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Repression of Proinflammatory Gene Expression by Lipid Extract of *Nostoc commune* var *sphaeroides* Kützing, a Blue-green Alga, via Inhibition of Nuclear Factor-κB in RAW 264.7 Macrophages

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Abstract

We investigated whether lipid extract from a blue-green alga, *N. commune*, modulates proinflammatory gene expression in RAW 264.7 macrophages. The cells were incubated with *N. commune* lipid extract (0–100 μg/mL) and subsequently activated by LPS (100 ng/mL). Quantitative real-time PCR analysis showed that mRNA abundance of proinflammatory mediators, including TNF-α, COX-2, IL-1β, IL-6, and iNOS, was significantly reduced by *N. commune* lipid extract in a dose-dependent manner. Secretion of TNF-α and IL-1β into cell culture medium was also significantly decreased by *N. commune* lipid extract. Thin-layer chromatography-densitometry analysis showed that *N. commune* lipid extract contained approximately 15% of fatty acids. To determine whether the inhibition

of proinflammatory mediator production by *N. commune* lipid extract is primarily conferred by fatty acids in the lipid extract, macrophages were incubated with 100 μg/mL of *N. commune* lipid extract or 15 μg/mL of a fatty acid mixture, which was formulated to reflect the fatty acid composition of *N. commune* lipid extract. The fatty acid mixture significantly reduced RNA abundance of TNF-α and COX-2, but to a lesser extent than did the *N. commune* lipid extract, suggesting the presence of additional bioactive compounds with an anti-inflammatory property in the lipid extract. As NF-κB is a major regulator for the proinflammatory gene expression, we measured its DNA-binding activity. DNA-binding activity of NF-κB was significantly reduced by *N. commune* lipid extract. In conclusion, our study suggests that *N. commune* lipid extract represses the expression of proinflammatory genes in RAW 264.7 macrophages, at least in part, by inhibiting the activation of NF-κB pathway.

Keywords: blue-green algae, *Nostoc commune* var *sphaeroides*, anti-inflammatory, NF-κB, RAW 264.7 macrophages

Abbreviations: COX-2, cyclo-oxygenase 2; iNOS, inducible nitric oxide synthase; IL-1β, interleukin-1β; IL-6, interleukin-6, LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; *N. commune*, *Nostoc commune* var *sphaeroides* Kützing; TNF-α, tumor necrosis factor-α

1. Introduction

Chronic inflammation can result in an elevated risk of various chronic inflammatory diseases such as atherosclerosis, rheumatoid arthritis, chronic obstructive pulmonary disease, asthma, and inflammatory bowel disease. Pharmaceutical anti-inflammatory drugs are generally used to treat these diseases. In general, the anti-inflammatory drugs reduce inflammatory response by suppressing the production of proinflammatory mediators, such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and nitric oxide, which are involved in the pathogenesis of inflammatory diseases [1,2]. However, reports on the increased risk of cardiovascular disease with long-term use of several nonsteroidal anti-inflammatory drugs have raised concerns over using the drugs [3]. In addition, increasing the public's preference for natural and herbal medicines to drug therapies in preventing and treating chronic inflammatory diseases has prompted the discovery and development of new bioactive natural products with anti-inflammatory properties.

Blue-green algae, also termed *cyanobacteria*, are among the most primitive forms of life on Earth [4]. Bioactive compounds isolated from blue-green algae have been shown to be neuroprotective, cytotoxic, antibacterial, antifungal, antiviral, and anti-inflammatory [5–9]. Edible blue-green algae, such as *Spirulina* and *Aphanizomenon flos-aquae*, are currently marketed as dietary supplements with various health claims for immune function, inflammation, heart disease, and general well-being. Concerns over naturally grown blue-green algae, however, have been raised because of contamination from algal toxins and heavy metals. During the harvesting of natural blue-green algae, they are easily contaminated with toxin-producing algae such as *Microcystis aeruginosa*, which generates microcystins, potent hepatotoxins [10]. In addition, the quality of naturally grown blue-green algae can vary depending on the growth environments; for example, altitude, temperature, and sun exposure [11].

To develop a new natural source with health-enhancing properties, an edible blue-green alga, *Nostoc commune* var *sphaeroides* Kützing (*N. commune*), was cultivated. *N. commune* has been used as a food delicacy or herbal medicine in Asian, African, and South American countries for centuries [12]. *N. commune* has been used as an ingredient of Chinese medicine since the Eastern Jin Dynasty (317–420 AD) as recorded in *The Supplement to Compendium of Materia Medica* [13]. It has been historically suggested that *N. commune* can treat a variety of medical conditions, including inflammation, night blindness, digestion, burns, anxiety, indigestion, and chronic fatigue. In addition, a hypocholesterolemic effect of *N. commune* was suggested in rats fed *N. commune*, which was attributed to its high content of dietary fibers [14]. Although studies have also demonstrated various health benefits from *N. commune*, including cholesterol lowering, antiviral, anticancer, and anti-inflammatory activities [8,14–19], a mechanistic investigation on the biological functions of *N. commune* is very limited.

In the present study, we intended to elucidate the molecular mechanisms responsible for an anti-inflammatory function of cultivated *N. commune*, which is free of contamination of toxins and whose quality can be controlled by culture conditions. Our hypothesis was that lipid extract of *N. commune* exerts an anti-inflammatory property by repressing the expression of proinflammatory genes through the inhibition of nuclear factor-κB (NF-κB), a major transcription factor for proinflammatory response. As macrophages play an important role in the innate immune system, we performed our experiments using murine RAW 264.7 macrophages, as the macrophages are widely used as an in vitro model for studies on inflammatory pathways. Our data indicate that lipid extract of *N. commune* reduced the production of proinflammatory mediators, such as TNF-α, IL-1β, cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS), and IL-6. This repression is mediated, at least in part, by inhibiting the activation of NF-κB. Our study provides, for the first time, scientific support for *N. commune* to be developed as a nutritional food to reduce the risk of inflammatory diseases.

2. Methods and materials

2.1. Preparation of **N. commune** *lipid extract*

Fresh *N. commune* was obtained from Algaen Corporation (Winston Salem, North Carolina). *N. commune* was freeze-dried and ground for lipid extraction using the Bligh-Dyer method [20]. Briefly, 42 mL of chloroform/methanol (1:2 vol/vol) was added to approximately 3 g of dry *N. commune* and vortexed. After the addition of 14 mL of chloroform and 10.6 mL of distilled water, the sample was mixed and filtered using a Whatman filter no. 42 (Whatman, Inc., Ann Arbor, Michigan) to remove solids. Subsequently, 6.3 mL of 0.05% H_2SO_4 was added, and samples were vortexed. To separate phases, samples were centrifuged at 500 × *g* for 5 minutes and the lower liquid phase was recovered. Lower phase was dried to completion under nitrogen, weighed for lipid mass, and resuspended in chloroform. Tubes containing lipid extract were purged with nitrogen and tightly sealed. The lipid extract was stored at −80°C with an oxygen absorber Ageless (Mitsubishi Gas Chemical America, New York, New York) to prevent oxidation until use. To incorporate the lipid

extract into cell culture medium, chloroform was evaporated under nitrogen, and medium was added for subsequent sonication at room temperature for 5 minutes.

2.2. Compositional analysis of **N. commune** *lipid extract*

The simple lipids of *N. commune* lipid extract were determined by thin-layer chromatography (TLC)–densitometry using a solvent system of hexane/diethyl ester/acetic acid (85:15:2 by volume) as previously described [21]. Images of the plates were obtained with a Kodak Gel Logic 440 imaging system and Kodak ID image analysis software (Kodak, Rochester, New York), and the sample spots were compared against external standards for migration and net band intensity.

Fatty acid composition of *N. commune* lipid extract was determined by gas chromatography. An aliquot of *N. commune* lipid extract (2 mg in chloroform) was dried under nitrogen. Fatty acid methyl esters were prepared by the method of Metcalfe et al [22] and quantified using a 0.25 mm × 100 m CP-Sil-88 capillary column (Chrompack, Raritan, New Jersey) under the following conditions: initial temperature, 180°C for 20 minutes, increased to 250°C at 5°C/min; injector temperature, 270°C; flame ionization detector temperature, 300°C; helium carrier gas; and split ratio of 50:1.

The carotenoid content of lipid extract was determined by high-performance liquid chromatography as previously described [23].

2.3. Cell culture and treatment

Murine RAW 264.7 macrophages (ATCC no. TIB-71) were maintained in RPMI 1640 containing 10% FBS, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 2 × vitamins (from 100 × vitamin solution, MediaTech, Herndon, Virginia), and 2 mmol/L L-glutamine in a humidified chamber at 37°C with 5% carbon dioxide. All cell culture supplies were purchased from MediaTech.

Cells were plated at a density of 6×10^5 μg cells in a well of 12-well plates. When cells became approximately 90% confluent, increasing concentrations of *N. commune* lipid extract (0–100 μg/mL) were added to culture medium for 12 hours, after which 100 ng/mL of lipopolysaccharide (LPS) (*Salmonella enterica* serotype typhimurium; Sigma-Aldrich, St. Louis, Missouri) was added to the cell medium to activate cells for 18 hours. In separate experiments, cells were incubated with either *N. commune* lipid extract (100 μg/mL) or a fatty acid mixture (15 μg/mL), consisting of 26% palmitic acid, 24% palmitoleic acid, 7% oleic acid, 16% linoleic acid, and 25% linolenic acid, for 12 hours. After the incubation, LPS (100 ng/mL) was added to activate macrophages for 18 hours. Fatty acids were complexed with bovine serum albumin (BSA, approximate molar ratio of BSA/fatty acid = 1:2.5), and therefore, the same amount of BSA was added to all the treatments. For all cell culture experiments, cells that were not incubated with *N. commune* lipid extract served as a control.

2.4. Cytotoxicity measurement

Cytotoxicity of *N. commune* lipid extract was determined using In Cytotox crystal violet dye elution (CVDE) kit (Aniara, Mason, Ohio) according to the manufacturer's instructions. In brief, RAW 264.7 macrophages were plated at a density of 25,000 cells per well in

a 96-well plate and incubated for 24 hours, after which 0 to 200 μg/mL of *N. commune* lipid extract was added for 24 hours. Cells were washed twice with 200 μL per well of wash solution CVDE I and 100 μL of labeling solution CVDE II was added to each well. After incubation for 10 minutes at room temperature, the cells were washed 4 times with distilled water. The plate was air dried, and the cell layer was dissolved with 100 μL per well of solubilization solution CVDE III. The absorbance was measured at 540 nm. Sodium dodecyl sulfate (SDS; 0.5 mmol/L) was used as a positive control for cytotoxicity. Data are expressed as viability (%) relative to control that was not incubated with *N. commune* lipid extract.

2.5. Total RNA isolation and quantitative real-time PCR

Cells were washed twice with cold phosphate buffered saline (PBS) and 1 mL of Trizol reagent (Invitrogen, Carlsbad, California) was added to each well of a 12-well plate to isolate total RNA following the manufacturer's protocol. One microgram of total RNA was treated with DNase I (Promega, Madison, Wisconsin) to remove genomic DNA contamination, and subsequently, RNA samples were reverse transcribed by MMLV reverse transcriptase (Promega, Madison, Wisconsin). Realtime PCR analysis was performed using the Sybr Green procedure and an ABI 7300 instrument (Applied Biosystems, Foster City, California). Primers for TNF-α, COX-2, IL-1β, IL-6, iNOS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed according to GenBank database using the Primer Express 3.0 software provided by ABI and listed in Table 1. Expression of mRNA values was calculated using the threshold cycle (Ct) value, that is, the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed threshold. For each sample, $\Delta C_{t,\,sample}$ was calculated by subtracting the Ct value of GAPDH, a housekeeping gene, from that of each gene of interest to normalize the data. The expression levels relative to control were estimated by calculating $\Delta\Delta C_t(\Delta C_{t,\,sample} - \Delta C_{t,\,control})$ and subsequently using the $2^{-\Delta\Delta Ct}$ method [24].

Table 1. Real-time PCR primers

Gene	Forward primer	Reverse primer
TNF-α	5′-GGCTGCCCCGACTACGT-3′	5′-ACTTTCTCCTGGTATGAGATAGCAAAT-3′
IL-1β	5′-GTCACAAGAAACCATGGCACAT-3′	5′-GCCCATCAGAGGCAAGGA-3′
COX-2	5′-AAAGGTTCTTCTACGGAGAGAGTTCA-3′	5′-TGGGCAAAGAATGCAAACATC-3′
IL-6	5′-CTGCAAGAGACTTCCATCCAGTT-3′	5′-AGGGAAGGCCGTGGTTGT-3′
iNOS	5′-GCAGCTGGGCTGTACAAA-3′	5′-AGCGTTTCGGGATCTGAAT-3′
GAPDH	5′-TGTGTCCGTCGTGGATCTGA-3′	5′-CCTGCTTCACCACCTTCTTGAT-3′

2.6. Enzyme-linked immunosorbent assay for cytokines

Concentrations of TNF-α and IL-1β in cell medium were determined by commercial mouse enzyme-linked immunosorbent assay (ELISA) kits (eBiosciences, San Diego, California) following the manufacturer's protocols.

2.7. Nuclear extract preparation and NF-κB activity measurement

RAW 264.7 macrophages were incubated without or with *N. commune* lipid extract (100 μg/mL) followed by activation with 100 ng/mL of LPS for 1 hour. Cells were then washed twice with cold PBS, and nuclear fraction was prepared using Nuclear Extract kit (Active Motif, Carlsbad, California) following the manufacturer's instruction. Protein concentrations of samples were measured by BCA assay (Pierce, Rockford, Illinois) using BSA as a standard.

DNA-binding activity of p65 was assessed using an ELISA-based TransAM NF-κB p65 assay kit (Active Motif). Briefly, 20 μL of complete lysis buffer containing 3 μg of nuclear extract was added to a well of a 96-well plate coated with immobilized NF-κB consensus oligonucleotides and incubated for 1 hour with slow agitation. Primary NF-κB antibody was added and incubated for 1 hour, after which wells were washed 3 times with a washing buffer. Horseradish peroxidase-conjugated secondary antibody was added, and after 1-hour incubation, wells were washed 4 times with a washing buffer. Color was developed and optical density was measured by a spectrophotometer (Bio-Tek, Winooski, Vermont) at 450 nm with a reference wavelength of 655 nm. Positive and negative controls were run simultaneously to validate the assay.

2.8. Statistical analysis

Treatment effects were analyzed by one-way analysis of variance (one-way ANOVA), and statistically significant differences among treatments were identified using Tukey's pairwise comparison using GraphPad InStat 3 (GraphPad Software, Inc., San Diego, California). Differences were considered significant at $P < .05$. Data are expressed as means ± SEM.

3. Results

3.1. Lipid composition of **N. commune** *lipid extract*

Extractable lipid compounds present in *N. commune* were determined by TLC-densitometry. The lipid extract contained pigments, free fatty acids, triacylglycerol, vitamin E, and wax esters/steryl esters (Fig. 1). Although a large percentage of pigments were observed in this lipid extract, identification of the pigments was beyond the scope of this study. The fatty acid composition of lipid extract was approximately 26% palmitic acid (16:0), 24% palmitoleic acid (16:1), 16% linoleic acid (18:2), and 25% linolenic acid (18:3) (Table 2). Assuming that carotenoids contributed to the high level of pigmentation in the lipid extract, we attempted to quantify several carotenoids, including α-carotene, β-carotene, lutein, lycopene, cryptoxanthin, and zeaxanthin. Only β-carotene was detected with a concentration of 70 mg/100 g dry weight.

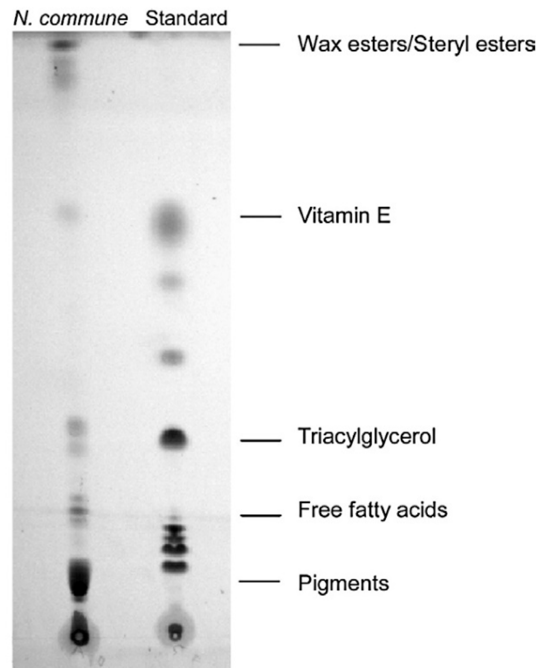

Figure 1. Lipid analysis of *N. commune* lipid extract by TLC-densitometry. Lipids of *N. commune* and lipid standards were fractionated on a silica gel plate and developed using hexane-diethyl ether-acetic acid (85:15:2, by volume). Bands were visualized by submerging the plate in a solution of 10% cupric sulfate and 8% phosphoric acid and subsequently by charring the dried plate at 165°C for 10 minutes. The TLC standard for lipids is shown in the right.

Table 2. Fatty acid composition of *N. commune* lipid extract[a]

Fatty acid	% of total fatty acids
14:0	0.62 ± 0.02
16:0	25.7 ± 0.38
16:1 Δ9	24.1 ± 0.40
18:0	2.2 ± 0.23
18:1 Δ9	4.3 ± 0.16
18:1 Δ11	2.2 ± 0.03
18:2	15.6 ± 0.18
18:3	25.3 ± 0.18

Values are mean ± SEM (n = 6).

a. Fatty acid composition of *N. commune* lipid extract was determined by GC analysis.

3.2. Cytotoxicity of **N. commune** *lipid extract in RAW 264.7 macrophages*

Cytotoxicity of *N. commune* lipid extract was assessed by incubating RAW 264.7 macrophages with increasing concentrations (0–200 μg/mL) of the lipid extract for 24 hours. Cells incubated with 25 to 200 μg/mL of *N. commune* lipid extract showed a comparable viability

with control (Fig. 2). This result indicates that *N. commune* lipid extract is not cytotoxic to RAW 264.7 macrophages within this range.

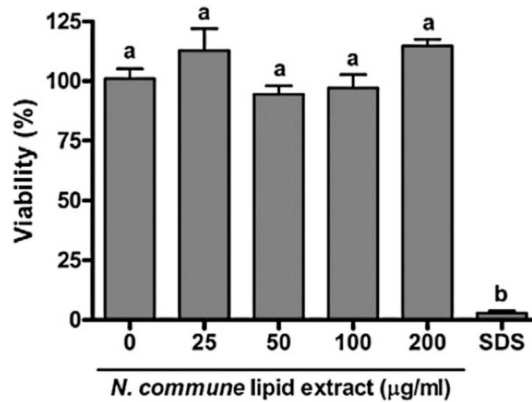

Figure 2. Cytotoxicity of *N. commune* lipid extract. RAW 264.7 macrophages were incubated with 0 to 200 μg/mL of *N. commune* lipid extract or 0.5 mmol/L SDS as a positive control for cytotoxicity for 24 hours. Cell viability was determined using In Cytotox crystal violet dye elution kit. Data are expressed as viability (%) relative to control that was not incubated with *N. commune* lipid extract. Bars without a common letter are significantly different ($P < .05$) using one-way ANOVA and Tukey's pairwise comparison. Values are means ± SEM ($n = 4–5$).

3.3. Repression of the expression of proinflammatory mediators by N. commune lipid extract

Despite that health benefits from *N. commune* have been recognized in Chinese medicine, there are very few studies in the scientific literature that have addressed this issue. To investigate whether lipid extract from *N. commune* could decrease the expression of proinflammatory mediators in RAW 264.7 macrophages, we incubated the cells with increasing concentrations of *N. commune* lipid extract (0–100 μg/mL) for 12 hours. Subsequently, the cells were activated with LPS (100 ng/mL), a component of gram-negative bacterial cell wall, for an additional 18 hours. *N. commune* lipid extract reduced mRNA abundance of proinflammatory mediators, including TNF-α, COX-2, IL-1β, IL-6, and iNOS, in a dose-dependent manner (Fig. 3A). Consistent with mRNA levels, secretion of TNF-α and IL-1β into culture medium was also significantly reduced by *N. commune* lipid extract (Fig. 3B). *N. commune* lipid extract appears to contain bioactive compounds that can antagonize the production of LPS-induced proinflammatory mediators.

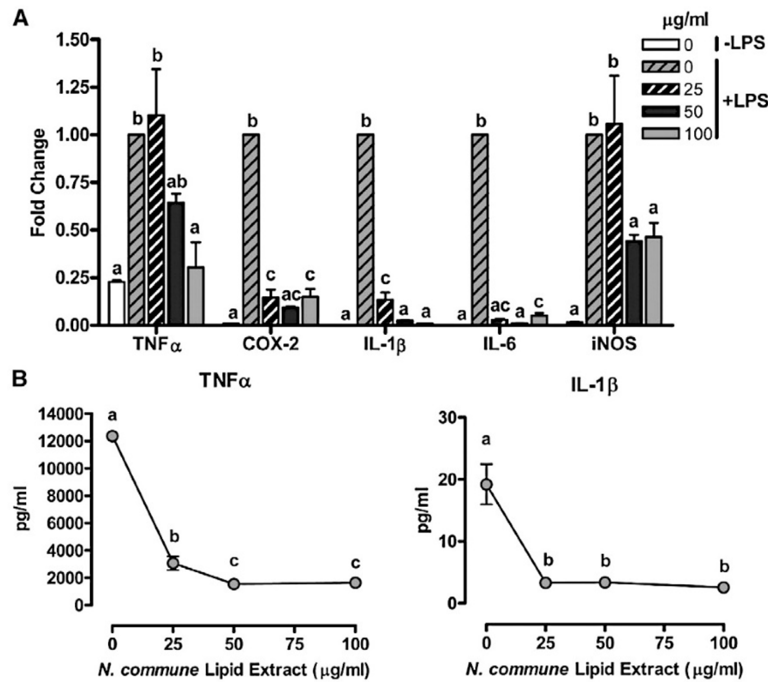

Figure 3. *N. commune* lipid extract inhibited the production of proinflammatory mediators in LPS-activated RAW 264.7 macrophages. Cells were incubated with increasing concentrations of *N. commune* lipid extract (0–100 μg/mL) for 12 hours, and LPS (100 ng/mL) was added to activate macrophages for 18 hours. (A) Real-time PCR analysis for mRNA expression of proinflammatory genes. Data are expressed as fold change relative to control treated with LPS. Bars without a common letter are significantly different ($P < .05$). Values are means ± SEM (n = 4–5). (B) Cytokine concentrations. TNF-α and IL-1β concentrations in the medium collected after the incubation with *N. commune* lipid extract and LPS were determined by ELISA. Data without a common letter are significantly different ($P < .05$) using one-way ANOVA and Tukey's pairwise comparison. Values are means ± SEM ($n = 6$).

3.4. Effect of fatty acid mixture on the expression of proinflammatory mediators

Palmitoleic acid, linoleic acid, and linolenic acid, which consist of approximately 75% of total fatty acids in *N. commune* lipid extract, inhibited the expression of proinflammatory genes in RAW 264.7 macrophages, whereas palmitic acid increased the expression of those genes (unpublished data). To address whether the unsaturated fatty acids in *N. commune* lipid extract play a major role in the reduced production of proinflammatory mediators by *N. commune*, we formulated a fatty acid mixture that reflects the same fatty acid composition of *N. commune* lipid extract. TLC-densitometry analysis showed that the *N. commune* lipid extract contains approximately 15% fatty acids (Fig. 1). Therefore, we incubated RAW 264.7 macrophages with *N. commune* lipid extract (100 μg/mL) or 15 μg/mL of a fatty acid mixture, which contains the same amount and composition of fatty acids as 100 μg/mL of the lipid extract, for 12 hours. Subsequently, the cells were activated by LPS for 18 hours. LPS drastically induced mRNA expression of TNF-α and COX-2 in control groups, whereas *N. commune* lipid extract inhibited the induction of TNF-α and COX-2 expression (Fig. 4).

Although the addition of 15% of fatty acid mixture significantly reduced the mRNA abundance of both genes compared with LPS-treated control, the degree of repression was less than that of *N. commune* lipid extract. The data suggest that in addition to unsaturated fatty acids, other bioactive compounds with an anti-inflammatory role are present in *N. commune* lipid extract that may inhibit the production of proinflammatory mediators in RAW 264.7 macrophages.

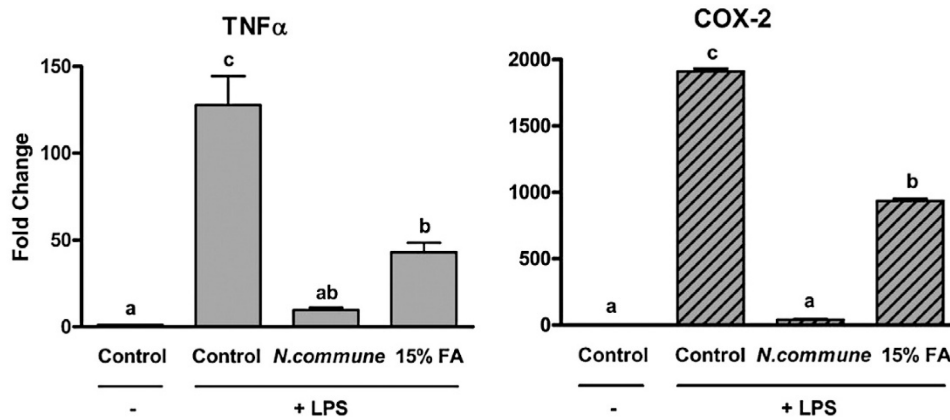

Figure 4. *N. commune* lipid extract contains bioactive compounds with an anti-inflammatory property. RAW 264.7 macrophages were incubated with *N. commune* lipid extract (100 μg/mL) or fatty acid mixture (15 μg/mL) for 12 hours, after which cells were activated by LPS (100 ng/mL) for 18 hours. Real-time PCR analysis was performed to measure mRNA abundance of TNF-α and COX-2. Data are a representative of 2 separate experiments and expressed as fold change relative to control without LPS treatment. Bars without a common letter are significantly different ($P < .05$) using one-way ANOVA and Tukey's pairwise comparison. Values are means ± SEM ($n = 3$).

3.5. Inhibition of NF-κB p65 DNA binding activity by **N. commune** *lipid extract*

To address whether *N. commune* lipid extract inhibits the production of proinflammatory mediators by blocking the NF-κB signaling pathway, we measured p65 DNA-binding activity in RAW 264.7 macrophages after the incubation with 100 μg/mL of *N. commune* lipid extract. Control experiments were run simultaneously for validity of assay using a positive control and wild-type and mutant oligos provided by the manufacturer (data not shown). In the absence of *N. commune* lipid extract, LPS significantly increased p65 DNA binding activity (Fig. 5). However, the addition of *N. commune* lipid extract inhibited LPS-induced NF-κB activation, indicating that the anti-inflammatory role of *N. commune* lipid extract is mediated, at least in part, through its inhibition of NF-κB activation.

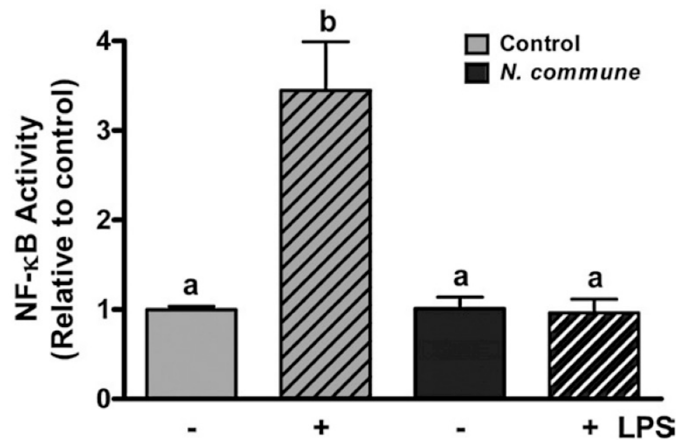

Figure 5. NF-κB p65 DNA-binding activity was inhibited by *N. commune* lipid extract. RAW 264.7 macrophages were incubated with 0 or 100 μg/mL of *N. commune* lipid extract for 12 hours, after which they were activated by LPS (100 ng/mL) for 1 hour. Nuclear extract was prepared, and p65 DNA-binding activity was measured using TransAM NF-κB p65 assay kit. Data are expressed as activity relative to control without LPS. Bars without a common letter are significantly different ($P < .001$) using one-way ANOVA and Tukey's pairwise comparison. Values are means ± SEM ($n = 6$).

4. Discussion

In this study, we investigated whether *N. commune* exerts an anti-inflammatory function and, if so, the mechanisms of action underlying such a function. We used cultivated *N. commune*, which is free of contamination of toxins and heavy metals and is under tight quality control during culture. Lipid extract from cultivated *N. commune* reduced the production of proinflammatory mediators by inhibiting, at least in part, the activation of NF-κB pathway in RAW 264.7 macrophages.

N. commune lipid extract contains pigments, free fatty acids, triacylglycerol, vitamins, and wax and steryl esters as demonstrated by TLC-densitometry analysis. Fatty acids have been implicated in modulating inflammatory responses [25] and they comprise approximately 15% of the total *N. commune* lipid extract. In addition, as approximately 75% of fatty acids in *N. commune* lipid extract are unsaturated fatty acids with a potential anti-inflammatory property, we reasoned that fatty acids could be the major bioactive components in *N. commune* lipid extract. As we expected, the fatty acid mixture that was constructed to reflect the same fatty acid composition in the *N. commune* lipid extract reduced the expression of proinflammatory mediators. The extent of the reduction, however, was less than that of *N. commune* lipid extract, indicating there are other bioactive components in the lipid extract that possess anti-inflammatory properties.

Various bioactive compounds from blue-green algae have been reported [26]. A well-known bioactive compound found in various blue-green algae is C-phycocyanin, a water-soluble protein-bound pigment that accounts for more than 20% of algal dry weight [27]. In animal models of inflammation, such as rats and mice treated with arachidonic acid on

ears and carrageenan injection in paws, C-phycocyanin reduced inflammation, and this effect was attributed to its antioxidative and oxygen free radical scavenging properties [7,8]. However, as C-phycocyanin is water-soluble, other unidentified bioactive components bearing an anti-inflammatory property are presumed to be present in *N. commune* lipid extract. It needs to be determined whether cultivated *N. commune* contains a high content of C-phycocyanin, similar to other blue-green algae. Bluegreen algae in general contain a high amount of carotenoids and the antioxidant properties of β-carotene could play a role in preventing inflammatory response. Studies have shown an inverse relationship between plasma β-carotene levels and makers of inflammation, such as sialic acid and C-reactive protein [28,29]. In LPS-stimulated RAW 264.7 macrophages, β-carotene prevented inflammatory gene expression by inhibiting NF-κB activation [30]. *N. commune* used in the present study contains approximately 70 mg/100 g (dry weight) of β-carotene, which is more than that of dried carrots (approximately 51 mg/100 g, US Department of Agriculture database). It is possible that the anti-inflammatory effect of *N. commune* lipid extract is conferred, in part, by its high β-carotene content. *N. commune* has been used for treating night blindness in Chinese medicine, and this effect could be attributed to high β-carotene content. Further chemical analysis for composition of *N. commune* lipid extract is necessary to identify additional bioactive compounds with an anti-inflammatory property.

Inflammatory response to LPS is primarily mediated through the toll-like receptor 4 (TLR-4) pathway [31,32]. Binding of LPS to TLR-4 and its coreceptors, such as cluster of differentiation 14 (CD14) and MD-2, triggers downstream signaling cascades, which lead to the activation of NF-κB. Recently, Macagno et al. [9] reported that LPS-like molecule extracted from a fresh-water cyanobacterium *Oscillatoria planktothrix FP1* strongly inhibited the expression of proinflammatory cytokines in human dendritic cells as well as endotoxin shock in mice. In the study, authors showed that the LPS-like molecule from the cyanobacterium competes with LPS for binding to the TLR-4–MD2 receptor complex, possibly blocking LPS-induced proinflammatory signaling cascades. The nature of the repressive effect of *N. commune* lipid extract on the production of proinflammatory mediators needs to be further examined. NF-κB is a dimeric transcription factor that consists of p65 (RelA), c-Rel, RelB, p50/105, and p52/100 [33]. Activity of NF-κB can be increased by phorbor esters, proinflammatory cytokines such as TNF-α and IL-1, and LPS [34]. In an unstimulated state, NF-κB is present in the cytoplasm bound with inhibitors of NF-κB (IκB) α/β, which masks the nuclear localization sequence of p65 [35]. In response to inflammatory stimulation, IκBα/β are phosphorylated, which are destined for ubiquitination and subsequent degradation by proteasomes [36–40]. Consequently, NF-κB becomes free to translocate from the cytoplasm to the nucleus where it binds to the κB element of target genes participating in the inflammatory and immune processes [41]. As transcription of various genes that encode proinflammatory cytokines, chemokines, and other effectors of the innate immune response is increased upon the activation of NF-κB, NF-κB has been a target for the treatment of inflammatory diseases [42]. Inhibition of NF-κB activation and the consequent repression of various proinflammatory mediators by *N. commune* lipid extract in our study suggest its potential to be used as an anti-inflammatory agent.

In conclusion, we have demonstrated for the first time that a lipid extract of a blue-green alga, *N. commune*, represses the expression of several genes involved in the proinflammatory response to inflammatory stimuli by the inhibition of signaling cascades leading to the activation of NF-κB. Because NF-κB regulates various genes involved in the inflammatory responses of cells, it has been a target for the treatment of acute or chronic inflammatory diseases such as sepsis, Crohn's disease, rheumatoid arthritis, and atherosclerosis [43,44]. Therefore, our study provides strong scientific evidence for *N. commune* to be developed as a new health-enhancing nutritional food for the prevention and treatment of chronic inflammatory diseases and further advances our knowledge in molecular nutrition by elucidating pathways for the functional food at a molecular level.

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