

Triptolide inhibits vascular endothelial growth factor expression and production in endothelial cells

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HU Ke-Bin, LIU Zhi-Hong¹, GUO Xiao-Hua, LIU Dong, LI Lei-Shi (Research Institute of Nephrology, Jinling Hospital, School of Medicine, Nanjing University, Nanjing 210002, China)

KEY WORDS *Tripterygium Wilfordii*; triptolide; proteinuria; endothelial growth factors; vascular endothelium; proto-oncogene proteins c-jun

ABSTRACT

AIM: To investigate the effects of triptolide on vascular endothelial growth factor (VEGF) expression and secretion by endothelial cells, and explore the mechanism of anti-proteinuric effect of triptolide on glomerulonephritis.

METHODS: A human umbilical endothelium derived cell line (ECV-304) from American Type Culture Collection (ATCC) was used in this study. The effects of triptolide on VEGF mRNA expression, production, and secretion induced by 12-*o*-tetradecanoyl-phorbol-13-acetate (TPA) were measured by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR), flow cytometry, and enzyme linked immunosorbent assay (ELISA) respectively. The endothelial *c-fos/c-jun* mRNA expression were also detected by RT-PCR after treatment of triptolide. **RESULTS:** VEGF mRNA expression was markedly up-regulated by TPA-stimulation. In addition, the production and secretion of VEGF in endothelial cells also increased in TPA treated cells. It was founded that triptolide inhibited VEGF mRNA expression, protein production and secretion in endothelial cells induced by TPA. Interestingly, TPA-induced *c-fos/c-jun* mRNA expression in endothelial cells was also inhibited by triptolide. **CONCLUSION:** Triptolide is a potent inhibitor of VEGF expression and production in endothelial cells. The inhibitory effects of triptolide on VEGF expression and production can contribute to its anti-proteinuric effect on glomerulonephritis. Down-regulation of *c-fos/c-jun* expression in endothelial cells by triptolide is one of the mechanisms of the inhibitory effect of triptolide on VEGF expression.

INTRODUCTION

Tripterygium Wilfordii Hook f (TW) has been used in the treatment of glomerulonephritis for more than 20 a^[1]. Previous studies demonstrated that TW could markedly decrease the urinary protein excretion in both animal models and clinical practice^[2,3]. Recently it was found that triptolide was the major active component of TW extracts^[4]. Our previous works showed that triptolide could induce apoptosis of activated T cells, decrease the synthesis of IL-2, and inhibit the activity of nuclear factor κ B (NF- κ B) in T cells^[4,5]. However, the exact mechanism for TW to attenuate proteinuria has not been well understood.

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is one of the most potent permeabilizing agents. In normal kidneys, VEGF expression is mainly localized to the podocytes of the glomeruli^[6], while VEGF receptors (VEGF-R1 and VEGF-R2) are expressed predominantly on glomerular endothelial cells^[7]. Recently VEGF has been identified as an important regulator of glomerular permeability and its increased production is the major cause of proteinuria^[8-13].

Based on these, we hypothesize that VEGF may be a target for the antiproteinuric effect of triptolide. Therefore, the effects of triptolide on VEGF mRNA expression, protein production, and mRNA expression of *c-fos/c-jun* were studied in the present study.

MATERIALS AND METHODS

Drug and agent Triptolide (purity >99.9%) was provided by Institute of Dermatology, Chinese Academy of Medical Science (Nanjing, China), and was prepared as previous described^[11]. 12-*o*-Tetradecanoyl-phorbol-13-acetate (TPA) was purchased from Sigma.

Cell culture A human umbilical vein derived endothelial cell line (ECV304) from ATCC (No CRL-1998) was cultured at 37 °C and 5% CO₂ in F12 medi-

¹ Correspondence to Prof LIU Zhi-Hong. Pkn 86-25-480-9112, Fax 86-25-458-1545. E-mail Zhihong@publied. ptt. js. cn

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um (Gibco, USA) with 10 % heat-inactivated fetal bovine serum (FBS), *L*-glutamine $2 \text{ mmol} \cdot \text{L}^{-1}$, NaHCO_3 $1.5 \text{ g} \cdot \text{L}^{-1}$, benzylpenicillin $100 \text{ KU} \cdot \text{L}^{-1}$, and streptomycin $100 \text{ mg} \cdot \text{L}^{-1}$. Media were changed every 2–3 d. After deprivation of serum for 24 h, cells were treated with TPA ($0.1 \mu\text{mol} \cdot \text{L}^{-1}$), vehicle (0.002% Me_2SO), or triptolide (5 or $10 \mu\text{g} \cdot \text{L}^{-1}$).

RNA isolation and reverse transcription Total RNA was isolated from endothelial cells with TRIzol reagent (Gibco, USA) according to the manufacturer's instructions. RNA samples were quantitated by measurement of optic absorbance (*A*) at 260 nm in a spectrophotometer, and the ratio of A_{260}/A_{280} ranged between 1.8–2.0. One μg of total RNA was reverse transcribed into cDNA in a volume of $20 \mu\text{L}$ by reverse transcription kit using AMV reverse transcriptase and poly (dT) primer (Promega, USA). The mixture was incubated at $42 \text{ }^\circ\text{C}$ for 60 min and heated to $95 \text{ }^\circ\text{C}$ for 10 min to stop reaction.

PCR procedures PCR was performed on a DNA thermal cycler (Model 2400, PE, USA) using primers specific for VEGF, *c-fos*, and *c-jun*. The sense primer for VEGF was 5'-CCTGGTGGACATCTTCCAGGAGT-ACC-3', and antisense primer was 5'-GAAGCTCATCTCTCCTATGTGCTGGC-3'^[15]. This pair of primers was designed to amplify all the four isoforms of human VEGF and predicted a product of 196 bp. For *c-fos*, the sequence of sense primer was 5'-GGAGAATCCGAAGGAAAGG-3'; and the antisense primer was 5'-GCTTGGGCTCAGGGTCATTG-3'^[16]. The product amplified by *c-fos* primers was 547 bp. For *c-jun*, the sense primer was 5'-GGAAACGACCTTCTATGACGATGCCCTCAA-3'; antisense primer was 5'-GAACCCCTCCTGCTCATCTGTACAGTTCTT-3', and predicted a product of 325 bp^[17]. To ensure that reverse transcriptase efficiencies and RNA input were comparable between test groups, the primers of VEGF, *c-fos*, or *c-jun* were added each with β -actin primers into the same eppendorf tube for amplification. The β -actin product size was 250 bp. Two μL each of reverse transcription solution was added to PCR mixture for amplification by Taq DNA polymerase (Promega, USA) in a volume of $50 \mu\text{L}$. The amplification profile of VEGF consisted of denaturation at $95 \text{ }^\circ\text{C}$ for 1 min, annealing at $60 \text{ }^\circ\text{C}$ for 1 min, and extension at $72 \text{ }^\circ\text{C}$ for 1 min for 35 cycles. For *c-fos/c-jun*, the profile was at $94 \text{ }^\circ\text{C}$ for 1.5 min, at $63 \text{ }^\circ\text{C}$ for 1 min, and at $72 \text{ }^\circ\text{C}$ for 1.5 min for 35 cycles. The products amplified were subjected to agarose gel elec-

trophoresis, and bands were visualized by staining with ethidium bromide. The density of each band was measured by densitometer. The ratio of VEGF, *c-fos*, and *c-jun* mRNA to β -actin mRNA were used to express the level of VEGF, *c-fos*, and *c-jun* mRNA abundance respectively.

Fluorescence flow cytometry (FCM) The intracellular VEGF protein expression was assayed by FCM as previous described with some modification^[18]. Cells from all experimental conditions were harvested with 0.25 % trypsin and 0.02 % edetic acid (1:1), then subjected to FCM assay. In brief, after fixed with 1 % formaldehyde in PBS for 15 min at room temperature, about 1×10^6 cells were permeabilized by incubation with 0.1 % saponin for 15 min at room temperature, and incubated with mouse anti-human VEGF monoclonal antibody (mAb) (1:100, Pharmingen) at $4 \text{ }^\circ\text{C}$ for 30 min. Then, cells were washed three times with PBS/0.1 % BSA and incubated in the dark at $4 \text{ }^\circ\text{C}$ for 30 min with a rabbit anti-mouse Ig fluorescein conjugate diluted 1:200 (DAKO). After the further three times wash, cells were analyzed for fluorescence by flow cytometer (EpicsXL, Coulter, USA). Each sample had a tube of cells serve as negative control, which underwent the same procedure mentioned above except that VEGF mAb was replaced by PBS/0.1 % BSA.

Enzyme linked immunosorbent assay (ELISA) Culture supernatants from all experimental conditions were collected, supplemented with 1 % FBS, centrifugated to remove cell debris, and stored at $-70 \text{ }^\circ\text{C}$ for analysis. The VEGF protein concentration was measured with a commercial available VEGF ELISA kit (R&D, USA) according to the manufacturer's guidelines. All protein results were adjusted for cell number.

Cell number determination Cells were harvested with 0.25 % trypsin and 0.02 % edetic acid (1:1) and the cell number was determined by a hemocytometer.

Statistical analysis All data were presented as $\bar{x} \pm s$ and assessed by *t*-test.

RESULTS

VEGF mRNA expression induced by TPA

After deprivation of serum for 24 h, cells were incubated with either TPA ($0.1 \mu\text{mol} \cdot \text{L}^{-1}$) or vehicle (0.002% Me_2SO) for 1, 2, 4, 8, and 12 h. The concentration of TPA was established in preliminary experiments. VEGF

mRNA level was increased time-dependently (Fig 1 and Tab 1). TPA could rapidly induce VEGF mRNA expression since the rise of VEGF mRNA was clearly evident within 2 h of incubation with TPA and reached the maximum by 12 h incubation.

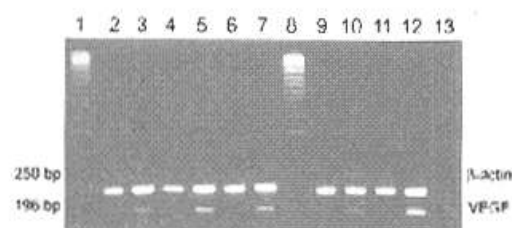


Fig 1. Time course of VEGF mRNA expression induced by TPA in endothelial cells detected by RT-PCR. Lane 1, 8: Marker; Lane 2, 4, 6, 9, 11: treated with vehicle for 1, 2, 4, 8, 12 h; Lane 3, 5, 7, 10, 12: treated with TPA $0.1 \mu\text{mol}\cdot\text{L}^{-1}$ for 1, 2, 4, 8, 12 h; Lane 13: negative control. Results were representative of three independent experiments.

Tab 1. VEGF mRNA expression in endothelial cells after incubation with TPA or vehicle for 1, 2, 4, 8, or 12 h. $n=3$ (triplicate). $\bar{x} \pm s$. $^*P < 0.01$ vs control.

	VEGF mRNA				
	1 h	2 h	4 h	8 h	12 h
TPA ($0.1 \mu\text{mol}\cdot\text{L}^{-1}$)	0.31 ± 0.05	$0.34 \pm 0.08^*$	$0.33 \pm 0.06^*$	$0.325 \pm 0.018^*$	$0.322 \pm 0.011^*$
Control	0.25 ± 0.11	0.12 ± 0.10	0.12 ± 0.11	0.063 ± 0.014	0.074 ± 0.017

VEGF mRNA level was detected by RT-PCR and expressed as the ratio of VEGF mRNA to β -actin mRNA.

Tab 2. VEGF production in endothelial cells after treated with TPA. $n=3$ (triplicate). $\bar{x} \pm s$. $^*P < 0.05$, $^*P < 0.01$ vs control.

	Control	TPA ($0.1 \mu\text{mol}\cdot\text{L}^{-1}$)		
		8 h	12 h	24 h
VEGF production (MFI)	15.4 ± 1.5	21.0 ± 0.6^b	$24.3 \pm 0.8^*$	18.8 ± 0.6^b

VEGF production was detected by FCM and expressed as mean fluorescence intensity (MFI).

Tab 3. VEGF secretion in endothelial cells after treated with TPA by ELISA. $n=3$ (triplicate). $\bar{x} \pm s$. $^*P < 0.01$ vs control.

	VEGF secretion ($10^{-1} \text{ng}\cdot\text{L}^{-1}\cdot\text{cell}^{-1}$)			
	8 h	12 h	18 h	24 h
TPA ($0.1 \mu\text{mol}\cdot\text{L}^{-1}$)	$12.8 \pm 1.4^*$	$14.7 \pm 1.5^*$	$30.3 \pm 1.5^*$	$22.7 \pm 1.2^*$
Control	4.9 ± 0.4	6.5 ± 0.4	7.8 ± 0.3	9.5 ± 0.4

Intracellular VEGF production induced by TPA There was a certain level of intracellular VEGF production in control cells. When cells were exposed to TPA ($0.1 \mu\text{mol}\cdot\text{L}^{-1}$) for 8 h, intracellular VEGF production was markedly increased and reached the maximum by 12 h stimulation (Tab 2).

VEGF protein secretion induced by TPA ELISA assay showed that TPA $0.1 \mu\text{mol}\cdot\text{L}^{-1}$ clearly stimulated VEGF protein secretion within 8 h, and VEGF protein secretion reached the maximum by 18 h, then gradually decreased (Tab 3).

Effect of triptolide on VEGF mRNA expression induced by TPA Cells were cultured in serum-deprived media for 24 h, then exposed to vehicle, TPA $0.1 \mu\text{mol}\cdot\text{L}^{-1}$, or TPA $0.1 \mu\text{mol}\cdot\text{L}^{-1}$ with triptolide 5 or $10 \mu\text{g}\cdot\text{L}^{-1}$ for 12 h. It was found that triptolide inhibited TPA-induced VEGF mRNA expression in a dose-dependent manner (Fig 2 and Tab 4).

Effect of triptolide on intracellular VEGF production induced by TPA Serum-deprived cells were exposed to vehicle, TPA $0.1 \mu\text{mol}\cdot\text{L}^{-1}$, or TPA

Tab 4. Effect of triptolide on VEGF mRNA expression, protein production, and secretion in endothelial cells. $n = 3$ (triplicate). $\bar{x} \pm s$. $^*P < 0.01$ vs control. $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ vs TPA.

	Control	TPA(0.1 $\mu\text{mol}\cdot\text{L}^{-1}$)	Triptolide(5 $\mu\text{g}\cdot\text{L}^{-1}$)	Triptolide(10 $\mu\text{g}\cdot\text{L}^{-1}$)
VEGF mRNA	0.26 \pm 0.03	0.64 \pm 0.01 [*]	0.530 \pm 0.016 [†]	0.169 \pm 0.010 [‡]
VEGF production (MFI)	14.8 \pm 0.6	24.7 \pm 0.3 [*]	20.4 \pm 0.3 [†]	17.5 \pm 0.7 [‡]
VEGF secretion ($\times 10^{-4}$ ng $\cdot\text{L}^{-1}\cdot\text{cell}^{-1}$)	6.3 \pm 0.4	22.4 \pm 0.6 [*]	10.6 \pm 0.6 [†]	8.9 \pm 0.5 [‡]

VEGF mRNA level and intracellular VEGF production was expressed as the ratio of VEGF mRNA to β actin mRNA and mean fluorescence intensity (MFI) respectively. VEGF mRNA level, intracellular VEGF protein production, and VEGF secretion was obtained by RT-PCR, FCM, and ELISA respectively.

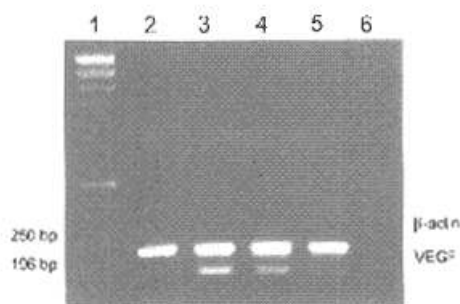


Fig 2. Effect of triptolide on VEGF mRNA expression in endothelial cells detected by RT-PCR. Lane 1: Marker; Lane 2: control; Lane 3: TPA 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ group; Lane 4: TPA 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ + triptolide 5 $\mu\text{g}\cdot\text{L}^{-1}$ group; Lane 5: TPA 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ + triptolide 10 $\mu\text{g}\cdot\text{L}^{-1}$ group; Lane 6: negative control. Results were representative of three independent experiments.

0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ with triptolide 5 or 10 $\mu\text{g}\cdot\text{L}^{-1}$ for 12 h. FCM analysis showed that triptolide inhibited TPA induced intracellular VEGF production dose-dependently (Tab 4).

Effect of triptolide on VEGF protein secretion induced by TPA After serum deprivation of 24 h, cells were exposed to vehicle, TPA 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$, or TPA 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ with triptolide 5 or 10 $\mu\text{g}\cdot\text{L}^{-1}$ for 18 h. Triptolide dose-dependently depressed TPA-induced VEGF protein secretion (Tab 4).

Effect of triptolide on *c-fos* mRNA and *c-jun* mRNA expression induced by TPA Serum deprived cells were exposed to vehicle, TPA 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ or TPA 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ with triptolide 5 or 10 $\mu\text{g}\cdot\text{L}^{-1}$ for 12 h. TPA markedly increased *c-fos* mRNA and *c-jun* mRNA expression, while triptolide dose-dependently inhibited the effects of TPA (Fig 3 and Tab 5).

DISCUSSION

The fact that VEGF is the major cause of proteinuria

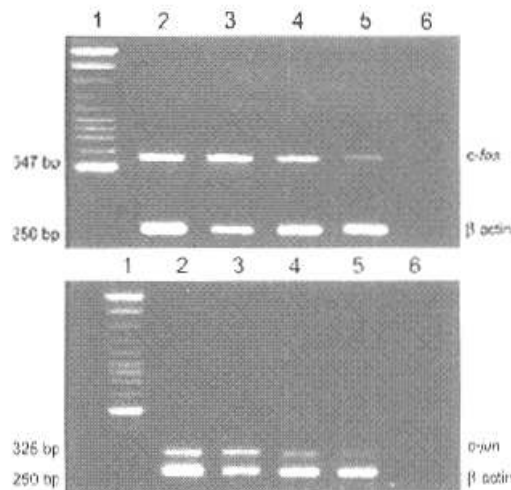


Fig 3. Effect of triptolide on *c-fos* and *c-jun* mRNA expression in endothelial cells detected by RT-PCR. Lane 1: Marker; Lane 2: control; Lane 3: TPA 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ group; Lane 4: TPA 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ + triptolide 5 $\mu\text{g}\cdot\text{L}^{-1}$ group; Lane 5: TPA 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$ + triptolide 10 $\mu\text{g}\cdot\text{L}^{-1}$ group; Lane 6: negative control. Results were representative of three independent experiments.

prompts us to explore whether the antiproteinuric effect of TW could be attributed to its inhibitory effects on the expression and production of VEGF.

To address our hypothesis, firstly we observed the time response of VEGF mRNA expression and protein production induced by TPA. TPA 0.1 $\mu\text{mol}\cdot\text{L}^{-1}$, the optimal concentration determined in preliminary experiments, markedly increased VEGF mRNA expression after 2 h incubation. This effect lasted for at least 12 h and reached the maximum by 12 h. Flow cytometry analysis showed that the intracellular VEGF protein production induced by TPA reached the maximum by 12 h. ELISA data demonstrated that the secreted VEGF protein level

Tab 5. Effect of triptolide on *c-fos* and *c-jun* mRNA expression in endothelial cells by RT-PCR. *n* = 3 (triplicate), *x* ± *s*. **P* < 0.01 vs control. ^a*P* < 0.05, ^b*P* < 0.01 vs TPA.

	Control	TPA (0.1 μmol·L ⁻¹)	Triptolide (5 μg·L ⁻¹)	Triptolide (10 μg·L ⁻¹)
<i>c-fos</i> mRNA	0.40 ± 0.09	0.61 ± 0.07 ^a	0.42 ± 0.05 ^b	0.38 ± 0.06 ^b
<i>c-jun</i> mRNA	0.59 ± 0.04	0.80 ± 0.03 ^a	0.66 ± 0.06 ^b	0.60 ± 0.03 ^b

Results were expressed as the ratio of *c-fos* and *c-jun* mRNA to β-actin mRNA.

reached the maximum by 18 h. Thus TPA not only induced VEGF mRNA expression, but also increased VEGF protein production.

In order to observe the effects of triptolide on VEGF mRNA expression and protein production, the mRNA expression of VEGF and its production were observed in endothelial cells at 12 h and 18 h respectively after the treatment of triptolide. It was found that triptolide markedly depressed the VEGF mRNA expression and protein production induced by TPA dose-dependently. This effect of triptolide was in absence of toxicity of the drug, which was confirmed by trypan blue exclusion test in the present study. These results indicated that the inhibitory effect of triptolide on VEGF expression and production might contribute to its antiproteinuric effect on glomerulonephritis.

To explore the mechanisms by which triptolide inhibited VEGF expression, the effect of triptolide on *c-fos/c-jun* expression in endothelial cells has also been observed. TPA activates protein kinase C (PKC) and ultimately induces the transcription of *c-fos* and *c-jun* genes which comprise the activator protein-1 (AP-1) transcription factor^[11]. It has been known that AP-1 is a promoter of the transcription of VEGF mRNA^[20]. Previous study localized the inhibitory effect of T2, an extract of TW, on mitogen-induced cytokine gene transcription to a step between PKC activation and gene transcription^[21]. So we observed the effect of triptolide on *c-fos/c-jun* expression. Interestingly, it was found that the up-regulation of *c-fos/c-jun* mRNA expression induced by TPA could be eliminated after the treatment of triptolide. These results indicated that triptolide could interfere with AP-1 formation by depressing *c-fos/c-jun* mRNA expression then to exert its inhibitory effect on VEGF mRNA expression. Since the regulation of VEGF expression is extremely complicated, further investigation is urged to elucidate the underlying mechanism of triptolide affecting VEGF expression and production.

VEGF is not only a potent vascular permeabilizing agent, but also a pro-angiogenic factor and plays a pivotal

role in angiogenesis both *in vivo* and *in vitro*. Rheumatoid arthritis, diabetic retinopathy, and psoriasis, as well as tumor growth, all involve in angiogenesis and therefore collectively have been called "angiogenesis diseases."^[22] It is evident that VEGF plays an important role in the pathogenesis of these "angiogenesis diseases."^[23] Our results of present study demonstrated that triptolide could be used as an anti-angiogenic agent in treating these diseases.

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雷公藤内酯抑制内皮细胞血管内皮生长因子表达与合成

胡可斌, 刘志红¹, 郭啸华, 刘 栋, 黎磊石
(南京大学医学院金陵医院肾脏病研究所, 南京 210002, 中国)

关键词 雷公藤; 雷公藤内酯; 蛋白尿; 内皮生长因子; 血管内皮; 原癌基因蛋白质 c-jun 类

目的: 研究雷公藤内酯对血管内皮细胞生长因子(VEGF) mRNA 表达及 VEGF 合成与分泌的影响, 进一步探讨雷公藤内酯降低肾小球肾炎患者尿蛋白的作用机制。 **方法:** 以人内皮细胞系 ECV-304 为研究对象, 利用半定量逆转录聚合酶链反应(RT-PCR), 流式细胞仪, 酶联免疫吸附法(ELISA)检测不同剂量雷公藤内酯对佛波脂(TPA)诱导的内皮细胞 VEGF mRNA 表达及 VEGF 合成与分泌的影响, 用 RT-PCR 检测雷公藤内酯对内皮细胞 *c-fos/c-jun* mRNA 表达的影响。 **结果:** TPA 能够明显上调 VEGF mRNA 表达, 蛋白合成与分泌, 而雷公藤内酯可以抑制 TPA 诱导的内皮细胞 VEGF mRNA 表达及 VEGF 蛋白合成与分泌, 该作用在 $10 \mu\text{g}\cdot\text{L}^{-1}$ 时更为明显。同样, 雷公藤内酯剂量依赖性地抑制 TPA 诱导的内皮细胞 *c-fos/c-jun* mRNA 的表达。 **结论:** 雷公藤内酯通过影响 *c-fos/c-jun* 基因转录而抑制内皮细胞 VEGF mRNA 表达及 VEGF 合成与分泌是雷公藤内酯降低肾小球肾炎患者尿蛋白的作用机制之一。

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