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Ethanol extract of *Callophyllis japonica* enhances nitric oxide and tumor necrosis factor-alpha production in mouse macrophage cell line, RAW 264.7 cells

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SUMMARY

Red seaweed (*Callophyllis japonica*) has long formed part of the diet of Asians, but the pharmacological properties of this plant have not been evaluated. In this study, we examined the effect of an ethanol extract of *C. japonica* on the generation of nitric oxide (NO) in RAW 264.7 cells. The *C. japonica* extract increased the generation of NO and tumor necrosis factor-α (TNF-α), which were detected by the Griess method and an enzyme-linked immunosorbent assay, respectively. The increased production of NO by *C. japonica* extract was inhibited by N^G-monomethyl-L-arginine (100 μM), a specific inhibitor of NO production in the L-arginine-dependent pathway, and by the nuclear factor-κB (NF-κB) inhibitor, pyrrolidine dithiocarbamate (10 - 100 μM) in a dose-dependent manner. These findings demonstrate that *C. japonica* extract stimulates the production of NO and TNF-α in RAW 264.7 cells through the activation of NF-κB and that this extract might also inhibit the growth of the human leukemic cells.

Key words: *Callophyllis japonica*; Red seaweed; Nitric oxide; RAW 264.7 cells; Tumor necrosis factor-α

INTRODUCTION

Nitric oxide (NO) is a highly reactive molecule that is produced from arginine by NO synthase (NOS) (Nathan *et al*., 1991). NO has received increasing attention as a potent macrophage-derived effecter molecule that is effective against a variety of bacteria, parasites, and tumors (Gantt *et al.,* 2001). For example, mice treated with a NOS inhibitor and inducible NOS knockout mice were reported to be more susceptible to bacterial infection than untreated and wild-type animals, respectively (Zhou *et al.,* 2002). Reactive oxygen and nitrogen intermediates produced in innate immune cells would be effective in the host defense mechanism against microbial pathogens. Although the precise mechanism by which NO antagonizes bacterial infection is not known, the mechanism may

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involve disruption of bacterial DNA, proteins, and signaling or the induction of apoptosis in macrophages that harbor mycobacteria (Chung *et al.*, 2002). In addition to the involvement of NO in the process of inflammation, vascular hemodynamics are greatly affected by NO.

Callophyllis japonica (C. japonica), a red seaweed, has been traditionally eaten in the oriental food for diet, the biological activity of *C. japonica* have been limited yet. Recently, we reported that *C. japonica* exhibits antioxidant properties through the increased activities of the cellular antioxidant enzymes, superoxide dismutase and catalase in Chinese hamster lung fibroblast line V79-4 (Kang *et al.*, 2005) and it has a hepatoprotective effect on chemical-induced liver injury (Park *et al.*, 2005).

The aim of this study is to examine the effects of an ethanol extract of *C. japonica* on NO production *in vitro*.

MATERIALS AND METHODS

Preparation of seaweed

Fresh *C. japonica* plants were obtained from a local marine market on Jeju Island, South Korea. The plants were confirmed to be *C. japonica* by a taxonomist (Prof. YP Lee, Cheju National University) and the specimens were kept for reference. The plants were washed several times with distilled water, soaked in 70% ethanol, and dried for 48 h at room temperature. The ethanol-soluble fraction was filtered and the supernatant was concentrated by rotary evaporation. The resulting concentrate was freeze-dried to yield a brown powder (5% yield, w/w), which was dissolved in phosphate-buffered saline (PBS) for *in vitro* experiments.

Cell cultures

A mouse macrophage-like cell line, RAW 264.7, was maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2. Cells were cultured in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml), and penicillin (100 U/ml).

Chemicals

Pyrrolidine dithiocarbamate (PDTC) and N^G-monomethyl-L-arginine (NMMA) were purchased from Calbiochem (San Diego, CA, USA). A mouse tumor necrosis factor (TNF)-α enzyme-linked immunosorbent assay kit was purchased from R & D Systems (Minneapolis, MN, USA). The remaining chemicals and reagents used were of analytical grade.

Measurement of nitrite concentrations and TNF-α release

RAW 264.7 cells were seeded at 1×10^5 cells/ml. After 16 h, the cells were treated with various concentrations of the *C. japonica* ethanol extract. The cells were then incubated for an additional 48 h at 37°C. NO synthesis by the cultured cells was measured using a microplate assay (Xie *et al.*, 1992). To measure the concentration of nitrite in the culture supernatant (an index of NO activity), aliquots (50 μl) of culture medium were harvested and were incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% N-(1-naphtyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance of nitrite was measured at 540 nm. The NO_2^- content was determined by using sodium nitrite as a standard. To measure the release of TNF-α, the culture supernatant from cells that had been stimulated with the *C. japonica* ethanol extract was collected and used with a mouse TNF-α enzyme-linked immunosorbent assay kit according to the manufacturer's instructions.

Western blot analysis

RAW 264.7 cells were seeded at 1×10^5 cells/ml. After 16 h, the cells were treated with 100 μg/ml of the *C. japonica* ethanol extract. The cells were then incubated for various periods at 37°C. Cells were then collected and lysed by boiling for 5 min in 500 μl of a solution of 120 mM NaCl, 0.1% NP 40,

and 40 mM Tris-HCl (pH 8.0). Aliquots of the lysates (which each contained ~40 μg of protein) were electrophoresed on a 10% sodium dodecyl sulfate–polyacrylamide gel before the proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was then incubated with primary mouse monoclonal antibody raised against inducible NOS (iNOS; Pharmingen, San Diego, CA) followed by incubation with a secondary goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Pierce, Rockard, IL, USA). The membrane was then exposed to X-ray film and protein bands were detected using an enhanced chemiluminescence Western blot detection kit (Amersham, Little Chalfont, Buckinghamshire, UK).

Viability assay

To determine the effect of the ethanol extracts on the viability of U937 human leukemic cells, cells were seeded in a 96-well plate at 1.0×10^5 cells/ml. Sixteen hours later, the cells were treated with various concentrations of the ethanol extract before being incubated for 72 h at 37°C. An aliquot of a stock solution of MTT reagent (50 μl; 2 mg/ml) was then added to each well (total reaction volume: 200 μl). The cells were incubated for 4 h, centrifuged at 800 ×*g* for 5 min, and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl dimethyl sulfoxide and the absorbance at 540 nm was quantified using a spectrophotometer.

RESULTS

Effects of ethanol extract of *C. japonica* on NO production

NO production in RAW 264.7 cells treated with the *C. japonica* extract was determined by quantifying nitrite concentrations in culture supernatants after 48 h of culture in the presence of the *C. japonica* extract. As shown in Fig. 1, the ethanol extract caused a significant increase in NO production in the cells in a dose-dependent manner.

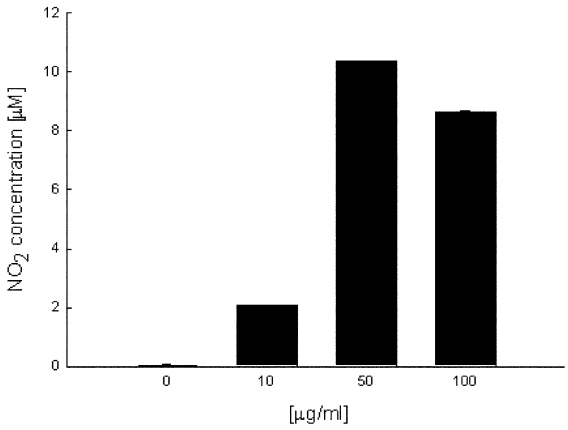

Fig. 1. Effect of an ethanol extract of *C. japonica* on the production of NO in RAW 264.7 cells**.** Cultured cells were incubated with various concentrations of the *C. japonica* extract (see Methods). After 48 h of incubation, NO release was measured by quantifying nitrite concentrations (the Griess method) in the culture medium. Data are the mean ± standard error (S.E.) of three independent experiments.

Effects of ethanol extract of *C. japonica* on iNOS expression

iNOS production in RAW 264.7 cells treated with the *C. japonica* extract was determined using Western blot analysis. As shown in Fig. 2, the ethanol extract increased iNOS expression in the cells in a time-dependent manner.

Inhibition of *C. japonica* extract-induced NO production by NMMA

To determine whether the *C. japonica* extract-induced NO production in RAW 264.7 cells involved an L-arginine-dependent signaling pathway, we examined the effect of NMMA (a specific inhibitor of L-arginine-dependent NO production) (Hinz *et al.*, 2000) on RAW 264.7 cells that were incubated for 48 h in the presence of the *C. japonica* extract. As shown in Fig. 3, *C. japonica* extract-induced NO production was inhibited by NMMA in a dose-dependent manner.

Inhibition of *C. japonica* extract-induced NO production by PDTC

To elucidate the signaling mechanism through

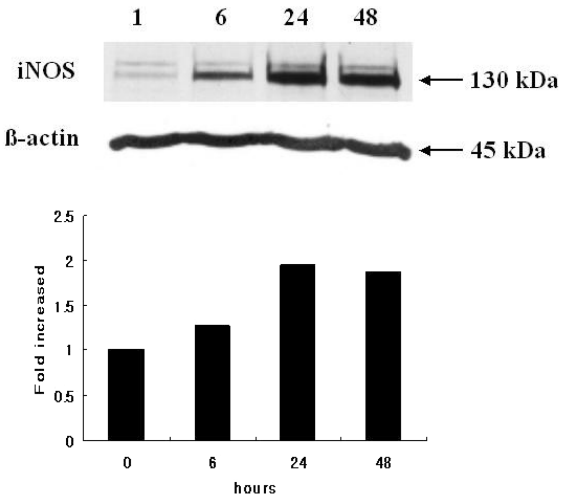

Fig. 2. Effect of an ethanol extract of *C. japonica* on the expression of iNOS in RAW 264.7 cells. Cultured cells were incubated with 100 μg/ml of the *C. japonica* extract. After various incubation times (see Methods), proteins were extracted and iNOS expression was quantified using Western blot analysis (A). The iNOS levels were quantified by densitometric analysis (B).

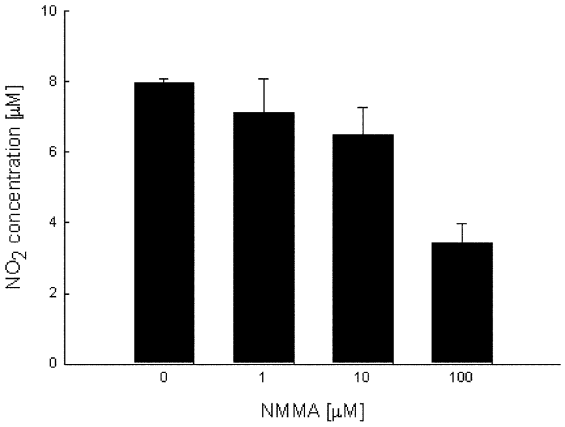

Fig. 3. Effect of NMMA on *C. japonica* extract-induced NO production in RAW 264.7 cells. Cultured cells were incubated with NMMA for 6 h. The cells were then treated with *C. japonica* extract (100 μg/ml) and cultured for an additional 48 h. NO release was measured by quantifying nitrite concentrations (the Griess method) in the culture medium. Data are the mean ± S.E. of three independent experiments.

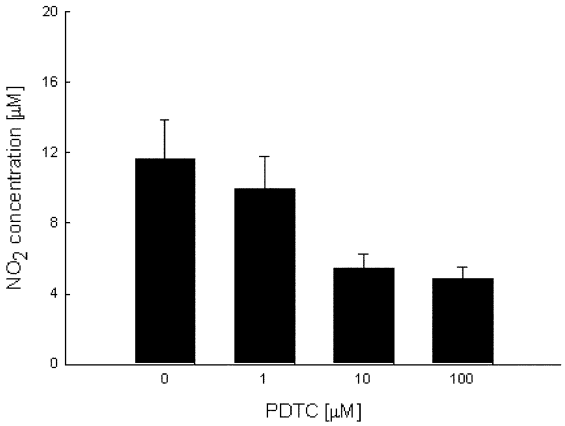

Fig. 4. Effect of PDTC on *C. japonica* extract-induced NO production in RAW 264.7 cells. Cultured cells were incubated with PDTC for 6 h. The cells were then treated with *C. japonica* extract (100 μg/ml) and cultured for an additional 48 h. NO release was measured by quantifying nitrite concentrations (the Griess method) in the culture medium. Data are the mean ± S.E. of three independent experiments.

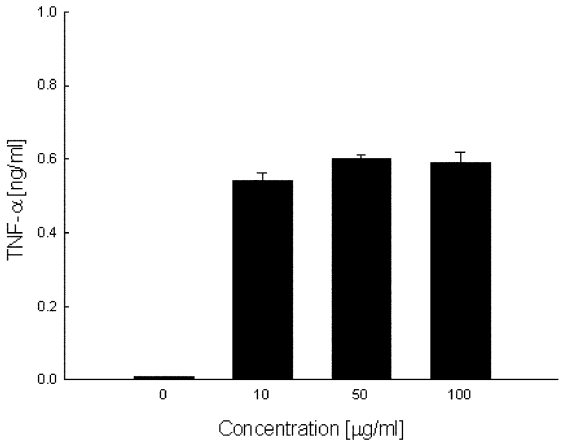

Fig. 5. Effect of *C. japonica* extract on the production of TNF-α in RAW 264.7 cells. Cultured cells were incubated with various concentrations of the *C. japonica* extract (see Methods). After 48 h of incubation, the amount of TNF-α secreted by RAW 264.7 cells was measured using an enzyme-linked immunosorbent assay. Data are the mean ± S.E. of three independent experiments.

which the *C. japonica* extract induced NO production in RAW 264.7 cells, we examined the effect of PDTC (an antioxidant compound that inhibits NF-κB activation) (Schreck *et al.*, 1992) on RAW 264.7 cells that were incubated for 48 h in the presence of the *C. japonica* extract. As shown in Fig. 4, *C. japonica* extract-induced NO production was inhibited by PDTC in a dose-dependent manner.

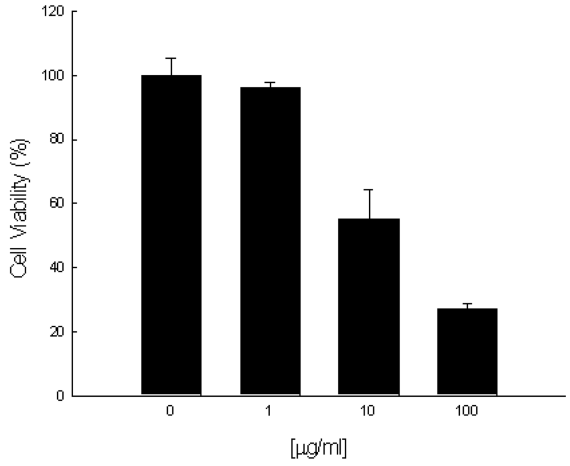

Fig. 6. Effect of *C. japonica* extract on the viability of human U937 leukemic cells. Cell viability was evaluated using a colorimetric MTT assay 72 h after the exposure of U937 cells to the *C. japonica* extract (see Methods). Data are the mean ± S.E. of three independent experiments.

Effect of *C. japonica* extract on TNF-α production

We examined the effect of the *C. japonica* extract on TNF-α production in RAW 264.7 cells. RAW 264.7 cells secreted very low levels of TNF-α after 48 h of incubation with medium alone, whereas the addition of the *C. japonica* extract caused an increase in TNF-α production in a dose dependent manner (Fig. 5).

Effect of the *C. japonica* extract on the viability of U937 cells

To test whether the *C. japonica* extract would affect the growth of human leukemic monoblasts, we evaluated the effect of the extract on the viability of U937 leukemic cells. As shown in Fig. 6, the ethanol extract caused direct inhibition of the viability of U937 cells in a dose dependent manner.

DISCUSSION

In the present study, we demonstrated that an ethanol extract of *C. japonica* caused an increase in the production of NO in cultured macrophage-like RAW 264.7 cells. The *C. japonica*-extract-induced NO production occurred through the induction of iNOS expression; this was supported by the observation that NMMA (an analogue of L-arginine) inhibited *C. japonica*-extract-induced NO production. At present, the precise physiological significance of the induction of NO production by *C. japonica* is unknown. However, NO synthesis is important in host defenses against pathogens and cancers (Gantt *et al.,* 2001). In addition, NO has emerged as an important intracellular and intercellular regulatory molecule with functions as diverse as vasodilation, neural communication, cell growth regulation, and host defense (Chung *et al.,* 2002, 2004). NO generation by iNOS also influences the cytotoxicity of macrophages and tumor-induced immunosuppression. The induction of NO production by *C. japonica* extract suggests that this plant contains a substance(s) that has antimicrobial, antitumoral, and antiviral activity. Indeed, we found that the ethanol extract of *C. japonica* inhibited cell growth of human U937 leukemic cells, which is a macrophage cell line. In a previous study, human granulocyte-macrophage colony-stimulating factor (GM-CSF) inhibited the colony growth of U937 cells in agar culture that was partially due to the presence of TNF (Cannistra *et al.,* 1987). It suggest that one of mechanisms that is inhibited U937 cells by extract of *C. japonica* may be the production of TNF-α (Eue *et al.,* 1995) and nitric oxide (Eue *et al.,* 1995; Bertholet *et al.,* 1999).

It has been reported that NO synthesis is greatly diminished by neutralization with an anti-TNF-α antibody (Kim *et al.,* 1999). *Chelidonium majus* and *Sinomenium acutum* stem (SSAE) increased the production of NO and TNF-α from rIFN-gamma mouse peritoneal macrophages (Kim *et al.*, 1999; Chung *et al.,* 2004). Our results suggest that the NO synthesis that is induced by *C. japonica* in RAW 264.7 cells could be synergistically associated with TNF-α secretion triggered by the *C. japonica* extract, even though the extract itself induced only a low level of TNF-α secretion.

NF-κB is expressed ubiquitously and plays a

major role in controlling the expression of proteins that are involved in inflammatory reaction and acute-phase immune responses (Baeuerle and Henkel, 1994). Expression of iNOS and TNF-α genes depends on the activation of NF-κB (Yu *et al*., 2004). We found in the present study that the addition of an inhibitor of NF-κB, PDTC, inhibited *C. japonica* extract-induced NO production. This suggests that *C. japonica* extract increases the production of NO and TNF-α through the activation of NF-κB.

In summary, *C. japonica* would appear to have important effects on immune-related cellular activities through the generation of NO and TNF-α. The precise mechanism by which *C. japonica* induces NO and TNF-α production remains to be elucidated.

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