**Effect of inducible nitric oxide synthase on intestinal microcirculation in endotoxic shock**

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**Abstract:** To investigate the changes of intestinal microcirculation in endotoxic shock and the effect of inducible nitric oxide synthase (iNOS) on intestinal microcirculation, endotoxic shock was induced by intravenous injection of lipopolysaccharide (LPS) in mice. Mean arterial pressure (MAP) was monitored throughout the experimental procedure. The velocity and flux of red blood cell (RBC) in villus tip arteriole and capillaries were measured by FITC-labeled erythrocytes and intravital microscopy. The effect of iNOS was determined by targeted disruption of mice iNOS-gene and administration of S-methylthiourea sulfate (SMT), a selective inhibitor of iNOS, before LPS injection. No significant differences in MAP, RBC velocity and flux at baseline were found among wild type mice, SMT pretreated mice and iNOS-gene knockout mice. LPS induced a dramatic fall of MAP in wild type mice. The decrease of MAP was significantly restored in iNOS-gene knockout mice and in wild type mice received SMT before LPS injection. The velocity and flux of RBC in villus tip arteriole and capillaries decreased markedly after LPS injection in wild type mice, while significantly higher velocity and flux of RBC were found in iNOS-gene knockout mice and SMT-pretreated mice both 60 and 120 min after LPS injection. The results demonstrate that iNOS plays an essential role in the intestinal microcirculation disturbance which occurs in endotoxic shock.

**Key words:** nitric oxide synthase; endotoxic shock; microcirculation

**诱导型一氧化氮合酶对内毒素休克小肠微循环的影响**

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**摘要:** 采用静脉注射脂多糖(lipopolysaccharide, LPS)的方法建立小鼠内毒素休克模型，探讨内毒素休克时小肠微循环的变化以及诱导型一氧化氮合酶(iNOS)对小肠微循环的影响。实验过程中连续监测小鼠平均动脉血压(mean arterial pressure, MAP)变化情况。利用FITC标记红细胞和活体显微方法直接观察并计算小鼠小肠绒毛尖端小动脉和毛细血管内红细胞的流速和流量，并观察敲除小鼠iNOS基因和选择性iNOS抑制剂S-methylthiourea sulfate (SMT)对实验过程中小肠微循环的影响。结果显示，给予LPS后，小鼠的MAP进行性下降。给予LPS前，应用SMT和敲除小鼠iNOS基因可以显著提高MAP;给予LPS后，小鼠小肠绒毛尖端小动脉和毛细血管内红细胞流速和流量显著下降，给予LPS前，应用SMT和敲除小鼠iNOS基因可以显著提高MAP;给予LPS后，小肠绒毛尖端小动脉和毛细血管内红细胞流速和流量显著下降。给予LPS前，应用SMT和敲除小鼠iNOS基因可以显著提高小肠绒毛尖端小动脉和毛细血管的红细胞流速和流量。结果表明，iNOS在内毒素休克小肠微循环衰竭的过程中发挥重要作用。

**关键词:** 一氧化氮合酶; 内毒素休克; 微循环

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Microcirculation failure is one of the most important pathophysiological features of endotoxic shock. The microcirculation disturbance of the gut mucosa may improve the translocation of intraluminal bacteria and toxins absorption, which plays a crucial role in the outcome of endotoxic shock. As noted by different investigators, nitric oxide (NO) is an essential mediator to induce vasodilation and catecholamine-resistant hypotension that occur in endotoxic shock. In addition, targeted ablation of inducible nitric oxide synthase (iNOS) gene can increase the microvascular responsiveness of mice in endotoxic shock and decrease the mortality [1]. However, little is known about the effects of iNOS on intestinal microcirculation of endotoxic shock.

Using intravital microscopy and fluorescent labeled erythrocytes, the current study was designed to measure microvascular red blood cell (RBC) velocity and flux simultaneously in order to reflect the RBC perfusion in intestinal villi directly. And the potential role of iNOS was determined by targeted disruption of iNOS-gene or pharmacological inhibition of iNOS.

1 MATERIALS AND METHODS

1.1 Animals
Adult male iNOS gene knockout mice (+/-) (B6, 129) and wild type mice (B6129PF2/J) with a genetic background as close as possible to the iNOS gene-knockout mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Body weight ranged from 25 to 35 g [2].

1.2 Fluorescent labeling of erythrocytes [3]
Erythrocytes were obtained from separate donor mice by heart puncture after injection of pentobarbital and anticoagulation with heparin. Erythrocytes were separated by centrifugation and washed four times in a phosphate-buffer of pH 7.4 (PBS, Sigma Diagnostics, USA) containing 100 mg/L ethylenediaminetetraacetic acid (EDTA). And then the erythrocytes were incubated in PBS (pH 8.0) containing FITC (Sigma Diagnostics, USA) at 25°C for 2 h in darkness. The labeled erythrocytes were again flushed with the PBS and centrifuged several times to free the supernatant from fluorescent dye. The labeled cells were stored in darkness in a refrigerator at 5°C no more than 48 h.

1.3 Surgical procedures [3]
Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). A tracheostomy was performed and the mice were mechanically ventilated using a rodent ventilator (SN-480-7, Shinano Manufacturing Co., Japan). The right jugular vein was cannulated with a polyethylene catheter for the injection of labeled erythrocytes and drugs. The right carotid artery was cannulated with Polyethylene-10 tubing to monitor mean blood pressure (MAP). Then the animal was placed in a lateral recumbent position on a specially designed microscope stage equipped with a glass slide to allow for transilluminating microscopy. An abdominal midline incision was done and a segment of 2 to 3 cm of the ileum was exteriorized. A segment of wire was put into the ileum to make it unfold as a semicircle. The ileum was opened along the antimesenteric border over a distance of 1 cm and placed on a specially designed pedestal to facilitate observation of the villi with transilluminating and fluorescent microscopy (250×). The bowel segment was gently fixed with two small pins on the outer fringes of each site to a frame with the mucosa on the upper side and the exposed intestine was gently covered by Saran-Wrap. The prepared bowel segment and mesentery were immersed in a modified Krebs solution (mmol/L: NaCl 118, KCl 5.9, MgSO4 0.5, NaH2CO3 28, KH2PO4 28, CaCl2 0.5, glucose 10) throughout the experiment (Fig. 1). The temperature of the solution was maintained at (37±0.5°C) with a heating device. FITC-labeled erythrocytes (15 µl) were administered intravenously 15 min before the measurement. All animals received 0.9% saline (0.1 ml/h) to compensate for fluid loss.

1.4 Measurement of the velocity and flux of RBC [3, 4]
Three villi were observed randomly in each mouse and 10 s video images were recorded at each time point which showed the velocity and flux of FITC-labeled RBC to traverse a chosen tip arteriole or capillary. At the end of observation, a drop of diluted blood with saline was placed on a covered microscope slide, and sites for cell counting were selected randomly using transillumination and calculated the number of total RBC. Then the number of FITC-labeled RBC was counted under fluorescent microscope and the fraction of FITC-labeled RBC was calculated.

To measure the velocity and flux of RBC, the videotape was replayed at a slow motion and the frame-to-frame analysis was performed with the help of computer. Velocity was calculated from the time required for a FITC-labeled RBC to traverse a chosen length of vessel, which was got from the length on screen divided by the magnification during videotape playback. To estimate the flux, the number of labeled RBC in a portion of capillary or tip arte-
role per unit time was counted. Flux was calculated by dividing this number by the fraction of labeled cell.

1.5 Experimental protocol
Lipopolysaccharide (LPS, Sigma Diagnostics, USA) and \(S\)-methylthiourea sulfate (SMT, Sigma Diagnostics, USA), a selective inhibitor of iNOS, were dissolved in 0.9% saline. LPS (10 mg/kg) or saline (0.1 ml) was injected intravenously 10 min after the surgical procedure and SMT (3 mg/kg) was administered intravenously 20 min before the surgical procedure. MAP, RBC velocity and flux were recorded at baseline (just before LPS or saline injection), 60 and 120 min after LPS or saline administration. Wild type mice were divided randomly into 4 groups (\(n=6\)): (1) WT, animals received only saline; (2) WT+LPS, animals received LPS; (3) WT+SMT, mice were pretreated with SMT and saline; (4) WT+SMT+LPS, mice were pretreated with SMT and LPS. iNOS-gene knockout mice were enrolled into another two groups (\(n=6\)); (5) iNOS(-/-) group, iNOS gene-knockout mice received saline; (6) iNOS(-/-)+LPS group, LPS was given to the iNOS gene-knockout mice.

1.6 Statistical analysis
All the values were expressed as mean±SD. These data were compared by analysis of variance (two-way ANOVA) and Student-Newman-Keuls post hoc analysis. Statistical significance was assumed at a \(P\) value of less than 0.05.

2 RESULTS

2.1 Mean arterial pressure
All the mice survived throughout the experimental procedure. MAP was monitored throughout the 120-min observation period. Figure 2 summarizes the MAP of the six groups of baseline, 60 and 120 min after LPS or saline injection. Baseline values for MAP of the six groups were not significantly different (\(P>0.05\)). MAP remained constant during the 120-min observation in group WT, WT+SMT and iNOS(-/-). MAP of WT+LPS group decreased significantly after LPS injection compared with that of WT group (\(P<0.05\)). And MAP of WT+SMT+LPS and iNOS(-/-)+LPS groups were also lower than that of WT group 60 and 120 min after LPS administration (\(P<0.05\)). Compared with WT+LPS group, MAP of iNOS(-/-)+LPS group was significantly higher 60 and 120 min after LPS administration (\(P<0.05\)). And 120 min after LPS injection, MAP of WT+SMT+LPS group were also markedly higher than that of WT+LPS group (\(P<0.05\)).

Fig. 2. MAP of all the experimental groups. * \(P<0.05\) vs WT group, * \(P<0.05\) vs WT+LPS group.

2.2 RBC velocity
At baseline, no differences among the six groups of RBC
velocities in villus tip arteriole and capillaries were found ($P > 0.05$). RBC velocities in villus tip arteriole and capillaries of WT, WT+SMT and iNOS(-/-) groups kept constant throughout the experimental procedure. sixty and 120 min after LPS administration, the RBC velocities of WT+LPS, WT+SMT+LPS and iNOS(-/-)+LPS groups in the two sites studied were markedly lower than those of WT group ($P > 0.05$). But significantly higher RBC velocities of WT+SMT+LPS and iNOS(-/-)+LPS groups in villus tip arteriole and capillaries were found compared with that in the WT+LPS group ($P < 0.05$) at the two time points after LPS injection, which is shown in Fig.3 and 4.

2.3 RBC Flux

As shown in Fig.5 and 6, there were no significant differences of RBC flux in villus tip arteriole and capillaries of baseline among all the six groups ($P > 0.05$). No marked changes in RBC flux at these two sites were observed in WT, WT+SMT and iNOS(-/-) groups throughout the experimental procedure. Although the RBC velocity in these two sites of WT+LPS, WT+SMT+LPS and iNOS(-/-)+LPS groups decreased markedly after LPS injection compared with that in the WT group, the RBC flux in villus tip arteriole and capillaries of WT+SMT+LPS and iNOS(-/-)+LPS groups were significantly higher than that of WT+LPS group both 60 and 120 min after LPS administration ($P < 0.05$).
3 DISCUSSION

Microcirculation failure is an important feature of endotoxic shock. RBC velocity and flux can be used to evaluate the microvascular perfusion directly. In this study, the endotoxic shock was induced in wild type mice by LPS injection, which elicited marked hypotension [1, 5]. Using intravital microscopy and FITC-labeled RBC, significant decrease of velocity and flux of RBC in villus tip arteriole and capillaries after LPS administration were found in wild type mice indicating microcirculation failure. Although LPS also induced a decrease in MAP, RBC velocity and flux in villus tip arteriole and capillaries in iNOS-gene knockout mice, these data were significantly higher than those of wild type mice. Similar outcome was also found in wild type mice received SMT before LPS injection.

Evidence to date strongly suggests that NO is one of the key mediators of the vasodilation and low resistance hypotension that occurs in septic shock [9]. NO is catalyzed by 3 isoforms of NO synthase (NOS), neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). The iNOS is present in macrophages, hepatocytes, and vascular smooth muscle cells. LPS as well as several host factors that circulate in high concentrations in endotoxic shock, including tumor necrosis factor (TNF), interleukin-1,2, and interferon, either alone or in combination, be capable of inducing iNOS expression and releasing a large amount of NO [7]. Studies in animals as well as human indicate that NO in endotoxic shock is mainly produced by iNOS[9]. Wild type mice injected with LPS accumulated high serum levels of nitrate plus nitrite, while LPS caused no increase in serum nitrate in iNOS-gene knockout mice[8], as in LPS-injected mice treated with a NOS inhibitor[9]. In the current study, either targeted abolition of iNOS gene or selective inhibition of iNOS could increase the blood pressure significantly after LPS injection. MacMicking also reported that the fall in central arterial blood pressure of endotoxic shock was markedly attenuated in iNOS-gene knockout mice[8]. It is also reported that pharmacological inhibitor did not fully restore arterial contraction after LPS, whereas iNOS gene deficiency did [11]. In contrast, our data suggested that pharmacological inhibitor induced the same recovery of hypotension as iNOS gene deficiency. It may be due to the different pharmacological inhibitor used in this study. SMT is a non-amino acid analog of L-arginine, which has been reported to highly selectively inhibit iNOS and has almost no effect on the other two types of NOS[10].

The most important finding in this study is that iNOS gene abolition or pharmacological inhibition of iNOS could effectively attenuate the microcirculation disturbance of intestinal villi indicated by an increase in RBC velocity and flux in villus tip arteriole and capillaries. But neither iNOS gene abolition nor pharmacological inhibition of iNOS could fully eliminate the effects of LPS and restore the microcirculation disturbance completely in the present study. Targeted abolition of iNOS gene may be the best method to directly investigate the function of iNOS. The current results indicate that iNOS plays an essential role in the intestinal microcirculation failure induced by LPS and inhibiting the function of iNOS may be helpful to attenuate the microcirculation disturbance in endotoxic shock. Mechanisms other than iNOS may be also contributive to the microcirculation disturbance in endotoxic shock as either abolition of iNOS gene or pharmacological inhibition of iNOS could only partly restore the microcirculation disturbance.

Mounting evidence including the current results have indicated that iNOS-induced NO release is one of the main factors related to the hypotension that occurs in endotoxic shock. Microcirculation failure in endotoxic shock is induced by multiple factors and hypotension possibly only plays a minor role in this procedure. It was reported that no significant microcirculation disturbance was found in hemorrhage shock mice while microcirculation failure occurred in endotoxic shock mice of a compared hypotension. In addition, a low dose of LPS could induce microcirculation disturbance while blood pressure remained unchanged [9]. So the protective effects on intestinal villi microcirculation induced by iNOS-gene abolition or iNOS inhibition may be independent of recovery of hypotension. Elimination the function of iNOS can block platelet and leukocyte adhesion to endothelial cells and also can modulate production of some cellular factors, which may contribute to the restore of microcirculation disturbance [11, 12]. Further investigations are necessary to elucidate the complete pathway by which iNOS affects intestinal microcirculation.

REFERENCES


