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Original Article

β-Hydroxyisovalerylshikonin promotes reactive oxygen species production in HCT116 colon cancer cells, leading to caspase-mediated apoptosis

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ABSTRACT

Although β-hydroxyisovalerylshikonin is suggested as a potential therapeutic agent for preventing various cancers, the underlying molecular mechanisms are not completely understood. In the present study, we investigated whether β-hydroxyisovalerylshikonin enhances apoptosis by triggering reactive oxygen species production in colon cancer HCT116 cells. β-Hydroxyisovalerylshikonin significantly inhibited the viability of HCT116 cells with maximum inhibition at 4 μM. Furthermore, treatment with β-hydroxyisovalerylshikonin subsequently increased sub-G_1 cells and annexin-V^+ cell population. Additionally, pretreatment with the caspase-8 inhibitor, z-IETD-fmk, and the caspase-9 inhibitor, z-LETD-fmk, significantly decreased β-hydroxyisovalerylshikonin-induced apoptosis, suggesting that β-hydroxyisovalerylshikonin promotes apoptosis through both the intrinsic and the extrinsic apoptotic pathways by activating caspase-8 and caspase-9. We also found that mitochondria played an important role in β-hydroxyisovalerylshikonin-mediated apoptosis via the intrinsic pathway. Accordingly, β-hydroxyisovalerylshikonin-induced reactive oxygen species production was evident after treatment with β-hydroxyisovalerylshikonin, and pretreatment with reactive oxygen species inhibitors, *N*-acetyl-L-cysteine and glutathione, significantly decreased β-hydroxyisovalerylshikonin-induced reactive oxygen species production, resulting in inhibition of apoptosis, which suggests that ROS generation is required for β-hydroxyisovalerylshikonin-mediated apoptosis. Taken together, these results demonstrated that the apoptotic effect of β-hydroxyisovalerylshikonin is enhanced in colon cancer HCT116 cells via reactive oxygen species generation and triggering of the caspase pathways, indicating that β-hydroxyisovalerylshikonin has potential as a therapeutic in the treatment of colon cancers.

Introduction

Reactive oxygen species (ROS) are natural byproducts of the normal metabolism of oxygen and play important roles in cell signaling, immunity, and homeostasis (West et al., 2011). ROS are classified into two groups; free-oxygen radicals and non-radical ROS. Free-oxygen radicals include superoxide ($O_2^{\bullet -}$), nitric oxide (NO•), hydroxyl radical (•OH), and organic radicals (R•), whereas hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and highly reactive lipid- or carbohydrate-derived carbonyl compounds are included in the non-radical ROS group (Birben et al., 2012). Normally, redox status is strongly balanced by the enzyme and non-enzyme systems (Wagener et al., 2013). However, redox balance is frequently disrupted by excessive ROS production and/or anti-oxidant depletion, leading to oxidative stress (Poljsak et al., 2013). In particular, aberrant ROS production is known as a potent mediator of inflammation, resulting in tissue injury and diseases such as cancer and neuronal disorder (Kehrer and Klotz, 2015). Thus, targeting ROS production is a promising therapeutic approach for inflammatory diseases and cancers. However, ROS also play an important role in apoptosis under both physiologic and pathologic conditions (Wagener et al., 2013). Enhanced

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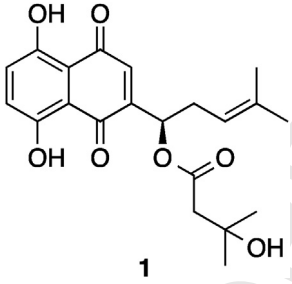
production of intracellular ROS triggered apoptosis by activating the mitochondrial-dependent cell death pathway via stimulation of the mitogen-activated protein kinase (MAPK) pathways and proapoptotic signals, and thus subsequently stimulated mitochondrial membrane potentials, resulting in cell death (Li et al., 2011). Therefore, ROS are crucial messengers in determining cell death or cell survival.

Apoptosis was described in terms of characteristic changes in cell morphology, including cell shrinkage, chromatin condensation, nuclear fragmentation, and membrane blebbing (Indran et al., 2011). Apoptosis is implicated in a variety of biological processes, such as embryogenesis, regulation of the immune system, and elimination of damaged cells (Guicciardi et al., 2013). The importance of apoptosis has been emphasized by recent demonstrations involving various chemotherapeutic anti-cancer agents. Indeed, current anti-cancer therapy using many chemotherapeutic agents as well as ionizing radiation therapy activated the apoptotic machinery to kill cancer cells (Zhang et al., 2015). The last decade has shown an extraordinary development in investigation of apoptosis and cancer treatments by regulating the redox system (Circu and Aw, 2010). Furthermore, the molecular mechanisms that control and execute apoptotic cell death are being identified. In the future, it seems likely that rational strategies to manipulate cell apoptosis will be focused novel therapies that are more beneficial than current treatment regimens.

To date, a series of novel shikonin-derivative analogs bearing oxygen-containing substituents were investigated, among which, β-hydroxyisovalerylshikonin (**1**, HIVS) has been highlighted for exhibiting the strongest apoptosis-inducing activity. β-Hydroxyisovalerylshikonin isolated from traditional Asian species, *Lithospermum radix* (*Lithospermum erythrorhizon* Siebold & Zucc., Boraginaceae), induced apoptosis in various types of human cancer cells (Kajimoto et al., 2008; Komi et al., 2009). It was also reported that HIVS showed great promise as a potent apoptotic agent in human leukemia cells (Masuda et al., 2003). So far, only a few studies have reported that HIVS induced apoptosis in various cancer cell lines. Therefore, in this study, we investigated the apoptosis mechanism of HIVS regulation via ROS generation in colon cancer HCT116 cells.



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Materials and methods

Plant material and β-hydroxyisovalerylshikonin

The roots of *L. erythrorhizon* Siebold & Zucc., Boraginaceae, were purchased in Jecheon Market (Jecheon, Republic of Korea). A voucher specimen has been deposited in Wood Chemistry & Microbiology Department, Korea Forest Research Institute (Seoul, Republic of Korea). HIVS (**1**) was isolated and characterized in our previous study (Jayasooriya et al., 2014).

Antibodies and reagents

Antibodies against caspase-3, caspase-8, caspase-9, Bad, Bcl-2, Bid, poly(ADP-ribose) polymerase (PARP), cytochrome *c*, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A caspase-8 inhibitor, z-IETD-fmk, and a caspase-9 inhibitor, z-LETD-fmk, were purchased from Calbiochem (San Diego, CA). Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulins were purchased from KOMA Biotechnology (Seoul, Republic of Korea). 6-Carboxy-2′,7′-dichlorofluorescein diacetate (DCFDA) and 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) were purchased from Molecular Probes (Eugene, OR). Glutathione (GSH), *N*-acetyl-L-cysteine (NAC), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO) and Roswell Park Memorial Institute Medium (RPMI), antibiotic mixture, and fetal bovine serum (FBS) were obtained from WelGENE Inc. (Daegu, Republic of Korea).

Cell line and growth assay

Human colon cancer HCT116 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI (WelGENE Inc., Daegu, Republic of Korea) supplemented with 10% FBS and antibiotics (WelGENE Inc.) at 37 °C in a 5% CO_2-humidified incubator. The cells were seeded at 1×10^5 cells/ml and then treated with the indicated concentrations of HIVS for 24 h in the presence of various inhibitors. MTT assay was performed to determine relative cell viability.

DNA fragmentation

HCT116 cells were treated with various concentrations of HIVS for 24 h and then lysed on ice in a buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 30 min. Lysates were vortexed and cleared by centrifugation at $10{,}000 \times g$ for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamylalcohol (25:24:1, v/v/v) and analyzed electrophoretically on a 1.5% agarose gel containing ethidium bromide.

Flow cytometry analysis

HCT116 cells were treated with various concentrations of HIVS for 24 h in the presence of NAC and GSH. The cells (1×10^6) were fixed in 70% ethanol overnight at 4 °C and washed in phosphate-buffered saline (PBS) with 0.1% BSA. Then, the cells were incubated with 1 U/ml RNase A (DNase free) and 10 μg/ml propidium iodide (PI, Sigma) for 30 min in the dark. The level of apoptotic cells containing sub-G_1 DNA content was determined as a percentage of the total number of cells. For annexin-V staining, live cells were washed with PBS and then incubated with annexin-V fluorescein isothiocyanate (R&D Systems, Minneapolis, MN) for 30 min, until the cells were analyzed using flow cytometry. A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) was used to determine the number of apoptotic cells, i.e., cells with sub-G_1 DNA that were annexin-V^+.

Western blot analysis

HCT116 cells were treated with various concentrations of HIVS for 24 h in the absence and the presence of NAC and GSH. The cells were lysed in buffer containing complete protease inhibitor mix (PRO-PREP) (iNtRON Biotechnology, Sungnam, Republic of Korea). After lysis for 30 min on ice, lysates were centrifuged at $14{,}000 \times g$ at 4 °C for 10 min. Supernatants were collected and protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Samples were stored at −80 °C or immediately used for Western blot analysis. Proteins were blotted onto

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