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Original article

Emodin targets mitochondrial cyclophilin D to induce apoptosis in HepG2 cells

Ling Zhang, Dian He, Kun Li, Hongli Liu, Baitao Wang, Lifang Zheng*, Jiazhong Li*

School of Pharmacy, Lanzhou University, Lanzhou 730000, China

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ABSTRACT

Emodin has demonstrated potent anticancer activity in human hepatocarcinoma cells and animal models, however, the cellular targets of emodin have not been fully defined. Here we report that emodin induces the dysfunction of mitochondria and the apoptosis in HepG2 cells through an enrichment in mitochondria. Specifically, A mitochondrial matrix protein (cyclophilin D, CyPD) is involved in emodin-induced apoptosis, and the inhibitor of CyPD (cyclosporin A) could almost completely suppressing the apoptosis; Moreover, as the expression of CyPD could be effectively inhibited by antioxidant N-acetyl-L-cysteine and epidermal growth factor (the activator of ERK), reactive oxygen species and ERK might be involved in the relevant role of CyPD. A further molecule-docking discloses the existence of three hydrogen-bonds in CyPD-emodin complex. Thus, target localization and CyPD in mitochondria provides an insight into the action of emodin in the treatment of liver cancer.

1. Introduction

Emodin is a naturally occurring anthraquinone from the roots and rhizome of *Rheum palmatum L*, which has been used for thousands of years in traditional Chinese medicine for the treatment of constipation jaundice, gastro-intestinal hemorrhage, and ulcers. Moreover, emodin has demonstrated potent anticancer activities such as arresting malignant cell growth, inducing cancer cell death, inhibiting cancer metastasis, and reversing multidrug resistance [1,2] etc. A number of molecular targets in various types of human cancer cells have been affected by emodin, including phosphatidylinositol 3-kinase (PI3K)/Akt [3], mitogen-activated protein kinase (MAPKs) [3], tyrosine kinase [4], peroxisome proliferator-activated receptor gamma (PPARγ) [5], p53 [6], protein kinase C [7], NF-κB [8], casein kinase II [9], Janus-activated kinase 2 [10], and DNA [11]. In human hepatoblastoma cells, emodin induces apoptosis via mitochondrial and death receptor-mediated pathways [3], and cause G2/M arrest, activate caspase-9, caspase-8, p53, and Fas [12,13]. Despite its undoubted anticancer efficacy, the primary cellular target and mode of action of emodin still need to be further explored.

Mitochondrial matrix-specific protein cyclophilin D (CypD), belonging to a family of highly homologous peptidylprolyl *cis*-*trans* isomerases (PPIases), regulates mitochondrial permeability transition pore (mPTP) and thus triggers cell death [14]. Evidently, mitochondria from CypD$^{-/-}$mice were more resistant to Ca^{2+}-induced mPTP opening than wild-type mice [15]. CypD is redox regulated. CypD in its reduced form is responsible for refolding of the newly imported proteins in mitochondria and contributes to maintain organelle integrity. And oxidized CypD induces the opening of mPTP and leads to mitochondrial-mediated cell death [16]. Hence, CypD has been widely studied in drug-induced apoptosis, such as sorafenib [17], esculetin [18], and berberine [19].

In the present study, the role of CypD in the emodin-induced apoptosis in human hepatoblastoma cells HepG2 was investigated. We found that emodin mainly localized in mitochondria to trigger mitochondrial dysfunction, elicited the cellular oxidative stress and eventually induced apoptosis. Cyclosporin A (CsA), an inhibitor of mPTP by binding to CypD [20], suppressed the apoptosis and the release of cytochrome c (Cyto-C). Expression of CypD was stimulated by emodin and attenuated by antioxidant N-acetyl-L-cysteine (NAC) and epidermal growth factor (EGF), an extracellular signal-regulated kinase (ERK) activator. Molecule docking study indicated that emodin can bind to CypD with good spatial and energy matching.

Abbreviations: CyPD, cyclophilin D; mPTP, mitochondrial permeability transition pore; CsA, cyclosporin A; Cyto-C, cytochrome c; NAC, *N*-acetyl-L-cysteine; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; MMP, mitochondrial membrane potential; MTDR, mito-tracker deep red; TMRE, tetramethylrhodamine ethyl ester; ROS, reactive oxygen species.

* Corresponding authors.
E-mail addresses: zhenglf@lzu.edu.cn (L. Zheng), lijiazhong@lzu.edu.cn (J. Li).

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2. Materials and methods

2.1. Chemicals and reagents

Emodin, EGF, CsA and NAC were from Sigma (Beijing, China). All antibodies were from Affinity Biosciences (OH, USA). For the subsequent assays, the final concentrations of each reactant are indicated in parentheses.

2.2. Cell culture

HepG2 cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. The cells were cultured in 1640 growth medium supplemented with 10% fetal bovine serum (Hyclone, Shanghai, China) in a humidified 5% CO_2 incubator at 37 °C.

2.3. Localization of emodin in mitochondria

HepG2 cells (5×10^4 per dish) were cultured on coverslips and treated with emodin (50 μM) for 15 min followed by staining with 100 nM Mito-Tracker deep red (MTDR, Thermo Fisher, Shanghai, China) for another 15 min. Then the cells were washed three times with phosphate-buffered saline (PBS, pH = 7.4) and photographed using a confocal microscopy (FV 1000MPE, Olympus, Japan). The colocalization coefficient is analyzed by the software along with the confocal microscope.

2.4. Mitochondrial membrane potential (MMP) detection

After incubation HepG2 cells (5×10^4 per well) with emodin (50, 100 μM) for 6, 12, 24, 48 h, the medium was replaced with fresh medium containing 100 nM of tetramethylrhodamine ethyl ester (TMRE, Biolite Biotech, Tianjin, China) and incubated for another 30 min at 37 °C. Cells were then washed with PBS three times. The fluorescence intensity was quantitatively measured using a microplate reader (Varioskan Flash, Thermo scientific, Finland) at 549 nm excitation and 574 nm emission.

2.5. Cellular ATP detection

Cellular ATP was detected by ATP Assay Kit (Beyotime, Shanghai, China) following the manufacturer's instruction. HepG2 cells were treated with emodin (50, 100 μM) for 6, 12, 24, 48 h, lysed with ice-cold lysis buffer, and centrifuged at 12000*g* for 10 min at 4 °C. The supernatant was used to assay the ATP level by the detection buffer, and luminescence was measured using a Thermo Varioskan Flash microplate reader.

2.6. Superoxide anion $O_2^{-\bullet}$ detection

After treatment HepG2 cells with emodin (50, 100 μM) for 6, 12, 24, 48 h, 50 μL of 2.5 μM MitoSOX probe working solution (Thermo Fisher, Shanghai, China) was added and incubation continued for another 15 min. Cells were then rinsed with PBS three times and the fluorescence intensity was determined by a microplate reader at 510 nm excitation and 580 nm emission.

2.7. Apoptosis assay

After the cells were pretreated with CsA (5 μM) for 1 h, emodin (50, 100 μM) was added and incubation continued for another 48 h. Then nuclear condensation was analyzed by Hoechst 33342 staining. The images were captured by an inverted fluorescence microscopy (DMI 4000B, Leica, Germany). The percentage of apoptotic cells were determined by the AnnexinV-FITC/PI kit (Beyotime, Jiangsu, China) using FACS Canto flow cytometer (Canto, Becton Dickinson, USA).

2.8. Western blots

After treatment, cytosol and mitochondrial fractions were isolated. Equal amounts of denatured proteins (20–40 μg) were separated by 12% SDS PAGE followed by electroblotting onto polyvinylidene difluoride membranes. Targeted proteins were detected with specific primary antibodies. Corresponding secondary antibodies were then utilized and immunoreactive bands were visualized by fully automatic chemiluminescence analysis system (Tanon, Shanghai, China).

2.9. Cell viability assay

After the cells were pretreated with NAC (5 mM), or EGF (10 ng/mL) for 1 h, emodin (50, 100 μM) was added and incubation continued for 6 h or 48 h respectively. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, sigma) assay was performed to assess the cell viability [21].

2.10. Molecule docking between emodin and CypD

The 3D structure of emodin was drawn in SYBYL 6.9 software [22]. Partial atomic charges were calculated using the Gasteiger-Hückel method, and energy minimization was performed using the Tripos force field with a convergence criterion of 0.01 kcal mol^{-1}. Then, the CDOCKER module of Discovery Studio 2.5 software [23] was used to dock emodin with CypD (PDB code: 2BIT). We defined the binding site sphere through receptor cavities, and set the radius of the sphere to 7 Å [24].

2.11. Statistical analysis

The data are expressed as the means ± SD of at least three independent experiments. Statistical differences between two groups were measured by one-way factorial analysis of variance (ANOVA), using Duncan's post-hoc test (SPSS 18.0). $p < 0.05$ versus control was used as the criterion for statistical significance.

3. Results

3.1. Emodin localized in mitochondria and triggered mitochondrial dysfunction

Analysis of the chemical structure of emodin (Fig. 1A) reveals that this compound has a planar polycyclic aromatic system, allowing it possessing the inherent fluorescence. Thus, we mapped its cellular localization in HepG2 cells by laser confocal microscopy (Fig. 1B). Upon selective excitation at 458 nm, the cells incubated with emodin exhibited bright green fluorescence in discrete subcellular distribution (A2), likely to be mitochondria around the nucleus. In order to prove this assumption, the image from Mito-tracker deep red-staining was performed by excitation at 633 nm (B2). The merging of A2 and B2 presents a clear yellow colocalization signal (C2) with a colocalization coefficient of 0.609, confirming emodin permeates into mitochondria and localizes in and around mitochondria. The highlighted part in the merged picture is enlarged in D2. Next, we investigated the impacts of emodin on mitochondrial functions. Drug induced mitochondrial dysfunction range from disruption of MMP, reduced ATP production, excessive production of ROS, deregulation of calcium to opening of mPTP and initiation of apoptosis [25]. As expected, emodin decreased MMP and ATP production in a dose- and time-dependent manner (Fig. 1C, D). Simultaneously, emodin-