Endotoxin Preconditioning Prevents Cellular Inflammatory Response During Ischemic Neuroprotection in Mice

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Background and Purpose—Tolerance to ischemic brain injury is induced by several preconditioning stimuli, including lipopolysaccharide (LPS). A small dose of LPS given systemically confers ischemic protection in the brain, a process that appears to involve activation of an inflammatory response before ischemia. We postulated that LPS preconditioning modulates the cellular inflammatory response after cerebral ischemia, resulting in neuroprotection.

Methods—Mice were treated with LPS (0.2 mg/kg) 48 hours before ischemia induced by transient middle cerebral artery occlusion (MCAO). The infarct was measured by 2,3,5-triphenyltetrazolium chloride staining. Microglia/macrophage responses after MCAO were assessed by immunofluorescence and flow cytometry. The effect of MCAO on white blood cells in the brain and peripheral circulation was measured by flow cytometry 48 hours after MCAO.

Results—LPS preconditioning induced significant neuroprotection against MCAO. Administration of low-dose LPS before MCAO prevented the cellular inflammatory response in the brain and blood. Specifically, LPS preconditioning suppressed neutrophil infiltration into the brain and microglia/macrophage activation in the ischemic hemisphere, which was paralleled by suppressed monocyte activation in the peripheral blood.

Conclusions—LPS preconditioning induces neuroprotection against ischemic brain injury in a mouse model of stroke. LPS preconditioning suppresses the cellular inflammatory response to ischemia in the brain and circulation. Diminished activation of cellular inflammatory responses that ordinarily exacerbate ischemic injury may contribute to neuroprotection induced by LPS preconditioning.

Key Words: cerebral ischemia, focal ■ inflammation ■ leukocytes ■ mice ■ microglia

Tolerance to ischemic brain injury is induced by several distinct preconditioning stimuli that confer neuroprotection, including brief periods of ischemia, cortical spreading depression, brief episodes of seizure, and exposure to anesthetic inhalants.1–4 Although the mechanisms that underlie the various forms of preconditioning are not well understood, they share a common link: small doses of an otherwise harmful stimulus induce protection against subsequent injurious challenge.

Preconditioning with low doses of endotoxin (lipopolysaccharide [LPS]) in the rat provides protection against subsequent challenge with injurious focal ischemia in the brain.5 The mechanisms involved in LPS preconditioning are incompletely understood; however, activation of inflammatory pathways appears to play a role. In particular, LPS-induced activation of tumor necrosis factor-α (TNF-α) and its downstream signaling mediator ceramide are important for neuroprotection against ischemic injury.6,7 Beneficial roles provided by superoxide dismutase (SOD) and endothelial NO synthase have also been postulated, which supports the critical involvement of inflammatory pathways in LPS preconditioning.8–10

Some evidence suggests that LPS preconditioning reduces ischemic injury without a corresponding decrease in inflammatory cell infiltration. Pretreatment of rats with low doses of LPS decreased ischemic infarct size despite increased numbers of inflammatory cells in the ischemic hemisphere.11 This is paradoxical because it is generally accepted that the degree of inflammatory infiltration correlates with the extent of ischemic injury.12,13 The observed reduction in ischemic injury, despite increased numbers of inflammatory cells, may reflect a condition in which the inflammatory cells exist in an altered state of activation at the time of ischemia. Such altered activity occurs in macrophages primed with a low dose of LPS, wherein they show reduced cytokine activity on subsequent challenge with LPS.14 We hypothesized that LPS preconditioning before ischemia renders peripheral macrophages and microglia hyporesponsive to activation by ischemia.

We investigated the effect of LPS preconditioning on the cellular inflammatory response after cerebral focal ischemia in mice. We report that LPS preconditioning induces significant neuroprotection against focal ischemic injury. LPS
preconditioning modulates the cellular inflammatory response to ischemia in the brain and peripheral circulation and leads to decreased cellular infiltration and suppressed microglia and monocyte activation, which may contribute to neuroprotection.

Materials and Methods

Mice
Male C57BL/6 mice (8 to 10 weeks of age, National Cancer Institute, Frederick, Md) were housed in an American Association for Laboratory Animal Care–approved facility. Procedures were conducted according to Oregon Health and Science University, Institutional Animal Care and Use Committee, and National Institutes of Health guidelines. Mice were given free access to food and water.

LPS Treatment
Mice were given an intraperitoneal injection of saline or LPS (0.2 mg/kg; Escherichia coli serotype 055:B5; Sigma) 48 hours before middle cerebral artery occlusion (MCAO). Mice administered LPS showed no differences in body weights and glucose levels. Mean body temperature fluctuated within a normal physiological range <37°C.

Ischemia Reperfusion Model
Mice were anesthetized with 4% halothane and subjected to 60 minutes of MCAO using the monofilament suture method described previously.15 Cerebral blood flow was monitored throughout surgery by laser Doppler flowmetry. Body temperature was maintained at 37°C with a thermostat-controlled heating pad after surgery.

Infarct Evaluation
A coronal brain section (2 mm) was removed at bregma and placed in 2% 2,3,5-triphenyltetrazolium chloride in saline at 37°C for 30 minutes.16 Stained sections were scanned and percentage of area of ischemic hemisphere was measured by a blind observer (NIH Image 1.62). We have documented that infarct area of this coronal section correlates highly ($r^2=0.96$) with infarct volume in this model.17

Preparation of White Blood Cells From Brain Tissue
Forty-eight hours after MCAO, mice were perfused transcardially with heparinized saline (2 U/mL heparin). A section was excised for infarct analysis as above, and cells were isolated from the ischemic and nonischemic hemispheres (excluding the olfactory bulb and cerebellum) as described.15 Tissue was dissociated (20 U/mL collagenase II and 25 U/mL DNase I) and cells were purified from Percoll gradients and counted using trypsin blue exclusion.

Preparation of White Blood Cells From Blood
Mice were anesthetized 48 hours after MCAO with isoflurance. For flow cytometry, blood was collected via the retroorbital sinus into tubes containing heparin (1000 U/mL). Red blood cells were lysed in buffer (0.15 mol/L NH4Cl, 1.0 mmol/L KHO3, and 0.1 mmol/L Na2EDTA) and resuspended in PBS containing 3% FBS. For total white blood cell (WBC) and platelet counts, truncal blood was collected into EDTA-coated microtainer tubes. Cell counts were measured using an automated Cell Dyne 3500R counter (Antech Labs).

Flow Cytometric Analysis
Antibody staining and flow cytometry were performed as described previously.15 Samples were treated with Fc Block (anti-CD16/CD32) and primary antibodies: anti-CD45 CyChrome-conjugated (WBCs), anti-CD11b R-phycocerythrin (PE)-conjugated (monocytes, macrophages, microglia, neutrophils), anti-CD45R/B220 fluorescein isothiocyanate (FITC)-conjugated (B lymphocytes), anti-CD3 PE-conjugated (T lymphocytes), and anti-Ly6G (neutrophils). An FITC-conjugated antibody was used to detect Ly6G. Antibodies were obtained from BD PharMingen, except anti-Ly6G (gift from T. Malek, University of Miami, School of Medicine, Miami, Fla).18 Flow cytometry was performed with a FACScan (Becton Dickinson); data were analyzed using Cell Quest software on equal numbers of CD45<sup>+</sup> cells.

CD11b-Immunofluorescence on Brain Tissue
Brain tissue was prepared for immunofluorescence as described.19 Brain sections were treated with anti-CD11b (BD PharMingen) and detected with an anti-FITC–conjugated antibody (Jackson Immunoresearch). Microglia were quantified from 10 randomly selected ×40 fields of view within the cortex for each individual mouse. Images were collected using a Leica microscope with an Optronics DEI-750 3-chip camera equipped with a BQ 8000 sVGA frame grabber and analyzed using Bioquant.

Statistical Analysis
Data are represented as mean±SEM and were analyzed using 2-factor (treatment and hemisphere) ANOVA. Post hoc analyses were performed using 1-way ANOVA or Student’s t test. Differences were considered statistically significant when $P<0.05$.

Results

LPS Preconditioning Is Neuroprotective Against Cerebral Ischemia in Mice
We examined the effects of low doses of LPS on ischemic outcome in pilot studies to determine the optimal dose of LPS that provides the most neuroprotection against MCAO.20 On the basis of these findings, mice were pretreated systemically with 0.2 mg/kg LPS 48 hours before MCAO. The extent of ischemic injury was assessed 48 hours after MCAO. LPS preconditioning showed a 31% infarct compared with saline controls (52%; Figure 1). Thus, LPS-preconditioned mice provide a 40% reduction in infarct size induced by MCAO.

LPS Preconditioning Attenuates Activation of Microglia After MCAO
Microglia are activated after ischemia and release inflammatory mediators that exacerbate injury. We quantified microglia in the ischemic brains of LPS-preconditioned mice using fluorescent immunocytochemistry to detect CD11b, a microglial/macrophage marker. After MCAO (24 hours), the number of microglia in the ischemic hemisphere was reduced by previous LPS treatment (6.4±1.0) compared with that of control, saline-treated mice (21.8±4.1; $P<0.05$; Table 1). LPS preconditioning alone did not alter the number of microglia. Immunocytochemical staining of CD11b-positive microglia detects activated microglia because of their high level of expression of CD11b but does not easily detect resting microglia. That LPS preconditioning reduces activation of microglia is analogous to the effects of LPS preconditioning on macrophage activation, which results in lack of macrophage responsiveness to subsequent LPS stimulation.14 We have demonstrated previously maximal activation of microglia at 48 hours after MCAO by flow cytometry.15 Because flow cytometry is a more sensitive means of assessing CD11b expression, we tested whether LPS preconditioning altered microglia activation after MCAO. Microglia populations were quantified as activated (CD11b-hi) or resting (CD11b-low). LPS preconditioning did not alter the total microglia population (resting+activated) in the ischemic
hemisphere after MCAO (Table 1). However, LPS-treated mice showed a marked reduction in an activated microglia population that ordinarily increases in response to MCAO (Figure 2A). The mean percentage of activated microglia of the total microglia population in the ischemic hemisphere increased significantly (57.2 ± 5.3; \( P < 0.05 \)) after MCAO compared with the nonischemic hemisphere (25.2% ± 3.0; Figure 2B, dashed line). In contrast, LPS-preconditioned mice showed no increase in the percentage of activated microglia in the ischemic hemisphere (29.9% ± 5.4) after MCAO compared with the nonischemic hemisphere (29.0% ± 4.6; Figure 2B, dashed line). This indicates that a neuroprotective dose of LPS reduces microglial activation after MCAO.

**LPS Preconditioning Reduces Neutrophil Infiltration in Brain After MCAO**

Inflammatory events can exacerbate injury after ischemia via recruitment of activated microglia and circulating WBCs. Neutrophils and lymphocytes present in brain were quantified by flow cytometry 48 hours after MCAO to determine whether LPS pretreatment alters infiltration of circulating WBCs. Comparisons of cell populations were made between ischemic and nonischemic hemispheres of each individual mouse after MCAO as described previously. Neutrophils in the brain were assessed using expression of CD45 and CD11b and distinguished from microglia by expression of Ly6G, a neutrophil-specific marker. Neutrophils were significantly increased (14.7% ± 2.1%) in the ischemic hemisphere compared with the nonischemic hemisphere (8.3% ± 1.4%; \( P < 0.05 \)) in saline-pretreated controls (Table 2). In contrast, LPS-preconditioned mice exhibited no increase in neutrophils in the ischemic hemisphere after MCAO compared with the control, nonischemic hemisphere (9.6% ± 1.6% and 9.4% ± 2.3%, respectively). Thus, neuroprotection is associated with reduced neutrophil recruitment after MCAO. B and T lymphocytes were assessed by expression of CD45 and either CD45R/B220 or CD3 expression, respectively, and were not altered by previous LPS treatment.

**LPS Preconditioning Alters WBCs in Blood After MCAO**

Based on altered peripheral cellular infiltration in ischemic brain in LPS-preconditioned mice, we postulated that LPS

| Table 1: Effect of LPS Preconditioning on No. of CD11b+ Cells in Ischemic Brain Tissue |
|---------------------------------|-----------------|-----------------|
|                                 | Saline          | LPS             |
| Before MCAO                     | 0.5 ± 0.2       | 0.8 ± 0.4       |
| 24 hours after MCAO             | 21.8 ± 4.1      | 6.4 ± 1.0*      |

Values are mean No. of CD11b microglia per field of view within the cortex (mean sum of 10 different fields of view for each mouse) ± SEM; * \( P < 0.05 \) vs saline controls; n=6 mice per group.

**Table 2: Effect of LPS Preconditioning on WBC Infiltration in Brain After MCAO**

<table>
<thead>
<tr>
<th></th>
<th>Nonischemic</th>
<th>Ischemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Microglia</td>
<td>55.2 ± 3.8</td>
<td>60.8 ± 3.9</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>8.3 ± 1.4</td>
<td>14.7 ± 2.1*</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>4.5 ± 0.9</td>
<td>4.0 ± 1.2</td>
</tr>
<tr>
<td>B lymphocytes</td>
<td>3.5 ± 1.0</td>
<td>4.5 ± 0.6</td>
</tr>
</tbody>
</table>

WBCs were quantified by flow cytometry 48 hours after MCAO. Equal numbers of CD45+ cells were gated, and values are mean percentage of CD45+ cells ± SEM; * \( P < 0.05 \) vs nonischemic hemisphere within a treatment; n=8–12 mice per group.
LPS Preconditioning Attenuates Monocyte Activation in Blood After Ischemia

Because the aforementioned studies do not distinguish resident microglia in the brain from peripheral macrophages infiltrating from the circulation (although both cell types would be expected to respond similarly to MCAO), we tested whether LPS preconditioning modulates monocyte activation in the blood after MCAO by flow cytometry. We found an activated population of monocytes identified as those CD45+ cells with high levels of CD11b expression present after MCAO (58±6.3%; Figure 3) but reduced in LPS-preconditioned mice (21.3±1.7%; P<0.05). This indicates that LPS modulates cellular activation of peripheral monocytes in parallel to that of microglia activation in the brain after cerebral ischemia.

Discussion

We investigated the effect of LPS preconditioning on infarct size and the cellular inflammatory response to ischemia. Administration of a low dose of LPS before MCAO conferred marked neuroprotection against subsequent cerebral focal ischemia (40% reduction in infarct size). LPS preconditioning before MCAO reduced neutrophils in the blood and caused a corresponding reduction of neutrophil infiltration into the brain. In addition, LPS preconditioning attenuated cellular activation of monocyte/macrophage and microglial populations in the peripheral circulation and ischemic hemisphere. Neutrophils are considered pathogenic in ischemic injury because of their ability to release inflammatory cytokines and free radicals that exacerbate tissue damage within the brain parenchyma as well as the microvasculature.12,13 Here, LPS-induced neuroprotection is associated with a significant reduction in neutrophil infiltration in the brain. Reduced neutrophils in the ischemic hemisphere of LPS preconditioned mice resulted in reduced neutrophil infiltration in the ischemic hemisphere of LPS preconditioned mice.

### Table 3. Effect of LPS Preconditioning on Circulating WBCs After MCAO

<table>
<thead>
<tr>
<th>Cell No.</th>
<th>Saline Control</th>
<th>Saline/MCAO</th>
<th>LPS Control</th>
<th>LPS/MCAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (×10³/mL)</td>
<td>3.5±0.31</td>
<td>2.2±0.45*</td>
<td>4.1±0.45</td>
<td>2.7±0.27*</td>
</tr>
<tr>
<td>Platelets (×10³/mL)</td>
<td>992.6±66.20</td>
<td>1204.57±87.72</td>
<td>851.2±69.44</td>
<td>1051.88±83.95</td>
</tr>
<tr>
<td>Monocytes (×10³/mL)</td>
<td>104.5±18.8</td>
<td>96±23.4</td>
<td>124.3±34.2</td>
<td>45.9±6†</td>
</tr>
<tr>
<td>Neutrophils (×10³/mL)</td>
<td>877.6±138.7</td>
<td>1117±355.2</td>
<td>626.4±105.4</td>
<td>517.3±58.4</td>
</tr>
<tr>
<td>Lymphocytes (×10³/mL)</td>
<td>2427±175</td>
<td>835.9±145.9*</td>
<td>3321±482</td>
<td>1824.1±320.4†</td>
</tr>
</tbody>
</table>

Blood was collected 48 hours after MCAO or 4 days after control injections for quantification of WBC and platelet numbers. Quantification of distinct percentage WBC populations was performed by flow cytometry. Values are mean±SEM. *P<0.05 vs saline or LPS control. †P<0.05 vs saline/MCAO; n=8–12 mice per group.

### Figure 3. Effect of LPS preconditioning on monocyte activation in blood after MCAO. Activated monocytes in blood were quantified by flow cytometry 48 hours after MCAO in LPS-preconditioned mice. A, Representative flow cytometric plot of blood sample from an individual mouse. Arrow indicates activated monocytes (CD11b+) in blood. B, Values are mean percentage of activated monocytes of total monocytes±SEM. *P<0.05 vs saline-treated mice; n=8 to 12 per group. Controls: saline injection (39.3±4.3%) and LPS injection (25.3±4.3%) vs saline/MCAO; P<0.05.
tioned mice may be the result of a corresponding decrease in neutrophils in the blood after MCAO. LPS preconditioning may also suppress neutrophil activity and adherence after ischemia, which would be consistent with the finding that LPS preconditioning promotes preservation of microvascular perfusion in MCAO.21 Alternately, reduced infiltration may simply reflect reduced ischemic injury. It is difficult to distinguish between these possibilities, particularly in vivo. Investigation of molecular mediators involved in cellular recruitment induced before infarct development may indicate whether the immune response is affected early, independent of neuronal injury.

We show that LPS pretreatment caused a marked attenuation in microglial activation in response to MCAO. This is noteworthy because microglial activation may exacerbate inflammatory injury in ischemia because of their participation in the inflammatory response.22 Mice preconditioned with LPS showed ~30% of the microglia are activated in the ischemic hemisphere after MCAO, which is comparable to the nonischemic hemisphere. In contrast, saline controls showed robust microglial activation (60% of microglia) in response to MCAO. Thus, the microglial response to LPS preconditioning results in a state that is refractory to activation by ischemia.

Inhibition of microglial activation may be associated with improved ischemic outcome. Rodents made hypothermic or preconditioned with TNF-α showed increased neuroprotection that corresponded to decreased microglial activation in response to MCAO.23 In addition, inhibition of microglia activation with minocycline reduces ischemic injury.6,24 Thus, a mechanism of LPS-induced neuroprotection may be suppression of microglial activation during ischemia. Interestingly, monocytes in the peripheral circulation of LPS-preconditioned mice also showed reduced activation after MCAO. This contrasts with larger doses of LPS that activate microglia and have a negative impact on neuronal injury. High doses of LPS increase the severity of neurodegeneration and attenuation of long-term potentiation associated with the pathology of amyotrophic lateral sclerosis and Alzheimer disease.25,26 Thus, dose and timing of LPS administration influence neurological outcome.

Hyporesponsiveness in macrophages occurs in endotoxin tolerance in which small doses of LPS provide protection against greater doses of LPS. Endotoxin tolerance is manifested by suppressed production of many proinflammatory cytokines (eg, TNF-α and interleukin 12 [IL-12]); however, other anti-inflammatory mediators (eg, IL-10 and IL-1R antagonist) are not inhibited.14 Thus, endotoxin tolerance is not due solely to unresponsiveness of macrophages but reflects a reprogramming of the cellular response to LPS signals.

Mechanisms that underlie endotoxin tolerance in macrophages may be similar to LPS-induced ischemic neuroprotection. Pretreatment with low-dose LPS may induce a similar reprogramming in microglia that alters their responsiveness to a subsequent ischemic insult. That LPS preconditioning renders microglia refractory to activation by ischemia supports this notion. Similar to LPS-tolerant macrophages, microglia exposed to a low dose of LPS may shift the balance between proinflammatory and anti-inflammatory mediators after ischemia. This scenario may also be envisioned for neutrophils given that endotoxin tolerance results in hyporesponsive neutrophil activity.27 Such reduced activity could extend to their ability to infiltrate the brain after MCAO in LPS-preconditioned mice. In addition to suppression of inflammatory responses, beneficial antioxidant responses may be enhanced with endotoxin preconditioning. For example, ischemic protection attributable to diphosphoryl lipid A pretreatment reduced neutrophil infiltration, and this reduction was accompanied by enhanced SOD activity.10 This implicates both responses as potential mechanisms involved in the neuroprotective process.

It is tempting to speculate that LPS preconditioning reprograms the cellular response to ischemia via genomic changes that render the brain refractory to ischemic injury. This is supported by our studies that suggest preconditioning by brief periods of noninjurious ischemia reprograms the genomic response to subsequent injurious ischemia.16 Similar studies to decipher the genomic response to LPS preconditioning are currently under way in our laboratory and should prove informative regarding the cellular and molecular events responsible for LPS-induced ischemic neuroprotection.

Acknowledgments

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References


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