Nitric Oxide and ATP-Sensitive Potassium Channels Mediate Lipopolysaccharide-Induced Depression of Central Respiratory-Like Activity in Brain Slices

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Respiratory activity · Lipopolysaccharide · Nitric oxide · ATP-sensitive potassium channels

Abstract
Infection may result in early abnormalities in respiratory movement, and the mechanism may involve central and peripheral factors. Peripheral mechanisms include lung injury and alterations in electrolytes and body temperature, but the central mechanisms remain unclear. In the present study, brainstem slices harvested from rats were stimulated with lipopolysaccharide at different doses. Central respiratory activities as demonstrated by electrophysiological activity of the hypoglossal rootlets were examined and the mechanisms were investigated by inhibiting nitric oxide synthase and ATP-sensitive potassium channels. As a result, 0.5 \textmu g/ml lipopolysaccharide mainly caused inhibitory responses in both the frequency and the output intensity, while 5 \textmu g/ml lipopolysaccharide caused an early frequency increase followed by delayed decreases in both the frequency and the output intensity. At both concentrations the inhibitory responses were fully reversed by inhibition of nitric oxide synthase with \textit{N}-nitro-L-arginine methyl ester hydrochloride (20 \textmu M), and by inhibition of ATP-sensitive potassium channels with glybenclamide (100 \textmu M). These results show that direct lipopolysaccharide challenge altered central respiratory activity in dose- and time-related manners. Nitric oxide synthase and ATP-sensitive potassium channels may be involved in the respiratory changes.

Introduction
Respiratory movement consists of a series of sequential physiological processes including rhythmogenesis in the medulla, transmission of excitatory drive from rhythmogenesis neurons to motoneurons, and control of respiratory muscles by respiratory motor nerves. Rhythmogenesis center plays a central role in these processes, and is regulated by a variety of physiological mechanisms including pulmonary mechanical and chemoreflexes, arte-
tio imaging.

Spinal fluid barrier (BCSFB) and blood-arachnoid barrier disruption of blood brain barrier (BBB), blood-cerebrovascular interactions, and so on, which have been the focus of many studies [3]. However, the central mechanism has not been well illustrated up to now.

According to the case reports presented by Hameed and Riordan [4], tachypnoea is an early feature of meningococcal disease, which might lead to misdiagnosis with bronchiolitis. Neuroinflammatory factors may also contribute to abnormalities in respiratory movement, including nitric oxide (NO) and prostaglandins, and some cytokines may be upregulated in the brain in several diseases, such as meningitis and septic encephalopathy. Some of these factors such as NO [5–18] and prostaglandins [19–21] have been found to alter the frequency and the output intensity of central respiratory activity under both physiological and pathological conditions. Lipopolysaccharide (LPS) and interleukin-1β induced meningitis will result in disruption of blood brain barrier (BBB), blood-cerebrospinal fluid barrier (BCSFB) and blood-arachnoid barrier (BAB), as demonstrated by gadolinium-enhancement ratio imaging [22]. It was also demonstrated that small amount of LPS might penetrate across BBB, though the concentration might be too low to invoke any pathophysiological changes [23]. Nevertheless, little has been known regarding the effect of LPS on respiratory center. The present study was therefore performed to investigate the effect of LPS challenge on electrophysiological activity of respiratory center and also the potential mechanism.

Materials and Methods

Slice Preparation

The 4–5-day-old Sprague-Dawley rats (Shanghai Institute for Family Planning) were anesthetized deeply with halothane and decapitated at the supracollicular level. The brain was submerged in cold (4°C) artificial cerebral spinal fluid (ACSF) of the following composition (in mM): NaCl 124, KCl 3.0, KH₂PO₄ 1.2, CaCl₂ 2.4, MgSO₄ 1.3, NaHCO₃ 26, D-glucose 10, constantly bubbled with 95% O₂-5% CO₂, pH 7.4. The cerebellum was removed and the hindbrain was isolated using a dissection microscope. The brainstem was set upwards and the dorsal surface was glued to an agar block facing the razor. The brainstem was sectioned serially in variable thickness in the transverse plane. Once the nucleus ambiguus was visible under the microscope, a single medulla slice of 600–800 μm thickness, of which one to two hypoglossal rootlets in each side were retained, was taken for experimentation. The thick medullary slice preparation generates rhythmic inspiratory-related discharge in hypoglossal rootlets [24]. The slice was transferred into the recording chamber and submerged in ACSF with a flow rate of 8–11 ml/min. The temperature was maintained at 23 ± 0.5°C, and the concentration of KCl in ACSF was increased to 10 mM to allow steady recording of the respiratory rhythm.

Electrophysiological Recording

The activity of the hypoglossal rootlets was recorded using a suction electrode and was amplified with a BMA-931 bioamplifier (5 kHz sampling frequency; 10–1,000 Hz band pass; 20,000 times), electronically integrated (τ = 200 ms) with an MA-1000 Moving Averager (CWE Inc., Pa., USA) before feeding into the computer. All animal procedures were performed in compliance with the institutional guidelines at Fudan University, and are in accordance with the internationally accepted principles in the care and use of experimental animals.

Drug Application

LPS (from Escherichia coli 055:B5) was dissolved in ACSF to make a fresh stock solution of 5 mg/ml, and was diluted to 0.5 or 5 μg/ml for use. LPS was usually applied continuously. In some slices LPS was applied for 30 min and the recovery process of respiratory responses to LPS application was watched. Glybenclamide, a selective inhibitor of ATP-sensitive potassium channels (KATP), was dissolved in DMSO to make a stock solution of 100 mM, and was diluted with ACSF to 10 or 100 μM for use. In experiments in which glybenclamide was used, DMSO was added to the perfusate throughout the experiment. No-nitro-L-arginine methyl ester hydrochloride (L-NAME), an inhibitor of NOS, was dissolved in ACSF to make a fresh stock solution of 10 mM, and was diluted to 20 μM for use. All drugs were purchased from Sigma-Aldrich (St. Louis, Mo., USA).

Data Analysis

The frequency of hypoglossal bursts was manually counted and at least a 2-minute recording was analyzed to get an average. The amplitude, the duration and the area were analyzed with Clampfit software (Axon Instruments, USA), and at least 10 continuous bursts were analyzed to get an average. Results are presented as mean ± SEM, and statistically compared with one-way ANOVA followed by Bonferroni’s correction. Significant difference was set at p < 0.05.

Results

0.5 μg/ml LPS Mainly Caused Inhibitory Responses of Central Respiratory Activity

In preliminary experiments, six slices were recorded for 5 h without application of LPS to observe the stability of the brainstem and then the perfusate throughout the experiment.
of hypoglossal bursts. A representative experiment is shown in figure 1A, which shows that the frequency and the amplitude had little changes within the first 3 h. Summarized data from all the six slices are presented in figure 1B–E, which shows that the frequency, the amplitude, the duration and the area did not change significantly with time in 150 min. In two slices, the frequency started to decline gradually after 3 h; however, in none of the six slices was cessation of hypoglossal bursts observed within 5 h.

In most (thirteen of fourteen) slices, application of 0.5 μg/ml LPS caused slow but progressive decreases in the frequency and the duration of hypoglossal bursts. In one slice, the frequency, as well as the amplitude, was transiently increased in a period from 5 to 20 min after LPS application, but the duration had shown a decrease even when the frequency was at its highest value. A typical experiment is shown in figure 2A, and a sample burst about 60 min after LPS application was compared with a control burst in figure 2B. In all the fourteen slices, the frequency and the duration were significantly lowered after 30 min LPS application, and were more profoundly inhibited after 60 and 90 min LPS application. However, the peak amplitude and area were not significantly altered at all the three time points after LPS application compared with control. The area at 90 min showed a significant decrease compared with the value at 60 min. Figure 2C–F shows the average values of the frequency, the peak amplitude, the duration, and the area of hypoglossal bursts, respectively, during control and 30, 60, and 90 min after LPS application.

In three of the fourteen slices, respiratory rhythm disappeared in 60–90 min after application of LPS. For statistic comparison, the frequency at 90 min in these three slices was regarded as zero, and the peak amplitude, the duration and the area were averaged from the last five
hypoglossal bursts after LPS application. 90 min after LPS application, in more and more slices, respiratory rhythm gradually disappeared with time and data from individual hypoglossal bursts were not available. Thus only the data within 90 min were analyzed and compared in this section of experiments.

5 μg/ml LPS Caused an Early Increase of the Frequency, and Delayed Inhibition of Both the Frequency and the Intensity of Central Respiratory Activity

Application of 5 μg/ml LPS initially caused inconsistent change of the frequency in individual slices. In a total of sixteen slices examined, five slices showed slowly growing frequency decrease; eleven slices showed an initial increase, followed by a delayed progressive decrease, of the frequency. The frequency increase in these eleven slices usually started in 5–10 min, reached the peak in several min, and mostly returned to baseline level within 25 min. In three slices, the frequency increase lasted for as long as 150 min. In all the sixteen slices, the peak frequency increase is statistically significant compared with control (fig. 3C). The initial response of the peak amplitude was also inconsistent in individual slices, which could be an increase, a decrease, or lack of change. In addition, the changes in the frequency and the peak amplitude were often asynchronous. A frequency increase could be accompanied by increased, decreased, or unaltered peak amplitude. In contrast, in all the sixteen slices, the duration showed progressive decrease, even in periods during...
which the frequency and/or the peak amplitude showed increases. A sample experiment that had typical biphasic frequency change is shown in figure 3A, and a sample burst about 90 min after LPS application was compared with a control burst in figure 3B. In all the sixteen slices, the frequency was significantly lowered by LPS application at 120 min (fig. 3C), the duration and area were significantly decreased at all the four time points analyzed (fig. 3E, F), but the peak amplitude was not significantly altered at all the time points analyzed (fig. 3D).

In four of the sixteen slices, respiratory rhythm disappeared in 100–115 min after application of LPS. For statistic comparison, the frequency at 120 min in these four slices was regarded as zero, and the peak amplitude, the duration and the area were averaged from the last five hypoglossal bursts after LPS application. 120 min after LPS application, in more and more slices, respiratory rhythm disappeared with time and data from individual hypoglossal bursts were not available. Thus only the data within 120 min were analyzed and compared in this section of experiments.

The responses of hypoglossal activity to LPS were largely irreversible. In four slices (two receiving 0.5 μg/ml and two receiving 5 μg/ml LPS) LPS application was discontinued after 30 min. All these four slices showed irreversible inhibition of hypoglossal respiratory activity in an observation period of 2.5–3.5 h.

**Glybenclamide Reversed the LPS-Induced Inhibition of Respiratory Frequency and the Intensity of Motor Output**

In twenty-one slices, after LPS (0.5 μg/ml in twelve slices; 5 μg/ml in nine slices) had been applied for over 120 min or the respiratory rhythm had been abolished, 10 μM glybenclamide was applied for 10 min. This protocol slightly increased the frequency of respiration in four
slices that retained respiratory rhythm, and evoked several spike-like hypoglossal bursts in one slice from a silent background. In most (seventeen of twenty-one) slices, 10 μM glybenclamide failed either to cause any change of respiratory frequency (in fourteen slices) or to evoke hypoglossal bursts from a silent background (in three slices). However, at a concentration of 100 μM, glybenclamide completely and repeatedly reversed the changes in the frequency, the peak amplitude, the duration, and the area induced by exposure of 5 μg/ml LPS, in all the six slices tested. A sample experiment is shown in figure 4A, and summary data are illustrated in figure 4B–E.

**Discussion**

There are two major findings in the present study. First, LPS caused dose- and time-related responses of central respiratory activity. At a concentration of 0.5 μg/ml, LPS mainly caused inhibitory responses in both the frequency and the output intensity. At a concentration of 5 μg/ml, LPS caused an early increase of the frequency and delayed decreases in both the frequency and the output intensity. Second, L-NAME, an inhibitor of NOS, and glybenclamide, a selective inhibitor of KATP, both completely reversed the inhibitory respiratory responses to LPS application, in both the frequency and the output intensity.

To our knowledge, this is the first study that investigated the effects of direct LPS challenge on the activity of isolated respiratory centers. The results suggest that the LPS-induced responses of central respiratory activity, at least the inhibitory ones, involves overproduction of NO and excessive opening of KATP.

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**Fig. 4.** Glybenclamide reversed the LPS-induced inhibition of central respiratory activity. A Recording of a sample experiment, which shows that glybenclamide (100 μM) reversibly restored rhythmic hypoglossal bursts from LPS-induced respiratory silence. B–E Averages of the frequency (B), peak amplitude (C), duration (D), and area (E) of hypoglossal bursts during control and immediately before, during, and 10 min after glybenclamide (glyb.) application. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with control.
The NO/cGMP/K\textsubscript{ATP} signaling pathway has been well documented as a preconditioning protective mechanism of the brain and many peripheral tissues in response to ischemic attack [25–27]. However, in sepsis, overproduction of NO, and as a result the excessive opening of K\textsubscript{ATP} in vascular smooth muscles and diaphragm, has been well known to be related to the genesis of hypotension [28–31] and diaphragmatic dysfunction [32–34]; and inhibition of NO production and/or blockade of K\textsubscript{ATP} opening have been suggested as prospective procedures for sepsis treatment. Neuronal NOS (nNOS) expression in local neurons or neuronal terminals has been identified in almost all respiratory-related medullary/spinal nuclei such as the rhythmogenesis center pre-Bötzinger complex [5], respiratory motoneurons [5, 8], and the sensory relay nuclei of the nucleus of solitary tract (NTS) [5–8]. In sepsis, NO in the brain can also originate both peripherally from immune cells and centrally from CNS-resident proinflammatory cells such as microglia [35]. Since it has been clearly proven that application of NO donors to brainstem slices caused inhibition of respiratory rhythm and output intensity, and caused opening of single K\textsubscript{ATP} channels in respiratory-related neurons in pre-Bötzinger complex [15], it is easy to understand that in sepsis, overproduction of NO and excessive opening of K\textsubscript{ATP} in respiratory centers would cause inhibition of respiratory rhythm and output intensity; and inhibition of NOS and blockade of K\textsubscript{ATP} would reverse the LPS-induced inhibitory respiratory responses. Thus the results from the present study have added new evidence for the beneficial effects of NOS inhibition and blockade of K\textsubscript{ATP} opening in sepsis.

Fig. 5. L-NAME repeatedly reversed the LPS-induced inhibition of central respiratory activity. A Recording of a sample experiment, which shows that L-NAME (20 μM) reversibly and repeatedly restored rhythmic hypoglossal bursts from LPS-induced respiratory silence. B–E Averages of the frequency (B), peak amplitude (C), duration (D), and area (E) of hypoglossal bursts during control and immediately before, during, and 10 min after L-NAME application. * p<0.05, ** p<0.01 compared with control.
mainly showed excitatory, but not inhibitory, respiratory responses. NO in the NTS has been well known to mediate the early excitatory respiratory responses to hypoxia in vivo, in both respiratory frequency and respiratory amplitude, suggesting that chemoreflex-related NTS neurons normally exert inhibitory action on central respiratory activity, and hyperpolarization of these NTS neurons by local NO production would result in disinhibition of rhythmogenesis neurons and/or respiratory motoneurons. Increasing NO supply by giving exogenous NO donors or endogenous NO biosynthesis substrate, L-arginine, increased the intensity and mostly increased the frequency of central respiratory activity; and reducing NO supply by inhibition of NOS or by scavenging of NO mainly resulted in inhibitory respiratory responses [8–14]. In one study, NO initially caused inhibitory respiratory responses in an in vitro preparation, but after mechanical isolation of a group of chemosensitive neurons in the ventrolateral medulla, the frequency response to NO was changed from inhibitory to excitatory [17], suggesting that these chemosensitive neurons normally exert excitatory action on rhythmogenesis neurons, and hyperpolarization of these chemosensitive neurons by local NO would result in inhibition of rhythmogenesis neurons. However, under severe pathological conditions, overproduction of NO in the brain might reach an extent that most or all respiratory-related neurons are silent or unresponsive, and regulatory inputs to rhythmogenesis neurons and motoneurons are nulled. This possibility might be able to explain the profound LPS inhibition of central respiratory activity in the present study, especially in the late period. Regarding the early excitatory respiratory responses of some slices to LPS, it is possible that during the initial period of LPS application, NO had not reached a very high concentration and it mainly had caused disinhibition of rhythmogenesis neurons and motoneurons by acting on regulatory neurons/nuclei that normally exert inhibitory effect on rhythmogenesis neurons and motoneurons.

In encephalopathy or LPS-challenged brain, a variety of neuroinflammatory factors such as NO, prostaglandins, interleukin-1β, TNF-α, etc., have increased release and have been suggested to be involved in the genesis of encephalopathy. These neuroinflammatory factors can be produced either from peripheral immune cells, or from CNS-resident proinflammatory cells such as microglia [35]. In experimental endotoxemia of mice and rats, prostaglandins, especially prostaglandin E2 and its EP3 type of receptors in brainstem ventral respiratory group, have been proven to account almost exclusively for causing the decrease of respiratory rate in vivo [20, 21]. However, in the present study, both inhibition of K_{ATP} and inhibition of NOS fully restored respiratory frequency and output intensity from LPS-induced depression, suggesting that direct LPS challenge of respiratory centers in vitro causes respiratory inhibition via different mechanisms from that of endotoxemia in vivo, and NO may be the common product of all the possible neuroinflammatory factors affecting respiratory centers in vitro, and K_{ATP} may be the final common effector.

The medullary respiratory centers include the basic rhythmogenesis center in the pre-Bötzinger complex and a variety of regulatory nuclei located from the rostral end to the caudal end. In the present study, the regulatory nuclei retained in the medullary slices might have subtle differences in the number and/or the integrity. These subtle structural differences might account for the differences in the responses to LPS. It is still under controversy whether the rhythmic activity recorded from perfused in vitro thick medulla slices or medulla-spinal preparations can represent the activity of respiratory centers in intact animals, especially because elevated potassium concentration is needed for steady recording of respiration in vitro. Even so, in vitro preparations are still widely used in studying the rhythmogenesis and regulation of respiratory centers. The main advantage is that the impact of peripheral chemo- and mechanical reflexes on respiratory centers can easily be excluded. Therefore, the present study revealed that NO and K_{ATP} may be involved in the pathogenesis of abnormalities of respiratory movement induced by LPS challenge.

In conclusion, direct LPS challenge of brainstem slices altered central respiratory activity in dose- and time-related manners, in both the frequency and the output intensity; and at least the inhibitory responses involved overproduction of NO and excessive opening of K_{ATP}. These results suggest that the neuroinflammatory changes in respiratory centers contribute to the alterations in respiratory movement in sepsis, and both NOS and K_{ATP} are prospective targets for treatment of respiratory dysfunctions in sepsis.

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