Bone Morphogenetic Protein-6 Reduces Ischemia-Induced Brain Damage in Rats

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Background and Purpose—Bone morphogenetic protein-6 (BMP6) and its receptors are expressed in adult and fetal brain. Receptors for BMP6 are upregulated in adult brain after injury, leading to the suggestion that BMP6 is involved in the physiological response to neuronal injury. The purpose of this study was to determine whether there was a neuroprotective effect of BMP6 in vivo and in vitro.

Methods—Lactate dehydrogenase and microtubule-associated protein-2 (MAP-2) activities were used to determine the protective effect of BMP6 against H$_2$O$_2$ in primary cortical cultures. The neuroprotective effects of BMP6 were also studied in chloral hydrate–anesthetized rats. BMP6 or vehicle was injected into right cerebral cortex before transient right middle cerebral artery (MCA) ligation. Animals were killed for triphenyl-tetrazolium chloride staining, caspase-3 immunoreactivity and enzymatic assays, and TUNEL assay. A subgroup of animals were used for locomotor behavioral assays.

Results—Application of H$_2$O$_2$ increased lactate dehydrogenase activity and decreased the density of MAP-2(+) neurons in culture. Both responses were attenuated by BMP6 pretreatment. Complementary in vivo studies showed that pretreatment with BMP6 increased motor performance and generated less cerebral infarction induced by MCA ligation/reperfusion in rats. Pretreatment with BMP6 did not alter cerebral blood flow or physiological parameters. There was decreased ischemia-induced caspase-3 immunoreactivity, caspase-3 enzymatic activity, and density of TUNEL-positive cells in ischemic cortex in BMP6-treated animals.

Conclusions—BMP6 reduces ischemia/reperfusion injury, perhaps by attenuating molecular events underlying apoptosis. (Stroke. 2001;32:2170-2178.)

Key Words: apoptosis • bone morphogenetic proteins • cerebral ischemia • neuroprotection • rats

Bone morphogenetic protein-6 (BMP6), a trophic factor in the transforming growth factor-β (TGF-β) superfamily, has been shown to control growth and differentiation of peripheral organs. BMP6 expression has been demonstrated in the brain as well. BMP6 is highly expressed in embryonic, neonatal, and adult brain. Most of the neurons in neonatal forebrain possess high levels of BMP6 mRNA, which is retained in selected areas in adult hippocampus and neocortex. BMP6 binds to BMPR-1A, BMPR-1B, and BMPR-II receptors in brain. BMPR-II receptors can be upregulated in the granule cells of dentate gyrus after transient global cerebral ischemia and brain contusion. These data suggest that BMP6 and its receptors might be involved in neuronal protection or regeneration during ischemia.

BMP6 has been found to have neurotrophic and neuroprotective activity. BMP6 was as potent as glial cell line–derived neurotrophic factor (GDNF) in augmenting the numbers of surviving midbrain dopamine neurons in vitro. BMP6 increased dopamine uptake in mesencephalic dopaminergic neuronal cultures. BMP6 also augmented the numbers of tryptophan hydroxylase–immunoreactive neurons and protected against 5,7-dihydroxytryptamine-induced lesioning of 5-HT neurons in vitro. Although these data suggest that BMP6 has protective effects against various toxins and may be involved in neuronal repair after brain injury, the effects of BMP6 in focal brain ischemia in vivo, and in complementary in vitro models, have yet to be documented.

Previous studies have suggested that the brain damage induced by ischemia involves the formation of reactive oxygen species. In the present study, we examined the neuroprotective effects of BMP6 against H$_2$O$_2$, a compound that generates superoxide and hydroxyl radicals, in vitro.
further examined the protective effects of BMP6 in vivo using middle cerebral artery (MCA) occlusion and measuring the extent of induced cerebral infarction and apoptotic markers. Our data suggest that BMP6 is neuroprotective against brain ischemia, possibly by interfering with proapoptotic mechanisms.

**Materials and Methods**

**In Vitro Primary Cortical Cultures**

Cultures were prepared from the cerebral cortex of gestation day 17 embryos from Sprague-Dawley rats. Pooled cortical tissues were dissociated by mechanical trituration in ice-cold calcium- and magnesium-free Hanks’ balanced salt solution (pH 7.4) with a supplement of 10 g/L glucose. After pelleting by centrifugation, cells were resuspended and plated (5 × 10^5 cells/0.5 mL/well) to 24-well cell culture plates precoated with poly-lysine (0.02 g/L). The culture medium consisted of Dulbecco’s modified Eagle medium with 10% heat-inactivated fetal bovine serum, 1 × 10^-3 mol/L pyruvate, 4.2 × 10^-3 mol/L sodium bicarbonate, 20 × 10^-3 mol/L HEPES, 3 g/L bovine serum albumin, 50 U/10^-5 L penicillin, and 50 × 10^-3 g/L streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Four days later, the cultures in each well were replenished with 0.5 × 10^-2 mL minimum essential medium with 0.5 g/L BSA and N-2 supplement, 0.5 × 10^-2 mol/L pyruvate, 50 U/10^-2 L penicillin, and 50 × 10^-2 g/L streptomycin. On the seventh day in vitro, the medium was changed to serum-free minimum essential medium containing 1 × 10^-3 mol/L pyruvate, 1 × 10^-3 mol/L glutamine, 0.5 g/L BSA, 50 U/10^-5 L penicillin, 50 × 10^-3 g/L streptomycin, 0.3 × 10^-3 mol/L KCl, and 1.25 × 10^-9 mol/L BMP6 (Creative BioMolecules) or vehicle. Twenty minutes later, H_2O_2 was added to the medium for 24 hours. Culture media were collected for lactate dehydrogenase (LDH) activity assay, and cells were examined for microtubule-associated protein-2 (MAP-2) immunoreactivity on the eighth day in vitro.
MAP-2 immunostaining, cell cultures were washed with 0.1 mol/L PBS (pH 7.4) and fixed for 20 minutes at room temperature in 40 g/L paraformaldehyde in PBS. After being washed with PBS, the fixed cultures were treated for 20 minutes with blocking solution (10 g/L BSA, 0.03% vol/vol Triton X-100, and 4% vol/vol appropriate serum in PBS). Cultures were incubated overnight at 4°C with a monoclonal antibody against MAP-2 (1:1000; Chemicon) and then were rinsed 3 times in PBS. The bound primary antibody was visualized by the avidin-biotin peroxidase complex method (ABC Elite kit; Vector Laboratories). Each assay was run in triplicate in different

Figure 2. Pretreatment with BMP6 increases locomotor activity in stroke rats. Animals were tested 48 hours after ischemia/reperfusion. Motor activity was examined 30 to 60 minutes after animals were placed in the chamber. Horizontal (A) and vertical (E) activities were measured by the sum of the total number of beam interruptions that occurred in the horizontal or vertical sensors, respectively, during the 30-minute testing period. Number of horizontal (B) and vertical (F) movements, total movement time (C), and distance traveled (H) were significantly increased in BMP6-pretreated rats. Vehicle-treated controls had significantly higher immobile times (D). *P<0.05.
The number cited in the cell culture results indicates the number of pregnant rats used.

In Vivo Brain Ischemia/Reperfusion

Adult male Sprague-Dawley rats (weight >300 g) were used for this study. Animals were subjected to right MCA ligation (see below) after intracerebral administration of BMP6 or vehicle. All surgical procedures were performed by sterile/aseptic techniques in accordance with institutional guidelines.

**Surgery**

Rats were anesthetized with chloral hydrate (0.4 g/kg IP) and were injected intracerebrally with BMP6 (4×10^-6 g in 4×10^-6 L, 3 sites) or vehicle (4×10^-6 L, 3 sites, 20×10^-3 mol/L acetate in 50 g/L mannitol buffer solution) through a Hamilton syringe into 3 cortical areas adjacent to the right MCA, 2.0 to 3.0 mm below the dura. The approximate coordinates for these sites were 1.0 to 2.0 mm anterior to the bregma and 3.5 to 4.0 mm lateral to the midline, 0.5 to 1.5 mm posterior to the bregma and 4.0 to 4.5 mm lateral to the midline, and 4.0 to 4.5 mm posterior to the bregma and 5.5 to 6.0 mm lateral to the midline. The needle was retained in place for 5 minutes after each injection. After injection, a piece of bone wax was applied to the skull defect to prevent leakage of the solution.

Thirty minutes after intracerebral administration of BMP6 or vehicle, the anesthetized animals were subjected to cerebral ischemia. Ligation of the right MCA and bilateral common carotids (CCAs) was performed by methods described previously. The CCAs were ligated with nontraumatic arterial clips. The right MCA was ligated with 10-0 suture. After 60 minutes of ischemia, the suture on the MCA and arterial clips on CCAs were removed to allow reperfusion. Core body temperature was monitored with a thermistor probe and maintained at 37°C with a heating pad during anesthesia. After recovery from the anesthesia, body temperature was maintained at 37°C with a heat lamp.

**Locomotor Behavioral Measurements**

Animals were placed in an Accuscan activity monitor 48 hours after ischemia for behavioral recording. The monitor contained 16 horizontal and 8 vertical infrared sensors spaced 25×10^-3 m apart. The vertical sensors were situated 105×10^-3 m from the floor of the chamber. Each animal was placed in a 420×420×310×10^-3 m Plexiglas open box for 1 hour. Motor activity was calculated with the number of beams broken by the animals from 30 to 60 minutes after placement in the chamber.

**Triphenyltetrazolium Chloride Staining**

Two days after reperfusion, some of the animals were killed. The brain tissue was removed and sliced into 2.0-mm-thick sections. Brain slices were incubated in 20 g/L triphenyltetrazolium chloride for 30 minutes at 37°C. The slices were then washed in phosphate buffer saline and mounted on slides for microscopic examination.

### Figure 3

Intracerebral administration of BMP6 markedly reduced cortical infarction induced by MCA ligation and reperfusion. The right MCA was ligated for 60 minutes after bilateral CCA ligation. A1 and A2, Two animals pretreated with vehicle were killed for TTC staining 48 hours after ischemia/reperfusion. Marked infarction (white areas) in the right cerebral cortex was found. B1 and B2, Pretreatment with BMP6 reduced the infarction area in 2 other animals. Calibration bar: 10 mm. C, The volume of infarction was significantly decreased in animals pretreated with BMP6 compared with vehicle control group. Volume of infarction (mm^3) = [sum of the infarction area in 7 consecutive brain slices (mm^2)]×[thickness of the slice, 2 mm]. The area of the largest infarction in a slice (D) and the number of infarcted slices (E) from each rat were also significantly diminished by BMP6 (*P<0.05).
(TTC) for 15 minutes at 37°C and then transferred into a 50 g/L formaldehyde solution for fixation. The area of infarction in each slice was measured with a digital scanner as described previously.11

Caspase-3 Immunostaining
Eight hours after ischemia, anesthetized animals were perfused with 40 g/L paraformaldehyde. The slices were incubated with primary antibody against caspase-3 (cleaved caspase-3 antibody, D175, Cell Signaling; 1:500 dilution in PB containing 10% normal rabbit serum) for 16 hours at 4°C, washed 3 times with PB, and then incubated with secondary antibody (biotinylated rabbit anti-goat IgG in the buffer) for 60 minutes, followed by incubation for 60 minutes with avidin-biotin-horseradish peroxidase complex. Staining was developed with 2,3-diaminobenzidine tetrahydrochloride.

Caspase-3 Enzymatic Activity
Caspase-3 enzyme activity was measured with the ApoAlert kit (Clontech). Eight hours after the onset of reperfusion, animals were killed. Brain tissue was removed, dissected, and homogenized in lysis buffer. Caspase-3 activity was determined fluorometrically by the formation of 7-amino-4-trifluromethyl coumarin (AFC) from Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC). The selective caspase-3 inhibitor DEVD-aldehyde (DEVD-CHO) was included at a concentration of 10^{-3} mol/L in some assays to ensure that the enzymatic reaction was specific.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Histochemistry
Animals were decapitated 24 hours after ischemia. The brains were taken out and cut into sections (30×10^{-6} m) in a cryostat. The sections were mounted on microscope slides, air-dried, then fixed with 4% paraformaldehyde for 30 minutes. A standard terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) procedure for frozen tissue sections, with minor modifications, was performed.12 Briefly, slide-mounted sections were rinsed in 0.1% Triton X-100 in 1 g/L sodium citrate for 2 minutes on ice. TUNEL reaction mixture (50×10^{-6} L) was then added to each sample followed by a 60-minute incubation at 37°C. Negative controls consisted of omission of the label solution (terminal deoxynucleotidyl transferase) in the TUNEL reaction mixture. The sections were counterstained with 1.25×10^{-3} mol/L propidium iodide. Positive signals were imaged with a Zeiss confocal LSM 410. Nuclei with fluorescein-labeled DNA strand breaks were excited at 488 nm, and the emission was collected at 510 to 525 nm. Propidium iodide was excited at 543 nm and the emission collected with a long-pass filter starting at 570 nm.

Cerebral Blood Flow
Cortical blood flow was measured continuously with a laser Doppler flowmeter (PF-5010, Periflux system) in anesthetized animals. A burr hole (1-mm diameter) was made in the right frontoparietal region to allow placement of the photodetectors. A probe (0.45 mm in diameter) was stereotaxically placed in the cortex (1.3 mm posterior, 2.8 mm lateral to bregma, and 1.0 mm below dura).

Blood Pressure, Heart Rate, Calcium, and Blood Gas Measurements
Physiological parameters were measured in 16 rats. Animals were anesthetized, and a polyethylene catheter was inserted into the right CCA. Mean arterial pressure was measured through a strain gauge transducer and recorded on a strip chart recorder. Arterial blood (0.3×10^{-3} L) was withdrawn from the artery 1 hour after BMP6 or vehicle injection. Blood was heparinized; blood pH, concentrations of CO_2 and O_2, serum electrolytes, and hemoglobin levels were analyzed by standard methods.

Results
Neuroprotective Effect of BMP6 in Primary Cortical Cultures
Exposure of primary cortical cultures to H_2O_2 alone caused significant cell death in a concentration-dependent manner, as measured by LDH activity in culture media (Figure 1A). The
degree of cell death in cultures receiving no pretreatment was not significantly different from that in those that received vehicle. Pretreatment with BMP6 (1.25×10^{-9} mol/L) significantly reduced LDH activity in cultures exposed to 10^{-4} or 10^{-5} mol/L H_2O_2 (P<0.001, F(5,78)=21.170, 1-way ANOVA; P<0.05, post hoc Newman-Keuls test). The survival of neurons was further examined with MAP-2 immunostaining. BMP6 (1.25×10^{-9} mol/L) significantly increased MAP-2(+) cell density (Figure 1B through 1D) in cortical cultures exposed to 10^{-5} mol/L and 10^{-4} mol/L H_2O_2 (P<0.001, F(5,34)=14.168, 1-way ANOVA; P<0.05, post hoc Newman-Keuls test), which suggests that BMP6 has a neuroprotective effect in vitro.

**Neuroprotective Effect of BMP6 In Vivo**

**Locomotor Behavior**

Two days after MCA ligation, animals (n=21) were individually placed in the behavioral chamber for 60 minutes. Locomotor activity did not show any differences during the first 30 minutes, possibly because of accommodation to the new test environment. However, from 30 to 60 minutes, animals pretreated with BMP6 (n=11) had significantly more...
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(P<0.05, t test) horizontal and vertical activity than vehicle-treated controls (n=10; Figure 2). BMP6-pretreated rats also spent less time immobile and traveled greater distances than vehicle-pretreated controls (Figure 2).

Cerebral Infarction
A total of 31 rats were killed for TTC staining after 48 hours of reperfusion. MCA ligation and reperfusion resulted in clear-cut infarction of the cortex in all vehicle-treated control animals studied. The 16 animals that received BMP6 pretreatment showed significantly smaller infarct volumes than vehicle controls (Figure 3C; n=15, P=0.003, t test). Typical TTC staining, demonstrating reduced cortical infarction in the BMP6-pretreated rat brain, is shown in Figures 3A and 3B. The area of largest infarction in a slice from each rat (Figure 3D; P=0.017, t test) and the number of infarcted slices in each rat (Figure 3E; P=0.018, t test) were also significantly reduced in BMP6-treated rats.

Caspase-3 Immunoreactivity
Rats (n=6) were killed 8 hours after MCA ligation for caspase-3 immunocytochemistry. Caspase-3 immunoreactivity was found mainly in cortex and striatum on the ischemic side in rats pretreated with vehicle (Figure 4, A1 and A2). In contrast, animals pretreated with BMP6 before MCA ligation showed fewer caspase-3(+) cells after reperfusion (Figure 4, B1 and B2).

Caspase-3 Enzymatic Activity
Twelve rats were killed at 8 hours after MCA ligation. The brains were sliced coronally at 2-mm intervals. We have demonstrated previously that the largest lesion, as examined by TTC staining, occurred in the fourth slice from the rostral end after 24 hours of reperfusion. We therefore examined caspase-3 enzymatic activity in the fourth slice from each animal. Caspase-3 activity in the ischemic versus the nonischemic side brain slice was significantly increased in vehicle-pretreated rats. Caspase-3 enzymatic activity was significantly reduced in the ischemic side brain slice from animals pretreated with BMP6. Ischemia-induced caspase-3 activity was significantly antagonized when DEVD-CHO, a selective caspase-3 inhibitor, was included in the medium as a control for specificity (Figure 4C; P<0.05, 1-way ANOVA and Newman-Keuls test).

TUNEL Staining
A total of 12 rats were used for TUNEL analysis. Six control animals that did not receive MCA ligation showed almost no TUNEL positivity in their brain sections (Figure 5A). The other 6 rats were treated with either BMP6 (Figure 5C) or vehicle (Figure 5B) before MCA ligation. Animals pretreated with vehicle had significantly more TUNEL-positive cells, mainly in the penumbra zone, than those pretreated with BMP6 (P<0.05, 2-way ANOVA; Figure 5D).

Blood Flow
A total of 16 rats were used for cerebral blood flow analysis. Cerebral cortical blood flow was measured continuously from 10 minutes before to 40 minutes after local BMP6 or vehicle injection (n=6) or 30 minutes before to 30 minutes after the onset of MCA ligation (n=10). Blood flow in each animal was normalized by comparison of blood flow before and after injection or MCA ligation (Figure 6). We found that local administration of a single dose of BMP6 or vehicle did not alter cerebral blood flow (P=0.726, F=0.447, 1-way ANOVA). An example is shown in Figure 6C. Cortical blood flow was significantly reduced to the same degree after MCA ligation in both vehicle and BMP6-treated animals. BMP6 did not enhance cortical blood flow during ischemia (P<0.05). B, A real-time tracing from 2 animals receiving vehicle pretreatment and 1 animal receiving BMP6 pretreatment, demonstrating that BMP6 did not increase cerebral blood flow during ischemia (arrows). C, Injection of BMP6 (arrow) did not alter cerebral blood flow in nonischemic animals.

Physiological Parameters
Sixteen animals were used for measurement of physiological parameters. Pretreatment with BMP6 compared with vehicle did not alter systemic blood pressure, blood gases, or serum electrolytes (Table).
Physiological Parameters Were Not Altered by BMP6 Pretreatment

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<th>Parameter</th>
<th>BMP6 (n=8)</th>
<th>Vehicle (n=8)</th>
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<tr>
<td>MBP, mm Hg</td>
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<td>HR, bpm</td>
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<td>Paco2, mm Hg</td>
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<td>Hematocrit, %</td>
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<td>K+ (10^-3 mol/L)</td>
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<td>Cl^- (10^-2 mol/L)</td>
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<td>Ca2+ (10^-2 g/L)</td>
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*MBP indicates mean blood pressure; HR, heart rate; and sat, saturation.

**Discussion**

In this study, we found that pretreatment with BMP6 (1.25×10^-9 mol/L) decreased cell death after exposure to H2O2 in primary cortical cultures. The density of MAP-2(+) cells, presumably neurons, was augmented in BMP6-pretreated cultures, which suggests that the protection mainly involves neurons. A similar protective response was found during hypoxic injury. We found that 24-hour hypoxia/24-hour reoxygenation significantly increased LDH activity in primary cortical cultures. This response was significantly attenuated by pretreatment with BMP6 (1.25×10^-9 mol/L). Previous studies have indicated that BMP6 or other BMPs at higher concentration (>12.5×10^-9 mol/L) induce cell differentiation.13,14 We found that at such a high concentration, BMP6 did not have protective effects against 10^-4 mol/L H2O2; however, BMP6 reduced H2O2-induced toxicity at a concentration of 10^-3 mol/L, which suggests that the neuroprotective and cell differentiation actions of BMP6 might represent a concentration-separable effect.

Previous studies have indicated that neurotoxic mechanisms in brain ischemia involve the formation of reactive oxygen species. Because BMP6 reduced toxicity induced by H2O2, a compound that generates superoxide and hydroxyl radicals, we further examined the protective effect of BMP6 in the ischemia/reperfusion model of stroke in vivo. We found that BMP6 pretreatment diminished ischemia/reperfusion-induced cerebral infarction. The volume of infarction, the area of largest infarction in a brain slice, and the total number of infarcted slices per rat were all reduced by BMP6 treatment. BMP6 pretreatment also allowed maintenance of a higher level of locomotor activity after stroke. We found BMP6 did not alter cerebral blood flow in nonischemic and ischemic cortex. Furthermore, because BMP6 did not alter blood gases, blood pressure, or serum electrolytes, its neuroprotective effect was not secondary to alterations in such systemic parameters. Taken together, these data suggest that BMP6 reduced ischemia/reperfusion injury in vivo by a direct action in the central nervous system.

We, and others, have previously reported that other proteins from the TGF-β superfamily can exert neuroprotective effects. For example, pretreatment with GDNF before MCA ligation attenuated ischemia-induced cortical infarction and behavioral deficits.11 Similarly, pretreatment with BMP7 before MCA ligation reduced motor impairment and attenuated the volume of infarction in the cortex.15 In situ hybridization studies have revealed increased expression of GDNF, BMP6, TGF-β, GDNF receptors,16–19 and BMP-2 receptors after ischemia.6,7 Because the present study showed that BMP6 was neuroprotective, upregulation of BMP6 receptors may enhance responsiveness to BMP6 and reduce ischemic damage as part of an endogenous neuroprotective pathway activated during ischemia.

Ischemia is known to induce production of reactive oxygen and nitrogen species, cleavage of poly(ADP-ribose) polymerase, and caspase activation, with subsequent DNA fragmentation. The involvement of caspase is supported by the observation that treatment with caspase inhibitors reduces ischemia-induced brain damage.20,21 Elevation of caspase-3 activity is evident as early as 8 hours after ischemia, whereas the density of TUNEL-positive cells peaks at 24 to 48 hours after ischemia.22 Moreover, previous studies demonstrated that GDNF-induced neuroprotection involves inhibition of apoptotic mechanisms. GDNF also decreases ischemia-induced reactive nitrogen species formation and caspase-3 immunoreactivity and reduces the density of TUNEL-positive cells in brain.23 These data suggest that apoptotic mechanisms are activated during ischemia and that inhibition of apoptosis reduces ischemic brain damage. In the present study, ischemia-induced caspase-3 immunoreactivity and caspase-3 enzymatic activity in the cerebral cortex were reduced by BMP6 8 hours after ischemia. Similarly, DNA fragmentation, as measured by TUNEL staining, was also attenuated 24 hours after ischemia by pretreatment with BMP6. Recent studies have indicated that the BMP antagonist noggin increased TUNEL-positive cell density in chick sympathetic ganglia.24 These data support the hypothesis that BMP6-mediated protection involves antiapoptotic mechanisms. We found that BMP6 induced a greater inhibition in caspase-3 activity at 8 hours after ischemia than the decrease of density of TUNEL labeling at 24 hours after ischemia. Such a discrepancy may be due to the fact that the peak responses of these 2 parameters occur at different time points after ischemia. It is also known that ischemia-induced programmed cell death can be both caspase dependent and independent.25,26 It is not known whether caspase-independent programmed cell death is involved in BMP6-induced neuroprotection.

In conclusion, our data indicate that pretreatment with BMP6 decreased H2O2-induced toxicity in vitro and ischemia/reperfusion-induced DNA fragmentation, caspase-3 activity, and cortical infarction in vivo. BMP6 also reduced motor deficits in stroke animals. Our data suggest that BMP6...
has neuroprotective effects against ischemic injury, possibly through inhibition of apoptotic pathways.

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