

Sesamol down-regulates the lipopolysaccharide-induced inflammatory response by inhibiting nuclear factor-kappa B activation

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We examined the effects of sesamol on the lipopolysaccharide (LPS)-induced inflammatory response. Sesamol inhibited serum tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and nitrite production in LPS-treated mice, and inhibited LPS-induced inducible nitric oxide synthase expression in mouse leukocytes. Sesamol also down-regulated TNF- α , IL-1 β , and nitrite production as well as inducible nitric oxide synthase expression in LPS-treated RAW 264.7 cells. Further, sesamol inhibited LPS-induced nuclear factor (NF)- κ B translocation and inhibitor (I) κ B- α phosphorylation in RAW 264.7 cells. By inhibiting TNF- α , IL-1 β , and nitrite levels, and interfering with the NF- κ B pathway, sesamol down-regulated the LPS-initiated inflammatory response.

Keywords: inflammation, lipopolysaccharide, nuclear factor- κ B, pro-inflammatory mediators, sesamol

INTRODUCTION

Lipopolysaccharide (LPS) is a major component of the Gram-negative bacterial cell wall. LPS is a potent stimulator of pro-inflammatory mediators, including tumor necrosis factor (TNF), interleukin (IL), and nitric oxide (NO), all of which are crucial in the pathogenesis of the systemic inflammatory response syndrome (SIRS) and sepsis.^{1–3} The activation of transcription factors, such as nuclear factor (NF)- κ B and activating protein (AP)-1, is involved in the release of LPS-initiated pro-inflammatory mediators.⁴ Of these transcription factors, NF- κ B is important in the LPS-induced inflammatory response.^{5,6}

Nuclear factor- κ B is a fundamental transcription factor vital for the expression of pro-inflammatory genes.^{5,7,8} Non-activated NF- κ B is retained in the cytoplasm by binding to an inhibitory protein, inhibitor κ B (I κ B)- α . During inflammation, LPS activates the NF- κ B pathway and phosphorylates I κ B, which leads to a disassociation of the I κ B/NF- κ B complex. Subsequently, free NF- κ B enters the nucleus and binds

to the DNA, thereby allowing rapid gene activation and the expression of pro-inflammatory mediators.^{9–11}

Sesamol (3,4-methylenedioxyphenol) is a potent antioxidant in sesame seed oil. Sesamol attenuates oxidative stress and multiple organ injury in septic¹² and endotoxemic¹³ rats. However, the effect of sesamol on LPS-induced inflammation has never been investigated. Therefore, we investigated the effects of sesamol on LPS-induced inflammatory response *in vivo* and *in vitro*.

MATERIALS AND METHODS

Materials

Lipopolysaccharide, derived from *Escherichia coli* serotype O55:B5, and sesamol were bought from Sigma-Aldrich (St Louis, MO, USA). Sesamol and LPS were dissolved in saline in *in vivo* experiments and in phosphate-buffered saline (PBS) in *in vitro* experiments.

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Animals

Male ICR mice, weighing 20–30 g, were obtained from, and housed in, our institution's Laboratory Animal Center. Mice were housed individually in a room with a 12-h dark/light cycle and with central air conditioning (25°C, 70% humidity). They were allowed free access to tap water and were fed a rodent diet (Richmond Standard, PMI Feeds, Inc., St Louis, MO, USA). The animal care and experimental protocols were in accordance with nationally approved guidelines. The mice were moved to cages equipped with wire mesh to prevent coprophagy; they were deprived of food but allowed free access to tap water for 24 h before the experiments.

Raw 264.7 cell line

The murine macrophage cell-line RAW 264.7 was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 U/ml) in a humidified atmosphere of 5% CO₂/95% air at 37°C.

*Experimental design***Experiment I**

Twenty-four mice were divided into four groups of six. Saline group mice were given one subcutaneous (s.c.) injection of saline (1 ml/100 g of body weight); SM group mice were given one injection of sesamol (10 mg/kg, s.c.); LPS group mice were given one intraperitoneal (i.p.) injection of LPS (5 mg/kg); and LSM group mice were given one injection each of LPS plus sesamol. Serum TNF- α was assessed 1 h after the LPS injection;¹⁴ IL-1 β was assessed 3 h after the LPS injection;¹⁴ and serum nitric oxide was assessed 6 h after the LPS injection.¹⁵ Inducible nitric oxide synthase (iNOS) expression was assessed 6 h after LPS in leukocytes had been assessed.

Experiment II

The RAW 264.7 macrophages were divided into four groups ($n = 6$). Twenty-four-well plates were used. Each well contained 8×10^5 cells in 1 ml medium. The PBS group macrophages were treated only with PBS; LPS group macrophages were treated only with LPS (100 ng/ml); LS100 group macrophages were treated with LPS (100 ng/ml) plus sesamol (100 μ M); and LS300 group macrophages were treated with LPS (100 ng/ml) plus sesamol (300 μ M). Cells and media were collected 24 h

after LPS treatment. The pro-inflammatory mediators TNF- α , IL-1 β and nitric oxide were evaluated in medium. Inducible nitric oxide synthase expression in the cells was assessed. Nuclear factor- κ B activation in nuclear extracts was assessed by examining p65 and p-I κ B- α expression in cells.

Measuring TNF- α and IL-1 β in serum and in medium

The amounts of TNF- α and IL-1 β in the serum and in the culture medium were determined using an enzyme-linked immunosorbent assay (ELISA) kit (DouSet; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Measuring nitrite concentration in serum and in medium

Briefly, the amounts of nitrite in serum and in medium were measured following the Griess reaction by incubating 100 μ l of sample with 100 μ l of Griess solution at 20–22°C for 20 min. The absorbancy was measured at 550 nm using a spectrophotometer (DU 640B; Beckman, Fullerton, CA, USA).¹⁶ Nitrite concentration was calculated by comparison with a standard solution of known sodium nitrite concentration.

Isolating leukocytes

An erythrocyte lysis buffer was used to isolate leukocytes from whole blood. Three milliliters of whole blood was collected in a sampling tube containing K₃EDTA and then centrifuged at 2500 rpm for 10 min. The upper layer was transferred to a tube, gently mixed with 6 ml of erythrocyte lysis buffer (8.3 g/l of ammonium chloride in 0.01 M of Tris-HCl buffer, pH 7.4), and then centrifuged at 2500 rpm for 10 min. The supernatant was discarded and the pellet resuspended with 1 ml of saline. The sample was transferred to a 1.5-ml Eppendorf tube and centrifuged again, and the supernatant was discarded. Leukocytes were stored at –80°C until use.

Assaying cell viability

Macrophage viability was analyzed using a variation of a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Twenty-four hours after each treatment, the macrophages (8×10^5 cells/ml) were renewed with a medium containing 0.5 mg/ml of MTT and cultured for 4 h. After dimethyl sulfoxide (equal in volume to the medium) had been added to the macrophage culture, it was spectrophotometrically measured at a wavelength of 470 nm.

Detecting iNOS, NF- κ B, and phosphorylated-I κ B expression

Expression levels of iNOS, NF- κ B, and I κ B were determined using Western blotting. Nuclear and cytoplasmic proteins were extracted using a kit (NE-PER; Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Twenty micrograms of that protein was separated using electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and then transferred to nitrocellulose sheets (NEN Life Science Products, Inc, Boston, MA). After the protein had been blocked, the blots were incubated with primary antibody against the target protein. The blots were then washed three times and incubated with secondary antibody conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories, Inc., Philadelphia, PA, USA). The immunoblots were developed using a reagent (BCIP/NBT; KPL [Kirkegaard & Perry Laboratories], Inc., Gaithersburg, MD, USA). Western blots were scanned and subjected to densitometry analysis using image analysis software (ImageJ; National Institutes of

Health; available at <<http://rsb.info.nih.gov/ij/>>; accessed on 3 January 2008).

Statistical analysis

Data are expressed as mean values \pm SD. Differences between groups were analyzed using Student's *t*-test or one-way analysis of variance (ANOVA) and then the Tukey Honestly Significant Difference *post-hoc* analysis. Statistical significance was set at $P < 0.05$.

RESULTS

Sesamol down-regulated the release of pro-inflammatory cytokines in LPS-treated mice

To examine the effect of sesamol on the release of LPS-induced pro-inflammatory cytokines in mice, we assessed serum levels of TNF- α and IL-1 β . Sesamol significantly reduced serum TNF- α (Fig. 1A) and IL-1 β (Fig. 1B) levels in LPS-treated mice (both $P < 0.05$).

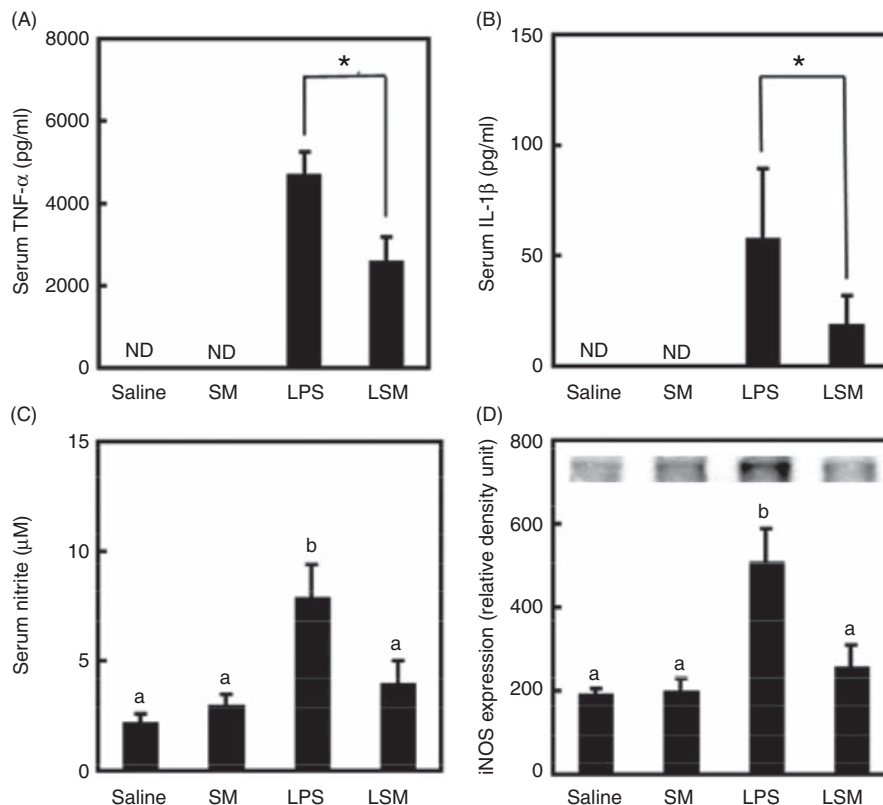


Fig. 1. The effect of sesamol (SM) on cytokine production in lipopolysaccharide (LPS)-treated mice. Twenty-four mice were divided into four groups of six. Saline group mice were given one subcutaneous (s.c.) injection of saline (1 ml/100 g of body weight); SM group mice were given one injection of SM (10 mg/kg; s.c.); LPS group mice were given one intraperitoneal (i.p.) injection of LPS (5 mg/kg); and LSM group mice were given one injection each of LPS plus SM. Blood was collected 1 h and 3 h after the LPS injection and analyzed for serum TNF- α (A) and IL-1 β (B). Serum nitrite levels (C) were determined in all mice 6 h after the LPS injection. Using Western blotting, iNOS expression (D) was assessed in the leukocytes of three arbitrarily selected mice 6 h after the LPS injection. Data are expressed as mean values \pm SD. Differences between groups were analyzed using Student's *t*-test or one-way analysis of variance (ANOVA). * $P < 0.05$ compared to the LPS group. ND, not detectable.

Sesamol down-regulated nitric oxide production in LPS-treated mice

We determined the effect of sesamol on nitrite production in serum and iNOS protein expression in leukocytes. Sesamol significantly reduced both serum nitrite levels compared to the LPS group (Fig. 1C) and leukocyte iNOS expression in LPS-treated mice (Fig. 1D) (both $P < 0.05$).

Sesamol down-regulated the release of pro-inflammatory mediators in LPS-treated macrophages

We assessed the effect of sesamol on the release of LPS-induced pro-inflammatory mediators in RAW 264.7 cells. Sesamol significantly and dose-dependently inhibited the release of LPS-induced TNF- α (Fig. 2A)

and IL-1 β (Fig. 2B; both $P < 0.05$). Sesamol also inhibited LPS-induced nitrite production (Fig. 2C) and iNOS protein expression (Fig. 2D) (both $P < 0.05$).

Sesamol did not affect macrophage viability

To examine the effect of sesamol on the main source of pro-inflammatory mediators during inflammation, we assessed the effect of sesamol on LPS-activated RAW 264.7 macrophage viability. There was no difference in macrophage viability between the LPS and LSM groups (Fig. 3).

Sesamol inhibited NF- κ B transduction and I κ B phosphorylation in LPS-treated macrophages

To examine what we hypothesize is the mechanism involved in sesamol-associated inhibition of

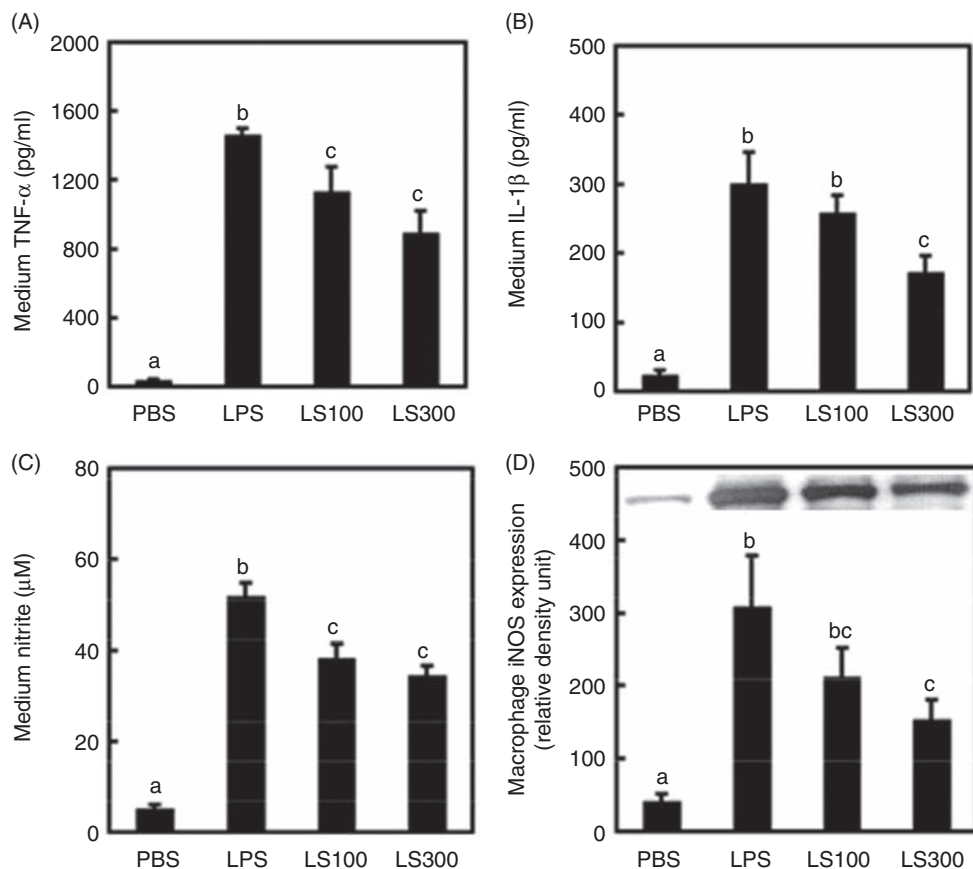


Fig. 2. The effect of SM (see Fig. 1 caption for abbreviations) on cytokine production in LPS-treated macrophages. RAW 264.7 macrophages were divided into four groups of six cells: PBS group macrophages were treated only with phosphate-buffered saline (PBS); LPS group macrophages were treated only with LPS (100 ng/ml); LS100 group macrophages were treated with LPS plus SM (100 μ M); and LS300 group macrophages were treated with LPS plus SM (300 μ M). Tumor necrosis factor- α (A), IL-1 β (B), and nitrite (C) production as well as iNOS expression (D) was assessed 24 h after LPS treatment ($n = 6$). Inducible nitric oxide synthase Western blotting data are shown as relative density units ($n = 3$). Data are expressed as mean values \pm SD. Differences between groups were analyzed using one-way ANOVA. Differences between treatments with different letters are statistically significant ($P < 0.05$).

pro-inflammatory mediator release, we assessed NF- κ B translocation and I κ B phosphorylation to evaluate the activation of the NF- κ B pathway. Sesamol significantly inhibited LPS-induced NF- κ B translocation (Fig. 4A) and I κ B phosphorylation (Fig. 4B) in macrophages (both $P < 0.05$).

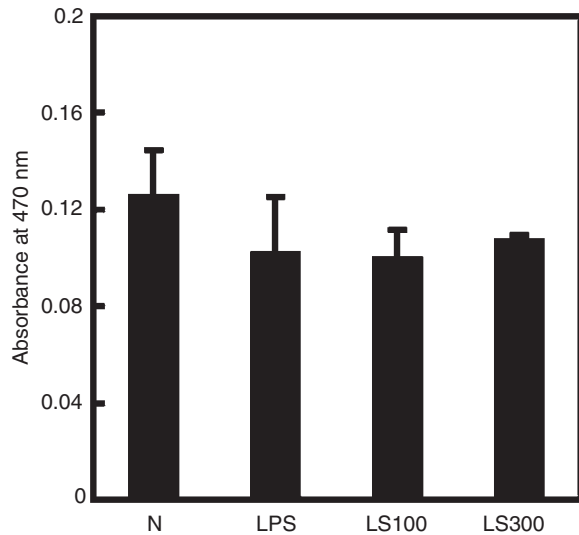


Fig. 3. Effect of SM (see Fig. 1 caption for abbreviations) on LPS-treated macrophage cell viability. RAW 264.7 macrophages were divided into four groups: N group macrophages were treated only with PBS; LPS group macrophages were treated only with LPS (100 ng/ml); LS100 group macrophages were treated with LPS plus SM (100 μ M); and LS300 group macrophages were treated with LPS plus SM (300 μ M). Cell viability was assessed 24 h after LPS treatment. Data are expressed as mean values \pm SD ($n = 6$). Differences between groups were analyzed using Student's t -test and compared with the LPS group.

DISCUSSION

Sesamol down-regulated the release of the LPS-induced pro-inflammatory mediators TNF- α , IL-1 β , and nitrite in mice and in RAW 264.7 macrophages. Further, sesamol down-regulated I κ B phosphorylation and NF- κ B translocation in LPS-treated RAW 264.7 cells. We hypothesize that the anti-inflammatory effects of sesamol occur because it partially blocks the NF- κ B pathway.

Sesamol's anti-inflammatory property might be responsible for its protection against experimental sepsis. During sepsis, inflamed cells produce various pro-inflammatory mediators, such as TNF- α , IL-1 β , IL-6, eicosanoids, platelet activating factor, nitric oxide, and reactive oxygen species.^{17–22} Tumor-necrosis factor- α , IL-1 β and nitric oxide are typical pro-inflammatory mediators; they are important for initiating systemic inflammation and sepsis-associated organ injury and death.^{17–22} Inhibiting the release of pro-inflammatory mediators attenuates the severity of sepsis.^{6,23–25} Because sesamol down-regulated the production of TNF- α , IL-1 β and nitric oxide in serum, and the expression of iNOS in leukocytes, and because it attenuated multiple organ injury and mortality in septic¹² and endotoxemic¹³ rats, inhibiting pro-inflammatory mediators may be important in the protection sesamol provides against sepsis.

Inhibiting NF- κ B activation may be important for sesamol's anti-inflammatory effect. LPS induces NF- κ B activation, which, in turn, induces the release of pro-inflammatory mediators in inflamed cells.^{5,26} Sesamol down-regulated the release of TNF- α , IL-1 β and nitric

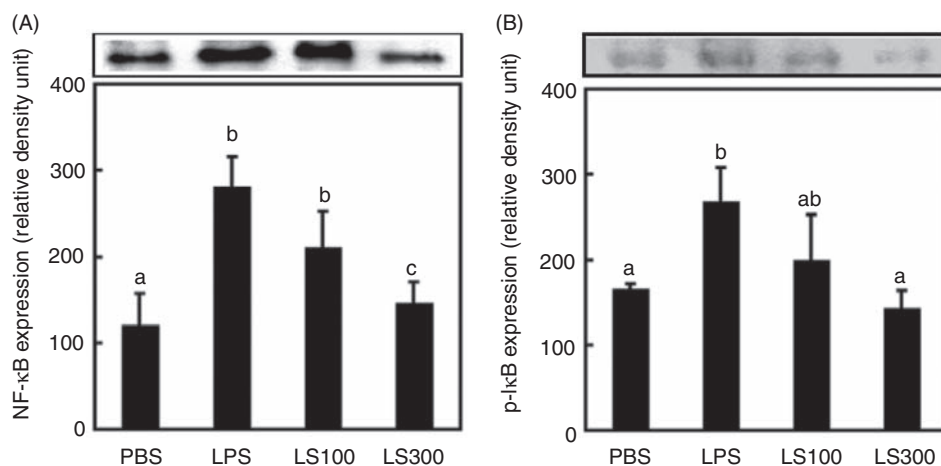


Fig. 4. The effect of SM on NF- κ B translocation and I κ B- α phosphorylation in nuclear extracts in LPS-treated macrophages. RAW 264.7 macrophages were divided into four groups of three cells. The PBS group macrophages were treated only with PBS; LPS group macrophages were treated only with LPS (100 ng/ml); LS100 group macrophages were treated with LPS plus SM (100 μ M); and LS300 group macrophages were treated with LPS plus SM (300 μ M). NF- κ B translocation (A) and I κ B- α phosphorylation (B) in nuclear extracts were assessed 24 h after LPS treatment. Protein Western blotting data are shown as relative density units. Data are expressed as mean values \pm SD. Differences between groups were analyzed using one-way ANOVA. Differences between treatments with different letters are statistically significant ($P < 0.05$).

oxide, and inhibited iNOS expression in LPS-treated macrophages and primary peritoneal macrophages (data not shown) without affecting macrophage viability. Further, sesamol suppressed NF- κ B translocation to the nucleus and down-regulated I κ B phosphorylation in LPS-treated macrophages. We hypothesize that sesamol, at least partially, blocks the NF- κ B pathway, thereby down-regulating LPS-induced inflammation. However, the mechanism of sesamol uses against sepsis requires more investigation.

Mechanisms other than anti-oxidation may be also involved in sesamol-induced inhibition of the LPS-induced inflammatory response. Although it has been suggested that oxidative stress is involved in the pathogenesis of LPS-associated organ injury,²⁷ it is not the only mechanism involved in LPS-induced inflammatory response. Some antioxidants such as *N*-acetylcysteine (NAC)²⁷ and melatonin²⁸ inhibit LPS-induced inflammation; however, other antioxidants, such as tocopherol²⁹ and dimethyl sulfoxide,³⁰ do not. Therefore, in addition to its anti-oxidative effect, other mechanisms may be also involved in sesamol's anti-inflammatory effect. On the other hand, LPS receptor activation and subsequent pro-inflammatory cytokine release may be important in LPS-associated inflammation. Therefore, in addition to its anti-oxidative effects, sesamol might reduce LPS-induced inflammation using some other mechanisms. More investigation is required to confirm this.

CONCLUSIONS

Sesamol reduces the LPS-induced inflammatory response by interfering, at least partially, with the NF- κ B pathway, which down-regulates TNF- α , IL-1 β and nitrite levels in mice.

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