Food and Chemical Toxicology 65 (2014) 97–104

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Induction of apoptosis by isoegomaketone from *Perilla frutescens* L. in B16 melanoma cells is mediated through ROS generation and mitochondrial-dependent, -independent pathway

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ARTICLE INFO

Article history:
Received 14 September 2013
Accepted 19 December 2013
Available online 29 December 2013

Keywords:
Isoegomaketone
Apoptosis
Reactive oxygen species
Apoptosis inducing factor
B16 melanoma cells

ABSTRACT

We have demonstrated for the first time the mechanism underlying ROS-mediated mitochondria-dependent apoptotic cell death triggered by isoegomaketone (IK) treatment in melanoma cells. We showed that IK induced apoptotic cell death and tumor growth inhibition using tissue culture and *in vivo* models of B16 melanoma. Furthermore, we observed that IK effectively induced apoptotic cell death, including sub-G1 contents up-regulation, nuclei condensation, DNA fragmentation, and caspase activation in B16 melanoma cells. Pretreatment with caspase inhibitor increased the survival rate of IK-treated B16 cells, implying that caspases play a role in IK-induced apoptosis. Furthermore, IK treatment generated ROS in melanoma cells. We also determined whether or not IK-induced cell death is due to ROS production in B16 cells. N-acetyl cysteine (NAC) inhibitedIK-induced Bcl-2 family-mediated apoptosis. This result indicates that IK-induced apoptosis involves ROS generation as well as up-regulation of Bax and Bcl-2 expression, leading to release of cytochrome c and AIF. Our data suggest that IK inhibits growth and induces apoptosis in melanoma cells via activation of ROS-mediated caspase-dependent and -independent pathways.

1. Introduction

Melanoma, one of the most common cancers in Western countries (Parkin et al., 2005), is the main factor responsible for deaths related to skin cancer (Jerant et al., 2000). In contrast, melanoma has not been recognized as a major health problem in Asian countries, including Korea. Despite advances in modern medicine, the incidence of melanoma is still increasing (Lucas et al., 2006). Specifically, the number of melanoma patients increases every year due to the continuing destruction of the environment by industrial development (Whited and Grichnik, 1998; Donohoe, 2003). The treatment of melanoma is centered on a combination regimen of surgery, radiation, and chemotherapy. These treatments cause many side effects in the body. Therefore, in the recent years, many studies have aimed for the prevention of cancer using non-toxic agents obtained from herbs and medicinal plants (Qiao et al., 2013; Wang et al., 2013).

Reactive oxygen species (ROS) are generated during cell metabolism, and they play important roles in cell signaling and homeostasis. Previous studies have shown that ROS induce depolarization of the mitochondrial membrane potential (MMP, $\Delta\psi m$) as well as release of cytochrome c from the mitochondria into the cytosol. The released cytochrome c triggers the activation of caspase-9 and initiates the caspase cascade, which induces apoptosis in cells (Chu et al., 2001). Recently, many reports have found that components from herbs and medicinal plants induce apoptosis of melanoma cells through production of ROS. Celastrol has been shown to induce apoptotic cell death in B16 mouse melanoma cells through a ROS-dependent mitochondrial pathway (Lee et al., 2012), and Jacaranone induces apoptosis in B16F10-Nex 2 melanoma cells via ROS-mediated down-regulation of Akt and p38 MAPK activation (Massaoka et al., 2012). The studies showed that components from natural compounds induce production of ROS, which can activate apoptotic signaling pathways via mitochondria-related mechanisms.

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http://dx.doi.org/10.1016/j.fct.2013.12.031

Perilla frutescens (L.) is an annual herbaceous and ornamental plant in the *Lamiaceae* family. Its leaves are consumed as food in China, Japan, and Korea, and its seeds are used to make edible oil in Korea (Seo and Baek, 2009). *P. frutescens* (L.) has many components, including rosmarinic acid, luteolin, apigenin, ferulic acid, catechin, caffeic acid, egomaketone, and isoegomaketone. *P. frutescens* (L.) has been reported to exhibit numerous biological, antioxidant, anti-inflammatory (Yang et al., 2013), and anti-allergic activities (Oh et al., 2011).

IK, an essential oil component in *P. frutescens*, has been shown to have anti-inflammatory activities in RAW 264.7 cells (Park et al., 2011). IK further has been shown to inhibit lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 macrophages (Jin et al., 2010). Although a recent study showed that IK induces apoptosis in human breast cancer cells (Cho et al., 2011), no such effect has been reported in melanoma cells. Especially, the involvement of IK-mediated ROS production in apoptotic signaling in B16 melanoma cells remains unknown.

In this study, we demonstrated that IK induced apoptosis in B16 mouse melanoma cells through mitochondria-dependent and -independent pathways. We further identified the potential mechanisms of IK-induced ROS-mediated apoptosis in mouse melanoma cells.

2. Materials and methods

2.1. Isolation of isoegomaketone

Isoegomaketone (IK) was prepared form *P. flutescens* (L.) Britt. cv. Chookyoupjaso, as described previously (Jin et al., 2010). In brief, the above-ground portion of *P. flutescens* (L.) Britt. cv. Chookyoupjaso was extracted with MeOH at room temperature over 3 days. After filtration, the MeOH extract was evaporated and partitioned using ethyl acetate, butanol and water. The soluble ethyl acetate fraction was separated by column chromatography on silica gel using a gradient of hexane–ethyl acetate. The fraction obtained was further separated to yield IK. The purity and concentration of the isolated IK were confirmed by spectroscopic analysis, and the final IK solution was prepared in sterilized DMSO (0.001% final concentration).

2.2. Cell culture

B16 mouse melanoma cell lines were purchased from the Korea Cell Line Bank, Seoul National University. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO®/Invitrogen™, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO®/Invitrogen™, Grand Island, NY, USA), penicillin (100 IU/mL), and streptomycin (100 μg/mL) in a humidified atmosphere with a 5% CO_2 incubator, at 37 °C.

2.3. Sulforhodamine B (SRB) assay

Cell viability was determined according to the method of Skehan et al. (1990). Briefly, IK was added at a range of 0–100 μM concentrations for 24 h. Cells were fixed with 50% trichloroacetic acid to terminate the reaction, after which 0.4% SRB in 1% acetic acid was added to each well. After 1 h of incubation, the plates were washed, and dyes were dissolved with 10 mM Tris buffer. Then, the 96-well plate was read using a micro-plate reader (540 nm) to obtain the absorbance density values.

2.4. Sub-G1 DNA content

Cells were seeded at a density of 1×10^6 cells in 6-well plates, and cultured for 24 h. After culturing, the cells were treated with the indicated concentrations of IK for 24 h. Then, the cells were harvested, washed with cold PBS, and processed for cell cycle analysis as described earlier (Park et al., 2005). Briefly, cells were collected and fixed in ice-cold 70% ethanol in media and stored at 4 °C overnight. After resuspension, the cells were washed and incubated with 1 μL of RNase (1 mg/mL) (Sigma–aldrich, St. Louis, MO, USA), 20 μL of propidium iodide (1 mg/mL) (Sigma–aldrich, St. Louis, MO, USA), and 500 mL of PBS at 37 °C for 30 min. After staining, flow cytometry was performed to analyze sub-G1 DNA content.

2.5. Morphological apoptosis

Characteristic apoptotic morphological changes were assessed by fluorescent microscopy using bis-benzimide (Hoechst 33258) staining (Ricote et al., 2006). Cells were seeded in 6-well plates at seeding densities of 5×10^5 cells, and then treated with IK for 24 h. After harvesting, the cells were washed twice with PBS, and then stained with 200 μL bis-benzimide (1 μg/mL) for 10 min at room temperature. Then 10 μL of this suspension was placed on a glass slide and covered with a cover slip. The cells were examined with a fluorescence microscope (Olympus Optical Co. Ltd., Tokyo, Japan) to determine nuclei fragmentation and chromatin condensation.

2.6. DNA fragmentation

The cells were seeded at a density of 2×10^6 cells in a 100 mm dish, and cultured for 24 h. After culturing, the cells were treated with the indicated concentrations of IK for 24 h, and then collected by centrifugation. The pellets were lysed by DNA lysis buffer (10 mM Tris–HCl, pH 7.5, 10 mM EDTA, pH 8.0, 0.5% Triton X-100, 20% SDS, 10 mg/mL proteinase K) and then centrifuged. The supernatant was fixed in ice-cold 70% ethanol and stored at 4 °C overnight. After extraction with phenol buffer (phenol–chloroform and phenol–chloroform–isoamylalcohol) the pellets were incubated with TE buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, pH 8.0) and RNase (2 mg/mL) for 1 h at 37 °C. Then, separation by electrophoresis was performed on 2% agarose containing ethidium bromide. The DNA bands were examined using a UV Transilluminator Imaging System (Kim et al., 2008).

2.7. In vivo antitumor studies

Male C57BL/6N mice at 5–6 weeks of age (approximately 20 g in weight) were purchased from Orient Bio (Seongnam, Gyunggido, Republic of Korea). B16 cells were detached and resuspended in phosphate-buffered saline. A single cell suspension (5×10^5 cells) was injected subcutaneously into the backs of mice. Mice were kept under a 12-h light/12-h dark cycle (light on at 8 a.m.) in an air-conditioned room (20 ± 2 °C and 50 ± 5% humidity under specific pathogen-free conditions) (Lee et al., 2010). Treatment was initiated on day 10 when tumors reached 100–200 mm^3 in volume. For treatment, the mice were randomly assigned to four groups: treatment with 5, 10 and 20 mg/kg/day of IK or a vehicle control for 20 days. IK was dissolved in vehicle (10% DMSO, 90% PBS) and administered through oral gavage every 2 days. Dosing solutions were prepared fresh each day. Tumor sizes were determined every other day via caliper measurements. Tumor volume was measured in two dimensions and calculated as follows: length/$2 \times \text{width}^2$. The study was conducted in accordance with Sunchon National University Guidelines for the Care and Use of Laboratory Animals.

For Western blotting, tumors were minced, ground, and lysed in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, and 1% PMSF). The lysates were centrifuged at 12,000*g* for 10 min at 4 °C to remove the insoluble components.

2.8. Caspase activity

This assay was based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate, Ac-DEVD-pNA (for caspase-3), Ac-IETD-pNA (for caspase-8), and Ac-LEHD-pNA (for caspase-9). The cells were seeded at a density of 2×10^6 cells in a 100 mm dish, and cultured for 24 h. After culturing, the cells were treated with the indicated concentrations of IK for 24 h, and then collected by centrifugation. The cells were incubated with the peptide substrate in lysis buffer for 30 min on ice, and centrifuged at 10,000*g* for 5 min at 4 °C. The protein content of the supernatant was measured using BCA protein assay reagent before analysis of the caspase-3, -8, and -9 activities. The supernatant that contained 50 μg of protein was mixed with DTT (dithiothreitol) in 2 × reaction buffer and a 10 μM concentration of the different substrates. After incubation, the release of *p*-nitroaniline was monitored at 405 nm (Kuo et al., 2005).

2.9. Caspase inhibitor activity

The cells were seeded at a density of 5×10^5 cells/well, and then cultured for 24 h. The cells were preincubated with pan-caspase inhibitor z-VAD-fmk for 2 h, followed by treated with the indicated concentrations of IK for 24 h. For the growth inhibition analysis and measurement of SRB assay, the cells were fixed with 50% trichloroacetic acid to terminate the reaction, after which 0.4% SRB in 1% acetic acid was added to each well. After 1 h of incubation, the plates were washed, and dyes were dissolved with 10 mM Tris buffer. Then, the 96-well plate was read using a micro-plate reader (540 nm) to obtain the absorbance density values.

2.10. Measurement of ROS

Production of intracellular ROS was detected by flow cytometry using dichlorofluorescein-diacetate (DCFH-DA) (Chen et al., 2007). Briefly, B16 cells were plated at a density 5×10^5 cells/well allowed to attach overnight, and exposed to IK for 30 min. Then, the wells were stained with DCFH-DA (10 μM) for 30 min at 37 °C, and the fluorescence intensity in the cells was determined using flow cytometry.