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# Edible blue-green algae reduce the production of pro-inflammatory cytokines by inhibiting NF-κB pathway in macrophages and splenocytes

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ABSTRACT

*Background:* Chronic inflammation contributes to the development of pathological disorders including insulin resistance and atherosclerosis. Identification of anti-inflammatory natural products can prevent the inflammatory diseases.
*Methods:* Anti-inflammatory effects of blue-green algae (BGA), i.e., *Nostoc commune* var. *sphaeroides* Kützing (NO) and *Spirulina platensis* (SP), were compared in RAW 264.7 and mouse bone marrow-derived macrophages (BMM) as well as splenocytes from apolipoprotein E knockout ($apoE^{-/-}$) mice fed BGA.
*Results:* When macrophages pretreated with 100 μg/ml NO lipid extract (NOE) or SP lipid extract (SPE) were activated by lipopolysaccharide (LPS), expression and secretion of pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα), interleukin 1β (IL-1β), and IL-6, were significantly repressed. NOE and SPE also significantly repressed the expression of TNFα and IL-1β in BMM. LPS-induced secretion of IL-6 was lower in splenocytes from $apoE^{-/-}$ fed an atherogenic diet containing 5% NO or SP for 12 weeks. In RAW 264.7 macrophages, NOE and SPE markedly decreased nuclear translocation of NF-κB. The degree of repression of pro-inflammatory gene expression by algal extracts was much stronger than that of SN50, an inhibitor of NF-κB nuclear translocation. Trichostatin A, a pan histone deacetylase inhibitor, increased basal expression of IL-1β and attenuated the repression of the gene expression by SPE. SPE significantly down-regulated mRNA abundance of 11 HDAC isoforms, consequently increasing acetylated histone 3 levels.
*Conclusion:* NOE and SPE repress pro-inflammatory cytokine expression and secretion in macrophages and splenocytes via inhibition of NF-κB pathway. Histone acetylation state is likely involved in the inhibition.
*General significance:* This study underscores natural products can exert anti-inflammatory effects by epigenetic modifications such as histone acetylation.



## 1. Introduction

Chronic inflammation is an underlying cause of several pathological disorders including insulin resistance, dementia, rheumatoid arthritis, atherosclerosis, and cancer [1,2]. Non-steroidal anti-inflammatory drugs (NSAID) are generally used to treat acute and chronic inflammatory conditions. However, due to their adverse side-effects and increased cardiovascular disease (CVD) risk associated with chronic use of several NSAID [3], there is a critical need to identify natural products with anti-inflammatory properties. Several anti-inflammatory bioactive compounds that have been extensively studied thus far include curcumin, resveratrol, anthocyanin, and green tea polyphenols [4–8].

Nuclear factor κ B (NF-κB) is a major transcriptional factors responsible for pro-inflammatory cytokine production, such as tumor necrosis factor α (TNFα), interleukins (ILs), inducible nitric oxide synthase and cyclooxygenase-2, under inflammatory conditions [9,10]. NF-κB exists as homo- or heterodimers consisting of five subunits of Rel family, i.e., p50, p52, p65, c-Rel and RelB [11]. In an unstimulated state, NF-κB is present in the cytoplasm bound with inhibitor of κB α (IκBα), which masks the nuclear localization sequence of p65 [12] and NF-κB activation largely depends on IκB kinase β (IKKβ). Extracellular inflammatory signals including lipopolysaccharide (LPS), pro-inflammatory cytokines, reactive oxygen species, advanced glycation end products, and oxidized low-density lipoprotein trigger the phosphorylation of IKKβ, which in turn phosphorylates IκBα for ubiquitination and degradation by proteasome [13–18]. This event liberates NF-κB to enter the nucleus for the induction of pro-inflammatory gene expression [19–24].

Blue-green algae (BGA), also known as cyanobacteria, are one of the most primitive forms of photosynthetic prokaryotes. They have

*Abbreviations:* $apoE^{-/-}$, apolipoprotein E knock out; BGA, blue-green algae; BMM, bone marrow-derived macrophages; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; C-PC, C-phycocyanin; CVD, cardiovascular disease; HAT, histone acetyltransferase; HDAC, histone deacetylases; IκBα, inhibitor of kappa B α; IL, interleukins; LPS, lipopolysaccharide; NLS, nuclear localization sequence; NO, *Nostoc commune var. sphaeroides* Kützing; NOE, NO lipid extract; SP, *Spirulina platensis*; SPE, SP lipid extract; TNFα, tumor necrosis factor α; TSA, trichostatin A

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been consumed as food or medicine for centuries and human consumption of BGA was recorded in the 14th century during the Aztec civilization [25]. They are recognized for their protective effects against viral and bacterial infections, cancer, allergy, diabetes, inflammation and hyperlipidemia [26–29]. At present, *Spirulina platensis* (SP) is the most commonly consumed and commercialized BGA species. We have previously reported that lipid extract of *Nostoc commune* var. *sphaeroides* Kützing (NO), another BGA species, inhibited NF-κB DNA binding activity and consequently repressed the pro-inflammatory gene expression in RAW 264.7 macrophages [30]. In this study, we compared anti-inflammatory effects of two BGA species using several model systems: RAW 264.7 macrophages, bone marrow-derived macrophages (BMM), and splenocytes isolated from BGA-fed apolipoprotein E knockout ($apoE^{-/-}$) mice. We found that in all of three systems, NO and SP either as lipid extract or as whole algae repressed pro-inflammatory gene expression and production.

## 2. Methods and materials

### 2.1. Preparation of BGA lipid extraction

BGA powder was kindly provided by Algaen Corporation (Winston Salam, NC) for NO and by Earthrise Nutritionals (Irvine, CA) for SP. Lipid extracts of BGA into chloroform/methanol (1:2) were prepared as previously described [30,31]. Lipid extracts were stored under $N_2$ gas at −20 °C for short term or at −80 °C for long term. The lipid extracts were dried down under $N_2$ to remove solvents and then dissolved in cell medium by sonication.

### 2.2. Bone marrow isolation and macrophage differentiation

BMM were isolated from C57BL/6J mice (Jackson Laboratory, Bar harbor, ME). Briefly, mouse legs were removed from the hip joint and cleaned, after which the femur and tibia were cut at the tip and subsequently bone marrow was collected by centrifugation. The bone marrow cells were differentiated into macrophages in low-glucose DMEM containing 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 300 U/mL penicillin, 300 μg/mL streptomycin, 20% FBS, and 30% L929 cell conditioned media. L929 cells were generously provided by Dr. John Parks (Wake Forest University School of Medicine, Winston-Salem, NC). BMM were incubated in a humidified chamber at 37 °C with 5% $CO_2$ and cell culture medium was changed every 3 days for 7 days until they became confluent.

### 2.3. Macrophage cell culture and treatment

RAW 264.7 macrophages (ATCC, Manasas, VA) and BMM were incubated in RPMI-1640 containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 x vitamins and 2 mmol/L L-glutamine in a 37 °C humidified chamber with a supply of 5% $CO_2$. Macrophages were incubated with 0–100 μg/mL of NO lipid extract (NOE) or SP lipid extract (SPE) for 12 h and subsequently activated by LPS (Sigma-Aldrich, St. Louis, MO) at 100 ng/mL concentration for additional 18 h. Cells and medium were collected for pro-inflammatory cytokine expression and secretion.

SN50, a cell permeable inhibitor specific for NF-κB nuclear translocation, was purchased from Enzo Life Science (Plymouth meeting, PA) and dissolved in sterile water. RAW 264.7 macrophages were incubated with 50 μg/mL of NOE or SPE for 11 h followed by 1 h incubation with 50 μg/mL of SN50. Subsequently, the cells were treated with LPS (100 ng/mL) in the presence of algal lipid extract and SN50 for additional 3 h. For the experiment with trichostatin A (TSA), a pan histone deacetylase (HDAC) inhibitor, RAW 264.7 macrophages were treated with algal lipid extract (100 μg/mL) and 50 nmol/L of TSA for 12 h prior to LPS stimulation for 7 h. All cell culture supplies were purchased from Thermo Scientific Hyclone (Logan, UT), unless stated otherwise.

For all cell culture experiments, cells without exposure to any algal extract were considered as control. Algal extracts were incorporated into cell culture medium by sonication and therefore no solvent was used as a vehicle.

### 2.4. Splenocyte isolation and culture

Spleens were harvested from male $apoE^{-/-}$ mice (Jackson Laboratory) fed a high fat/high cholesterol (15% fat, 0.2% cholesterol by wt) containing 5% NO or SP by wt for 12 wk from 8 wk of age. After being anesthetized with ketamine HCl (50 mg/kg)/xylazine (10 mg/kg) and subsequently euthanized by cardiac puncture and cervical dislocation, spleen of each mouse was excised aseptically and ground in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. After removal of red blood cells by a pre-warmed RBC lysis buffer (eBioScience, San Diego, CA), the cells were resuspended in the medium and centrifuged at 2000 rpm for 5 min. Cell pellet was resuspended in PBS and filtered through 40 μm strainer (BD Biosciences, San Jose, CA). After washing with PBS, cells were resuspended in RPMI-1640 complete media and plated at a density of $1\times10^6$/0.5 mL for experiments. Cells were incubated with LPS (500 ng/mL) for 20 h and IL-6 concentrations in the media were measure by ELISA (eBioScience) according to the manufacturer's instruction. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Connecticut.

### 2.5. Quantitative realtime PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Life technology, Carlsbad, CA) and cDNA synthesis was performed as previously described [30,31]. qRT-PCR was conducted using Bio-Rad CFX96 Real-Time system (Bio-Rad, Hercules CA). Primers were designed according to GenBank database and the sequences were previously published [30].

### 2.6. Cytokine array

RAW 264.7 macrophages were pretreated with 100 μg/mL of NOE or SPE for 12 h, after which they were incubated with LPS for 18 h. Conditioned medium was collected and centrifuged at 12,000 ×*g* for 5 min to remove any cell debris or dead cells. Secretion of cytokines was assessed by RayBio Mouse Cytokine Antibody Array (RayBiotech, Norcross, GA) according to the manufacturer's protocol. Signals were captured using ChemiDoc XRS+ system (Bio-Rad, Hercules, CA).

### 2.7. NF-κB translocation by Western blot analysis

RAW 264.7 macrophages incubated with 100 μg/mL of NOE or SPE for 12 h were activated by LPS for 1 or 2 h. Nuclear and cytoplasmic fractions of the cells were separated by using Cayman nuclear extraction kit (Ann Arbor, MI) and Western blot analysis was conduct as we previously described [30,31]. Polyclonal anti-p65 and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody against TATA binding protein was obtained from Abcam (Cambridge, MA) and used as a loading control for nuclear fraction. Protein expression were detected using Westpico horseradish peroxidase chemiluminescence (Pierce, Rockford, IL) and imaged using a Chemidoc XRS+ system (Bio-Rad) and Image Lab software (Bio-Rad).