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Inhibitory effects of nitric oxide and interleukin-10 on production of tumor necrosis factor α, interleukin-1β, and interleukin-6 in mouse alveolar macrophages

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KEY WORDS nitric oxide; alveolar macrophages; inflammation; interleukin-1; interleukin-6; interleukin-10; tumor necrosis factor; lipopolysaccharides; enzyme-linked immunosorbant assay

ABSTRACT

AIM: To observe the effects of nitric oxide and interleukin-10 (IL-10) on inflammatory reaction in mouse alveolar macrophages (AM). **METHODS**: AM from mice were stimulated by lipopolysaccharides (LPS) 10 $mg \cdot L^{-1}$ and nitric-oxide synthase inhibitor, *S*-methylisothiorea sulfate (SMT) or nitric-oxide donor, *S*-nitroso-*N*-acetyl-*D*,*L*-penicillamine (SNAP). The production of tumor necrosis factor α (TNFα), IL-1β, IL-6, and IL-10 by AM were measured by ELISA. **RESULTS**: After LPS-stimulation, TNFα, IL-1β, and IL-6 peaked at 6, 12, and 24 h, respectively by AM. SMT inhibited LPS-induced nitric oxide release and increased IL-1β and IL-6 secretions in AM, but the TNFα levels remained unchanged. SNAP had inhibitory effects on IL-1β and IL-6 secretions in a concentration-dependent manner, but exerted no effect on TNFα release. TNFα, IL-1β, and IL-6 secretions were inhibited by recombinant IL-10, but the cytokines release was upregulated by anti-IL-10 monoclonal antibody. **CONCLUSION**: Both endogenous and exogenous nitric oxide and IL-10 had inhibitory effects on the LPS-induced TNFα, IL-1β, and IL-6 secretions in mouse AM.

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INTRODUCTION

The inflammatory response is a crucial part of the normal host defense mechanism and, as such, has a protective role[1]. However, when this response is excessive or uncontrolled, pathological consequences may occur[2,3]. Inapprociate inflammatory response has been implicated as the causal mechanism in the adult respiratory distress syndrome (ARDS). Tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), and IL-6 are the important proinflammatory mediators of the inflammatory response directly inducing neutrophil influx and capillary endothelial cell permeability[2]. To control the overproduction of the proinflammatory cytokines may be beneficial to modulate the inflammatory response.

IL-10 was formerly known as cytokine synthesis inhibitory factor. A human form was soon identified to have a regulatory role on inflammatory response[4,5]. Endogenous nitric oxide was first identified as a vasodilating factor in vascular endothelial cells, served an important role in lung as a regulator of pulmonary circulation[3]. Nitric oxide has regulatory effects on coagulation and inflammatory response[6]. But the effects of nitric oxide and IL-10 on the

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inflammatory response have not been clearly elucidated.

The current study was to investigate the effects of nitric oxide on the production of inflammatory cytokines and the role of IL-10 in the proinflammatory cytokines release in alveolar macrophages (AM).

MATERIALS AND METHODS

Kunming mice (♂, $n = 160$, 18 – 22 g) were obtained from the Experimental Animal Institute of Chinese Academy of Medical Sciences, Grade Ⅱ, Certificate № 01-3001. AM were obtained by bronchoalveolar lavage, which was performed by repeated instillation with total 15 mL of Mg^{2+}- and Ca^{2+}-free Hanks' solution containing edetic acid 0.6 $mmol \cdot L^{-1}$. Isolated cells were pooled and washed twice with RPMI-1640 (Gibco) containing 5% fetal calf serum to remove edetic acid. Washed cells were suspended in culture medium (RPMI-1640, 10 % fetal calf serum, *L*-glutamine 2 $mmol \cdot L^{-1}$, Pen/Strep). The isolated cells (> 95 % AM by acid phosphatase staining, > 95 % viable by trypan-blue staining) were plated in 24-well plates (Nunc) at a cell concentration of 5×10^5 cells in 1 mL culture medium and cultured for 24 h with lipopolysaccharides (LPS, *E coli* 0127: B8, Sigma) with or without indicated concentrations of the nitric-oxide synthase inhibitor, *S*-methylisothiourea sulfate (SMT, Sigma), or nitric-oxide donor, *S*-nitroso-*N*-acetyl-*D*,*L*-penicillamine (SNAP, Sigma). The cells were also cultured for 24 h with recombinant mouse IL-10 (Biosource Co, USA) or rat anti-mouse IL-10 monoclonal antibody (Biosource Co, USA). The culture medium was collected, centrifuged (2000 × *g*, 4 ℃, 10 min), divided in aliquots, and stored at – 20 ℃ until assay.

TNFα, IL-1β, IL-6, and IL-10 in culture supernatants were measured by ELISA (TNFα, IL-1β, IL-6, and IL-10 ELISA kit were obtained from Biosource Co, USA). Nitrite in culture supernatants was measured by a colorimetric assay based on the Griess reaction (Nitrite kit from Biotin Co, Beijing).

Cytotoxicity of culture procedures was analyzed by trypan-blue inclusion. Briefly, 5×10^5 AM were plated in microtiter plate and cultured in 1-mL culture medium with various concentrations of LPS with or without SMT 250 $\mu mol \cdot L^{-1}$. Following 24-h culture, cultured medium was removed, Hanks' solution 1 mL and 0.1 % trypan-blue 10 μL were added to the wells. The plate was observed under microscope and the necrosis cells was calculated.

Data were expressed as $\bar{x} \pm s$ and compared with ANOVA.

RESULTS

Effects of LPS on cytokine and nitric oxide release The levels of TNFα, IL-1β, and IL-6 peaked at 6, 12, and 24 h after AM were stimulated by LPS 10 $mg \cdot L^{-1}$. The nitrite levels maintained increase at 24 h after LPS-stimulation (Fig 1).

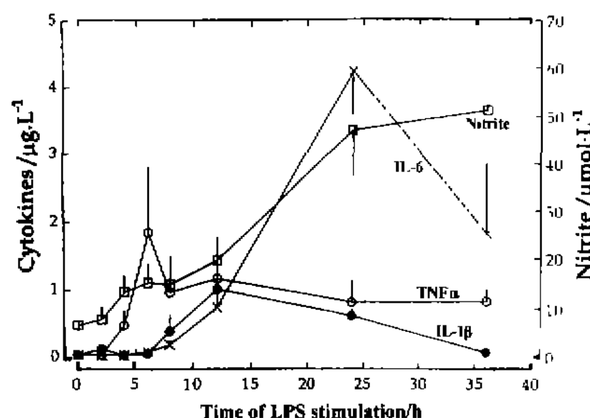

Fig 1. Nitrite and cytokine releases after LPS-stimulation. $n = 7$ mice. $\bar{x} \pm s$.

Effects of nitric-oxide synthase inhibition on cytokine production SMT inhibited nitrite production in a concentration-dependent manner in LPS-stimulated AM (Tab 1).

Tab 1. Effects of *S*-methylisothiourea sulfate (SMT) on nitrite production by LPS-stimulated alveolar macrophages. $n = 7$ mice. $\bar{x} \pm s$. $^{b}P < 0.05$ *vs* SMT 0 μmol·L⁻¹.

SMT/μmol·L⁻¹	Nitrite levels/μmol·L⁻¹
0	61 ± 16
1	57 ± 15
10	47 ± 14[b]
100	36 ± 10[b]
250	17 ± 5[b]
500	12 ± 5[b]
1000	9 ± 5[b]

Inhibition of nitrite production by SMT 250 μmol·L⁻¹ led to a marked increase of IL-1β and IL-6 production by AM stimulated by LPS 10 mg·L⁻¹ at 24 h, IL-10 production decreased, but the levels of TNFα in medium were not affected (Tab 2). No differences were observed in the cellular viability.

Tab 2. Effects of *S*-methylisothiourea sulfate (SMT) and *S*-nitroso-*N*-acetyl-*D*, *L*-penicillamine (SNAP) on cytokine production by LPS-stimulated alveolar macrophages. $n = 4$ mice. $\bar{x} \pm s$. $^{b}P < 0.05$ *vs* LPS 10 mg·L⁻¹.

	Cytokines levels/ng·L⁻¹			
	TNFα	IL-1β	IL-6	IL-10
Saline	104 ± 24[b]	0[b]	368 ± 172[b]	70 ± 13[b]
LPS 10 mg·L⁻¹	1 212 ± 110	632 ± 97	3 590 ± 921	1 500 ± 139
SMT 250 μmol·L⁻¹	1 664 ± 505	1 094 ± 437[b]	5 385 ± 1 005[b]	960 ± 115[b]
SNAP 50 μmol·L⁻¹	1 040 ± 69	99 ± 36[b]	1 524 ± 603[b]	822 ± 136[b]

Effects of SNAP on cytokine production SNAP 10 – 1000 nmol·L⁻¹ inhibited the increases of IL-1β and IL-6 productions in a concentration-dependent manner, when the LPS-induced production of endogenous nitric oxide was blocked by SMT 250 μmol·L⁻¹. There was no effect on TNFα production (Fig 2).

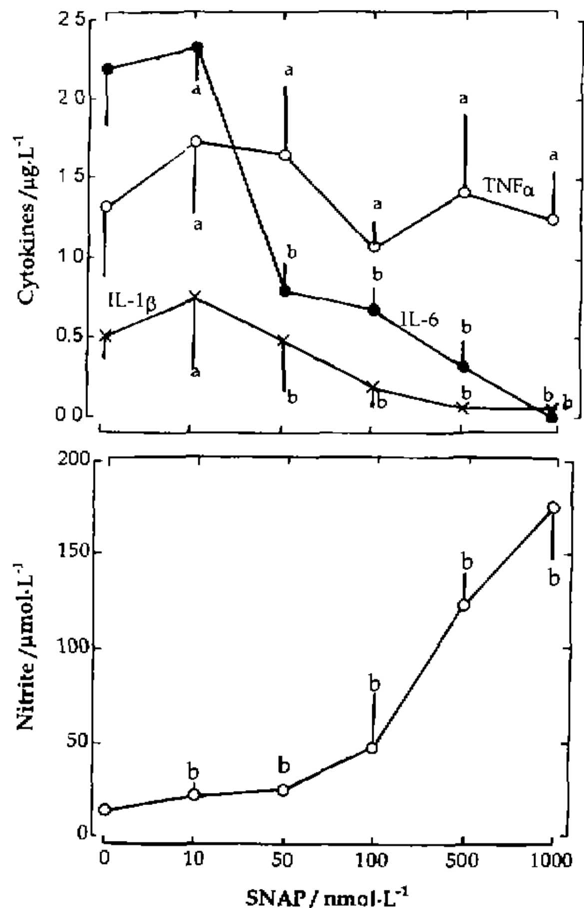

Fig 2. Effects of *S*-nitroso-*N*-acetyl-*D*, *L*-penicilamine (SNAP) on LPS-induced cytokine production. $n = 4$ mice. $\bar{x} \pm s$. $^{b}P < 0.05$ *vs* SNAP 0 nmol·L⁻¹.

LPS 10 mg·L⁻¹ stimulated AM with SNAP 50 μmol·L⁻¹. Compared with LPS control, the levels of IL-1β, IL-6, and IL-10 decreased markedly, TNFα concentration remained unchanged (Tab 2).

Effects of IL-10 on LPS-stimulated cytokine production Addition of IL-10 led to a marked decrease of TNFα, IL-1β, and IL-6 production by AM upon stimulation with LPS 10 mg·L⁻¹ at 24 h, while IL-10 antibody led to a marked increase of TNFα, IL-1β, and IL-6 production by AM (Tab 3).

DISCUSSION

The present study showed that LPS-

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Tab 3. Effects of recombinant IL-10 and IL-10 antibody on LPS-stimulated cytokine production. $n = 4$ mice. $\bar{x} \pm s$. $^bP < 0.05$ vs LPS 10 mg·L^{-1}.

Drug/ mg·L^{-1}		Cytokines levels/ng·L^{-1}		
		TNFα	IL-1β	IL-6
LPS	10	1 212 ± 110	632 ± 97	4 300 ± 698
IL-10	0.05	586 ± 49^b	100 ± 36^b	2 601 ± 460^b
Anti-IL-10	0.15	1 689 ± 364^b	1 200 ± 253^b	5 269 ± 1 127

stimulated IL-1β, and IL-6 production was increased by nitric oxide synthase inhibitor, SMT, and the effects were reversed in a concentration-dependent manner by addition of nitric oxide donor, SNAP, at the same time in AM. SMT had no effect on AM viability, indicating that SMT did not decrease viability. These observations suggested that endogenous nitric oxide might serve as an endogenous antiinflammatory mediator in LPS-stimulated AM.

Furthermore, addition of SNAP had an inhibitory effect on LPS-induced IL-1β and IL-6 release by AM, confirming that the production of IL-1β and IL-6 was attenuated by exogenous nitric oxide.

In our study, nitric oxide had no effect on LPS-induced TNFα production, that was different from other reports[7-9]. The apparent discrepancy between these reports and our results might depend on differences in experimental setting such as type of stimulus, dose of LPS, animal species, time of TNFα assay.

The mechanism of SMT-induced upregulation of IL-1β, and IL-6 production was unclear. NF-kB binding sequence (kB) is a common structural characteristics of proinflammatory cytokines (eg, IL-1β and IL-6) in the 5′-flanking region at DNA level, which plays a critical role in the regulation of many early response genes associated with the host response to infection and tissue damage[10]. Nitric oxide increased the mRNA expression of IkBα (NF-kB inhibitor) and stabilized the mRNA of IkBα[11]. IkBα inhibited NF-kB action by binding with NF-kB, and then inhibited gene expression of the proinflammatory cytokines. It is possible that inhibition of nitric oxide may upregulate proinflammatory cytokine synthesis through the upregulation of NF-kB activation.

Our results showed that IL-10 had inhibitory effects on TNFα, IL-1β, and IL-6 production by LPS-stimulated AM, while IL-10 antibody increased the proinflammatory cytokines. It suggested that IL-10 could reduce the inflammatory response in this situation and lead to the speculation that increased levels of IL-10 would be beneficial. Thus, IL-10 might be important in autoregulation of proinflammatory cytokine production.

The present study demonstrated that *in vitro* LPS-stimulated AM not only produced proinflammatory mediators (such as TNFα, IL-1β, and IL-6), but also released endogenous antiinflammatory mediators (IL-10 and nitric oxide). But the time course was different. After LPS-stimulation, TNFα, IL-1β, and IL-6 peaked at 6, 12, and 24 h, but nitrite peaked at 24 h later in our study. IL-10 peaked at 48 h by LPS-stimulated monocytes[5]. It suggested that the production of antiinflammatory mediators was later than that of proinflammatory mediators, addition of nitric oxide and IL-10 at earlier stage of inflammatory response or ARDS may be effective to control the overproduction of proinflammatory mediators.

Interestingly, the present study showed that both SNAP and SMT had inhibitory effects on LPS-induced IL-10 production. But its mechanism is less clear.

This work demonstrates that both nitric oxide and IL-10 exhibit an inhibitory effect on inflammatory cytokines in AM, nitric oxide and IL-10 added exogenously may be beneficial to

control overproduction of proinflammatory mediators.

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一氧化氮和白细胞介素-10 对小鼠肺泡巨噬细胞产生肿瘤坏死因子 α、白细胞介素-1β 和白细胞介素-6 的抑制作用

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关键词 一氧化氮; 肺泡巨噬细胞; 炎症; 白细胞介素-1; 白细胞介素-6; 白细胞介素-10; 肿瘤坏死因子; 脂多糖; 酶联免疫吸附测定

目的: 观察一氧化氮和 IL-10 对肺泡巨噬细胞炎症反应的调节作用. **方法**: 小鼠肺泡巨噬细胞(AM)受脂多糖(LPS) 10 mg·L^{-1}刺激同时, 加入一氧化氮合酶抑制剂 *S*-硫酸甲基异硫脲(SMT)或一氧化氮供体 *S*-亚硝基乙酰青霉胺(SNAP). ELISA 法测定上清液中 TNFα、IL-1β、IL-6 和 IL-10 浓度. **结果**: AM 受 LPS 刺激后, TNFα、IL-1β 和 IL-6 释放峰值分别在 6、12 和 24 小时. SMT 抑制一氧化氮释放, 但促进 IL-1β 和 IL-6 释放, 对 TNFα 无影响. SNAP 对 IL-1β 和 IL-6 释放有明显的抑制作用, 呈剂量依赖效应. 重组 IL-10 抑制 TNFα、IL-1β 和 IL-6 释放, 而 IL-10 单克隆抗体促进上述因子释放. **结论**: 内源及外源性一氧化氮和 IL-10 均对 LPS 诱导的炎症性细胞因子释放有抑制作用.

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