Original Article

Honokiol induces cell cycle arrest and apoptosis via p53 activation in H4 human neuroglioma cells

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Abstract: Objective: To investigate the signal pathway of honokiol-induced apoptosis in H4 human neuroglioma cells and to evaluate whether p53 signaling and cell cycle arrest were involved in honokiol-treated H4 human neuroglioma cells. Methods: The cell viability was detected by the CCK8 assay. The cell apoptosis was assessed by annexin V-PI double-labeling staining and hoechst 33342 staining. The protein expression of cell cycle regulators and tumor suppressors were analyzed by western blotting. Results: Treatment of H4 human neuroglioma cells with honokiol induced cell death in a dose-and time-dependent manner by using CCK8 assay. Consistent with the CCK8 assay, the flow cytometry results showed that the proportion of the apoptosis cells increased after honokiol when compared with untreated group. Moreover, H4 human neuroglioma cells exposed to honokiol, resulted in an accumulation of cells in S and G2/M phase. Apoptotic bodies were clearly observed in human neuroglioma cells when treated with honokiol and then stained with Hoechst 33342. The expression of Cyclin B1, CDC2 and cdc25C were downregulated, however, the expression of p-CDC2 and p-cdc25c was significantly upregulated when the neuroglioma cells were exposed to honokiol. Moreover, p53, p21 and Bax/Bcl-2 were significantly upregulated by honokiol treatment. Conclusions: These results confirmed that honokiol could induce apoptosis in human neuroglioma cells, the underlying molecular mechanisms, at least partially, through activation p53 signaling and induction of cell cycle arrest.

Keywords: Honokiol, neuroglioma, p53/p21, cell cycle arrest

Introduction

Honokiol is a biphenolic phytochemical extracted from the bark and/or seed cones of Magnolia plants. The crude extracts have been extensively used in the traditional herbal medicines in China [1]. Individual constituents of Magnolia have been reported by many investigators to have anti-cancer effects [2]. Honokiol, a small molecular weight natural product isolated and purified from the Magnolia officinalis, has been shown to possess potent anti-oxidation [3], anti-inflammatory [4], ameliorate body fat accumulation and insulin resistance [5], anti-neoplastic [6, 7] and anti-angiogenic properties [1, 8, 9]. Functional studies reveal that honokiol can induce cell apoptosis in human chondrosarcoma cells in vitro and reduce tumor volume in vivo [9]. Moreover, honokiol significantly inhibit cyclosporine A-induced and Ras-mediated survival of renal cancer cells through the down-regulations of vascular endothelial growth factor (VEGF) and cytoprotective enzyme HO-1 [10]. Interestingly, honokiol analogs show much higher growth inhibitory activity in A549 human lung cancer cells and significant increase of cell population in the G0/G1 phase [11]. In human glioblastoma cell, honokiol induces cell apoptosis through upregulation intracellular [Ca2+] [6]. Moreover, honokiol inhibits U87MG human glioblastoma cell invasion through endothelial cells by regulating membrane permeability and the epithelial-mesenchymal transition [12]. Moreover, honokiol may serve to augment T cell-mediated cancer immunotherapy through inhibition of PI3K/mTOR pathway and immunoresistance in glioma, breast and prostate carcinoma [13]. However, the pharmacological functions of honokiol are rarely conducted in human neuroglioma cell growth and anti-cancer efficacy.
Cell cycle control is the major regulatory mechanisms of cell growth, and achieving cell cycle control is an ultimate goal in the treatment of diseases characterized by uncontrolled cell proliferation [14]. Cell division cycle 2 (CDC2) is always overexpressed in malignant glioma cells and is correlated with chemosensitivity. Knockdown of CDC2 expression inhibits proliferation, enhances apoptosis, and increases chemosensitivity to temozolomide in glioblastoma cells [15]. The phosphorylation of cdc25C, a cycle regulatory protein, is involved in arresting effect of glioblastoma cells on the cell cycle at G2/M phase [16]. Interestingly, inhibition of Hsp90 function by ansamycins causes down-regulation of CDC2 and cdc25C and G2/M arrest in glioblastoma cell lines [17]. Moreover, activation of p53, a tumor suppressor protein, is involved in the regulation of cell cycle arrest and apoptosis [18]. The p53 protein normally responds to different forms of cellular stress by binding the consensus sites of target genes, such as p21CIP1 and pro-apoptotic Bax protein, that inhibit cell cycle progression and trigger apoptotic cell death, respectively [19, 20]. In U87 and U251 human glioma cell lines, activating p53 signaling pathway inhibits glioma cell growth in vitro and in vivo by targeting epidermal growth factor [21, 22]. According to recent reports, honokiol was able to induce cell cycle arrest and cell apoptosis [23, 24], suggesting a strong possibility that it could be an effective drug for the treatment of pancreatic cancer and breast cancer, including neuroglioma.

In the present study, we demonstrated in H4 human neuroglioma cell that honokiol induced cell death through activation p21/p53 and suppression CDC2/cdc25C signaling. These data suggested that honokiol might be an effective adjuvant therapy drug for patients with neuroglioma.

Materials and methods

Cell culture

The H4 human neuroglioma cells were obtained from the Chinese Academy of Sciences (Institute of Shanghai Cell Biology and Chinese Type Culture Collection, China), and maintained in DMEM (Dulbecco’s modified Eagle’s medium; Invitrogen), supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) at 37°C in a humidified, 5% CO₂, 95% air atmosphere. The medium was replenished every day. Confluent cells were treated with various concentrations of honokiol (0-50 μM).

Cell viability detection by CCK8

The H4 human neuroglioma cells (1.0 × 10⁴/well) were plated and treated in 96-well plates (three wells per group) with honokiol (0-50 mg/ml) for 24 or 48, respectively. 10 μL of CCK8 (Dojindo, Kumamoto, Japan) was added to the cells, and the viability of the cells was measured at 490 nm using an ELISA reader (BioTek, Winooski, VT, USA) according to the manufacturer’s instructions.

Quantification of apoptosis by flow cytometry

Apoptosis was assessed using annexin V, a protein that binds to phosphatidylserine (PS) residues which are exposed on the cell surface of apoptotic cells. Cells were treated with vehicle or honokiol for indicated time intervals. After treatment, cells were washed twice with PBS (pH = 7.4), and re-suspended in staining buffer containing 1 μg/ml PI and 0.025 μg/ml annexin V-FITC. Double-labeling was performed at room temperature for 10 min in the dark before the flow cytometric analysis. The H4 human neuroglioma cells were immediately analyzed using FACScan and the Cellquest program. Quantitative assessment of apoptotic cells was also assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) method, which examines DNA-strand breaks during apoptosis by using BD ApoAlert<sup>TM</sup> DNA Fragmentation Assay Kit. Briefly, the H4 human neuroglioma cells were incubated with honokiol. The H4 human neuroglioma cells were trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton-X-100 in 0.1% sodium citrate. After being washed, the H4 human neuroglioma cells were incubated with the reaction mixture for 60 min at 37°C. The stained cells were then analyzed with flow cytometer (FC500, Beckman Coulter, FL, USA).

Cell cycle assays

The H4 human neuroglioma cells (1.0 × 10⁵/well) were plated and treated in 6-well plates (three wells per group) with vehicle, DMSO or honokiol (10 μM) for 48 h. After treatment with honokiol, the cells were harvested and subject-
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Hoechst 33342 staining analysis

The H4 human neuroglioma cells were seeded into 6-well plates, and then treated with vehicle, DMSO or honokiol (10 μM) for 48 h, stained with 0.1 μg/ml Hoechst 33342 (Sigma) for 5 min, then observed by fluorescence microscopy using appropriate filters for blue fluorescence.

Western blotting

The H4 human neuroglioma cells were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 50 μg of protein were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with the following antibodies: p53, p21, Bax, Bcl-2, CDC2, P-CDC2, cdc25C and P-cdc25C (Santa Cruz Biotechnology, CA, USA), at dilutions ranging from 1:500 to 1:2,000 at 4°C over-night. After three washes with TBST, membranes were incubated with secondary immunoglobulins (Igs) conjugated to IRDye 800CW Infrared Dye (LI-COR), including donkey anti-goat IgG and donkey anti-mouse IgG at a dilution of 1:10,000-1:20,000. After 1 hour incubation at 37°C, membranes were washed three times with TBST. Blots were visualized by the Odyssey Infrared Imaging System (LI-COR Biotechnology). Signals were densitometrically assessed (Odyssey Application Software version 3.0) and normalized to the β-actin signals to correct for unequal loading using the mouse monoclonal anti-β-actin antibody (Bioworld Technology, USA).

Statistical analysis

The data from these experiments were reported as mean ± standard errors of mean (SEM) for each group. All statistical analyses were performed by using PRISM version 4.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA, and followed by Tukey’s multiple comparison test as a post test to compare the group means if overall \( P < 0.05 \). Differences with \( P \) value of < 0.05 were considered statistically significant.

Results

Cell growth inhibition

The H4 human neuroglioma cell viability was measured when cells were exposed to various concentrations of honokiol (0-50 μM) for 24 h and 48 h. Treatment of H4 human neuroglioma cell with honokiol induced cell growth inhibition in a dose-dependent manner by using CCK8
Honokiol induces apoptosis in human neuroglioma cell assay. As shown in Figure 1A, the concentrations at which honokiol inhibited cell growth by 50% (IC50) was 30 μM for 24 h. The IC50 was 10 μM when the cells were exposed to honokiol for 48 h. To evaluate the time-dependent effect of honokiol on the cell viability, the cells were exposed to 10 μM honokiol for various times. As shown in Figure 1B, the cell viability was significantly decreased with increasing durations.

Effects of honokiol on cell apoptosis and cell cycle arrest

We next investigated whether honokiol induced cell death through an apoptotic mechanism. Annexin V-PI double-labeling was used for the detection of PS externalization, a hallmark of early phase of apoptosis. Consistent with the CCK8 assay, the results showed that growth inhibition was accompanied with an increasing in apoptotic cells, as determined by flow cytometry. The proportion of apoptosis cells increased after honokiol treatment as compared with control group (Figure 2A and 2B). To gain insights into the mechanism of the anti-proliferative activity of honokiol, its effect on cell-cycle distribution was determined via a flow cytometry assay. As shown in Figure 2C, the H4 human neuroglioma cells exposed to honokiol for 48 h resulted in an accumulation of cells in S and G2/M phase. As shown in Figure 3, apoptotic bodies were clearly observed in H4 human neuroglioma cells that had been treated with honokiol for 48 h and then stained with Hoechst 33342. These results were consistent with the Annexin V assay and cell cycle analysis, and confirmed that honokiol could induce apoptosis in human neuroglioma cells. These results suggested that the effects of honokiol suppressed H4 human neuroglioma cell proliferation, at least in part, through cell cycle arrest and cell apoptosis.

Figure 2. Effect of honokiol on cell apoptosis and cell cycle arrest. The H4 human neuroglioma cells were treated with vehicle, DMSO or honokiol (10 μM) for 48 h, the percentage of apoptotic cells was also analyzed by flow cytometric analysis of annexin V/PI double staining (A) and bar graphs represent the percentage of apoptotic cells (B). The percentage of cell cycle phase was analyzed by flow cytometry analysis after cells exposure to honokiol for 48 h (C). Values are expressed as mean ± SEM, n = 3 in each group. * P < 0.05 versus control group.
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Figure 3. Human neuroglioma cells were treated with vehicle, DMSO or honokiol (10 μM) for 48 h. The morphologic changes in the H4 human neuroglioma cells were evaluated using hoechst 33342 staining.

Figure 4. Effects of honokiol on tumor suppressors and apoptosis-related proteins. H4 human neuroglioma cells were treated with various concentrations honokiol (0, 5, 10, 20 and 30 μM) for 48 h, and the expression levels of p53 and p21 were determined by western blotting (A) and densitometric analyses (B). The expression levels of BAX and Bcl-2 were determined by western blotting (C) and densitometric analyses (D). Values are expressed as mean ± SEM, n = 3 in each group. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group.

Effect of honokiol on p53, p21, BAX and Bcl-2

Significant changes in the protein levels of tumor suppressors were observed in H4 human neuroglioma cells with honokiol-treated. As shown in Figure 4A and 4B, p53 and p21 were significantly upregulated by honokiol treatment at doses of 5 μM or more. Moreover, the apoptotic response was further investigated by measuring apoptosis-related proteins expression. Treatment of H4 human neuroglioma cells with honokiol significantly increased the pro-apoptotic Bax level and decreased the anti-apoptotic Bcl-2 level at doses of 5 μM or more (Figure 4C and 4D). These results indicated that honokiol might induce cell death through activation tumor suppressors signaling pathway and upregulation of the Bax/Bcl-2 ratio.

Effect of honokiol on the cell cycle regulated protein

To evaluate the potential molecular mechanism by which honokiol causes a G2/M arrest, we
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analyzed the steady-state levels of proteins involved in the G2/M checkpoint. The results found that Cyclin B1, CDC2 and cdc25C were downregulated upon honokiol treatment in H4 human neuroglioma cells (Figure 5A-D). However, we found that the expression of p-CDC2 and p-cdc25C was significantly upregulated when the neuroglioma cells were exposed to honokiol at doses of 10 μM or 20 μM (Figure 5A-D).

Discussion

The anti-cancer activity of honokiol is confirmed in many tumor models [6, 7, 9, 12, 23], but there is mostly sparse reports of the anticancer activity of honokiol on human nerve tumor, especially on human neuroglioma. This study showed that honokiol, an active component isolated from the herb Magnolia officinalis, exerted anticancer effects in H4 human neuroglioma cells through the regulation of cell cycle regulated proteins and p53 signaling, which induced apoptotic cell death. In addition, we suggested that honokiol induces apoptotic cell death in neuroglioma cells through the upregulation of the Bax/Bcl-2 ratio.

P53 is a crucial protein in cellular stress response, and p53 was an important component of the cell cycle arrest [25]. Wild-type p53 was considered to participate in apoptosis in response to DNA damage in many tumor cells [26]. When cells received UV or ionizing radiation and were exposed to anticancer drugs, p53 protein was accumulated [27]. The p53 pathway has been reported to be involved in histone acetyltransferase inhibitor (HATi) II induces apoptosis in glioma cell lines, and upregulation of p53 signaling pathway-related genes in HATi II-treated cells is confirmed by quantitative RT-PCR and Western blotting [22]. In our study, human neuroglioma cell exposure to honokiol could upregulate the expression of p53 and p21, and the apoptotic cell proportion was increased in honokiol-treated group compared with control group. Thus it is necessary to evaluate the level of Bax and Bcl-2. Our results showed that honokiol-induced apoptosis of neuroglioma cells was accompanied with upregulation of Bax and downregulation of Bcl-2. Pro-apoptotic Bax is a p53 downstream target, which supports the view that honokiol probably trigger the p53/Bax mediated apoptosis pathway.

Figure 5. Effects of honokiol on G2/M checkpoint proteins. H4 human neuroglioma cells were treated with honokiol (0, 10 or 20 μM) for 48 h, and the expression levels of Cyclin B1, CDC2 and p-CDC2 were determined by western blotting (A) and densitometric analyses (B). The expression levels of cdc25C and p-cdc25C were determined by western blotting (C) and densitometric analyses (D). Values are expressed as mean ± SEM, n = 3 in each group. *P < 0.05, **P < 0.01 versus control group.
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P53 transcriptional function is considered as a marker of large-scale differently expressed genes, which are involved in cell cycle arrest [28]. Reprimo appears to induce cell cycle arrest by inhibiting CDK1 activity and nuclear translocation of the CDC2/cyclin B1 complex, and may be involved in regulation of p53-dependent G2 cell cycle arrest [29]. In the present study, we demonstrated that honokiol-treated caused the downregulation of both CDC2 and cdc25C, however, the expression of p-CDC2 and p-cdc25c was significantly upregulated when the human neuroglioma cells were exposed to honokiol. CDC2 is the cyclin-dependent kinase responsible for the entry and exit from G2 and mitosis. It forms a complex with cyclin B1 or cyclin A. It can be inactivated by wee-1 and myt-1 when it is phosphorylated in T15 [30]. In human esophageal cancer cells, jaridonin results in G2/M phase arrest through upregulation phosphorylation of cdc25C and activation of checkpoint kinases Chk1 and Chk2 [31].

In conclusion, honokiol could induce apoptosis and cell cycle arrest in H4 human neuroglioma cell, the underlying molecular mechanisms, at least partially, through activation p53 signaling and downregulation CDC2/cdc25C expression. In view of the results of this experiment, it seemed reasonable to highlight the possibility of honokiol in the clinical treatment of neuroglioma.

Disclosure of conflict of interest

None.

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