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Effects of esculentoside A on production of interleukin-1, 2, and prostaglandin E_2[1]

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KEY WORDS esculentoside A; interleukin-1; interleukin-2; prostaglandins E; interferon-gamma

ABSTRACT

AIM: To investigate the influence of esculentoside A (EsA) on immunological function and its mechanism of anti-inflammation. **METHODS:** Interleukin-1 production was measured by thymocyte co-stimulating assay; the radioactivity of [^{3}H]arachidonic acid (AA) was used to evaluate the release of AA; prostaglandin E_2 production was measured with radioimmunoassay (RIA); IL-2 and IFN-γ were detected by ELISA method. **RESULTS:** EsA (3-12 μmol/L)could potently inhibit the production of IL-1 and PGE_2 from both silent and LPS induced macrophages. EsA had no significant effect on the release of AA from murine macrophages. EsA could inhibit the production of IL-2 from murine lymphocytes induced by ConA, but not affect the production from silent lymphocytes. EsA showed no effect on the production of IFN-γ from both silent and ConA induced lymphocytes. **CONCLUSION:** EsA could affect the immunological function through inhibiting the production of IL-2 from activated splenocytes and the inhibition of production of IL-1 and PGE_2 might be one of the anti-inflammation mechanisms of EsA.

INTRODUCTION

Esculentoside A (EsA), a kind of saponin isolated from the Chinese herb *Phytolacca esculenta*, which was identified as 3-*O*-[β-*D*-glucopyranosyl-(1,4)-β-*D*-xylopyranosyl]phytolaccagenin (Fig 1). Previous experiments showed that it had strong anti-inflammatory effects and significantly decreased the production of tumour necrosis factor (TNF) and platelet activating factor (PAF) from macrophages[1-4]. EsA has been shown to inhibit antibody production and phagocytic activity in mouse macrophages and has the positive curative effect on autoimmunity in the mouse model[5,6]. Prostaglandin E_2 and cytokines, such as IL-1, IL-2, and IFN-γ, play important roles in the inflammatory and immunological response. To gain a better understanding of the anti-inflammatory and immuno-regulating effects of EsA, we studied the effects of EsA on the release of arachidonic acid (AA), IL-1, and PGE_2 from macrophages as well as IL-2 and IFN-γ production from mouse lymphocytes.

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MATERIALS AND METHODS

Reagent Esculentoside A (purity >99 %) was provided by Prof Yang-hua YI (Second Military Medical University, Shanghai, China). Zymosan, Lipopolysaccharides (Escherichia coli 055:B5) and concanavalin A (Con A) were purchased from Sigma (USA). RPMI-

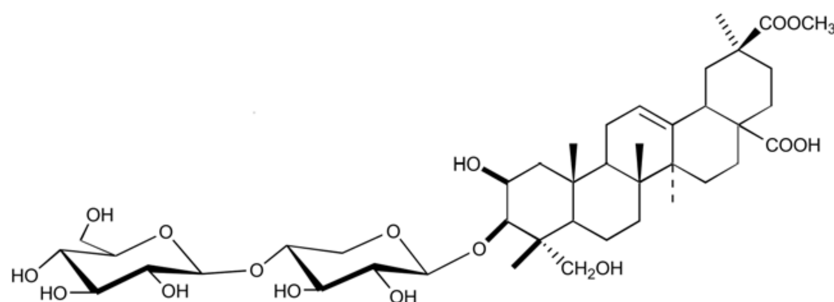

Fig 1. Structure of esculentoside A

1640, MEM and fetal calf serum (FCS) were obtained from GIBCO (USA). [^{3}H]Arachidonic acid (8029 GBq/mmol) was purchased from Amersham company (UK). PGE_2 radioimmunoassay (RIA) kit was obtained from the Chinese Academy of Medical Sciences. Thioglycolate was obtained from the Shanghai Biological Research Institute. [^{3}H]Thymidine was obtained from Shanghai Institute of Nuclear Research, Chinese Academy of Sciences. Monoclonal antibody against IL-1β, mouse IL-2, and IFN-γ ELISA kit were purchased from R&D systems, Inc (USA).

Animal and cell line BABL/C mice, both sex, weighing 18-22 g, were from the Animal Center of Second Military Medical University (Grade II, Certificate No 02-25-7, China). ICR mice, both sex, weighing 18-22 g, were purchased from Shanghai Experimental Animal Center (Grade II, Certificate No 153, China).

Preparation of murine macrophages Thioglycolate medium (1 mL, 3 % w/v) was injected intraperitoneally into the ICR mice. Four days later the cells in the peritoneal cavity were harvested with D-Hanks' solution, washed twice with RPMI-640 and adhered at 37 ºC in a culture bottle (Nunclon, Denmark) for 2 h. The non-adherent cells were decanted and the adherent cells were harvested through policeman. The adherent cell suspension was adjusted to 1×10^{10} cells/L with culture medium and disposed at 1 mL/well in 24-well plates (Nunclon, Denmark).

IL-1 bioassay Various concentrations of esculentoside A with or without 10 mg/L LPS were added to above mouse macrophages to induce IL-1. After 18 h of incubation at 37 ºC in 5 % CO_2, the supernants were collected, centrifuged at 2000 r/min for 10 min, dialyzed at 4 ºC in PBS for 24 h, and stored at -70 ºC for IL-1 bioassay. For control group, cells were treated without EsA.

In brief, newly isolated mouse thymocytes from BABL/C mice were used to measure IL-1 activity in cell supernants according to our previously reported methods[7]. IL-1 activity was expressed as [^{3}H]thymidine incorporation by ConA 2.5 mg/L stimulated thymocytes at 1:8 dilution. Monoclonal antibody against IL-1β was used to confirm IL-1 activity by bioassay, and the results demonstrated that addition of IL-1β monoclonal antibody decreased ConA-induced thymocyte proliferation by 85 % in samples. ConA 2.5 mg/L contributes to 15 % of the proliferation of thymocyte proliferation.

PGE_2 determination The macrophages (1×10^9/L) in 1 mL medium (MEM containing 10 % FCS) was seeded in wells and incubated for 24 h. The supernatants were decanted and the cells were washed with MEM three times. EsA at different concentrations in the presence or absence of 10 mg/L LPS were added to cells. For control group, cells were treated without EsA. After a 24-h incubation, the supernatants were adjusted to pH 3.5 with 10 % HCOOH and extracted with 2 mL ethyl acetate twice. The organic section was evaporated and the residual was reconstituted with 200 μL RIA assay buffer. The PGE_2 content was determined according to the RIA kit instruction and expressed as ng per 1×10^6 macrophages.

[^{3}H]arachidonic acid determination The determination of [^{3}H]AA was carried out as previously reported[8]. Briefly, 1×10^7 macrophages in 1 mL medium (RPMI-1640 containing 10 % FCS) were added to culture dishes (Clontech, USA) for 2 h. Non-adherent cells were washed away by D-Hanks' solution. [^{3}H]AA (18 500 Bq) was added into each dish. Four hours later, the supernatant was decanted and the cells were washed twice. Zymosan 400 mg/L was added to each dish after EsA was incubated with macrophages for 20 min. The supernatants were collected at 2, 5, and 15 h after zymosan addition, and the radioactivity of [^{3}H]AA in the supernatants was determined by β-scintillator (Institute of Atomic Nucleus, China). For control group, cells were treated without EsA.

IL-2 and IFN-γ detection Single cell suspension of spleens was prepared from BABL/C mice. Spleen cells (1.5×10^6/well) were seeded in 24-well plate and incubated with different concentrations of EsA in the presence or absence of 5 mg/L ConA at 37 ºC in 5 % CO_2. Control cells were treated without EsA. After 48 h, the supernatants were harvested and stored at -20 ºC. IL-2 and IFN-γ levels were measured by enzyme-linked immunosorbent assay according to instruction of mouse IL-2 ELISA kit and mouse IFN-γ ELISA kit. The absorbance was measured at 450 nm in Bio-RAD microplate spectrophotometer. According to the standard curve, the IL-2 and IFN-γ levels were calculated and expressed as μg/L.

Statistics Data were expressed as mean±SD. Each experiment was repeated at least three times. Differences were considered statistically significant when $P<0.05$ as analyzed by ANOVA and unpaired *t*-test.

RESULTS

Effects of EsA on IL-1 production The macrophages secreted IL-1 when stimulated with 10 mg/L LPS ($P<0.01$). Different concentrations of EsA in the presence or absence of LPS were assessed for their effects on the production of IL-1. The results showed that IL-1 production from both silent and LPS activated mouse macrophages was markedly inhibited by esculentoside A from 3 to 12 μmol/L (Tab 1).

Tab 1. Effect of EsA on IL-1 production from different states of macrophages. n=6. Mean±SD. $^cP<0.01$ *vs* control without LPS group. $^fP<0.01$ *vs* control with LPS group.

Groups		Thymocyte proliferation /Bq	
		without LPS	with LPS
Control	-	253±27	325±31
EsA/μmol·L^{-1}	3	124±15^c	152±24^f
	6	96±14^c	144±13^f
	12	78±12^c	116±16^f

Effects of EsA on PGE_2 production Macrophages stimulated with LPS could produce more PGE_2 than those without LPS ($P<0.01$). PGE_2 production was decreased concentration-dependently by EsA. Without LPS, the inhibitory rate of EsA at the concentration of 3, 6, and 12 mmol/L was 30 %, 53 %, and 63 %, respectively. When macrophages were stimulated with LPS, the inhibitory rate of EsA at the concentration of 3, 6, and 12 mmol/L was 13 %, 27 %, and 40 %, respectively. The results indicated that EsA significantly inhibited PGE_2 production from different states of mouse macrophages (Tab 2).

Tab 2. Effect of EsA on PGE_2 production from different states of macrophages. n=6. Mean±SD. $^cP<0.01$ *vs* control without LPS group. $^fP<0.01$ *vs* control with LPS group.

Groups		PGE_2 content /ng·10^{-6} macrophages	
		without LPS	with LPS
Control	-	9.1±0.7	14.2±0.6
EsA/μmol·L^{-1}	3	6.2±0.5^c	11.8±0.8^f
	6	4.1±0.3^c	9.9±0.3^f
	12	3.2±0.2^c	8.1±0.4^f

Effects of EsA on the release of AA Macrophages will take [^{3}H]AA when co-cultured with [^{3}H]AA. Zymosan 400 mg/L could stimulate macrophage to release [^{3}H]AA. At different times, the release of AA from zymosan-treated macrophages was more than that from normal macrophages. The results illustrated that EsA from 3 to 12 μmol/L had no effect on the release of AA from zymosan-treated macrophages (Tab 3).

Tab 3. Effects of EsA on the release of AA at different times. n=4. Mean±SD. $^cP<0.01$ *vs* normal group.

Groups		Arachidonic acid radioactivity/Bq		
		3 h	5 h	15 h
Normal	-	197±6	262±8	360±13
Control (zymosan treated)		360±12^c	475±18^c	623±22^c
EsA/μmol·L^{-1}	3	328±10	443±15	498±16
	6	336±11	436±16	593±17
	12	344±10	445±19	599±20

Effects of EsA on IL-2 and IFN-γ production When splenocytes were stimulated with 5 mg/L ConA, IL-2 and IFN-γ production were about 8 times and 20 times higher than those of untreated cells, respectively. EsA had no effect on IFN-γ production neither in the presence nor absence of ConA. EsA also did not affect IL-2 production in the absence of ConA. However,

EsA effectively lowered the secretion of IL-2 from ConA stimulated splenocytes in a concentration-dependent manner (Tab 4).

Tab 4. Effect of EsA on IL-2 and IFN-γ production in splenocytes treated with or without ConA. n=4. Mean±SD. ^{c}P<0.01 *vs* control.

Groups		Without ConA/μg·L^{-1}		With ConA/μg·L^{-1}	
		IL-2	IFN-γ	IL-2	IFN-γ
Control	-	61±7	36±4	521±32	720±57
EsA/μmol·L^{-1}	3	62±8	31±4	391±27^{c}	722±48
	6	62±4	34±3	324±22^{c}	723±56
	12	58±7	32±5	245±18^{c}	731±53

DISCUSSION

Chinese people have utilized the herb *Phytolacca esculenta* for the therapy of various diseases for more than a thousand years. This herb has been proven to have very satisfactory therapeutic effects on a number of diseases such as rheumatoid arthritis, edema, and tumors. Among various compounds purified from the root of this ancient herb, saponins and polysaccharides were major components that might account for the pharmacological effects of this herb. EsA is the first saponin found to have obvious anti-inflammatory effects, whose mechanisms are different from commonly used anti-inflammatory drugs. EsA inhibited the swelling of rat hind paws induced by carrageenan and suppressed the proliferation of granuloma by cotton pellet in a pituitary-adrenal-independent manner[1]. Previous results showed that EsA could reduce the production of TNF, IL-6, and PAF by macrophages *in vitro*[2-4]. Macrophages are very important cells in inflammatory processes and produce many kinds of mediators including cytokines, PAF, NO, leukotrienes, *etc*. So we assumed that EsA had anti-inflammatory effects through inhibiting inflammatory mediators secreted by macrophages.

In this experiment we found that EsA could not only inhibit the production of IL-1 by macrophages within bigger concentration range than before, but also in a concentration-dependent manner markedly suppressed the production of PGE_2 from both the normal and LPS induced macrophages. The present results confirmed our hypothesis that EsA had effects on the production of inflammatory mediators secreted by macrophages. Moreover, we examined effects of EsA on the release of AA. EsA had no significant effect on the release of AA. It suggested that EsA exerted its anti-inflammation effect not through inhibiting the release of AA.

Previous experiments showed that EsA had the positive curative effect on autoimmunity in a mouse model and might inhibit cellular immunity since it could accelerate the apoptosis of activated thymocytes[6]. To gain a better understanding of the effects of EsA on immunological function, we studied the effect of EsA on production of IL-2 and IFN-γ from mouse spleen lyphmocytes. The present results showed that EsA had no effect on the production of IFN-γ from neither silent nor ConA treated splenocytes. It suggested that the immuno-regulating effects of EsA were not caused by the inhibition of production of IFN-γ. EsA inhibited the production of IL-2 from mouse splenocytes activated by ConA, but did not affect IL-2 production from silent ones. The lack of IL-2 could lead to lymphocyte apoptosis since IL-2 was an important cytokine that is essential for T lymphocyte existing and called T lymphocyte growth factor[10]. Inhibition of IL-2 production by EsA is consistent with the previous results that EsA accelerated the apoptosis of activated thymocytes and decreased splenocyte proliferation[6].

T lymphocytes play important roles in inflammatory responses especially in the initial phase of the inflammation, and certain agents, such as immunosuppressive agents, nicotine or corticosteroids, exert anti-inflammatory effects via inhibition of T cells[11]. Our results demonstrated that EsA could affect both the silent and activated macrophages, but only influenced the activated T lymphocytes. It suggested that EsA might have stronger effect on macrophages, and exert anti-inflammatory effects mainly through influencing the function of macrophages.

In summary, our study clearly demonstrated that EsA inhibited the production of IL-1 and PGE_2 by macrophages, which might be one of the mechanisms of its anti-inflammatory effects. In addition, we previously found that EsA could reduce the production of TNF, IL-6 and PAF[2-4]. Taken together, the inhibition of inflammatory mediators from macrophages might be mainly contributed to the anti-inflammatory mechanisms of EsA. Moreover, EsA could inhibit the production of IL-2 from ConA-treated lymphocytes without effect on normal lymphocytes, which suggested that EsA might have immunomodulatory effects. These findings sug-

gest that EsA possesses anti-inflammation and immunoregulation actions, which might expand the knowledge of distinct therapeutic effects of EsA.

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