Induction of tolerance against ischemia/reperfusion injury in the rat brain by preconditioning with the endotoxin analog diphosphoryl lipid A

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Object. Inflammatory responses and oxygen free radicals have increasingly been implicated in the development of ischemic brain injury. In some cases, an attenuation of inflammation or free-radical injury can provide tissue protection. Diphosphoryl lipid A (DPL) is a detoxified derivative of a lipopolysaccharide (endotoxin) of Salmonella minnesota strain R595, which is capable of stimulating the immune system without eliciting direct toxic effects. In this study the authors examined the influence of preconditioning with DPL on ischemia/reperfusion injury in rats.

Methods. Sprague–Dawley rats were injected intravenously with either DPL or vehicle. Twenty-four hours later, some animals were tested for superoxide dismutase (SOD) activity. Others were subjected to a 3-hour period of focal cerebral ischemia and, after a reperfusion period of 24 hours, were killed. Infarction volume, SOD activity, and myeloperoxidase (MPO) activity were assayed in the posts ischemic animals.

Pretreatment with DPL produced significant reductions in cerebral infarction and MPO activity in the ischemic penumbra. A significant enhancement of basal SOD activity was observed 24 hours after DPL treatment (that is, before ischemia), and a further enhancement of SOD activity was seen in the ischemic penumbra 24 hours after reperfusion.

Conclusions. These data provide the first evidence of a neuroprotective effect of preconditioning with DPL in an in vivo model of cerebral ischemia. Although the precise mechanisms through which DPL exerts its neuroprotective influence remain to be established, an inhibition of the complex inflammatory response to ischemia and an enhancement of endogenous antioxidant activity are leading candidates.

KEY WORDS • tolerance • cerebral ischemia • neuroprotection • endotoxin • free radical • rat
Materials and Methods

Animal Model

All experimental protocols were approved by the University of Virginia Animal Research Committee. Male Sprague-Dawley rats weighing 280 to 330 g were given free access to food and water before and after surgery. The animals were anesthetized initially with a gas mixture of 4% halothane in oxygen. Injections of 0.1 mg/kg atropine sulfate were administered intramuscularly, and orotracheal intubation was performed. The halothane concentration was reduced to 1% after intubation, and the rats were maintained on a gas mixture of 4% halothane in oxygen. Injections of 0.1 mg/kg atropine sulfate were administered intramuscularly, and orotracheal intubation was performed. The halothane concentration was reduced to 1% after intubation, and the rats were maintained on a mechanical ventilator. The femoral artery was cannulated for blood pressure monitoring and for serial blood gas analyses. Both common carotid arteries were exposed, taking care to preserve the vagus nerve. The common carotid arteries were occluded with a loop of Silastic tubing at the time of MCA occlusion.

Both exposure and occlusion of the MCA were performed as described by Hiramatsu, et al.20 The animal was placed in a right lateral decubitus position and a 1-cm incision was made between the left margin of the orbit and the tragus. The exposed temporals muscle was dissected from the cranium to reveal the inferotemporal fossa. A craniectomy preserving the zygomatic arch was made slightly anterior to the foramen ovale by using an electric drill; this procedure was visualized using a surgical microscope. The dura was opened carefully and reflected with a 30-gauge needle. This manipulation exposed the MCA, which was isolated and occluded with a Sundt No. 1 microclip. The clip site was proximal to the MCA bifurcation, but distal to the origin of the lenticulostrate arteries. Interruption of blood flow was confirmed visually, and occlusion of the MCA and both carotid arteries was maintained for 3 hours. Fifty units of heparin sodium was administered intravenously before induction of ischemia and 90 minutes after ischemia. Blood flow was reestablished by removing the arterial clips; blood flow in the MCA was verified visually by using the surgical microscope. The wound was closed with a No. 5-0 running suture and irrigated. Digital thermometers were used to monitor rectal and temporal muscle temperatures continuously throughout the surgical procedure. The animals’ body temperature was maintained at 37 ± 0.5°C.

Experimental Protocols and Treatment With DPL

In the first experiment, the effect of preconditioning with DPL on MPO and SOD activity was examined 24 hours after ischemic reperfusion. The effect of DPL on infarction volume was examined 48 hours after reperfusion. Animals were assigned to one of three groups: 1) vehicle-treated (saline containing 0.2% triethylamine, eight animals); 2) DPL-treated (100 µg/kg, eight animals); and 3) DPL-treated (200 µg/kg, eight animals). The DPL was dissolved in saline containing 0.2% triethylamine and the volume of the injection was 0.6 ml. A single injection of vehicle or DPL was administered 24 hours before ischemia was induced; injections were made into the tail vein for a period of 3 minutes.

In the second experiment, the effect of DPL treatment on SOD activity was determined in animals that were not subjected to ischemia. In this experiment, rats were assigned to the same vehicle or drug treatment groups as described previously (six animals per group) and SOD activity was examined 24 hours after vehicle or drug injection; no ischemia was induced in these animals. The time of SOD analysis in the second experiment corresponded to the time at which animals in the first experiment were subjected to ischemia. The dosages of DPL used in this study were selected on the basis of a pilot study in which 100 and 200 µg/kg DPL, but not 50 µg/kg DPL, appeared to be effective in limiting cerebral infarction. Higher dosages were not tested because of their potential toxicity. The selection of the 24-hour pretreatment time point was based on previous evidence indicating that MPL treatment immediately before cardiac ischemia59 or 2 hours before ischemia20 is not effective in inducing protection, but that treatment 24 hours before ischemia is protective.42,59–61 In addition, previous findings demonstrate that the maximum enhancement of endogenous antioxidant activity occurs approximately 24 hours after injection of MPL.42

Determination of Infarction Size

The infarction size was measured 48 hours after ischemic reperfusion. Animals were decapitated after deep anesthesia was induced, their brains were removed, and 2-mm-thick coronal sections were cut with a tissue slicer. Sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride in 0.9% phosphate-buffered saline, incubated for 30 minutes at 37°C, and placed in buffered 10% formalin. As described previously, the boundaries of the infarct could be visually identified.20,51,52 These boundaries were outlined by hand, and area measurements were performed using a computerized image analysis system. Total infarct volume was calculated by multiplying infarcted areas in each slice by slice thickness and totaling the values for all slices.

Activity of MPO

We used MPO activity as a marker of PMNL accumulation in the tissue. This enzyme is found within the azurophilic granules of PMNLs and monocytes, but not in lymphocytes.6 In a number of recent studies, investigators have confirmed the reliability of MPO as an index of PMNL accumulation and have used MPO to quantify the accumulation of PMNLs in postischemic brain.4,44 As described previously,45 MPO activity was measured 24 hours after ischemic reperfusion. Animals were perfused transcardially with 200 ml of physiological saline. The brains were removed and 4-mm-thick sections were cut through the MCA distribution. Three samples were chosen from these sections (Fig. 1): one from the center of the infarction (core sample), one from the area adjacent to the infarction (penumbra sample), and one from the central area of the striatum (basal ganglia sample). The samples were weighed on an electronic analytical balance to obtain the wet weight.

The procedure described by Bradley, et al.,4 with minor modification, was used for the analysis of MPO. Brain tissue was homogenized in seven volumes of 50 mmol/L potassium phosphate buffer (pH 6) containing 0.5% hexadecyltrimethylammonium bromide. The specimens were freeze-thawed and sonicated three times (10 seconds each at 25°C). Samples were incubated at 4°C for 20 minutes and centrifuged at 16,000 G for 15 minutes. Ten milliliters of the supernatant was mixed with 250 µL of 50 mmol/L phosphate buffer (pH 6) containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The levels of MPO were then assayed spectrophotometrically. The change in absorbance at 450 nm was measured, and MPO activities in tissue were calculated using human MPO as a standard. Although it has been demonstrated in a recent study that hemoglobin can confound the measurement of MPO in tissue samples,60,62 this was not a major complication in our
Endotoxin analog and induction of ischemic tolerance

physiological variables in rats treated with DPL with or without ischemia*

<table>
<thead>
<tr>
<th>Group</th>
<th>3VO &amp; Vehicle</th>
<th>100 μg/kg</th>
<th>200 μg/kg</th>
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<td>preischemic</td>
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<tr>
<td>MABP (mm Hg)</td>
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<td>102.0 ± 2.0</td>
<td>104.0 ± 3.0</td>
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<td>BT (˚C)</td>
<td>36.1 ± 0.1</td>
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<tr>
<td>pH</td>
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<tr>
<td>P O 2 (mm Hg)</td>
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<td>38.8 ± 1.6</td>
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<tr>
<td>P O 2 (mm Hg)</td>
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<td>MABP (mm Hg)</td>
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<td>P O 2 (mm Hg)</td>
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<tr>
<td>P O 2 (mm Hg)</td>
<td>110.0 ± 6.0</td>
<td>116.0 ± 5.0</td>
<td>122.0 ± 4.0</td>
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*Values are the means ± SEM. No significant differences were observed among groups for the physiological variables. Abbreviations: BT = body temperature; MABP = mean arterial blood pressure; TMT = temporal muscle temperature; 3VO = three-vessel occlusion.

Results

Physiological Values

Physiological parameters for the individual treatment groups are summarized in Table 1. There were no significant differences among groups in body or temporal muscle temperatures monitored during the surgical procedure.

Infarction Volume

Transient focal ischemia for 3 hours produced consistent infarction in the rat neocortex when examined after 24 hours of reperfusion (Fig. 2). Pretreatment with DPL attenuated cerebral infarction. The volume of cerebral infarction was reduced by 15% and 31% in animals receiving 100 μg/kg and 200 μg/kg DPL, respectively. This effect achieved statistical significance in the group receiving 200 μg/kg (p < 0.05).

Activity of MPO

Low levels of MPO activity (< 5 U/g wet weight) were observed in samples prepared from sham-operated animals (Fig. 3). The MPO activity was increased in the core and penumbral regions of the cortex following ischemia/reperfusion, but not in the basal ganglia. The increase in MPO activity induced by ischemia/reperfusion was substantially greater in the core than in the penumbra (Fig. 3). Preconditioning with DPL tended to inhibit ischemia-induced increases in MPO activity compared with those in vehicle-treated animals (Fig. 3). In the core region, treatment with 100 or 200 μg/kg DPL inhibited ischemia-induced MPO activity by 37% and 36%, respectively; however, neither of these differences achieved statistical significance. In the penumbra, preconditioning with 100
or 200 μg/kg DPL inhibited the ischemia-induced increase in MPO activity by 31% and 78%, respectively; the decrease observed in the group treated with 200 μg/kg DPL achieved statistical significance (p < 0.01).

**Effect of DPL Administration on SOD Activity**

**Nonischemic Brain.** The basal activity of SOD in the cortex of control animals was 4.4 ± 0.7 U/mg protein. Increases in SOD activity were observed 24 hours after administration of DPL. The SOD activity in the groups receiving 100 and 200 μg/kg DPL was 9.8 ± 6.3 and 18.7 ± 6.3 U/mg protein, respectively (Fig. 4). The increase in the group receiving 200 μg/kg achieved statistical significance (p < 0.05).

**Ischemic Brain.** The SOD activity was elevated significantly 24 hours after ischemia/reperfusion in the penumbra (106.2 ± 25.6 U/mg protein, p < 0.05 compared with nonischemic control). In contrast, SOD activity was not affected in the core region and only slightly (but not significantly) increased in the basal ganglia (Fig. 5). Pretreatment with DPL enhanced the increase in SOD activity induced by ischemia/reperfusion in the penumbra. The SOD activity in the penumbra was increased to 131.9 ± 35.2 U/mg protein (p > 0.05 compared with the vehicle-treated ischemic group) and 232.8 ± 67.6 U/mg protein (p < 0.05) in the 100 and 200 μg/kg treatment groups, respectively (Fig. 5A). In contrast, no significant changes in the induction of SOD activity were observed in the core region or basal ganglia of either DPL-treated group (Fig. 5B and C).

**Discussion**

In this study we demonstrate that preconditioning with the endotoxin analog DPL reduces cerebral infarction after transient focal ischemia. Systemic treatment with DPL is likely to induce a variety of cellular and molecular mechanisms that could plausibly contribute to the phenomenon of ischemic tolerance. Although the underpinnings of this form of induced tolerance remain to be established unequivocally, our findings implicate two mechanisms in this process: a reduction in cell-mediated inflammation and an enhancement of endogenous antioxidant activity.

Considerable evidence supports a role for cell-mediated inflammation in the elaboration of ischemic injury. Polymorphonuclear leukocytes have been linked to the pathogenesis of ischemia, and in particular to the elaboration of focal ischemia/reperfusion injury. The number of PMNLs in the brain increases substantially after cerebral ischemia, and these cells may exert deleterious effects by means of multiple mechanisms. The intravascular adherence and accumulation of PMNLs can contribute to secondary ischemia via capillary plugging. In addition, PMNLs release a variety of cytokines and free radicals, which can directly and/or indirectly trigger cellular damage. This damage may occur at the level of both the cerebral microvasculature and the brain parenchyma. In several recent studies it has been demonstrated that antibodies or synthetic compounds blocking neutrophil adhesion and/or infiltration are capable of attenuating cerebral
ischemia/reperfusion injury. Our findings demonstrate that the postischemic elevation of MPO levels, which provides an index of the number and activity of PMNLs in the tissue, is reduced in animals pretreated with DPL. Thus, our observations indicate that cell-mediated inflammation after ischemia/reperfusion is attenuated after DPL treatment. It is possible that this reduction in cellular inflammation is the result of an unrelated protective influence provided by DPL and thus reflects a secondary manifestation of neuroprotection. However, it is also conceivable that the reduction in cell-mediated inflammation is a direct source of the protective influence of DPL preconditioning.

As discussed previously, DPL acts as an immunostimulant, suppresses the infiltration of neutrophils, and limits cellular injury in other tissues. This is a somewhat paradoxical finding because treatment with endotoxin analogs can stimulate cytokine production, which can lead to increased cellular infiltration and inflammation. However, in pretreatment paradigms, a conditioning challenge with subinjurious levels of endotoxin analogs attenuates the subsequent inflammatory response to an injurious challenge. For instance, pretreatment with endotoxin analogs results in decreased leukocyte infiltration after subsequent injurious insults such as myocardial infarction or septic shock. It is therefore plausible that a similar suppression of neutrophil infiltration could underlie the protective influence of DPL against cerebral injury after focal ischemia/reperfusion.

A second mechanism through which DPL may exert a neuroprotective effect is an induction of antioxidant activity. Oxygen free radicals are thought to play a direct role in cellular injury after ischemia/reperfusion. Free radicals are produced during partial ischemia and following ischemia/reperfusion. In addition, oxidative damage is a common feature of ischemic brain injury. It has been demonstrated in numerous studies that free radical scavengers and antioxidant enzymes can inhibit ischemic brain injury. Although the evidence concerning a protective role of exogenous antioxidants has not been uniform, the bulk of the findings indicate an important role for free radical–mediated injury in ischemia/reperfusion injury. An attenuation of free radical–induced damage could be responsible in part for the protective effects observed after DPL preconditioning. Levels of the endogenous antioxidant SOD were significantly increased in DPL-treated animals by the time of the ischemic challenge. These levels were further enhanced in DPL-treated animals 24 hours after transient ischemia. These findings raise the possibility that DPL-induced increases in endogenous antioxidant activity contribute to the neuroprotective effect of DPL preconditioning.

It is also conceivable that the effects of DPL on antioxidant activity and cell-mediated inflammation are directly related to one another. Becker, et al., have recently demonstrated that SOD inhibits the postischemic adhesion of PMNLs to vascular endothelium. It is thus possible that a sequence of events involving the induction of SOD, an inhibition of cellular adhesion, and a resultant suppression of inflammatory injury is responsible for the protective actions of DPL.

Another potential effect of DPL that could theoretically contribute to neuroprotection is hyperthermia; hyperthermic preconditioning has previously been shown to attenuate ischemic injury to the brain. However, the available evidence in the literature and the results of the present study indicate that intravenous administration of DPL is not strongly pyrogenic. In our present study, we measured body temperature at 30 minutes and 24 hours after DPL administration and did not observe a significant change from baseline temperature. In an earlier study, rats receiving a relatively high dose of DPL (500 µg/kg) did not exhibit changes in body temperature at 30 minutes, 1, 2, or 5 hours after injection of DPL. Together, these findings indicate that, at the dosages used in this study, DPL is not strongly pyrogenic and that hyperthermia does not contribute to the induction of tolerance.

Over the last decade, ischemic tolerance has been found to occur in response to a variety of preconditioning stimuli, including brief ischemia, spreading depression, and hyperthermia. It is possible that some or all
of these forms of ischemic tolerance share underlying protective mechanisms. For instance, recent evidence from our laboratory indicates that ischemia-induced ischemic tolerance is associated with substantial increases in SOD activity.1 When brief periods of preconditioning ischemia were administered 24 hours before a more profound (test) period of ischemia, cerebral infarction was attenuated only in conjunction with those protocols in which SOD levels also increased significantly. Although purely correlative in nature, these findings indicate that the induction of SOD could be a common feature of multiple forms of ischemic tolerance. An important goal of future research will be to determine whether the induction of SOD activity translates into a suppression of ischemia-induced oxygen radicals; this outcome would be expected if the increased activity of SOD is indeed causally related to the phenomenon of ischemic tolerance.

Finally, it will be important to explore the potential therapeutic value of chemically-induced ischemic tolerance. It is conceivable that the prophylactic use of tolerance-inducing agents could be of benefit to surgical candidates and/or individuals at high risk for stroke.1 Future studies establishing optimal dose–response relationships and the precise time course for the induction and maintenance of ischemic tolerance will be essential for evaluating these concepts.

Conclusions
Preconditioning with the endotoxin analog DPL induces ischemic tolerance in a rat model of focal cerebral ischemia. Enhancement of endogenous antioxidant activity and suppression of cellular inflammation may play critical roles in the expression of this form of ischemic tolerance.

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