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Low dose of resveratrol enhanced immune response of mice[1]

FENG Yong-Hong, ZHOU Wen-Liang, WU Qing-Li, LI Xiao-Yu, ZHAO Wei-Min, ZOU Jian-Ping[2]

Lab of Immunopharmacology, Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

KEY WORDS resveratrol; T-lymphocytes; interleukin-12; interleukin-10; interferon type II; delayed hypersensitivity; ethanol

ABSTRACT

AIM: To study the immune modulating effect of low dose of resveratrol. **METHODS:** Concanavalin A (ConA) and Staphylococcus aureus Cowan (Sac) were used to induce the activation of T lymphocyte and antigen presenting cell and cytokine production. [^{3}H]-Thymidine incorporation was used to evaluate the proliferation of lymphocyte. Cytokine production was detected by ELISA method. Dinitrofluorobenzene (DNFB) was used to induce mice delayed type hypersensitivity (DTH, delayed hypersensitivity) response and ear swelling was used as an evaluating indicator. Changes of lymphocyte subtypes were detected by flow cytometry. **RESULTS:** Resveratrol (0.75-6 μmol/L) concentration-dependently promoted lymphocyte proliferation and IL-2 production induced by ConA. Sac induced IL-12 and IFN-γ (interferon type II) production were also concentration-dependently enhanced by resveratrol, while IL-10 production was inhibited. Resveratrol (4 mg/kg, ig) promoted DTH response of mouse, which was suppressed by ethanol (16 %, w/v) consumption. Resveratrol treatment had no significant influence on lymphocyte subtypes in mice, however it could reverse the suppressive effect of ethanol both on macrophage percentage and on macrophage MHC-II molecule expression. **CONCLUSION:** Low dose resveratrol enhanced cell-mediate immune response. Promoting Th1 cytokine production and influencing on macrophage function might be its mechanisms.

INTRODUCTION

Resveratrol, one of the most important polyphenol fraction found in red wine, has been proved to possess many functions in modulating physiological and pathological reactions of body[1], such as anti-cancer, anti-mutagenesis, and cardioprotection, *etc*. The functional mechanisms of resveratrol included acting as a free radical scavenger, and as a modulator of many key enzymes in cell life, such as cyclooxygenases (COX-1, COX-2), lipoxygenase, iNOS, protein kinase C (PKC), protein tyrosine kinase (PTK), ribonucleotide reductase, and P450, *etc*[2].

In immune system, resveratrol was reported to inhibit production of nitric oxide (NO) and tumor necrosis factor (TNF-α) from macrophages[3,4]. We found that the effect of resveratrol on TNF-α release from macrophage was correlated with time course: resveratrol inhibited TNF-α production from 2 h of endotoxin-activated macrophage, however, it slightly enhanced TNF-α production from the macrophage after 24 h

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[2] Correspondence to Prof ZOU Jian-Ping.
Phn 86-21-6431-1833, ext 351. Fax 86-21-6437-0269.
E-mail JpZuo@mail.shcnc.ac.cn
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stimulation.

Many researches indicated that resveratrol possessed anti-inflammatory function, and almost all reports stated its immune-suppressive effect. However, few reports were about the effect of resveratrol on lymphocyte function. In this paper, we evaluated the effect of low dose of resveratrol on the immune response in mice.

MATERIALS AND METHODS

Reagents Con A (Concanavalin A), LPS (*Escherichia coli* 055:B5), *trans*-resveratrol, and TMB (3,3',5,5'-tetramethylbenzidine) were Sigma products. Sac (Staphylococcus aureus Cowan strain 1) was from Pansorbina cells, Biosciences, Inc (La Jolla, CA 92039, USA). RPMI-1640 and fetal bovine serum (FBS) were obtained from Gibco. Purified rat anti-mouse IL-2, IL-10, IL-12p40, IFN-γ, biotinylated anti-mouse IL-2, IL-10, IL-12p40, IFN-γ, R-phycoerythrin (PE)-anti-mouse L3T4 (CD4), FITC-anti-mouse Lyt-2 (CD8), FITC-anti-mouse B220, and FITC-anti-mouse-CD11b(Mac-1) were Pharmingen products.

Resveratrol (M_r 228.2) was dissolved in 100 % ethanol as a stock of 0.2 mol/L stored at -20 °C and diluted to needed concentrations with RPMI-1640 before use. The final concentration of ethanol in the culture medium was less than 0.001 %, which had no influence on the immune index we detected.

Animals Inbred Balb/c mice, 6-8 weeks of age, were provided by Shanghai Experimental Animal Center of Chinese Academy of Sciences (Certificate No 99-003). The mice were housed in specific pathogen-free (SPF) conditions with room temperature of (24±2) °C, 12-h light/dark cycle, and provided with sterile food and water *ad libitum.*

Preparation of splenocytes Splenocytes were prepared aseptically from Balb/c mice at the age of 6-8 weeks and cultured in RPMI-1640 supplemented with 10 % endotoxin-free and heat-inactivated fetal bovine serum, supplemented with benzylpenicillin 100 kU/L, and streptomycin 100 mg/L.

[^{3}H]-Thymidine incorporation to the splenic lymphocytes For splenocyte proliferation assay, 5×10^5 cells each well were inoculated into 96-well plate with ConA (5 mg/L) and different concentrations of resveratrol in a final volume of 200 μL. Cells were cultured in 5 % CO_2 incubator at 37 °C for 48 h. Cells were pulsed with 1.9×10^4 Bq/well of [^{3}H]-thymidine 12 h before harvest. After the culture finished, cells were harvested onto glass fiber filters and the incorporated radioactivity was counted by a Beta Scintillator (MicroBeta Trilux, PerkinElmer Life Sciences).

Induction of cytokine production from splenocytes For splenocyte cytokine production assay, 5×10^6 cells per well was used with or without ConA (5 mg/L) or Sac (1:10000) and resveratrol in a final volume of 2 mL. After 24 h culture, cell-free supernatant was collected and frozen at -80 °C for ELISA.

Cytokine analysis IL-2, IL-12p40, IL-10, and IFN-γ levels were measured by ELISA. TMB was used to develop color reaction. The absorbance was measured at 450 nm by BioRad microplate reader. Concentrations were calculated based on standard curve using standard murine cytokines (rIL-2, rIL-12, rIL-10, and rIFN-γ).

DNFB-induced delayed type hypersensitivity (DTH) response Female Balb/c mice were used with 8 mice each group. Mice were sensitized with 0.5 % DNFB solution (20 μL) in absolute acetone-olive oil (4:1) on each hind foot on d 0 and d 1. After initial sensitization, resveratrol (4, 16 mg/kg) was administered orally to the mice once per day until experiment finished. At the same time, ethanol (16 %, w/v) was added in the water bottle and let the mice drink freely, according to reference[5]. Five days after initial sensitization, mice were challenged with 0.2 % DNFB (10 μL) on both sides of left ear. The right ear was treated with vehicle alone. Ear swelling was expressed as the difference between the weight of the left and right ear patch made by a specific 8-mm punch 24 h after the second challenge and after euthanasia.

Flow cytometry Splenocytes were washed in cold PBS (staining buffer, containing 0.1 % NaN_3, 1 % FBS, pH 7.2). Cells were resuspended at 2.0×10^{10}/L in cold staining buffer. Optimal concentrations of each fluorochrome-labeled antibody were added to 50 μL cells. Fc receptors were blocked using 2.4G2 monoclonal antibody. Cells were incubated in the dark at 4 ℃ for 30 min, washed twice with 2.0 mL staining buffer and resuspended in 0.5 mL of PBS. Cells were stored in the dark at 4 ℃ and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Data were analyzed by CellQuest™ Software.

Statistical analysis Results were expressed as mean±SD, independent two-tailed *t*-test was performed, and *P* values less than 0.05 were considered to be significant. Each experiment was repeated at least three

times.

RESULTS

Promoting effects of resveratrol on murine lymphocytes proliferation and IL-2 production induced by ConA After 24 h of culture, resveratrol (0.75-6 μmol/L) concentration-dependently enhanced lymphocytes proliferation induced by ConA. Resveratrol (0.75-6 μmol/L) also concentration-dependently promoted IL-2 production from ConA activated murine splenic lymphocytes (Tab 1).

Tab 1. Resveratrol enhanced lymphocyte proliferation and IL-2 production induced by ConA stimulation. *n*=3. Mean±SD. [b]*P*<0.05, [c]*P*<0.01 *vs* control.

Group	Concentration/ $\mu mol \cdot L^{-1}$	$[^3H]$-Thymidine incorporation/Bq No stimulation	ConA (5 mg/L)	IL-2/ $\mu g \cdot L^{-1}$
Control	-	2.0±0.0	632±12	4020±14
RES	0.75	2.0±0.0	792±10[c]	4102±4
	1.5	2.0±0.0	1026±30[c]	4216±1[b]
	3	1.7±0.0	1048±14[c]	4841±5[c]
	6	1.7±0.0	1091±7[c]	5074±11[c]

Effects of resveratrol on IL-10, IL-12P40, and IFN-g production from Sac stimulated murine splenocytes As shown in Fig 1, 2, and 3, Sac stimulation induced marked increasing of IL-10, IL-12P40, and IFN-γ production from murine splenocytes compared with resting splenocytes. Resveratrol (0.75-6 μmol/L) concentration-dependently inhibited IL-10 release, but enhanced IL-12P40 and IFN-γ production from Sac stimulated splenocytes.

Resveratrol reversed the suppression of mouse DTH response induced by ethanol consumption Resveratrol (4, 16 mg/kg, ig) promoted mouse DTH response, although only in the dose of 16 mg/kg, the data showed statistical difference. However, ethanol induced suppression of DTH response (*P*=0.035, compared with control group) was significantly up-regulated by resveratrol (*P*=0.033 for 4 mg/kg group and *P*=0.036 for 16 mg/kg group, compared with ethanol group, respectively (Fig 4).

We also detected the changes of lymphocyte sub-

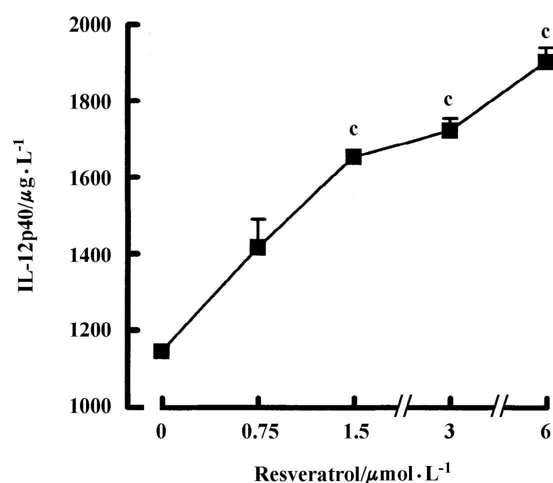

Fig 1. Resveratrol concentration-dependently promoted IL-12p40 production from Sac (0.01 %) stimulated murine splenocytes. *n*=3. Mean±SD. [c]*P*<0.01 *vs* control.

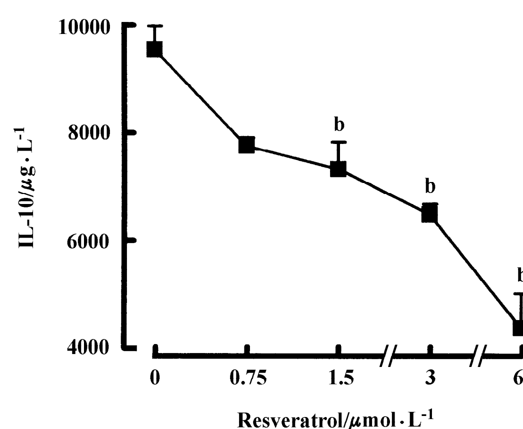

Fig 2. Resveratrol concentration-dependently inhibited IL-10 production from Sac (0.01 %) stimulated murine splenocytes. *n*=3. Mean±SD. [b]*P*<0.05 *vs* control.

types in DTH mice. Administration of resveratrol had no apparent influence in lymphocyte subtypes, while ethanol (16 %, w/v) consumption down-regulated the macrophage number in splenocytes (12.6 % in DTH control group, 9.1 % in ethanol group), and the number was up-regulated by resveratrol (4 mg/kg) to 11.9 %. The most significant influence of ethanol and resveratrol was on the antigen presenting molecule MHC class II expression. As in Tab 2, Ethanol decreased the MHC-II molecule expression, and this suppressive effect of ethanol on macrophage function was antagonized by resveratrol. However, both resveratrol and ethanol up-regulated the MHC-I molecule expression on macrophages, when used together, the effect changed little compared with ethanol sole used group.

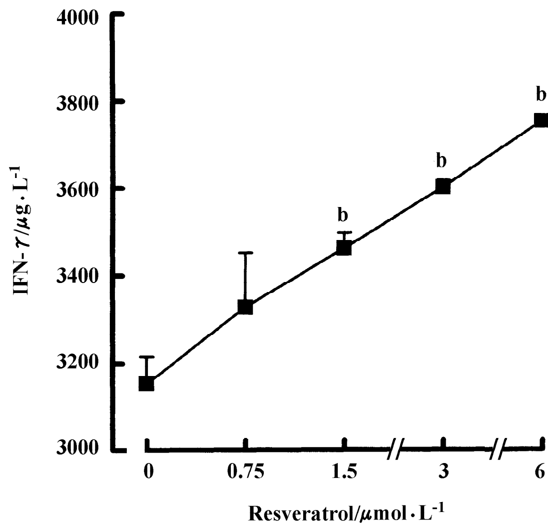

Fig 3. Resveratrol promoted IFN-g production from Sac (0.01 %) stimulated murine splenocytes. n=3. Mean±SD. [b]P<0.05 *vs* control.

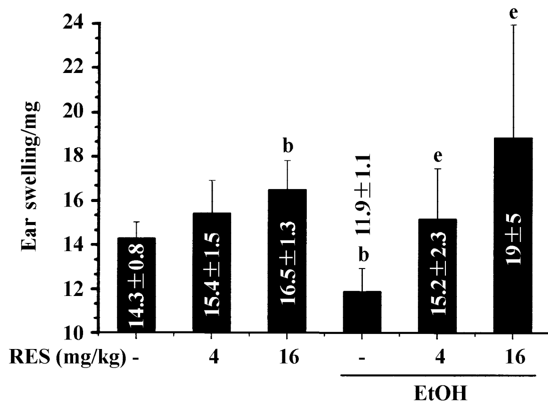

Fig 4. Resveratrol antagonized the ethanol-induced suppression of DTH ear swelling in Babl/c mice. n=8. Mean±SD. [b]P<0.05 *vs* control group. [e]P<0.05 *vs* ethanol treated only group.

DISCUSSION

The anti-inflammatory effect of resveratrol has been emphasized in many reports and proposed to be one of its health-care mechanisms (eg, anti-cancer)[6]. In contrast to previous reports, we confirmed in this paper, that low dose of resveratrol had capability to enhance the function of lymphocyte and could promote immune response.

Th1/Th2 cytokine balance is one of the most important regulatory mechanisms in immune system. Th1 cytokines include IL-2, IFN-γ, and IL-12, *etc*. Increasing of Th1 cytokine production is correlated with enhancement of T lymphocyte proliferation in adaptive immunity and initiating and supporting LAK and NK cell differentiation in innate immunity. However Th2 cytokines including IL-4, IL-10, and TGF-β are correlated with humoral immune response and can suppress the function of Th1 cytokines[7].

Tab 2. Resveratrol (4 mg/kg) antagonized the ethanol-induced down-regulation of macrophage MHC class II molecule expression on Balb/c DTH model mice.

Group	Mean channel fluorescence	
	H-2K^d (MHC-I)	I-A^d/I-E^d (MHC-II)
Control	2041	2185
RES	2412	2690
EtOH	2914	1841
EtOH+RES	2943	2558

Splenocytes 1×10^6 from three mice of each group were stained with anti-Mac-1/FITC and anti-H-2K^d or anti-I-A^d/I-E^d-phycoerythrin. The cells were gated to on Mac-1$^+$ cells to show the difference on MHC-I and MHC-II molecule expression. The data were representative of three experiments.

Resveratrol (0.75-6 μmol/L) promoted the proliferative response of T lymphocyte. Consistent with this, resveratrol also induced the cytokine balance of antigen stimulated spleen cells to Th1 type: that was, enhanced IL-2, IFN-γ, and IL-12 production, while suppressed IL-10 production. This may be one explanation of the promoting effect of resveratrol on DTH response of mouse, the traditional T cell mediated immune response, which is enhanced by Th1 cytokines.

We further tested the effect of resveratrol on animal model of ethanol-induced suppression of DTH response. As many researches have indicated that alcohol consuming impaired antigen-specific, cell-mediated immune response, which mostly related to the impairment of macrophage (or monocyte) functions[8]. Our data also showed that ethanol consuming significantly down-regulated the percentage of macrophage in mouse spleen, and the expression of MHC class II, the main antigen-presenting functional molecule of macrophage. Most interesting was that, resveratrol could markedly promote MHC-II molecule expression; this might be one of molecular mechanism of resveratrol in antagonizing the immune suppressive effect of ethanol. We also noticed the promoting effect of resveratrol and ethanol on macrophage MHC class I molecule expres-

sion. IFN-γ can up-regulate the expression of MHC class I molecules, this may be one explanation of the effect of resveratrol. However, the physiological meaning of effects of resveratrol and ethanol on MHC-I expression needs further investigation.

As for the contradictory of our finding and others, which almost all stated the immune suppression effect of resveratrol, we ascribed the inconsistence to the doses of resveratrol used. In our testing system, the immune promoting effect of resveratrol was found to be limited to a relatively lower concentration (less than 12 μmol/L). At higher concentration (24 μmol/L), resveratrol significantly inhibited the lymphocyte proliferation (induced by both mitogens and mixed lymphocyte reaction (data not provided) and pro-inflammatory cytokine (TNF-α, IL-6, *etc*) production. The result was consistent with recent findings[9].

Our study showed another aspect that is noticeable to evaluate the function of resveratrol, as low dose of resveratrol may be more often to encounter in daily wine consuming.

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