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The anti-inflammatory effects of ethyl acetate on Lipopolysaccharide/D-galactosamine challenged mice and Lipopolysaccharide activated RAW264.7 cells

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ABSTRACT

Ethyl acetate (EA) is an ordinary organic compound in fruits, wine and cosmetics, and used as a solvent frequently. With the recent observation in our experiment, we suspected that EA could affect immune function, in particular macrophage activity. In this paper, we tested EA's protect effect against death in Lipopolysaccharide/D-galactosamine (LPS/D-GalN)-induced endotoxic shock model in mice. And also found EA decreased the LPS-induced mRNA expression of mediators of inflammation including cyclooxygenase 2 (COX2), inducible NOS (iNOS), and tumor necrosis factor α (TNF α) in RAW264.7 cells. Consequently, EA decreased the production of, TNF α and the inflammatory agent nitric oxide (NO) in RAW264.7 cells treated with LPS. Other pro-inflammatory cytokines such as IL-1h and IL-6 were similarly decreased by EA treatment of RAW264.7 cells. The potential mechanism may associate with NF-κB activity as we shown. Taken together, these results suggest that EA has anti-inflammatory properties.

1. Introduction

Ethyl acetate (EA) is an ester of ethanol and acetic acid, and can be hydrolyzed in acidic or basic conditions to become acetic acid and ethanol. EA is present in confectioneries, perfumes, and fruits [1]. It is also the main ester occurring in wine with concentrations from 50 to 200 mg/L [2] and can enter the body through breathing, touching or swallowing. It is important to know the physiological effects of EA. The toxic effects of EA evaluated in earlier documents [3,4], indicated its low toxicity (LD50 was 2.6 g/kg body weight), and maximum non-toxic dose (LD50 was 0.8 g/kg body weight) for white mice, s.c. [5]. However, little information has been released on the anti-inflammatory effect of EA. It is reasonable to consider the contribution of ethanol and acetate when exploring the physiological effects of EA, because EA can rapidly metabolize into ethanol and acetate [6]. Acute ethanol can down regulate production of pro-inflammatory factors [7–9]. Von Haefen et al. reported recently that ethanol suppressed the LPS-induced gene expression of Foxp3, RORγt and T-bet, inhibited the production of TNF-α and IL-1β in supernatant of PBMCs, while levels of IL-10 and IL-6 remained unchanged following ethanol exposure [10]. An impressive number of papers have described the anti-inflammatory effects of SCFAs in recent years [11–14]. Butyrate seems to be more powerful than propionate and acetate [11,14–17], but acetate also shows anti-inflammatory effects [12,18]. Our recent work showed that three types of SCFAs (acetate, propionate and butyrate) reduced the production of pro-inflammatory factors (TNF-α, IL-1β, IL-6 and NO), while inhibiting the vitality of iNOS, and enhancing the production of anti-inflammatory cytokine (IL-10) at low concentrations (1–1200 μmol/L) [19].

Ethyl pyruvate (EP), a compound formed from pyruvate and ethanol has been broadly reported to be an anti-inflammatory agent [20]. Ulloa et al. [21] reported that EP inhibited LPS-induced secretion of the pro-inflammatory cytokine, tumor necrosis factor in RAW 264.7 cells. EA is a simple ester from acetate and ethanol, and has similar structure with EP. Logically, EA maybe have an anti-inflammatory effect like EP. Infact, we recently reported that EA (0.6 g/kg body weight) significantly decreased LPS/D-galactosamine-induced mice mortality, and alleviated the level of NO and alanine transaminase (ALT) levels in serum [22]. These findings drive us to explore the anti-inflammatory effects of EA and the underlying mechanisms further.

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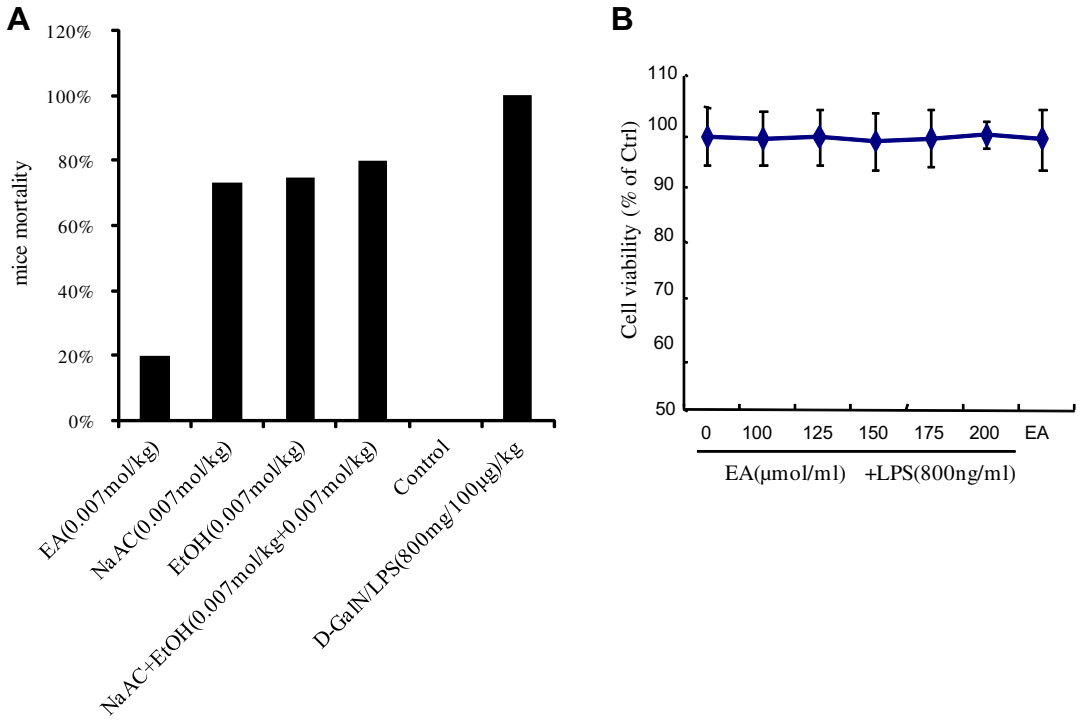

Fig. 1. (A) EA reduced LPS/D-GalN-induced mice mortality. The LPS/D-GalN-induced murine endotoxic shock model was used to test the protective effect of EA. Mice were pretreated with EA, NaAc and EtOH (30 min prior to LPS). Twelve hour-death rate was recorded. (B) No reductive effects of treatments on cell viability. RAW 264.7 cultures treated with different concentrations of EA for 30 min, followed by LPS (800 ng/mL) for 24 h. Cell viability was measured by MTT assay. Results are the mean ± SEM of three independent experiments.

2. Materials and methods

2.1. Chemicals

LPS (from *Escherichia coli,* 0111:B4), D-galactosamine (D-GalN) and MTT were purchased from Sigma. EA, DMSO, phosphoric acid, ethanol, sodium nitrite ($NaNO_2$), formaldehyde, H_2O_2 and methanol were purchased from Tianjin Kermel Chemical Reagent Company. Sulfanilamide and *N*-(1-naphthyl) ethylenediamine dihydrochloride were purchased from Aldrich Chemical Company. RIPA cell lysate, DMEM from Beijing Solarbio Science & Technology Co. Ltd., Mouse IL-1β, IL-6, TNF-α and iNOS ELISA kits were purchased from Boster biological technology company. Rabbit polyclonal anti-NF-κB p65 IgG, goat anti-rabbit monoclonal IgG-bionormal, goat IgG-HRP, anti-β-actin, and histone H1 from Santa Cruz Biotech-nology Inc. TRIZOL was purchased from Invitrogen Life Technologies Company. RNA fixer was bought from Generay biotechnology.

2.2. Animal treatments

Eight-week male (20–21 g) KM mice were obtained from Hebei laboratory animal center (license no: SCXK (ji) 2008-1-003. certificate Numbers: 911098; 912043; 912110; 1206028). The animals were housed in an air-conditioned room with controlled temperature (22 ± 1 °C) for 3 days and feed on a standard diet and tap water ad libitum, fasted 12 h before the experiment. Endotoxic shock was induced by administering a single intraperitoneal dose of LPS/D-GalN (100 μg/800 mg/kg) in saline. To compared the protective effect of EA, ethanol and acetate on experimental endotoxic shock, same dose (7 mmol/kg) of EA, NaAc, ethanol and NaAc mixed with ethanol were administrated subcutaneously 30 min before LPS/D-GalN injection. Control group received the same volume of saline. Record the death time and number of mice.

2.3. RAW264.7 cell culture and treatments

The RAW264.7 cells were maintained at a 37 °C in a humidified atmosphere of 5% CO_2, 95% air, in DMEM supplemented with 100 U/mL of penicillin, 100 g/mL of streptomycin and 10% placental bovine serum. For experimental purpose, the cells were harvested in log phase, and were plated at the density of 1×10^6 cells/mL medium in 6-, 24- or 96-well sterile plate. Then, cells were pre-treated with vehicle or EA solutions for 20 or 30 min before treatment with LPS (800 ng/mL).

The supernatants and cells were collected at different time points for ELISA and real-time PCR.

2.4. MTT cell viability assay

The cells in log phase were seeded in 96-well plates and incubated for 12 h, then treated with different treatment media. After 20 h, 10.0 μL of MTT (5.0 μg/mL) was added, and the cultures were incubated for an additional 4 h. The medium was removed and 100 μL of DMSO was added. The absorbance was read at 490 nm with a Bio-Tek microplate Reader.

2.5. Cytokines assay and nitrite assay

The release of TNF-α, IL-6 and IL-1β in supernatant was determined using ELISA kits according to the manufacturer's instructions.

The accumulation of nitrite, an indicator of NO production in the culture supernatant, was determined with a colorimetric assay with the Griess reagent (0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H_3PO_4) at 24 h. Equal volumes of culture supernatant and Griess reagent were mixed, incubated for 10 min at room temperature, and absorbance read at 540 nm. The concentrations of nitrite in the samples were determined from a sodium nitrite standard curve.

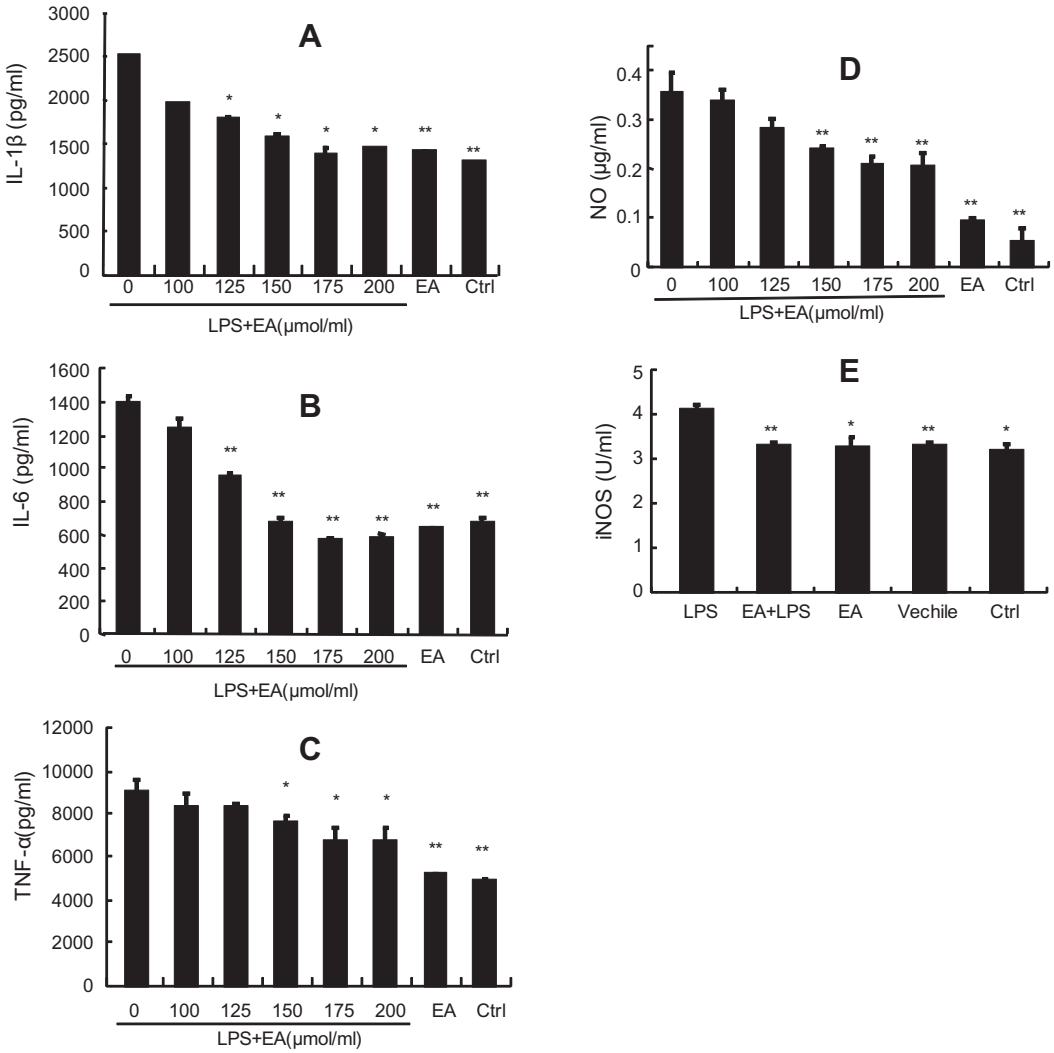

Fig. 2. Effects of EA on release of IL-1β, IL-6, TNF-α, NO, and iNOS. Cultures were pretreated for 30 min with vehicle or the indicated concentrations of EA before stimulation with 800 ng/mL LPS. Supernatants were removed at 6 h for measurement of IL-1β, IL-6 and TNF-α. Cells were harvested at 24 h for iNOS detection; supernatants were removed at 24 h for measurement of NO. The results are the mean ± SEM of three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with LPS-treated cultures. (A) IL-1; (B) IL-6; (C) TNF-α. (D) NO (different concentrations); (E) iNOS (175 μmol/mL).

2.6. iNOS assay

Supernatant was collected from cells treated with vehicle, LPS or EA (175 μmol/mL) for 12 h, and then broken with an ultrasonic cell disruptor (280 W, 5s-5s, 6 min) in RIPA cell lysate solution, and centrifuged at 12,000 r/min for 40 min. The level of iNOS was measured using ELISA kits according to the manufacturer's instructions.

2.7. Real Time RT-PCR analysis

Cells were harvested 6 h post LPS treatment and stored in a RNA fixer. Total RNA was extracted with TRIZOL reagent, and purified with RNAeasy columns (Shanghai Sangon Biotech Company, Shanghai, China). Expression of the selected genes was quantified using real-time RT-PCR analysis as previously described [23].

2.8. NF-κB activation assay by Western blotting

Cells were pretreated with 175 μmol/mL of EA, and then stimulated with LPS (800 ng/mL). Cell nuclear lysates were obtained by using Nuclear and Cytoplasmic Extraction Kit (cwbiotech, Beijing, China). Equal amounts of protein were separated by SDS–PAGE gel electrophoresis and transferred onto PVDF. The membrane was blocked for 2 h at room temperature (RT) with TBS containing 5% fat-free dried milk. Next, the membrane was incubated overnight at 4 °C with the primary antibody diluted by 1:100–1000, and immune complexes were incubated for an hour at RT with horseradish peroxidase-conjugated antibody diluted by 1:2000–5000. After application of the secondary antibody, triplicate washes were followed with TBS-T, and developed for visualization using ECL auto-radiographed and the intensity of the bands was determined using gray scale analysis.

2.9. Immunofluorescence assay on NF-κB p65

For immunolocalization of NF-κB, the cells were grown on coverslips placed in 6-well plate and were either pretreated or non-pretreated with EA (175 μmol/mL) for 30 min, then, stimulated with LPS (800 ng/mL) for 24 h before fixation. The cells were immunofluorescence-labeled using a Cellular NF-κB Translocation Kit (Beyotime Biotech). Briefly, after washing and fixing, cells were incubated with a blocking buffer for 1 h to suppress non-specific binding. Next, cells were incubated with the primary NF-κB p65 antibody for 1 h, followed by incubation with a Cy3-conjugated

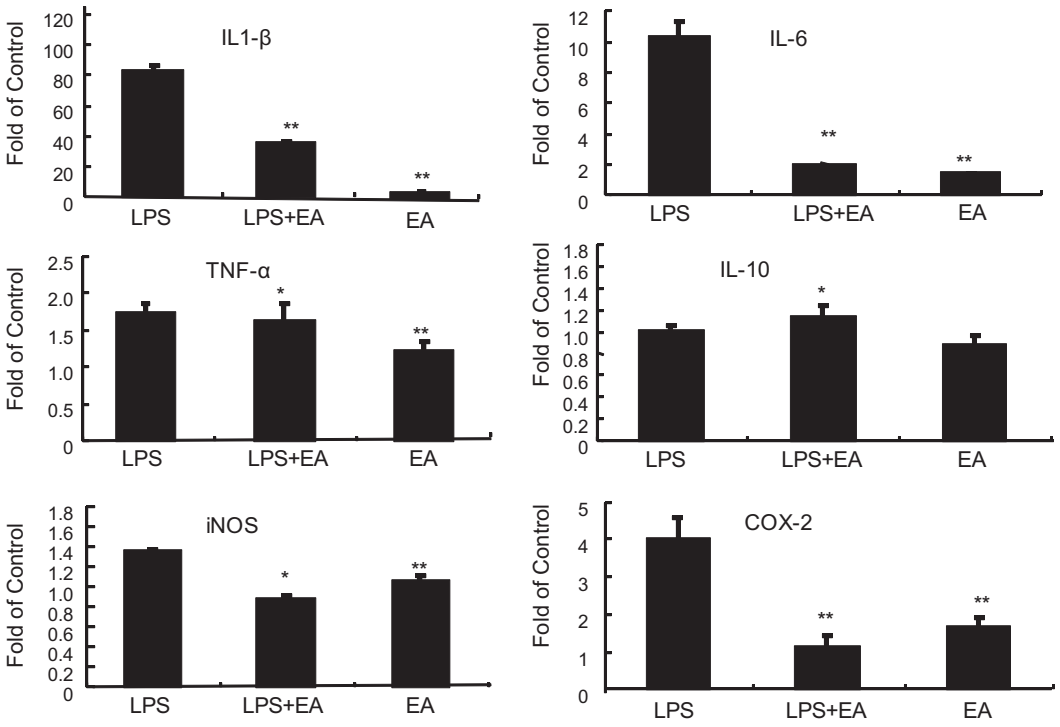

Fig. 3. Effects of EA on the expression of IL-1β, IL-6, IL-10, iNOS, TNF-α and COX-2 in RAW264.7 cell. Cells pretreated 30 min with EA (175 μmol/mL), followed by 6 h LPS treatment, and then harvested for real time RT-PCR Analysis. The results are the mean ± SEM of three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with LPS-treated cultures.

secondary antibody for 1 h, then with DAPI, for 5 min before observation, p65 protein and nuclei fluoresce red and blue, respectively, and can be simultaneously viewed by a confocal microscope (Olympus IX81-FV1000, Japan). To create a two-color image, the red and blue images were overlaid, producing purple fluorescence in areas of co-localization.

2.10. Statistical analysis

Data are expressed as mean ± SEM. Statistical significance was assessed with ANOVA followed by *t*-test using SPSS Statistics 12.0. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. EA reduced LPS/D-GalN-induced mice mortality

The LPS/D-GalN-induced murine endotoxic shock model was used to test the protective effect of EA. EA pretreatment (30 min prior to LPS) decreased mice mortality. Twelve hour-death rate was 40% in EA (7 mmol/kg) treatment group. Mice mortality is shown in Fig. 1A.

3.2. Effects of EA on LPS-induced release of IL-1β, IL-6 and TNF-α releasing

A MTT assay was done and showed that cell viability was formal in all treatments (Fig. 1B), which ruling out the reduction of inflammatory factors coming from the depression of cell viability. RAW 264.7 cell cultures were pretreated with a series of EA or vehicle to evaluate the effects of EA on release of these cytokines. Three target cytokines were markedly increased in LPS-stimulated RAW 264.7 cells; the increase was dramatically diminished by EA at 150, 175 and 200 μmol/mL concentrations. When the concentration of EA was 175 μmol/mL, the level of IL-1β, IL-6 and TNF-α was decreased by 44.56%, 59.71% and 26.41% respectively (Fig. 2A–C). EA alone had no effect on cytokines release compared with control.

3.3. Effect of EA on LPS-induced production of NO and iNOS

EA pretreatment suppressed NO production in a concentration-dependent manner from 100–200 μmol/mL, and was reduced by 5.34%, 20.92%, 32.58%, 41.85% and 43.12% respectively (Fig. 2D). The iNOS levels were detected by pretreating cultures with 175 μmol/mL EA. The result showed EA can down-regulate iNOS to control level after the stimulation of LPS (Fig. 2E).

3.4. Real-time PCR analysis of expression of genes of interest

To further examine the effects of EA on LPS-activated RAW264.7 cells, pertinent gene expression was examined with real-time PCR (Fig. 3A). EA (175 μmol/mL) significantly diminished the LPS induced increase in the expression of IL-1β, IL-6, TNF-α, iNOS and COX-2 genes. EA reduced mRNA production of IL-1β, IL-6, TNF-α, iNOS and COX-2 by 57.66%, 82.14%, 6.67%, 36.05% and 72.45% respectively. Interestingly, EA greatly enhanced the expression of IL-10, increasing by 14.08% ($p < 0.01$).

3.5. Inhibitory effect of EA on LPS-induced translocation of NF-κB

In order to further investigate the molecular mechanism of EA attenuating the LPS-induced inflammatory responses, the effect of EA on transcription activities of NF-κB was examined by Western blotting and Immunofluorescence assays. The results of Western blot showed EA could reduce LPS-induced p65 protein translocating into nucleus (Fig. 4A). The level of p65 in nuclear is significantly reduced in EA pro-treated group. And the level of p65 in cytoplasmic extract of EA pro-treated group is up-regulation.

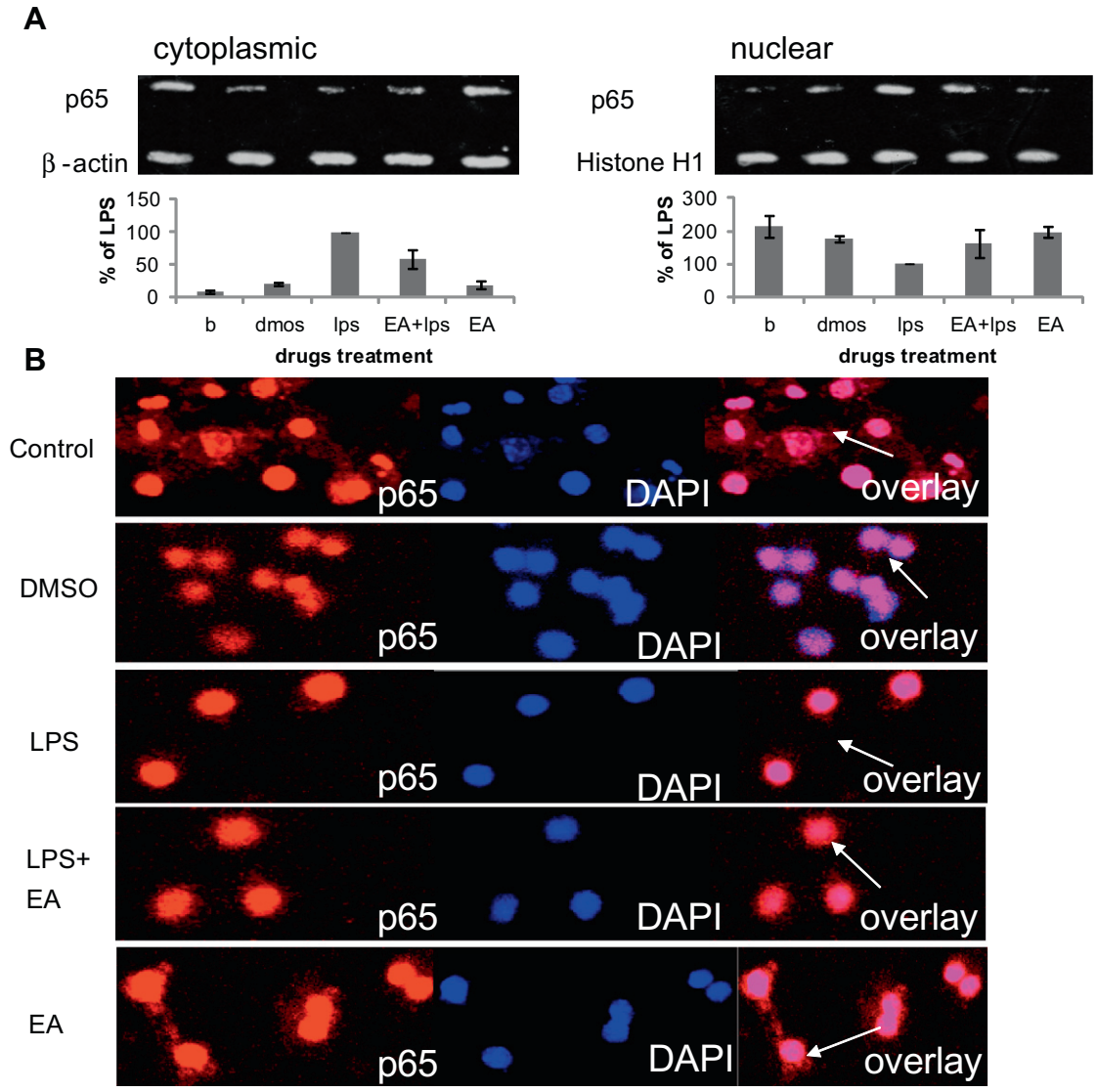

Fig. 4. Effects of EA on p65 transposition. (A) Western blot assay: cells were pretreated with 175 μmol/mL of EA and then stimulated with LPS (800 ng/mL). Cell nuclear and cytoplasmic lysates were obtained at 24 h for p65 level detection using Western blot analysis. The assay was repeated 3 times, and the results point to the same trend; the chart stands for the mean ± SEM optical densitometry from 3 independent experiments. (B) Immunofluorescence assay: approximately 175 μmol/mL of EA prevents p65 entering nuclear in LPS-treated (800 ng/mL) RAW 264.7 cells. Control, cells incubated in DMEM medium without any drugs. DMSO, cells incubated in DMSO without any drugs. LPS, cells stimulated by 800 ng/mL LPS. EA + LPS, cells stimulated by 175 μmol/mL EA and 800 ng/mL LPS. EA, cells stimulated by 175 μmol/mL EA.

Immunofluorescence experiments indicated that EA inhibited LPS induced p65 protein translocating into nucleus, consistent with their effects Western blot results (Fig. 4B). It suggested that EA inhibited LPS-stimulated p65 entry into cell nucleus in RAW264.7 cells.

4. Discussion

We compared the protective effects of same dose of EA, Acetate, Ethanol and combination of acetate and ethanol on LPS/D-GalN-induced endotoxic shock, and demonstrated that EA itself contributes to its protective effect, rather than its catabolite-acetate and ethanol. In addition, we explored the possible anti-inflammation mechanism of EA by using LPS-activated macrophage-like cell line RAW 264.7, and certificated EA could significantly diminish the release of pro-inflammatory factors including TNF-α, IL-1β, IL-6 and NO by inhibiting NF-κB activity.

Inflammation is a complex biological process in which macrophages play a critical role in the initiation, maintenance, and resolution, including the overproduction of pro-inflammatory cytokines and inflammatory mediators such as TNF-α, IL-1β, IL-6, NO and prostaglandin(PGE2) [24–26]. TNF-α and IL-1β are pro-inflammatory cytokines which mediate both acute and chronic inflammation by triggering a cascade of inflammatory mediators, like platelet-activating factor and leukotrienes, prostaglandins, NO, IL-6 and IL-8 [27,28]. Suppressing production of TNF-α and IL-1β *in vitro* and *in vivo* has been widely applied to screen anti-inflammatory agents [26,29]. IL-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine [30,31]. Although IL-6 can down-regulate the synthesis of IL-1 and TNF-α [31], overproduction of IL-6 has been shown to underlie a number of autoimmune and inflammatory diseases, and blocking of IL-6 signaling is considered to be therapeutic in diseases characterized by pathological IL-6 overproduction [32]. The agents which can reduce production of IL-6 are thought to be anti-inflammatory

[11,12]. IL-10 is a cytokine with potent anti-inflammatory properties, repressing the expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1 by activated macrophages, IL-10 exerts its anti-inflammatory effects on various cell types (TH1 cells, monocytes/macrophages) [31,33]. Inflammatory stimulations like LPS and cytokines can induce macrophages to express iNOS and produce large amounts of NO as a defense mechanism. There is a large body of evidence that a massive amount of NO production is involved in several inflammatory disorders [34–37]. In present study, EA could adjust the cytokines and NO production induced by LPS in RAW264.7 cell, which including significantly reduced release of pro-inflammatory factors such as TNF-α, IL-1β, IL-6 and NO (Fig. 3) by down-regulating gene expression of related cytokines and iNOS, while up-regulating IL-10 (Fig. 3). The results showed that decrement of IL-1β, IL-6 and iNOS correlated with the their rRNA reduction, but the decrease of TNF-α was not in proportion to the production of its rRNA, maybe for TNF-a, posttranscriptional events are relatively more important than transcriptional activation in determining the quantity of TNF-a produced [38].

Current study and our recent reports clearly indicated that EA could inhibit LPS-stimulated inflammatory responses *in vitro* and *in vivo* [22]. What is the underlying mechanism? Could the effect of EA come from its metabolite, acetate and ethanol? It is well known that EA metabolizes into acetate and ethanol. Acetate [12,18] or ethanol [7–9] can reduce production of inflammatory factors. Our *in vivo* results showed that EA (7 mmol/kg body weight) significantly decreased LPS/D-GalN induced mice mortality. Acetate, ethanol or combination administration of acetate and ethanol did not show any protective effect (Fig. 1A). The results of acetate and ethanol were consistent with what has been previously reported [10,39]. In previous reports, only acute ethanol at a dose greater than 2.9 g/kg body weight can reduce mortality. The effective dose of acetate was 2.3 g/kg body weight. However the dose of EA was only 0.6 g/kg body weight [22]. Current results apparently showed that the metabolites of EA, acetate and ethanol were not the only contributors in this *in vivo* experiment, suppose that EA could rapidly hydrolyze into acetate and ethanol *in vivo,* 7 mmol EA will produce approximately 7 mmol ethanol and 7 mmol acetate. The potency of 7 mmol/kg dose acetate, or ethanol or combination of acetate and ethanol could not diminish the mortality, but same dose of EA (7 mmol/kg) significantly reduced the mortality (Fig. 1A). It suggests EA itself to be anti-inflammatory.

In cell experiment, EA significantly reduced release of proinflammatory factors including TNF-α, IL-1β, IL-6 and NO in LPS-activated RAW264.1 cells. But the effective concentrations of EA were more than 125 μmol/mL (Fig. 2). Our recent report showed that *in vitro* efficient concentrations of acetate were only nmol/mL grade [19]. The effective concentrations of ethanol were also 0.1–0.4% (about 17.7–70.8 μmol/mL) [40,41]. It is in conflict with *in vivo* lower dose than acetate or ethanol. Could the hydrolysis products of EA be the effector molecules *in vitro?* Firstly, EA could not rapidly hydrolyze into acetate and ethanol *in vitro* [6], secondly, short time treatment of RAW 264.7 by EA which washed out after 20 min, could also suppress release of IL-1β, IL-6, TNF-α and NO (date not show). We guess that using a high concentration of EA *in vitro* experiment was due to its high volatile. The high performance liquid chromatograph assay showed that 175 μmol/mL EA solution becomes approximately 35 μmol/mL after 24 h in 37 °C (data not show). EA was lost more than 80%. So the real work concentration of EA should be less. Therefore, EA itself also was the effector molecules *in vitro*.

In summary, our data showed EA could significantly reduce release of pro-inflammatory factors including TNF-α, IL-1β, IL-6, COX-2 and NO in LPS activated RAW264.1 cells, and can also inhibit the production of iNOS associated with down-regulating gene expression of TNF-α, IL-1β, IL-6, iNOS and COX-2, while up-regulating IL-10 by adjusting NF-κB activation.

EA maybe is an anti-inflammatory or an immunosuppressive agent. The underlying mechanisms rest with reduction of pro-inflammatory factors by adjusting NF-κB activation and down-regulating gene expression of these factors. Our results maybe particular interest from a human life standpoint, because EA is a component in fruits, wine, confectionery and cosmetics.

Acknowledgments

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