Postischemic Brain Injury Is Exacerbated in Mice Lacking the Kinin B2 Receptor

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Abstract—Kallikrein cleaves low molecular weight kininogen to generate vasoactive kinins, which bind to the kinin B2 receptor, triggering a host of biological effects. Kallikrein gene delivery has been shown previously to reduce ischemia/reperfusion-induced cerebral infarction. In this study, we tested the hypothesis that the kinin B2 receptor plays a protective role in ischemic brain injury using kinin B2 receptor gene knockout (B2R-KO) mice subjected to middle cerebral artery occlusion (MCAO). The mortality rate and neurological deficit scores of B2R-KO mice (n=48) after MCAO were significantly increased compared with wild-type (WT) mice (n=40) when examined over a 14-day period. In addition, the infarct volume in B2R-KO mice was significantly larger than in WT mice at days 1 and 3 after MCAO. Similarly, apoptotic cells, detected by TUNEL labeling counterstained with propidium iodide, and caspase-3 activity in the ischemic brain increased significantly in B2R-KO mice when compared with WT mice and was associated with elevated tumor necrosis factor α expression. These alterations in B2R-KO mice correlated with decreased NO levels, Akt, and glycogen synthase kinase-3β phosphorylation and increased nicotinamide-adenine dinucleotide oxidase activity. These results indicate that the kinin B2 receptor promotes survival and protects against brain injury by suppression of apoptosis and inflammation induced by ischemic stroke. (Hypertension. 2006;47:752-761.)

Key Words: kinins ■ stroke ■ apoptosis ■ cerebral ischemia ■ receptors, bradykinin

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inins are peptides formed in plasma and peripheral tissues from cleavage of kininogen substrates by kal-
lkrein. The actions of kinins are mediated through stimulation of 2 subtypes of 7-transmembrane G protein–coupled receptors, denoted B1 and B2. The kinin B1 receptors are not normally expressed in healthy tissues, whereas the kinin B2 receptors are constitutively expressed and widely distributed throughout central and peripheral tissues. Most of the physio-
logical actions of kinin are believed to be mediated by stimulation of the kinin B2 receptors.1 Binding of kinins to their receptors transduces signals through NO-cGMP and prostacyclin-cAMP pathways, thereby modulating a broad spectrum of cellular functions.2 Murone et al3,4 have demonstrated the distribution of B2 receptors in the brain and spinal cord of guinea pigs and sheep by autoradiography. Chen et al5 have also shown that the B2 receptor is widely expressed within the rat brain. The broad distribution pattern of the B2 receptor indicates a functional importance of kinins in the mammalian brain.

The tissue kallikrein–kinin system has been shown previ-
ously to exert protective effects in heart after ischemic injury.6,7 In addition, intracerebroventricular injection of the kallikrein gene has been shown to reduce cerebral infarct size and promote glial cell migration and survival after ischemic stroke.8 The effects of kallikrein gene delivery were associated with enhanced NO formation, decreased oxidative stress, and activation of cell survival signaling pathways. Our previous studies have shown that the benefits of kallikrein gene delivery in the ischemic heart can be abolished by the administration of a kinin B2 receptor antagonist.7 Therefore, the kinin B2 receptor may also play an important role in protecting the brain from ischemia/reperfusion (I/R) injury.

To determine whether kallikrein/kinin provides neuropro-
tective effects against ischemic stroke via the kinin B2 receptor, we used wild-type (WT) and kinin B2 receptor knockout (B2R-KO) mice subjected to 90-minute occlusion of the middle cerebral artery followed by reperfusion. We then examined the potential effects of kinin B2 receptor on cell death and inflammatory cell accumulation in the ischemic brain. Our results showed that neurological deficit scores, infarct volume, and mortality rate were significantly elevated in B2R-KO mice in association with increased severity of apoptosis and inflammation in the ischemic brain.

Methods

Animals and Treatments
Mice (male and female, body weight 25 to 30 g), including WT mice (B6129SF2/J) and B2R-KO mice (derived from breeding pairs of...
C57BL/6 mice on a 129/J genetic background), were purchased from Jackson Laboratories (Bar Harbor, ME). All of the procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals. Transient focal cerebral ischemia was induced by right middle cerebral artery (MCA) occlusion (MCAO) with a modified intraluminal filament technique. Briefly, mice were anesthetized with 1.5% isoflurane using a RC2 Rodent Anesthesia System (VetEquip Inc). A 5-0 monofilament surgical nylon suture with a heat-blunted tip was introduced into the right internal carotid artery through the stump of the external carotid to the base of the right MCA to stop blood flow to MCA. After 90 minutes of ischemia, the filament was withdrawn, blood reperfusion was established, and the skin was sutured. The body temperature of animals was maintained at 37°C during the surgery until they recovered from anesthesia.

Survival Rate and Behavioral Test Measurement
A total of 88 mice were used for the survival rate study (WT, n=20 male, n=20 female; B2R-KO, n=28 male, n=20 female). This measurement was terminated 14 days after MCAO. Mice were tested for neurological deficits after MCAO as described previously. Neurological deficit scores were determined as follows: no neurological deficit (value = 0); left forelimb flexion when suspended by the tail or failure to extend right forepaw fully (value = 1); left shoulder adduction when suspended by the tail (value = 2); reduced resistance to lateral push toward the left side (value = 3); spontaneous movement in all directions with circling to the left exhibited only if pulled by tail (value = 4); circle or walk spontaneously only to the left (value = 5); walk only when stimulated (value = 6); no response to stimulation (value = 7); and stroke-related death (value = 8). Mice with neurological deficit scores of ≥5 after fully recovering from anesthesia were used in the experiment.

Cerebral Infarction Stained by TTC
At days 1 and 3 after MCAO, brains (n=6 of each group) were processed for histology as described. Serial coronal brain sections (~2 mm in thickness) were immersed in normal saline containing 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 30 minutes at 37°C. To minimize artifacts produced by postischemic edema in the infarcted area, the infarct volume was calculated using a technique as described previously. Briefly, the infarction area in the ipsilateral hemisphere was indirectly measured by subtracting the noninfarcted area in the ipsilateral hemisphere from the total intact area of the contralateral hemisphere.

Immunohistochemistry and TUNEL Staining
At days 1 and 3 after MCAO, animals (n=8 of each group) were euthanized. Sections (4 μm) of the paraffin-embedded brain were subjected to immunohistochemistry using a Universal Elite ABC kit (Vector) according to the manufacturer’s instructions. Purified anti-mouse neutrophils antibody (1:200 dilution, Serotec Inc) and anti-mouse tumor necrosis factor (TNF) α antibody (1:200 dilution, R&D Inc) were used for immunostaining. The number of neutrophils was determined by counting the whole cross-section of brain. Cerebral apoptosis was analyzed by TUNEL assay kit (Roche Diagnostic) with propidium iodide counterstaining according to the manufacturer’s instructions. The TUNEL-positive (apoptotic) cells and total cells were counted in 10 microscopic fields of each brain section at ×200 magnification. The extent of apoptosis was calculated and expressed as a ratio of TUNEL-positive cells versus total cells.

Western Blot Analyses of Akt and Glycerogen Synthetase Kinase-3β and Caspase-3 Activity Assay
Antibodies against total and phosphorylated Akt and glycerogen synthetase kinase (GSK)-3β (Cell Signaling Technology, 1:1000 dilution) were used for Western blot. Caspase-3 activity was measured as described previously.
group (day 7, 5.83±0.69 versus 3.2±0.44, n=12 in B2R-KO, n=20 in WT, P<0.01; day 8, 3.67±0.33 versus 2.5±0.24, n=9 in B2R-KO, n=18 in WT, P<0.05; Figure 2). There was no significant difference in neurological deficits between the surviving WT and B2R-KO mice at 14 days after MCAO (data not shown). Examination of infarct size, via TTC staining (Figure 3A), paralleled the observed exacerbation of neurological impairments in that the total infarct volume in the B2R-KO group was significantly increased compared with the WT group at day 1 (30.9±10.2 versus 12.8±7.3 mm³/rat; P<0.05; n=6) and day 3 (67.55±5.2 versus 29.3±8.5 mm³/rat; P<0.005; n=6) after MCAO (Figure 3B).

Increased Cerebral Apoptosis in B2R-KO Mice
Cerebral apoptosis was evaluated by TUNEL staining together with propidium iodide counterstaining (Figure 4A). When compared with the WT group, the ratio of TUNEL-positive cells to total cells in B2R-KO mice was significantly increased at day 1 (45.4±9.1 versus 14.6±3.7%; n=5; P<0.05) and day 3 (48.4±4.2 versus 16.2±2%; n=5; P<0.001) after MCAO (Figure 4B). Caspase-3 activity was also markedly increased in B2R-KO mice brain at day 1 (5.85±0.56 versus 4.29±0.41 pmol/min per milligram of protein; n=8; P<0.05) and day 3 (6.44±0.41 versus 3.62±0.26 pmol/min per milligram of protein; n=6 or 8; P<0.05) after MCAO as compared with WT mice (Figure 4C).

Neutrophil Accumulation and TNF-α Expression in B2R-KO Mice
To evaluate the effect of the kinin B2 receptor on inflammatory cell accumulation, anti-mouse neutrophils and anti-mouse TNF-α antibodies were used to detect the accumulation of
neutrophils and expression of TNF-α by immunohistochemistry (Figure 5A). Three days after MCAO, the number of neutrophils in B2R-KO mice was higher (156.7±35.3 versus 66.0±13.5 per cross-section; n=7; P<0.05) compared with mice in the WT group (Figure 5B). TNF-α immunostaining increased in B2R-KO mice after MCAO compared with WT mice. Similarly, RT-PCR showed that TNF-α mRNA levels were elevated in B2R-KO mice 3 days after MCAO (Figure 5C). Quantitative real-time PCR analysis showed a significant increase in relative TNF-α mRNA versus GAPDH mRNA levels in B2R-KO mice (Figure 5D).

Biochemical Differences in the Brains of WT and B2R-KO Mice

Figure 6A shows that cerebral nitrate/nitrite levels in B2R-KO mice were significantly reduced compared with WT mice at day 1 (0.52±0.06 versus 0.76±0.08 nmol/mg protein; n=8 or 9; P<0.05) and day 3 (0.50±0.06 versus 0.75±0.09 nmol/mg protein; n=8; P<0.05) after MCAO. In contrast, cerebral NADH oxidase activity levels in B2R-KO mice markedly increased compared with WT mice at day 1 (14.06±2.46 versus 7.72±1.60 RLU/mg protein per minute; n=8; P<0.001) but not at day 3 (19.56±2.0 versus 20.53±4.89 RLU/mg protein per minute; n=8; P=0.84) after MCAO (Figure 6B). Western blot (Figure 6B, top) and quantitative analyses (Figure 6B, bottom) showed reduced Akt and GSK-3β phosphorylation in B2R-KO mice compared with WT mice (Figure 6C and 6D). Quantitative analysis showed that the ratio of phosphorylated to total Akt in the infarcted brain of B2R-KO mice significantly decreased after MCAO (ratio of phospho-Akt to total-Akt, 0.98±0.03 versus 0.48±0.09; n=3 or 4; P<0.05) as compared with that of WT mice (Figure 6C). Similarly, GSK-3β phosphorylation also markedly decreased in the ischemic brain of B2R-KO mice as compared with WT mice (ratio of phospho-GSK-3β to total-GSK-3β, 0.90±0.07 versus 0.61±0.03; n=5; P<0.05; Figure 6D).

Discussion

In this study, we provide convincing evidence for a protective role of the kinin B2 receptor against ischemic stroke. Mice lacking the kinin B2 receptor subjected to MCAO had marked increases in mortality rate, cerebral infarct size, and infarct volume compared with WT mice after focal cerebral ischemia. Moreover, neurological deficit scores and apoptosis were more pronounced in B2R-KO mice than in WT mice. This observation is consistent with our previous study that local tissue kallikrein gene delivery into the brain reduces neurological deficit, cerebral infarction, and apoptosis induced by ischemic stroke.8 Taken together, these results indicate that kallikrein, through the endogenous kinin B2 receptor, plays a crucial role in protection against focal cerebral ischemic injury induced by MCAO.

Our present study showed an increased mortality rate in B2R-KO mice from day 1 to day 14 after MCAO when compared with WT mice. Similarly, the neurological deficit scores of B2R-KO were worse than WT. WT mice recovered and functioned better after stroke compared with the mice lacking the B2 receptor. This could partially be explained by the protective effect of the receptor against cerebral infarction. Because WT mice had smaller infarcts, this would lead to increased survivability and neurological function, decreased apoptosis, and, hence, overall improved condition compared with the B2R-KO mice. However, reduced survival in B2R-KO mice is in contrast with the findings of a recent study that showed prolonged survival, improved motor function, and smaller cerebral infarcts in B2R-KO after experimental stroke.16 The reason for the discrepancy of the study by Groger et al16 versus ours is not clear at the present time but may be explained by differences in the MCAO model (45 minutes versus 90 minutes of ischemia); the total number of animals used for measuring the survival rate (20 versus 88); and in the timing of behavioral, histological, and survival rate evalu-
vation (7 days versus 14 days). In addition, our recent study showed that intravenous injection of the kallikrein gene protects against ischemic brain injury after MCAO by inhibiting apoptosis and inflammation and promoting angiogenesis and neurogenesis. Additionally, the neuroprotective effects of kallikrein were abolished by icatibant, a kinin B2 receptor antagonist. These findings are consistent with our present report. Taken together, these results support the notion that kinin B2 activation mediates the neuroprotection of tissue kallikrein. Because kinin B2 receptor levels in the brain fluctuate over the course of stroke progression, the time frame chosen to evaluate kinin effects on stroke parameters is likely to influence the outcome. Indeed, more pronounced neurological impairments and increased mortality rate were seen in the B2R-KO mice when examined at later periods after

Figure 3. (A) Representative images of serial coronal brain sections with TTC staining at days 1 and 3 after MCAO. (B) Quantitative analysis of infarct volume, n=6 per group.
MCAO. We chose 90-minute ischemia to produce a significant infarction, a time frame which has been published previously.18–21 Furthermore, our previous study showed that kallikrein gene delivery reduced neurological dysfunction, cerebral infarction, and apoptosis induced by ischemic stroke.8 It has also been observed that kallikrein exerts beneficial effects in the ischemic heart, which were blocked by icatibant, indicating a kinin B2 receptor–

Figure 4. Brain cell apoptosis at days 1 and 3 after MCAO. (A) Representative TUNEL-positive staining (with propidium iodide counterstaining). (B) Quantitative analysis presented as percentage of TUNEL-positive nuclei. (C) Caspase-3 activity measurement.
mediated event. These combined results provide strong evidence that kallikrein, through the kinin B2 receptor, plays a protective role in cerebral ischemic injury.

Previous reports have shown that early activation of the kallikrein–kinin system after cerebral ischemia increases brain vessel permeability, edema, and spread of the ischemic lesion. Also, early administration of a kinin B2 receptor antagonist was shown to improve neurological recovery after focal cerebral I/R. Moreover, a recent study showed increased brain edema in kinin B2 receptor–deficient mice.

Figure 5. Neutrophil infiltration and TNF-α expression in ischemic brain of WT and B2R-KO mice at day 3 after MCAO. (A) Representative neutrophil-positive staining in infarct area of brain (top) and TNF-α staining (bottom). (B) Quantitative analysis of neutrophils. The number of neutrophils was determined by counting the whole cross-section of brain. (C) Representative RT-PCR products of TNF-α mRNA in WT and B2R-KO mice were detected by ethidium bromide–stained agarose gels, with RT-PCR products of GAPDH as an endogenous control. (D) Quantitative real-time-PCR analysis of TNF-α mRNA levels in WT (n=8) and B2R-KO (n=6) mice brain after MCAO.
Thus, it appears that kinin aggravates cerebral injury at an early stage by promoting edema. However, our recent results showed that delayed kallikrein gene delivery protects against I/R-induced neurological dysfunction and cerebral infarct size in rats after MCAO, and icatibant abrogated these effects (data not shown). These results indicate that kinin may have a protective role in the later stages of ischemic brain injury. Similarly, treatment of VEGF exerted neuroprotection after focal cerebral ischemia, whereas early administration of VEGF induced edema. A possible explanation for the differential effects of early versus late interventions is that the timing of brain inflammation occurs within several days/weeks after stroke, including early injury and late posts ischemic repair processes. These results indicate that kinin, through B2 receptor activation, has a detrimental effect in increasing vessel permeability and, thus, inflammatory cell infiltration in the early stage of ischemic stroke but has a protective effect in the later stage. In this study, we showed that neutrophil accumulation increased in the ischemic brain of B2R-KO mice after MCAO when compared with WT mice. Taken together, these results indicated a role of kinin B2 receptor in the inflammatory response.

Inflammatory cells induce damage in the ischemic brain by adhesion to endothelial cells and transmigration into brain parenchyma where they release oxygen-derived free radicals, phospholipases, proteases, and proinflammatory cytokines, such as interleukin 1 and TNF-α. Activated neutrophils...
have been shown to be the main source of reactive oxygen species (ROS) during reperfusion.²⁹ Inflammatory cells can cause cellular necrosis and promote oxidative stress in the early course of ischemic organ damage. Beneficial effects have been observed from therapeutic strategies designed to reduce neutrophil accumulation or function in experimental models of acute ischemic tissue injury.³⁰,³¹ Indeed, our present study indicates that the kinin B2 receptor exerts neuroprotection through suppression of inflammatory cell recruitment, ROS formation, and proinflammatory cytokines, such as TNF-α, after ischemic stroke. After stimulation by proinflammatory mediators, neutrophils are recruited to the site of injury and produce ROS via NADH oxidase. Increases in these cytokines and subsequent elevation of ROS leads to cellular apoptosis.

Our previous investigation showed that intracerebroventricular injection of tissue kallikrein gene reduced brain cell apoptosis at day 8 after ischemic stroke via activation of Akt-Bcl-2 cell survival pathway.³² In this study, we observed elevated apoptosis in kinin B2 receptor–deficient mice after MCAO. Our results reveal reduced NO levels and decreased Akt and GSK-3β phosphorylation in B2R-KO mice. It is well known that activation of the kinin B2 receptor leads to increased endothelial NO synthase activity and, hence, an increase in NO. Without the kinin B2 receptor in place, NO levels were likely suppressed compared with WT mice, leading to reduced activation of the survival factors Akt and GSK-3β phosphorylation. These data indicate a role of kinin B2 receptor in protection against ischemia-induced apoptosis through Akt-GSK-3β signaling cascades. In addition, we showed that absence of the B2 receptor resulted in increased activity of caspase-3, which probably exacerbated the cerebral infarcts. Because apoptosis and inflammation largely contribute to the transformation of the ischemic penumbra into becoming a part of the infarct core, modulation of apoptotic- and inflammatory-related pathways may limit ischemic injury. In conclusion, we demonstrate here that deletion of the kinin B2 receptor intensified apoptotic cell death and elevated inflammation, which likely mediated the observed worsening of the stroke outcomes.

Perspectives
Ischemic stroke is the third leading cause of death in the United States and is frequently associated with long-term disability. In this study, we showed that absence of the kinin B2 receptor aggravates posts ischemic brain injury, as neurological dysfunction, cerebral infarction, and mortality rate are significantly elevated in kinin B2-deficient mice in association with increased severity of apoptotic cell death and inflammatory cell infiltration in the ischemic brain. Because apoptosis and inflammation largely contribute to the transformation of the ischemic penumbra into becoming a part of the infarct core, modulation of apoptotic- and inflammatory-related pathways could limit ischemic injury. Under cerebral ischemia, activation of the kinin B2 receptor–mediated signaling pathways may promote cell survival and prevent the damaging effects of inflammatory responses. Our results illustrate that kinin B2 activation may be especially advantageous in the treatment of ischemic stroke.

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