Osteogenic Protein-1 Protects Against Cerebral Infarction Induced by MCA Ligation in Adult Rats

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**Background and Purpose**—Osteogenic protein-1 (OP1) not only possesses trophic activity on bone tissue but also influences neuronal survival and differentiation in vitro. Specific receptors for OP1 are present in brain and spinal cord and can be upregulated during cerebral contusion. OP1 is a member of the transforming growth factor-β superfamily, several of whose members possess neuroprotective activity. In this study, the neuroprotective effect of OP1 in cerebral ischemia was evaluated in adult animals.

**Methods**—Adult male Sprague-Dawley rats were anesthetized with chloral hydrate. OP1 or vehicle was administered intracortically or intracerebroventricularly to the rats. Thirty minutes, 24 hours, or 72 hours after OP1 injection, the right middle cerebral artery (MCA) was ligated for 90 minutes. Twenty-four hours after reperfusion, animals were tested for motor behavior. The animals were subsequently anesthetized with urethane and perfused intracardially with saline. Brain tissue was removed, sliced, and incubated with 2% triphenyltetrazolium chloride to localize the area of infarction.

**Results**—Only animals pretreated with OP1 24 hours before MCA ligation showed a reduction in motor impairment. OP1, given 30 minutes or 72 hours before MCA ligation, did not reduce cortical infarction. In contrast, pretreatment with OP1 24 hours before MCA ligation significantly attenuated the volume of infarction in the cortex, in agreement with the behavioral findings.

**Conclusions**—Intracerebral administration of OP1 24 hours before MCA ligation reduces ischemia-induced injury in the cerebral cortex. (Stroke. 1999;30:126-133.)

**Key Words:** bone morphogenetic proteins ■ cerebral infarction ■ cerebral ischemia ■ neuroprotection ■ rats

Osteogenic protein-1 (OP1), also known as bone morphogenetic protein 7 (BMP7), a member of the transforming growth factor-β (TGF-β) superfamily, has been documented to be a trophic factor for bone and cartilage. OP1 increases proteoglycan synthesis and initiates formation of cartilage and differentiation of bone. However, recent studies indicate that effects of OP1 are not limited to skeletal system. OP1 is found in developing embryos and fetuses as are other proteins in the TGF-β superfamily. OP1 is highly expressed in fetal kidney, heart, teeth, eye, bone, and intestine in perinatal neuronal tissues, such as hippocampus, cortex, and cerebellum. Exogenous application of OP1 to rat mesencephalic cell cultures increases numbers of tyrosine hydroxylase (+) cells and dopamine uptake in vitro.

Two classes of receptors are known to be involved in OP1-induced responses: type I and type II. Type I receptors are highly expressed in fetal ependymal epithelium and in sympathetic ganglia. Type II receptor mRNA is also found in the fetal neuronal tissues, such as spinal cord and neuroepithelium. Unlike type I receptors, type II receptors are present in adult cortex, dentate, hippocampus, and substantia nigra. Binding of type II receptors with ligands cooperatively enhances type I receptor binding. Recent studies have indicated that the type II receptor mRNA in the dentate gyrus is upregulated after mild cerebral contusion injury in adult rat brain, suggesting that type II receptors may be involved in neuronal plasticity or repair after acute brain injury. The presence of OP1 and its receptors in the perinatal brain suggests that OP1 is involved in the regulation of neuronal development. However, because type II receptors for OP1 are also present and can be regulated in the adult brain, it is possible that OP1 has actions other than during maturation, as is true for other TGF-β superfamily members.

There are some data indicating that OP1 has protective effects in the CNS. Intraperitoneal injection of OP1 before general hypoxia, at a dose of 20 to 50 µg, reduces brain infarction volume and mortality in neonatal rats. However, previous studies have shown that age, body weight, or both play an important role in the degree of ischemia-induced infarction and that young rats are much more resistant to ischemic damage. It is thus more relevant to human disease to determine whether OP1 has protective effects in CNS ischemia.
mia in adult animals. Moreover, it is critical to evaluate behavioral and histological correlates of cerebral ischemia.

In the present study, we examined the effects of OP1 in adult rats. We found that intracerebral administration of OP1 reduces the volume of ischemia-induced cortical infarction. We also found that OP1 improves the middle cerebral artery (MCA) occlusion–induced behavioral deficits in these animals.

Materials and Methods

Animals and Drugs
Two sets of animals were used in this study. A first group of 26 adult male Sprague-Dawley rats (400.8 ± 11.3 g) was used for evaluating motor behavior and the area of infarction induced by ischemia/reperfusion. These animals were divided into 4 groups: (1) OP1/0d (n = 6)—OP1 was given intracerebroventricularly (10 × 10⁶ L, 30 × 10⁻⁶ mol/L, 30 minutes before MCA ligation) and directly into the cortex (5 × 10⁻⁶ L, 30 × 10⁻⁶ mol/L, X3 sites, 5 to 10 minutes before MCA ligation), adjacent to the right MCA; (2) OP1/1d (n = 11)—OP1 (30 × 10⁻⁶ L, 30 × 10⁻⁶ mol/L) was given intracerebroventricularly 24 hours before MCA ligation; (3) OP1/3d (n = 3)—animals received intracerebroventricular injection of OP1 (30 × 10⁻⁶ L, 30 × 10⁻⁶ mol/L) 72 hours before MCA ligation; and (4) controls (n = 6), animals received intracerebroventricular injection of vehicle (30 × 10⁻⁶ L, 20 mmol/L acetal/5% mannitol buffer solution pH 4.5) 24 hours before MCA ligation.

A second set of adult male Sprague-Dawley rats (n = 16) was evaluated for OP1-induced physiological responses (eg, blood pressure, heart rate, blood glucose, and blood gases). These animals received either OP1 or vehicle 24 hours before MCA ligation and were studied both before and after ligation. Separate groups (n = 8) were used for each study. OP1 and vehicle were supplied by Creative Biomolecules. OP1 was dissolved in vehicle (20 mmol/L acetate/5% mannitol buffer solution pH 4.5) at a concentration of 30 × 10⁻⁶ mol/L.

Intracerebral Injection
The animals, anesthetized with chloral hydrate (400 mg/kg, IP), were injected intracerebroventricularly with 30 × 10⁻⁶ L (except for animals in group 1) vehicle or OP1 over 20 minutes (~0.8 mm AP, 1.5 mm ML relative to bregma, and 3.5 mm below the dural surface). After injection, a piece of bone wax (W810; Ethicon) was applied to the skull defect to prevent the leakage of the solution. In the OP1/0d animals, OP1 was given intracerebroventricularly and directly into cortical areas (AP: 0.0 mm, 1.5 mm, and 3.0 mm; ML: 5.0- to 5.5-mm relative to bregma; 1.0 mm below the dural surface).

MCA Ligation
Rats were anesthetized with chloral hydrate (400 mg/kg, IP). Ligation of the right MCA and bilateral common carotids (CCAs) was performed with methods previously described. The bilateral CCAs were identified and isolated through a ventral midline cervical incision. The CCAs were ligated with nontraumatic arterial clips. A craniotomy of approximately 4 mm² was made in the right squamosal bone. The right MCA was ligated with a 10–0 suture for 90 minutes. The craniotomy was then covered with gelfoam. Sutures were removed 90 minutes later because ligation of this duration induces maximal infarction in rats at this age. After recovery from anesthesia, the animals were returned to their home cage for 24 hours to allow reperfusion of blood to the ischemic brain area.

Behavioral Measurements
Behavioral assays were carried out after 24 hours’ reperfusion. A modified rating scale adapted from Bederson et al was used to evaluate the behavioral deficits in the stroke rats. Animals were classified into 2 groups according to their neurological deficits: behaviorally impaired, rats keep their left forelimb to the breast and extend the right forelimb straight or twist the upper half of their body when suspended 1 m above the floor or show decreased resistance to lateral push and behaviorally normal, rats extend both forelimbs straight and none of the observable deficits described for behaviorally impaired rats above were present.

TTC Staining
One day after reperfusion, animals were euthanized and perfused intracardially with saline. The brain tissue was then removed, immersed in cold saline for 5 minutes, and sliced into 2.0-mm-thick sections. The brain slices were incubated in a 2% triphenyltetrazolium chloride (TTC; Research Organics Inc), dissolved in saline for 30 minutes at 37°C, and then transferred to 5% formaldehyde solution for fixation. The area of infarction in each slice was measured with a digital scanner and ImageTools programs (University of Texas Health Sciences Center). The volume of infarction in each animal was obtained from the product of average slice thickness (2 mm) and sum of infarction areas in all brain slices examined. To minimize any artifacts induced by postischemic edema in the infarcted tissue, the area of infarction was also calculated with the use of a second approach described by Lin et al. The infarction area in the right cortex was indirectly measured by subtracting the noninfarcted area in the right cortex from the total cortical area of the left hemisphere.

Blood Pressure, Heart Rate, Blood Glucose, and Blood Gas Measurements
One day after the intracerebroventricular administration of OP1 or vehicle, animals were anesthetized with chloral hydrate. The femoral artery was cannulated with polyethylene catheters (model PE-50; Dural Plastics and Engineering). Mean arterial pressure was recorded through a strain gauge transducer (model P23 ID; Statham) and recorded on a strip-chart recorder (model RS 3600; Gould).

Arterial blood (0.3 to 0.5 mL) was withdrawn from the femoral artery 24 hours after intracerebroventricular administration of OP1 or vehicle in the “nonstroke” animals or 85 minutes after the onset of MCA ligation in the “stroke animals.” Blood was heparinized; blood pH and concentrations of CO₂ and O₂ were analyzed with a blood gas analyzer (model BGA3; Radiometer). Blood glucose and hemoglobin levels were examined by Dri-Chem 100( Fuji Film) and ABL3 Radiometer.

Body Temperature
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 further maintained at 37°C with the use of a heat lamp.

Results

Behavioral Measurements
Previous studies have indicated that MCA ligation and reperfusion induces neurological deficits in rats. We also found that 4 of 6 rats pretreated with vehicle developed these symptoms. Pretreatment with OP1 either 30 minutes (OP1/0d) or 3 days (OP1/3d) before the ligation did not reduce the incidence of behavioral abnormalities. Four of 6 OP1/0d rats and 3 of 3 OP1/3d rats developed neurological symptoms. On the other hand, only 1 of 11 animals pretreated with OP1 1 day (OP1/1d) before ligation developed these symptoms. The incidence of neurological symptomatology in the OP1/1d group was significantly less than that in the vehicle-treated control group (P < 0.05, Fisher’s exact test).

Brain Infarction
We and others have previously reported that ligation of the MCA for 90 minutes and reperfusion for 24 hours elicits cortical infarction in rats. In the present study, we found...
that MCA ligation and reperfusion resulted in a clear-cut infarction of the cortex in all the solvent-treated control animals studied (n=6, Figures 1, 2). The incidence of infarction, expressed as the number of animals developing infarction/total number of animals studied, in control rats was not different from those in rats pretreated with phosphate-buffered saline or in rats without any pretreatment as reported in our previous work.19 The volume of infarction, the product of the infarcted area in all slices and thickness of each slice (2 mm), was 202.9±32.1 mm³ (mean±SEM).

Six rats were treated with local and intracerebroventricular injections of OP1 on the day of MCA ligation. The volume (168.5±32.1 mm³) and incidence of infarction were not different from those in control animals (Figures 2 and 3). Similarly, the 3 rats that received OP1 3 days before MCA ligation also showed no reduction in infarction volume (206.0±11.6 mm³, Figures 2, 3). In contrast, 6 of 11 rats that received OP1 pretreatment given 24 hours before MCA ligation showed mild (<40 mm³) or no infarction after MCA ligation. The incidence of infarction (>40 mm³) in the OP1/1d rats was significantly lower than that in the control animals (P<0.05, Fisher’s exact test, Figure 3D). The area of the largest infarction in a given slice from each rat was also significantly diminished, from a control value of 21.5±2.4 mm² to 9.6±2.2 mm², after the 24-hour OP1 pretreatment (Figure 3B, P<0.05, 1-way ANOVA and Bonferroni’s test). Furthermore, the number of infarcted slices in each rat was significantly reduced (Figure 3C), from 6.5±0.3 slice per rat in solvent-treated animals to 3.5±0.8 slice/rat in the OP1/1d rats (P<0.05, 1-way ANOVA and the Dunn test).

Taken together, these data suggest that 24-hour pretreatment with OP1 diminishes not only the volume but also the extent of infarction in the ischemic brain.

The volumes of edema and infarction were further analyzed with measurement techniques used previously.23 In agreement with previous reports,23 we found that the MCA ligation causes brain edema. The volume of edema was 36.7±12.9 mm³, or 25.5±9.3% of the total infarction, in control animals. Pretreatment with OP1 did not significantly alter the percentage of edema in the infarcted area (25.4±5.7%, P=0.9933, t test) in the OP1/1d rats. Moreover, the volume of infarction in the OP1/1d animals, measured with this method, was still significantly less than that in the control animals (control: 167.8±15.4 mm³; OP1/1d: 49.1±14.6 mm³; P<0.05, t test).

Physiological Responses to OP1

We found that pretreatment with OP1 did not alter a number of physiological parameters before or during ischemia. OP1, given 1 day earlier, did not significantly change mean blood pressure, blood glucose, hemoglobin, or blood gas values before MCA ligation (n=8, Figure 4). The blood PO₂, PCO₂, and pH values in the OP1- or vehicle–treated rats were also not different from those in the urethane-anesthetized rats, without any pretreatment, as reported by our group previously.24 Similarly, 85 minutes after the onset of MCA ligation, these parameters were not significantly altered by OP1 in an additional 8 animals studied (Figure 5). In both groups, OP1 pretreatment elicited a small reduction in heart rate, which was of borderline significance.
Discussion

Previous experiments have demonstrated that intraperitoneal administration of OP1 reduces cerebral infarction induced by general hypoxia in the neonatal rat. However, several trophic factors that have neuroprotective effects, such as glial cell line–derived neurotrophic factor (GDNF) and nerve growth factor, are found at significant levels in fetuses and neonates but not in adults. The protective effects of OP1 in the neonates may be partially attributed to the synergistic effects of these factors. In the present study, we found...
that intracerebroventricular pretreatment with OP1, but not vehicle, reduced motor dysfunction and decreased MCA ligation–induced cerebral infarction in the adult rats, suggesting that OP1 has protective effects against neuronal ischemia in adult animals.

Our behavioral methods were modified from the method of Bederson et al.22 Bederson et al used irreversible cauterization and transection of MCA and found that animals developed neurological symptoms 24 hours after surgery. The severity of symptoms was graded from 0 to 3. Grade 3 deficits, as seen in the article by Bederson et al, primarily occur when infarction is present in the basal ganglia or the basal ganglia plus the cortex. In our study, we used MCA ligation/reperfusion and found that the area of infarction was mainly limited to the cortex. Our stroke animals did not show grade 3 symptoms, such as circling. Furthermore, some animals with higher grade neurological deficits from the Bederson et al scale, such as decreased resistance of the forearm to lateral push (grade 2), did not show lower grade symptoms, such as forelimb flexion when animals were suspended above the floor (grade 1) in our study. We thus combined grade 1 and grade 2 as “behaviorally impaired” and used grade 0 from the Bederson et al scale as “behaviorally normal.”

We found that OP1 had to be administered 24 hours before ischemia to show neuroprotection. We have previously reported that GDNF, another TGF-β superfamily member, given on the day of MCA ligation, can protect the brain from ischemia-induced nitric oxide release and infarction. There are several possible reasons for such a temporal difference. It is known that GDNF diffuses rapidly in the CNS.29 Although the diffusion rate for OP1 has not yet been determined, the rate for GDNF is certainly much greater than for other trophic factors such as those in the neurotrophin family. In addition, it has been reported that OP1 stimulates bromodeoxyuridine incorporation into glial cells, resulting in the proliferation of immature glial cells and increasing astrocyte numbers in vitro. Inhibition of bromodeoxyuridine incorporation into the glial cells abolishes OP1-induced trophic effects on dopamine neurons.8 These data suggest that BMPs have trophic effects on dopaminergic neurons that are indirectly mediated through activation of glial-derived factors.8 Similar findings were reported, that BMPs selectively promote the differentiation of oligodendroglial-astroglial progenitor cells into astrocytes.30 It is thus also possible that the neuronal protection by OP1 reported here is indirectly mediated through the activation of astroglia, which delays its onset of action. It has been reported that activation of BMP receptors on cell membranes causes phosphorylation of SMAD-1 protein at carboxy terminal serine residues,31 which later associate with SMAD-4 in cytoplasm and translocate to the nucleus to modulate tran-
TGF-β superfamily trophic factor for central dopaminergic neurons, spinal cord motorneurons, and kidney. It has been shown to protect dopaminergic neurons from damage induced by neurotoxins that elevate intracellular free radicals and produce damage to mitochondrial respiratory enzymes. We and others have reported that ligation of the MCA induces nitric oxide release from the ischemic cortical area. Pretreatment with GDNF protects against cortical infarction and nitric oxide formation. Recent studies have further indicated that GDNF pretreatment markedly reduces terminal deoxynucleotidyl transferase labeling in the cortex during stroke. Taken together, these data suggest that TGF-β superfamily molecules may have neuroprotective effects during acute ischemia.

We found that 1 of 11 OP1/1d rats had behavioral deficits. However, 6 of these 11 rats showed minimal infarctions. Kawamata et al have recently reported that OP1, when administered 24 hours after a permanent MCA occlusion, produces a significant improvement in the recovery of motor skills without an effect on infarction volume. The recovery of motor skills was first evident 48 hours after the administration of OP1 and continued for at least 1 month. It was suggested that this effect may have been due to a dendritic outgrowth–promoting activity of OP1. It is thus possible that OP1 may have additional mechanisms that improve motor behavior in stroke animals, beyond anatomic changes in infarct size. However, it is not clear whether there is a relationship between these 2 mechanisms. The molecular events that limit infarct size may or may not underlie the longer-term effects that result in motor skills recovery.

Previous studies have indicated that MCA ligation–induced brain infarction can be reduced by lowering the body temperature or increasing the arterial blood pressure. We found that pretreatment with OP1 did not alter these parameters either before or during ischemia, suggesting that the protective effect of OP1 is probably not indirectly mediated through an alteration of these physiological parameters in the stroke animals.

In conclusion, our data indicate that OP1 has protective effects in the CNS and that OP1 can reduce ischemia-induced injury in the adult cerebral cortex. In future studies it will be important to evaluate whether OP1 can reduce infarction when administered subsequent to ischemia and whether this molecule is part of an endogenous protective mechanism.

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References
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TGF-β has been extensively shown to exert neuroprotective roles. Osteogenic protein-1 (OP1) is a member of the TGF-β superfamily that is prominently expressed in fetal but not adult brain. In the preceding article, OP1 is reported to exert neuroprotective effects in an adult rat MCAO model. Results show OP1 reduces impairment of motor function and infarct volumes. The therapeutic window, however, is restricted. Only when it was given 24 hours before injury were the neuroprotective effects of OP1 noted. In addition to neuroprotective effects, neurotrophic factors such as OP1 may also enhance repair or regeneration after ischemic brain injury. It is conceivable that any beneficial effects of OP1 in enhancing regeneration could be conducted in a posttreatment paradigm with specific measures that more specifically address repair and functional recovery, as has been so elegantly done with basic fibroblast growth factor.

References
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