

## Original Research Article

# Anti-inflammatory Effects of (-)-Epicatechin in Lipopolysaccharide-Stimulated Raw 264.7 Macrophages

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### Abstract

**Purpose:** To investigate the protective effects of (-)-epicatechin on lipopolysaccharide (LPS)-induced inflammation in Raw 264.7 murine macrophages and the possible underlying mechanisms.

**Methods:** The effects of epicatechin on LPS-stimulated production of inflammatory mediators in Raw 264.7 cells were evaluated by enzyme-linked immunosorbent assay.

**Results:** Epicatechin in doses of 5, 25 and 50  $\mu$ M remarkably ( $p < 0.05$ ) inhibited the production of pro-inflammatory mediators including nitric oxide (NO) and prostaglandin E2 (PGE<sub>2</sub>), as well as the production of pro-inflammatory cytokines including tumor necrosis factor (TNF)-alpha and interleukin (IL)-6 in LPS-induced Raw 264.7 macrophages.

**Conclusion:** The results suggest that epicatechin can inhibit inflammatory response and may be a potential therapeutic candidate for the treatment of chronic inflammatory diseases.

**Key words:** Inflammation, Epicatechin, Cytokines, Nitric oxide, Prostaglandin E2, Macrophages

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## INTRODUCTION

Inflammation is one of the body's self-defense mechanisms which is characterized by redness, pain, swelling and a sensation of heat. The inflammatory responses play an important role in host survival although it can also lead to chronic inflammatory diseases such as asthma [1], cancer [2], rheumatoid arthritis [3], Crohn's disease [4] and ulcerative colitis (UC) [5]. Inflammation can be initiated by a microbial pathogen such as lipopolysaccharide (LPS). LPS is a prototypical endotoxin, which can directly activate macrophages [6]. The production of inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-alpha), and other inflammatory mediators including nitric oxide (NO) and prostaglandin E2 (PGE<sub>2</sub>) is

increased during the processes of inflammation in activated macrophages [7].

Natural flavonoids are a group of polyphenolic compounds that are present in tea, grapes and other plants as well as in wine and cocoa [8-10]. Many studies show that consumption of flavonoid-rich foods exhibited regression of inflammatory diseases [11]. These beneficial effects of flavonoids might be partially attributed to their anti-oxidative capacity. Epicatechin is a natural phenolic compound found widely in plants, and possesses anti-oxidative, anti-cancer and other biological activities [12,13].

Therefore, the objective of the present study was to evaluate the anti-inflammatory activities of epicatechin on Raw 264.7 mouse macrophages.

## EXPERIMENTAL

### Chemicals and reagents

Dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS, *Escherichia coli* 055:B5) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). (-)-Epicatechin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Jilin, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were from Invitrogen Gibco BRL (Grand Island, NY). Mouse TNF-alpha and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Biolegend (San Diego, CA). All other chemicals were used of reagent grade.

### Cell culture

Raw 264.7 mouse macrophages cell line was obtained from Cell Storehouse of Chinese Academy of Science (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum and 1 % of penicillin and streptomycin. The cells were incubated in a humidified incubator with 5 % CO<sub>2</sub> atmosphere at 37 °C.

### Cell viability assay

MTT assay was used for evaluating the effects of (-)-epicatechin on the cell viability. In brief, the cells were seeded into 96-well plates at a density of 1 x 10<sup>5</sup> cells/ml. Cells were treated with different concentrations (0 – 50 μM) of (-)-epicatechin for 1h, followed by stimulation with LPS (1 μg/ml) for 24 h. Then, 30 μl of MTT (5 mg/ml) were added into each well, and incubated for another 4 h. After replacing the culture supernatant with 100 μl of DMSO, the optical density of plates was read at a wavelength of 570 nm using a microplate reader (TECAN, Austria).

### NO and PGE<sub>2</sub> production

Mouse macrophages Raw 264.7 cells were pre-treated with various concentrations (5, 25 and 50 μM) of epicatechin for 24 h before stimulating with LPS (1 μg/ml). After 18 h incubation, the levels of nitrate and PGE<sub>2</sub> in the culture supernatant were calculated and determined by Griess and PGE<sub>2</sub> ELISA kits, respectively.

### Cytokine assay

The cell-free supernatants were collected after treatment with (-)-epicatechin and LPS for 24 h, and measured for the pro-inflammatory cytokines (TNF-alpha and IL-6) production by commercial ELISA kits (R & D Systems), following the manufacturer's protocol.

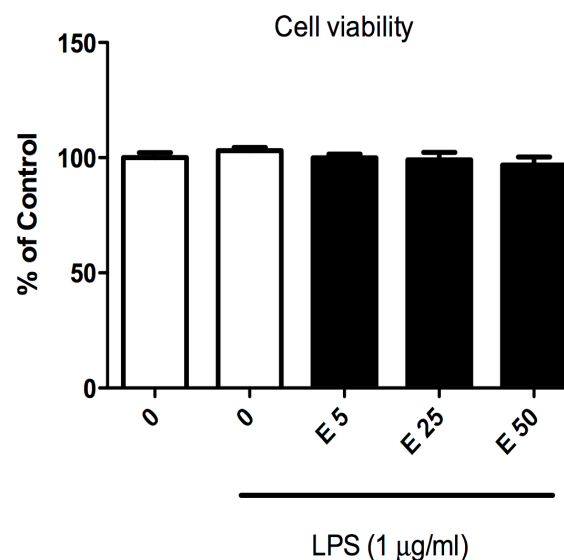
### Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analyses and significance, as measured by one-way analysis of variance (ANOVA), were performed using GraphPad PRISM software version 5.0 (GraphPad Software, USA). In all comparisons, *p* < 0.05 was considered statistically significant.

## RESULTS

### Effect of (-)-epicatechin on cells viability in Raw 264.7 macrophages

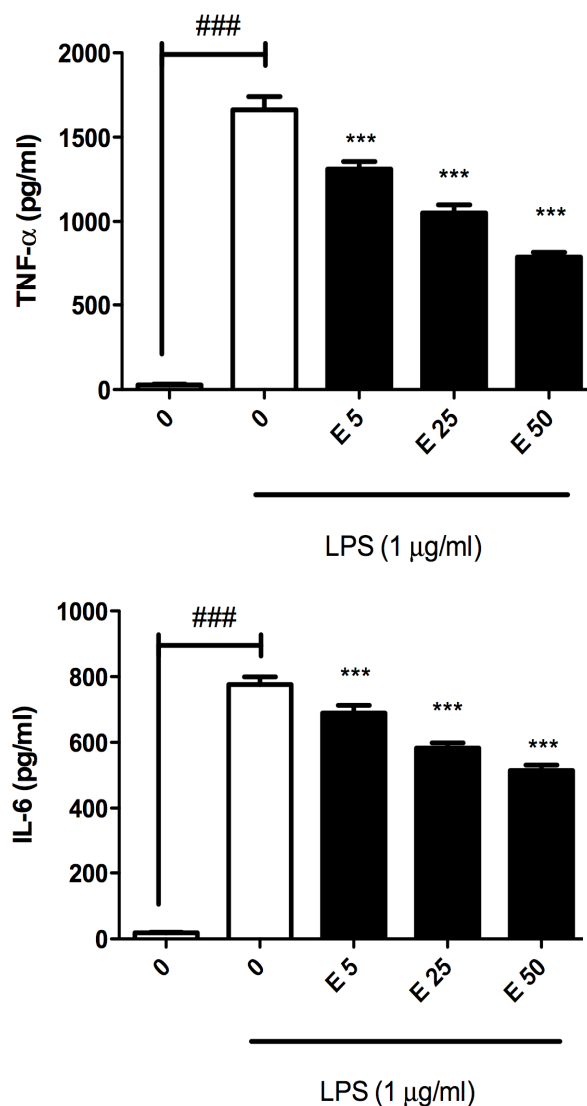
The results of MTT assays show that there were no significant changes in cell viabilities by epicatechin treatment with tested concentrations (Fig 1). Therefore, we used non-toxic concentrations (5, 25 and 50 μM) for the entire experiment.



**Fig 1:** Effects of (-)-epicatechin on cell viability in LPS-treated Raw 264.7 mouse macrophage cells. Raw 264.7 cells were cultured with (-)-epicatechin in the presence of LPS (1 μg/ml) for 24 h. Cell viability was tested by MTT reduction assays. Values are presented as mean ± standard deviation (SD) of three independent experiments

### Effects of (-)-epicatechin on the production of pro-inflammatory cytokines in LPS-stimulated macrophages

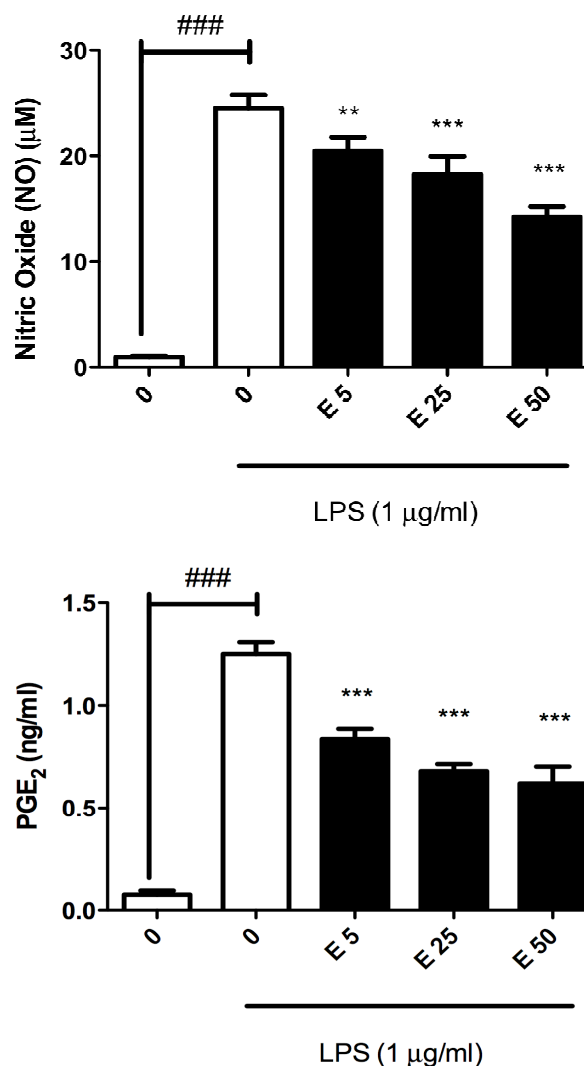
Treatment with LPS alone in Raw 264.7 cells resulted in a significant increase of TNF-alpha and IL-6 production compared with the control group (Fig 2). However, treatment with epicatechin (5, 25 and 50  $\mu\text{M}$ ) remarkably inhibited LPS-induced TNF-alpha and IL-6 production in a dose-dependent manner (Fig 2).



**Fig 2:** Effect of (-)-epicatechin on the production of TNF-alpha and IL-6 in LPS-stimulated Raw 264.7 cells. The cells were treated with LPS alone or LPS plus various concentrations (5, 25, and 50  $\mu\text{M}$ ) of (-)-epicatechin for 24 h. Values represent mean  $\pm$  SD of three independent experiments. The differences between mean values were assessed by student's *t*-test. ###  $p < 0.001$  indicates significant differences from the unstimulated control group; \*\*\*  $p < 0.001$  versus the LPS alone treatment control

### Effects of (-)-epicatechin on NO and PGE<sub>2</sub> production in LPS-stimulated macrophages

NO production was significantly inhibited by (-)-epicatechin treatment compared to LPS alone-treatment. Besides, the inhibitory levels of (-)-epicatechin on NO and PGE<sub>2</sub> production also showed a dose-dependent pattern (Fig 3).



**Fig 3:** Effects of (-)-epicatechin on NO and PGE<sub>2</sub> production in LPS-stimulated Raw 264.7 macrophages. Cells were incubated with LPS (1  $\mu\text{g/ml}$ ) for 18 h in the absence or presence of (-)-epicatechin. Cells were treated with (-)-epicatechin 1 h prior to addition of LPS. Nitrite and PGE<sub>2</sub> production in the medium was determined using the Griess reagent and PGE<sub>2</sub> EIA Kit. Data show the mean  $\pm$  SD of three independent experiments. The differences between mean values were assessed by student's *t*-test. ###  $p < 0.001$  indicates significant differences from the unstimulated control group; \*\*\*  $p < 0.001$  versus the LPS alone treatment control

## DISCUSSION

In the present study, we investigated the effects of (-)-epicatechin on the production of pro-inflammatory mediators in LPS-stimulated Raw 264.7 mouse macrophages. The results demonstrate that (-)-epicatechin effectively inhibited LPS-induced release of TNF- $\alpha$ , IL-6, NO, and PGE<sub>2</sub> production in LPS-stimulated macrophages, suggesting that (-)-epicatechin possesses potent anti-inflammatory effects.

Chronic inflammation is one of the major inducers of various diseases. Many pro-inflammatory cytokines such as TNF- $\alpha$  and IL-6, as well as other inflammatory mediators including NO and PGE<sub>2</sub> are produced during the inflammation process [2]. These mediators drive the recruitment and initiation of macrophages and other immune cells to complete a full cycle of inflammation and interfere with metabolic functions [14]. These inflammatory mediators are risk factors that can stimulate angiogenesis and therefore promote tumor growth in the inflammatory lesion [15].

TNF- $\alpha$  and IL-6 are pro-inflammatory cytokines produced by various immune cells including macrophages, monocytes and lymphocytes in response to inflammation and infection [16]. Growing evidence demonstrate that TNF- $\alpha$  and IL-6 played a central role in the inflammatory process during cancer development, leading a new direction of therapeutics, the pro-inflammatory cytokines blocking agents [15]. NO and PGE<sub>2</sub> also work as pro-inflammatory mediators that are produced by inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) respectively, which are involved in the innate response in activating macrophages [17].

In our present study, (-)-epicatechin showed that it could significantly suppress the releases of TNF- $\alpha$  and IL-6, and the production of NO and PGE<sub>2</sub> in LPS-stimulated macrophages in a dose-dependant manner. The potencies of the inhibitory activities of (-)-epicatechin on Raw 264.7 mouse macrophages are comparable to those of epigallocatechin gallate (EGCG), another anti-inflammatory compound isolated from green tea [12].

## CONCLUSION

The results obtained here demonstrate that (-)-epicatechin is able to inhibit various pro-

inflammatory mediators in antigen-activated macrophages, and also shows a capacity to attenuate cytokine-mediated inflammation. These results also suggest that (-)-epicatechin-containing diets have beneficial effects in preventing various inflammation-related diseases including cancers and IBD.

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