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Evodiamine, a plant alkaloid, induces calcium/JNK-mediated autophagy and calcium/mitochondria-mediated apoptosis in human glioblastoma cells

Ann-Jeng Liu [a,b], Sheng-Hao Wang [c], Ku-Chung Chen [c], Hsiu-Ping Kuei [d], Yung-Luen Shih [d,e], Sz-Ying Hou [c,f], Wen-Ta Chiu [a,g], Sheng-Huang Hsiao [b,1], Chwen-Ming Shih [c,f,h,i,*,1]

[a] Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, ROC
[b] Department of Neurosurgery, Taipei City Hospital Ren-Ai Branch, Taipei, Taiwan, ROC
[c] Department of Biochemistry, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, ROC
[d] Department of Pathology and Laboratory Medicine, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan, ROC
[e] School of Medical Laboratory Science and Biotechnology, College of Medicine, Taipei Medical University, Taipei, Taiwan, ROC
[f] Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, Taiwan, ROC
[g] Department of Neurosurgery, Taipei Municipal Wan-Fang Hospital, Taipei, Taiwan, ROC
[h] Traditional Herbal Medicine Research Center, Taipei Medical University Hospital, Taipei, Taiwan, ROC
[i] Center for Reproductive Medicine and Sciences, Taipei Medical University Hospital, Taipei, Taiwan, ROC

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ABSTRACT

Glioblastomas, the most common primary gliomas, are characterized by increased invasion and difficult therapy. Major clinical medicines for treating gliomas merely extend the survival time for a number of months. Therefore, development of new agents against gliomas is important. Autophagy, a process for degrading damaged organelles and proteins, is an adaptive response to environmental stress. However, the role of autophagy in glioblastoma development still needs to be further investigated. Evodiamine, a major alkaloid isolated from *Evodia rutaecarpa* Bentham, has various pharmacological activities, such as inhibiting tumor growth and metastatic properties. However, the effects of evodiamine on glioblastomas and their detailed molecular mechanisms and autophagy formation are not well understood. In this study, we observed that evodiamine induced dose- and time-dependent apoptosis in glioma cells. Blockade of calcium channels in endoplasmic reticulum (ER) significantly reduced evodiamine-induced cytosolic calcium elevation, apoptosis, and mitochondrial depolarization, which suggests that evodiamine induces a calcium-mediated intrinsic apoptosis pathway. Interestingly, autophagy was also enhanced by evodiamine, and had reached a plateau by 24 h. Pharmacological inhibition of autophagy resulted in increased apoptosis and reduced cell viability. Inhibition of ER calcium channel activation also significantly reduced evodiamine-induced autophagy. Inactivation of c-Jun N-terminal kinases (JNK) suppressed evodiamine-mediated autophagy accompanied by increased apoptosis. Furthermore, evodiamine-mediated JNK activation was abolished by BAPTA-AM, an intracellular calcium scavenger, suggesting that evodiamine mediates autophagy *via* a calcium-JNK signaling pathway. Collectively, these results suggest that evodiamine induces intracellular calcium/JNK signaling-mediated autophagy and calcium/mitochondria-mediated apoptosis in glioma cells.

1. Introduction

Glioblastoma multiforme (GBM) and anaplastic astrocytoma, the most common and serious primary gliomas in adults, are highly mobile, invasive, and difficult to completely resect through surgery [1]. Therefore, radiation and chemotherapy are generally initiated following surgical treatment as adjuvant therapies. The alkylating agent, temozolomide (TMZ), is the major chemotherapeutic drug used clinically to treat malignant gliomas [2]. However,

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; 3-MA, 3-methyladenine; AIF, apoptosis-inducing factor; AVO, acidic vesicular organelle; $[Ca^{2+}]_i$, intracellular calcium; Evo, evodiamine; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide; LC3, microtubule-associated protein 1 light chain 3; FITC, fluorescein isothiocyanate; PI, propidium iodide; ROS, reactive oxygen species.

* Corresponding author at: Department of Biochemistry, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, ROC. Tel.: +886 2 27361661/3151; fax: +886 2 27356689.
E-mail address: cmshih@tmu.edu.tw (C.-M. Shih).
[1] Equal corresponding author.

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the effect of TMZ against gliomas is modest since it just increases the median survival time of 2 months for patients [3]. Therefore, development of new agents for treating gliomas is important.

Evodiamine (Evo) is a quinozole alkaloid isolated from the dried, unripe fruit of *Evodia rutaecarpa* Bentham [4]. It is widely used in Chinese herbal medicine, with variable effects. Evo shows pharmacological effects of antitumor growth [5], anti-metastatic [6], anti-anoxic [7], antinociceptive [8] and vasorelaxant [9] properties. An *in vitro* study showed that Evo is able to induce apoptosis in HeLa cells through modulating reactive oxygen species (ROS), altering mitochondria, and activating caspases [10]. However, the effects of Evo on glioma development and autophagy induction are unclear.

Programmed cell death is classified into two types, namely apoptosis (type I) and autophagy (type II) [11]. Both processes are important in the development and tissue homeostasis of multicellular organisms. Apoptotic cells usually exhibit unique morphological and biochemical characteristics, such as membrane blebbing, chromatin condensation, DNA fragmentation, mitochondrial depolarization, release of apoptotic factors from the mitochondria, and caspase activation [12,13]. In contrast, autophagy is an evolutionarily conserved mechanism that regulates the turnover of long-lived cellular proteins and damaged organelles, and is a response to environmental stimuli such as nutrient depletion [14]. The formation of double-membrane vesicles (autophagosomes) and processing of microtubule-associated protein 1 light chain 3 (LC3) are two hallmarks of autophagy [15]. Disruption of the autophagic pathway may result in cancer development, bacterial and viral infections, neurodegenerative disorders, and cardiovascular diseases [16,17]. The role of autophagy in cell death, and its cross-reaction with apoptosis have not been well defined. It was demonstrated that survival of cells deprived of nutrients depends on the induction of autophagy. Inhibition of autophagy also enhances cell apoptosis [18,19]. However, autophagic cell death during oxidative stress was also reported to be independent of apoptosis [20]. As was mentioned above, autophagy and apoptosis might interact or might occur independently. Such results might reflect a complicated interaction in cells receiving different types of stimuli.

In this study, we examined the effects of Evo on a U87-MG human malignant glioblastoma cell line. We found that inhibition of Evo-induced autophagy resulted in an increase of apoptosis. Both autophagy and apoptosis were induced following an increase in intracellular calcium ($[Ca^{2+}]_i$). Inhibition of calcium release from endoplasmic reticulum (ER) resulted in decreased autophagy, apoptosis, and mitochondrial depolarization, suggesting that Evo induces a calcium-mediated cellular response in U87-MG cells. Blockade of c-Jun N-terminal kinase (JNK) activation reduced the percentage of Evo-induced autophagy accompanied by an increase in apoptosis. An intracellular calcium scavenger abrogated JNK activation. Finally, all of the results demonstrated that Evo induced a calcium/JNK-mediated autophagy and calcium/mitochondria-mediated apoptosis.

2. Materials and methods

2.1. Cell culture, treatment, and chemicals

Human glioblastoma U87-MG cells were purchased from the American Type Culture Collection (Manassas, VA) and grown at 37 °C in culture medium. The medium consisted of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 200 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 mM sodium pyruvate, and 1% non-essential amino acids. The mixture was kept in a humidified atmosphere containing 5% CO_2. For the drug-responsiveness experiments, U87-MG cells were either pretreated (treatment group) or not (control group) with an indicated inhibitor for 1 h. They were then incubated with 6 μM Evo for the rest of the experimental period. DMEM, FBS, and non-essential amino acids were purchased from Hyclone (Logan, UT), and phenol red-free RPMI, L-glutamine, penicillin–streptomycin, and sodium pyruvate were obtained from Gibco (Grand Island, NY). Evo, bovine serum albumin (BSA), acridine orange (AO), propidium iodide (PI), 3-methyladenine (3-MA), Fluo-3 AM, 5,5,6,6,-tetrachloro-1,1,3,3,-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), SP600125, 1,2-bis(2-amino-phenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid (BAPTA-AM), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). 2-Aminoethoxydiphenyl borate (2-APB) was from Calbiochem (San Diego, CA). Rabbit polyclonal anti-LC3 was obtained from MBL International (Nagoya, Japan). Rabbit polyclonal anti-GAPDH, anti-phosphorylated-JNK (p-JNK) was obtained from Cell Signaling (Beverly, MA). Goat polyclonal anti-JNK1 (total (t)-JNK1) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Annexin-V-FITC reagent was supplied by Biovision (Mountain View, CA). The secondary antibodies, including horseradish peroxidase (HRP)-conjugated goat anti- rabbit immunoglobulin G (IgG), were purchased from Pierce (Rockford, IL). Polyvinylidene difluoride (PVDF) membranes were supplied by Millipore (Bedford, MA), and the Protein Assay Dye Reagent was from Bio-Rad Laboratories (Hercules, CA).

2.2. Measurement of cell viability

Cell viability was measured with an MTT assay, which is based on the conversion of a tetrazolium salt to colored formazan, as described in our previous report [15]. In brief, 10 μl of an MTT solution (5.5 mg/ml in PBS) was added to each well of a 96-well plate containing 100 μl medium and cells at 4 h before the end of incubation. The supernatant was then discarded, and 100 μl DMSO was added to dissolve the formazan. The absorbance was measured at 570 nm using a Thermo Varioskan Flash Reader (Thermo Electron Corporation, France).

2.3. Measurement of apoptosis and necrosis

Apoptosis was analyzed by detecting phosphatidylserine externalization, using a flow cytometer with a two-color analysis of FITC-labeled annexin V/PI double-staining, as described in our previous report [21]. In brief, trypsinized adherent cells and suspended cells in the medium were collected in HEPES buffer containing 10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$. Subsequently, cells were stained with annexin V (1 μg/ml) and PI (0.2 ng/ml) for 15 min and then analyzed by flow cytometry using CellQuest software (Becton Dickinson, San Jose, CA). The percentage of total apoptosis was the sum of primary apoptosis (annexin V+/PI−) and late apoptosis (annexin V+/PI+). The ratio of necrosis was indicated in the upper-left quadrant (annexin V−/PI+).

2.4. Measurement of acidic vesicular organelles (AVOs)

The percentage of autophagy was analyzed by flow cytometry with AO dye according to published procedures [15]. After Evo treatment, cells were stained with AO (1 μg/ml) for a period of 20 min. Trypsinized adherent cells and cells suspended in the medium were collected in phenol red-free RPMI growth medium. Green (510–530 nm) and red (650 nm) fluorescence emissions from 10^4 cells illuminated with blue (488 nm) excitation light were measured with a flow cytometer using CellQuest software (Becton