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Research article

Induction of Nrf2/ARE-mediated cytoprotective genes by red ginseng oil through ASK1–MKK4/7–JNK and p38 MAPK signaling pathways in HepG2 cells

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ABSTRACT

Background: The induction of cellular defensive genes such as phase II detoxifying and antioxidant enzymes is a highly effective strategy for protection against carcinogenesis as well as slowing cancer development. Transcription factor Nrf2 (nuclear factor E2-related factor 2) is responsible for activation of phase II enzymes induced by natural chemopreventive compounds.
Methods: Red ginseng oil (RGO) was extracted using a supercritical CO_2 extraction system and chemical profile of RGO was investigated by GC/MS. Effects of RGO on regulation of the Nrf2/antioxidant response element (ARE) pathway were determined by ARE–luciferase assay, western blotting, and confocal microscopy.
Results: The predominant components of RGO were 9,12-octadecadienoic acid (31.48%), bicyclo[10.1.0] tridec-1-ene (22.54%), and 22,23-dihydrostigmasterol (16.90%). RGO treatment significantly increased nuclear translocation of Nrf2 as well as ARE reporter gene activity, leading to upregulation of heme oxygenase-1 and NAD(P)H:quinone oxidoreductase 1. Phosphorylation of the upstream kinases such as apoptosis signal-regulating kinase (ASK)1, mitogen-activated protein kinase (MAPK) kinase (MKK)4/7, c-Jun N-terminal kinase (JNK), and p38 MAPK were enhanced by treatment with RGO. In addition, RGO-mediated Nrf2 expression and nuclear translocation was attenuated by JNK inhibitor SP600125 and p38 MAPK inhibitor SB202190.
Conclusion: RGO could be used as a potential chemopreventive agent, possibly by induction of Nrf2/ARE-mediated phase II enzymes via ASK1–MKK4/7–JNK and p38 MAPK signaling pathways.

1. Introduction

Carcinogenesis is a multistep process including initiation, promotion, and progression, in which the accumulation of genetic alterations leads to transformation of an initiated cell into a population of preneoplastic cells and eventually a tumor with invasive and metastatic capacities [1]. Recently, many dietary phytochemicals have exhibited beneficial effects on health including prevention, delay, and inhibition of cancer progression [2]. Cancer chemoprevention is defined as the use of natural, synthetic, or biological chemical agents to prevent the early precancerous stage of carcinogenesis [3]. Among the underlying mechanisms of chemopreventive agents is the induction of phase II detoxifying/antioxidant enzymes involved in carcinogen detoxification, and antioxidants, such as heme oxygenase (HO)-1, NAD(P)H:quinone oxidoreductase (NQO)1, aldo–keto reductase (AKR), and

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glutathione S-transferase (GST). Accumulating evidence shows that transcription factor Nrf2 (nuclear factor-E2-related factor 2) is involved in the regulation of phase II enzymes through the activation of antioxidant response element (ARE), which is located in the promoter region genes encoding these enzymes [4–6]. Under physiological conditions, Nrf2 is sequestered in the cytoplasm by binding to Keap1 (Kelch-like ECH-associated protein 1, cytosolic repressor). Upon stimulations, however, Nrf2 dissociates from Keap1 and translocates into the nucleus where it binds to ARE to transcriptionally induce defensive genes [7,8]. Studies of Nrf2-deficient mice have highlighted the importance of phase II detoxifying/antioxidant enzymes in the deactivation of chemical carcinogens, as these mice were more susceptible to carcinogenesis than normal mice were [9,10].

Many studies have demonstrated that activation of the Nrf2/ARE pathway is related to the upstream kinases, including mitogen-activated protein kinases (MAPKs), protein kinase C, phosphatidylinositol 3-kinase (PI3K), or transmembrane kinase [11]. MAPK families, including c-Jun N-terminal kinase (JNK), extracellular signal-regulating kinases (ERKs) and p38 MAPK are key signaling molecules that respond to mitogenic stimulation or environmental stress, resulting in expression of target proteins [12]. Specific MAPK inhibitors have been shown to block the induction Nrf2 as well as phase II detoxifying/antioxidant enzymes [13,14]. MAPKs are regulated by their upstream kinases: ERK is activated by MAPK kinase kinase (MKK)1 and MKK2, JNK by MKK4 and MKK7, and p38 MAPK kinase by MKK3, MKK4, and MKK6, which are activated by upstream MAPK kinase kinases (MAPKKKs) [15,16].

Ginseng (*Panax ginseng* Meyer) is a representative herbal, which has been widely used in Korea, China, and Japan for about 2,000 years. Red ginseng is made by steaming and drying fresh ginseng, a process that chemically transforms components and acquires special physiological activities such as antioxidant [17], antidiabetic [18], antiobesity [19], and anticarcinogenic [20] effects. Although several pharmacological effects of red ginseng have been studied, they are mostly water-soluble fractions. Moreover, the biological activities and molecular mechanisms of lipid-soluble moieties of red ginseng have been poorly characterized. Lipophilic fractions from red ginseng extracted with hydrophobic organic solvents such as petroleum ether and hexane have been shown to have antithrombotic and antitumor effects [21,22]. Our group has recently reported anti-inflammatory and hepatoprotective mechanisms of supercritical CO_2-extracted red ginseng oil (RGO) *in vitro* and *in vivo* [23,24]. In addition, our previous study has demonstrated the safety of RGO in an acute toxicity study using male and female Sprague–Dawley rats [25].

In the present study, we examined the chemical profile of supercritical CO_2-extracted RGO and its underlying mechanisms in the induction of cellular defense system in HepG2 cells.

2. Materials and methods

2.1. Chemicals

Primary antibodies against Nrf2, NQO1, β-actin, and Lamin B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); HO-1, phospho (p)-ERK1/2, p-JNK, p-p38, p-Akt, p-MAPK/ERK kinase (MEK)1/2, p-MKK3/MKK6, p-B-Raf, mixed lineage protein kinase (MLK)3, stress-activated protein kinase/ERK kinase-1 (SEK1)/MKK4, p-MKK7, phospho-apoptosis signal-regulating kinase (p-ASK)1, and phospho-transforming growth factor β-activated kinase (p-TAK1) antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). Peroxidase-conjugated secondary anti-rabbit and anti-goat antibodies were purchased from Santa Cruz Biotechnology, and Alexa-Fluor-555-conjugated secondary antibody was from Cell Signaling Technology (Boston, MA, USA). Specific inhibitors including U0126 (MEK1/2 inhibitor), SP600126 (JNK inhibitor), SB202190 (p38 MAPK inhibitor), and LY294002 (PI3 kinase inhibitor) were obtained from Cell Signaling Technology. Triton X-100, polyethylene glycol and other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of RGO

RGO was prepared as previously described [24]. Dried red ginseng powder was placed into the extraction vessel of a pilot-scale supercritical fluid extraction system (Ilshin Autoclave Co. Ltd., Daejeon, Korea). Extractions with supercritical CO_2 were operated at 6,500 psi (relative to 450 bar) in combination with temperature at 65°C. Extracted constituents were collected in a vial that was prefilled with a trapping solvent and maintained at 4°C during the extraction step.

2.3. GC/MS

Analysis of RGO was performed using an HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm; Agilent Technologies, Santa Clara, CA, USA) in a Gas chromatography–mass spectrometry (GC/MS) (5,975C; Agilent Technologies). Samples were injected into the column and run using split mode (split ratio = 10:1). The helium carrier gas was programmed to maintain a constant flow rate of 1 mL/min. Oven temperature was initially 80°C for 3 min and then finally raised to 300°C at 4°C/min. Compounds were tentatively identified by comparing mass spectra of the peaks with those in the mass spectrum library of NIST 11.

2.4. Cell culture

Human hepatoma HepG2 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). HepG2-C8 cell line was established by the stable transfection of HepG2 cells with p-ARE-T1-luciferase reporter gene as previously described [26]. Both cell lines were maintained in F-12 medium with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 1% essential amino acids, 1% GlutaMAX, and 0.1% insulin. The cells were maintained in a humidified incubator at 37°C in 5% CO_2.

2.5. Cell viability assay

HepG2 cells were plated at 10^5 cells/well in 24-well plates and treated with vehicle (polyethylene glycol: dimethyl sulfoxide, 1:1, v/v) or various concentrations of RGO for 24 h. After incubation, each well was washed twice with phosphate-buffered saline (PBS) followed by incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h at 37°C. Dark blue formazan crystals were dissolved with dimethyl sulfoxide and absorbance was measured with a PowerWave XS microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 570 nm. The percentage of viable cells was estimated by comparison with vehicle-treated control cells.

2.6. ARE–luciferase activity assay

HepG2-C8 cells were plated at 10^6 cells/well in 12-well plates and incubated for 24 h. After starvation overnight, cells were treated with RGO for 12 h, washed twice with PBS, and lysed with the reporter lysis buffer supplied for luciferase assay system (Promega, Madison, WI, USA). After centrifugation at 3,000 *g* for 10 min, a 10-μL aliquot of the supernatant was assayed for luciferase activity with a GloMax luminometer (Promega). The