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Suppressive Effect of Chlorella Methanol Extract on Oxidative Stress and NFκB Activation in RAW 264.7 Macrophages

Ji-Young Park, Hyo-Sun Lee and Young-Sun Song†

School of Food and Life Science and Biohealth Product Research Center, Inje University, Kimhae 621-749, Korea

Abstract

This study was designed to investigate whether a methanol extract of chlorella can suppress oxidative stress and nuclear factor κB (NFκB) activation in lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage cells. Treatment of RAW 264.7 cells with chlorella methanol extract (25, 50, and 100 μg/mL) significantly reduced LPS-stimulated nitric oxide production in a dose-dependent manner. Treatments with chlorella methanol extract at all concentrations also reduced thiobarbituric acid-reactive substances accumulation and enhanced glutathione level at 50 and 100 μg/mL levels. The specific DNA binding activities of NFκB on nuclear extracts in cells treated with 50 μg/mL and 100 μg/mL chlorella methanol extracts were significantly suppressed. These results suggest that chlorella methanol extract has mild antioxidative activity and the ability to suppress intracellular oxidative stress and NFκB activation.

Key words: chlorella methanol extract, oxidative stress, NFκB, NO, macrophages

INTRODUCTION

Nuclear factor κB (NFκB), inducible transcription factor, exists in a latent form in the cytoplasm of unstimulated cells, comprising a transcriptionally active dimer bound to inhibitor protein, IκB. NFκB is activated in response to various extracellular stimuli, including cytokines (1,2), lipopolysaccharide (LPS) (3), and oxidative stress (4). A recent publication on the NFκB activation pathway suggested that IκB kinase is a novel pathway to NFκB activation by LPS in monocytes (5), while reactive oxygen species (ROS) are known to be a central pathway to NFκB activation (1-4). It has been reported that NFκB is sensitive to oxidative modification of a particular cysteine at position 62 in p50, which is essential for DNA binding (6). Furthermore, antioxidants such as organosulfur compounds (7), anthocyanins (8), selenium (9), and carnosol (10) have been reported to suppress NO production in macrophages and their inhibition mechanisms are based on their ability to inhibit the activation of NFκB. Numerous animal studies have demonstrated that intraperitoneal injection of LPS elevates oxidative stress and pretreatment with antioxidants before an LPS challenge ameliorates oxidative damage, especially in decreasing the production of ROS (11-14). These studies suggest a role of oxidative stress in the intracellular signaling of LPS to activate NFκB.

Foodstuffs or food components with the capacity to attenuate oxidative stress have been extensively studied by several researchers (15,16). Free-radical scavenging, as well as anti-inflammatory and anti-tumor activities of chlorella have been demonstrated in *in vitro* studies (17-19). Chlorophyll and phenolic compounds in chlorella have been suggested as the active components with antioxidative activity (20-22), while hydrosoluble components or sterols are reported to have the anti-inflammatory activity (17,18). However, little information is available about the effect of chlorella extract on the NFκB, a ROS-sensitive transcriptional factor, which regulates the expression of inflammatory cytokines in LPS-stimulated murine macrophages. This study was performed to investigate the effect of chlorella methanol extract on the production of nitric oxide (NO), thiobarbituric acid-reactive substances (TBARS), NFκB activation, and glutathione (GSH) levels in LPS-stimulated RAW 264.7 macrophges.

MATERIALS AND METHODS

Preparation of chlorella methanol extract

Dried, powdered chlorella (*Chlorella ellipsoidea*) was obtained from Korea Chlorella Company (Kimhae, Korea). The protein content of the chlorella was 34.8%, lipid 0.5%, dietary fiber 10.6%, ash 21.1%, and carbohydrate 21.4%. Chlorella was extracted with methanol (20-fold) three times by stirring for 12 hrs, and was then evaporated in

†Corresponding author. E-mail: fdsnsong@inje.ac.kr
Phone: +82-55-320-3235, Fax: +82-55-321-0691

a rotary vaccum evaporator (Buchi B-490, Switzerland) at 45°C until dry and then dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co., USA) for the experiment.

Cell culture and treatment

The murine macrophage cell line RAW 264.7 was obtained from ATCC (#TIB-71) and cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Cells in 10 mm dishes (5×10^6 cells/dish) or 24 well plates (8×10^5 cells/well) were pre-incubated with and without indicated concentrations of chlorella methanol extract (25, 50, 100 μg/mL) for 2 h, and then incubated with LPS (2 μg/mL) for 20 h. Untreated means negative control without LPS, while control means positive control treated with LPS.

Cell viability

Cell viability was assessed by measuring the uptake of the supravital dye, neutral red, by viable cells according to the procedure of Fautz et al. (23). After culturing the cells as described previously, the medium was removed and replaced with 0.5 mL of fresh medium containing 1.14 mmol/L neutral red. After incubation for 3 h, the medium was removed and the cells were washed twice with phosphate buffered saline solution (PBS, pH 7.4). The incorporated neutral red was released from the cells by incubation for 15 min at room temperature in the presence of 1 mL of the cell lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L dithiothreitol (DTT) and Triton X-100 (1%, v/v)] containing acetic acid (1%, v/v) and ethanol (50%, v/v). To measure the dye taken up, the cell lysis products were centrifuged and absorbance of the supernatant was measured spectrophotometrically at 540 nm.

NO production

The nitrite accumulated in the culture medium as an indicator of NO production was measured according to the Griess reaction (24). One hundred μL of each medium supernatant was mixed with 50 μL of 1% sulphanilamide (in 5% phosphoric acid) and 50 μL of 0.1% naphthylenediamine dihydrochloride and then incubated at room temperature for 10 min. The absorbance at 550 nm was measured with $NaNO_2$, serial dilutions were performed and a standard curve generated and used for the determination of nitrite production.

TBARS production

Lipid peroxidation was quantified by measuring TBARS production as described by Fraga et al. (25). Two hundred μL of each medium supernatant was mixed with 400 μL of TBARS solution and then boiled at 95°C for 30 min. The TBARS values were expressed as nmol of malondialdehyde equivalents using a standard curve of 1,1,3,3-tetraethoxypropane.

Glutathione (GSH) concentration

GSH was measured by an enzymatic recycling procedure by Tietze (26), in which GSH is sequentially oxidized by 5,5″-dithiobis (2-nitrobenzoic acid) and reduced by NADPH in the presence of glutathione reductase. The medium was removed from the cultured RAW cells and the cells were washed twice with PBS. One mL of PBS was added and cells were scraped. Cell suspensions were sonicated 3 times for 5 s each time on ice and then cell sonicates were centrifuged at 4,500 rpm for 10 min. Four hundred μL of cell supernatant was mixed with 200 μL of 5% sulfosalicylic acid and then centrifuged at 4,500 rpm for 10 min. Fifty μL of supernatant was mixed with 100 μL of reaction mixture [100 mM sodium phosphate buffer with 1 mM EDTA (pH 7.5), 1 mM dithiolnitirobezene, 1 mM NADPH, 1.6 U glutathione reductase]. The rate of 2-nitro-5-thio-benzoic acid formation was monitored spectrophotometrically at 412 nm. GSH content was determined by comparision of the rate to a standard curve generated with known concentrations of GSH.

Electrophoretic mobility shift assay (EMSA)

Nuclear protein was extracted by the method of Dignam et al. (27) with slight modification. Cells in 10 mm dishes were lysed with buffer containing 0.6% igepal, 0.15 M NaCl, 10 mM Tris pH 7.9, 1 mM EDTA and 0.1% protein inhibitor cocktail, vortexed, kept on ice for 5 min, and centrifuged at 2,300 rpm for 5 min at 4°C. Pelleted nuclei were resuspended in 50 μL of extraction buffer [10 mM Hepes, pH 7.9, 0.1 mM EGTA, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 1 mM DTT, and 0.33% protein inhibitor cocktail]. Following gentle mixing and keeping on ice for 20 min, samples were centrifuged at 2,300 rpm for 5 min at 4°C. The supernatant fraction was transferred to new tubes and stored at -70°C. Protein concentration was determined by Bradford assay (28). For the EMSA, NFκB-specific oligonucleotide was end-labeled with [γ-^{32}P]-ATP using T_4 polynucleotide kinase (Promega, Madison, WI, USA) and purified using a microspin G-25 column (Amersham Inc., Piscataway, NJ, USA). EMSA was performed according to the instruction manual of Promega. Five mg of nuclear protein, binding buffer, ^{32}P-labeled NFκB, and loading buffer were incubated for 30 min at room temperature. In competition assays, 100-fold excess unlabeled NFκB were added 10 min before addition of the radio-labeled probe. For the supershift assay, nuclear proteins were incubated with 2 μg of anti-p50 and anti-p65 at room temperature. DNA-protein complexes were separated from unbound DNA probe by electrophoresis through 4% polyacrylamide gel by using 0.5×

Tris-Borate EDTA buffer as the running buffer. The gels were dried and exposed to an X-ray film for 2 h at room temperature and the bands were quantitated by phospho imager (Packard, USA).

Statistical analysis

All data are expressed as the means ± SD. The statistical analyses were performed on an SPSS program. One-way ANOVA and Duncan's multiple range test were used to evaluate the significance of differences between groups. P values <0.05 were considered significant, if not otherwise stated.

RESULTS AND DISCUSSION

Earlier research has suggested a role for ROS in LPS-stimulated NFκB activation, which was largely based on the suppression of oxidative stress and NFκB activation by antioxidants (1,3,4). This study was designed to investigate the suppressive effect of chlorella methanol extract on intracellular oxidative stress and NFκB activation in LPS-stimulated RAW 264.7 cells, a murine macrophage cell line. The antioxidant activity of chlorella methanol extract was evaluated by using it to treat LPS-stimulated RAW 264.7 cells and measuring oxidative stress markers, such as levels of NO, TBARS, and GSH. LPS treatment of cells significantly elevated NO generation to 16 times higher than in LPS-untreated negative control, which has been well-documented by several investigators (7-10,29). Chlorella methanol extract was found to suppress NO production in a dose-dependent manner ($p<0.05$) (Fig. 1). NO is involved in phagocytosis at physiological levels, but it can also lead to amplification of inflammation and tissue injury when NO is produced in large amounts. NO reacts rapidly with superoxide to form peroxynitrite, a powerful oxidant, that damages many biological molecules, including DNA, and upregulates DNA-binding activity of NFκB in macrophages activated with inflammatory stimulants (29).

Treatment with LPS for 20 h increased TBARS levels, an indicative of lipid peroxidation. However, pre-incubation with chlorella methanol extract significantly reduced the LPS induced increase in TBARS at all levels used ($p<0.05$) (Fig. 2), suggesting that lipid peroxidation was attenuated in LPS-stimulated macrophages by treatment with chlorella methanol extract. Since the GSH plays an important role in the protection of cells against oxidative stress, we also measured the level of GSH in LPS-stimulated RAW 264.7 cells. As shown in Fig. 3, exposure of RAW 264.7 cells to LPS induced a significant increase in GSH levels. Depletion of GSH by oxi-LDL treatment and recovery from the oxidative injury by the pre-incubation of aged garlic extract possessing antioxidative activity was reported in cultured endothelial cells (30). However, our finding that GSH was elevated by LPS alone was comparable with those of Yen and Lai (31), who treated RAW 264.7 cells with peroxynitrite, a powerful oxidant formed by the reaction of NO and superoxide. These observations might be validated by the finding that GSH synthesis was induced by a nano-molar concentration of NO due to enhanced expression of the rate-limiting enzyme for GSH synthesis, glutamylcysteine synthetase (32). GSH levels were further enhanced in LPS-treated RAW 264.7

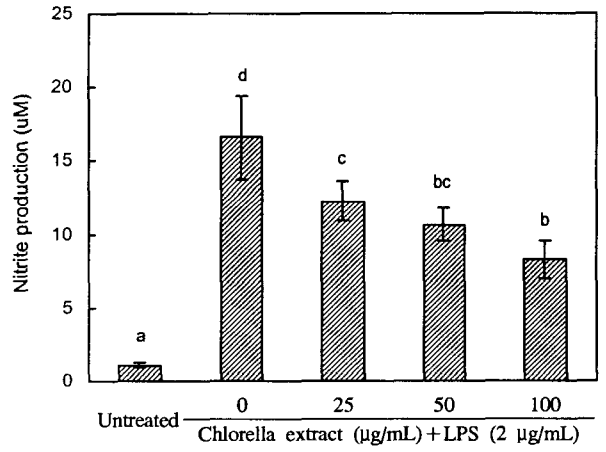

Fig. 1. Inhibitory effect of chlorella methanol extract on NO production in LPS-stimulated RAW 264.7 cells. Cells (8×10^5 /plate) in 24-well plates were pre-incubated with and without indicated concentrations of chlorella methanol extract for 2 h, and then incubated with LPS (2 μg/mL) for 20 h. Untreated is negative control without LPS treatment. Data represent the mean ± SD of triplicate experiments. A value sharing same superscript is not significantly different at $p<0.05$.

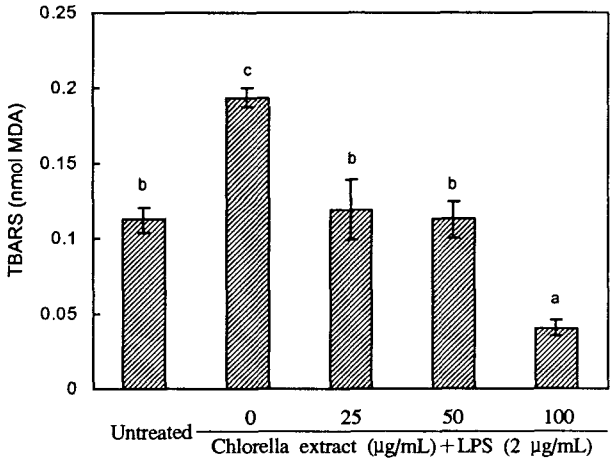

Fig. 2. Inhibitory effects of chlorella methanol extract on TBARS generation in LPS-stimulated RAW 264.7 cells. Cells (8×10^5 /plate) in 24-well plates were pre-incubated with and without indicated concentrations of chlorella methanol extract for 2 h, and then incubated with LPS (2 μg/mL) for 20 h. Untreated is negative control without LPS treatment. Data represent the mean ± SD of triplicate experiments. A value sharing same superscript is not significantly different at $p<0.05$.

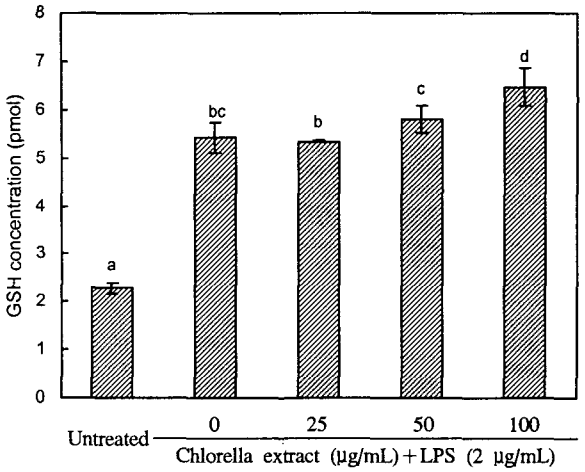

Fig. 3. Effects of chlorella methanol extract on GSH concentration in LPS-stimulated RAW 264.7 cells. Cells (5×10^6 cells) in 10 mm dishes were pre-incubated with and without indicated concentrations of chlorella methanol extract for 2 h, and then incubated with LPS (2 μg/mL) for 20 h. Untreated is negative control without LPS treatment. Data represent the mean±SD of triplicate experiments. A value sharing same superscript is not significantly different at $p<0.05$.

cells pre-incubated with 50 μg/mL and 100 μg/mL of chlorella methanol extract, as compared with that of LPS-only treated cells. Thus, an increase in GSH content by chlorella methanol extract treatment could lead to decreased intracellular oxidative stress, and thus could be part of the mechanism for the antioxidative effect of chlorella methanol extract.

Specific DNA binding of NFκB using EMSA showed that LPS treatment of RAW 264.7 cells enhanced NFκB activation (Fig. 4B, lane 2). NFκB bands induced by LPS were identified by specific and nonspecific competitors, and antibody supershift assay (Fig. 4A). Bands of the nuclear extract were competitively inhibited by a 100-fold excess of unlabelled NFκB oligonucleotide (Fig. 4A, lane 2), but not by the nonspecific oligonucleotide, sp-1 (Fig. 4A, lane 3). In the supershift assay, nuclear extracts from RAW 264.7 cells were incubated with 2 μg of antisera p50 and p65, and normal rabbit serum. As shown in Fig. 4A, normal rabbit serum did not affect complex formation (lane 6). Antibody specific for the p50 and p65 subunit of NFκB supershifted the upper band but not the lower band (lane 4 and 5), indicating that the upper band is active NFκB composed of p65 and p50 subunits. The lower band could represent inactivated NFκB bound with IκB or the monomeric p52 component of NFκB (33).

Pre-incubation of cells in the presence of chlorella methanol extract at 25~100 μg/mL did not show an inhibitory effect on NFκB activity in a dose-dependent manner. Chlorella methanol extract at 50 μg/mL and 100 μg/mL inhibited the activation of NFκB induced by LPS significantly (Fig. 4B and 4C). The concentration of chlorella methanol

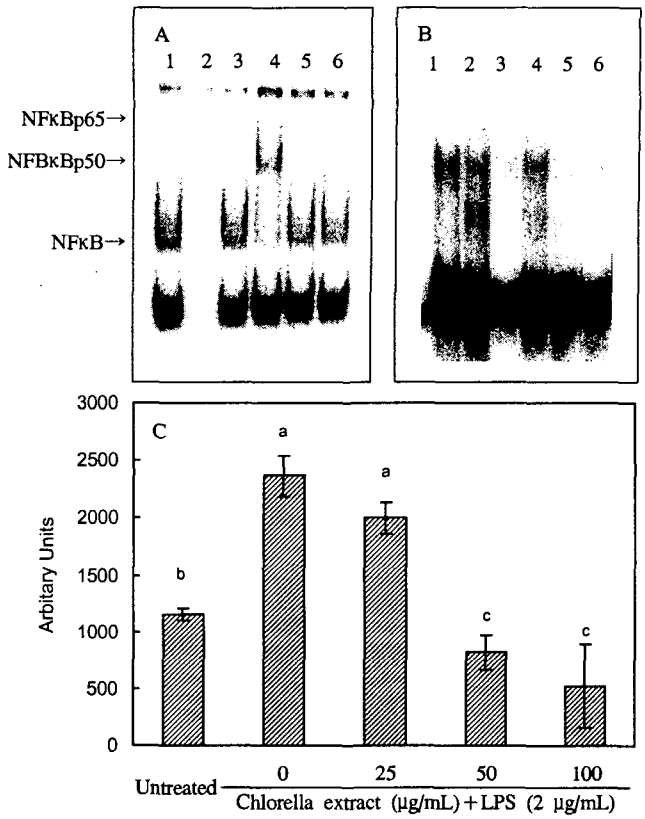

Fig. 4. Suppressive effect of chlorella methanol extract on NFκB activation in LPS-stimulated RAW 264.7 cells. (A) competitive and supershift assay. Lane 1=Nuclear extract (NE) from RAW cells without LPS treatment, Lane 2=NE incubated with 100-fold NFκB specific oligonucleotide, Lane 3=NE incubated with nonspecific oligonucleotide sp-1, Lanes 4~6=NE incubated with anti-p50, anti-p65, and normal rabbit sera, respectively. (B) Cells (5×10^6 cells) in 10 mm dishes were pre-incubated with and without indicated concentrations of chlorella methanol extract for 2 h, and then incubated with LPS (2 μg/mL) for 20 h. Lane 1=Untreated, Lane 2=Control, Lane 3=Control NE incubated with 100-fold NFκB specific oligonucleotide, Lane 4~6=NE from chlorella methanol extract 25 μg/mL, 50 μg/mL, and 100 μg/mL treatments, respectively. (C) Arbitary units of NFκB activation. Untreated is negative control without LPS treatment. Data represent the mean±SD of triplicate experiments. A value sharing same superscript is not significantly different at $p<0.05$.

extract used in this study did not affect cell viability. We used 5 μg of nuclear protein extracted from macrophages treated with different levels of chlorella methanol extract and LPS. Thus, the decreased level of NFκB activity is not due to cell death. Based on the observations made in this study, the inhibitory effect of chlorella methanol extract on NFκB activation induced by LPS in RAW 264.7 cells can be explained by the attenuated intracellular oxidative stress in the signaling pathway of LPS leading to NFκB activation. Indeed, the suppressive effect of chlorella methanol extract on NFκB activation at 50 μg/mL and 100 μg/mL coincides with attenuated NO, TBARS, and elevated GSH generations at these concentrations.

Previous studies also observed antioxidant activities of chlorella extract (20,21). Furthermore, it has been reported that carnosol (7), an antioxidant in rosemary, suppresses NFκB activation by LPS; and supplementation of epigallocatechin-3-gallate (34) and vitamin E (13) suppressed oxidative stress in macrophages and brain of rats. These studies suggest that suppressed NO production and NFκB activation by chlorella treatments might be attributed in part to the antioxidant properties of chlorella (20,21,35). Several lines of evidence have suggested that the anti-inflammatory and free radical scavenging activities of chlorella may be due to the presence of chlorophyll (21,22), sterols (17), or phenolic compounds (20,22). On the contrary, Guzman et al. (18) reported that hydrophilic components present in water extract of chlorella exhibited anti-inflammatory and free radical scavenging activities. However, water extracts of chlorella showed no inhibitory effects on NFκB in this study (data not shown). Based on this evidence, we postulated that several compounds in chlorella, both lipophillic and hydrophilic, may combat oxidative stress.

In summary, chlorella methanol extract may attenuate intracellular oxidative stress by directly scavenging NO and peroxide radicals, which reduces the consumption of cellular GSH and results in the suppression of NFκB activation by LPS.

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