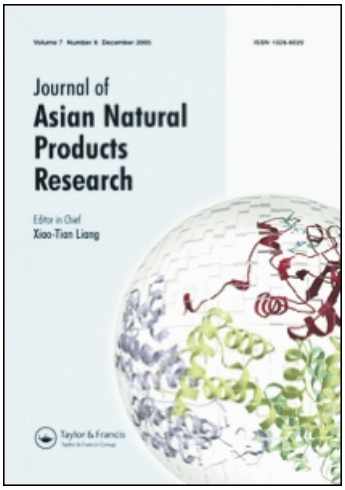

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Suppression of IL-8 gene transcription by resveratrol in phorbol ester treated human monocytic cells

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SUPPRESSION OF IL-8 GENE TRANSCRIPTION BY RESVERATROL IN PHORBOL ESTER TREATED HUMAN MONOCYTIC CELLS

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Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), a natural phytoalexin found in grapes and other food products, has promising anti-inflammatory and anticancer effects. To observe the modulation of interleukin-8 (IL-8) production in human monocytic cells by resveratrol and explore its mechanism at the gene transcription level, U937 cells were stimulated with phorbol 12-myristate 13-acetate (PMA) for 24 h. IL-8 protein in supernatants was measured by radioimmunoassay. The cytotoxicity of PMA, dexamethasone and resveratrol was accessed by MTT cell proliferation assay. The RNA level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and IL-8 were detected by RT-PCR using specific primers. DNA binding activities of NF-κB and AP-1 were examined by electrophoretic mobility shift assay (EMSA). 0.01–100 nM PMA could significantly induce IL-8 production in U937 cells; 10 μM Dexamethasone and 10, 1, 0.1 μM resveratrol could inhibit PMA-induced IL-8 protein production and mRNA accumulation. The cytotoxicity did not contribute to their inhibitory effect. The DNA binding activity of AP-1 was inhibited by dexamethasone and resveratrol, but resveratrol has little effect on PMA-induced NF-κB activation. Resveratrol could inhibit PMA-induced IL-8 production in U937 cells at protein and mRNA levels. The suppression of IL-8 gene transcription by resveratrol was, at least partly, due to inhibition of AP-1 activation.

Keywords: IL-8; U937; Resveratrol; NF-κB; AP-1

INTRODUCTION

There is a great deal of evidence that resveratrol (3,4′,5-trihydroxy-*trans*-stilbene, Fig. 1), a natural phytoalexin found in grapes and other food products, has promising anti-inflammatory and anticancer effects. These findings are consistent with epidemiological studies that defined the so-called "French paradox" [1]. Recently, resveratrol has also been shown to possess chemopreventive activity by inhibiting cellular events associated with tumor initiation, promotion, and progression [2]. However, the precise mechanisms of its anti-inflammatory, antitumorigenic or chemopreventive activities remain largely unknown.

Interleukin-8 (IL-8) is a member of the CXC chemokine family and plays an important role during inflammation. It is induced by many proinflammatory cytokines, *i.e.*, tumor

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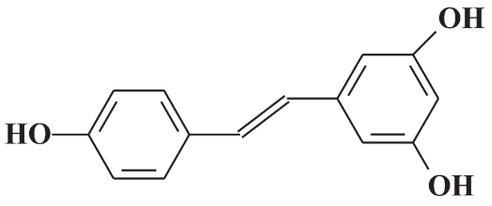

FIGURE 1 Structure of resveratrol.

necrosis factor (TNF-α), IL-1, IL-2, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) and produced by a variety of cells including monocytes, neutrophils, fibroblasts, endothelial cells, lung epithelial cells, mast cells and keratinocytes [3]. As a potent leukocyte activating cytokine, IL-8 is chemotactic for neutrophils and induces the release of metalloproteinases, shedding of L-selectin, the upregulation of the β2-integrin, leukocyte function-associated antigen-1 (LFA-1) and transendothelial migration of neutrophils [4]. Overproduction of IL-8 happens in many inflammations and autoimmune diseases [5]. Activation of NF-κB is the most crucial step for IL-8 gene transcription in most cells, but NF-IL-6 and AP-1 binding sites are also required for IL-8 transcription [6]. Tacrolimus (FK-506), cyclosporin A [7] and glucocorticoid [8] are known to inhibit the gene transcription as well as the production of IL-8 at the step of the activation of transcription factors. In this study, we tried to observe the modulation of IL-8 production in human monocytic cells by resveratrol and explore its mechanism at the gene transcription level.

RESULTS AND DISCUSSION

Induction of IL-8 Biosynthesis by PMA

The IL-8 protein in supernatants of U937 cells was greatly induced by PMA after 24 h (Table I). The maximum induction was 24.3-fold in a 100 nM PMA group *vs.* a control group. The induction of IL-8 biosynthesis by 10% fetal bovine serum was also observed after serum starvation (data not shown). Thus serum were omitted in all treatments in order to eliminate this influence.

Inhibition of IL-8 Biosynthesis by Dexamethasone and Resveratrol

10 μM Dexamethasone could inhibit IL-8 production in 0.01 μM PMA treated U937 cells with an inhibitory ratio of 82.14%. Resveratrol at concentrations of 10, 1 and 0.1 μM also

TABLE I Induction of IL-8 biosynthesis in U937 cells treated with different concentration of PMA for 24 h; $n = 6$, $\bar{x} \pm SD$

	Concentration (nM)	*IL-8 protein ($\mu g\,L^{-1}$)*	*Fold of induction*
Control		0.59 ± 0.20	
PMA	100	14.36 ± 2.74*	24.3
	10	12.69 ± 2.35*	21.5
	1	7.70 ± 1.79*	13.1
	0.1	6.06 ± 1.99*	10.3
	0.01	4.85 ± 1.12*	8.2

* $P < 0.01$ *vs.* control group.

TABLE II Inhibition of IL-8 biosynthesis by dexamethasone and resveratrol in U937 cells treated with 0.01 μM PMA for 24 h; $n = 6$, $\bar{x} \pm SD$

	Concentration (μM)	*IL-8 protein (μg L^{-1})*	*Inhibitory ratio (%)*
Control		0.30 ± 0.13	
PMA	0.01	11.19 ± 1.72*	
Dexamethasone+10 nM PMA	10	2.24 ± 0.24*,†	82.14
Resveratrol+10 nM PMA	10	5.36 ± 0.82*,†	53.54
	1	7.00 ± 1.23*,†	38.46
	0.1	9.05 ± 1.22*,‡	19.67

* $P < 0.01$ vs. control group.
† $P < 0.01$ *vs.* PMA group.
‡ $P > 0.05$ *vs.* PMA group.

exhibits an inhibitory effect on IL-8 biosynthesis (Table II). The maximum inhibitory ratio of resveratrol was 53.54% ($P < 0.01$ *vs.* PMA group).

Toxicity of Dexamethasone and Resveratrol

0.01 μM PMA alone could inhibit U937 cell proliferation (Table III, $P < 0.01$ *vs.* control group), while dexamethasone or resveratrol alone showed no toxicity to U937 cells (data not shown). Dexamethasone or resveratrol combined with 0.01 μM PMA showed no additional toxicity to U937 cells *vs.* PMA group (Table III). The cytotoxicity did not contribute to the inhibition of IL-8 biosynthesis.

Inhibition of IL-8 mRNA Transcription by Dexamethasone and Resveratrol

Semi-quantitative RT-PCR was performed to observe the relative difference in IL-8 mRNA level. IL-8 mRNA accumulation can be observed after 24 h in 0.01 μM PMA-treated U937 cells. 10 μM Dexamethasone could inhibit PMA-induced IL-8 mRNA transcription significantly (Fig. 2). Resveratrol showed a lower inhibition of IL-8 mRNA transcription compared to dexamethasone (Fig. 2).

Modulation of NF-κB and AP-1 Activity by Dexamethasone and Resveratrol

There was a basal NF-κB activity in U937 cells, especially for the low molecular-weight complex. Two bands of NF-κB specific DNA binding could be seen in 0.01 μM PMA-treated U937 cells. The high molecular-weight complex of NF-κB was induced by PMA

TABLE III Toxicity of dexamethasone or resveratrol to U937 cells treated with 0.01 μM PMA for 24 h; $n = 6$, $\bar{x} \pm SD$

	Concentration (μM)	*OD_{570}*
Control		0.970 ± 0.017
PMA	0.01	0.717 ± 0.018*
Dexamethasone+10 nM PMA	10	0.742 ± 0.016*,†
Resveratrol+10 nM PMA	10	0.741 ± 0.022*,†
	1	0.749 ± 0.022*,†
	0.1	0.726 ± 0.029*,†

* $P < 0.01$ *vs.* control group (Me_2SO).
† $P > 0.05$ *vs.* PMA group.

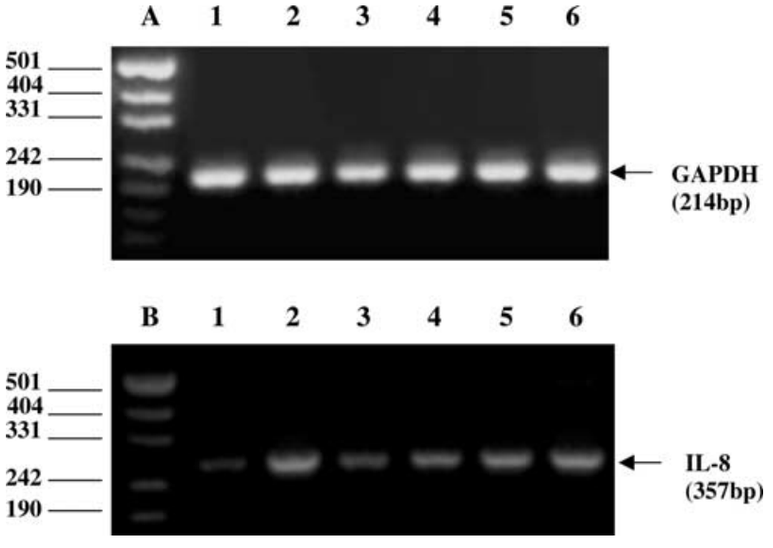

FIGURE 2 Inhibition of IL-8 mRNA transcription by dexamethasone and resveratrol. (A) GAPDH: lane 1, control; lane 2, PMA; lane 3, PMA + 10^{-5} M dexamethasone; lane 4, PMA + 10^{-5} M resveratrol; lane 5, PMA + 10^{-6} M resveratrol; lane 6, PMA + 10^{-7} M resveratrol. (B) IL-8: lane 1, control; lane 2, PMA; lane 3, PMA + 10^{-5} M dexamethasone; lane 4, PMA + 10^{-5} M resveratrol; lane 5, PMA + 10^{-6} M resveratrol; lane 6, PMA + 10^{-7} M resveratrol.

dramatically. PMA-induced NF-κB activation could be inhibited by 10 μM dexamethasone, while resveratrol had little effect.

Induction of AP-1-specific DNA binding activities was also observed in PMA-treated U937 cells. Dexamethasone and resveratrol, at different concentrations, could both inhibit PMA-induced AP-1 activity (Fig. 3).

DISCUSSION

Recently, resveratrol has been found to act as an antioxidant, antimutagen, anti-inflammatory and promising chemopreventive agent [9]. It has been shown to inhibit cyclooxygenase and hydroperoxidase [10], suppress the expression of inducible nitric oxide synthase [11] and cyclooxygenase-2 [12]. It can induce human promyelocytic leukemia cell differentiation [13], inhibit the development of preneoplastic lesions in carcinogen-treated mouse mammary glands in culture and tumorigenesis in a mouse skin cancer model [14]. Our previous study showed that resveratrol could inhibit IL-6 biosynthesis induced by calcimycin and fMLP in mouse peritoneal macrophages [15]. Holmes-McNary's data [16] also show that resveratrol is a potent inhibitor of NF-κB nuclear translocation and IκB-α degradation induced by TNF-α or lipopolysaccharide. Furthermore, these effects are mediated through the inhibition of IκB kinase (IKK), the key regulatory complex required for NF-κB activation of gene transcription. In this study, resveratrol exhibited inhibition of IL-8 mRNA and protein biosynthesis in human monocytic cells induced by PMA. The induction of AP-1 DNA binding activities by PMA could be inhibited by resveratrol, while that of NF-κB was not significantly influenced even at a concentration of 10 μM. The modulation of IL-8 gene transcription by resveratrol was, at least partly, due to inhibition of AP-1 activation.

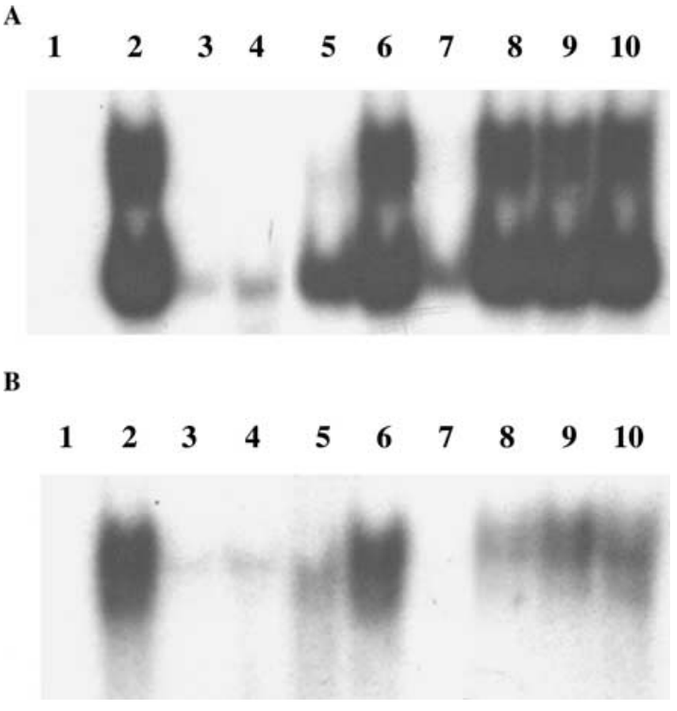

FIGURE 3 Modulation of NF-κB and AP-1 activity by dexamethasone and resveratrol. (A) NF-κB: lane 1, no probe; lane 2, NF-κB probe; lane 3, NF-κB probe + competitive none ^{32}P-labeled probe; lane 4, NF-κB mut probe; lane 5, control; lane 6, PMA; lane 7, PMA + 10^{-5} M dexamethasone; lane 8, PMA + 10^{-5} M resveratrol; lane 9, PMA + 10^{-6} M resveratrol; lane 10, PMA + 10^{-7} M resveratrol. (B) AP-1: lane 1, no probe; lane 2, AP-1 probe; lane 3, AP-1 probe + competitive none ^{32}P-labeled probe; lane 4, AP-1 mut probe; lane 5, control; lane 6, PMA; lane 7, PMA + 10^{-5} M dexamethasone; lane 8, PMA + 10^{-5} M resveratrol; lane 9, PMA + 10^{-6} M resveratrol; lane 10, PMA + 10^{-7} M resveratrol.

These differences could be interpreted as the result of different stimulation and the lower concentration of resveratrol. TNF-α or lipopolysaccharide can induce a rapid activation of NF-κB in approximately 30 min, while PMA treatment will cause a late-phase accumulative activation in 24 h. We also need to mention that the concentration of resveratrol used in our study was ten to fifty times lower than that other used in other studies. A tentative explanation of this contradiction could be that resveratrol, at a lower concentration, could interact with a regulatory factor other than IKK in PMA-treated human monocytic cells. This hypothesis, if proven, could explain the different modulation of NF-κB and AP-1 transcriptional activity by resveratrol and broaden our knowledge of the molecular targets of resveratrol.

EXPERIMENTAL

Chemical and Reagents

Resveratrol, isolated from *Gnetum montanum*, *G. pendulum*, *G. parvifolium*, *G. hainanse* or *G. montanum f. megalocarpum*, was a kind gift from Professor Lin Mao, Institute of Materia

Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, 100050, China.

Dexamethasone, phorbol 12-myristate 13-acetate (PMA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma. M-MLV reverse transcriptase, and Trizol reagent were from GIBCO-BRL. Random hexamers were from Promega. dNTPs mixture and TaKaRa Tag were from TaKaRa. The IL-8 radioimmunoassay kit was from the 301 Hospital of PLA.

Cell Culture and Drug Treatment

Human monocytic cells (U937) were obtained from the Cell Center, Chinese Academy of Medical Sciences & Peking Union Medical College. Cells were maintained in RPMI-1640 supplemented with 10% (v/v) fetal calf serum, $100\,\mathrm{u\,ml^{-1}}$ penicillin and $100\,\mathrm{mg\,ml^{-1}}$ streptomycin. Before each experiment, cells were harvested, washed by D-Hanks' balanced salt solution and then seeded in a 10 cm cell culture dish (Costar) at a cell density of $4 \times 10^5\,\mathrm{cell\,ml^{-1}}$ in RPMI-1640 without serum for 10 h. U937 cells were incubated with a test compound at different concentrations or solvent (Me_2SO) for 1 h and were stimulated with PMA for 24 h. All incubation procedures were performed with 5% CO_2 in humidified air at 37°C.

IL-8 Radioimmunoassay

The IL-8 protein in supernatants was measured with an IL-8 radioimmunoassay kit according to the manufacturer's guide. The inhibitory ratio was calculated as $\mathrm{IR}\% = 100[(C_s - C_t)/(C_s - C_c)]$ where C_s, C_t, and C_c refer to the IL-8 concentration in supernatants of PMA, test compound, and control groups, respectively.

MTT Cell Proliferation Assay

MTT was added at a final concentration of $0.1\,\mathrm{mg\,ml^{-1}}$ after supernatants were collected. Four hours later, cell lysate was prepared using 10% SDS, 50% dimethylformamide. Cell viability was assessed by absorbance at 570 nm.

Semi-quantitative RT-PCR

Total RNA was isolated by Trizol reagent and reverse transcribed using M-MLV reverse transcriptase and random hexamer primers as described previously [17]. Equal amounts of synthesized cDNA were determined by PCR amplification of GAPDH (sense primer: 5′-GAG GGG CCA TCC ACA GTC TTC-3′; antisense primer: 5′-CAT CAC CAT CTT CCA GGA GCG-3′). For IL-8 mRNA semi-quantitative analysis, a gene-specific primer (sense primer: 5′-CCG GAA GAA CCA TCT CAC T-3′; antisense 5′-CCA GTT TTC CTT GGG GTC C-3′) was used for PCR amplification. The amplified cDNA were solved on 2% (w/v) agarose gel electrophoresis and visualized by ethidium bromide.

Nuclear Extracts

Nuclear extracts were prepared as described previously [18]. U937 cells were washed twice with D-Hanks' balanced salt solution, resuspended in Buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% Triton X-100) and then disrupted in Pestle B. After centrifugation, nuclei were resuspended in Buffer C (20 mM HEPES, 25% glycerol, 420 mM

NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) and lysed on ice. Supernatants were collected, diluted with Buffer W (20 mM HEPES, 20 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, 1 mM PMSF, 17% glycerol) and stored at −70°C.

EMSA

Nuclear extracts were incubated with ^{32}P-labeled oligonucleotides, solved on non-denaturing 8% polyacrylamide gel and visualized by autoradiograph. Oligonucleotides used in gel shift assay were as follows: NF-κB (5′-AGT TGA GGG GAC TTT CCC AAG GC-3′, 5′-GC CTT GGG AAA GTC CCC TCA ACT-3′), NF-κB mut (5′-AGT TGA GGC GAC TTT CCC AAG GC-3′, 5′-GCC TTG GGA AAG TCG CCT CAA CT-3′), AP-1 (5′-CGC TTG ATG AGT CAG CCG GAA-3, 5′-TTC CGG CTG ACT CAT CAA GCG-3′) and AP-1 mut (5′-CGC TTC ATC AGT CAG CCG GAA-3′, 5′-TTC CGG CTG ACT GAT GAA GCG-3′).

Statistical Analysis

Data were expressed as the mean ± SD of more than five independent experiments. Statistical significance was examined with Student's *t*-tests.

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