Suppressive Effect of *Punica granatum* on the Production of Tumor Necrosis Factor (Tnf) in BV2 Microglial Cells

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While the anti-oxidant properties of *Punica granatum* methanol extract (PGME) are well documented, little is known concerning the anti-inflammatory effect of *Punica granatum*. PGME was pretreated in BV2 microglial cells and cells were stimulated to induce inflammation by lipopolysaccharide (LPS). The effect of PGME on the production and expression of tumor necrosis factor (Tnf) was determined by enzyme-linked immunosorbent assay (ELISA), western blotting, and reverse transcription-polymerase chain reaction (RT-PCR). In addition, the expression of nuclear factor kappa b (Nfκb) was measured using an electrophoretic mobility shift assay (EMSA). By ELISA, PGME at the concentrations of 1, 5, 10, and 50 μg/ml inhibited Tnf production in LPS-stimulated cells by 30.2, 42.3, 57.6, and 88.4%, respectively, compared to LPS-stimulated cells. The EMSA-stimulated Tnf production was reduced with a dose-dependent manner. Immuno blot and RT-PCR analyses revealed that PGME of 5 and 50 μg/ml inhibited the expression of both protein and mRNA levels of Tnf compared to LPS-stimulated cells. EMSA revealed that PGME of 5 and 50 μg/ml blocked the LPS-stimulated activation of Nfκb. These data suggest that PGME may suppress LPS-stimulated Tnf production through inhibition of Nfκb in BV2 microglia cells.

**Key words** *Punica granatum*; tumor necrosis factor (Tnf); nuclear factor kappa b (Nfκb); lipopolysaccharide (LPS); BV2 microglial cell

Increasing evidence indicates that fruit and natural extracts consumption is associated with reduced risk of diseases, including cardiovascular disease, stroke, cancer and inflammation related diseases.1,2) *Punica granatum*, commonly known as pomegranate, belongs to the Punicaceae family and consumed around the world as edible fruit.3) *Punica granatum* (Pomegranate) showed various biological effects including cardiovascular protection and anti-cancer.4,5) Also, it was reported that pomegranate fruit extract possess effect of anti-carcinogenesis6,7) and skin tumorigenesis8) and pomegranate wine also inhibited production of Tnf-α.9) In several study, Pomegranate fruit extract were shown to possess antioxidant activity, its antioxidant activity was higher than well-known natural antioxidant such as vitamin E (α-tocopherol) and flavonoid.10–13)

Microglia is the immunocompetent, macrophage-like cells that reside in the central nervous system.14) However, the over-activation of microglial cells and their release of neurotoxic inflammatory mediators can also contribute to neuronal cell death in many neurodegenerative diseases.15) Therefore, controlling microglial activation may have therapeutic benefit. Upon their activation, microglial cells release a number of pro-inflammatory cytokines including tumor necrosis factor (Tnf), that have been suggested to induce tissue damage and is considered to be important mediators of the inflammatory response.16,17) The expression of these cytokines depends on the activation of the transcriptional factor, nuclear factor kappa b (Nfκb) which is a critical intracellular mediator of the inflammatory reaction.18,19)

This study was to investigate effects of PGME on the production Tnf in LPL-stimulated BV2 microglia cells.

**MATERIALS AND METHODS**

**Preparation of Extract** *Punica granatum* was purchased from Kyungdong market (Seoul, Korea) and was authenticated by College of Oriental Medicine, Semyung University. Methanol extract of *Punica granatum* (yield; 19.7% of dry weight) was obtained by 48 h maceration at room temperature and was filtered through a 0.45 μm filter (Osmonics, Minnetonka, MN, U.S.A.), lyophilized, and kept at 4 °C.

**Cell Culture and Treatment** The BV2 microglial cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mm l-glutamine, streptomycin and penicillin (Gibco, Grand Island, NY, U.S.A.). Cells were treated with PGME for the indicated time and harvested for further analysis.

**Enzyme-Linked Immunosorbent Assay (ELISA)** For the analysis of Tnf, the BV2 microglial cells were plated onto 24 well culture plates. Cells were (1×10⁵ cell/ml) serum-starvated for 2 h and were pretreated with 1, 5, 10 and 50 μg/ml of PGME for 2 h and then treated with lipopolysaccharide (LPS, 1 μg/ml). After incubation with LPS for 24 h, supernatants were collected and immediately frozen at −70 °C. Harvested supernatants were tested for Tnf by ELISA. The plates were coated overnight with 2 μg/ml anti-Tnf capture monoclonal antibody (R&D systems, Minneapolis, MN, U.S.A.) in 0.1 M Na₂HPO₄, pH 9 buffer and blocked with phosphate buffered saline (PBS)-Tween 20. A biotin-labeled 1 μg/ml anti-Tnf detecting antibody (R&D systems, Minneapolis, MN, U.S.A.) was used. The plates were developed using streptavidin- horseradish peroxidase (Vector, Burlingame, CA, U.S.A.) and 2,2-azino-bis substrate (Sigma, St. Louis, MO, U.S.A.).

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Western Blotting  Forty micrograms of proteins were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose membrane (Schleicher & Schuell, Middlesex, U.K.). The membrane was blocked with 5% skin milk in 10 mM Tris–HCl containing 150 mM NaCl and 0.5% Tween 20 (TBS-T). After brief washing with TBS-T the membrane was then incubated with primary antibody (1:1000) that recognizes Tnf protein (Cell Signaling, Beverly, MA, U.S.A.). After thorough washing with TBS-T, horseradish peroxidase-conjugated secondary antibody (New England Biolabs, Beverly, MA, U.S.A., 1:2000 dilution in TBS-T) was applied to the membrane and the blot was developed using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ, U.S.A.).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) The cells were cultured with or without PGME and/or LPS (1 μg/ml) for 8 h. Total cellular RNA was isolated by RNA isolation kit (Zymo Research, Orange, CA, U.S.A.). The levels of Tnf mRNA were determined with Reverse Transcription System (Promega, Madison, WI, U.S.A.) in a 25 μl reaction volume using 1 μg total RNA and 10 units of reverse transcriptase. Samples were incubated at 42 °C for 60 min, and the reaction was terminated by heating to 95 °C for 5 min. PCR reactions included 5 min at 94 °C for denaturation and 35 cycles (30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C), followed by incubation for 10 min at 72 °C for final extension before holding at 4 °C. Five microliter aliquot of each sample was analyzed by electrophoresis in 1.5% agarose gel in the presence of 5 ng/ml ethidium bromide and visualized under ultraviolet light.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA) Nuclear extracts from microglia cells were prepared as follows; cells (2×10⁶ cells on a 100 mm dish) were treated with 1 ml of lysis buffer (10 mM Tris–HCl, pH 7.9, 10 mM NaCl, 3 mM MgCl₂, 1% NP-40) on ice for 4 min. After 10 min of centrifugation at 15000 g, the pellet was resuspended in 50 μl of extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 1.5 mM MgCl₂, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM phenylmethyl sulfonyl fluoride (PMSF) and incubated on ice for 30 min. After centrifugation at 12000 g for 5 min, the supernatant was harvested as the nuclear protein extract and stored at −70 °C. Protein concentration was determined with a Lowry assay reagent from Bio-Rad (Hercules, CA, U.S.A.). Ten micrograms of the nuclear proteins were incubated with 3P-labeled Nfκb probe on ice for 30 min and resolved on 5% acrylamide gel.

Statistical Analysis Statistical analysis was performed using Student's t-test and one way analysis of variance (one way-ANOVA). The accepted level of significance was preset as p value <0.05. Data are represented as means±S.E.M.

RESULTS

The Production of Tnf Was Increased by LPS As shown in Fig. 1, the amount of Tnf secreted into extracellular space was gradually increased at 2, 6, and 12 h incubations. After 24 h incubation with LPS the secreted concentration of Tnf was markedly increased almost to 2 ng/ml. Since the production of LPS-stimulated Tnf was greatest at 24 h incubation, throughout the experiment, the incubation time with LPS was set to 24 h.

Production of LPS-Stimulated Tnf Was Decreased by PGME To investigate anti-inflammatory effect of PGME, we determined the production of Tnf, a potent inflammatory mediator, by ELISA (Fig. 2). The cells were pre-incubated with 1, 5, 10, or 50 μg/ml of PGME for 2 h and then with LPS (1 μg/ml) for 24 h. As shown in Fig. 2, Tnf production by PGME was significantly inhibited about 50% compared to LPS-stimulated Tnf production (100%, p<0.01). The inhibitory effect of PGME on Tnf production depends on the concentration range from 1 to 50 μg/ml. At the concentrations of 1, 5, 10, and 50 μg/ml, Tnf productions by PGME were similar to that of control. This result showed the inhibitory effect of Tnf production by PGME was observed at 50 μg/ml.

Tnf Protein Expressions Were Decreased by PGME Western blot analysis was executed to determine protein level of Tnf in BV2 microglial cells (Fig. 3). The result was that Tnf protein expressions by PGME were decreased at both 5 and 50 μg/ml. The expression level of Tnf by PGME at 50 μg/ml was similar to that of control. This result showed the inhibitory effect of PGME on the LPS-stimulated Tnf production.

Tnf mRNA Expression Was Decreased by PGME To elucidate the mechanism responsible for the inhibitory effect of PGME on Tnf production, we determined the level of Tnf mRNA by RT-PCR analysis. When the cells were pretreated with PGME for 8 h, the Tnf mRNA level induced by LPS (1 μg/ml) was greatly decreased (Fig. 4). Pretreatment of
PGME (5, 50 μg/ml) decreased Tnf mRNA expressions to near basal level.

**Nfκb Binding Activities Were Decreased by PGME**

We next examined the influence of PGME on the NfκB DNA binding activity by EMSA (Fig. 5). NfκB is a well known transcription factor to transduce extracellular signals to the increased expression of inflammatory cytokines including Tnf. As shown in Fig. 5, the incubation of cells with LPS (1 μg/ml) increased the NfκB binding activity and pretreatment of BV2 microglia cells with PGME decreased the LPS-stimulated DNA binding activity of NfκB at both 5 and 50 μg/ml. Densitometer analysis revealed that PGME at concentrations of 5 and 50 μg/ml decreased the levels of LPS-stimulated NfκB DNA binding activity to 60.8 and 89.9%, respectively.

**DISCUSSION**

The proinflammatory and anti-inflammatory immune responses of the healthy body are maintained in a carefully controlled dynamic equilibrium. Tnf, a kind of cytokines which mediate inflammation pathway, is major target of therapeutic strategy on in many chronic inflammatory conditions. The induction of inflammatory mediators is regulated by transcription factors, such as NFκB. Systemic injection of a sublethal LPS dose induces acute inflammation in susceptible strains of rats and mice. LPS triggers the generation of reactive oxygen intermediates as well as the secretion of Tnf, which activates the DNA binding ability of NFκB. It is known that Tnf production, induced by LPS, plays an important role in inflammatory conditions.

In this study, PGME decreased the production of Tnf dose-dependently at 1, 5, 10, and 50 μg/ml in LPS-stimulated microglial cells (Fig. 2). By western blot analysis, the LPS-stimulated protein expression of Tnf was decreased by PGME (Fig. 3) and the present study also showed that PGME inhibited the expression of Tnf mRNA in LPS-stimulated BV2 microglial cells (Fig. 4). These results suggest that PGME suppresses Tnf mRNA expression, and thus contributes to decreasing the production of Tnf.

Antioxidant properties of PGME have been proposed to influence beneficially on local inflammatory and tissue damaging, which were triggered by oxidative stress.

Ginko and grape seed extracts, which has high antioxidant ability, also show inflammatory inhibition effect. Ilieva et al. reported that ginkgo exerted an anti-inflammatory effect on inflammatory cells by suppressing the production of active oxygen. Vitseva et al. suggested that grape seed extracts inhibited inflammation by decreasing release of reactive oxygen intermediates.

Immune and inflammatory reactions are regulated by NFκB through the NFκB binding sites in their promoter regions. We examined whether increased Tnf production is mediated via NFκB mediated pathway by EMSA (Fig. 5) and EMSA analysis revealed that the DNA binding ability of NFκB was inhibited by PGME. In conclusion, LPS-stimulated PGME suppresses Tnf in BV2 microglial cells, the mechanism of which, at least in part, might involve the inhibition of NFκB.

**REFERENCES**


![Fig. 3. Effect of PGME on Lipopolysaccharide (LPS)-Stimulated Expression of Tnf Protein](image-url)

**B**V2 microglial cells were pretreated with PGME (5, 50 μg/ml) and treated with LPS (1 μg/ml). After 24 h of incubation, the cell lysates (40 μg/ml) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and then developed using the chemiluminescence kit. Con, control; LPS, lipopolysaccharide; PG, *Punica granatum*. Tnf, tumor necrosis factor; Actb, beta actin.

![Fig. 4. Effect of PGME on LPS-Stimulated Expression of Tnf mRNA](image-url)

**B**V2 microglial cells were pretreated with PGME (5, 50 μg/ml) and treated with LPS (1 μg/ml). After 8 h of incubation, total RNA was prepared and Reverse transcription-polymerase chain reaction (RT-PCR) was performed. The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. Con, control; LPS, lipopolysaccharide; PG, *Punica granatum*. Tnf, tumor necrosis factor; Gapdh, glyceraldehyde-3-phosphate dehydrogenase. Gapdh was used as an internal control.

![Fig. 5. Effect of PGME on Nuclear Factor κB (NfκB) DNA Binding Activity](image-url)

**B**V2 microglial cells were pretreated with PGME (5, 50 μg/ml) and treated with LPS (1 μg/ml) for 8 h. Nuclear extracts were isolated and used in an electrophoretic mobility shift assay with 32P-labeled NfκB oligonucleotide as a probe. The arrow indicates the p65 NfκB binding complex. Con, control; LPS, lipopolysaccharide; PG, *Punica granatum*; nuclear factor κB, NfκB.