The Effects of iNOS on the Lymphatic Microcirculation in Endotoxemia

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Abstract **Objective** To study the effects of inducible nitric oxide synthase（iNOS）on the lymphatic microcirculation in endotoxemia. **Methods** Rats were injected with lipopolysaccharides(LPS) and SMT (S-Methyliscthiourea sulfate). Using inverted microscopy and digital image processing to observe and measure the diameter and motive frequency of the rat mesenteric lymphatic vessels. Inducible nitric oxide synthase（iNOS） was assessed by immunohistochemiscal stain. **Results** The diameters of lymphatic vessels increased and frequency decreased in comparison with those before injection in LPS group. Administration of LPS significantly increase the expression of iNOS in epithelial cells of mesenteric lymphatic vessels. There were no obvious alteration of diameter and frequency in LPS+SMT group. SMT can decrease iNOS expression. **Conclusions** iNOS may have an effect on regulating the motion of lymphatic vessels. SMT may have a protective effect by reducing the activity of iNOS during endotoxemia.

Key Words Nitric oxide synthase; Lipopolysaccharides; Mesenteric lymphatic vessels; Microcirculation; Rats; Inbred strains

Endotoxin induced septic shock, one of the most frequent causes of death in the intensive care unit, is represented by hypotension and multiple systems organ failures. In the early 1990s, Nitric oxide (NO) emerged as a potentially important player in the pathogenesis of sepsis. NO, a relatively unstable radical under acrobic conditions, has been identified as a pleiotropic mediator in various physiologic responses to external stimuli: (1)accounting for the activity of endothelium-derived relaxing factor; (2) being a major defense molecule of immune cells against parasites, bacteria, and tumor cells; (3) acting as a neurotransmitter and (4) preventing platelet aggregation[10,11].

The present study is to investigate the effects of inducible nitric oxide (iNOS) on the lymphatic microcirculation in endotoxemia and the alteration of iNOS expression after inhibitor of iNOS- SMT(S-Methylisothiourea sulfate) is employed.

MATERIALS AND METHODS

Animals and groups

Adult male Wistar rats (200-300g) were used in this study. They were maintained in individual cages of the Animal Research Center of Shandong university (Jinan, China) under 12h light, 12h dark conditions with free access to food and water. The animals were divided into three groups (18 rats for each group): Control group (Control) - normal saline (5ml/kg) was injected through femoral vein. the rats were killed at 1h, 3h, 6h after the injection respectively; lipopolysaccharides group (LPS) -LPS (5mg/kg) was injected through femoral vein, the other treatment were the same as Control; LPS+SMT group (LPS+SMT) - LPS (5mg/kg)and SMT(10mg/kg) were injected through femoral vein, the other treatment were the same as Control. All experiment in the present study were carried out following the national institute of health guidelines for the care and use of laboratory animals.

Dynamic observation

The rats were anesthetized with 20% Urethane (7ml/kg). We opened the abdomen, took out the mesentery, looked for a free lymphatic vessel and placed on special watching -box which was kept the temperature of 32℃ or about. Inverted microscope and high- resolution -closed -circuit -television video system were used to watch and record the lymphatic motions. Each rat which was needed to watch for 6 h was stabilized for 5-10 min before injection and recorded as normal motion. The rats were killed at 6h after injection.

According to the data recorded, we used computer -picture -analysis system for measuring the lymphatic diameter and contractile frequency. We measured the same lymphatic vessel in each rat, choosing the time -stages for measure as such: before injection, after injections 1, 2, 3, 4, 5, 6h. The parameters which were needed to be recorded include the mean diameter of lymphatic vesses (d), the maximal diastolic diameter (b), the minimal contractile diameter (c) and contractile frequency (a). According to the formula of Yasuda[1] and Goto[2], we calculated contractive index[the Index I=

$(b^2-c^2)/b^2$], total contractive index [IndexII= $(b^2-c^2)a/b^2$] and lymphatic dynamic index[LD-Index=$(b-c)100a/d^2$].

Immunohistochemistry

After the rats been killed, mesentery were taken out and washed in PBS for 30 min at RT, free floating mesentery were quenched with 3% H_2O_2 in PBS for 10 min and blocked with PBS containing 10% bovine serum albumin (BSA) for 30 min at RT. iNOS was immunostained by incubation with rabbit anti-iNOS antiserum at a dilution of 1:100 in PBS at 4℃ overnight followed by 30 min incubation at room temperature with biotinylated goat anti-rabbit IgG and avidin-biotinylated horseradish peroxidase complex in PBS. The color reaction was performed by using diaminobenzidine as a substrate. The immunostained sections were mounted on gelatin-coated glass slides, dehydrated, and covered with coverslip. The primary antisera were omitted from the staining procedures as control experiment.

Five slides were chosen from each group, three fields of view were observed in each slide and count the positive cells number in every visual field. Results were expressed as mean±standard error mean. One -way and two-way analyses of variance (ANOVA) with one repeated measure were employed to assess differences in indexes between and within groups. A P-value less than 0.05 was considered significant.

RESULTS

Alteration of lymphatic vessel mean diameter(Table 1.)

Lymphatic vessels of LPS dilated significantly at 4 h after injection in comparison with before injection(p<0.05). There is no significant difference in lymphatic vessel mean diameter between and within Control, LPS+SMT(p>0.05).

Alteration of lymphatic vessel maximal diastolic diameter in each group (Table 2.)

The maximal diastolic diameter of lymphatic vessels in LPS increased significantly at 4 h after injection in comparison with that before injection(p<0.05). There is no significant difference in lymphatic vessel maximal diastolic diameter between and within Control, LPS+SMT(p>0.05).

Alteration of lymphatic vessel minimal contractile diameter in each group (Table 3)

Table 1. The mean diameter of lymphatic vessel in each group ($\bar{x}$±S, unit: picture element, n=6)

group	Before injection	After injection					
	oh	1h	2h	3h	4h	5h	6h
Control	68.06±17.49	67.67±17.73	67.85±25.66	69.87±18.71	66.47±22.12	70.28±19.75	71.48±18.17
LPS▲▲	57.44±13.41	64.40±9.56	65.64±12.91	61.37±16.69	71.53±11.46**	71.91±13.99**	76.51±14.29**
LPS+SMT	59.33±11.63	55.04±6.36	56.41±7.61	53.16±7.04	53.55±10.35	55.63 ±7.09	56.49 ±5.67

▲▲ p<0.01 LPS compared with other groups; *P<0.05; **P<0.01 compare with oh within group.

Table 2 Maximal diastolic diameter of lymphatic vessel in each group (±S,unit: picture element，n=6))

group	Before injection	After injection					
	0h	1h	2h	3h	4h	5h	6h
ontrol	74.17±16.25	76.83±14.46	79.00±27.00	79.67±18.12	75.50±19.61	80.50±15.22	81.67±14.88
LPS▲▲	73.17±16.94	77.83±17.70	76.67±15.15	72.17±18.37	79.17±15.25*	82.00±18.32*	83.33±16.28*
LPS+SMT	63.50±7.23	64.33±6.35	63.33±7.53	59.33±6.92	62.17±13.61	61.83±8.16	62.50±6.12

* p<0.05 compared with 0h, ▲▲ p<0.01 compared with Control, LPS+SMT

Table 3 Minimal contractile diameter of lymphatic vessel in each group ($\bar{x}$±S，unit: picture element, n=6)

group	Before injection	After injection					
	0h	1h	2h	3h	4h	5h	6h
Control	60.67±19.79	59.83±18.24	59.17±26.66	60.50±17.76	59.00±21.27	59.67±17.58	63.83±18.52
LPS▲▲	43.83±9.66	49.00±6.13	51.00±9.57	48.17±18.86	57.00±13.67*	59.77±14.21**	66.00±17.82**
LPS+SMT	50.17±15.77	47.33±8.87	45.33±12.23	42.17±10.30	45.67±9.67	46.33±7.37	47.17±7.05

*p<0.05 compared with 0h, ** p<0.01 compared with 0h. ▲▲ p<0.01 compared with Control, LPS+SMT

The minimal contractile diameter of lymphatic vessels in LPS increased significantly at 3 h after injection in comparison with before injection (p<0.05). There is no significant difference in lymphatic vessel minimal contractile diameter between and within Control, LPS+SMT(p>0.05).

Immunohistochemistry results

There was no iNOS –ir cells in mesentery lymphatic vessels and blood vessels of Control group.

There was no iNOS-ir positive cells in mesentery lymphatic vessels and blood vessels of LPS group at 1 h after injection. At 3 h after injection, Macrophages around lymphatic vessels and blood vessels were iNOS-ir cells. A small quantity of endothelial cells of lymphatic vessels and blood vessels were also iNOS-ir.At 6 h after injection, the number of iNOS-ir cells were increased greatly, the positive cells were endothelial cells and macrophages in or around the lymphatic vessels and blood vessels (Fig.1).

In LPS+SMT group, there was no iNOS-ir cells in or around the lymphatic vessels and blood vessels at 1 h after injection. iNOS-ir cells were all seen at 3 h and 6 h after injection. These cells were also situated in or around the lymphatic vessels and blood vessels. But the number of iNOS-ir cells was less than LPS (Fig.2).

Image analysis

Our results showed that iNOS-ir cells number of LPS at 6 h after injection was increased in comparison with that of at 3 h after injection (p<0.01). There is no difference between LPS+SMT at 3 h and at 6h after injection (p>0.05). The number of iNOS-ir cells in LPS at 3 h after injecton was greater than that of LPS+SMT at 3 h after injection (p<0.05). The number of iNOS-ir cells in LPS at 6 h after injecton was also greater than that of LPS+SMT at 6 h after injection (p<0.05).

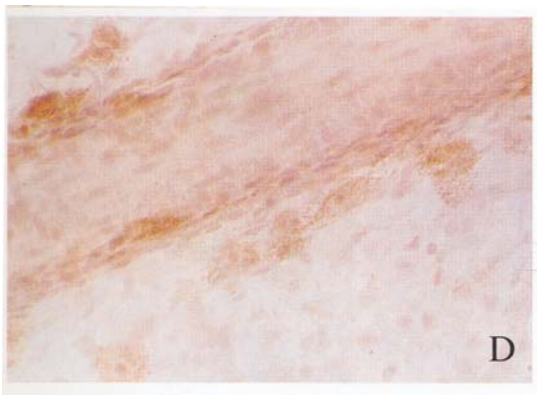

Fig 1 After injecting LPS 3h, lots of iNOS epithelial cells on the mesenteric lymphatic vessels（×400）

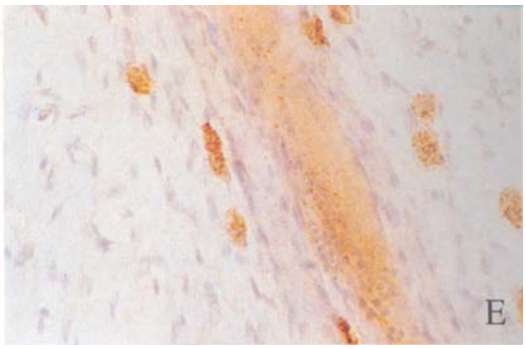

Fig 2 After injecting the LPS+SMT 3h, a little iNOS epithelial cells on the mesenteric lymphatic vessels（×400）

DISCUSION

The lymphatic system vessels form a network throughout the body almost like blood vessels. This system is usually thought of as being part of the cardiovascular system. One of lymph fluid's primary functions is to supply a fluid environment between the cells and the tissues [3]. It is recently found that NO took part in the regulation of the lymphatic system[4,5]. It is reported that the overproduction of NO induced by inducible nitric oxide synthase (iNOS) plays an important role in circulatory failure and diffuse tissue injury[6-8]. Neural (nNOS or NOS1) and endothelial (eNOS or NOS3) NOS is always present under physiological condition, but relatively inactive until intracellular calcium levels rise. The small amounts of NO produced by the calcium-dependent cNOS are involved in various physiologic processes, including neurotransmission[9] and vasodilation. The induction of iNOS mRNA is dependent on calcium.

In the present study, we found that the alteration of contractile frequency, Index-Ⅰ, Index-Ⅱ and LD- Index was not significant (p>0.05) in control rats. The function of valves was also well. But such parameters above in LPS rats were altered significantly (p<0.01). The valves of lymphatic vessels were closed and the lymphatic fluid decreased. This may be due to the overproduction of NO induced by iNOS that are activated by LPS in endothelial cells and macrophages. Overproduced NO can stimulate guanosine 3'5'-cyclic monophosphate (cGMP) which mediate smooth muscle relaxation release from lymphatic smooth muscle. Lymphatic vessels relaxation may result in the high permeability and decrease of blood flow. All these factors may responsible for the initial hypotension after endotoxemia.

Our results showed that SMT can significantly but partly inhibit the production of NO induced by iNOS. i-

NOS mainly expressed in macrophages, and a large quantity of macrophages were around the lymphatic vessels in LPS rats. So inflammatory response must be inhibited in order to decrease the production of NO induced by iNOS. Our data also showed that SMT has an selective effect on iNOS, which can decrease the overexpression of NO and maintain cNOS and NO at a certain level. In conclusion, the administration of selective inhibitor of iNOS is profitable in endotoxemia. But the mechnisim need to be further investigated.

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