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Triptolide: a potent inhibitor of NF-κB in T-lymphocytes

LIU Hao, LIU Zhi-Hong, CHEN Zhao-Hong, YANG Jun-Wei, LI Lei-Shi[1]
(*Research Institute of Nephrology, Jinling Hospital, Nanjing University School of Medicine, Nanjing 210002, China*)

KEY WORDS triptolide; NF-κB; T-lymphocytes; IκBα

ABSTRACT

AIM: To study the effect of triptolide on the activity of nuclear factor-κB (NF-κB) and gene expression of IκBα, a major inhibitor of NF-κB, in human Jurkat T cell line. **METHODS**: Jurkat cells treated with or without PMA/PHA were incubated with varied doses of triptolide for different time periods. The activity of NF-κB in Jurkat cells was measured with electrophoretic mobility shift assays (EMSA), and the mRNA expression of IκBα in Jurkat cells was detected by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). **RESULTS**: PMA/PHA treatment was found to induce NF-κB activation rapidly. Triptolide treatment decreased the activity of NF-κB in both PMA/PHA treated and untreated Jurkat cells. This effect of triptolide was in a dose-dependent manner and was more obvious in cells treated with PMA/PHA. The mRNA expression of IκBα were upregulated by triptolide, while this effect was more potent in cells without PMA/PHA treatment. **CONCLUSION**: Triptolide is a potent inhibitor of NF-κB activation in T lymphocyte and this effect is partly due to the upregulation of IκBα mRNA expression.

INTRODUCTION

Tripterygium wilfordii Hook f (TWHf) has been used in traditional Chinese medicine for centuries[1]. The extract of the herb was found to have unique and potent immunosuppressive and anti-inflammatory activity. It was recently demonstrated that triptolide was one of the most potent components extracted from the TW[2]. Triptolide was able to block mitogen-induced T cell proliferation, induce apoptosis of activated T cells, and decrease the synthesis of IL-2 in T cells[2-4]. However, the molecular mechanisms of the immunosuppressive activity of triptolide are still not well understood.

Nuclear factor κB (NF-κB) is an important transcription factor regulating the transcription of quite a number of genes. Most genes known to be activated by NF-κB are involved in the immune and inflammatory responses. Their products include cytokines, chemokines, cell adhesion molecules, and immunoreceptors. In addition, the change of NF-κB activity in the immune cells plays an important role in cell activation, proliferation, and apoptosis[5,6]. Upon activation, NF-κB release from IκB, the major inhibitor of NF-κB, then translocate into the nucleus where they bind specific DNA motifs in the promoter/enhancer regions of target genes and activate transcription[7-9]. Thus, IκBα plays an important role in the regulation of NF-κB activity.

Since triptolide is a potent immunosuppressive and anti-inflammatory agent and NF-κB is a central mediator of immune responses, we hypothesize that NF-κB may be a target for triptolide-mediated immunosuppression. Therefore, the effects of triptolide on the activity of NF-κB and the mRNA expression of IκBα in human Jurkat T cells were observed in the present study.

MATERIALS AND METHODS

Reagents Purified triptolide was provided by Institute of Dermatology, Chinese Academy of Medical Sciences (Nanjing, China), and prepared as previously described[4]. Phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (USA).

Cell culture and treatments Human Jurkat T cell line was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured and treated in RPMI-1640 (Gibco, USA), supplemented with 10 % heat-inactivated fetal bovine serum (FBS), glutamine 2 mmol/L, 2-mercaptoethanol 10 mmol/L, benzylpenicillin 100 kU/L, streptomycin 100

[1] Correspondence to Prof LI Lei-Shi.
Phn 86-25-480-9112. Fax 86-25-458-1545.
E-mail LSLi1226@public1.ptt.js.cn
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kU/L. Before incubation with triptolide, Jurkat cells were divided into two groups at random: stimulated group and normal culture condition group, with or without PHA/PMA treatment respectively. In normal culture condition group, cells were incubated with triptolide 0, 2 μg/L, 10 μg/L for 12 h; while in the stimulated group, cells were incubated with PMA 25 μg/L and PHA 2 mg/L plus triptolide 0, 2 μg/L, 10 μg/L for 1 h to examine the activity of NF-κB. To examine the mRNA level of IκBα, both groups were treated with triptolide 10 μg/L for varied time. Cellular viability was measured by trypan blue exclusion before and after the treatment of triptolide as previously described.

Nuclear protein extraction At the appropriate time, Jurkat cells were collected and washed twice with cold phosphate-buffered saline (PBS), resuspended in 400 μL of cold buffer A (HEPES 10, KCl 10, edetic acid 0.1, DTT 1, PMSF 0.1 mmol/L, 0.5 % Triton X-100, 0.2 % NP-40, pH 7.9). After being incubated on ice for 15 min and vortexed vigorously, the lysate was centrifuged at 4 ℃ for 10 min. The resulting nuclear pellet was resuspended in 50 μL buffer B (HEPES 20, NaCl 420, edetic acid 0.1, $MgCl_2$ 1.5, DTT 1, PMSF 0.1 mmol/L, 25 % glycerol, pH 7.9), then incubated on ice for another 20 min and vortexed occasionally. Cell debris was pelleted by centrifugation at 4 ℃ for 15 min, the supernatant used for electrophoretic mobility shift assay (EMSA) was stored at – 80 ℃ until used. The protein content of the extracts was determined by the Bradford method.

Electrophoretic mobility shift assay (EMSA) EMSA was performed with a commercial kit (Promega, USA). Briefly, 3.5 pmol of the appropriate consensus oligonucleotide was ^{32}P end-labeled by incubation at 37 ℃ for 10 *min* with 10 U of T4 polynucleotide kinase in a reaction containing 0.37 MBq γ-^{32}P-ATP. The reaction was stopped by edetic acid. Identified quantity of nuclear protein extracts (6 – 8 μg) were equilibrated for 10 min at room temperature in a buffer containing 4 % glycerol, $MgCl_2$ 1, edetic acid 0.5, DTT 0.5, NaCl 50 mmol/L, poly (dI-dC) 0.05 g/L, Tris-HCl 10 mmol/L, pH 7.5. When a competition assay was performed, a 100-fold molar excess of the cold oligonucleotide (unlabelled probe) was added to the reaction 10 min prior to the addition of the labeled probe. Subsequently, 35 fmol of the labeled probe were added to the reaction and incubated for another 20 min at room temperature. The reactions were run on a non-denaturing, 4 % acrylamide (80:1 acrylamide to bisacrylamide) gel. The gel was dried and exposed to an X-ray film for about 11 – 14 h[10].

The following oligonucleotides were used: 1) NF-κB: 3′-TCA ACT CCC CTG AAA GGG TCC G-5′ and 5′-AGT TGA GGC GAC TTT CCC AGG C-3′; 2) SP-1: 5′-ATT CGA TCG GGG CGG GGC GAG C-3′ and 3′-TAA GCT AGC CCC GCC CCG CTC G-5′.

Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) Total RNA was isolated from Jurkat cells by the single-step method of acid guanidium thiocyanate-phenol chloroform extraction[11]. RNA samples were quantified by absorbance (A) at 260 nm and the ratio of A_{260}/A_{280} ranged between 1.8 – 2.0.

First-strand cDNA was synthesized from 2 μg of total RNA by priming with oligo (dT) using a cDNA synthesis kit (Promega, USA). The reaction mixture was incubated at 42 ℃ for 45 min and heated at 95 ℃ for 5 min to stop the reaction. The sample was then stored at – 20 ℃ before subsequent amplification. One μL cDNA was amplified with both primers for IκBα and GAPDH in the same eppendorf tube by a DNA Thermal Cycler (Model 2400; PE corp, USA) for 35 cycles. The primers for IκBα were: the upstream primer 5′-GCC TGG ACT CCA TGA AAG AC-3′, the downstream primer 5′-CAA GTG GAG TGG AGT CTG CTG CAG CTT GTT-3′, this pair of primers predicted a production of 253 bp[12]. The primers for GAPDH were: the upstream primer 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′; the downstream primer 5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′, this pair of primers predicted a production of 598 bp. PCR reaction system was 25 μL, including primers for IκBα and GAPDH 12.5 pmol each, Mg^{2+} 2.0 mmol/L, AmpliTaq Gold DNA polymerase(PE corp, USA) 0.5 unit, the reaction mixture was heated at 95 ℃ for 10 min before amplification. The amplification profile was 94 ℃ 45 s (denaturing), 56 ℃ 45 s (annealing), 72 ℃ 2 min (extension), the final cycle included a further 5 min at 72 ℃. The PCR products were quantified by 3 % agarose gel electrophoresis and photographed, and the relative densities of the bands (IκBα over GAPDH) were determined by a computerized laser densitometer.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and analyzed by t test.

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RESULTS

Effect of PMA/PHA on NF-κB activation We initially investigated the effect of PMA/PHA on the activity of NF-κB in Jurkat cells. Baseline activity of NF-κB was detected in Jurkat cells cultured in RPMI-1640/10 % FBS. In response to PMA/PHA treatment, a rapid induction of NF-κB activity was observed at 0.5 h, which became more obvious at 1 h (Fig 1).

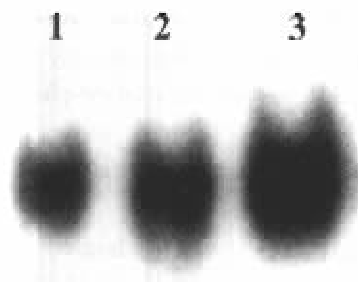

Fig 1. Time course of NF-κB activity on PMA/PHA treated Jurkat cells. Lane 1: control. Lane 2: treated with PMA 25 μg/L and PHA 2 mg/L for 30 min. Lane 3: treated with PMA 25 μg/L and PHA 2 mg/L for 60 min. Results are representatives of three independent experiments.

The specificity of the shift bands in EMSA was verified by competition assays: all the shift bands were suppressed by incubation with a 100-fold excess of unlabeled κB probe and unchanged by competition with a similar amount of another unrelevant oligonucleotide (SP1, Fig 2).

Effect of triptolide on NF-κB activation We next assessed the effect of triptolide on NF-κB activation in Jurkat cells. In the stimulated group, Jurkat cells were treated with PMA/PHA and different doses of triptolide for 1 h. The triptolide concentration (2 – 10 μg/L) was determined from our previous work showing significant effects in Jurkat cells without toxicity, which has been confirmed by trypan blue exclusion in the present study. It was found that in the presence of triptolide, the PMA/PHA-induced NF-κB activation was markedly inhibited. This inhibitory effect of triptolide was in a dose-dependent manner (Fig 3).

In the unstimulated group, Jurkat cells were only incubated with different doses of triptolide for 12 h. Similarly, the activity of NF-κB in Jurkat cells was decreased by triptolide in a dose-dependent manner too (Fig 3). As the activity of NF-κB in unstimulated cells was very

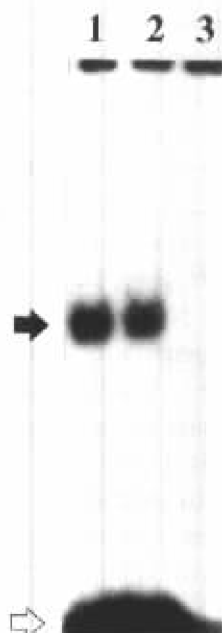

Fig 2. Results of competitive electrophoretic mobility shift assay for NF-κB activity. Lane 1: nuclear extraction from Jurkat cells. Lane 2: nuclear extraction from Jurkat cells + 100-fold molar excess of unlabeled SP1 probe. Lane 3: nuclear extraction from Jurkat cells + 100-fold molar excess of unlabeled NF-κB probe. ➡ bound probe. ⇨ free probe. Results are representatives of two independent experiments.

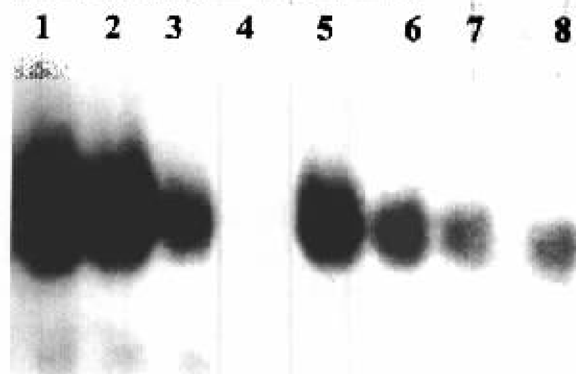

Fig 3. Effect of triptolide on the activity of NF-κB in Jurkat cells. Lane 1: treated with PMA/PHA for 1 h. Lane 2: treated with PMA/PHA in the presence of triptolide 2 μg/L for 1 h. Lane 3: treated with PMA/PHA in the presence of triptolide 10 μg/L for 1 h. Lane 4: treated with PMA/PHA and triptolide 2 μg/L + 100-fold molar excess of unlabeled NF-κB probe. Lane 5: treated with PMA/PHA and triptolide 2 μg/L + 100-fold molar excess of unlabeled SP1 probe. Lane 6: control. Lane 7: treated with triptolide 2 μg/L for 12 h. Lane 8: treated with triptolide 10 μg/L for 12 h. Results are representatives of three independent experiments.

low compared with that in stimulated cells, the inhibitory

effect of triptolide in the unstimulated group was not so obvious as that in the stimulated group.

Effect of triptolide on the mRNA expression of IκBα In the unstimulated group, the mRNA expression of IκBα in the cells treated with triptolide increased gradually at 0.5 h and peaked at 2 h (Fig 4, Fig 5).

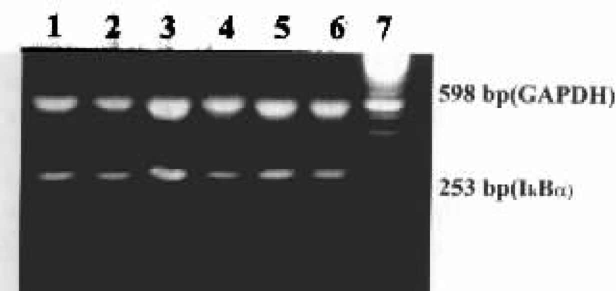

Fig 4. Results of IκBα mRNA expression in Jurkat cells detected by RT-PCR. Lane 1: control. Lane 2: treated with triptolide 10 μg/L for 30 min. Lane 3: treated with triptolide 10 μg/L for 60 min. Lane 4: treated with triptolide 10 μg/L for 120 min. Lane 5: treated with PMA/PHA for 60 min. Lane 6: treated with PMA/PHA in the presence of triptolide 10 μg/L for 60 min. Lane 7: Marker. Results are representatives of three independent experiments.

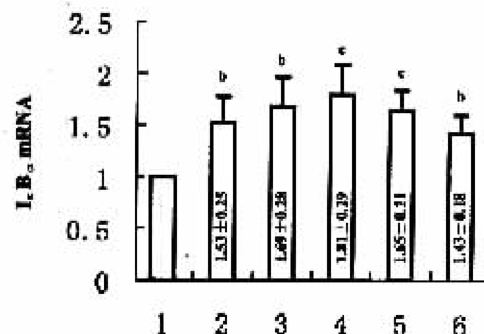

Fig 5. Effect of PMA/PHA and triptolide on the mRNA expression of IκBα in Jurkat cells. 1: Control. 2: treated with triptolide 10 μg/L for 30 min. 3: treated with triptolide 10 μg/L for 60 min. 4: treated with triptolide 10 μg/L for 120 min. 5: treated with PMA 25 μg/L and PHA 2 mg/L for 60 min. 6: treated with PMA/PHA in the presence of triptolide 10 μg/L for 60 min. $n=3$. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control.

In the stimulated group, treatment with PMA/PHA combined with or without triptolide was found to increase the mRNA levels of IκBα in Jurkat cells compared with untreated cells. This effect seemed to be more potent in the group treated with PMA/PHA only (Fig 4, Fig 5).

DISCUSSION

The fact that NF-κB is a central mediator in the immune and inflammatory response prompted us to probe whether triptolide exerted its immunosuppressive and anti-inflammatory effects through inhibiting the activity of NF-κB. To test our hypothesis, the activity of NF-κB in Jurkat cells treated with different doses of triptolide was detected by EMSA. As our previous work demonstrated that the effects of triptolide on T cells were related with the status of cell activation, the observations were made in Jurkat cells treated with or without PMA/PHA respectively.

The results of EMSA showed that: (1) A low baseline activity of NF-κB was observed in Jurkat cells cultured in RPMI-1640/10 % FBS, while treatment with PMA/PHA could induce NF-κB activation rapidly and markedly. (2) Incubation with triptolide could decrease the activity of NF-κB in both groups treated with or without PMA/PHA, and this effect was in a dose-dependent manner. The inhibitory effect of triptolide on the activity of NF-κB was more obvious in PMA/PHA treated cells. These results demonstrated that triptolide could inhibit the activity of NF-κB in T cells, and this effect was more obvious in the activated T cells induced by stimulus.

As IκBα was an important factor regulating the activity of NF-κB in most cells, we further examined IκBα gene expression in Jurkat cells treated with triptolide by semi-quantitative RT-PCR. Similar to EMSA, we set two groups for our investigation: stimulated group and unstimulated group. The present data revealed that, in unstimulated group, treatment of triptolide elevated the gene expression of IκBα in a time-dependent manner, and the effect peaked at 2 h. Interestingly, upregulation of IκBα mRNA expression was also observed in the PMA/PHA treated cells, while treatment with PMA/PHA plus triptolide could not induce a further elevation of IκBα mRNA expression compared with cells only treated with PMA/PHA or triptolide for the same time. These data suggested that triptolide could upregulate the gene transcription of IκBα in Jurkat cells and the potency of this effect correlated with the activation status of Jurkat cells. Upregulation of the gene transcription of IκBα may subsequently increase the production of IκBα proteins and the binding with NF-κB in cells, this may result in the inhibitory effect of NF-κB activity[13,14]. Although triptolide elevates the gene expression of IκBα, the upregulation of gene transcription of IκBα seems to be one of the many mechanisms mediating the inhibitory effect of triptolide on NF-κB activation. Since the inhibitory

effect of triptolide on the activity of NF-κB was more obvious in the PMA/PHA stimulated cells, the expression of IκBα gene did not change much in these stimulated cells.

Regarding the cellular signal transduction pathways, many factors are involved in the regulation of NF-κB activity, and these regulation mechanisms may be varied in the cells treated with different stimulus[15-18]. Further investigation is needed to elucidate the underlying mechanisms of triptolide on the activity of NF-κB in different cells.

REFERENCES

1 Li LS, Liu ZH. The prospect of *Tripterygium wilfordii* application in the field of nephrology. Chin J Nephrol Dialysis Transplant 1997; 6: 203 - 4.
2 Yang YL, Liu ZH, Tolosa E, Yang JW, Li LS. Triptolide induces apoptotic death of T lymphocyte. Immunopharmacology 1998; 40: 139 - 49.
3 Liao CX, Li JS. Low dose cyclosporin A and *Tripterygium wilfordii* inhibited porcine intestinal allograft rejection. Chin Med J (Engl) 1994; 107: 669 - 72.
4 Yang JW, Dai CS, Chen ZH, Liu ZH, Li LS. Triptolide inhibits T lymphocyte activation by increasing cellular level of cAMP and activity of protein kinase A. Chin J Nephrol Dialysis Transplant 1998; 7: 106 - 13.
5 Patrick AB, Thomas H. Function and activation of NF-κB in the immune system. Annu Rev Immunol 1994; 12: 141 - 79.
6 Baldwin AS Jr. The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol 1996; 14: 649 - 83.
7 Patrick AB, David B. NF-κB: ten years after. Cell 1996; 87: 13 - 20.
8 Chiao PJ, Shigeki M, Verma IM. Autoregulation of IκBα activity. Proc Natl Acad Sci USA 1994; 91: 28 - 32.
9 Kopp E, Ghosh S. Inhibition of NF-κB by sodium salicylate and asprin. Science 1994; 265: 956 - 9.
10 Andersen RD, Taplitz SJ, Oberbauer AM, Calame KL, Herschman HR. Metal-dependent binding of a nuclear factor to the rat metallothionein-1 promoter. Nucl Acids Res 1990; 18: 6049 - 55.
11 Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156 - 9.
12 Jobin C, Haskill S, Mayer L, Panja A, Sartor RB. Evidence for altered regulation of IκBα degradation in human colonic epithelial cells. J Immunol 1997; 158: 226 - 34.
13 Scheinman RI, Rosenbloom CL, Lofquist AK, Baldwin AS. Role of transcriptional activation of IκBα in mediation of immunosuppression by glucocorticoids. Science 1995; 270: 283 - 6.
14 Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis. Science 1995; 270: 286 - 90.
15 Auwardt RB, Mudge SJ, Chen CG, Power DA. Regulation of nuclear factor κB by corticosteroids in rat mesangial cells. J Am Soc Nephrol 1998; 9: 1620 - 8.
16 Christian W, Susanne L, Guido A, Roland MS. Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. J Clin Invest 1998; 101: 1163 - 74.
17 Brostjan C, Anrather J, Csizmadia V, Stroka D, Soares M, Bach FH, *et al*. Glucocorticoid-mediated repression of NF-κB activity in endothelial cells does not involve induction of IκBα synthesis. J Biol Chem 1996; 271: 19612 - 6.
18 Ardite E, Panes J, Miranda M, Salas A, Elizalde JJ, Sans M, *et al*. Effects of steroid treatment on activation of nuclear factor κB in patients with inflammatory bowel disease. Br J Pharmacol 1998; 124: 431 - 3.

雷公藤内酯：一种 T-淋巴细胞核因子-κB 的强效抑制剂

刘 浩，刘志红，陈朝红，杨俊伟，黎磊石[1]
(南京金陵医院肾脏病研究所，南京大学医学院，南京 210002，中国)

关键词 雷公藤内酯；核因子-κB；T-淋巴细胞；IκBα

目的：探讨雷公藤内酯醇对 T 淋巴细胞中核因子-κB 活力及其抑制分子 IκBα 的影响，以进一步阐明其免疫抑制效应的分子机制． **方法**：将不同浓度的雷公藤内酯醇处理普通培养状态的和同时使用 PMA/PHA 激活的 Jurkat 细胞，以凝胶迁移率实验(EMSA)检测细胞 NF-κB 活力的改变，并以逆转录-半定量 PCR 方法检测处理前后细胞中 IκBα mRNA 水平的改变． **结果**：(1)在普通培养状态下的 Jurkat 细胞中存在有一定活力的 NF-κB，使用 PMA/PHA 处理可显著升高 Jurkat 细胞中 NF-κB 的活力，雷公藤内酯醇可以降低两种培养状况下的 Jurkat 细胞中 NF-κB 活力，并以激活状态下更为显著． (2)雷公藤内酯醇可以上调 Jurkat 细胞中 IκBα mRNA 的转录水平． **结论**：雷公藤内酯醇对 T 细胞内 IκBα 基因转录的调控以及抑制 T 细胞内 NF-κB 的活力是雷公藤免疫抑制效应的分子机制之一．

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