Enhanced endogenous antioxidant activity and inhibition of cerebral vasospasm in rabbits by pretreatment with a nontoxic endotoxin analog, monophosphoryl lipid A

Tomikatsu Toyoda, M.D., Aij-Lie Kwan, M.D., Murad Bavbek, M.D., Neal F. Kassell, M.D., John E. Wanebo, M.D., and Kevin S. Lee, Ph.D.

Department of Neurological Surgery, University of Virginia Health Sciences Center, Charlottesville, Virginia

Objective. Monophosphoryl lipid A (MPL) and diphosphoryl lipid (DPL) are derivatives of the lipopolysaccharide (endotoxin) of Salmonella minnesota strain R595. Monophosphoryl lipid A is relatively nontoxic and can stimulate the natural defense or immune system. Diphosphoryl lipid is relatively toxic; however, at higher concentrations, it can also stimulate an immune response. The purpose of the present study was to determine the effects of these endotoxin analogs on cerebral vasospasm after the onset of subarachnoid hemorrhage (SAH) in rabbits.

Methods. Intrathecal administration of MPL or DPL (5 μg/kg) was performed immediately before and 24 hours after induction of SAH in New Zealand White rabbits. Forty-eight hours after induction of SAH, the animals were killed by perfusion fixation for morphometric analyses of vessels or perfused with saline and assayed for superoxide dismutase (SOD) activity. Additional rabbits were administered MPL or DPL and killed 24 hours later for assessment of SOD activity; no SAH was induced in these animals.

Experimental SAH elicited spasm of the basilar arteries in each group. Vasospasm was markedly attenuated in animals treated with MPL (p < 0.01 compared with vehicle-treated animals), but not in animals treated with DPL. A substantial reduction in SOD activity in the basilar artery accompanied the vasospasm; this loss of activity was significantly blocked by treatment with MPL, but not DPL. In animals that were not subjected to experimental SAH, MPL elicited a significant increase in SOD activity over basal levels, whereas DPL was ineffective.

Conclusions. These data provide evidence of a marked protective effect of the endotoxin analog MPL against vasospasm. Although the mechanism(s) responsible for the protective effect of MPL remains to be verified, an enhancement of basal antioxidant activity and an inhibition of SAH-induced loss of this activity are attractive candidates. An MPL-based therapy could represent a useful addition to current therapies for SAH-induced cerebral injury.

Key Words • cerebral vasospasm • monophosphoryl lipid A • diphosphoryl lipid A • superoxide dismutase • rabbit
Endotoxin analog attenuates cerebral vasospasm

impact of SAH. In the present study, we examined the effects of pretreatment with two endotoxin analogs on the activity of the antioxidant enzyme SOD and on cerebral vasospasm following SAH.

Materials and Methods

Animal Preparation

All experimental protocols were approved by the University of Virginia Animal Research Committee. New Zealand White rabbits weighing 3 to 4 kg each were subjected to experimental SAH by injecting autologous blood, as described in detail later in this section. The animals received intracisternal injections of drug or vehicle (saline) immediately before and 24 hours after induction of SAH and were killed 48 hours after the onset of SAH by perfusion fixation. Forty rabbits were randomly assigned to one of five groups: 1) control (without SAH); 2) SAH only; 3) SAH plus vehicle (saline); 4) SAH plus MPL (5 μg/kg); or 5) SAH plus DPL (5 μg/kg). The MPL and DPL were dissolved in saline, thoroughly vortexed, and sonicated before administration.

Experimental SAH

Subarachnoid hemorrhage was produced in the following manner. The rabbits were anesthetized with an intramuscular injection of a mixture of 9 mg/kg xylacaine (Rompun) and 55 mg/kg ketamine (Ketaset). The animals were intubated with an endotracheal tube. Three milliliters of arterial blood was taken from the ear artery and injected into the cisterna magna by using a 23-gauge butterfly needle. The animals were positioned in ventral recumbency for at least 15 minutes after injection of the blood to allow ventral clot formation. The rabbits were monitored closely for respiratory distress and ventilated as needed, after which they were extubated and returned to the vivarium when they were fully awake.

Morphometric Studies

Forty-eight hours after SAH occurred, perfusion fixation was performed in the following manner. All animals were reanesthetized, intubated, and ventilated with a small animal ventilator. The animals were paralyzed by an intramuscular injection of pancuronium bromide (0.3 mg/kg). The ear artery was catheterized to the vivarium when they were fully awake.

Arterial cross sections from each animal were analyzed by an arterial computer-assisted morphometric analysis. The luminal areas of five cross sections from a given artery were averaged to provide a single value for each animal.

Measurement of SOD Activity

Basal SOD Activity. Twenty-four rabbits were divided into three groups and treated with: 1) saline; 2) MPL (5 μg/kg); or 3) DPL (5 μg/kg). The animals in this series were not subjected to SAH. Twenty-four hours after intracisternal drug administration, these animals were killed.

Superoxide Dismutase Activity After SAH. Another series of 18 animals was subjected to SAH and analyzed for SOD activity. These animals were divided into three groups receiving: 1) saline; 2) MPL (5 μg/kg); or 3) DPL (5 μg/kg). The induction of SAH and drug treatments were performed in the manner described earlier in the morphometric series. The animals were killed 48 hours after induction of SAH.

Superoxide Dismutase Activity Assay. The animals were anesthetized and perfused transcardially with saline, after which the basilar arteries were removed from the brainstem. The activity of SOD was assayed as described in detail elsewhere.125 Briefly, the basilar artery was homogenized in 10 vol of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediamine tetraacetic acid (EDTA). The samples were incubated at 4°C for 20 minutes and centrifuged at 16,000 G for 15 minutes. The SOD activity was measured by assaying the ability of endogenous SOD to inhibit superoxide radical-dependent cytochrome C reduction. The assay medium contained 20 μM cytochrome C (Type III from horse heart), 100 μM xanthine, and sample supernatant, with a final volume of 1 ml. The reaction was initiated by adding 16.7 μl of xanthine oxidase solution (Grade I from buttermilk), which was sufficient to produce 0.02 to 0.03 absorbance U/minute, as measured spectrophotometrically at 550 nm in the absence of sample. The rate of cytochrome C reduction was recorded for each sample using a standard spectrophotometer over 1 minute at a wavelength of 550 nm. One unit of SOD is defined as the amount of SOD in brain homogenate required to inhibit 50% of the rate of cytochrome C reduction. The protein was assayed by using the phenol reagent method and the SOD activity is presented as units per milligram of protein.

Statistical Analysis

All data are presented as the mean ± standard error of the mean (SEM). Differences among groups in arterial area and SOD activity were compared by factorial analysis of variance (ANOVA) with Fisher’s protected least significant differences (LSD) test. Probability values that were less than 0.05 were considered significant.

Sources of Supplies and Equipment

The MPL and DPL were purchased from RIBI ImmunoChem Research (Hamilton, MT). The HBSS, cytochrome C, xanthine, xanthine oxidase, and EDTA were obtained from Sigma Chemical Co. (St. Louis, MO). Cross sections of basilar arteries were cut with an Ultracut E ultramicrotome (Reichert, Vienna, Austria). To maintain the animals, we used a ventilator (model 683) available from Harvard Apparatus Co. (South Natick, MA). The spectrophotometer used in the SOD activity assay (model U2000) was obtained from Hitachi Instruments, Inc. (San Jose, CA).

Results

General Observations

A thick subarachnoid clot was observed over the basal surface of the brainstem in each animal subjected to SAH. Table 1 displays the physiological parameters, measured at the time of perfusion fixation, for the groups of animals in the morphometric studies. There were no significant differences among the groups with respect to blood pH,
PCO₂ or PO₂. Basilar arteries in animals subjected to SAH were constricted relative to control animals (Fig. 1). In the SAH-only group, constriction of the basilar arteries was severe, and substantial corrugation of the internal elastic lamina was observed. Constriction and corrugation of the basilar artery were attenuated in animals treated with MPL (Fig. 1).

**Cross-Sectional Area Measurements**

**Effects of Endotoxin Analogs on Vasospasm After SAH.** Experimental SAH constricted the basilar arteries in each group of animals. Average cross-sectional areas of basilar arteries were reduced from control levels by 68% and 62% in the SAH-only group and the SAH plus vehicle group, respectively. The SAH-induced reduction in the size of basilar arteries was attenuated in animals treated with MPL (Fig. 2). The average cross-sectional area was reduced from control levels by 32% in the group receiving 5 μg/kg MPL; this effect achieved statistical significance (p < 0.01 compared with the SAH plus vehicle group; Fisher’s protected LSD test). The average cross-sectional area in the DPL-treated group did not differ significantly from that of the SAH plus vehicle group.

**Superoxide Dismutase Activity**

**Effects of Endotoxin Analogs on Basal SOD Activity.** The basal SOD activity of basilar arteries in control animals was 8647.7 ± 3290.3 U/mg protein. Twenty-four hours after intracisternal administration of 5 μg/kg MPL, SOD activity was increased to 50,849.4 ± 17,214.5 U/mg protein (p < 0.05) (Fig. 3 left). The level of basal SOD activity in the group receiving 5 μg/kg DPL was higher than control levels (15,587.1 ± 9258.2); however, this difference did not achieve statistical significance.

**Effects of Endotoxin Analogs on SOD Activity After SAH.** The SOD activity was significantly reduced from control levels 48 hours after SAH occurred in all groups. In the SAH plus vehicle group, SOD levels were 52 ± 17.2 U/mg protein (p < 0.01 compared with basal SOD activity). Treatment with MPL attenuated the reduction of SOD activity after SAH (Fig. 3 right); the SOD activity in the SAH plus MPL group was 262.6 ± 111.3 U/mg protein (p < 0.05 compared with SAH plus vehicle group). The SOD activity in the SAH plus DPL group (54.4 ± 25.2 U/mg protein) did not differ significantly from that observed in the SAH plus vehicle group.

**Discussion**

Oxygen free radicals appear to be major contributing factors to the pathogenesis of cerebral vasospasm. Oxygen radicals and lipid peroxides, through the peroxidation of membrane phospholipids, can compromise the structure and function of cells in the arterial wall. Administration of exogenous antioxidants, such as SOD and catalase, have been shown to inhibit oxyhemoglobin-induced contractions of cerebral arteries. Moreover, free radical scavengers exert significant protective effects in both clinical and experimental cerebral vasospasm.

---

**TABLE 1**

Summary of physiological parameters in 40 rabbits used in morphometric studies of the effects of MPL and DPL.

<table>
<thead>
<tr>
<th>Group</th>
<th>pH</th>
<th>PCO₂ (mm Hg)</th>
<th>PO₂ (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>7.46±0.01</td>
<td>36.9±1.2</td>
<td>116±10</td>
</tr>
<tr>
<td>SAH only</td>
<td>7.46±0.01</td>
<td>35.4±1.3</td>
<td>104±11</td>
</tr>
<tr>
<td>SAH plus vehicle</td>
<td>7.46±0.01</td>
<td>35.0±2.0</td>
<td>107±7</td>
</tr>
<tr>
<td>SAH plus MPL (5 μg/kg)</td>
<td>7.45±0.02</td>
<td>37.0±2.9</td>
<td>106±7</td>
</tr>
<tr>
<td>SAH plus DPL (5 μg/kg)</td>
<td>7.44±0.01</td>
<td>33.9±1.7</td>
<td>115±5</td>
</tr>
</tbody>
</table>

* Values are expressed as the mean ± SEM. Eight animals were assigned to each study group.

---

Fig. 1. Photomicrographs showing the effect of MPL on basilar arteries. Basilar artery cross sections are shown for animals from the following groups: SAH only (A), SAH plus vehicle (B), and SAH plus MPL (C). Bar = 250 μm.
Endotoxin analog attenuates cerebral vasospasm

Although SOD activity has been shown to be affected in the brain parenchyma after SAH, the role of endogenous antioxidant systems in SAH-induced injury to cerebral arteries has remained poorly understood. The present findings indicate that SAH substantially reduces the endogenous antioxidant activity of SOD. This reduction presumably occurs as a result of increased oxidative challenge in the vessel. Pretreatment of cerebral vessels with a relatively nontoxic derivative of endotoxin, MPL, attenuates the reduction in SOD activity elicited by SAH. In addition, MPL by itself enhances basal SOD activity substantially. The upregulation of basal SOD activity and the attenuation of SAH-induced reductions in SOD by MPL are associated with a significant amelioration of cerebral vasospasm. In contrast, another more toxic endotoxin derivative, DPL, did not significantly alter basal SOD activity or the reduction of SOD activity in response to SAH. The DPL was also ineffective in blocking SAH-induced cerebral vasospasm. These observations are consistent with the concept that an impairment of endogenous antioxidant enzyme activity may contribute to SAH-induced vasospasm. In addition, the results suggest that the upregulation of endogenous SOD activity may provide a protective influence against SAH-induced vascular damage.

Monophosphoryl lipid A is a purified endotoxin product obtained by acid and alkaline hydrolysis of Salmonella minnesota R595 lipopolysaccharide. The MPL is 100 to 10,000 times less toxic than endotoxin but retains many of the immunostimulatory features of its parent toxin. Treatment with MPL has been shown to provide protective effects against endotoxic shock and cardiac ischemia–reperfusion. These findings have stimulated considerable interest in the potential clinical value of MPL. In healthy human patients, MPL appears to be well tolerated.

Fig. 2. Bar graph displaying the effect of endotoxin analogs on cross-sectional areas of basilar arteries. The average luminal area (mean ± SEM) of cross sections of rabbit basilar arteries is shown for each group of animals. The degree of vasospasm was reduced significantly in the group treated with 5 µg/kg MPL (SAH plus MPL) (p < 0.01, for comparisons with the vehicle-treated group [SAH plus vehicle] using ANOVA with Fisher’s protected LSD test [F4,35 = 18.739]). DPL = SAH plus DPL group.

Fig. 3. Left: Bar graph showing the effects of endotoxin analogs on basal SOD activity 24 hours after intracisternal drug administration. The SOD activity was elevated 5.9-fold (p < 0.05 compared with vehicle) in rabbits receiving 5 µg/kg MPL. The increase in the DPL-treated group was only slight (1.8-fold) and nonsignificant. Values are expressed as the mean SOD activity ± SEM. Significance was determined by ANOVA with Fisher’s protected LSD test (F2,21 = 3.914). There were eight rabbits in each group. Right: Bar graph showing the effects of endotoxin analogs on SOD activity 48 hours after induction of SAH. The level of SOD activity was markedly reduced 48 hours after SAH (1/166-fold, p < 0.01 using the Mann–Whitney U-test between two vehicle groups). Treatment with 5 µg/kg MPL (SAH plus MPL group) significantly enhanced SOD activity (p < 0.05 using ANOVA with Fisher’s protected LSD test [F2,15 = 3.291]). No elevation in the DPL-treated (SAH plus DPL) group was observed. There were six animals in each group.
tolerated and current targets for the development of MPL therapy include: 1) septic shock; 2) ischemic injury associated with coronary artery surgery; and 3) ischemia as an adjuvant for vaccines. The present study suggests that MPL-based therapy could also be of value in the treatment of cerebral vasospasm following SAH. Treatment with MPL immediately before and 24 hours after SAH provides a substantial and significant attenuation of cerebral vasospasm in the rabbit. This type of therapeutic approach could also be of value in human patients who have suffered SAH. Following SAH in humans, there is a delay of a day or more before the onset of angiographic or clinical vasospasm. Based on the present study, it is conceivable that early intervention with MPL could suppress the progression of the vasospastic response within this time frame. An important goal of future studies will be to identify the precise time course over which MPL treatment is effective and to continue evaluating the mechanisms underlying this unique form of cerebral protection.

Acknowledgment

The authors thank Jennifer L. Collins for her assistance.

References


T. Toyoda, et al.

J. Neurosurg. / Volume 88 / June, 1998
Endotoxin analog attenuates cerebral vasospasm


Manuscript received June 30, 1997.
Accepted in final form February 5, 1998.
This work was supported by Grant No. HL49396 from the National Institutes of Health.

Address reprint requests to: Kevin S. Lee, Ph.D., Department of Neurological Surgery, Box 420, Health Sciences Center, University of Virginia, Charlottesville, Virginia 22908. email: ksl3h@virginia.edu.