Intravenous Brain-Derived Neurotrophic Factor Reduces Infarct Size and Counterregulates Bax and Bcl-2 Expression After Temporary Focal Cerebral Ischemia

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Background and Purpose—Pretreatment with intraventricular brain-derived neurotrophic factor (BDNF) reduces ischemic damage after focal cerebral ischemia. In this experiment we studied the effect of intravenous BDNF delivered after focal cerebral ischemia on neurological outcome, infarct size, and expression of proapoptotic and antiapoptotic proteins Bax and Bcl-2, respectively.

Methods—With the use of the suture occlusion technique, the right middle cerebral artery in rats was temporarily occluded for 2 hours. Thirty minutes after vessel occlusion, BDNF (300 μg/kg per hour in vehicle; n = 12) or vehicle alone (n = 13) was continuously infused intravenously for 3 hours. After 24 hours the animals were weighed and neurologically assessed on a 5-point scale. The animals were then killed, and brains underwent either 2,3,5-triphenyltetrazolium chloride staining for assessment of infarct volume or paraffin embedding for morphology and immunohistochemistry (Bax, Bcl-2).

Results—Physiological parameters (mean arterial blood pressure, P O 2, P CO 2, pH, body temperature, glucose) and weight revealed no difference between groups. Neurological deficit was improved in BDNF-treated animals versus controls (P <0.05, unpaired, 2-tailed t test). Mean±SD infarct volume was 229.7±97.7 mm 3 in controls and 121.3±80.2 mm 3 in BDNF-treated animals (P <0.05, unpaired, 2-tailed t test). Cortical infarct volume was 155.5±78.5 mm 3 in the placebo group and 69.9±50.2 mm 3 in the BDNF-treated group (P <0.05, unpaired, 2-tailed t test). Subcortical infarct volume was 74.1±30.6 mm 3 in the placebo group and 51.1±26.8 mm 3 in the BDNF-treated group (P =NS). Bax-positive neurons were significantly reduced in the ischemic penumbra in BDNF-treated animals (P <0.05, unpaired, 2-tailed t test), whereas Bcl-2–positive neurons were significantly increased in this area (P <0.001, unpaired, 2-tailed t test).

Conclusions—This study demonstrates a neuroprotective effect of BDNF when delivered intravenously after onset of focal cerebral ischemia. As shown here, one possible mechanism of action of neuroprotection of BDNF after focal ischemia appears to be counterregulation of Bax/Bcl-2 proteins within the ischemic penumbra. (Stroke. 2000;31:2212-2217.)

Key Words: brain-derived neurotrophic factor ■ middle cerebral artery occlusion ■ proteins ■ rats

Brain-derived neurotrophic factor (BDNF) and the biologically similar nerve growth factor act on a set of high-affinity receptor kinases to promote survival, differentiation, and neurite extension in many types of mammalian central nervous system neurons.1,2 BDNF, acting mainly through the tyrosine kinase receptor TrkB, displays in vitro trophic effects on a wide range of neuronal cells, including hippocampal, dopaminergic, cerebellar, and cortical neurons.3–10 In vivo, BDNF rescues motoneurons and substantia nigra dopaminergic cells from traumatic and toxic brain injury.11,12 After transient forebrain ischemia, BDNF treatment prevented hippocampal neuronal death in rats.13–15 Intraventricular BDNF given before and intraparenchymal infusion of BDNF after focal cerebral ischemia significantly reduced infarct volume, primarily in the cortex.16,17

In this study we extended our previous findings and administered BDNF intravenously after middle cerebral artery (MCA) occlusion (MCAO) and assessed the effects on neurological outcome, infarct volume, and morphological analysis. A potential role of BDNF for attenuation of apoptosis was assessed by immunostaining of the proapoptotic protein Bax and the antiapoptotic protein Bcl-2.

Materials and Methods

Male Wistar rats weighing 300 to 315 g had access to food and water ad libitum. All animals were randomly assigned before surgery to one of the following groups: BDNF-treated group (group B; 2,3,5-
BDNF was delivered as a concentrated stock solution (15 mg/mL) as a generous gift from Amgen Inc and stored at 8°C before use. BDNF was delivered intravenously at a rate of 300 μg/kg per hour for 3 hours beginning 30 minutes after MCAO. Controls received similar infusions of the vehicle alone.

Temporary ischemia of the MCA for 2 hours was induced by the suture occlusion technique.18,19 Briefly, the right common carotid artery and the right external carotid artery were exposed through a midline neck incision. A 4-0 monofilament nylon suture (Ethicon) coated with silicon (Bayer) was inserted through an arteriectomy in the common carotid artery, gently advanced into the internal carotid artery, and positioned approximately 17 mm from the carotid bifurcation. With the use of this technique, the tip of the suture occludes unilaterally the proximal anterior cerebral artery, the origin of the MCA, and the posterior communicating artery. A large infarct in the territory of the MCA is typically produced.18 After surgery, the catheters were removed, and the animals were allowed to recover from the anesthesia and given food and water ad libitum. Sham-operated animals received the same surgical procedure without insertion of the occluder filament.

After 24 hours the animals were weighed and neurologically assessed (rating scale: 0=no deficit to 4=spontaneous circling).20 Animals that were to undergo TTC staining were then reanesthetized with chloral hydrate (400 mg/kg) and decapitated. The brains were removed and then coronally sectioned into five 2-mm coronal slices, incubated for 30 minutes in a 2% (wt/vol) solution of TTC at 37°C, and fixed by immersion in 10% (wt/vol) phosphate-buffered paraformaldehyde. TTC stains viable brain tissue red, while infarcted tissue remains unstained. TTC-stained brain sections were photographed with a charge-coupled device camera (EDC-1000HR Computer Camera, Electrim Corporation). The infarct volumes were calculated with the investigator blinded to the treatment given (W.Z.), and in each of the 5 slices the infarct size was quantified with the use of an image processing software package (NIH Image, Apple, Macintosh). To compensate for the effect of brain edema, the corrected infarct volume was calculated as previously described in detail: corrected infarct area equals left hemisphere area minus (right hemisphere area minus infarct area).21 Cortical and subcortical infarct areas were calculated separately. Then the mean, cortical, and subcortical infarct areas on each slice were added together and multiplied by slice thickness to give the infarct volumes. Animals that died prematurely were replaced and not included for calculation of infarct volumes (n=3, controls).

The values presented in this study are mean±SD. After all the data were acquired, the randomization code was broken. Volumes of infarction and the neurological scores between groups were compared by an unpaired, 2-tailed t test. Nonparametric data were compared by Mann-Whitney U test. Continuous data were analyzed by ANOVA and Scheffé’s test.

For morphological analysis, animals were transcardially perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer. The brains were removed from the skull, postfixed overnight in the same fixative at 4°C, and then sectioned at 8 μm corresponding to stereotaxic coordinates −14.6, −12.0, −9.4, +11.2, +13.8, +16.4, +19.0, and +21.6 anterior and posterior to the bregma, respectively. After paraffin embedding, 1-μm-thick sections of each level were cut and used for Nissl and Luxol fast blue staining and immunohistochemical analysis.

Immunohistochemistry was performed with antisera against Bax (DAKO, Carpinteria) and Bcl-2 (Upstate Biotechnology). For anti-Bax/immunohistochemistry, sections for Bax and Bcl-2 immunohistochemistry were heated for 20 minutes in 10 mmol/L citrate buffer at 99°C. Sections were then incubated in normal swine serum (10% in phosphate-buffered saline) for 30 minutes followed by the primary antisera overnight at 4°C (Bax, Bcl-2). The primary antibodies were diluted 1:100 (Bax) and 1:200 (Bcl-2). Immunoreactivity was visualized by the avidin-biotin complex method (Vectastain, Vector Laboratories). Sections were developed in 0.02% diaminobenzidine with 0.02% hydrogen peroxide. The reaction product was intensified by addition of 0.02% cobalt chloride and nickel ammonium sulfate. Neither preabsorption of Bcl-2 and Bax antibodies with the respective peptides nor omission of the primary antisera in a subset of control slides resulted in no immunostaining (not shown).

For quantitative analysis of nuclear Bax and Bcl-2 immunoreactivity in the peri-infarct tissue, morphologically intact cortical areas at the level of the ventral hippocampal commissure in close proximity to the infarct border zone dorsally (upper parietal cortex) and ventrally (lateral preoptic area) were used. At a magnification of ×500, 3 microscopic fields were taken, and all neurons, including neurons with nuclear immunoreactivity, were counted. The mean values of the percentage of neurons with nuclear immunoreactivity were taken for further processing. The values presented are mean±SD. After all the data were acquired, the randomization code was broken. The data were then compared by an unpaired, 2-tailed t test.
Results

Physiological variables, presented in the Table, showed no significant differences when the 2 groups were compared. The percent body weight decline was 16% for BDNF-treated animals and 17% for controls (P=NS). Premature death occurred in 3 animals of the control group and 0 animals of the BDNF-treated group; thus, the premature mortality rate was 20% versus 0% (P=NS). The mean neurological score after 24 hours was 3.5 in the placebo group and 2.7 in the BDNF-treated group (P<0.05, t test). The mean volume of infarction was 229.7±97.7 mm³ in the control group and 121.3±60.2 mm³ in the BDNF-treated group (P<0.05, t test) (Figure 1). Cortical infarct volume was 155.5±78.5 mm³ in the control group and 69.9±50.2 mm³ in the BDNF-treated group (P<0.05, t test). Subcortical infarct volume was 74.1±30.6 mm³ in the control group and 51.1±26.8 mm³ in the BDNF-treated group (P=NS) (Figure 1).

The extent of striatal and cortical infarction in Nissl-stained brains correlated with corresponding TTC-stained brain sections in both groups (not shown). The ischemic lesion, clearly discernible from the surrounding brain, involved the basal ganglia, a portion of the thalamus, and part of the cortex. Infarcts were, on average, smaller in BDNF-treated animals than in controls; in particular, cortical infarctions appeared distinctly smaller in BDNF-treated animals. Moreover, examination of brain sections showed no apparent differences in vascular proliferation or reactive gliosis in the brain parenchyma surrounding the ischemic lesion between BDNF-treated animals and controls.

In both experimental groups, Bax immunoreactivity in the ischemic core was absent or restricted to nuclei of shrunken degenerated cells. The adjacent border zone exhibited shrunken neurons with strong nuclear Bax immunoreactivity. BDNF treatment significantly reduced the number of nuclear Bax immunoreactivity–positive neurons in cortical areas in close proximity to the ventral and dorsal ischemic zone, where neurons were morphologically intact (dorsal, 3.8±1.9 [9±6.9%] versus 10.2±6.2 [23.6±10.2%] of nuclear Bax-positive neurons, P<0.05; ventral, 4.7±2.8 [9±3.4%] versus 12.7±7.2 [18.7±18.5%] of nuclear Bax-positive neurons, P=NS) (Figures 2 and 3). In the contralateral hemisphere, neuronal Bax immunoreactivity was widely restricted to the cytoplasm. Only a few neurons exhibited positive nuclear Bax immunoreactivity without any statistical difference between the groups (dorsal, group B, 2.4±1.2 [4.7±2.5%] versus group C, 10.4±6.5 [20±25.3%] of nuclear Bax-positive neurons, P=NS; ventral, group B, 2.1±1.1 [5.3±3%] versus group C, 2.4±1.6 [5.3±3.2%] of nuclear Bax-positive neurons, P=NS).

Bcl-2 immunoreactivity in the ischemic core was absent or restricted to nuclei of shrunken degenerated cells. The adjacent border zone exhibited shrunken neurons with strong nuclear Bcl-2 immunoreactivity. The whole overlying cortex visibly exhibited strong nuclear Bcl-2 immunoreactivity. BDNF treatment significantly upregulated the number of nuclear Bcl-2–positive neurons in cortical areas in the prox-

Figure 1. Infarct volume after 24 hours of focal cerebral ischemia in BDNF-treated animals (B) and controls (C). Bar indicates SD. Mean and cortical infarct volumes were significantly smaller in BDNF-treated animals than in the control group, whereas no difference was found in subcortical infarct volume between both groups. *P<0.05, unpaired, 2-tailed t test.

Figure 2. Number of Bax/Bcl-2–positive neurons after 24 hours of temporary focal cerebral ischemia or sham operation in the dorsal and ventral peri-infarct zone. Percentage of Bax/Bcl-2–positive neurons from all neurons in BDNF-treated animals (B) and control group (C) is shown (*P<0.05, **P<0.01, ***P<0.001, unpaired, 2-tailed t test).

Figure 3. Bax/Bcl-2 immunoreactivity 24 hours after temporary focal cerebral ischemia in BDNF-treated animals and controls (Co). The figure demonstrates a part of the upper parietal cortex adjacent to the infarct (arrow). a, Immunostaining shows more Bax-positive neurons in the control group than in BDNF-treated animals (b). Note the moderate cortical Bcl-2 expression in the control group (c) compared with the strong upregulation of Bcl-2 in the BDNF-treated group (d). Bar=50 μm.
inity of the ventral and dorsal border zone, where neurons were morphologically intact (dorsal, $56.3 \pm 8.9 \% [73.8 \pm 6.6 \%]$ versus $11.5 \pm 6.6 \% [18.6 \pm 11.9 \%]$ of nuclear Bcl-2–positive neurons, $P<0.001$; ventral, $56.3 \pm 20.2 \% [73.5 \pm 6.7 \%]$ versus $14.4 \pm 2.3 \% [24.9 \pm 1.4 \%]$ of nuclear Bcl-2–positive neurons, $P<0.0001$) (Figures 2 and 3). In the contralateral hemisphere, neuronal Bcl-2 immunoreactivity was widely restricted to the cytoplasm. Only a few neurons exhibited positive nuclear Bcl-2 immunoreactivity, without any statistical difference between the groups (dorsal, group B, $14.4 \pm 10.1 \% [15.3 \pm 21.2 \%]$ versus group C, $5.7 \pm 2.9 \% [9.1 \pm 9.9 \%]$ of nuclear Bcl-2–positive neurons, $P=NS$; ventral, group B, $22 \pm 13.3 \% [1.6 \pm 0.37 \%]$ versus group C, $8.7 \pm 3.5 \% [12.2 \pm 8.9 \%]$ of nuclear Bax–positive neurons, $P=NS$). BDNF-treated and sham-operated animals exhibited strong nuclear Bcl-2 immunoreactivity in the dorsal cortex, whereas sham-operated controls exhibited only a few neurons with positive nuclear Bcl-2 immunoreactivity ($36.4 \pm 8.1 \% [61.1 \pm 11.8 \%]$ versus $6.8 \pm 3.1 \% [15.3 \pm 10.7 \%]$, $P<0.01$).

**Discussion**

The results of this study demonstrate to our knowledge for the first time that a physiologically relevant application of BDNF after focal cerebral ischemia reduced infarct volume (50%) and improved neurological outcome in a model of temporary MCAO in rats. The infarct-reducing effect was significant only for the cortex and not for subcortical structures. Furthermore, BDNF treatment significantly reduced expression of the proapoptotic Bax protein in cortical neurons in the penumbra, whereas it increased upregulation of the antiapoptotic protein Bcl-2 in this area. Bcl-2 expression was also upregulated in the cortex of sham-operated controls after BDNF infusion. No differences in physiological parameters such as mean arterial blood pressure, blood gases, glucose, and body temperature and no difference in weight loss were seen between the groups during the experiment, as previously reported. 

The dose of BDNF chosen for the present intravenous study was higher than the one used in our previous studies of intraventricular administration. It was also higher than doses of other growth factors, such as basic fibroblast growth factor (bFGF), used for intravenous treatment of focal cerebral ischemia. We have chosen this relatively high dose to maximize any potential neuroprotective effects. A lower daily dose (60 to 80 µg/d) has been demonstrated to be not neuroprotective when given intravenously after transient forebrain ischemia, although a conjugated form of the same dose of BDNF with enhanced transport through the blood-brain barrier achieved significant neuroprotection. However, the forebrain ischemia model produces only mild ischemia with good survival and no disruption of the blood-brain barrier. Therefore, not enough of the nonconjugated form of the growth factor may have entered the brain to achieve significant neuroprotection. Disruption of the blood-brain barrier occurs 2 to 4 hours after occlusion by the suture occlusion model and is thought to reflect a major way of entry of growth factors into the brain, as demonstrated by Fisher et al for bFGF. However, BDNF has been reported to cross the blood-brain barrier after intravenous infusion with a rapid (10 minutes) and saturable parenchymal uptake in the cortex.

Many studies have documented the neuroprotective capability of BDNF after a variety of brain insults. BDNF prevents in vitro degeneration of dentate granule cells and hippocampal, striatal, septal, and cortical neurons against hypoglycemic/hypoxic insults. In vivo BDNF reduced neuronal death after transient forebrain ischemia. In focal cerebral ischemia, intraventricular BDNF before and intraparenchymal BDNF after permanent ischemia reduced the final lesion size. Neuroprotection in this study predominantly occurred in the cortex, as suggested previously. In stroke models in which the cortex represents large parts of the penumbra, an attractive explanation for the mechanism of action of the neuroprotective effect of BDNF could be the prevention of apoptotic cell death. Apoptotic cell death has been confined to the penumbral border of the infarction, where cells are not so rapidly and severely damaged that they can undergo an apoptotic cell death rather than necrosis. Presence and anatomic location of apoptotic cells in particular after temporary focal cerebral ischemia suggest that apoptosis may contribute to the final infarct size. Expression of endogenous BDNF mRNA at these peripheral areas of the evolving infarct suggests that BDNF could protect neurons carrying the corresponding TrkB receptor. Furthermore, BDNF in vitro has been shown to prevent apoptosis. As demonstrated in this study, BDNF treatment reduced expression of the proapoptotic protein Bax and counterregulated the antiapoptotic protein Bcl-2 in neurons located at the ischemic border zone. Neurons destined to develop apoptosis have been reported to express upregulation of the proapoptotic Bax protein and nuclear translocation paralleled by Bcl-2 decrease in the same neurons. On the other hand, Bcl-2 is expressed in cortical neurons that survive focal cerebral ischemia. In vitro treatment with bFGF and insulin-like growth factor-1 counteracts Bax upregulation, increases Bcl-2 expression, and reduces apoptosis. Bcl-2 can be upregulated by BDNF and other growth factors, such as nerve growth factor, and is necessary for survival of BDNF-dependent neurons. Possibly, neurotrophins (and particularly BDNF) promote survival of neurons, in part through a mechanism involving CREB family transcription factor–dependent expression of genes encoding prosurvival factors such as Bcl-2. However, as shown by others, BDNF did not reduce the number of terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL)–positive neurons in the penumbra after permanent focal cerebral ischemia. TUNEL–positive neurons include cells destined to develop apoptosis as well as necrosis, and therefore TUNEL staining is not an exclusive marker for cells that undergo delayed neuronal cell death.

Another mechanism of neuroprotection achieved by growth factors after hypoxic/ischemic events is probably prevention of excitotoxicity. Glutamate-triggered excitotoxicity with subsequent Ca²⁺ overload of cells is thought to be the main cause of cellular death after ischemia. BDNF protects in vitro neuronal cells against glutamate-induced neurotoxicity and the subsequent high intracellular calcium level. By inducing an antioxidant defense...
system, BDNF suppresses the glutamate-triggered peroxide accumulation, which contributes to the loss of Ca\textsuperscript{2+} hemostasis.\textsuperscript{45} BDNF may also protect neurons from glutamate neurotoxicity by reducing cytokotoxic action of NO.\textsuperscript{46} Another growth factor, bFGF, has been demonstrated to protect in vivo against N-methyl-D-aspartate–induced excitotoxicity.\textsuperscript{47} However, BDNF treatment was also found to potentiate necrotic death of cortical neurons induced by oxygen-glucose deprivation or N-methyl-D-aspartate exposure.\textsuperscript{32}

In conclusion, intravenous treatment with BDNF significantly reduced infarct size and neurological outcome after temporary focal cerebral ischemia. BDNF induced counter-regulation of Bax and Bcl-2 protein expression and probably achieved neuroprotection after focal cerebral ischemia, at least in part, through a Bax/Bcl-2–dependent mechanism. Further studies are necessary to clarify other mechanisms of neuroprotection after BDNF treatment of focal ischemia, including prevention of glutamate-induced excitotoxicity as well as cytoskeletal protein degradation. Further exploration for potential clinical use includes the testing of different species and different models of ischemia as well as dose-finding studies.

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References


In the accompanying article, Schabitz and colleagues show that intravenous administration of BDNF, initiated after the onset of ischemia, reduces infarct volume and neurological disability in a model of focal cerebral ischemia and reperfusion in rats. Moreover, BDNF treatment appeared to downregulate expression of the pro-apoptotic protein Bax and upregulate expression of the anti-apoptotic protein Bcl-2 in neurons in the ischemic penumbra, suggesting that the mechanisms of infarct reduction by BDNF may depend on antagonism of apoptosis in the penumbra. These data are similar to recent data on other growth factors and hormones (notably bFGF and estrogen), showing that these factors upregulate expression of Bcl-2 (or at least prevent its downregulation) in the ischemic penumbra after focal ischemia.1,2

The findings of Schabitz et al suggest that intravenous BDNF may represent a viable cytoprotective strategy for the treatment of acute stroke. Clearly, the development of such a treatment would require considerably more preclinical data, including dose-response, time window of efficacy, biodistribution, and extensive toxicology studies. Recent clinical trials in acute stroke with another growth factor, bFGF, emphasize the point that the dose and timing of administration of such factors are critical. In a North American trial,3 intravenous administration of 5 or 10 mg of bFGF over 8 hours was toxic, whereas in a European-Australian trial,4 administration of the same doses over 24 hours were not. Moreover, an interim analysis of the European-Australian trial showed a trend toward bFGF efficacy at the 5-mg dose, with some interesting subgroup data observed.4

Trophic growth factors are endogenous molecules that support cell survival during normal growth and development. Therefore, it makes sense that they might also have value as exogenous treatments that antagonize cell death in pathological circumstances such as stroke.

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