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The *in vivo* effect of lipopolysaccharide on the spontaneous release of transmitter from motor nerve terminals

[1]*Shing H. Liu, *Tzong J. Sheu, **Ruey H. Lin & *Shoei Y. Lin-Shiau

Institutes of *Toxicology and Pharmacology, College of Medicine, National Taiwan University, Taipei and **Department of Pharmacology, Chung Shan Medical and Dental College, Taichung, Taiwan

1 The *in vivo* effect of *E. coli* lipopolysaccharide (LPS) on the spontaneous release of transmitter was studied in the isolated phrenic nerve-diaphragm preparation of the mouse.

2 The resting membrane potential was decreased and frequency of miniature endplate potentials (m.e.p.ps) was increased by treatment with LPS.

3 Pretreatment of diaphragms with ouabain markedly increased the frequency of m.e.p.ps in control group but not in the LPS group.

4 When mice were treated with polymyxin B (a LPS neutralizer), pentoxifylline (an inhibitor of tumour necrosis factor-α formation) and N^G-nitro-L-arginine (an inhibitor of nitric oxide (NO) synthase) the effects of LPS were reversed.

5 These results suggest that LPS increases the spontaneous transmitter release through, at least in part, the pathways of tumour necrosis factor-α and NO followed by an inhibition of the Na^+-pump activity in the endplate area.

Keywords: Lipopolysaccharide; miniature endplate potential; sodium pump; polymyxin B; pentoxifylline; N^G-nitro-L-arginine

Introduction

Lipopolysaccharide (LPS or endotoxin) is the major component of the outermost membrane of Gram-negative bacteria. It is generally believed that LPS induction of cytokine release, mainly produced by mononuclear phagocytes, is probably the central event in the pathophysiology of Gram-negative bacterial septicaemia (Nathan, 1987; Pruzanski & Vadas, 1991).

Many studies have shown that cytokines can affect the nervous system (Calvert & Gresser, 1979; Bocci, 1988; d'Arcangelo *et al.*, 1991; Caratsch *et al.*, 1994). Recent studies have also shown that CNS microglia, akin to macrophages, induced with bacterial endotoxin or with combinations of cytokines, release large quantities of nitrite (Aoki *et al.*, 1991; Chao *et al.*, 1992; Mollace & Nistico, 1992). Moreover, nitric oxide as an intercellular messenger in CNS function has been widely studied (Garthwaite, 1991; Murphy *et al.*, 1993). However, the exact mechanisms of action of cytokines on the nervous system have not yet been established.

The aims of the present study were to investigate the influence of LPS on neuromuscular transmission occurring during endotoxicity by observation of the changes in miniature endplate potentials and to study its possible mechanism of action and the relationship between altered neuromuscular transmission and the cytokines involved.

Methods

Mouse phrenic nerve-diaphragm preparation

Mice (ICR strain) of either sex, weighing 20–25 g, were used. The phrenic nerve-diaphragm preparation was isolated according to the method of Bülbring (1946). A modified Krebs solution was used having the following composition (mM): NaCl 130.6, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 12.5 and glucose 11.1.

[1] Author for correspondence at: Institute of Toxicology, College of Medicine, National Taiwan University, No. 1, Jen Ai Road, 1st Section, Taipei, Taiwan.

Intracellular recording

Conventional microelectrode recording techniques (Fatt & Katz, 1951) were used. Glass-microelectrodes, which were used for intracellular recording of superficial fibres of muscle, were filled with 3 M KCl; their resistance ranged from 6 to 20 MΩ. An Axoclamp-2 preamplifier and Tektronix 2221A oscilloscope were used to record intracellular responses. Miniature end-plate potentials (m.e.p.ps) were cumulatively recorded with a tape recorder (Neuro Data, model DR390). A Data 6100 waveform analyzer (Data Precision) was used to store and analyze waveforms.

Drugs

Lipopolysaccharide (*E. coli*, 055:E5), ouabain, pentoxifylline, polymyxin B and N^G-nitro-L-arginine were purchased from the Sigma Chemical Company (U.S.A.).

Statistics

The number of the experiments for each group was more than four and significance of difference was assessed by using one-way analysis of variance (ANOVA) as indicated in the Results. The difference between the control and each test group was also assessed by Student's *t* test.

Results

Changes of m.e.p.ps response and resting membrane potential induced by LPS

The mice were pretreated intraperitoneally with LPS (7.5 mg kg^{-1}) at various time intervals before isolation of the diaphragm. The frequency, amplitude and duration of m.e.p.ps were measured in normal Krebs solution. The frequency of m.e.p.ps was increased by treatment with LPS, a maximum being reached at 24 h after injection of LPS (Figure 1). The increase in frequency of m.e.p.ps induced by LPS was unlikely to result from the postsynaptic action because the amplitude was unaffected by LPS (Figure 2). The average m.e.p.ps amplitude and half decay time of the control and

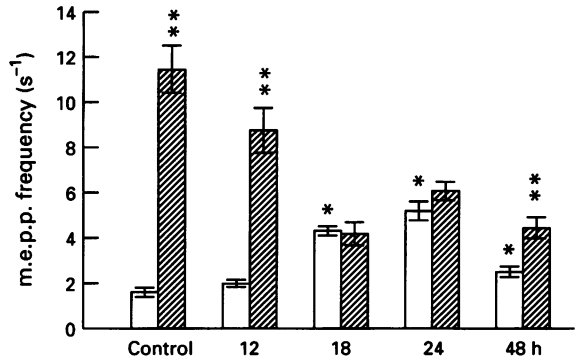

Figure 1 Alteration of frequency of miniature endplate potentials (m.e.p.ps) in the diaphragm of mice treated with lipopolysaccharide (LPS) at various time intervals. Data are presented as mean ± s.e. obtained from 3 to 6 preparations in the absence (open columns) or presence of 0.1 mM ouabain (hatched columns); for each preparation 10–12 endplates were studied. *$P < 0.05$ as compared with control in the absence of ouabain. **$P < 0.05$ as compared with preparations without ouabain in the same group.

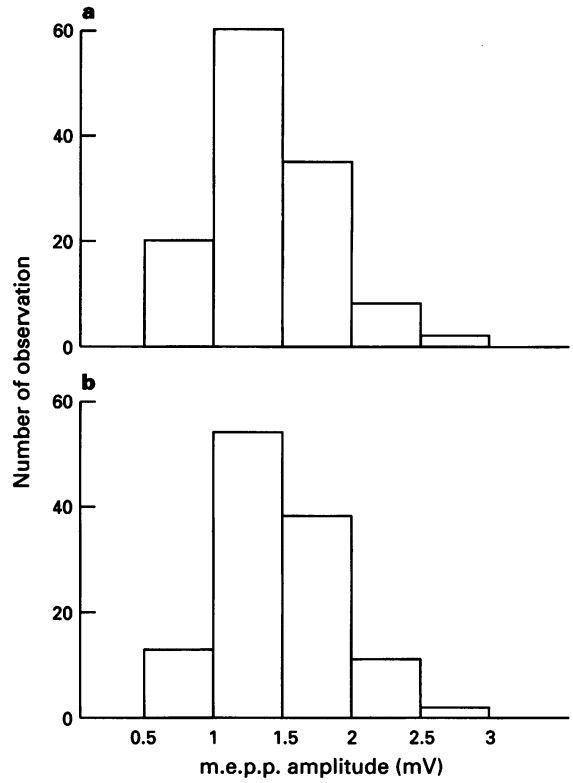

Figure 2 Diagram showing amplitude distribution of miniature endplate potentials (m.e.p.ps) in the diaphragm of normal and lipopolysaccharide (LPS)-treated mice. Miniature endplate potentials were recorded from 10–12 endplates in 3–6 diaphragm preparations of normal (a) or LPS-treated (b) mice.

LPS-treated group were 1.25 ± 0.05 mV, 1.91 ± 0.10 ms ($n = 5$ preparations) and 1.33 ± 0.06 mV, 1.58 ± 0.08 ms ($n = 5$ preparations), respectively; the differences between the two groups were not statistically significant.

The diaphragms from mice which had been injected 24 h previously with LPS (7.5 mg kg^{-1},i.p.) had membrane potentials at the endplate regions which were significantly reduced compared with control animals from −75.33 ± 1.89 mV to −69.78 ± 0.32 mV (Table 1).

Effects of ouabain on the m.e.p.ps response in diaphragms of normal and LPS-treated mice

Ouabain (0.1 mM) was bath-applied to the diaphragms isolated from normal mice or mice treated with LPS for various time periods. Pretreatment with ouabain for 30 min, markedly increased the frequency of m.e.p.ps in the control group but not in the LPS group (Figure 1, 3a(ii) and Table 1). The amplitude and half decay time of m.e.p.ps were unaffected by ouabain in either the control or LPS group.

Effects of polymyxin B, N^G-nitro-L-arginine and pentoxifylline on the increase of frequency of m.e.p.ps induced by LPS

Mice were injected with either polymyxin B (7.5 mg kg^{-1},i.p.), N^G-nitro-L-arginine (175 mg kg^{-1},i.p.) or pentoxifylline (100 mg kg^{-1},i.p.) 15 min before treatment with LPS (7.5 mg kg^{-1},i.p.) for 24 h. Polymyxin B, which is an LPS neutralizer, antagonized not only the increased frequency of m.e.p.ps but also the decreased resting membrane potential induced by LPS and also reversed the effects of ouabain (Figure 3b(iii) and Table 1). Similarly, N^G-nitro-L-arginine, which is an inhibitor of NO synthase, antagonized the effects of LPS (Figure 3b(iii) and Table 1). Pentoxifylline, which is an inhibitor of tumour necrosis factor-α (TNF-α) formation, antagonized the decreased resting membrane potential and markedly restored the effects of ouabain on the frequency of m.e.p.ps in diaphragms of LPS-treated mice, although it had less antagonistic effect on the increased frequency of m.e.p.ps induced by LPS (Table 1).

Table 1 Alteration of the resting membrane potential and m.e.p.p. frequency in the diaphragm of lipopolysaccharide (LPS)-treated mice

Treatment[a]	*Resting membrane potential*[b] *(−mV)*	*m.e.p.p. frequency*[c] *(s^{-1})*
Control	75.33 ± 1.89	1.57 ± 0.19
Ouabain (0.1 mM)	67.86 ± 0.55**	11.44 ± 2.04**
LPS	69.78 ± 0.32*	5.13 ± 0.42*
Ouabain (0.1 mM)	64.23 ± 0.86**	6.52 ± 0.39*
LPS + polymyxin B	73.33 ± 0.56***	1.71 ± 0.14***
Ouabain (0.1 mM)	63.53 ± 0.64**	7.10 ± 0.34**
LPS + pentoxifyllin	73.55 ± 0.58***	4.87 ± 0.70*
Ouabain (0.1 mM)	69.25 ± 0.53**	13.89 ± 1.39**
LPS + N^G-nitro-L-arginine	71.53 ± 0.24*,***	2.76 ± 0.29*,***
Ouabain (0.1 mM)	66.53 ± 0.48**	13.78 ± 2.01**

[a]The mice were treated with either LPS (7.5 mg kg^{-1}) or normal saline (control) for 24 h before isolation of the diaphragm preparation. In other experiments, the mice were pretreated with either polymyxin B (7.5 mg kg^{-1}), pentoxifyllin (100 mg kg^{-1}) or N^G-nitro-L-arginine (175 mg kg^{-1}) for 15 min prior to the application of LPS. Ouabain (0.1 mM) was applied to the diaphragm preparation from each treatment group.

Data are presented as mean ± s.e. from 3–6 preparations, for each preparation 10–15 muscle fibres (b) or 6–12 endplates (c) were studied. *$P < 0.05$ as compared with control. **$P < 0.05$ as compared with the condition without ouabain in each group. ***$P < 0.05$ as compared with the LPS group.

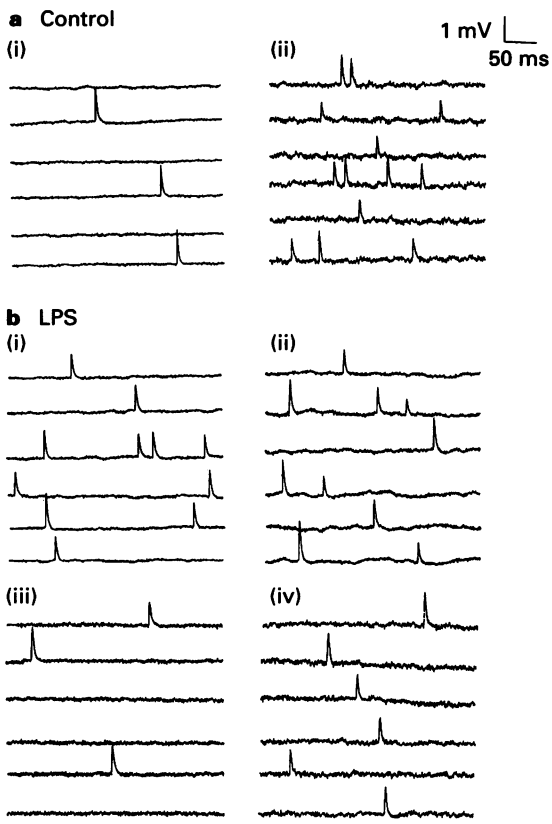

Figure 3 Alteration of frequency of miniature endplate potentials in the diaphragm of lipopolysaccharide (LPS)-treated mice and antagonism by polymyxin B and N^G-nitro-L-arginine. Ouabain (0.1 mM) increased the frequency of m.e.p.ps in control group (a(ii)) but not that of LPS group (b(ii)). The mice were treated with either polymyxin B (7.5 mg kg^{-1}) or N^G-nitro-L-arginine (175 mg kg^{-1}) 15 min before the application of LPS (7.5 mg kg^{-1}) for further 24 h. The increased frequency of m.e.p.ps group (b(i)) was reversed by polymyxin B (b(iii)) and N^G-nitro-L-arginine (b(iv)).

Discussion

In this study, we have found that frequency of m.e.p.ps of diaphragms isolated from LPS-treated mice was markedly increased. This effect of LPS was time-dependent reaching a maximum at 24 h treatment. The possible mechanism of action was investigated.

Na^+-K^+-ATPase is in charge of various important cell functions like cationic equilibrium and recovery of resting membrane potential in neurones (Hernandez, 1992). Ouabain has been shown to inhibit Na^+-K^+-ATPase and could accelerate the transmitter release (Vizi, 1972; Baker & Crawford, 1975; Vizi & Oberfrank, 1992). In this paper, we found that ouabain decreased the resting membrane potential accompanied by an increase in the frequency of m.e.p.ps in the diaphragm of normal mice, but not that of LPS-treated mice. Similarly, the phenomena of decreased resting membrane potential and increased frequency of m.e.p.ps were also shown in the diaphragm of LPS-treated mice. Moreover, we have also found that Na^+-K^+-ATPase activity was decreased in the sciatic nerve of LPS-treated mice (Liu *et al.*, unpublished data). From these results, we infer that suppression of sodium pump activity in the endplate area contributes to the increased frequency of m.e.p.ps in the diaphragm of LPS-treated mice.

Polymyxin B is a polycationic antibiotic which can prevent the generalized Schwartzman reaction and reduce endotoxin lethality (Craig *et al.*, 1974). Polymyxin B directly binds to the anionic lipid A portion of LPS, presumably accounting for its ability to neutralize LPS (Morrison & Jacobs, 1976). Therefore, the antagonistic action of polymyxin B on the increased frequency of m.e.p.ps and the decreased resting membrane potential induced by LPS, implies a pathophysiological role of LPS in the endplate area.

As LPS applied directly to the mouse diaphragm did not affect the frequency of m.e.p.ps and resting membrane potential (data not shown), we supposed that the effects of LPS may be due to a secondary mediator (probably some cytokines) affecting the function of endplate area. LPS is known to induce the release of cytokines including TNF-α, interleukins, interferons, platelet-activating factor and others, as well as having a key role in inflammatory and immune responses (Beutler & Cerami, 1988; Pruzanski & Vadas, 1991; Lynn & Golenbock, 1992). Recently, Caratsch *et al.* (1994) have observed that interferon-α,β and TNF-α enhanced the frequency of m.e.p.ps at rat neuromuscular junction, suggesting a possible regulatory role for spontaneous synaptic neurotransmitter release. In this paper, we found that the inhibitory action of treatment of LPS on the increased frequency of m.e.p.ps induced by ouabain and the decrease of resting membrane potential by LPS itself were reversed by pentoxifylline, an inhibitor of endogenous TNF-α formation (Strieter *et al.*, 1988; Zabel *et al.*, 1993). These findings suggest that alteration of neuromuscular transmission by LPS may be in part through TNF-α formation.

It is known that nitric oxide (NO), induced by bacterial lipopolysaccharide or cytokines, plays an important role in killing of cells by macrophages (Hibbs *et al.*, 1987; Stuehr & Nathan, 1989). NO release has been described in cerebellar neurones in response to glutamate and from other neuronal types in response to various stimuli (Bredt *et al.*, 1990; Bult *et al.*, 1990; Garthwaite, 1991). Recent study has also shown that certain cytokines such as TNF-α and interferon-r were capable of inducing neuroblastoma cell differentiation and NO may be an important mediator (Munoz-Fernandez *et al.*, 1994). In this paper, we show that the increased frequency of m.e.p.ps and the decreased resting membrane potential in the diaphragm of LPS-treated mice can be reversed by pretreatment with N^G-nitro-L-arginine, an NO synthase inhibitor. These results indicate that NO may be a mediator in the action of LPS on the neuromuscular transmitter. However, the relationship among cytokines, NO and the sodium pump in the endplate area still requires further study.

In conclusion, the induction of the increased frequency of m.e.p.ps and the decreased membrane potential in the diaphragm of LPS-treated mice is related to the impairment of Na^+-pump. TNF-α, at least in part, is involved in this reaction and NO may be an important mediator.

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