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 (H₂O). 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 upstream 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.

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),
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n-butanol (BuOH, 13.358 g), and water (H₂O, 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).

**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 H₂O₂ 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