ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY 39 (2015) 982–989

Available online at www.sciencedirect.com

ScienceDirect

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

Esculetin induces death of human colon cancer cells via the reactive oxygen species-mediated mitochondrial apoptosis pathway

Areum Daseul Kim[1], *Xia Han*[1], *Mei Jing Piao*, *Susara Ruwan Kumara Madduma Hewage*, *Chang Lim Hyun*, *Suk Ju Cho*[*], *Jin Won Hyun*[*]

School of Medicine, Jeju National University, Jeju 690-756, Republic of Korea

ARTICLE INFO

Article history:
Received 25 September 2014
Received in revised form
1 March 2015
Accepted 3 March 2015
Available online 10 March 2015

Keywords:
Esculetin
Human colon cancer
Apoptosis
Reactive oxygen species
Signal pathway

ABSTRACT

The present study investigated the apoptotic effects of esculetin, a coumarin derivative, on the human colon cancer cell line HT-29. Esculetin had cytotoxic effects on HT-29 cells in a dose- and time-dependent manner; treatment with 55 μg/mL esculetin reduced cell viability by 50%. Esculetin induced apoptosis, as evidenced by apoptotic body formation, an increased percentage of cells in sub-G_1 phase, and DNA fragmentation. Moreover, esculetin increased mitochondrial membrane depolarization, released cytochrome c into cytosol, and modulated the expression of apoptosis-associated proteins, resulting in reduced expression of B cell lymphoma-2, increased expression of Bcl-2-associated X protein, and activation of caspase-9 and caspase-3. Esculetin induced the formation of reactive oxygen species; however, treatment with an antioxidant reduced the apoptotic cell death induced by esculetin treatment. In addition, esculetin activated mitogen-activated protein kinases and specific inhibitors of these kinases abrogated the reduction in cell viability induced by esculetin treatment.

1. Introduction

Colon cancer is the third most frequently diagnosed cancer in males and the second most frequently diagnosed cancer in females worldwide (Jemal et al., 2011). Modifiable risk factors for colon cancer include smoking, physical inactivity, overweightness and obesity, red and processed meat consumption, and excessive alcohol consumption (Ferrari et al., 2007). The survival and prognosis of colorectal cancer patients are dependent on the tumor stage at the time of detection. Unfortunately, about 50% of patients display regional or distant metastases at the time of diagnosis (Figueredo et al., 2008). Therefore, treatment of colon cancer has become a focus of research worldwide.

Previous attempts have been made to identify effective drugs and improve drug delivery systems for colorectal cancer (Balin-Gauthier et al., 2008). Herbal therapies, in particular plant-based products, have shown suppressive effects on colon cancer development (Gali-Muhtasib et al., 2004; Lepri et al., 2014).

* *Corresponding authors.*
E-mail addresses: sukjucho@gmail.com (S.J. Cho), jinwonh@jejunu.ac.kr (J.W. Hyun).
[1] These authors equally contributed to this work.

http://dx.doi.org/10.1016/j.etap.2015.03.003
1382-6689/

Of these natural products, esculetin (6,7-dihydroxycoumarin) is a type of coumarin. Coumarin (1,2-benzopyrone) consists of fused benzene and α-pyrone rings, and is an important low molecular weight phenolic group (Fylaktakidou et al., 2004). Esculetin has anticancer activity in N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats (Matsunaga et al., 1998), and inhibits oxidative stress-induced cell damage via scavenging reactive oxygen species (ROS) (Kim et al., 2008).

Apoptosis, a process of programmed cell death, can be activated by the death receptor pathway or the mitochondrial pathway (Huang et al., 2015). In the mitochondrial pathway, apoptotic signals are mediated by Bcl-2 family members, including the anti-apoptotic proteins B cell lymphoma-2 (Bcl-2) and B cell lymphoma-extra-large (Bcl-xL), and the pro-apoptotic proteins Bcl-2-associated X protein (Bax), Bcl-2 homologous antagonist/killer (Bak), and Bcl-2-associated death promoter (Bad) (Reuter et al., 2008). Bcl-2 family proteins regulate the mitochondrial apoptosis pathway mainly by controlling the release of cytochrome c and other intermembrane mitochondrial proteins from the mitochondria into the cytosol (Balaban et al., 2005), which is followed by the activation of caspases (Zamzami et al., 2000). The activated forms of caspase-9 and caspase-3 are among the main mediators of apoptosis. These enzymes cleave a wide range of important proteins, including other caspases (Nicholson, 1999). In addition, ROS, which are by-products of normal cellular oxidative processes, are mainly generated in mitochondria. ROS regulate apoptotic signal transduction and induce depolarization of the mitochondrial membrane, leading to increased levels of pro-apoptotic molecules in the cytosol (Jin et al., 2014).

The mitogen-activated protein kinase (MAPK) family, comprising c-Jun-N-terminal kinase (JNK), p38 MAPK, and extracellular-regulated protein kinase (ERK) are crucial mediators of signal transduction from the cell membrane to nucleus and are activated by various extracellular stimuli. MAPKs regulate a series of physiological processes, including cell growth, differentiation, and apoptosis (Ahmed-Choudhury et al., 2006). Much evidence suggests that anticancer agents can regulate the activities of MAPK family members in most cancer cell lines.

Herein, we show that esculetin induces apoptosis in a human colon adenocarcinoma cell line (HT-29) via mitochondrial apoptosis pathway regulation, ROS generation, and MAPK activation.

2. Materials and methods

2.1. Materials

Esculetin (6,7-dihydroxycoumarin) was purchased from Wako Pure Chemicals (Tokyo, Japan) and dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO did not exceed 0.02% when esculetin was added to cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342, propidium iodide (PI), and 2′,7′-dichlorofluorescein diacetate (DCF-DA), primary anti-actin antibody were purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine chloride (JC-1) was purchased from Molecular Probes (Eugene, OR, USA). Primary anti-cytochrome c, -COX4, -Bcl-2, -Bax antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas,TX, USA). Primary anti-caspase-9, -caspase-3, -poly ADP ribose polymerase (PARP), -JNK, -phospho JNK, -p38 MAPK, -phospho p38 MAPK, -ERK, and -phospho ERK antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). SP600125, SB203580, and U0126 were purchased from Calbiochem (San Diego, CA, USA).

2.2. Cell culture

Human colon adenocarcinoma cells (HT-29) purchased from the American type culture collection (Rockville, MD, USA) were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO_2 and cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/mL), and penicillin (100 units/mL).

2.3. Cell viability assay

The effects of esculetin on cell viability were determined using the MTT assay, which is based on the reduction of a tetrazolium salt by mitochondrial succinate dehydrogenase in viable cells (Carmichael et al., 1987). Briefly, cells were seeded in a 96-well plate at a density of 1.0×10^5 cells/mL, treated with 0, 20, 40, 60, or 80 μg/mL esculetin, and incubated at 37 °C for 48 h. Thereafter, MTT stock solution (50 μL, 2 mg/mL) was added to each well to attain a total reaction volume of 250 μL. After incubation for a further 4 h, the supernatants were aspirated and the formazan crystals in each well were dissolved in 150 μL of DMSO. The absorbance was measured at 540 nm using a scanning multi-well spectrophotometer (Sunrise Tecan, Durham, NC, USA).

2.4. Nuclear staining with Hoechst 33342

Cells were incubated with the DNA-specific fluorescent dye Hoechst 33342 (1.5 μL, 10 mg/mL) for 10 min at 37 °C and visualized using a fluorescence microscope equipped with a CoolSNAP-Pro color digital camera (Media Cybernetics, Silver Spring, MD, USA).

2.5. Detection of sub-G_1 hypodiploid cells

Cells were treated with esculetin for 48 h, harvested, washed with phosphate-buffered saline (PBS), and fixed in 70% ethanol for 30 min at 4 °C. Subsequently, the cells were incubated in the dark for 30 min at 37 °C with a solution containing 50 mg/mL PI and 50 μg/mL RNase A. Cells were then examined in a FACSCalibur flow cytometer (Becton Dickinson, East Rutherford, NJ, USA). The number of sub-G_1 hypodiploid cells was assessed based on histograms generated by the CellQuest and Mod Fit computer programs (Nicoletti et al., 1991).

2.6. Detection of DNA fragmentation

DNA fragmentation was examined and quantified using a cytoplasmic histone-associated DNA fragmentation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.