Effects of ifenprodil, a polyamine site NMDA receptor antagonist, on reperfusion injury after transient focal cerebral ischemia

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Polyamines and N-methyl-D-aspartate (NMDA) receptors are both thought to play an important role in secondary neuronal injury after cerebral ischemia. Ifenprodil, known as a noncompetitive inhibitor of polyamine sites at the NMDA receptor, was studied after transient focal cerebral ischemia occurred. Spontaneously hypertensive male rats, each weighing between 250 and 350 g, underwent 3 hours of tandem middle cerebral artery (MCA) and common carotid artery occlusion followed by reperfusion for a period of 3 hours or 21 hours. Intravenous ifenprodil (10 µg/kg/minute) or saline infusion was started immediately after the onset of MCA occlusion and continued throughout the ischemic period. Physiological parameters including blood pressure, blood gas levels, blood glucose, hemoglobin, and rectal and temporal muscle temperatures were monitored. Six rats from each group were evaluated at 6 hours postocclusion for brain water content, an indicator of brain edema, and Evans blue dye extravasation for blood-brain barrier breakdown. Infarct volume was also measured in six rats from each group at 6 and 24 hours postocclusion. Ifenprodil treatment significantly reduced brain edema (82.5 ± 0.4% vs. 83.5 ± 0.4%, p < 0.05) and infarct volume (132 ± 14 mm³ vs. 168 ± 25 mm³, p < 0.05) compared with saline treatment, with no alterations in temporal muscle (brain) or rectal (body) temperature (35.9 ± 0.4°C vs. 36.2 ± 0.2°C; 37.7 ± 0.4°C vs. 37.6 ± 0.6°C; not significant). These results demonstrate that ifenprodil has neuroprotective properties after ischemia/reperfusion injury in the absence of hypothermia. This indicates that antagonists selective for the polyamine site of the NMDA receptors may be a viable treatment option and helps to explain some of the pathophysiological mechanisms involved in secondary injury after transient focal cerebral ischemia has occurred.

KEY WORDS • blood-brain barrier breakdown • cerebral edema • cerebral ischemia • ifenprodil • reperfusion • rat

A mong the possible mechanisms involved in the development of ischemic brain injury, the overactivation of N-methyl-D-aspartate (NMDA) receptors has been thought to play an important role. In various animal models of cerebral ischemia, extracellular glutamate and aspartate levels increase massively after the ischemic event. This increase permits Ca⁺⁺ and water influx into neurons via the NMDA receptors and results in enhanced cellular swelling and neuronal death. It has been shown that the degree of ischemic injury correlates well with the amount of glutamate released during ischemia. In addition, endogenous polyamines may enhance activation of the NMDA receptors by acting at a novel binding site distinct from the recognition sites for glutamate or glycine. Ifenprodil, an antagonist at the polyamine site of the NMDA receptor, has been widely investigated as a possible regulator of NMDA activation and is reported to reduce brain damage and ischemic injury volume after global or permanent focal cerebral ischemia. Although its precise mechanism of action remains unclear, it has been shown that the polyamine, spermidine, significantly reversed the ifenprodil-induced protection against glutamate and NMDA cytotoxicity. This finding indicated that the neuroprotective effects of ifenprodil may be caused by an antagonistic effect on polyamine modulatory sites of NMDA receptors.

Focal cerebral ischemia induced by middle cerebral artery (MCA) occlusion in rat models has been commonly used to evaluate the effects of various drugs on ischemic brain damage. Among these ischemia models, the transient MCA occlusion model is rather close to the common clinical mechanism of cerebral ischemia. Although there are several studies indicating the effectiveness of ifenprodil administration after permanent focal cerebral ischemia has occurred, results derived by using a model of temporary focal cerebral ischemia and reperfusion have not been reported. The purpose of the present study was to determine the effect of ifenprodil therapy on posts ischemic brain edema, blood-brain barrier (BBB) breakdown, and infarction size after reperfusion by using a transient MCA occlusion model in rats.

Materials and Methods

In this study, we carefully adhered to the animal welfare guidelines set forth in the Guide for the Care and Use of Laboratory Animals (United States Department of Health and Human Services Publication 85–23, 1985). The animals underwent operation in ran-
dom order, and outcome assessments were performed by investigators blinded to the experimental group.

Preparation of Animals

Spontaneously hypertensive male rats, each weighing between 250 and 350 g, were anesthetized with an induction dose of 2% halothane and a maintenance dose of 1.2% halothane plus 50% N2O and 50% O2. The animals were mechanically ventilated through an endotracheal tube by a rodent ventilator (PE-240). The PaO2 was maintained between 100 and 200 mm Hg, and the PaCO2 was kept between 30 and 40 mm Hg. The left femoral artery was cannulated for continuous arterial blood pressure (ABP) monitoring and to obtain measurements of pH, PaCO2, PaO2, hemoglobin, and the blood glucose concentration. The left femoral vein was used for drug infusion. Temporal muscle and rectal temperature probes were inserted and the cranial and body temperatures were maintained by a heating blanket and lamp in a physiological range during the period of 3 hours of ischemia and 3 hours of reperfusion. The animals were then allowed to recover from anesthesia and were returned to their cages for the remainder of the study.

Regional Cerebral Blood Flow

Changes in regional cerebral blood flow (rCBF) at the surface of the left parietal cortex were recorded using laser Doppler flowmeter (LDF) probes attached to a laser flowmeter device. After rats were placed in a stereotactic frame, a craniectomy over the MCA territory (5 mm in diameter, 4–6 mm lateral and 1–2 mm caudal to the bregma) was performed with extreme care; a surgical blade was used to shave the bone gently. The dura was left intact. The probe of the LDF was advanced steadily, perpendicular to the cortical surface, by means of a micromanipulator (standard electrode micromanipulator). Care was taken to avoid placing the probe above the pial arteries and veins; although it touched, it did not indent the dura mater. To facilitate optimum LDF recording, the dura was kept moist with warmed saline. Changes in rCBF were expressed as the percentage difference from baseline rCBF assessed before drug administration.

Focal Ischemia Study

The left MCA and ipsilateral common carotid artery (CCA) of the rats were occluded according to a modification of the Brint method as previously described. Briefly, the left cervical CCA was exposed and a No. 3-0 silk suture was positioned under it for the purpose of occlusion. Animals were placed in the lateral position in a stereotactic frame, and a 1-cm skin incision was made at the midpoint between the left lateral canthus and anterior pinna. A 3-mm craniectomy was performed at the junction of the zygoma and squamosal bone, and the dura overlying the MCA was pierced. An 80-μm stainless steel hook was inserted under the MCA by using a micromanipulator, without interrupting blood flow, just superior to the inferior cortical vein.

After the LDF probe was placed over the cortex and a stable baseline was reached, the left CCA was occluded by an aneurysm clip and the ipsilateral MCA was immediately occluded by raising the MCA 0.5 mm above the cortical surface by means of the hook attached to the micromanipulator. The MCA/CCA occlusion was reversed 3 hours later by removing the carotid clip and releasing the hook under the MCA.

Experimental Protocol

All rats underwent 3 hours of ischemia with 3 hours or 21 hours of reperfusion. To measure early BBB breakdown and cerebral edema formation, animals were studied after 3 hours of reperfusion. Assessment of ischemic injury size was performed at 3 and at 21 hours of reperfusion.

Treatment was initiated immediately after the induction of ischemia. An intravenous infusion of either ifenprodil (10 μg/kg/minute) or vehicle (physiological saline) was continued over the next 3 hours. All animals received the same amount of fluid (1.5 ml). Wet-Dry Method for Cerebral Edema Measurements

After the animals were killed, the brain was removed and left and right cortex tissues were dissected and weighed immediately to yield the wet weight. After drying in a desiccating oven at 70°C for 48 hours, the tissues were reweighed to determine water content. Water content was expressed as the percentage of H2O, which was calculated as (wet weight − dry weight/wet weight) × 100.

Evaluation of BBB Integrity

The integrity of the BBB was investigated with Evans blue dye extravasation, according to the method of Uyama, et al. Briefly described, Evans blue dye (2% in saline, 3 ml/kg) was injected intravenously 4 hours after the onset of the ischemic event. Halothane anesthesia was administered 2 hours later and each animal’s chest was opened. The rats underwent saline perfusion through the left ventricle at 100 cm of water pressure until colorless perfusion fluid was obtained from the right atrium. After decapitation the brains were removed, and the left and right hippocampi and cortex were dissected. Each tissue sample was weighed, homogenized in 2 ml of 50% trichloroacetic acid (w/v), and centrifuged at 10,000 rpm for 20 minutes. The supernatant was diluted fourfold with ethanol. For fluorescence measurement, an aliquot was diluted with solvent (50% trichloroacetic acid/ethanol, 1:3). Tissue levels of Evans blue dye were then quantitated using a spectrofluorometer at an excitation wavelength of 620 nm and an emission wavelength of 680 nm. Sample values were compared with those of Evans blue dye standards mixed with the solvent (100–1000 ng/ml).

Measurement of the Volume of Ischemic Brain Injury

The animals were killed 6 or 24 hours after the onset of ischemia. Their brains were quickly removed, placed in cold saline solution for 10 minutes, and cut using a rodent brain matrix into $8 \times 1.5$–mm coronal slices. Sections were stained with 2% 2,3,5-triphenyltetrazolium chloride monohydrate (TTC) as previously described. The volume of ischemic brain injury was measured using a computer programmed with the public domain National Institutes of Health (NIH) Image software. The volume of ischemic brain injury was calculated by the numerical integration of data from individual slices.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD) and were analyzed by analysis of variance followed by Scheffé’s F test or Student’s t-test. A probability value of less than 0.05 was considered statistically significant.

Sources of Supplies and Equipment

Ifenprodil was obtained from Research Biochemical International, Natick, MA, and the rodent ventilator was purchased from Harvard Apparatus, Inc., S. Natick, MA. The Laserflo blood perfusion monitor, model BPM 403A, was manufactured by TSI, Inc., St. Paul, MN. The micromanipulator, model 960, and the small animal stereotactic frame, model 900, with model 924 rotational rat adapter, were obtained from David Kopf Instruments, Tujunga, CA. The Gilford spectrofluorometer, model IV, was obtained from CIBA Corning Diagnostic Corp., Medford, MA. The rodent brain matrix, model RMB 4000C, was manufactured by Activation Systems, Warren, MI. The NIH Image software was written by Wayne Rasband and is available from the Internet via anonymous file transfer protocol from zippy.nimh.nih.gov or as a floppy disk from National Technical Information Service, 5285 Port Royal Road, Springfield, VA 22161, part number PB93-504868.

Results

Physiological Parameters

There were no significant alterations in PaCO2, PaO2, or pH during ischemia and reperfusion; there was no significant difference in average values in the ifenprodil- and saline-treated groups. In the ifenprodil-treated group, temporal muscle temperature was maintained at $35.9 \pm 0.4°C$.
Effects of ifenprodil on reperfusion injury

TABLE 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Saline (24 animals)</th>
<th>Ifenprodil (24 animals)</th>
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<tbody>
<tr>
<td>pH</td>
<td>7.41 ± 0.04</td>
<td>7.43 ± 0.03</td>
</tr>
<tr>
<td>PaCO₂ (mm Hg)</td>
<td>35.7 ± 2.5</td>
<td>35.6 ± 2.8</td>
</tr>
<tr>
<td>PaO₂ (mm Hg)</td>
<td>138 ± 17</td>
<td>132 ± 21</td>
</tr>
<tr>
<td>hemoglobin (g/dl)</td>
<td>15.5 ± 0.7</td>
<td>15 ± 1.1</td>
</tr>
<tr>
<td>glucose (mg/dl)</td>
<td>88.5 ± 9.5</td>
<td>91 ± 10</td>
</tr>
<tr>
<td>brain temp (°C)</td>
<td>36.2 ± 0.2</td>
<td>35.9 ± 0.4</td>
</tr>
<tr>
<td>body temp (°C)</td>
<td>37.6 ± 0.6</td>
<td>37.7 ± 0.4</td>
</tr>
<tr>
<td>mean ABP (mm Hg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>preischemia</td>
<td>127 ± 12</td>
<td>124 ± 13</td>
</tr>
<tr>
<td>posts ischemia</td>
<td>119 ± 13</td>
<td>117 ± 15</td>
</tr>
</tbody>
</table>

* Values are expressed as the mean ± SD. There was no significant difference between the groups for any physiological parameter.

and body temperature at 37.7 ± 0.4°C; in the saline-treated group temperatures were 36.2 ± 0.2°C and 37.6 ± 0.6°C, respectively. Ifenprodil treatment did not cause a significant decrease in mean ABP during the 3-hour periods of ischemia and reperfusion, respectively (Table 1).

Regional Cerebral Blood Flow

After MCA occlusion, rCBF fell to 12 ± 7% of baseline in the ischemic hemisphere of the saline-treated rats. In the ifenprodil-treated group, occlusion reduced rCBF to 12 ± 6% of baseline in the ischemic hemisphere. Reperfusion restored rCBF to 89 ± 54% of baseline in the saline-treated group and 80 ± 40% of baseline in the ifenprodil-treated group at 5 minutes of reperfusion. There was no significant change in the rCBF between the ifenprodil- and saline-treated rats during the experiment (Fig. 1).

Cerebral Edema and BBB Permeability

After 3 hours of reperfusion, the brain water content in both groups (six animals each) was found to be significantly higher in the ischemic cortex than in the contralateral cortex. However, in the ischemic hemisphere of the ifenprodil-treated group, the cortical water content was significantly lower than in the saline-treated group (82.5 ± 0.4% and 83.5 ± 0.4%, respectively, p < 0.05) (Fig. 2).

The Evans blue dye content of the ischemic cortex in both groups was significantly higher than that of the contralateral nonischemic hemisphere (299 ± 113 μg/g in the ischemic and 24 ± 16 μg/g in the nonischemic cortex, respectively, in the saline-treated group; 280 ± 128 μg/g in ischemic and 19 ± 6 μg/g in nonischemic cortex, respectively, in the ifenprodil-treated group) (Fig. 2). There were no statistical differences between the Evans blue dye content of saline-treated and ifenprodil-treated groups in the ischemic cortex.

Ischemic Injury Volume

The total volume of ischemic brain injury in the saline-treated group at 6 and 24 hours after the ischemic event (six animals tested for each time period), as assessed from TTC-stained brain slices, was 168 ± 25 mm³ and 186 ± 29 mm³, respectively. In this method, the striatum was mostly protected from ischemic injury (Fig. 3). Treatment with 10 μg/kg/minute of ifenprodil for 3 hours postocclusion reduced the ischemic brain injury volume to 132 ± 14 mm³ and 143 ± 21 mm³, respectively, at 6 and 24 hours after ischemia (six animals for each time period). A statistically significant difference (p < 0.05) existed between the two groups for both time periods (Fig. 4). The total volume of ischemic brain injury at 24 hours postocclusion was reduced by 23% with ifenprodil treatment.

Discussion

Although numerous animal models have been developed, no single experimental model of focal cerebral ischemia in rodents is ideal for answering all questions about focal stroke. It is widely accepted that the transient MCA occlusion model more closely resembles the pathological changes observed in human stroke. Blood flow restoration may limit to some extent the size of the resulting infarction and improve other measures of outcome such as edema formation. However, this may depend on the duration of the ischemic period. It was observed that reperfusion after 3 hours of MCA occlusion may exacerbate brain injury and edema formation, particularly the vasogenic component, compared with the same duration of permanent occlusion.

Examining the important components of the phenomenon of reperfusion injury has assumed greater importance since researchers have found that recanalization may occur spontaneously in human embolic stroke as well as by intentional thrombolytic therapy, and temporary occlusion may be required for some cerebrovascular surgery and embolizations as well. Ifenprodil, an antagonist at the polyamine site of the NMDA receptor, has been shown to decrease ischemic brain injury and edema formation. However, the published reports demonstrating beneficial effects of ifenprodil are limited to global or focal perma-
ment cerebral ischemia. The present study clearly demonstrates that the postischemic systemic administration of ifenprodil reduces the ischemic injury resulting from prolonged ischemia even after reperfusion. In addition to the infarct size, ifenprodil reduced the edema formation without any effect on the BBB breakdown.

There are some possible mechanisms to explain the neuroprotective effect of ifenprodil on the size of the ischemic injury and cytotoxic edema. It is well known that glutamate plays an important role in the development of cytotoxic edema in astrocytes and neuronal loss, which is mediated by NMDA receptors.12,13,15,32,41 These receptors possess three distinct sites that are sensitive to glutamate, glycine, and the polyamines and that modulate the opening of a channel permeable to Na+, Ca++, and K+ ions.24,37 Selective antagonists for the various sites have been identified.19,30,45 It has been demonstrated that ifenprodil reduces the edema formation without any effect on the BBB breakdown.

The blockade of each site (channel or modulatory) of the NMDA receptor is likely to produce distinct functional consequences in the central nervous system. It has been demonstrated consistently that NMDA channel blockers increase local cerebral glucose use in specific areas in the rat brain.11,29 Cudennec, et al.,18 have reported that eliprodil, a synthetic ifenprodil derivative, did not induce metabolic activation in any region. These findings suggest that ifenprodil prevents NMDA-mediated cytotoxicity via a selective antagonism at the polyamine modulatory site.

In addition to its activity as an NMDA receptor antagonist, ifenprodil has been shown to bind to sigma receptors.17,25 There are some reports indicating that the modulation of the NMDA receptor channel by ifenprodil may occur via its binding to the sigma site.38 However, studies on hippocampal cell cultures showed that the activity of ifenprodil did not appear to result from sigma receptor sites.40 Poignet, et al.,36 have also demonstrated that sigma
Effects of ifenprodil on reperfusion injury

![Graph showing effect of ifenprodil](image)

**Fig. 4.** Bar graph showing the effect of ifenprodil on the volume of ischemic brain injury at the 6th and 24th hours. Values are expressed as the mean ± SD. A statistically significant difference existed between the two groups at both time periods. The total volume of ischemic brain injury was reduced by 23% with ifenprodil treatment at the 24th hour. *p > 0.05 versus saline-treated group.

ligands had no neuroprotective effects in a mouse model of focal cerebral ischemia.

Ifenprodil has also been demonstrated to have some vasodilator effects via α-adrenoceptors,9 and it is also known to constrict pial vessels.48 These properties may result in an inverse steal phenomenon in which blood flow is diverted from the normal tissue toward the ischemic focus.21 However, we did not observe any change in mean ABP or CBF between saline- and ifenprodil-treated groups during ischemic and reperfusion periods. The data derived from this model do not support vascular effects as mechanisms for the neuroprotective properties of ifenprodil.

Ischemic edema is a mixed form of brain lesion with contributions from cytotoxic and vasogenic edema.26 In this study, we used the wet/dry method and Evans blue dye extravasation tests to study both the cytotoxic and vasogenic components of brain edema. Although ifenprodil decreased brain edema formation in the ischemic hemisphere in the present study, we did not observe any beneficial effect of ifenprodil on the BBB breakdown. This finding is in agreement with the lack of NMDA receptors in brain endothelium.3 Although Başkıaya, et al.,2 demonstrated the protective effect of ifenprodil on vasogenic edema after permanent focal cerebral ischemia in cats, this effect might be caused by some mechanisms other than NMDA receptors. These results, along with the other reports, suggest that the pathophysiological mechanisms of the BBB breakdown after recirculation are quite different from those of permanent ischemia.6,47

It has been demonstrated that after 3 hours of temporary MCA occlusion, infarction volumes measured at 6 hours were identical to those observed at 24 hours.14 In this study we measured these time points to see if ifenprodil treatment would delay the evolution of infarction. Hypothermia has been shown to be an important factor in reducing infarction volume after temporary MCA occlusion in rats.35 In the present study the brain temperature was regulated to 35.9 ± 0.4°C and 36.2 ± 0.2°C; and the body temperature to 37.7 ± 0.4°C and 37.6 ± 0.6°C in the ifenprodil- and saline-treated groups, respectively. Our findings demonstrated that the reduction of the ischemic volume was 21% and 23% at the 6th and 24th hours, respectively, despite maintaining the physiological level of brain and body temperature. Because spontaneously hypertensive rats have fewer collateral vessels and are more refractory than Sprague–Dawley rats to treatments aimed at reducing stroke size, our findings indicate that the protection afforded by ifenprodil is substantial.

**Conclusions**

Ifenprodil, an antagonist at the polyamine site of the NMDA receptor, provided protective effects against ischemic injury size and brain edema formation. This was achieved without affecting systemic blood pressure, rCBF, or temperature after ischemic and reperfusion injury. It seems likely that an ifenprodil–receptor interaction at the polyamine site may be responsible for these neuroprotective effects. The clinical relevance of ifenprodil treatment remains to be evaluated, especially in postreperfusion administration.

**References**


A. Doğan, et al.

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