Systemic Administration of Lipopolysaccharide Induces Release of Nitric Oxide and Glutamate and c-fos Expression in the Nucleus Tractus Solitarii of Rats

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Abstract—There is increasing recognition that communication pathways exist between the immune system and brain, which allows bidirectional regulation of immune and brain responses to infection. The endotoxin lipopolysaccharide (LPS) has been reported to elicit release of cytokines and expression of inducible nitric oxide synthase (iNOS) in peripheral organs. Whereas LPS given systemically causes endotoxic shock, little is known about its central nervous system action, particularly the induction of iNOS. Nitric oxide (NO) and glutamate in the nucleus tractus solitarii (NTS) are important mediators of central cardiovascular regulation. We have previously demonstrated that intravenous injections of LPS increased the NO precursor L-arginine–induced depressor effect in the NTS. The present study investigated further the effects of LPS on the release of NO and glutamate in the NTS and the expression of c-fos, an immediate early response gene product, in neural substrates for central cardiovascular control. In vivo microdialysis coupled with chemiluminescence and electrochemical detection techniques were used to measure extracellular levels of NO and glutamate in the rat NTS. Immunohistochemistry was used for the examination of c-fos protein expression. We found that intravenous infusion of LPS (10 mg/kg) produced a biphasic depressor effect, with an early, sharp hypotension that partially recovered in 15 minutes and a secondary, more prolonged hypotension. In the NTS, a progressive increase of extracellular glutamate and NO levels occurred 3 and 4 hours after LPS was given, respectively. The effects of LPS on the induction of delayed hypotension and NO formation in the NTS were abolished by pretreatment with the iNOS inhibitor aminoguanidine. Finally, c-fos protein expression in the NTS and related structures for cardiovascular regulation was observed after LPS challenge. Taken together, these data suggest that an endotoxin given systemically can elicit delayed increases of glutamate release and iNOS-dependent NO production in the NTS and activate the central neural pathway for modulating cardiovascular function. (Hypertension. 1999;33:1218-1224.)

Key Words: nitric oxide synthase ■ endotoxin ■ cardiovascular system ■ microdialysis

Nitric oxide (NO), a free-radical gas produced from L-arginine by the enzyme NO synthase (NOS), is a remarkable regulatory molecule that plays an important role in a variety of physiological functions. Several isoforms of NOS have been isolated and purified. The NOS in endothelial (eNOS) and neuronal (nNOS) cells are expressed constitutively, and their activities are regulated by changes in intracellular calcium. In contrast, activation of macrophages with endotoxins and/or cytokines results in the de novo biosynthesis of a calcium-independent (inducible) isoform of NOS (iNOS). The iNOS-mediated NO formation induced by endotoxins is responsible for the fall of blood pressure in endotoxic shock.

The endotoxin lipopolysaccharide (LPS) is a unique glucosamine-based phospholipid that makes up the monolayer of the outer membrane of Gram-negative bacteria. LPS produces changes in behavior, endocrine function, and immune function. Many of these effects of LPS are secondary to the synthesis and release of cytokines such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and interleukin-6 (IL-6). Thus, elevation in serum TNF and IL-1 levels has been found in animal models of endotoxin-induced shock, and these elements exhibit potent immunologic and inflammatory properties. Additionally, cytokines are key messengers in associating the immune system with brain signaling. In this context, brain stem nuclei are intensively involved in the adaptive responses to peripheral immunologic signals. This idea is supported by the observation that brain stem nuclei may mediate central actions of LPS given peripherally.

Specifically, LPS activates the hypothalamic-pituitary-adrenocortical axis and brain stem nuclei, including the locus ceruleus and nucleus tractus solitarii (NTS). Moreover,
discrete brain stem nuclei are differentially activated by peripheral LPS, with the broadest range of effects in the NTS, affecting norepinephrine, dopamine, and 5-HT activities. However, the change of NO in the brain during the course of infection or inflammation is less well defined. Recent studies revealed that mRNA for iNOS is upregulated in the brain during several viral infections. Additionally, brain-derived glial cells were found to produce NO in response to LPS given alone or in combination with cytokines.

The NTS, located in the dorsal medial part of the medulla oblongata, is the predominant site of termination of peripheral vagal afferents. We have previously demonstrated that NO in the NTS is involved in central cardiovascular control. Thus, unilateral injection of the NO precursor L-arginine in this region produced prominent dose-dependent hypotension and bradycardia and reduced renal sympathetic nerve activity. Glutamate is the major neurotransmitter for cardiovascular modulation in the NTS and is released by vagal afferent stimulation. Recent studies indicated that peripheral inflammatory signals may enter the brain through the NTS. Our earlier data indicated that LPS given systemically enhanced the cardiovascular responses induced by L-arginine in the NTS.

In the present study, we addressed further the possibility that LPS given systemically may influence NO formation and glutamate release in the NTS. We also examined the induction of c-fos protein in the NTS and related neural substrates involved in central cardiovascular regulation after LPS was given systemically.

**Methods**

**Experimental Procedure**

Male Sprague-Dawley rats (250 to 300 g; National Laboratory Animal Breeding and Research Center, Taipei, Taiwan, R.O.C.) were obtained and housed in the animal room of the National Defense Medical Center, Taipei, Taiwan. Rats were anesthetized with urethane (400 mg/kg) mixed with α-chloralose (40 mg/kg IP). The preparation of animals for intra-NTS microdialysis and the methods used in the localization of NTS have been described previously.

After insertion of microdialysis probes into the NTS, perfusion was maintained at 1.1 μL/min with an artificial cerebrospinal fluid (aCSF: 140 mmol/L NaCl, 1.2 mmol/L CaCl₂, 3 mmol/L KCl, 1 mmol/L MgCl₂, 7 mg ascorbic acid, pH 6.3), and following a 3-hour equilibration period, perfusates were collected every 1 hour for NO and glutamate analysis. The microdialysis probes used in this study were a concentric type made specifically for this experiment. The body of the probe was attached to a dialysis membrane in a straight direction. In general, the straight dialysis membrane probe (ID 220 μm, OD 310 μm; 5000 molecular weight cutoff) was glued at one end of the fused silica (ID 75 μm, OD 150 μm, Polymicro Technologies Inc) with a 1.2-mm length of active membrane in the NTS.

Basal levels of extracellular NO and glutamate were defined by use of the mean of the first 3 samples immediately before the drug was given. The levels of NO and glutamate in the NTS were continuously monitored for 12 hours after animals received an intravenous infusion of LPS (10 mg/kg, Sigma Chemical Co) or saline. In an additional group of animals, aminoguanidine hemisulfate (AMI, 15 mg/kg IV, Research Biochemicals International Co), an inhibitor of iNOS, was given 15 minutes before LPS was given. After a 12-hour perfusion following drug injections, fractional collection of perfusates was discontinued, and animals were perfused intracardially with normal saline and 4% paraformaldehyde. Placement of the microdialysis probe in the NTS was then verified in sections stained with cresyl violet (Figure 1).

**Measurements of NO**

For the measurement of NO in the microdialysis perfusates, we used the NO/ozone chemiluminescence technique (NO-Analyzer 280, Sievers Research Inc). We measured the oxidation products of NO with a reaction vessel that contained a reducing agent (0.1 mol/L vanadium chloride, Aldrich Co) dissolved in 8% HCl, in which the sample was injected and NO was generated from nitrite in an equimolar manner. A continuous stream of helium (99%) purged the resultant NO from the reaction vessel to the chemiluminescence chamber. Detection of NO was based on its reaction with ozone, which leads to the emission of red light (NO + O₃ → NO₂ + O₂; NO₂ → NO + hv). The photons from this reaction were detected and transformed to an electrical signal by a photomultiplier tube (PMT). The current from the PMT was converted from analog to digital and fed into a personal computer for analysis. The calculated area under the curve of the PMT current for each determination paralleled the amount of NO. Standard curves were made immediately before each measurement (0, 1, 3, 5, 10, 30, and 50 μmol/L NO), which was produced with the use of freshly prepared solutions of sodium nitrite (10 μL) in distilled water.

**High-K⁺ Stimulation**

Extracellular levels of glutamate reflect both metabolic and neuronal activities. High-K⁺ stimulation has been applied to ascertain the neuronal origin of substances measured by the microdialysis technique. High K⁺ can depolarize presynaptic nerve terminals, which causes neurotransmitter release by way of exocytosis. To test whether our fabricated microdialysis probes could reflect glutamate release of neuronal origin, we monitored the extracellular glutamate level after perfusion with high K⁺ in the microdialysis probe. Two hours after infusion of original aCSF, aCSF that contained 100 mmol/L KCl was perfused for 60 minutes in 4 rats. After the high K⁺-challenge, the probe was perfused again with the original aCSF for 90 minutes. Samples were collected every 30 minutes for glutamate analysis.

**Detection of Glutamate**

Separation and analysis of glutamate were performed by high-performance liquid chromatography with electrochemical detection. A programmable solvent delivery system (BAS 480) with an electro-
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Immunocytochemical Procedure
All animals were perfused transcardially with 200 mL of 0.9% saline followed by 400 mL of fresh 4% paraformaldehyde in phosphate buffer (pH 7.4) and 30% sucrose. Brains were postfixed and then placed into 30% sucrose for 1 to 2 days before they were sectioned. After fixation, the brain stem and thoracic spinal cord from T1 through T4 were removed and serial 40-μm coronal sections were cut on a cryostat. All the sections were transferred to a separate retention chamber filled with 0.1 mol/L Tris-HCl buffer (pH 7.35). The tissue-bound peroxidase was then visualized by incubating the sections in 0.1 mol/L Tris buffer and sequentially incubated in biotinylated goat anti-rabbit IgG (1:200) for 1 hour and the avidin-biotin-peroxidase complex reagent (1:100 Vector Laboratories) at room temperature. Between these 2 groups. A single injection of LPS (10 mg/kg IV at time 0, n=8), saline (n=8), and pretreatment with AMI (15 mg/kg IV 15 minutes before LPS, n=5). AMI significantly inhibited the fall of MBP by LPS given systemically. Data are expressed as mean±SEM. *P<0.05, LPS vs saline group; **P<0.05, LPS vs AMI group, at the same time point by Dunnett’s t test after an overall ANOVA.

Data Analysis
The time-course data were expressed as mean±SEM. Results were analyzed by ANOVA with a post hoc Dunnett’s t test. Brain sections taken at comparable levels of NTS, rostral ventrolateral medulla (RVL), caudal ventrolateral medulla (CVL), and intermediolateral column (IML) of each animal were used to indicate the number of c-fos–labeled cells for each group. All cells labeled in a single section were counted, regardless of the intensity of staining, in a standardized manner through a microscope with a grid reticule. Results for c-fos staining were analyzed by unpaired Student’s t test. Data were considered significant when P<0.05.

Results
LPS-Induced Delayed Hypotension Is Inhibited by AMI
Baseline values for mean blood pressure (MBP) and heart rate (HR) of the saline- and AMI-pretreated animal groups were 98±4 and 105±3 mm Hg and 365±8 and 379±11 bpm, respectively, and were not significantly different between these 2 groups. A single injection of LPS (10 mg/kg IV, n=8) produced a biphasic hypotension; an immediate and transient decrease in MBP (65±8 mm Hg, P<0.005), which partially recovered within 15 minutes, followed by a delayed and prolonged hypotension (66±12 mm Hg, 5 hours after LPS infusion, P<0.01; Figure 2A). HR was also significantly increased (450±17 bpm, 5 hours after LPS infusion, P<0.05, versus saline group, Figure 2B). Pretreatment with AMI 15 minutes before (15 mg/kg IV, n=5) did not change the transient, early hypotension produced by LPS. However, the secondary, prolonged hypotension was significantly attenuated by pretreatment with AMI because the MBP remained unchanged 3 hours after LPS was given (F=3.721, P<0.01). AMI did not significantly alter changes of HR induced by LPS administration.

NO Production by LPS Is Attenuated by AMI
Basal levels of extracellular NO in the NTS were 4.8±0.4 μmol/L, which was consistent with the values measured by Kashihara et al.16 After intravenous LPS (10 mg/kg), an increase of extracellular NO levels in the NTS occurred 4 hours later (159±14%), then a progressive potentiation of increase was noted that lasted for 12 hours (F=4.585, P<0.001, LPS versus saline group, n=8, Figure 3). Pretreatment with AMI 15 minutes before LPS administration (15 mg/kg IV, n=5) significantly inhibited the increase of NO induced by LPS given systemically (10 mg/kg IV, n=8). Results are expressed as mean±SEM. *P<0.05, LPS vs AMI group, at the same time point by Dunnett’s t test after an overall ANOVA.
3). It was noted that 12 hours after intravenous LPS, NO concentration increased up to 501 \( \pm \) 119% compared with the basal level. AMI given 15 minutes before LPS infusion significantly attenuated the delayed increase of extracellular NO by LPS stimulation (\( F = 2.912, P < 0.01 \), LPS versus AMI group, \( n = 5 \), Figure 3).

**Glutamate Release Is Enhanced by High K\(^+\) and LPS**

The basal level of extracellular glutamate in the NTS, defined as the mean of the first 3 samples immediately before the drug was given, was 14.6 \( \pm \) 2.2 pmol/20 \( \mu \)L. After perfusion with aCSF that contained 100 mmol/L KCl for 60 minutes, extracellular glutamate increased up to 327 \( \pm \) 46% (\( F = 2.603, P < 0.01 \), high-K\(^+\) versus control group, \( n = 4 \) for each group, Figure 4A). Concentrations returned to basal levels 60 minutes after perfusion had reverted to normal aCSF. Glutamate release in the NTS began to increase 3 hours after intravenous LPS (10 mg/kg) was given (176 \( \pm \) 23%), then a progressive increase of glutamate release was noted that lasted for 12 hours (\( F = 5.153; P < 0.001 \), LPS versus saline group, \( n = 6 \), Figure 4B). The elevation of extracellular glutamate levels in the NTS after a single dose LPS was given was in a time-dependent fashion (465 \( \pm \) 41% of basal levels for 12-hour sample).

**c-fos Protein Expression Is Induced by LPS in the Brain Stem Nuclei and IML**

Figure 5A illustrates the large number of labeled neurons focused in the NTS, RVL, CVL, and IML of rats 6 hours after peripheral injection of LPS (10 mg/kg IP), relative to animals injected with vehicle solution. The basal number of c-fos--
labeled cells in the NTS, RVL, CVL, and thoracic IML were 8±3, 35±5, 12±2, and 0.4±0.2, respectively. Compared with vehicle-injected controls, there were dramatic increases in the number of c-fos-positive neurons localized in these regions of the brain stem and thoracic IML after injection of LPS. The number of c-fos-labeled neurons in the NTS, RVL, CVL, and IML were significantly increased by 1940±38% (n=10, P<0.001, unpaired t test), 245±10% (n=10, P<0.001), 761±31% (n=10, P<0.001), and 1500±139% (n=10, P<0.001), respectively, compared with the saline group (Figure 5B).

**Discussion**

The major findings of the present study were that (1) although initial hypotension was resistant to pretreatment with the iNOS inhibitor AMI, this agent significantly abolished the delayed depressor effect of LPS given peripherally; (2) with a slow onset, a progressive increase of NO production in the NTS was noted after LPS was given; this effect was blocked by AMI; (3) LPS given systemically also elicited a delayed and prolonged increase of glutamate release in the NTS; and (4) c-fos protein was induced in the NTS, RVL, and CVL regions of the brain stem and thoracic IML after systemic injection of LPS.

In this study, consistent with a previous report, LPS given peripherally produced a biphasic reduction in blood pressure in anesthetized rats. The early hypotensive response, recovered partially within 15 minutes, was unaffected by pretreatment with AMI, which implies that an iNOS-independent mechanism is responsible for the initial action of LPS. The mediators responsible for the LPS-elicited initial depressor effect were not identified. However, increased levels of other vasodilators, such as platelet-activating factor, prostaglandin, serotonin, bradykinin, and histamine, have been demonstrated. Conversely, the secondary, more prolonged hypotension after LPS was given was significantly attenuated by pretreatment with the iNOS inhibitor AMI. iNOS expression is not normally expressed under physiological conditions but can be induced with inflammatory stimuli. NO generated from iNOS in endotoxic shock plays an important role in vascular hyporeactivity and tissue damage through its cytotoxic function. NO production in the NTS by systemic LPS. Also, AMI given systemically has been shown to ameliorate iNOS-induced cerebral ischemic damage. It is documented that formation of NO by way of iNOS closely correlates with the expression of iNOS protein and activity. In addition, the present results are supported by our previous observations that depressor and bradycardic effects of L-arginine in the NTS were significantly increased 3 to 5 hours after intravenous injection of LPS. However, the observation that AMI blocked NO release in the NTS did not necessarily prove that its effect took place exclusively within this region. Alternatively, AMI may simply have caused removal of the excitatory input to the NTS by inhibition of iNOS action in the periphery. Some studies claim that during sepsis, iNOS is induced throughout the body except in the brain. However, Hom et al reported that LPS could induce expression of iNOS mRNA and protein in the brain, and nNOS and eNOS mRNA were quite low compared with iNOS in LPS-treated rats. Additionally, Koprowski et al detected low-level iNOS mRNA in rat brain associated with brain viral infections or autoimmune disease by the extremely sensitive method of polymerase chain reaction. Further evidence suggests that glial cells may contribute to iNOS expression under inflammatory conditions.

Glutamate represents not only the major excitatory neurotransmitter in the central nervous system (CNS) but also a primary mediator of afferent sensory information. Glutamate receptor activation is implicated in the mediation of the central effects of endotoxins. Thus, the NMDA glutamate antagonist MK-801 blocked the expression of c-fos protein in the rat brain by systemic LPS. Consistent with this result, our data revealed an increased glutamate release, possibly of neuronal origin, in the NTS. However, this effect did not occur until 3 hours after intravenous LPS injection. Similarly, Mascarucci et al demonstrated that intravenous injection of LPS (10 μg/rat) failed to cause glutamate release in 3 hours. Conversely, the authors found that an intraperitoneal injection of LPS or IL-1β elicited an early (first-hour) increase of glutamate release in the NTS, which was attributed to the activation of the vagal glutamatergic pathway. Unfortunately, these investigators did not perform experiments that lasted longer than 3 hours, as done in this work, for a parallel comparison.

Glutamate and NO are important substrates for autonomic control in the NTS, and we have recently found that they reciprocally augment each other in the modulation of cardiovascular responses. Thus, the depressor response to glutamate was attenuated by NO inhibitors; prior administration of MK-801 significantly decreased the depressor effect evoked by the NO precursor L-arginine. In fact, glutamate can induce NO formation through Ca2+-dependent nNOS activation in the brain. Conversely, NO can serve as a retrograde messenger by which presynaptic glutamate release is enhanced. In the present study, both glutamate and NO in the
NTS increased by several-fold after LPS was given systemically. It is conceivable that these effects of LPS are mediated by positive interactions between glutamate and NO in the NTS.

The potential mechanisms whereby LPS activates neurons in the NTS await further investigation. Because LPS and immune mediators such as IL-1 are large molecules, it is unlikely that they cross the blood-brain barrier and act directly on the brain. Thus, the circumventricular organs (CVOs), which lack a blood-brain barrier, are possible entry sites for LPS and blood-borne immune signals into the brain. Specifically, LPS can act directly in the brain by way of CVOs at the hypothalamic level to stimulate corticotropin-releasing factor release.29 Another possibility is that LPS activates immune mediators such as IL-1β, TNF-α, and IL-6, which in turn act centrally. Cytokines may enter the brain by use of several pathways, including passive diffusion through the fenestrated capillaries in the CVO, carrier-mediated transport, and binding to the cerebral vascular endothelium, which thereby induces the generation of central mediators such as prostaglandins.6 LPS given systemically has been shown to induce IL-1 mRNA and TNF mRNA expression in specific brain regions as well as increase the levels of bioactive cytokines in the CNS.30 Conversely, primary sensory afferents may serve as an anatomic substrate that conveys immune messages triggered by locally released cytokines to the brain. Specifically, the vagus contains a large number of sensory afferent fibers, and subdiaphragmatic vagotomy attenuates a number of central actions induced by peripheral LPS.31 In this study, because a delayed onset (3 to 4 hours after LPS injection) of NO and glutamate release in the NTS was observed, it is less likely that these effects of LPS were mediated by activation of vagal afferents, which was implicated in the glutamate release that occurred in the first hour after LPS was given.25

The expression of immediate early response gene products, such as c-fos, has become a powerful tool for structural and functional analysis of the nervous system and may serve as a metabolic marker in response to neuronal activation.32 Although the precise function of c-fos protein in the CNS remains unclear, the neuronal expression of c-fos protein provides a dynamic view of complex neural regulation at the cellular level. Several lines of evidence suggest that c-fos mRNA expression is prominent in the brain-stem catecholaminergic nuclei such as the locus coeruleus and NTS after a single injection of LPS.33 In the present study, we extended earlier studies and systematically examined c-fos induction after LPS injection in the neuronal pathway for modulation of cardiovascular function. The NTS is the major brain stem structure that receives baroreflex afferents from peripheral receptors, and it consecutively innervates both the RVL and CVL. The CVL serves as a modulatory action on RVL, and it consecutively innervates both the RVL and CVL. These substrates contribute to the cardiovascular homeostasis by tuning afferent as well as efferent cardiovascular signals.34 The present study showed that a large number of neurons of the NTS, RVL, CVL, and IML were positive for c-fos immunoreactivity in LPS-challenged rats, and the number of c-fos-immunoreactive cells in the NTS was greater than that in the RVL, CVL, and IML of the thoracic spinal cord. These results support the idea that the neuronal pathway for central cardiovascular regulation is activated after peripheral administration of an endotoxin.

Notably, the NTS, RVL, and CVL are among the regions of the brain that are responsive to peripheral injection of bacterial endotoxins.35 These brain stem regions provide massive projections to the paraventricular nucleus, the pathways believed to play a role in the mediation of the effects of immune challenges and systemic cytokines on hypothalamic neuroendocrine response.36 Wan et al7 demonstrated that prostaglandin synthesis and visceral-vagal afferents are involved in the LPS-activated c-fos protein expression in specific autonomic and neuroendocrine nuclei in the brain. It is tempting to speculate that NO and glutamate may be at least partially responsible for the induction of c-fos protein in the brain stem nuclei. In fact, the involvement of NO in the activation of brain structures that express c-fos protein has been shown recently.37 Also, activation of the glutamate NMDA receptor is proposed to represent a common pathway for the induction of brain c-fos protein induced by LPS.7 Conversely, the present study does not exclude the possibility that reflex activation also contributes to the induction of c-fos expression in response to hypotension induced by LPS. Li and Dampney38 observed expression of c-fos–immunoreactive cells in brain stem nuclei after sustained blood-pressure change in conscious rabbits. They proposed that alterations in baroreceptor input would be a major factor to induce c-fos expression. Thus, the observed c-fos expression in the present study may be the combined results of an indirect effect related to LPS-induced hypotension and of the effects of LPS on activation of NO, glutamate, and other mediators, such as prostaglandins.

In summary, the present study reports that LPS given systemically can induce a delayed onset and progressive increase of glutamate release and iNOS-dependent NO production in the NTS. Moreover, systemic challenge with an endotoxin seems to activate neural substrates involved in central autonomic regulation.

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