Lipopolysaccharide Induces Early Tolerance to Excitotoxicity via Nitric Oxide and cGMP

Marcello Orio, MD; Alexander Kunz, MD; Takayuki Kawano, MD, PhD; Josef Anrather, VMD; Ping Zhou, PhD; Costantino Iadecola, MD

Background and Purpose—Sublethal injury induces tolerance to a subsequent lethal insult, a phenomenon termed preconditioning (PC). PC occurs within hours (early tolerance) or days (delayed tolerance) after the inducing stimulus. In the brain, delayed tolerance has been studied extensively, but very little is known about early tolerance. We investigated whether the proinflammatory agent lipopolysaccharide (LPS), a well-established inducer of delayed tolerance, can also induce early tolerance and, if so, whether nitric oxide (NO) is involved in its mechanisms.

Methods—In C57BL/6 mice, LPS was administered and N-methyl-D-aspartate (NMDA) was microinjected into the neocortex 30 minutes to 24 hours later. Lesion volume was assessed 24 hours after NMDA administration in thionine-stained sections.

Results—LPS reduced NMDA lesions when administered 1 hour (−25±1%; P<0.05, n=5 per group) or 24 hours (−25±4%; P<0.05, n=5 per group) before NMDA application. LPS administration 30 minutes or 2 to 4 hours before NMDA administration was not neuroprotective (P>0.05). The protection at 1 hour was independent of protein synthesis and was blocked by inhibition of neuronal NO synthase or soluble guanylyl cyclase. Furthermore, early protection was not observed in neuronal or endothelial NO synthase–null mice, but it was present in inducible NO synthase–null mice.

Conclusions—The data demonstrate that LPS induces both early and late tolerance. At variance with delayed tolerance, which depends on inducible NO synthase and peroxynitrite, early tolerance is mediated by endothelial and neuronal NO through production of cGMP. The findings suggest that LPS can trigger signaling between endothelial cells and neurons, leading to NO production and cGMP-dependent neuroprotection. (Stroke. 2007;38:2812-2817.)

Key Words: basic science ■ eNOS-null mice ■ experimental ■ iNOS-null mice ■ nitric oxide ■ NMDA ■ nNOS-null mice ■ preconditioning

Preconditioning (PC), or tolerance, is a phenomenon in which a sublethal noxious stimulus confers transient resistance to a subsequent lethal insult.1,2 In the brain, as in other organs, PC can be induced by numerous stimuli, including transient cerebral ischemia and proinflammatory mediators, for example.1 In the PC induced by cerebral ischemia, tolerance develops in 2 different time frames: early and delayed. Early tolerance occurs within minutes to hours after the inducing stimulus and is independent of protein synthesis.1,2 Delayed tolerance occurs with a latency of hours to days and requires protein synthesis.3 The mechanisms of PC have been investigated extensively, but most efforts have focused on delayed tolerance, and relatively little is known on the factors inducing early tolerance.1 For example, transient ischemia induces early tolerance to cerebral ischemic injury,4,5 but it is not known whether other well-established PC stimuli, such as lipopolysaccharide (LPS), can also induce early tolerance. Furthermore, it is not known whether early tolerance can protect the brain from injury modalities other than ischemia.

The mechanisms underlying early tolerance in the brain are also unclear. Although some studies have implicated A1 adenosine receptors,5,6 other investigations have suggested a role for nitric oxide (NO).4 NO is of particular interest because this mediator has been implicated in both early and delayed PC.3 However, the involvement of NO in early tolerance is not as firmly established as in delayed tolerance.

In this study, we sought to provide further insight into the mechanisms of early PC in the brain. Using neocortical injection of the excitatory amino acid N-methyl-D-aspartate (NMDA) as an injury model, we investigated whether the proinflammatory mediator LPS can protect the brain from NMDA lesions with temporal characteristics and mechanisms consistent with early tolerance. Then, we used pharmacologic and nonpharmacologic approaches to determine whether NO plays a role in the induction of early tolerance and to define its enzymatic source(s).
Materials and Methods

Animals
All experimental procedures were approved by the institutional animal care and use committee of Weill-Cornell Medical College. Experiments were performed in male C57BL/6 mice and in mice lacking inducible NO synthase (iNOS), neuronal NO synthase (nNOS), or endothelial NO synthase (eNOS). Mice were obtained from in-house colonies and were studied at age 2 to 3 months. All null mice were congenic with the C57BL/6 strain, and C57BL/6 mice were used as wild-type controls.

LPS PC
As described in detail elsewhere, Salmonella typhimurium LPS (0.5 mg/kg, lot No. 054K4010; Sigma-Aldrich, St. Louis, Mo) was administered intraperitoneally and mice were returned to their cages. This dose of LPS does not significantly alter mouse behavior and produces small and transient reductions in body temperature that do not account for the PC. Mice treated with vehicle (saline) served as controls. Mice were subjected to NMDA microinjection in the neocortex at different time points after LPS administration.

NMDA Microinjection Into the Neocortex and Lesion Volume Measurement
Procedures for microinjection of NMDA into the cortex were identical to those described previously. In brief, in isoflurane-anaesthetized mice, the dura overlying the parietal cortex was exposed, and NMDA (20 nmol in 140 nL of sterile 0.1 mol/L phosphate-buffered saline, pH 7.4) or vehicle was loaded in a glass micropipette (tip, 40 to 50 μm) connected to a microinjection device. The micropipette was inserted into the parietal cortex at a site 1.5 mm caudal to bregma, 4.0 mm from the midline, and 0.8 mm below the dural surface, and NMDA was injected. Twenty-four hours after NMDA injection, mice were killed and their brains were removed. Lesion volume was determined 24 hours after NMDA injection. As illustrated in Figure 2A, lesion volume was reduced 1 hour after LPS (Figure 2B; P<0.05 versus vehicle, ANOVA and Tukey test), but it was not different from vehicle-treated mice at 2, 3, and 4 hours (P>0.05). However, lesion volume was reduced again 24 hours after LPS (−25±4%, P<0.05). Thus, LPS induces both early (1 hour) and delayed (24 hours) tolerance to NMDA lesions.

Drug Treatment Protocols
The drug treatment protocol is summarized in Figure 1. In experiments involving early PC, the NOS inhibitor N7-nitroindazole (N7-NI), 50 mg/kg IP; Cayman Chemical), the iNOS inhibitor aminoguanidine (AG), 100 mg/kg IP; Sigma-Aldrich), or the protein synthesis inhibitor anisomycin (50 mg/kg IP; Sigma-Aldrich) was administered at the time of the LPS injection (Figure 1A). The concentrations of AG and N7-NI used are effective in selectively inhibiting iNOS or nNOS, respectively. Furthermore, we used a concentration of anisomycin previously shown to block protein synthesis in vivo. NMDA was microinjected into the neocortex 1 hour after LPS, and mice were humanely killed 24 hours later (25 hours after LPS; Figure 1A). In some experiments, the guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ), 42 pmol in 140 nL of dimethyl sulfoxide; Cayman Chemical), the slow-release NO donor, spermine-NONO-ate (1.36 μmol in 140 nL of Na2HPO4/phosphate-buffered saline, pH 7.2; Cayman Chemical), or the cGMP analog 8-bromo-cGMP (42 pmol in 140 nL; Sigma-Aldrich) was microinjected into the neocortex with NMDA. In experiments involving delayed PC, NMDA was injected 24 hours after LPS, and mice were humanely killed 24 hours later (48 hours after LPS; Figure 1B). In these experiments, as in a previous study, AG was administered at the time of LPS and 6 hours later (Figure 1B). To ensure sustained protein synthesis inhibition in delayed-PC experiments, anisomycin was administered with LPS and 2 hours later.

Data Analysis
All data are expressed as mean±SEM. Comparisons between 2 groups were statistically evaluated by Student t test. Multiple comparisons were evaluated by one-way ANOVA, followed by Tukey test. Differences were considered significant at P<0.05.

Results

LPS Induces Early Tolerance to NMDA Lesions
Our first goal was to determine whether LPS induces early tolerance to NMDA lesions. Mice received LPS and were subjected to neocortical injection of NMDA 30 minutes or 1, 2, 3, or 4 hours later (n=5 per group). Lesion volume was determined 24 hours after NMDA injection. As illustrated in Figure 2A, lesion volume was reduced 1 hour after LPS (−25±1%; P<0.05 versus vehicle, ANOVA and Tukey test), but it was not different from vehicle-treated mice at 2, 3, and 4 hours (P>0.05). However, lesion volume was reduced again 24 hours after LPS (−25±4%, P<0.05). Thus, LPS induces both early (1 hour) and delayed (24 hours) tolerance to NMDA lesions.

LPS-Induced Early Tolerance Is Independent of Protein Synthesis
Delayed tolerance requires the synthesis of new proteins, but early tolerance does not. To determine whether the protection induced by LPS is independent of protein synthesis, we used the protein synthesis inhibitor anisomycin. Anisomycin did not affect tolerance to NMDA lesions observed 1 hour after LPS (Figure 2B; P>0.05 versus vehicle, n=5 per group), but it abolished the PC at 24 hours (Figure 2B). These
findings indicate that the early protection induced by LPS does not require protein synthesis.

**nNOS and eNOS, but Not iNOS, Are Involved in LPS-Induced Early Tolerance**

NO derived from de novo expression of iNOS plays a key role in delayed tolerance to NMDA lesions induced by LPS.14 Because early tolerance does not require new protein synthesis, we hypothesized that iNOS was not involved in the protective effect of LPS. In support of this hypothesis, the iNOS inhibitor AG did not affect the early tolerance induced by LPS (P<0.05 versus LPS vehicle, n=5 per group; Figure 3A), but it blocked delayed tolerance (P<0.05 versus vehicle, n=5 per group; Figure 3A). Furthermore, LPS was able to induce early PC in iNOS-null mice (Figure 3B). Next, we focused on the role of the constitutive isoforms of NOS, nNOS and eNOS. To study the role of nNOS, we used the nNOS inhibitor 7-NI and nNOS-null mice. The early tolerance induced by LPS was abolished by 7-NI (P<0.05 versus LPS vehicle, n=5 per group; Figure 4A) and was not observed in mice lacking nNOS (P>0.05 versus saline, n=5 per group; Figure 4B). 7-NI did not reduce NMDA lesions when administered without LPS (Figure 4A). We then used eNOS-null mice to explore the role of eNOS. LPS was unable to induce early tolerance to NMDA in eNOS-null mice (P>0.05 versus saline, n=5 per group; Figure 4C). Thus, early PC induced by LPS is abolished by inhibition and/or genetic inactivation of nNOS or eNOS.

**NO, Guanylyl Cyclase, and cGMP Are Involved in Early Tolerance**

To provide additional evidence that NO is involved in the mechanisms of LPS PC, we tested whether exogenous NO could rescue the early tolerance in mice treated with the nNOS inhibitor 7-NI. As before, 7-NI blocked the early tolerance induced by LPS (P<0.05 versus vehicle, n=5; Figure 5A). However, neocortical microinjection of the NO donor, spermine NONO-ate, together with NMDA reestablished LPS PC in 7-NI–treated mice (P<0.05 versus vehicle, n=5; Figure 5A). To explore the role of cGMP, the second messenger mediating many of the biologic effects of NO,20 we used the soluble guanylyl cyclase inhibitor ODQ.18 ODQ completely blocked LPS PC (P<0.05 versus vehicle, n=5; Figure 5B), an effect reversed by the cell-permeable cGMP analog 8-bromo-cGMP (P<0.05 versus vehicle, n=5; Figure 5B). Thus, NO, guanylyl cyclase, and cGMP are needed for the early PC induced by LPS. However, the concentrations of NO and cGMP mediating the early protective effects of LPS remain to be defined.

**Discussion**

We examined whether LPS confers early tolerance to brain lesions induced by NMDA. We found that LPS reduces the lesions produced by NMDA, an effect observed 1 hour after the PC stimulus. The reduction in injury was unaffected by anisomycin, suggesting that, as in other models of early PC, the protection is independent of new protein synthesis. The
protective effect of LPS was abrogated by pharmacologic inhibition of nNOS and was not observed in nNOS-null mice. Furthermore, LPS-induced neuroprotection was not found in mice lacking eNOS. Administration of the NO donor, spermine NONO-ate, reestablished early tolerance after nNOS inhibition. In addition, tolerance was blocked by the soluble guanylyl cyclase inhibitor ODQ, an effect counteracted by the cGMP analog 8-bromo-cGMP. These new findings collectively provide evidence that (1) LPS protects the brain from excitotoxicity in a protein synthesis–independent manner and within a time frame consistent with early tolerance and (2) the mechanisms of this neuroprotection involve eNOS- and nNOS-derived NO, soluble guanylyl cyclase activation, and cGMP.

The findings of the present study cannot be attributed to differences in experimental conditions among the different groups. Depth and duration of anesthesia, important variables in studies of PC, were closely monitored and kept constant in all experiments. Body temperature has powerful effects on NMDA-mediated brain injury. Although low-dose LPS can induce mild hypothermia, which can be neuroprotective, LPS does not alter rectal temperature up to 4 hours after LPS administration. Therefore, it is unlikely that the early neuroprotective effect observed 1 hour after LPS was secondary to hypothermia. Furthermore, body temperature was carefully controlled up to 4 hours after NMDA injection, the critical period during which the lesion develops.

Although delayed PC in the brain is relatively well studied, less is known about early PC. Previous studies in vivo have established that rapid protection from cerebral ischemic injury can be induced by short periods of focal ischemia. However, it was not known whether early tolerance could be induced also by other stimuli well known to induce delayed tolerance, such as LPS. We found that LPS is able to protect the brain from NMDA lesions 1 hour after administration. The protection was lost 2 hours after LPS and was independent of protein synthesis. These observations establish that LPS can also induce early tolerance.
We then investigated the mechanisms of the early tolerance induced by LPS. We found that the early neuroprotection by LPS requires eNOS and nNOS. This finding is in agreement with the observations of Atochin et al., indicating that the rapid tolerance induced by focal ischemia could not be obtained in eNOS- or nNOS-null mice. Therefore, at variance with delayed PC, which depends on iNOS-derived NO,13,14 early PC induced by LPS requires NO derived from eNOS and nNOS. The fact that both eNOS and nNOS are needed suggests that these enzymes act sequentially in the signaling pathway leading to early tolerance. One possibility, supported by studies in endothelial cell cultures,27 is that LPS activates eNOS through phosphorylation by phosphatidylinositol-3 kinase/Akt, leading to an increase in NO production. Endotheliually derived NO, or a related species, could diffuse into the brain parenchyma, leading to nNOS activation and production of neuronal NO. Neuronal effects of eNOS-derived NO are well established and have been described in the hippocampus, brainstem autonomic nuclei, and optic nerve.28–30 However, this possibility remains speculative, and additional studies are needed to investigate the interaction between eNOS and nNOS in early PC. Irrespective of the mechanisms governing the cross-talk between eNOS and nNOS, the present findings indicate that vascular-neuronal signaling is a key step in the early tolerance induced by LPS.

NO exerts many of its biologic effects by activating soluble guanylyl cyclase and increasing cGMP.20 Our finding with the soluble guanylyl cyclase inhibitor ODQ indicates that early tolerance requires cGMP. This is in contrast with the delayed tolerance induced by LPS, which depends on peroxynitrite, the reaction product of NO with superoxide, and not on cGMP.14 The cGMP concentrations needed for the early tolerance by LPS and the signaling pathways downstream of cGMP remain to be defined. Studies in models of early PC in the myocardium indicate that activation of mitochondrial ATP-sensitive potassium channels by cGMP and protein kinase G could play a role.31 However, it is not known whether these mechanisms are involved in early PC in the brain, and future studies are needed to address this issue.

In conclusion, we found that LPS is able to confer protection from NDMA excitotoxicity, an effect that occurs 1 hour after LPS and is independent of protein synthesis. Such early tolerance is mediated by NO derived from eNOS and nNOS through the second-messenger cGMP. Although the ultimate signaling events leading to neuroprotection remain to be defined, the evidence indicates that the interaction between vascular and neuronal NO is a key factor in the neuroprotective mechanisms underlying tolerance. Unraveling these mechanisms may lead to new strategies to protect the brain at risk for injury.

Sources of Funding
This work was supported by National Institutes of Health grant NS34179 and Deutsche Forschungsgemeinschaft (KU 1990/1-1, A.K.). C.I. is the recipient of a Javits award from National Institutes of Health/National Institute of Neurological Disorders and Stroke.

Disclosures
None.

References
22. Kapinya KJ, Lowl D, Futterer C, Maurer M, Waschke KF, Isaev NK, Dinnagg U. Tolerance against ischemic neuronal injury can be induced by...
volatile anesthetics and is inducible NO synthase dependent. Stroke. 2002;33:1889–1898.


Lipopolysaccharide Induces Early Tolerance to Excitotoxicity via Nitric Oxide and cGMP
Marcello Orio, Alexander Kunz, Takayuki Kawano, Josef Anrather, Ping Zhou and Costantino Iadecola

Stroke. 2007;38:2812-2817; originally published online August 30, 2007;
doi: 10.1161/STROKEAHA.107.486837
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/38/10/2812

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/