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Anti-inflammatory activity of c-phycocyanin in lipopolysaccharide-stimulated RAW 264.7 macrophages

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Abstract

C-phycocyanin (C-PC), found in blue green algae, is often used as a dietary nutritional supplement. C-PC has been found to have an anti-inflammatory activity and exert beneficial effect in various diseases. However, little is known about its mechanism of action. Overproduction of nitric oxide (NO) derived from inducible nitric oxide synthase (iNOS) plays an important role in the pathogenesis of inflammation. The aim of this study was to determine whether C-PC inhibits production of nitrite, an index of NO, and iNOS expression in lipopolysaccharide (LPS)-treated RAW 264.7 macrophages. Our results indicated that C-PC significantly inhibited the LPS-induced nitrite production and iNOS protein expression accompanied by an attenuation of tumor necrosis factor-α (TNF-α) formation but had no effect on interleukin-10 production in macrophages. Furthermore, C-PC also suppressed the activation of nuclear factor-κB (NF-κB) through preventing degradation of cytosolic IκB-α in LPS-stimulated RAW 264.7 macrophages. Thus, the inhibitory activity of C-PC on LPS-induced NO release and iNOS expression is probably associated with suppressing TNF-α formation and nuclear NF-κB activation, which may provide an additional explanation for its anti-inflammatory activity and therapeutic effect.

Keywords: C-phycocyanin; Nitric oxide; TNF-α; Nuclear factor-κB; Lipopolysaccharide; Macrophages

Introduction

C-phycocyanin (C-PC), a biliprotein found in blue green algae such as *Spirulina platensis*, is often used as a dietary nutritional supplement in many countries due to its therapeutic value, including hepatoprotective, anti-aggregatory, neuroprotective and reactive oxygen species (ROS)-scavenging actions (Kay, 1997; Romay et al., 2003; Vadiraja et al., 1998; Bhat and Madyastha, 2000; Chiu et al., 2006). In addition, the anti-inflammatory activity of C-PC has been demonstrated in various in vitro studies and in vivo experimental models such as mice with arthritis or sepsis (Romay et al., 1998; Remirez et al., 1999; Romay et al., 2001). However, little is known about its mechanism of action, warranting further investigation.

Some possible anti-inflammatory effects of C-PC have been reported, including inhibition of cyclooxygenase-2 activity and leukotriene B_4 formation in arachidonic acid-induced mouse ear inflammation test (Reddy et al., 2000; Romay et al., 1999). In addition, nitric oxide (NO), synthesized by the enzyme nitric oxide synthase (NOS), also plays an important regulatory/modulatory role in many physiological and pathological conditions (Moncada et al., 1991). Two NOS isoforms have been found: constitutive Ca^{2+}/calmodulin-dependent NOS (cNOS) and inducible Ca^{2+}/calmodulin-independent NOS (iNOS) (Forstermann et al., 1991). The release of NO by cNOS regulates many important physiological functions (Moncada et al., 1991). However, the high amount of NO produced by inducible NOS (iNOS) stimulated by pro-inflammatory cytokines, free radicals and lipopolysaccharide (LPS) may be a critical mediator in the pathogenesis of inflammatory diseases (Vane et al., 1994; Szabó

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and Thiemermann, 1995). In sepsis and other inflammation-related diseases, attenuation of iNOS/NO pathway has been proven beneficial (Blantz and Munger, 2002; Chou et al., 2001; MacMicking et al., 1995). Furthermore, it is well known that activated macrophages play an important role in the regulation of inflammation and immune response through producing various inflammatory mediators, including pro-inflammatory cytokines, NO and reactive oxygen species (ROS), which are then released into the general circulation to exert systemic effects (Fujii et al., 1998; West et al., 1994; Williams and Shacter, 1997). Accordingly, agents that inhibit the overproduction of NO derived from iNOS in macrophages may have an anti-inflammatory activity. However, the effect of C-PC on LPS-induced NO production and iNOS induction in active macrophages is still unreported. Therefore, the present study was to investigate whether the anti-inflammatory activity of C-PC was associated with the suppression of iNOS/NO pathway, cytokine formation and activation of nuclear transcription factor-κB (NF-κB), a key regulator of the expression of many inflammatory genes, including iNOS (Baeuerle and Henkel, 1994; Sun and Andersson, 2002; Xie et al., 1994), in LPS-stimulated RAW 264.7 macrophages.

Materials and methods

Cell culture

Murine macrophage cell line RAW 264.7 (American Type Culture Collection ATCC, TIB71, Rockville, MD, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) without phenol red, supplemented with 10% fetal calf serum, 4 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were grown at 37 °C under 5% CO_2 in fully humidified air and used for experiments in passages 5 to 10.

Cell viability assay

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) to formazan. The extent of reduction of MTT to formazan with cells was quantified spectrophotometrically by measurement at OD_{550}.

Nitrite measurements

After incubation of 60–250 μg/ml C-PC (with a purity of A_{620}/A_{280} >3.5, Sigma, St. Louis, MO, USA) with RAW 264.7 cells at 37 °C for 24 h in response to LPS (1 μg/ml), the culture medium was collected for the determination of nitrite concentration, an index of NO production. Nitrite was measured by the Griess reaction.

iNOS Western blot analysis

Total cellular protein of cells was extracted using lysis buffer [50 mM Tris–HCl, pH 7.4, 0.5 M NaCl, 1 mM EDTA, 0.05% SDS, 0.5% TritonX-100, 1 mM phenylmethylsulphonyl fluoride (PMSF)]. Then the cell lysates were centrifuged at 15,000 *g* for 10 min at 4 °C to collect the supernatant. The protein (12 μg) was separated on 7.5% sodium dodecylsulphate (SDS)-polyacrylamide minigels and then transferred to polyvinylidene difluoride (PVD) membranes (Millipore, Bedford, MA, USA). Nonspecific IgGs were blocked with 5% (w/v) skim milk in Tris-buffered saline (TBS; 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2.5 mM KCl) and incubated for 1 h at room temperature. Then, the membranes were incubated with specific antibodies against iNOS (1:1000 dilution, Transduction Laboratory, Lexington, KY, USA) or α-tubulin (1:5000 dilution, Santa Cruz, San Francisco, CA, USA). The bands were visualized by exposure of the membrane to a horseradish peroxidase-conjugated secondary antibody (1:5000 dilution, Transduction Laboratory, Lexington, KY, USA) followed by addition of ECL reagent (Amersham International Plc., Buckinghamshire, UK). The relative optical density of bands was quantified by densitometry and normalized with respect to α-tubulin.

Cytokine measurements

The concentrations of tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) in medium were measured by using EIA kits, respectively (Genzyme Corporation, Cambridge, MA, USA).

IκB-α Western blot analysis

To determine whether C-PC affects IκB-α degradation, the level of IκB-α in cytosol was determined. After incubation with LPS for 1 h in the presence or absence of C-PC, the protein of cells was extracted and separated on 10% SDS-polyacrylamide gels and then transferred to PVD membranes as described above. After blocking with 5% (w/v) skim milk, membranes were incubated overnight at 4°C with primary antibody IκB-α at a dilution of 1:1000 in 5% skim milk in TBS. Then, membrane was washed three times with 0.1% (v/v) Tween-20 in TBS (T-TBS) at room temperature, and incubated for 1 h at room temperature with a secondary antibody, anti-rabbit IgG conjugated to horseradish peroxidase (Sigma, St. Louis, MO, USA), at a dilution of 1:5000 in 5% (w/v) skim milk powder in TBS, followed by the addition of ECL reagent. The relative optical density of bands was quantified by densitometry and normalized with respect to α-tubulin.

NF-κB activation assay

After incubation with LPS for 1 h in the presence or absence of C-PC, the nuclear protein fractions of cells were collected as described previously (Chang et al., 2005). Briefly, the cells were suspended in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM DTT, 1.5 mM $MgCl_2$, 1 mM PMSF, 0.1% TritonX-100) for 15 min on ice and vortexed for 10 s. The nuclei were pelleted by centrifugation at 15,000 *g* for 10 min and then resuspended in hypertonic buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.2 mM ETDA, 1 mM PMSF) for 30 min on ice. The supernatants