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Effects of tripcholorolide on inflammatory reaction of mouse alveolar macrophages *in vitro*

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KEY WORDS *Tripterygium wilfordii*; dexamethasone; interleukin-1; interleukin-6; interleukin-10; tumor necrosis factor; nitric oxide; gene expression; alveolar macrophages

ABSTRACT

AIM: To observe the effects of tripcholorolide (T4) on inflammatory reaction of mouse alveolar macrophages. **METHODS**: RT-PCR was used to investigate tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), IL-6, IL-10, and inducible nitric-oxide synthase (iNOS) mRNA expression in alveolar macrophages after LPS 10 mg/L and T4 500 μg/L treatment. ELISA was used to detect TNFα, IL-1β, IL-6, and IL-10 protein expression. Nitrite was measured by Griess reaction. **RESULTS**: TNFα, IL-1β, IL-6, IL-10, and nitrite increased in supernatants, when alveolar macrophages were stimulated by LPS 10 mg/L at 24 h. Both T4 500 μg/L and dexamethasone 100 μmol/L had inhibitory effects on the production of TNFα, IL-1β, IL-6, IL-10, and nitric oxide. The mRNA expression of TNFα, IL-6, IL-10, and iNOS increased at 5 h after LPS stimulation which was decreased on addition of T4 500 μg/L or dexamethasone 100 μmol/L. T4 had no effect on stability of LPS-induced mRNA expression in TNFα, IL-6, and IL-10. **CONCLUSION**: T4 had inhibitory effects on the expression of proinflammatory and antiinflammatory mediators.

INTRODUCTION

Activation of alveolar macrophages (AM) is a major cause of acute lung injury[1]. Both proinflammatory and antiinflammatory cytokines released by AM are involved in inflammatory response of acute lung injury. Tumor necrosis factor (TNF), interleukin-1 (IL-1), and IL-6 are the important proinflammatory mediators of the inflammatory response directly inducing neutrophil influx and capillary endothelial cell permeability[2]. IL-10, formerly known as cytokine synthesis inhibitory factor, was identified to have a regulatory role in the inflammatory response[3,4]. Several studies have suggested that nitric oxide has regulatory effects on coagulation and inflammatory response[5]. To control or regulate the inflammatory response in lung may be an effective approach to improve acute lung injury[1,2].

Tritcholorolide (T4) is a single active ingredient prepared from *Tripterygium wilfordii* Hook and it was found to have immunosuppresive and antiinflammatory actions[6]. *In vitro* experiments have revealed that T4 reduced the production of IL-6 in endothelial cells[7]. Zhang, *et al* have reported that T4 inhibited proliferation of cultured mesangial cells and IL-1 production[8]. But there are no data about the effect of T4 on LPS-induced cytokine gene expression in AM.

The current study was to investigate the effects of T4 on the gene expression of proinflammatory and antiinflammatory mediators in AM.

MATERIALS AND METHODS

Mice and alveolar macrophages Kunming mice (♂, 18-22 g) were supplied by the Experimental Animal Center of Nanjing General Hospital of PLA, Grade II, Certificate № 97001. AM were obtained by bronchoalveolar lavage, which was performed by repeated instillation of total 9 mL of Mg^{2+}- and Ca^{2+}-free Hanks' solution containing edetic acid 0.6 mmol/L. 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/L, 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

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cell concentration of 1×10^6 cells in 1 mL culture medium and cultured with lipopolysaccharides (LPS, *E coli* 0127:B8, Sigma) with or without the indicated concentrations of the T4 (T4 was generously provided by Institute of Materia Medica, Chinese Academy of Medical Sciences), or dexamethasone for 5 h or 24 h. The culture medium was collected, centrifuged ($2000 \times g$, 4 ℃, 10 min), divided in aliquots, and stored at −20 ℃ until assay. The cells were also harvested for RNA isolation.

Cytokine and nitrite 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 kits were obtained from Biosource Co, USA). Nitrite in culture supernatants was measured by a colorimetric assay based on the Griess reaction (Nitrite kit was purchased from Biotin Co, Beijing).

RNA extraction and reverse transcription After supernatants were removed, TRIZOL solution (Gibco/BRL) 1 mL was added to each well. Total cellular RNA was isolated from AM with TRIZOL (Gibco/BRL) according to the manufacturer's guidelines. For cDNA synthesis, 13.5 μL reverse transcription mixture containing 12.5 μL (1 μg) total RNA temple and 1 μL Oligo $(dT)_{18}$ primer. The mixture was incubated at 70 ℃ for 2 min, and then incubated at 0 ℃ for 2 min, according to the guidelines of advantage RT-POR PCR kit (Clontech). One μL each of dNTP 10 mmol/L, 0.5 μL recombinant RNase inhibitor, 0.5 μL MMLV reverse transcriptase, and 4 μL 5 × reaction buffer were added. The mixture was incubated at 42 ℃ for 60 min and then the reverse transcriptase was inactivated by heating the reaction mixture to 94 ℃ for 5 min.

PCR procedure Semiquantification PCR was used to observe the effects of T4 on cytokines and inducible nitric-oxide synthase (iNOS) mRNA levels. The forward primers for TNFα, IL-6, IL-10, and iNOS were 5′-GCG-ACG-TGG-AAC-TGG-CAG-AAG-3′, 5′-CCA-GTT-GCC-TTC-TTG-GGA-CTG-3′, 5′-GCT-ATG-CTG-CCT-GCT-CTT-AC-3′, and 5′-CTG-CAG-GTC-TTT-GAC-GCT-CGG-3′, respectively, and the reverse primers were 5′-GGT-ACA-ACC-CAT-CGG-CTG-GCA-3′, 5′-GGT-AGC-TAT-GGT-ACT-CCA-3′, 5′-CTG-GGG-CAT-CAC-TTC-TAC-C-3′, 5′-GTG-GAA-CAC-AGG-GGT-GAT-GCT-3′, respectively. The PCR product of TNFα, IL-6, IL-10, and iNOS were 372, 328, 274, and 741 bp. The primers were generated by the Beijing SBS Biotechnology Co. G3PDH primers were purchased from Clontech and the PCR product was 938 bp. TNFα, IL-6, IL-10, iNOS, and G3PDH PCR were carried out in the same procedure. One μL each of reverse transcription solution was added to PCR mixture containing 5 μL 10 × buffer, 3 μL $MgCl_2$, 1 μL 10 mmol/L dNTP, 1 μL each of the primers (50 pmol/L) and 0.2 μL (1 U) Taq DNA polymerase (Promega Co) in a total volume of 50 μL. PCR was performed on a thermal cycler (Perkin-Elmer, USA) with the following thermocycle parameters: a 3-min initial denaturation at 94 ℃ followed by 25 cycles of 45 s denaturation at 94 ℃, 30 − 45 s annealing at 58 − 60 ℃, 90 − 120-s extention at 72 ℃, and finally a 7-min extension at 72 ℃. Five μL PCR products were fractionated on a 2 % agarose gel and stained by ethidium bromide. The bands were visualized on a UV light box and photographed. The photograms were scanned with a laser densitometer and area-integrated in order to quantitate the relative mRNA levels.

Stability of mRNA Following incubation with LPS 10 mg/L for 5 h, actinomycin D (Sigma) 5 mg/L was added and AM were lysed at 0, 2, 4, and 8 h following the addition of actinomycin D[9]. RNA was isolated as above.

Statistical analysis Data were expressed as $\bar{x} \pm s$, and compared with paired t-test.

RESULTS

Effects of T4 on LPS-induced cytokine and nitric oxide release The levels of TNFα, IL-1β, IL-6, IL-10, and nitrite in supernatants increased significantly at 24 h after LPS 10 mg/L stimulation (Tab 1). Both T4 500 μg/L and dexamethasone 100 μmol/L inhibited the production of TNFα, IL-1β, IL-6, IL-10, and nitrite markedly. But T4 5 μg/L inhibited the production of IL-6, IL-10, and nitrite markedly. Dexamethasone 0.1 μmol/L reduced IL-10 and nitric oxide production.

Effects of T4 on LPS-induced mRNA expression At 5 h of LPS 10 mg/L stimulation, mRNA expression of TNFα, IL-6, IL-10, and iNOS in AM were increased significantly. Compared with LPS control, dexamethasone 100 μmol/L decreased the mRNA levels of TNFα, IL-6, IL-10, and iNOS markedly, T4 500 μg/L inhibited the mRNA levels of TNFα, IL-10, and iNOS significantly (Fig 1, Tab 2).

Effects of T4 on stability of mRNA Compared with LPS control, T4 had no effect on the decay of TNFα, IL-6, and IL-10 mRNA (Tab 3).

Tab 1. Effect of T4 on LPS-induced cytokines and nitric oxide production. $n=5$. $\bar{x} \pm s$. $^bP<0.05$ *vs* LPS 10 mg/L.

	TNFα/ng·L^{-1}	IL-1β/ng·L^{-1}	IL-6/ng·L^{-1}	IL-10/ng·L^{-1}	Nitrite/μmol·L^{-1}
Saline	104 ± 24^b	0^b	3.7 ± 1.7^b	70 ± 10^b	17 ± 4^b
LPS 10 mg/L	762 ± 401	489 ± 123	12.8 ± 2.1	1501 ± 139	59 ± 13
Dexamethasone					
0.1 μmol/L	498 ± 147	402 ± 116	6 ± 6	441 ± 198^b	34 ± 11^b
100 μmol/L	222 ± 170^b	151 ± 174^b	3.6 ± 1.9^b	558 ± 76^b	26 ± 4^b
T4					
5 μg/L	450 ± 214	380 ± 18	9.7 ± 0.9^b	652 ± 92^b	37 ± 7^b
500 μg/L	199 ± 32^b	14 ± 16^b	6.2 ± 0.1^b	518 ± 221^b	32 ± 8^b

Tab 2. Effect of T4 on mRNA expression of cytokines and iNOS in alveolar macrophages. The ratio of $A_{TNF\alpha}/A_{G3PDH}$, $A_{IL\text{-}6}/A_{G3PDH}$, $A_{IL\text{-}10}/A_{G3PDH}$, and A_{iNOS}/A_{G3PDH} were used to express the levels of TNFα, IL-6, IL-10, and iNOS mRNA in alveolar macrophages, respectively. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* LPS 10 mg/L.

	n	TNFα/ng·L^{-1}	IL-6	IL-10	iNOS
Normal saline	3	0.38 ± 0.07^b	0.22 ± 0.09^b	0.26 ± 0.12^c	0.30 ± 0.06^b
LPS 10 mg/L	3	2.5 ± 0.3	2.2 ± 0.6	2.1 ± 0.4	1.9 ± 0.4
Dexamethasone					
0.1 μmol/L	3	0.76 ± 0.24^b	0.8 ± 0.3^b	1.88 ± 0.23	1.42 ± 0.26
100 μmol/L	3	0.18 ± 0.05^b	0.35 ± 0.08^b	0.25 ± 0.07^b	0.34 ± 0.09^b
T4					
5 μg/L	3	2.0 ± 0.4	1.7 ± 0.5	1.14 ± 0.19^b	1.65 ± 0.22
500 μg/L	3	1.70 ± 0.08^b	1.6 ± 0.3	0.33 ± 0.11^b	0.23 ± 0.09^b

Tab 3. Effect of T4 on stability of mRNA. The rate of TNFα, IL-6, and IL-10 mRNA decay were presented as percentage and mRNA level at 0 h was 100 %.

Time/h	TNFα/%				IL-6/%				IL-10/%			
	0	2	4	8	0	2	4	8	0	2	4	8
LPS 10 mg/L	100	92	62	15	100	67	44	7	100	66	31	24
T4 500 μg/L	100	79	48	6	100	61	32	3	100	87	43	9

DISCUSSION

The present study showed that LPS-stimulated TNFα, IL-1β, and IL-6 production in AM was increased significantly, indicating that the proinflammatory cytokines may play an important effect on LPS-induced lung injury. It has been known that TNFα, IL-1β, and IL-6 expression in lung tissue increased significantly in LPS-induced lung injury, but the source of proinflammatory cytokines was not very clear[1]. The present results suggested that AM might be one of the major sources of proinflammatory cytokines in acute lung injury.

In previous studies, it was demonstrated that IL-10 and nitric oxide had inhibitory effects on LPS-induced TNFα, IL-1β, and IL-6 secretion in human AM[3,10] and mouse AM[11]. Our study showed 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). It suggested that endogenous antiinflammatory mediators might have protective effects on acute lung injury by inhibiting the overproduction of proinflammatory mediators.

As an immunosuppressant, dexamethasone may inhibit the production of several proinflammatory cytokines including TNFα, IL-1β, IL-6, and IL-8 in acute lung

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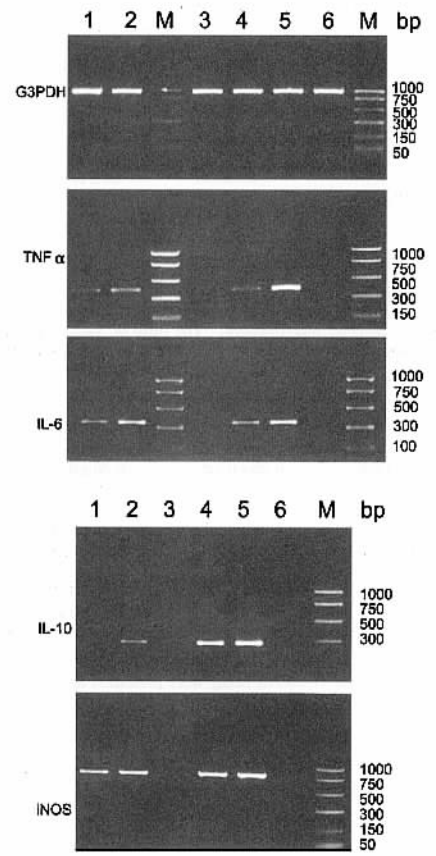

Fig 1. Expression of TNFα, IL-6, IL-10, iNOS, and G3PDH mRNA in alveolar macrophages treated with LPS for 5 h. RT-PCR was carried out for TNFα, IL-6, IL-10, iNOS, and G3PDH after 25 cycles. PCR products were run and separated by 2 % agarose gel. Lane 1. Dexamethasone 100 μmol/L; Lane 2. Dexamethasone 0.1 μmol/L; Lane 3. T4 500 μg/L; Lane 4. T4 5 μg/L; Lane 5. LPS 10 mg/L; Lane 6. Saline control; Lane M. Molecular weight marker.

injury and sepsis[1]. In our study, both LPS-induced TNFα, IL-1β, and IL-6 release and mRNA expression were inhibited by dexamethasone. The results indicated that dexamethasone had inhibitory effects on proinflammatory response in acute lung injury. Of course, dexamethasone might not simply inhibit immune responses but might enhance some immune functions. Upregulation of receptors for IL-1, IFN-γ, and TNF in various cell types by glucocorticoids has been demonstrated[12]. Our work showed that dexamethasone had inhibitory effects on IL-10 and nitric oxide expression in a dose-dependent manner. It suggested that the inhibitory effect of dexamethasone on IL-10 and nitric oxide might prevent severe immunosuppression and optimize the inflammatory response in acute lung injury.

The present experiments revealed that a Chinese traditional medicine, T4 which is an immunosuppressant, had inhibitory effects on the production of the proinflammatory cytokines in AM. Inhibitory effect on proinflammatory mediators in cultured mesangial cells[8], endothelial cells[7] by T4 have been demonstrated, but there were no data about the effect of T4 on AM. The results indicated that T4, like dexamethasone, might play an important role in regulation of proinflammatory cytokine production.

There is no report about the effect of T4 on IL-10 and nitric oxide. T4 has the same effects as dexamethasone in AM, which inhibits IL-10 and nitrite release in a dose-dependent manner. It suggests that T4 also has an inhibitory effect on antiinflammatory mediators in AM. T4 not only had an inhibitory effect on proinflammatory response, but also could suppress antiinflammatory mediators. Thus, T4 might optimize the inflammatory response in acute lung injury. Of course, the results that T4 had the same effects as dexamethasone in AM were from *in vitro* experiment. It is necessary to observe the effect of T4 in *in vivo* experiment.

Furthermore, the effect of T4 on the stability of the mRNA was observed. Actinomycin D 5 mg/L was added at 5 h after LPS stimulation. Actinomycin D at this dose has been shown to inhibit translation of mRNA[6]. Our results showed that TNFα, IL-6, and IL-10 mRNA decay had no difference between T4 and LPS. Thus T4 inhibits gene expression of TNFα, IL-6, and IL-10 at gene transcription level.

This work demonstrates that T4, like dexamethasone, has inhibitory effects on the expression of proinflammatory and antiinflammatory mediators in AM. T4 may be an effective therapeutic approach for acute lung injury.

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雷公藤氯内酯醇对小鼠肺泡巨噬细胞体外炎性反应的影响

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关键词 雷公藤；地塞米松；白介素-1；白介素-6；白介素-10；肿瘤坏死因子；一氧化氮；基因表达；肺泡巨噬细胞

目的：探讨雷公藤单体雷公藤氯内酯醇（tripcholorolide，T4）对肺泡巨噬细胞（AM）炎症反应的影响及机制．**方法**：小鼠AM受脂多糖（LPS）10 mg/L刺激的同时，加入T4 500 μg/L或地塞米松100 μmol/L；ELISA法测定上清液中TNFα、IL-1β、IL-6及IL-10浓度；RT-PCR检测上述因子及iNOS基因mRNA的表达．**结果**：AM受10 mg/L LPS刺激24小时后，上清液中TNFα、IL-1β、IL-6、IL-10及NO释放均明显增加．T4 500 μg/L及地塞米松100 μmol/L对上述介质均有不同程度的抑制作用．LPS刺激5小时后，AM中TNFα、IL-6、IL-10和iNOS的mRNA表达均明显增加．T4和地塞米松对上述介质的mRNA表达均有明显抑制作用．另外，T4对TNFα、IL-6、IL-10 mRNA的稳定性无明显影响．**结论**：T4具有抑制AM中促炎介质和抗炎介质表达的作用．

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