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Induction of apoptosis by esculetin in human leukemia U937 cells through activation of JNK and ERK

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Abstract

Esculetin is a phenolic compound that is found in various natural plant products and induces apoptosis in several types of human cancer cells. However, the underlying mechanisms of its action are not completely understood. In the present study, we used human leukemia cells to gain further insight into the mechanism of esculetin-induced anti-proliferative action and apoptosis. It was found that esculetin inhibits cell viability by inducing apoptosis, as evidenced by the formation of apoptotic bodies, DNA fragmentation, and the accumulation of cells in the sub-G1 phase. Esculetin-induced apoptosis was correlated with mitochondrial dysfunction, leading to the release of cytochrome *c* from the mitochondria to the cytosol, as well as the proteolytic activation of caspases. The z-DEVD-fmk caspase-3 inhibitor and the ectopic expression of anti-apoptotic Bcl-2 significantly inhibited esculetin-induced apoptosis, demonstrating the important role of caspase-3 and mitochondrial proteins in the observed cytotoxic effect. Furthermore, esculetin selectively increased the phosphorylation of extracellular-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), but not that of other kinases such as Akt and p38 activation. In addition, an ERK-specific inhibitor, PD98059, and a JNK-specific inhibitor, SP600125, showed inhibited sub-G1 phase DNA content, DNA fragmentation, caspase activation, and mitochondrial dysfunction induced by esculetin treatment. These results indicated that the JNK and ERK pathways were key regulators of apoptosis in response to esculetin in human leukemia U937 cells.

Keywords: Esculetin; Apoptosis; Bcl-2; ERK; JNK

Introduction

Many studies have shown that polyphenols in medicinal and edible plants have important pharmacological activities, such as anti-inflammatory/antioxidant and anti-carcinogenic activities (Surh, 1999; Sang et al., 2005). Esculetin is a coumarin derivative that is found in various natural plant products and has been reported to have beneficial pharmacological and biochemical activities. For example, esculetin has been shown to have an anti-inflammatory effect in the croton oil ear test (Tubaro et al., 1988), anti-proliferative effects on vascular smooth muscle cells (Huang et al., 1993), and inhibitory action on *N*-methyl-*N*-nitrosourea-induced mammary carcinoma (Matsunaga et al., 1998; Hecht et al., 1999). In addition, it is a scavenger of oxygen-free radicals (Martin-Aragon et al., 1998; Lin et al., 2000). It has also been shown to have inhibitory effects on 5- and 12-lipoxygenases of cloned mastocytoma cells (Neichi et al., 1983), induce apoptosis of 3T3-L1 adipocytes (Yang et al., 2006), and inhibit Ras-mediated cell proliferation (Pan et al., 2003). However, the anti-proliferative effect of esculetin in human leukemia cells has not yet been reported.

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Responses to numerous types of extracellular signals are mediated by mitogen-activating protein kinases (MAPKs), members of the serine/threonine kinase family. Several studies have revealed that c-Jun N-terminal kinase (JNK)1/stress-activated protein kinase (SAPK) and/or p38 MAPK activation were involved in apoptosis induced by a variety of different stimuli, such as γ-radiation-induced apoptosis in Jurkat T-cells (Chen et al., 1996a,b), glutamate-induced apoptosis in rat cerebellar granule cells (Kawasaki et al., 1997), membrane IgM-induced apoptosis in human B lymphocytes (Graves et al., 1996), or apoptosis induced by genotoxic stresses such as ultraviolet radiation, X-rays, H_2O_2, heat shock (Sachsenmaier et al., 1994; Chen et al., 1996a,b; Butterfield et al., 1997; Zanke et al., 1996; Verheij et al., 1996), and cell surface receptor Fas (Brenner et al., 1997; Goillot et al., 1997). Various well-known chemotherapeutic drugs, including adriamycin, vinblastine, VP-16, and CPT, are also capable of activating JNK. These drugs are critical in triggering apoptosis in different cell lines (Chau et al., 1998; Osborn and Chambers, 1996; Seimiya et al., 1997; Shiah et al., 1996), which suggests that the JNK signaling cascade may be the dominant participant in apoptosis. Previous studies have shown that esculetin could exert an anti-proliferation effect together with an inhibitory effect on the activation of p42 and p44 extracellular signal-regulated kinases (ERKs) and p38 MAPK in HepG2 cells (Kuo et al., 2006), and the SP600125 (a specific inhibitor of the JNK MAP kinase pathway) reduced esculetin-induced apoptosis by inhibiting the release of cytochrome *c* (Yang et al., 2006), suggesting that esculetin-induced apoptosis is associated with the ERK and JNK signaling pathways. However, these results must be investigated further using specific inhibitors in cancer cells.

Although the induction of apoptosis by esculetin has been observed in some cancer cell lines, the mechanisms by which esculetin induces apoptosis are generally unknown. In the current report, we use the esculetin in the human leukemia cell line as a model system with which to investigate the effects of esculetin-induced apoptosis. Our observations demonstrate that treatment with esculetin may induce apoptosis, inhibit the activations of ERK and JNK, and upregulate mitochondrial dysfunction and caspase activation. In addition, Bcl-2 overexpression reduced esculetin-induced apoptosis in U937 cells.

Materials and methods

Reagents. Propidium iodide (PI), 4,6-diamidino-2-phenylindole (DAPI), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphnyl-2H-tetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO). Caspase activity assay kits were obtained from R&D Systems (Minneapolis, MN). An enhanced chemiluminescence (ECL) kit was purchased from Amersham (Arlington Heights, IL). z-DEVD-fmk (caspase-3 inhibitor), PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), and SB203580 (p38 inhibitor) and specific mitochondrial dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were obtained from Calbiochem (San Diego, CA). RPMI 1640 medium was purchased from Invitrogen Corp. (Carlsbad, CA) and fetal bovine serum (FBS) was purchased from GIBCO-BRL (Gaithersburg, MD). Esculetin (6,7-dihydroxycoumarin, 98% purity) was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO, vehicle). All other chemicals not specifically cited here were purchased from Sigma.

Antibodies. Antibodies against XIAP, Bcl-2, Bax, Bid, cytochrom *c*, PARP, PLCγ-1, caspase-3, caspase-8, caspase-9, Akt, p38, and ERK were purchased form Santa Cruz Biotechnology (Santa Cruz, CA), and JNK, phospho-Akt, phospho-p38, phospho-ERK, and phospho-JNK were purchased from Cell Signaling (Beverly, MA). Antibody against actin was from Sigma. Peroxidase-labeled donkey anti-rabbit and sheep anti-mouse immunoglobulin were purchased from Amersham.

Cell culture. Human leukemia lines (U937, HL-60 and K562) were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL) at 37 °C and 5% CO_2. The Bcl-2 overexpressing U937 cells were a generous gift from Dr. T. K. Kwon (Department of Immunology, Keimyung University School of Medicine, Taegu, Korea) and were maintained in a medium containing 0.7 μg/ml geneticin (G418 sulfate, Calbiochem).

Cell viability and growth. The cells were grown to 70% confluence and treated with the indicated concentrations or times of esculetin. Control cells were supplemented with complete media containing 0.1% DMSO (vehicle control) for various time points. Following treatment, cell number and viability were determined by Trypan blue exclusion and MTT assays, respectively.

Cell cycle analysis. The cells were serum-starved for 24 h to synchronize them in the G0 phase of cell cycle. Synchronous populations of cells were subsequently treated in the absence or presence of esculetin for various time points. The cells were washed twice with cold PBS and then centrifuged. The pellet was fixed in 75% (v/v) ethanol for 1 h at 4 °C. The cells were washed once with PBS and resuspended in cold PI solution (50 μg/ml) containing RNase A (0.1 mg/ml) in PBS (pH 7.4) for 30 min in the dark. Flow cytometry analyses were performed using FACSCalibur (Becton Dickinson, San Jose, CA). Forward light scatter characteristics were used to exclude the cell debris from the analysis. The sub-G1 population was calculated to estimate the apoptotic cell population.

DNA fragmentation assay. Cells (2×10^5) were lysed in 100 μl of 10 mM Tris–HCl buffer (pH 7.4) containing 10 mM EDTA and 0.5% Triton X-100. After centrifugation for 5 min at 15,000 rpm, supernatant samples were treated with RNase A and proteinase K. Subsequently, 20 μl of 5 M NaCl and 120 μl isopropanol were added to the samples and kept at −20 °C for 6 h. Following centrifugation for 15 min at 15,000 rpm, the pellets were dissolved in 20 μl of TE buffer (10 mM Tris–HCl and 1 mM EDTA) as loading samples. To assay the DNA fragmentation pattern, samples were loaded onto 1.5% agarose gel, and electrophoresis was carried out.

Nuclear staining. After treatment with esculetin, the cells were harvested, washed with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde (Sigma) in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with 2.5 μg/ml DAPI solution for 10 min at room temperature. The cells were washed 2 more times with PBS and analyzed via a fluorescent microscope.

Preparation of mitochondrial proteins. Cells were treated with esculetin and were harvested with ice-cold PBS. The mitochondrial and cytosolic fractions were isolated using a mitochondrial fractionation kit (Activemotif, Carlsbad, CA). Cell lysates (30 μg protein per lane) were fractionated in SDS–polyacrylamide gels before transfer to the nitrocellulose membranes (Schleicher and Schuell, Keene, NH) using standard protocol.

Protein extraction and Western blot analysis. Cells were harvested, washed once with ice-cold PBS, and gently lysed for 2 min in 80 μl ice-cold lysis buffer (20 mM sucrose, 1 mM EDTA, 20 μM Tris–Cl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). Supernatants were collected and protein concentrations determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Samples were stored at −80 °C or immediately used for immunoblotting. Aliquots containing 30 μg of total protein were separated on SDS–polyacrylamide gels and transferred to nitrocellulose membranes for immunoblot analysis using the indicated primary antibodies. HRP-conjugated secondary antibodies were detected using an ECL detection system.