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Hesperidin promotes programmed cell death by downregulation of nongenomic estrogen receptor signalling pathway in endometrial cancer cells

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ABSTRACT

Endometrial carcinoma (EC) is the most common malignant gynecologic tumor in women. EC is thought to be caused by increasing estrogen levels relative to progesterone in the body. Hesperidin (Hsd), a biologically active flavonoid, could be extracted from *Citrus* species. It has been recently shown that Hsd could exert anticarcinogenic properties in different cancer types. However, the effects of Hsd and its molecular mechanisms on EC remain unclear. In this study, the antiproliferative, apoptotic and genomic effects of Hsd in EC and its underlying mechanisms were identified. We found that Hsd significantly suppressed the proliferation of EC cells in dose and time dependent manner. Mechanistic studies showed that Hsd could contribute apoptosis by inducing externalization of phosphatidyl serine (PS), caspase-3 activity and loss of mitochondrial membrane (MMP). Furthermore, we examined that Hsd could also significantly upregulate the expression of proapoptotic Bax subgroup genes (Bax and Bik) while downregulating the anti-apoptotic protein Bcl-2 in EC cell lines. According to GO enrichment and KEGG pathway analysis of differentially expressed genes in Hsd treated EC cells, we identified that Hsd could promote cell death via downregulation of estrogen receptor I (ESRI) that was directly related to ERK/MAPK pathway. Taken together, our study first showed that Hsd could be an antiestrogenic compound that could modulate nongenomic estrogen receptor signaling through inhibition of EC cell growth. Our findings may provide us a novel growth inhibitory agent for EC treatment after verifying its molecular mechanism with *in vivo* studies.

1. Introduction

Endometrium cancer (EC) is the most common gynecological malignancy type women in the world [1]. The major risk factors associated with EC are, nulliparity, early or late age of menopause, polycystic ovarian syndrome, obesity and imbalanced estrogenic stimulation [2,3]. EC tumors classify into two main categories due to their molecular and histopathological features [4,5]. Approximately 80% of EC tumors belong to Type I EC (endometrioid EC,EEC) which are estrogen-dependent, well-differentiated and characterized by a good prognosis [6]. However, the molecular events included in the development and progression of ECC is not well-elucidated [7,8].

Flavonoids are a group of naturally occurred plant-based compounds which exist abundantly in the fruits, vegetables and beverages [9]. Citrus fruits are the most consumed phenol-rich source containing numerous glycoside derivatives of flavonoids [10]. Hesperidin (Hsd) is the most active flavanone glycoside in citrus flavonoids which is extracted from fruit and peels of citrus species [11]. Hsd has been reported to show important biological features such as antioxidant, anti-inflammatory, antiproliferative and anticarcinogenic activity in various cancers such as lung, colon, bladder, breast cancers [12–20]. In our previous study, we found that Hsd could suppress proliferation and induce apoptosis in non-small lung cancer cell lines (NSLCC). In addition, we also showed that FGF and NF-κB were the most statistically significant pathways in Hsd treated NSLCC [19]. Furthermore, Hsd could promote apoptotic and autophagic signals through suppression of Aurora kinases by mediating PI3K/AKT and mTOR pathways in experimental colon carcinogenesis model [23].

The impacts of Hsd on the diseases such as cancer have recently been a focus of attention because of its pharmacological properties.

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Although Hsd is thought to exhibit anti-proliferative effects against many cancer types, its certain mechanism of action remains unclear. The biologically beneficial activities of Hsd have been reminded to consider its potential usage as therapeutic agent on EC treatment. In this study, we aimed to elucidate signal transduction pathways that were played effective and functional roles in Hsd treated EC progression to understand whether hesperidin could use as a new therapeutic approach on endometrium cancer treatment.

2. Material and methods

2.1. Cell culture

Cell culture media and additional substances were obtained from GIBCO (Thermo Fisher, USA). ECC-1 endometrium cancer cells (CRL-2923) was purchased from American Type Culture Collection. The ECC-1 cells were grown and maintained into RPMI 1640 medium advanced by heat inactivated fetal bovine serum (FBS) (%1) and antibiotics (penicillin/streptomycin, 1%) under 37 °C temperature and 5% CO_2 conditions.

2.2. Hormone depletion with charcoal stripping

To improve accuracy and reliability of analyses, the effects of estrogen mimicking compounds eliminated from cell culture conditions by using phenol red free RPMI 1640 enhanced with charcoal-stripped FBS (5%). Dembinski protocol used to make charcoal-stripped or hormone depleted FBS according to the manuscript [21]. Briefly, activated charcoal (Sigma, C9157) and Dextran T-70 (Sigma, D4751) were added to heat inactivated FBS. Then, the mixture was incubated for an hour at 25 °C by using a platform shaker. After this process, charcoal was pelleted by centrifugation (12.000xg for 15 min). The stripped FBS was sterilized by vacuum filtration (0.2 μM sterile filter), aliquoted and stored at −20 °C prior to use.

2.3. Hesperidin treatment

The purified hesperidin compound was provided from SCBT (Santa Cruz Biotechnology, USA). The ECC-I endometrium cancer cell lines were transferred to appropriate cell culture vessels including phenol red free RPMI 1640 media enhanced with charcoal-stripped FBS for 24 h prior to experimental studies. After 24 h incubation period, ECC-1 cells were treated with DMSO (1%) alone as a vehicle or increasing doses of hesperidin solution (5, 10, 25 and 50 μM) dissolved in DMSO (1%).

2.4. Cell viability assay

Cell proliferation (WST-I) and ELISAPLUS (Cell Death Detection) reagents were obtained from Sigma Aldrich (USA). The influence of hesperidin on ECC-I cell growth was determined using WST-1 assay. As a first step, EC cancer cells were transferred in 96 well multiwell plate (seeding density; 1×10^4 cells per well) and incubated overnight. After this period, EC cancer cells were exposed to increasing doses of hesperidin or DMSO (1%) alone as a vehicle control for various time periods. WST-1 colorimetric cell proliferation assay was carried out after each incubation period. Briefly, WST-1 solution (10 μl) was transferred into wells and incubated for appropriate conditions (4 h, 37 °C). The quantification of changes in the rate of cell proliferation was determined at 450 nm wavelength using microplate reader (Thermo Fisher, Germany). Cell proliferation ratio of Hsd treated EC cancer cells was computed by comparing OD values and given as a percentage of proliferation versus untreated cells (control, 100%).

2.5. Cell cytotoxicity assay

CytoTox 96® Cytotoxicity assay was obtained from Promega (USA). The possible cytotoxic impact of hesperidin on the viability of ECC-I cell was determined using CytoTox 96R NonRadioactive Cytotoxicity Assay kit. Firstly, cells were seeded in 96 well multiwell plate (seeding density; 1×10^4 cells per well) and incubated overnight. After this period, EC cancer cells were exposed to increasing doses of hesperidin or DMSO (1%) alone as a vehicle control for various time periods. For each incubation period, the 10X lysis solution was transferred into wells and incubated at appropriate conditions (an hour, 37 °C). After an hour, supernatants from each well (50 μl) and substrate solution (50 μl) were transferred to the enzymatic microtiter plate and then incubated according to manufacturer's instructions (30 min, 25 °C in dark condition). As a final step in this experiment, the stop solution (50 μl) was inserted into each well and development of color was determined at 490 nm wavelength using microplate reader (Thermo Fisher, Germany). Cell cytotoxicity calculated as a percentage of cell inhibition versus untreated cells (control).

2.6. Impact of Hsd on apoptosis

2.6.1. Apoptotic nucleosomal DNA release assay

To examine whether hesperidin has possible apoptotic effects on the growth of EC cancer cells, Cell Death Detection ELISA^{PLUS} kit used for determination of histone associated DNA fragments out of cytoplasm after stimulation of apoptosis. These cells transferred in 96 well multiwell plate (1×10^4 cells per well) and incubated overnight. Then, EC cancer cells incubated with increasing doses of hesperidin or DMSO (1%) alone as a vehicle control for various time periods. After incubation, EC cancer cells were centrifuged at 200xg for 10 min. Following to removal of supernatants, cells suspended with lysing buffer (200 μl) and incubated for appropriate conditions (30 min, 25 °C). The lysates of EC cancer cells were centrifuged at $200 \times g$ speed at 10 min and supernatants transferred to streptavidin covered microtiter plates. Afterwards, immunoreagent (80 μl) was inserted into each well and incubated on multiwell shaker for two hours at 25 °C and dark condition. After incubation process, solution removed through tapping and then wells were rinsed 3X with incubation buffer (250 μl). Following to the removal of incubation buffer carefully, ABTS solution (100 μl) added and incubated at a multiplate shaker at 250xg speed until developing color was enough for the photometric analysis (15 min). In the final step, ABTS stop solution (100) μl pipetted into each well and development of color determined at 405 nm wavelength via ELISA plate reader (Thermo Fisher).

2.6.2. Caspase-3 enzyme activity assay

The colorimetric caspase 3 assay was obtained from Bio Vision (USA). Previous studies showed that apoptosis could be initiated by the activation of ICE family caspases in mammalian cells. In this study, the caspases-3 colorimetric assay was used to investigate whether dose and time-dependent Hsd had possible effects that was leaded to apoptosis by determining changes in caspase-3 activity. Briefly, 5×10^4 cells were pelleted by centrifugation and resuspended by adding chilled Cell Lysis Buffer (50 μl). After 10 min incubation period on ice, cells were centrifuged again for a minute at 10.000xg. Following transfer of supernatants to fresh tubes, 2X reaction buffer and DEVD-pNA substrate were added to each tube then waited for two hours incubation. Changes in caspase-3 activity were determined by reading samples at 405 nm in a plate reader.

2.6.3. Determination of mitochondrial membrane potential (MMP)

The JC-I dye was obtained from Cayman Chemicals (Michigan, USA). The JC-I assay was used to investigate changes in mitochondrial function against Hsd on EC cancer cells. Briefly, EC cancer cells (5×10^5/2 ml) were centrifuged (10 min, 1000 rpm) as a first step. After removal of supernatants, each sample was resuspended in media (200 μl) plus JC-I dye (20 μl), incubated at appropriate conditions (37 °C, 5% CO_2, 30 min) and centrifuged again. Each sample was