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Effects of resveratrol and ethanol on production of pro-inflammatory factors from endotoxin activated murine macrophages[1]

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KEY WORDS resveratrol; ethanol; macrophages; nitric oxide; tumor necrosis factor; interleukin-1; interleukin-6; lipopolysaccharides

ABSTRACT

AIM: To study the interaction of resveratrol and ethanol on the production of pro-inflammatory factors from activated murine peritoneal macrophages (MPM). **METHODS:** NO production was measured with Griess assay; IL-1 production was measured through thymocyte co-stimulating assay; IL-6 and TNF-α were detected by ELISA method. **RESULTS:** Resveratrol (6.25, 12.5, 25 μmol/L) and ethanol (0.2 %, 0.8 %) synergistically inhibited the 24 h production of NO from lipopolysaccharide (LPS, 1 mg/L) and IFN-γ (5 kU/L) stimulated MPM; resveratrol at higher dose (25 μmol/L) also inhibited IL-6 production. Ethanol additively strengthened this effect. Ethanol had no significant influence on 24 h MPM IL-1 production, but it promoted the ability of resveratrol on enhancing the IL-1 release from activated MPM. Low doses of ethanol inhibited 24 h production of TNF-α, however, both dose of ethanol enhanced the promoting effect of resveratrol on TNF-α production. **CONCLUSION:** Resveratrol and ethanol can interact to influence the production of macrophage function molecules, which is noteworthy in evaluating the health-care effect of wine consumption.

INTRODUCTION

There are increasing epidemiological studies reveal that wine, when consumed in moderate amounts, may confer many beneficial effects (cardioprotection and neuroprotection, *etc*) to human health[1,2]. The health-care effects of wine are mostly ascribed to the presence of its phenolic components, while the effect of ethanol is debatable.

Resveratrol, one major polyphenol component in red wine, has been proved to possess multifunctions like anti-cancer, anti-mutagenesis and cardioprotection[3]. The functional mechanisms of resveratrol include acting as free radical scavenger, and as modulator of many key enzymes in cell life, such as cyclooxygenase (COX-1, COX-2), lipoxygenase, iNOS, protein kinase C (PKC), protein tyrosine kinase (PTK), ribonucleotide reductase, and P450. Regulation on the activity of

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nuclear factor-kappa B (NF-κB) may explain its multi-functions in part.

Contrast to many detailed however separate researches done on the physiological or pharmacological effects of ethanol and wine beneficial components, there are few reports focusing on the interaction between them. In this experiment, taking primary cultured murine peritoneal macrophages (MPM) as the cell model, we tested how production of macrophage effector mediators (NO, TNF-α, IL-1, IL-6) changed by resveratrol and ethanol. We especially noticed how the secretion mode of macrophage changed in the presence of both of them.

MATERIALS AND METHODS

Reagents Trans-resveratrol, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], LPS (Lipopolysaccharide, from *Escherichia coli* Serotype 055:B5) and ABTs [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] were Sigma products. RPMI-1640 and fetal bovine serum (FBS) were obtained from GIBCO. Purified rat anti-mouse IL-6, purified rabbit anti-mouse TNF-α, and biotin rat anti-mouse IL-6, TNF-α were PharMingen products.

Cell preparation Inflammatory peritoneal macrophages were elicited by ip male Balb/c mice, 6-8 weeks old, with 1 mL 10 % sterilized thioglycollate. Cells were harvested aseptically from the peritoneal cavity 4d after the injection and were cultivated in RPMI-1640 containing 10 % heat-inactivated fetal bovine serum. Cells with density of 3.5×10^6/well were used throughout the whole experiment.

Nitrite detection Griess reagent (1 % sulfanamide in water and 0.1 % naphthylenediamide in 2.5 % phosphoric acid) was used to detect nitrite accumulation[4]. That was, 100 μL of macrophage culture supernatant was collected and mixed with 100 μL Griess reagent, after 15 min reaction, the absorbance value was read at 570 nm with a Bio-Rad microplate reader, and the relative nitrite concentration was calculated with the standard curve of nitric sodium.

TNF-a and IL-6 production measurement Macrophage TNF-α, IL-6 levels were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using purified anti-mouse antibody (PharMingen). ABTs was used to develop color reaction. The absorbance was measured at 405 nm.

IL-1 detection IL-1 was detected with thymocyte co-stimulation assay[5]. Balb/c mouse thymocyte (1×10^6/well) suspension with 50 μL ConA (2 mg/L) and 50 μL collected supernatant in a total volume of 200 μL was incubated for 48 h at 37 °C and 5 % CO_2. Cell proliferation was assayed by MTT method[6]. Briefly, 4 h before the ending of culture, 20 μL MTT was added to each well (final MTT concentration was 0.5 g/L), at the end of the culture, 100 μL per well of culture supernatant was moved out, and 100 μL dissolving solution (10 % SDS-50 % *N,N*-dimethylformamide) was added in, then the plate was incubated for further 6–7 h in the incubator to let the purple formazan dissolve. Absorbance was measured at 570 nm, and the relative IL-1 amount was expressed as the proliferation promoting rate (PPR) calculated by the formula below:

$$\%PPR = \frac{A_{570\ nm\ (detected\ well-basal\ well)}}{A_{570\ nm\ (basal\ well)}} \times 100\ \%$$

Statistical analysis Results were expressed as mean±SD, independent two-tailed Student's *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

Synergistic effects of resveratrol and ethanol on inhibition of NO release from endotoxin activated macrophages NO production from macrophages caused by endotoxin (LPS 1 mg/L) reached peak after 24 h of stimulation. While no direct cytotoxic effect were observed (MTT method, data not provided), resveratrol (6.25, 12.5, and 25 μmol/L) and ethanol (0.2 %, 0.8 %) both inhibited the LPS induced NO release from macrophages. The inhibitory rate were 5.3 %, 25.8 %, 62.2 % for resveratrol and 5.1 %, 43.1 % for ethanol, respectively. Their effects were synergistic when used together, the inhibition rate increased to 19.4 %, 52.8 %, 77.6 % (resveratrol with 0.2 % ethanol) and 70.7 %, 80.0 % and 96.5 % (resveratrol plus 0.8 % ethanol) respectively (Fig 1).

Effects of resveratrol and ethanol on macrophage IL-6 release Under our testing system, 25 μmol/L resveratrol showed weak inhibitory effect on activated MPM IL-6 production, the inhibitory rate was 15 %, while other doses of resveratrol showed no apparent effect (data not provided). Ethanol could inhibit the MPM IL-6 production; the inhibitory rate was 13 % for 0.2 % ethanol and 24 % for 0.8 % ethanol. Adding of resveratrol

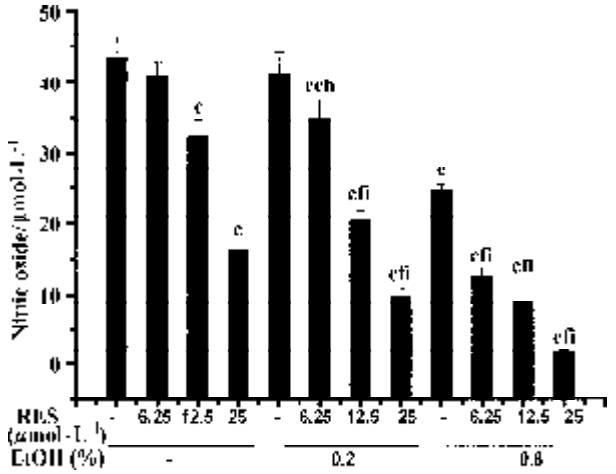

Fig 1. Resveratrol and ethanol synergistically inhibit 24 h NO release from LPS stimulated MPM. *n*=4. Mean±SD. $^{c}P<0.01$ *vs* control. $^{e}P<0.05$, $^{f}P<0.01$ *vs* related ethanol (EtOH) group. $^{h}P<0.05$, $^{i}P<0.01$ *vs* related resveratrol (RES) group.

significantly strengthened the ethanol's inhibitory effect (Tab 1).

Tab 1. Effects of resveratrol and ethanol on IL-6 release from 24 h culture of LPS and IFN-g stimulated murine peritoneal macrophages. *n*=3. Mean±SD. $^{b}P<0.05$ *vs* control group. $^{e}P<0.05$ *vs* related ethanol group. $^{h}P<0.05$, $^{i}P<0.01$ *vs* related resveratrol group.

Resveratrol/μmol·L^{-1}	Ethanol/%	IL-6/μg·L^{-1}
-	-	64.6±1.2
25	-	54.7±0.4^{b}
-	0.2	56.1±1.6^{b}
25	0.2	42.3±0.4^{bei}
-	0.8	48.9±0.6^{b}
25	0.8	42.0±1.4^{beh}

Effects of resveratrol and ethanol on IL-1 production After stimulated by LPS 1 mg/L plus IFN-γ5 kU/L, MPM produced large amount of IL-1 in 1-2 h, which significantly promoted mouse thymocytes proliferation with the co-action of ConA. While after 24 h of stimulation, the IL-1 production decreased significantly (data not provided). As shown in Tab 2, resveratrol apparently dose dependently increased the 24 h IL-1 production, and its effect was enhanced by ethanol additively. Ethanol itself also slightly increased IL-1 production, but the results had no statistical significance.

Tab 2. Effects of resveratrol and ethanol on 24 h IL-1 release from LPS and IFN-g stimulated murine peritoneal macrophages. *n*=4 for IL-1 detction, *n*=3 for TNF-a detection. Mean±SD. $^{c}P<0.01$ *vs* control. $^{e}P<0.05$, $^{f}P<0.01$ *vs* related ethanol group. $^{h}P<0.05$ *vs* related resveratrol group.

Resveratrol/ μmol·L^{-1}	Ethanol/%	Proliferation promoting rate/%	TNF-α/μg·L^{-1}
-	-	10.1±2.3	6.8±0.1
6.25	-	19.7±3.5^{c}	10.1±0^{c}
12.5	-	34.7±3.2^{c}	10.2±0.1^{c}
25	-	50.8±8.9^{c}	7.3±0.1^{c}
-	0.2	12.8±2.8	4.0±0.0^{c}
6.25	0.2	27.4±1.6^{cf}	12.9±0.1^{ce}
12.5	0.2	45.5±3.1^{cfh}	12.2±1.1
25	0.2	56.7±4.2^{cf}	6.0±0.0^{bh}
-	0.8	14.9±4.2	11.4±0.0^{cb}
6.25	0.8	26.5±1.7^{ce}	16.6±0.8^{be}
12.5	0.8	49.0±4.4^{cfh}	14.6±0.3^{beh}
25	0.8	63.8±7.9^{cfh}	9.0±0.2^{beh}

Effects of resveratrol and ethanol on macrophage TNF-a release LPS and IFN-γ treatment also induced large amount of MPM TNF-α release. Six hours after stimulation, TNF-α production reached its peak concentration. Resveratrol dose dependently suppressed the TNF-α production. When used with ethanol, resveratrol antagonized the inhibitory effect of ethanol on TNF-α production (data not provided). After 24 h stimulation, while low concentration of ethanol maintained the inhibitory effect, higher concentration (0.8 %) of ethanol enhanced the production of TNF-α, and both doses of ethanol could enhance the promoting effect of resveatrol on MPM TNF-α production (Tab 2).

Considering the contradictory results between ours and previous reports about the effect of resveratrol on macrophage TNF-α production may be caused by different testing time point selected, we also checked the effect of resveratrol and ethanol on early phase (1 h) activated MPM TNF-α production. Consistent with effects on MPM NO production, resveratrol and ethanol both dose dependently and synergistically inhibited TNF-α production from 1 h endotoxin activated MPM (data not shown).

DISCUSSION

Under the stimulation of bacterial endotoxin (especially with the co-effect of IFN-γ), macrophage released large amounts of functional molecules (NO, TNF-α, IL-6, IL-1). Acute ethanol treatment suppressed macrophage NO production, this was similar to previous *in vivo* and *in vitro* reports on alveolar macrophages[7] and Kupffer cells[8]. Wine phenolic component resveratrol also dose-dependently suppressed NO production. When used together, resveratrol and ethanol synergistically inhibited NO production. Our result was similar to Chan's work[9]. However, while the concentrations of ethanol used were similar, we used dose of 0.2 % and 0.8 %, they 0.1 %-0.75 %, they found no direct or minimal inhibitory effect of ethanol on NO production and iNOS gene expression of Raw 264.7 macrophage. The difference may be caused by different cell studied.

At the same doses that showed significant inhibition on MPM NO production, the effect of resveratrol on IL-6 production was weak. However, it could strengthen the inhibitory effect of ethanol on IL-6 production. Ethanol (at low dose) maintained its inhibitory effect on 24 h TNF-α production, while it had no apparent influence on IL-1 production. However, resveratrol enhanced the production of both of the cytokines, and its effect on IL-1 production was promoted by addition of ethanol.

We have noticed most of the effects of resveratrol and ethanol on production of pro-inflammatory factors were synergistic or additive. Ethanol enhanced the incorporation of resveratrol into cells, so to promote the effect of resveratrol may be one possible explanation; however, research is needed to find out the molecular targets of resveratrol and ethanol in signal transduction of endotoxin activated macrophage, which induced inflammatory cytokines production.

Another noticeable point in our result was that, the inhibitory effect of ethanol on 24 h TNF-α production in moderate dose was not augmented at higher dose; On the contrary, higher dose of ethanol could promote TNF-α production. We also noticed this phenomenon in the influence of ethanol on the production of other inflammatory cytokine (IL-12, data not provided), while the physiological or pathological meaning of this phenomenon needs further investigation.

Cytokines influence the production of each other, which compose a complex immuno-regulation network. For example, nitric oxide can attenuate the production of IL-1β through modifying the structure of IL-1β converting enzyme; TNF-α recently was found to play protective roles during inflammation by regulating of pro-inflammatory factors (COX-2, exogenous IL-1β induced IL-1β and IL-6) gene expression, TNF-α also could attenuate its own expression[10]. In our test system, wine component resveratrol and ethanol may act together to restrict some inflammatory factors (like NO and IL-6) production. However, they also cooperate to enhance the production of other inflammatory/immunostimulatory factors (IL-1). Our experiment provided useful data to better recognize the effect of wine component, resveratrol, and to rationally evaluate the possible influence of wine consumption on human health.

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