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Full Length Research Paper

Anti-inflammatory effects of *Portulaca oleracea* L. on the LPS-induced RAW 264.7 cells

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***Portulaca oleracea* L. (Portulacaceae) has been widely used as a folk medicine in many countries. The present study investigated the effects of aqueous extract of *P. oleracea* (PO) on pro-inflammatory mediators secreted from lipopolysaccharide (LPS) - activated macrophage cells (RAW 264.7) as an established inflammation model. The reference drug indomethacin was used for comparison purpose. PO did not show cytotoxic effects at the concentrations tested. When RAW 264.7 macrophages were treated with PO together with LPS, a significant concentration, dependent inhibition of nitrogen oxide (NO) production was detected. Western blotting revealed that PO blocked protein expression of iNOS in LPS - stimulated RAW 264.7 macrophages, significantly. The change in the contents of PGE_2, IL-6 and TNF-α were monitored by enzyme linked immunosorbent assay (ELISA). Compared with indomethacin, PO has much more potency and inhibited the production of PGE_2, IL-6 and TNF-α in LPS- induced RAW 264.7 cells at concentrations of 0.05, 0.1, and 0.2 mg/ml ($p < 0.05$). These results suggested that PO might have a potential therapeutic effect by inhibiting the inflammation process such as arthritis.**

Key words: *Portulaca oleracea,* inflammation, extract, therapeutic effect.

INTRODUCTION

Inflammation preserves the host against tissue injury and microbial invasion. An inflammatory reaction is self-limiting and includes the decrease of pro-inflammatory protein expression and increased reaction of anti-inflammatory proteins that promote the innate immune responses (Murakami et al., 2007). Drugs had been prescribed as steroidal anti-inflammatory drugs (SAID) and non-steroidal anti-inflammatory drugs (NSAID) to treat acute inflammatory diseases, but these ordinary drugs have not been successful against chronic inflammatory diseases such as rheumatoid arthritis (RA), and atopic dermatitis (AD). Anti-inflammatory activity has been studied by the activity of inflammatory mediators and pro-inflammatory cytokines in the inflammation of

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RAW 264.7 cell (Hong et al., 2012). Macrophages are fundamental cells in cell-mediated innate immune reaction with reduced functions and the capacity to start acute inflammatory response (Gordon, 2007). The stimulation of macrophages with lipopolysaccharide (LPS) results in a number of functional reaction production of nitric oxide (NO) and cytokines increased (Olszanecki et al., 2002).

During cell signaling, materials such as NO, cytokine, growth factor, PGE_2 and other inflammatory mediators are precise subjects in immunologic and inflammatory study. IL-1β, IL-6 and TNF-α are known to make fever, inflammation, and tissue destruction (Dinarello, 2000). However, overproduction of these inflammatory mediators leads to many diseases such as rheumatoid arthritis, atherosclerosis, asthma, pulmonary fibrosis. Indomethacin is used to relieve pain and inflammation in a wide range of musculoskeletal conditions, including various forms of arthritis and gout. Indomethacin blocks the production of prostaglandins and is therefore effective in reducing inflammation and pain (Shakeel et al., 2010).

Portulaca oleracea (PO) is from the Portulacaceae family, which has the highest rates of omega-3 fatty acids and anti-oxidant vitamins (Rahdari et al., 2012). PO has pharmacological effects including antibacterial (Leite et al., 2007), analgesic (Terra et al., 2007), skeletal muscle-relaxant (Chang et al., 2006) and wound-healing (Clancy et al., 1998). PO has high resistance to environmental stress such as drought and it is used as a medical plant in low-raining regions (Rahdari et al., 2012). Most of the biologically active compounds associated with the referred multi-pharmacological effects of PO have not been shown thus far. The present study investigates the inhibitory effects of PO water extract on the production of pro-inflammatory mediators including NO, iNOS, PGE_2, IL-6 and TNF-α in LPS - induced RAW 264.7 macrophages.

MATERIALS AND METHODS

Macrophage RAW 264.7 cells were purchased from Amerocal Type Culture Collection (ATCC, USA). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin solution were obtained from Gibco (Invitrogen, USA). Lipopolysaccharide (LPS, from Escherichia coli 0111: B4) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (USA). Param assay kit was purchased from R&D systems (Minneapolis, USA). The antibodies (Abs) used in this study included: anti-INOS mAb, anti-β-actin mAb and anti-IgG-HRP, rabbit polyclonal Ab were purchased from Santa Cruz (USA).

Sources of plant materials

Total aerial parts of PO was purchased from Kyung Dong Market in Seoul, Republic of Korea, and then specimens were taxonomically identified by a oriental doctor, S.W. Lee at the National Institute of Horticultural and Herbal Science, RDA. The voucher specimen (HPR-209) was deposited at the herbarium of Herbal Crop Research Institute (Eumsung, Republic of Korea).

Preparation of plant extracts

PO has been extracted by water traditionally in oriental medicine, PO (100 g) was extracted with 4 L of boiling water for 2 h, filtered and then lyophilized (25%, wt/wt). The powered extract was dissolved in saline and then filtered through a 0.22 μm syringe filter.

Cell culture

Murine macrophage RAW 264.7 cells (ATCC) were cultured at 37°C in Dulbecco's modified eagles's medium containing 10% fetal bovine serum, 2 mM glutamate, 100 unit/ml of penicillin and 100 μg/ml of streptomycin in a humidified incubator with 5% CO_2. Cells were incubated with 1 μg/ml LPS along with various concentrations of plant extracts for 24 h as indicated.

Protein isolation and western blot analysis

Raw 264.7 cells were stimulated in the presence of LPS (1 ug/ml) with and without PO and indomethacin for 10 h. Protein (80 μg) was loaded onto polyacrylamide gels. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Criterion system (Bio-Rad) at a constant voltage of 90 V. Proteins were subsequently transferred to poly(vinylidene fluoride) (PVDF) membrane (Millipore, Billerica, MA, USA) at a constant voltage of 15 V for 30 min and identified by using relative antibodies.

Anti-inflammatory activity

To investigate NO formation, nitrite (NO_2^-) is measured since it is a stable and nonvolatile breakdown product of NO. The determination of nitrite relies on a diazotization reaction with the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid). Therefore, the nitrite accumulated in culture medium is a direct indicator of NO production (Green et al., 1982). A 100 μl aliquot of each supernatant from the 96-well plate was mixed with 100 μl Griess reagent and incubated at room temperature for 15 min. The concentration of total nitrite was determined by reading the absorbance at 550 nm and then calculated by a $NaNO_2$ dilution standard curve. The culture medium was collected after LPS treatment for PGE_2, TNF-α and IL-6 assays, respectively. The time points for PGE_2, TNF-α and IL-6 level were determined by a time course assay (data not shown). Levels of PGE_2, TNF-α and IL-6 in the culture media were determined with commercial enzyme linked immunosorbent assay (ELISA) assay kits (Park et al., 2004).

RESULTS AND DISCUSSION

Cell viability was tested throughout the experiments using MTT assay. At concentrations up to 0.2 mg/ml, no significant cytotoxicity was observed for any of the extract (data not shown). PO produced a dose-dependent inhibition of LPS-induced NO production (Figure 1A).

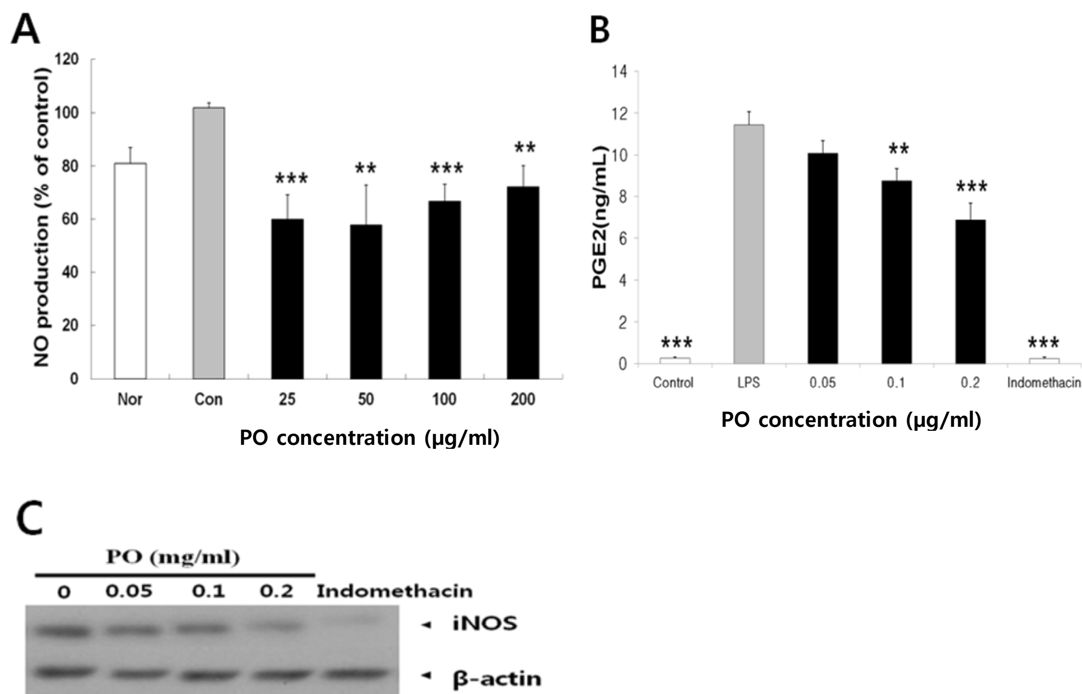

Figure 1. Effects of PO on NO, PGE_2 and iNOS in LPS-stimulated RAW 264.7 macrophages. (A) The normal group (Nor) was treated with media only. The control group (Con) was treated by the Griess reaction assay and expressed as a percentage of the control group. B. RAW 246.7 cells were treated with LPS (1 μg/ml) without and with PO (0.05, 0.1, and 0.2 mg/ml), and indomethacin (0.25 mM) for 12hrs prior to PGE_2 concentration being measured. C. Cells were incubated with of PO (0.05, 0.1 and 0.2 mg/ml), and indomethacin (0.25 mM) in the presence of LPS (1 μg/ml) for 10 hrs prior to analyzing iNOS protein (140 kDa) expression in RAW246.7 cells. Values are the mean±SEM of the three independent experiments. *** $p < 0.001$, ** $p < 0.01$ compared with LPS-stimulated group.

0.25 mM of indomethacin was used as a positive control in further studies to allow a comparison with PO anti-inflammatory activity and has often been used as a iNOS gene inhibitor (Terra et al., 2007). Figure 1A shows that the amount of NO released were 57.8 ± 3.3, 55.6 ± 3.9, 49 ± 6.1, 43.6 ± 8.2 and 0 μM for 1, 5, 25, 125 and 625μg/ml, respectively. Whereas, LPS-treated cells produced a large amount of NO. PO IC_{50} was 380 ± 20.3 μg/ml. Distinctly, the effect on NO inhibition indicates that PO has a great potency against inflammation. During the inflammatory process, large amounts of nitric oxide and PGE_2 are released by a wide variety of tissues and cells. LPS stimulation significantly increased PGE_2 production. An abnormal level of PGE_2 via COX activity is known to mediate inflammation. The results showed that the inhibition of PGE_2 synthesis were 10.1 ± 0.6, 8.8 ± 0.6 and 6.9 ± 0.8 ng/ml, for 0.05, 0.1 and 0.2 mg/ml, respectively (Figure 1B). The induction of PGE_2 was decreased by dose dependent of PO but can be mostly abolished by 0.25 mM of indomethacin.

PO was less potent than indomethacin in the terms of inhibiting PGE_2. The expression of iNOS and the release of NO by macrophages are esteemed to play a significant action in the pathogenesis of various inflammatory diseases. LPS is the major component of endotoxin, arrests macrophage proliferation and activation of pro-inflammatory factors (Morrison and Ryan, 1987). Raw 264.7 cells were incubated for 12 h with LPS (1 μg/ml) in the absence or presence of PO (0.05 to 0.2 mg/ml). When the effect of PO on iNOS protein was examined in LPS-induced cells by Western blotting, the PO inhibited LPS-induced protein expression level of iNOS in a dose-dependent manner as shown in Figure 1C. PO inhibited iNOS expression in similar way as shown in LPS-induced nitric oxide synthesis. Therefore, the inhibition of iNOS expression may establish an effective new therapeutic agent for the medicine of inflammation and the prevention of inflammatory disease. A number of iNOS inhibitors have been known to reduce the production of inflammatory cytokines such as IL-6 and TNF-α. The mechanism where IL-6 and TNF-α act in concert to stimulate prostaglandin production, is however not well

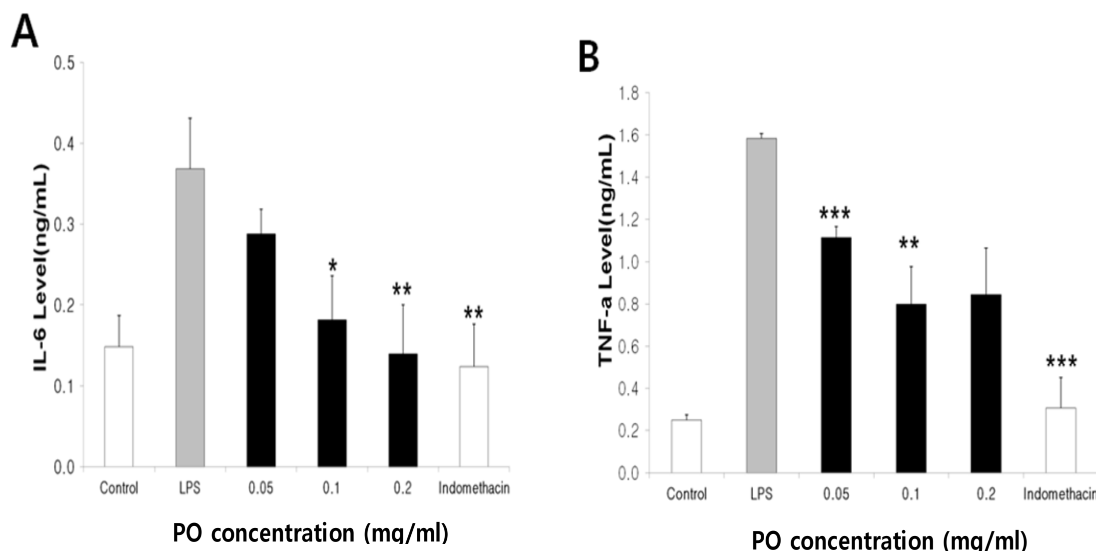

Figure 2. Effects of PO on (A) IL-6 and (B) TNF-α production. RAW 246.7 cells were treated with LPS (1 μg/ml) with and without PO (0.05-0.2 mg/ml), or indomethacin (0.25 mM) for 3 hrs prior to TNF-α and IL-6 concentration being measured. Values are the mean ± SEM of the three independent experiments.**P* < 0.05, ***P* < 0.01, and ****P* < 0.001 compared with LPS-stimulated group.

not well known. They are involved in bone resorption, IL-6 and TNF-α elevated levels can be found in many acute and chronic inflammatory diseases (Diehl et al., 2002). Figure 2A and B showed that LPS significantly stimulated IL-6 and TNF-α production. PO and indomethacin have only slight influence on TNF-α level inhibition in LPS stimulated RAW 264.7cells. The results showed that the inhibition of IL-6 synthesis were 0.29 ± 0.03, 0.18 ± 0.05 and 0.14±0.06 ng/ml, for 0.05, 0.1 and 0.2 mg/ml, respectively. The inhibition LPS-induced increases in IL-6 and TNF-α has been used to impose the potential anti-inflammatory effects of drug (Kumar et al., 2004) and medicinal herb (Kim et al., 2005). In addition, IL-6 plays a pivotal role in controlling the immune system so IL-6 has become a promising outstanding target for immunomodulatory anti-rheumatic therapy (Nishimoto and Kishimoto, 2006).

Although the precious mechanisms regulating the anti-inflammatory activity of PO are not yet known, in this study it was demonstrated that PO inhibits LPS-induced production of pro-inflammatory mediators including NO, PGE2, iNOS, IL-6 and TNF-α in RAW 264.7 mouse macrophages for the first time. These results suggest that PO possesses anti-inflammatory properties and could control macrophage-mediated inflammatory stimulation. Although the detailed mechanism remains to be elucidated, the PO may be a potential candidate as a remedy against inflammatory disorders such as rheumatoid arthritis.

Conclusion

Although the important mechanisms regulating the anti-inflammatory activity of PO is not yet known, in this research it was shown that PO inhibits LPS-stimulated production of pro-inflammatory mediators in RAW 264.7 mouse macrophages. These findings suggest that PO has anti-inflammatory effects and could regulate macrophage-mediated inflammatory stimulation. Further studies are needed to verify the precious mechanism regulating anti-inflammatory activities of PO.

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Conflicts of interest

Authors declare that there are none.

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