhibitor R-715 (500 µg/kg body weight or 1 mg/kg body weight, respectively) 1 hour after tMCAO compared to untreated controls. A, Brain infarct volumes (lower panel) at day 1 in mice treated with Hoe-140 (0.2 mg/kg body weight, n=10) and R-715 (500 µg/kg body weight, n=10) or R-715 (1 mg/kg body weight, n=10) 1 hour after tMCAO or untreated controls (n=10). B, Neurological Bederson score and grip test score as assessed at day 1 after tMCAO in untreated controls, R-715–treated or Hoe-140–treated mice (n=10/group). ***P<0.0001, **P<0.01, 1-way ANOVA compared to untreated controls, Bonferroni post hoc test.
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 (n=4/group). ***P<0.0001. Nonparametric Mann-Whitney test compared to WT controls. 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=8/group). Note that endothelin-1 expression was strongly induced in WT mice but not B1R KO mice after tMCAO. **P<0.01, 2-way ANOVA, Bonferroni post hoc test.

BIR 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 IL-1β 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 25.2±6.3 induction, respectively; P<0.001).

Figure 4. Extent of brain edema formation 24 hours after tMCAO in WT and B2R and B1R KO mice. A, Brain water content as a measure of brain edema of the ischemic hemisphere of WT (n=8) and B2R (n=4) and B1R KO (n=8) 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 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 (n=4/group). ***P<0.0001. Nonparametric Mann-Whitney test compared to WT controls. 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=8/group). Note that endothelin-1 expression was strongly induced in WT mice but not B1R KO mice after tMCAO. **P<0.01, 2-way ANOVA, Bonferroni post hoc test.

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 \( \beta-1 \) expression sustained until day 1. Tumor necrosis factor-\( \alpha \) 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. 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, 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.

Previous studies could show that the blockade of the B1R protects from ischemic tissue damage, eg, in models of myocardial or renal infarction. 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 inhibitors for clinical application during the acute phase of ischemic stroke in humans.

The kallikrein–kinin system plays an important role in regulating blood pressure, 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. Moreover, B1R antagonists did not change blood pressure in normotensive rats. 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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{42,43} Mice overexpressing endothelin-1 developed more brain edema and larger cerebral infarctions after tMCAO.\textsuperscript{44} Moreover, pharmacological blockade of the endothelin type A receptor attenuated ischemic brain injury, edema formation, and blood–brain barrier blockade of the endothelin type A receptor attenuated ischemic brain injury, edema formation, and blood–brain barrier disruption in rats.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{52} The cells producing these cytokines after tMCAO need to be further characterized but might include neurons, microglia, or invading immune cells.\textsuperscript{47}

Previous investigations using B2R-deficient mice in experimental stroke produced contradictory results. Gröger et al\textsuperscript{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.\textsuperscript{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,\textsuperscript{12} Xia et al\textsuperscript{53} postulated that postischemic 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.\textsuperscript{12,54,55} Infarct size in WT mice after 90 minutes of MCAO was surprisingly small in the study by Xia et al,\textsuperscript{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 others\textsuperscript{27,28,55,56} could demonstrate that 60 minutes of occlusion of the MCA already causes infarct volumes between 50 and 80 mm\textsuperscript{3} at day 1 after MCAO, eg, in C57BL/6, SV129, or Swiss mice. In the article by Xia et al,\textsuperscript{53} 90 minutes of occlusion only led to infarct volumes of 12.8±7.3 mm\textsuperscript{3} 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.\textsuperscript{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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\textbf{Disclosures}

None.

\textbf{References}


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