

## The role of nitric oxide and protein kinase C in lipopolysaccharide-mediated vascular hyporeactivity

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**Abstract PURPOSE:** Overactivation of nitric oxide and protein kinase C (PKC) pathway has been reported to play a role in the pathogenesis of vascular hyporesponsiveness of endotoxic shock. In this study we investigated the role of nitric oxide and PKC in lipopolysaccharide (LPS) mediated vascular hyporeactivity. **METHODS:** Contraction to phenylephrine and endothelium-dependent and independent vasodilation in the presence and absence of a nonspecific NO inhibitor (L-NAME) and potent PKC inhibitor (chelerythrine) were examined. **RESULTS:** In LPS treated rats, contractile response of aortic rings to phenylephrine and relaxation in response to acetylcholine were reduced, but relaxation induced by sodium nitroprusside remained unchanged. The attenuation of contractile response to phenylephrine in the presence of L-NAME and chelerythrine was more pronounced in aortic ring isolated from LPS treated rats than control. L-NAME decreased acetylcholine -dependent vasodilation in both group but it was more pronounced in LPS treated rats. Chelerythrine pretreatment improved maximal relaxation to acetylcholine in aortic ring isolated from LPS treated rats. **CONCLUSION:** These data indicate that the vascular hyporesponsiveness to phenylephrine and acetylcholine after treatment with LPS may be related to an enhanced NO production in the smooth muscle cells and PKC plays a role as an intracellular mediator of LPS-induced NOS activity and vascular suppression.

### INTRODUCTION

Septic shock is the most common cause of death in medical and surgical intensive care units (1-2). In

humans, it is characterized by a high cardiac output and decreased systemic peripheral resistance due to dilation of resistance arteries (3-4). This cause progressive systemic hypotension that is resistant to vasoconstrictors (5-6) and lead to abnormal organ perfusion and so organ damage and failure (7-9).

The most Common cause of septic shock is infection with gram-negative bacteria, resulting in the release of bacterial lipopolysaccharid (LPS) (10-11). Many mediators which markedly influence cardiovascular function are generated in response to LPS. These vasoactive substances include catecholeamines, histamine, thromboxanes, leukoterines, angiotensines, various as yet uncharacterized 'shock toxins' and oxygen derived free radicals (12-13). Nitric oxide (NO) is perhaps the latest mediator to be scrutinized with reference to the pathophysiology of sepsis (5, 14). LPS is known to express an inducible isoform of NOS (iNOS), followed by production of large amount of NO in various cells, including vascular smooth muscle, endothelial cells and macrophage, which contributes importantly to several key feature in pathophysiology of septic shock, such as hypotension and vascular hyporeactivity to vasoconstrictor agent (12,15) and vasodilators (16-17).

Protein kinase C plays a role as intracellular mediator of LPS-induced NOS activity and vascular suppression (18-20). Lipopolysaccharid induce time-dependent increase in PKC isotype mRNA expression and isotype-specific PKC activation and synthesis in vascular tissues (21). It has shown that PKC plays an important role in LPS-induced NO formation (22-23).

In this study, we investigated the effect of NO pathway, by blocking NO synthase with L-NAME and of PKC pathway, by blocking with chelerythrine, on the responsiveness of the isolated aortic rings of LPS-treated rats to vasoconstrictor agent (Phenylephrine) and an endothelium-dependent vasodilator (Acetylcholine) and an endothelium-independent vasodilator sodium nitroprusside.

### MATERIALS AND METHODS

#### *Animals*

Adult male Sprague Dawley rats, (Razi Institutes, Mashhad, Iran) weighing between 250-300 g were used. Animals were housed in temperature and humidity controlled, light-cycled quarters. The protocols used conformed to guidelines of the conduct approved by the committee on the ethics of animal experiments in Mashhad University. Animals

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were randomly divided into two groups including control and LPS treated. Control rats received saline injection ( $1\text{ ml kg}^{-1}$  i.p.  $n=5$ ), whereas septic rats received a bolus injection of LPS ( $10\text{ mg kg}^{-1}$  i.p.  $n=5$ ) 5 hours before examination.

### Preparation of rat thoracic aortic rings

Five hours after saline or LPS injection, animals were anesthetized with sodium pentobarbital ( $60\text{ mg kg}^{-1}$  i.p.) and thoracic aorta was immediately removed and placed in physiologic salt solution (containing (in mM) : NaCl, 130; KCl, 4.7; CaCl<sub>2</sub>, 1.6; MgSO<sub>4</sub>, 1.17; KH<sub>2</sub>PO<sub>4</sub>, 1.18; NaHCO<sub>3</sub>, 14.9; Dextrose, 5.5; and EDTA-Ca,Na<sub>2</sub>, 0.03) bubbled with mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. After cleaning the tissue of fat and other adhering tissues, the vessel were cut into 3 mm long rings, with special care to avoid damaging the endothelium. The preparation were mounted on a pair of stainless-steel hooks; one of each was fixed to a L-shaped rod inside the chamber and other to an isometric force transducer (F-60, Narco Biosystems, Inc., TX, USA) connected to a polygraph (MK III-S, Narco Biosystems, Inc., TX, USA). Tissues were allowed to equilibrate under an optimum final force of 1.5 g for a period of 60 min in a water-jacketed tissue bath (10 ml) containing oxygenated physiologic salt solution at 37°C (final pH of 7.4), renewing the buffer every 15 min. After stabilization vascular smooth muscle integrity was assessed by standard contraction obtained with 40 mM KCl. Endothelium integrity was assessed with  $1\mu\text{M}$  acetylcholine.

### Experimental procedure

All of the following experiments were conducted on the aortic rings of both LPS-treated and control rats.

*First.* Concentration response was assessed by adding cumulative concentration of phenylephrine ( $0.1\text{ nM}$  to  $1\mu\text{M}$ ). The concentration-contraction curves were studied for: (1) intact aortic rings; (2) aortic rings incubated with L-NAME ( $10\mu\text{M}$ ) for 20min; (3) aortic rings incubated with chelerythrine ( $10\mu\text{M}$ ) for 20min.

*Second.* Relaxation response to acetylcholine, an endothelium-dependent vasorelaxant, was assessed by adding cumulative concentration of acetylcholine ( $1\text{ nM}$  to  $1\text{ mM}$ ) on the aortic rings precontracted with phenylephrine  $1\mu\text{M}$ . The similar concentration-relaxation curves were also done on: (1) the aortic rings incubated with L-NAME ( $10\mu\text{M}$  for 20min); (2) aortic rings incubated with chelerythrine ( $10\mu\text{M}$  for 20min). Relaxation response curves to sodium nitroprusside (SNP) were also constructed by adding

cumulative concentration ( $1\text{ nM}$  to  $10\text{ nM}$ ) to phenylephrine-precontracted aortic rings.

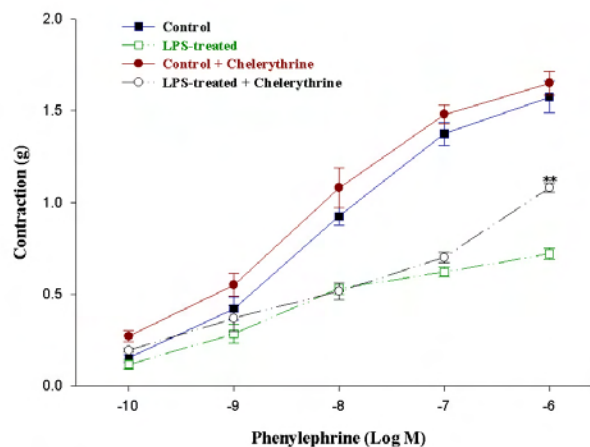
### Statistical Analysis of Data

Results are expressed throughout as means  $\pm$  S.E.M. and were analyzed by one way analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparison test (for comparison of responses to phenylephrine and acetylcholine in aortic). P value of less than 0.05 was considered to be significant.

## RESULTS

### Phenylephrine-induced contraction responses

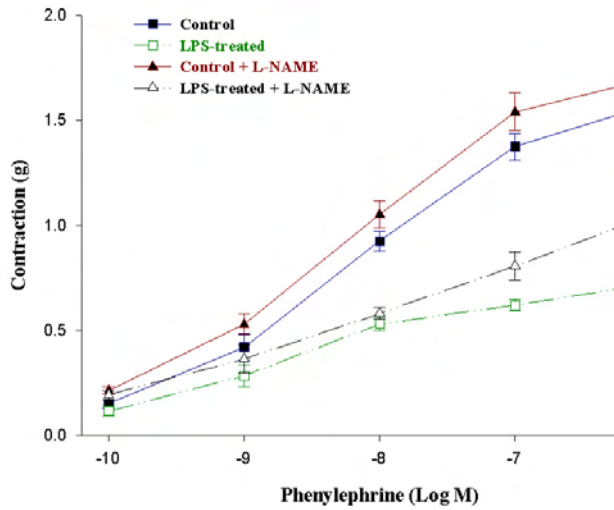
Phenylephrine produced a concentration-dependent contraction in aortic rings. Contractile responses to phenylephrine were significantly decreased in aortic rings isolated from LPS treated rats (Figure 1).



**Fig.1.** Dose-response curves of the contraction of aortic rings of the control and LPS treated rats to phenylephrine. It also shows the effect of incubation with chelerythrine ( $10\mu\text{M}$  for 20min). ( $n = 5$ , mean  $\pm$  SEM, (\*\*))  $P < 0.01$  compared to the value of LPS-treated).

Inhibition of PKC by chelerythrine had no significant effect on the maximal response ( $R_{\text{max}}$ ) of aortic rings of the control rats. However, in the aortic rings of LPS treated groups, chelerythrine incubation caused significant increase on  $R_{\text{max}}$  compared to respective intact rings (Figure 1).

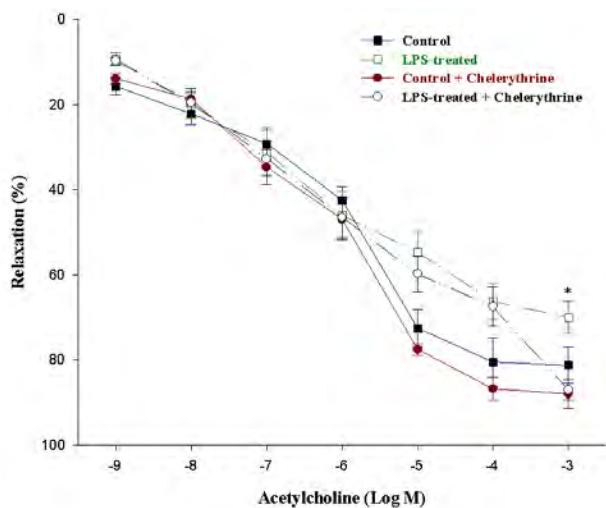
Preincubation with L-NAME significantly increased responses to phenylephrine in the aortic rings of LPS treated rats compared to control (Figure 2).



**Fig.2.** Dose-response curves of the contraction of aortic rings of the control and LPS treated rats to phenylephrine. It also shows the effect of incubation with L-NAME (10 $\mu$ M for 20min). (n = 5, mean  $\pm$  SEM, (\*) P < 0.05 compared to the value of LPS-treated).

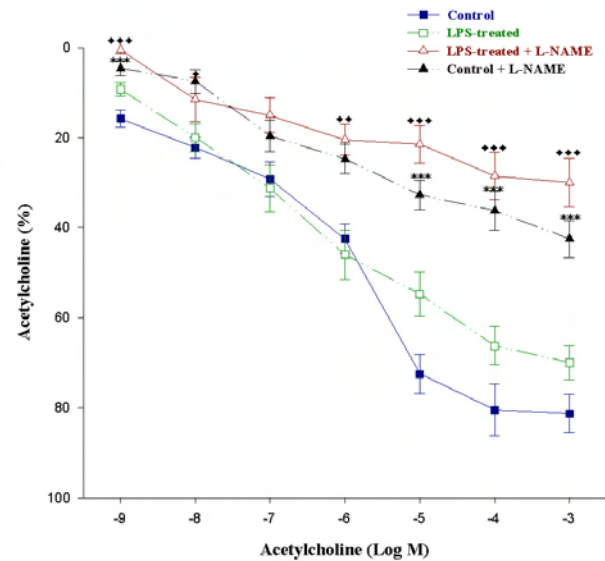
**Acetylcholine -induced relaxation responses**

Acetylcholine caused a concentration-dependent relaxation in the precontracted aortic rings. In the aortic rings of LPS treated rats the relaxation responses were significantly less than control groups (figure 3).



**Fig.3.** Acetylcholine-induced relaxation response curves of aortic rings of the control and LPS treated rats. It also shows the effect of incubation with chelerythrine (10 $\mu$ M for 20min). (n = 5, mean  $\pm$  SEM, (\*) P < 0.05 compared to the value of LPS-treated + chelerythrine).

Preincubation with chelerythrine increased the maximal relaxation response of the aortic rings of LPS treated animals. However, there was no significant change in the concentration-response curve of acetylcholine in control groups (figure 3). Incubation with L-NAME caused significant attenuation of acetylcholine induced relaxation of aortic rings in both groups (figure 4).



**Fig.4.** Acetylcholine-induced relaxation response curves of aortic rings of the control and LPS treated rats. It also shows the effect of incubation with L-NAME (10 $\mu$ M for 20min). (n = 5, mean  $\pm$  SEM, (♦) P < 0.05, (♦♦) P < 0.01, (♦♦♦) P < 0.001 compared to the value of LPS-treated and (\*\*\*) P < 0.001 compared to the value of control).

**Sodium nitroprusside-induced relaxation responses**

Sodium nitroprusside induced a concentration-dependent relaxation in the precontracted aortic rings (data not showed). There was no significant difference in sodium nitroprusside-induced relaxation of the aortic rings between the LPS treated and control rats.

**DISCUSSION**

The data presented here show that LPS can induce vascular hyporesponsiveness to vasoactive factors. Different hypothesis for the vasodilation and resistance to vasopressors that occur in this syndrome had been pronounced such as activation of potassium channels ( $K_{ATP}$  and  $K_{Ca}$  channels) in the plasma membrane of vascular smooth muscle and activation of the inducible form of nitric oxide synthase (24-25).

In our experiments the pressor response to phenylephrine was impaired in LPS-treated rats and blocking NO pathway improved it. This may be due to activation of the inducible form of nitric oxide synthase in aorta of LPS treated rats.

Our data also showed that LPS reduced endothelium-dependent vascular reactivity to receptor-mediated agonists such as acetylcholine. The effect of LPS on the aortic NO-mediated component of acetylcholine-induced relaxation was not caused by a reduction in the sensitivity of vascular smooth muscle to NO, because responses to SNP were unaltered. This finding confirms the theory that by the time iNOS is up regulated in endotoxic shock, there is a corresponding down-regulation in the endothelial constitutive NOS (eNOS) (26-28).

Excessive iNOS activation and NO production is not the main cause of these defects and other factors like PKC directly or indirectly may contribute to vascular hypocontractility. In our study chelerythrine showed beneficial effect on maximal contractile and dilatory response of LPS-treated rat which can suggest the probable effect of PKC in reducing response of aorta to phenylephrine and acetylcholine. This is in agreement with previous studies which showed that LPS activates PKC and induce iNOS transcription and NO formation (20, 21, 29). The relationship between LPS stimulation, PKC activation, iNOS expression and NO formation are complex, and it is likely that PKC influence this process at several sites in the cell. Those observations suggest that multiple intracellular pathways, some of which are PKC independent, can transduce an LPS signal into iNOS expression and PKC activity is necessary for NO formation after iNOS induction. It can be concluded that PKC and NO pathways are altered in aortic rings of LPS treated rats. The exact nature of the altered vascular responses needs further detailed studies.

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