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Anti-inflammatory effects of ethanol extract from *Orostachys japonicus* on modulation of signal pathways in LPS-stimulated RAW 264.7 cells

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In this study, powder of *Orostachys japonicus* A. Berger (*O. japonicus*) was extracted with 95% ethyl alcohol and fractionated using a series of organic solvents, including n-hexane (hexane), dichloromethane (DCM), ethylacetate (EtOAc), n-butanol (BuOH), and water (H_2O). We investigated the anti-inflammatory effects of these *O. japonicus* extracts on lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Their effects on the expression of inflammatory mediators and transcription factors were analyzed by Western blotting. DCM fraction significantly inhibited formation of reactive oxygen species (ROS) as well as nitric oxide (NO) in LPS-stimulated RAW 264.7 cells. Phosphorylation of the pro-inflammatory transcription factor complex nuclear factor-kappa B (NF-κB) p65 and expression of inducible nitric oxide synthase (iNOS), one of its downstream proteins, were also suppressed by DCM fraction. These effects were regulated by upsteam proteins in the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase/Akt (PI3K/Akt) signaling pathways. Taken together, our data suggest that *O. japonicus* could be used as a potential source for anti-inflammatory agents. [BMB reports 2011; 44(6): 399-404]

INTRODUCTION

Orostachys japonicus, a perennial herbaceous plant belonging to the family Crassulaceae, is traditionally used as a folk medication. Previous studies on *O. japonicus* have revealed the presence of friedelin, epi-friedlanol, grutinone, glutinol, triterpenid, β-sitosterol, campesterol, fatty acid ester, kaempferol, quercetin, flavonoid, and aromatic acid (1-8). However, more scientific research on *O. japonicus* is required due to the lack of fundamental data on the signaling pathway regarding its physiological activity. Inflammation is caused by a variety of factors, including physical and chemical factors, the immune response, and tissue necrosis. Molecules that play a crucial role in the inflammatory response include active species such as nitric oxide (NO) and reactive oxygen species (ROS, superoxide anion, hydrogen peroxide, and hydroxyl radical), as well as enzymes such as inducible nitric oxide synthase (iNOS), which is known as an index of inflammation. ROS are known for reinforcing repair systems and limiting tissue injury. However, due to their destructive nature, excess ROS can inflict major damage to cells and tissues (9-14). When extracellular stimuli such as stress, UV radiation, and lipopolysaccharide (LPS) reach the cell membrane, typical signaling pathways are activated that translocate membrane proteins such as mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase/Akt (PI3K/Akt) to the nucleus (15, 16). When joined with cytoplasmic p50 and p65, nuclear factor-kappa B (NF-κB) becomes a major transcription factor that controls the expression of genes related to apoptosis, oncogenesis, cell proliferation, inflammation, and the immune response (17, 18). Cytoplasmic NF-κB is inhibited by inhibitory kappa B alpha (IκBα), which is phosphorylated by the activation of MAPKs and PI3K/Akt (19). Phosphorylated IκBα activates NF-κB by causing its translocation to the nucleus. The cytoplasmic bonds of IκBα are cleaved by a protein called ubiquitin and then degraded by a massive protein called the proteasome. Once in the nucleus, NF-κB binds to its κB site, resulting in target gene expression of iNOS, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) (20-25). This study aimed to assess the physiologically active substances extracted from *O. japonicus* and observe their anti-inflammatory activities. *O. japonicus* extract was separated into six solvent fractions, which were then each analyzed for their effects on signaling pathways related to inflammation.

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RESULTS

O. japonicus fractionation system and cell viability

O. japonicus was extracted with 95% ethanol (EtOH) and systematically fractioned with n-hexane (hexane, 2.386 g), dichloromethane (DCM, 2.814 g), ethylacetate (EtOAc, 6.806 g),

n-butanol (BuOH, 13.358 g), and water (H_2O, 6.221 g). To determine their potential toxicity, we examined the effects of the systematic fractions of *O. japonicus* extract on macrophage cell proliferation. We found no fraction had an effect on macrophage survival and proliferation in a dose-dependent manner (Fig. 1).

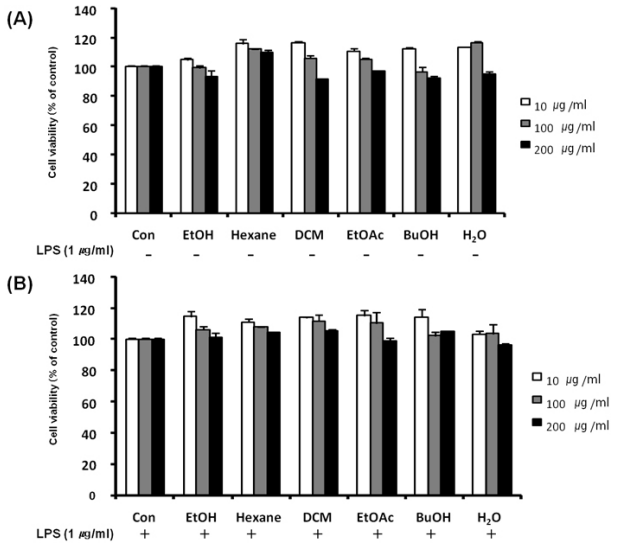

Fig. 1. Effects of *O. japonicus* solvent fractions on viability of RAW 264.7 cells using MTS assay. The cells were treated with *O. japonicus* solvent fractions for 1 h and then again for an additional 24 h (A: LPS untreated; B: LPS treated). The values are expressed as the means ± S.D. of three individual experiments.

Inhibition of ROS and NO formation

To investigate the anti-inflammatory activities of *O. japonicus* at the cellular level, we measured ROS and NO formation in LPS-treated macrophages in each solvent fraction. We observed the inhibition of H_2O_2 production in solvent fractions of *O. japonicus* extract and found differing degrees of concentration-dependent inhibition in every fraction. We also found that NO formation decreased in a concentration-dependent manner in the hexane and DCM solvent fractions compared to controls. Specifically, the DCM fraction demonstrated the lowest level of NO formation, followed by the hexane fraction (Fig. 2).

Inhibition of iNOS expression

The stress-induced production of NO in RAW 264.7 cells is related to inflammation. Stressed macrophages are known to generate excessive iNOS, which forms NO as a part of the inflammatory response that causes oxidative DNA damage. Therefore, we examined the effect of each solvent fraction that demonstrated significant NO inhibition on the expression of iNOS, which is considered a molecular index of inflammation. To observe their effects, we treated macrophages with LPS, which promoted differential protein expression depending on

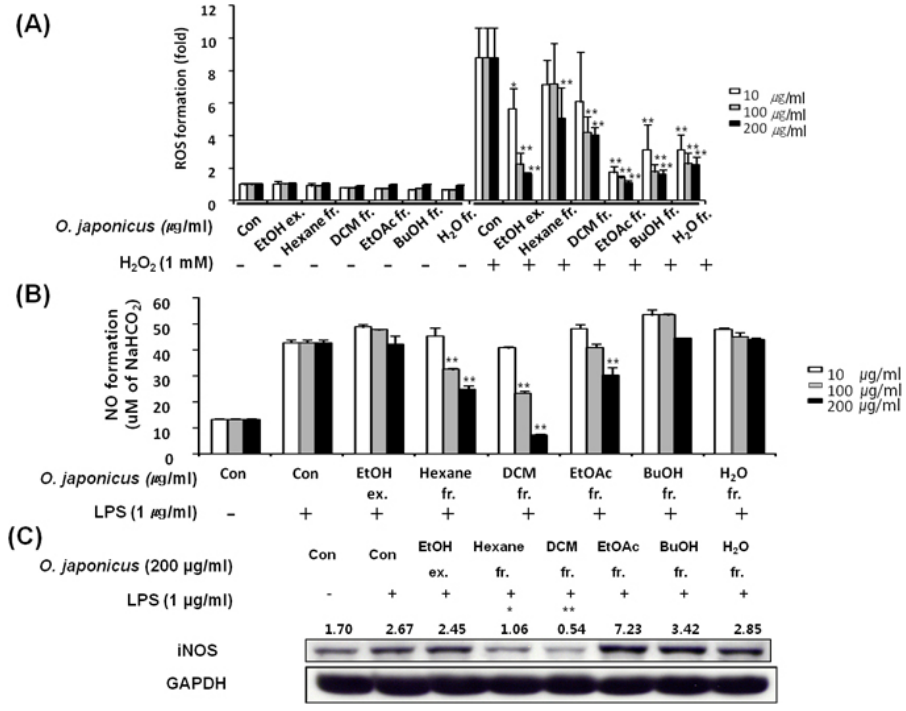

Fig. 2. Effects of *O. japonicus* on H_2O_2/LPS-induced ROS formation, NO formation, and LPS-induced iNOS protein expression in RAW 264.7 cells. (A) ROS formation. Cells were treated with *O. japonicus* solvent fractions for 1 h and then incubated with H_2O_2 (1 mM) for an additional 16 h. (B) NO formation. Cells were pretreated with the indicated concentrations of various *O. japonicus* solvent fractions for 1 h and treated with LPS (1 μg/ ml). After 16 h incubation, the amount of NO in the culture supernatants was measured by Griess reaction. (C) iNOS expression. After the NO formation experiment, total protein lysates were subjected to Western blot analysis. The values are expressed as the means ± S.D. of three individual experiments. Values of *P < 0.05, **P < 0.01 were considered statistically significant.

the solvent fraction. We found that expression of iNOS was inhibited in the hexane and DCM fractions, but most significantly in the DCM fraction (Fig. 2).

Inhibition of inflammatory mediators

We used Western blotting to analyze the effects of *O. japonicus* solvent fractions on the phosphorylation of the transcription factors IκBα and NF-κB p65 (p65). Our results indicate that the phosphorylation of IκBα and NF-κB p65 was inhibited by the hexane and DCM fractions (Fig. 3).

Inflammation-related upstream signaling pathways

When MAPKs such as C-Jun N-terminal kinases (JNK), Extracellulor signal-regulated kinases 1/2 (ERK), and p38 mitogen- activated protein kinases (p38) as well as PI3K/Akt are stimulated by stress, UV, or LPS, they act as receptors and activate certain transcription factors. Therefore, we measured activation of the MAPK and Akt pathways to identify the upstream signaling pathways of the following transcription factors. ERK is activated by increased cellular levels of growth factors, oxidizing stressors, and calcium as well as by the stimulation of glutamate receptors. When testing the phosphorylation of upstream signaling pathways that control the expression of inflammation-related molecular indices such as iNOS and p65, we observed that several fractions inhibited this process. Specifically, the DCM fraction demonstrated the most representative result (Fig. 3). This suggests that the upstream signaling pathways MAPK and Akt may control the inhibition of iNOS expression as well as phosphorylation of IκBα and p65 in the DCM fraction.

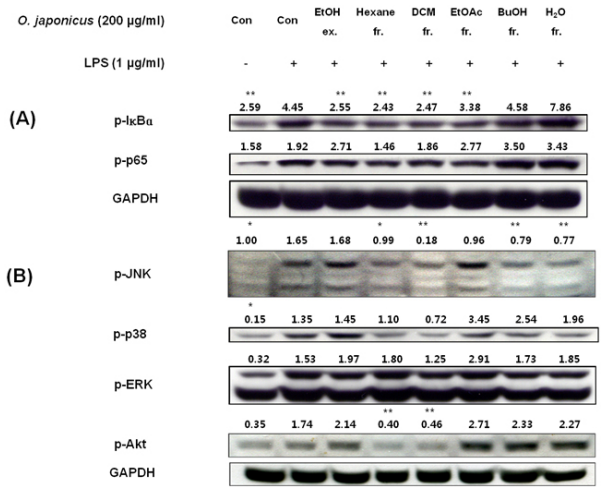

Fig. 3. Effects of *O. japonicus* on LPS-induced phosphorylation of pro-inflammatory transcription factors and MAPKs and Akt in RAW 264.7 cells. (A) Phosphorylation of pro-inflammatory transcription factor (p65) and degradation of IκB. Cells were pretreated with the indicated concentrations of various *O. japonicus* solvent fractions for 1 h and then incubated with LPS (1 μg/ml) for an additional 16 h. (B) Phosphorylation of MAPKs and Akt. Cells were treated with the indicated concentrations of various *O. japonicus* solvent fractions for 1 h and then incubated with LPS (1 μg/ml) for an additional 1 h. Total protein lysates were subjected to Western blot analysis. The values are expressed as the means ± S.D. of three individual experiments. Values of *P < 0.05, **P < 0.01 were considered statistically significant.

DISCUSSION

Inflammation is a complex process mediated by inflammatory and immune cells such as macrophages and monocytes (26). LPS stimulation of macrophages activates several extracellular signaling pathways, including the IκB kinase NF-κB pathway, MAPK pathway, and PI3K/Akt pathway (27). The NF-κB family of transcription factors is involved in the expression of numerous genes related to growth, development, apoptosis, inflammation, and oncogenesis. NF-κB is primarily composed of the proteins p50 and p65, and it is inactive when present in the cytosol bound to the inhibitory protein IκB. After induction by a variety of agents, IκB becomes phosphorylated and triggers its own proteolytic degradation, releasing NF-κB, which then translocates to the nucleus. Other investigators have reported an alternative mechanism of NF-κB activation. They observed increased NF-κB activity with nuclear localization in airway epithelial cells, which strongly express pro-inflammatory cytokines such as chemokinase and iNOS (28-32). Our results provide the first evidence that *O. japonicus* extract possesses anti-inflammatory activity. Of the various solvent fractions of *O. japonicus*, DCM fraction appeared to be the most active fraction, significantly inhibiting the phosphorylation of IκBα, p65, and proteins downstream of NF-κB, including iNOS. NO and ROS are known as important inflammatory mediators. NO is also a labile, gaseous-free radical known to participate in both physiological and pathological conditions (33). We found that the hexane and DCM fractions of *O. japonicus* inhibited NO formation in a dose-dependent manner. Besides NO, ROS including H_2O_2 can result in oxidative stress in cells, inflicting serious damage to membrane lipids, proteins, and nucleic acids. Accmulation of such damage can play an important role in the pathogenesis of many diseases, including inflammation, eye diseases, and cancer (33-35). We observed that H_2O_2 formation was inhibited by each of the six solvent fractions. Among them, the EtOAc and BuOH fractions both significantly inhibited H_2O_2 formation in a dose-dependent manner. This result suggests that *O. japonicus* fractions could inhibit the formation of H_2O_2 as well as NO. Further, we investigated the effects of various *O. japonicus* solvent fractions on LPS-induced activation of the MAPK and Akt pathways in RAW 264.7 cells. The DCM fraction inhibited the phosphorylation of MAPKs and Akt. These results suggest that the anti-inflammatory activity of the DCM fraction is due to the down-regulation of iNOS via suppression of p65 activation. We also found that the anti-inflammatory activity of the DCM fraction was related to the inhibition of JNK, ERK, p38, and Akt

Table 1. Composition of DCM fraction from *O. japonicus*

Peak	Retention time (min)	Area (%)	Component
1	8.798	1.63	Unknown
2	12.820	0.98	Unknown
3	16.648	2.32	Unknown
4	20.239	1.54	Unknown
5	23.480	2.55	Unknown
6	26.484	3.47	Unknown
7	29.316	5.27	Unknown
8	31.980	6.71	Unknown
9	34.470	5.24	Unknown
10	26.793	7.76	Kaempferol
11	38.974	6.51	Quercetin
12	40.073	2.36	Unknown
13	41.039	1.12	Unknown
14	42.415	52.53	Campesterol

signaling. Therefore, we believe that the DCM fraction of *O. japonicus* has treatment potential to modulate and regulate macrophage activation in a variety of inflammatory diseases. The DCM fraction also appears to be relatively safe, even at maximal concentrations. After analyzing the DCM fraction by GC-MS, 14 peaks were identified. Eleven peaks were unknown, but three peaks were identified as kaempferol, quercetin, and campesterol (Table 1). It was thus concluded that components such as kaempferol, quercetin, and campesterol (36, 37) contribute to superior anti-inflammation activity. However, further experiments on component analysis are needed.

In conclusion, we observed and analyzed the anti-inflammatory activity of several fractions of *O. japonicus* extract. We investigated the mechanisms underlying the inflammation pathways using distinct extract fractions. Among each fraction, DCM fraction showed the highest activity. Expression of IκBα, p65, and iNOS was decreased by the DCM fraction. Furthermore, the MAPK and Akt pathways were also inhibited by the DCM fraction. This study provides a useful foundation for investigating and developing new anti-inflammatory substances based on these extracts.

MATERIALS AND METHODS

Cell line and reagents

The cell line and reagents were a murine macrophage RAW 264.7 cell line from the American Type Culture Collection (ATCC, Manassas, VA); Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin from Hyclone; 2′-7′-dichlorodihydrofluorescein diacetate (DCF-DA) from Molecular Probe Inc. (OR, USA); monoclonal antibodies against iNOS, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), p-JNK, p-ERK, p-p38, p-Akt, p-IκBα, and p-p65 from Cell Signaling Technology Inc. (OR, USA); peroxidase-conjugated secondary antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); and LPS (*Escherichia coli* 0127:B8) from Sigma-Aldrich Co. (MO, USA). All other reagents used in this study were of the highest grade available. Cell viability was measured using a CellTiter 96 Non-radioactive Cell Proliferation Assay Kit (Promega, Madison, USA) according to manufacturer's instructions.

Plant material and extraction

O. japonicus was provided by Geobugiwasong Ltd. (Miryang, Korea). *O. japonicus* were dried and sliced, and powdered. *O. japonicus* (200 g) was extracted by boiling with 95% EtOH for 3 h three times. The EtOH extract was concentrated by rotary evaporation at 40°C. The concentrate was suspended in water and then fractioned with the following sequence of organic solvents: hexane, DCM, EtOAc, BuOH, and H_2O. Each solvent fraction was concentrated by rotary evaporation at 40°C and stored at -20°C until use.

GC-MS analysis

DCM fraction was analyzed by a GC-MS system, which included a Shimadzu 2010 series (Shimadzu, Kyoto, Japan) GC equipped with an AOC-20S automatic liquid sampler (Shimadzu, Kyoto, Japan) and interfaced directly to a QP2010 mass selective detector controlled via an accompanying data system. A 30 mmDB-5MS capillary column (0.25 mm id, 0.25 μm film) was used. The following GC temperature program was applied: hold at 50°C for 5 min, heat at a temperature ramp of 4°C/min up to 300°C, and maintain this temperature for 30 min. The injection volume was 1 μl in splitless mode. Helium was used as the carrier gas with a 1 ml/min flow rate. The temperature of the injector was maintained at 250°C. A solvent delay of 5 min was maintained throughout. Tuning was performed using the autotune feature with perfluorotributylamine (PFTBA); the electron multiplier voltage was nominally kept at 1,400 V. All data were obtained by collecting the full-scan mass spectra within the scan range of 200-550 amu. The GC-MS interface line and MS inlet temperature were 250°C, and the ion-source temperature was 280°C.

Cell culture

Murine macrophage RAW 264.7 cells were cultured at 37°C with 5% CO_2 in DMEM containing 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The cells were subcultured every 5 to 7 days at 1 : 5 split ratios. The medium was changed every 2 days. Cells were cultured for 24 h (approximately 80 to 90% confluency) and then treated either with vehicle (0.1% DMSO) or with various concentrations of *O. japonicus*, followed by stimulation with 1 μg/ml of LPS.

Cell viability assay

Cell viability was measured by a CellTiter 96 Non-radioactive Cell Proliferation Assay Kit according to manufacturer's instructions. In brief, macrophages were seeded in 24-well plates

and incubated at 37°C for 24 h (approximately 80 to 90% confluency), followed by treatment with solvent-soluble fractions of *O. japonicus* alone or in combination with LPS for 24 h. After incubation, the medium was removed and culture media containing MTS (3-[4,5-dimethylythiazol-2-yl]-5-[3-carboxy-methoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) was added and cultured for 1 h at 37℃ in a humidified atmosphere of 5% CO_2. Absorbance was measured at 490 nm with a microplate reader (PowerWaveXS, BioTek, USA). This assay was repeated three times with triplicate samples for each measurement.

ROS assay

Intracellular ROS production was measured using the cell permeable probe DCF-DA. This method measures the formation of ROS generated by an oxidative metabolic burst. In this assay, viable cells acetylate DCF-DA to 2′-7′-dichlorofluorescein, which is not fluorescent. When this compound reacts quantitatively with intracellular oxygen species, fluorescent 2′-7′-dichlorofluorescein is produced, which remains intracellular and can be measured to provide an index of intracellular oxidation. To study the effects of the solvent-soluble fractions of *O. japonicus* extract on ROS production, cells were loaded with 20 μM DCF-DA dissolved in DMSO for 1 h at 37°C in darkness. After washing out excess probe, cells were treated for 1 or 24 h. Medium was then removed and cells washed twice with 1 × ice-cold PBS. Fluorescence was measured at 485/20 nm excitation and 528/20 nm emission with a microplate reader.

NO assay

RAW 264.7 cells were seeded at a density of 1×10^5 cells/well in 24-well plates and then incubated for 16 h with or without 1 μg/ml of LPS in the presence or absence of various concentrations of *O. japonicus* fractions. NO levels in the culture media were determined by Griess reaction (38) and were presumed to reflect NO levels. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (equal volumes of 1% w/v sulfanilamide in 5% v/v phosphoric acid and 0.1% w/v naphthyl ethylenediamine-HCl) and incubated for 10 min at room temperature. Absorbance was then measured at 550 nm using a microplate reader. Fresh culture medium was used as a blank in all of the experiments. Nitrite levels in samples were determined from a standard sodium nitrite curve.

Western blot analysis

RAW 264.7 cells were treated with various concentrations of *O. japonicus* solvent fractions, stimulated with LPS, washed twice with ice-cold PBS (pH 7.4), and harvested using a cell scraper. The cell pellets were resuspended in lysis buffer on ice for 1 h, and cell debris was removed by centrifugation at 10,000 × g for 10 min. Protein concentrations were determined by BCA protein assay (Pierce, Rockford, USA). Equal amounts of protein were mixed with 2 x Laemmli loading buffer and preheated at 95°C for 5 min. Samples were then loaded onto 10% SDS-polyacrylamide gels and transferred onto a PVDF membrane for 1 h with a semidry transfer system (Bio-Rad). Membranes were blocked with 5% nonfat milk in PBST with 0.1% Tween 20 for 1 h at room temperature and then incubated overnight with primary antibodies. After hybridization with primary antibodies, membranes were washed for 5 min with PBST three times. The membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and washed for 5 min with PBST three times. Final detection was performed with Immunostar horseradish peroxidase Western blotting reagents (Pierce, Rockford, USA).

Statistical analysis

The data are expressed as mean ± standard deviation (SD). Comparisons between multiple groups were performed using the one-way analysis of variance (ANOVA). Values of $*P < 0.05$, $**P < 0.01$ were considered statistically significant.

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