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Regulation of virus-induced inflammatory response by *Dunaliella salina* alga extract in macrophages

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ABSTRACT

Previous reports have suggested that many constituents within various algal samples are able to attenuate LPS-induced inflammatory effects. To date no report has been published on the regulation of virus-induced inflammatory response of *Dunaliella salina* carotenoid extract. In the present study, the anti-inflammatory effect of *D. salina* carotenoid extract on pseudorabies virus (PRV)-infected RAW 264.7 macrophages was investigated.

We evaluated the anti-inflammatory effect of *D. salina* carotenoid extract on PRV-infected RAW 264.7 cells by measuring cell viability, cytotoxicity, production of inflammatory mediators such as NO, iNOS, COX-2, pro-inflammatory cytokines and anti-virus replication by plaque assay.

We found down-regulation of the expression of the iNOS, COX-2 and pro-inflammatory genes IL-1β, IL-6, TNF-α, and MCP-1 in a dose-dependent manner. Although there was no effect on viral replication, there were tendencies toward lower virus titer and tendencies toward higher cell survival. Most importantly, we found that inhibition of TLR9, PI3K and Akt phosphorylation plays a crucial role in the extract-mediated NF-κB regulation by modulating IKK-IκB signaling in PRV-infected RAW264.7 cells. These results indicate that *D. salina* carotenoid extracts inhibited inflammation by inhibition of NF-κB activation by TLR9 dependent via PI3K/Akt inactivation.

1. Introduction

Dunaliella salina (*Chlorophyceae*) is a halophilic unicellular micro-alga that has a mucus surface coat but no cell wall. The alga contains notable amounts of carotenoids (especially all-trans-ß-carotene, 9-cis-ß-carotene and 9′-cis-ß-carotene) (Hu et al., 2008; Lin et al., 2010b; Yang et al., 2013). Previous reports and our earlier study have suggested that many constituents within various algal samples are able to attenuate LPS-induced inflammatory effects; examples of these algae include blue–green algae (Ku et al., 2013), the red alga *Porphyridium cruentum* (Jiang et al., 2012), crude polysaccharide from marine brown alga *Turbinaria ornata* (Ananthi et al., 2010), the brown alga *Ecklonia cava* (Islam et al., 2013), the blue–green alga *Nostoc commune* var *sphaeroides* Kützing (Park et al., 2008) and the micro-alga *D. salina* (Yang et al., 2013). There have been, however, no reports exploring the effects of algal carotenoid extract on viral-induced inflammatory properties of cells.

Toll-Like Receptors (TLRs) have been shown to be important to the innate immune response, and the expression levels of these receptors may reflect the sensitivity of immune cells to infections. This recognition event leads to activation of macrophage effector functions important in host defense, such as enhanced microbial killing and production of chemokines, cytokines, and mediators which coordinate the inflammatory response. TLRs mediate cellular responses to microbial lipopeptides (TLR1, -2, and -6), LPS (TLR4), flagellae (TLR5), and nucleic acids (TLR7, -8, and -9) and are among the most potent activators of macrophage inflammatory responses (Lester and Li, 2014). Previous studies have also

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shown that the role of TLR9-mediated antiviral responses have observed TLR9 recognition of several double-stranded DNA viruses such as CMV, HSV-1, HSV-2, and poxviruses (Ma and He, 2013). In this study, we investigated the role of TLR9 signaling in regulating the production of an inflammatory response by PRV infection using RAW264.7 cells.

An infection with Herpes Simplex Virus (HSV) is always observed in humans who are suffering from hypoimmunity. HSV is the most common infectious agent responsible for sporadic acute encephalitis, and, when left untreated, has a mortality rate of 70%. Pseudorabies virus (PRV) is a neurotropic alphaherpes virus that, after intranasal infection of adult mice, enters peripheral neurons and propagates into the central nervous system. Due to the fact that its pathogenesis and immunobiology are similar to that of HSV, many studies have used PRV to mimic HSV infection (Ambagala et al., 2003; Lin et al., 2010a, 2012).

NF-κB is an inducible transcription factor that regulates the expression of many genes involved in immune and inflammatory responses (Baldwin, 2001). The transcription factor NF-κB is activated downstream during the T-cell activation via signaling induced by various inflammatory cytokines, such as TNF-α or IL-1β, bacterial lipopolysaccharide (LPS), viruses (Lin et al., 2012) and other innate immune response effectors. NF-κB is most often regulated by the IKK complex via phosphorylation of IκB proteins, which then results in subsequent ubiquitination and degradation; this promotes the nuclear accumulation of NF-κB dimers (Yang et al., 2013). Additionally, phosphorylation of the RelA/p65 subunit is associated with transcriptional activity of the NF-κB subunit (Hayden and Ghosh, 2004). Therefore, the development of inhibitors that target the various different enzyme molecules present in the virus signaling cascades is an attractive strategy when developing therapies targeting inflammatory diseases.

Hence, the aim of this study was to investigate the anti-inflammatory effects of *D. salina* carotenoid extract on PRV-infected RAW264.7 cells and explore the molecular mechanisms associated with the anti-inflammatory effects of *D. salina* carotenoid extract.

2. Materials and methods

2.1. D. salina sample

D. salina sample Spray dried powder of *D. salina* algae cultivated in Taiwan in 2011 was from Gong Bih Enterprise Co., Ltd. (Wunlin, Taiwan).

2.2. Materials and reagents

Solvents used for preparation of algal carotenoid extract included acetonitrile (ACN), methanol (MeOH), methylene chloride (CH_2Cl_2), ethanol (EtOH) and *n*-hexane from Merck Co. (Darmstadt, Germany). Distilled deionized water (H_2O) was prepared with an Ultrapure water purification system (Lotun Co., Ltd., Taipei, Taiwan). Potassium hydroxide (KOH) was from Merck Co. (Darmstadt, Germany). All-*trans*-α-carotene and all-*trans*-β-carotene, glutamine, penicillin, streptomycin, dimethyl sulphoxide (DMSO), Griess reagent (1% sulphanilamide, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 2.5% phosphoric acid), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), ethylenediaminetetraacetic acid (EDTA), leupeptin, phenylmethanesulphonyl fluoride (PMSF), phosphate-buffered saline (PBS), sodium chloride (NaCl), sodium nitrite ($NaNO_2$), Triton X-100, and Tris were from Sigma Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), RPMI1640, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco/In vitrogen Co. (Carlsbad, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for the measurement of TNF-α, IL-1β, MCP-1 and IL-6 were from eBioscience (San Diego, CA).

2.3. Cell culture and virus

Porcine kidney 15 (PK 15) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, Utah). The RAW264.7 cell line was cultured in RPMI1640 supplemented with 10% FBS. The stock of wild type (wt) PRV (strain TNL) used in this study was amplified from PK15 cells and the titer was determined by standard plaque assay in PK15 cells.

2.4. Preparation of D. salina carotenoid extract

D. salina extract was prepared according to the method of Hu et al. (2008). The algal sample (10 g) was extracted for 2 h at room temperature in 250 mL of hexane/acetone/ethanol (2:1:1, v/v/v) with a shaker. Saponification was then performed through adding 10 ml of 40% methanolic KOH at 25 °C for 16 h. After filtering, the extract was transferred to a separatory funnel and washed with 250 ml of distilled water 3 times. The solvent was evaporated to dryness in a rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan) to yield carotenoid extract. Composition of carotenoids in the extract was measured by high performance liquid chromatography (HPLC) with the conditions established in the previous report (Hu et al., 2008): column, YMC C30 (250 × 4.6 mm, 5 μm) (Waters Co., Milford, MA, U.S.A.); mobile phase, MeOH–ACN–H_2O (84/14/2, v/v/v) /CH_2Cl_2 = 75/25 (v/v); flow rate, 1 ml/min; detection, 210–650 nm at a rate of 1.00 spectrum/s. The equipment was a *PrimeLine*™ Gradient Model 500G HPLC pump system (Analytical Scientific Instruments, Inc., El Sobrante, CA, U.S.A.) and a S-3210 photodiode-array detector (PDA) (Schambeck SFD GmbH, *Bad Honnef, Germany*). The extract was then dissolved in DMSO for the experiments.

2.5. Nitrite assay

The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction (Yoon et al., 2009). RAW264.7 cells were plated at a density of 1×10^6 cells/well into 24-well plates. After 4 h incubation, the cells were pre-treated with DMSO (0.1%) or different concentrations (25, 50 or 100 μl) of *D. salina* carotenoid extract (DMSO concentration in each well was 0.1%) for 1.5 h and then incubated for 3, 6, 9, 12 or 24 h with PRV (0.1 MOI, Multiplicity of Infection). The supernatant (100 μl) was mixed with 100 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylethyylene-diamine dihydrochloride, 2.0% phosphoric acid) and incubated for 10 min at room temperature and the absorbance of the mixture at 595 nm was determined with an ELISA plate reader (Multiskan Spectrum, Thermo Co., Vantaa, Finland). All measurements were performed in triplicate. The concentration of NO_2^- was determined using a standard curve generated with $NaNO_2$.

2.6. Determination of IL-1β, IL-6, TNF-α and MCP-1 levels secreted from RAW264.7 cells

RAW264.7 cells were pre-treated with DMSO (0.1%) or various concentrations of *D. salina* carotenoid extract (DMSO concentration in each well was 0.1%) for 1.5 h before an infection with 0.1 MOI of PRV for 24 h, and the absorbance of the mixture at 450 nm was determined with an ELISA plate reader (Multiskan Spectrum, Thermo Co., Vantaa, Finland). The amounts of IL-1β, IL-6, TNF-α and MCP-1 in the supernatant were detected by ELISA kits (eBioscience, San Diego, CA, U.S.A.). All the experiments were done in triplicate.

2.7. Western blotting analysis

RAW264.7 cells were untreated or pre-treated with DMSO or 100 μM of *D. salina* carotenoid extract for 1.5 h before an infection with 0.1 MOI of PRV. After 3, 6, 12 or 24 h, the cells were washed with PBS and lysed in a lysis buffer [50 mM Tris–HCl (pH7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfone fluoride (PMSF), 25 μg/ml leupeptin] and kept on ice for 30 min. Cell lysates were centrifuged at 12,000×g at 4 °C for 15 min, and the supernatants were stored at −70 °C until analyses. Protein concentrations were measured using a protein assay kit (Bio-Rad, Laboratories, Inc., Hercules, California, USA). Twenty micrograms of whole cell lysate protein or nuclear lysate protein were resolved in loading buffer, electrophorezed on SDS/polyacrylamide gels, and electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked in Tris-buffered saline (TBS)-Tween 20 solution containing 5% non-fat dry milk and incubated overnight at 4 °C with specific antibodies against iNOS, COX-2, NF-κB p50, NF-κB p65, IKK, p-IKK, IKB, p-IKB, TLR-9, Akt, p-Akt, β-actin and GAPDH (Santa Cruz, CA, USA). Proteins were visualized using goat anti-rabbit or mouse antibody conjugated to horseradish peroxidase and a chemiluminescence western blotting detection system (ECL PlusTM Western Blotting Reagents, Amersham Biosciences, Boston, MA, USA). Protein band densities were quantified using AlphaImager 2200 software.

2.8. Determination of cytotoxicity and cell viability

RAW264.7 cells were pre-treated with DMSO (0.1%) or various concentrations of *D. salina* carotenoid extract (DMSO concentration in each well was 0.1%) for 1.5 h before an infection with 0.1 MOI of PRV for 24 h. The cells were centrifuged at 12,000 rpm for 3 min at room temperature and the medium carefully removed from each well in order to determine lactate dehydrogenase (LDH) activity using an LDH cytotoxicity detection kit (Promega, Madison, WI, USA). Briefly, 100 μl of reaction mixture were added to each well, and the reaction incubated for 30 min at room temperature in the dark. After incubation, 50 μl of 1 N HCl was added to each well to stop the enzymatic reaction. The absorbance of each well was