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Lactucopicrin ameliorates oxidative stress mediated by scopolamine-induced neurotoxicity through activation of the NRF2 pathway

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(-)-Scopolamine hydrobromide trihydrate (PubChem CID: 20055509)
Retinoic acid (PubChem CID: 444795)
2′,7′-Dichlorofluorescein diacetate (PubChem CID: 104913)
Rhodamine 123 (PubChem CID: 65217)
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ABSTRACT

Cholinergic activity plays a vital role in cognitive function, and is reduced in individuals with neurodegenerative diseases. Scopolamine, a muscarinic cholinergic antagonist, has been employed in many studies to understand, identify, and characterize therapeutic targets for Alzheimer's disease (AD). Scopolamine-induced dementia is associated with impairments in memory and cognitive function, as seen in patients with AD. The current study aimed to investigate the molecular mechanisms underlying scopolamine-induced cholinergic neuronal dysfunction and the neuroprotective effect of lactucopicrin, an inhibitor of acetylcholine esterase (AChE). We investigated apoptotic cell death, caspase activation, generation of reactive oxygen species (ROS), mitochondrial dysfunction, and the expression levels of anti- and pro-apoptotic proteins in scopolamine-treated C6 cells. We also analyzed the expression levels of antioxidant enzymes and nuclear factor (erythroid-derived 2)-like 2 (NRF2) in C6 cells and neurite outgrowth in N2a neuroblastoma cells. Our results revealed that 1 h scopolamine pre-treatment induced cytotoxicity by increasing apoptotic cell death via oxidative stress-mediated caspase 3 activation and mitochondrial dysfunction. Scopolamine also downregulated the expression the antioxidant enzymes superoxide dismutase, glutathione peroxidase, and catalase, and the transcription factor NRF2. Lactucopicrin treatment protected C6 cells from scopolamine-induced toxicity by reversing the effects of scopolamine on those markers of toxicity. In addition, scopolamine attenuated the secretion of neurotrophic nerve growth factor (NGF) in C6 cells and neurite outgrowth in N2a cells. As expected, lactucopicrin treatment enhanced NGF secretion and neurite outgrowth. Our study is the first to show that lactucopicrin, a potential neuroprotective agent, ameliorates scopolamine-induced cholinergic dysfunction via NRF2 activation and subsequent expression of antioxidant enzymes.

1. Introduction

Cholinergic neuronal activity plays a prominent role in the basal forebrain and is essential for cognitive functions, such as learning and memory. Cognitive impairment is negatively correlated with levels of acetylcholine in the cerebrospinal fluid of patients with dementia (Tohgi et al., 1996). Acetylcholine receptor-deregulating compounds such as scopolamine have been used to create AD models for studies into the pathophysiological mechanisms of AD and drug development (Hasselmann, 2014). Scopolamine, from *Giovanni Scopoli*, is a broad-spectrum, non-specific, and well-known cholinergic antagonist with high binding affinity for

Abbreviations: ROS, reactive oxygen species; AD, Alzheimer's disease; AChE, acetylcholine esterase; CNS, central nervous system; NGF, neurotrophic nerve growth factor; $\Delta\psi_m$, mitochondrial membrane potential; SOD, superoxide dismutase; GPx, glutathione peroxidase; NRF2, nuclear factor (erythroid-derived 2)-like 2; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; FBS, fetal bovine serum; mAChR, muscarinic acetylcholine receptor; FITC, fluorescein isothiocyanate; PI, propidium iodide; ANOVA, analysis of variance; *P*, probability; *F*, degree of freedom; BcL-2, B-cell CLL/lymphoma 2; Bax, BcL-2-associated X Protein; N2a cell, mouse neuroblastoma cell; C6 cell, rat glioma cell; ERK, extracellular signal-regulated kinase; GSK-3β, glycogen synthase kinase-3β; BcL-xl, B-cell lymphoma extra-large; Bad, Bcl-2-associated death promoter; Cyto C, cytochrome C.

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muscarinic receptors (Konar et al., 2011). Bajo et al. and others reported that treatment of scopolamine might induce AD. Therefore it has been used to develop *in vitro* and *in vivo* AD models (Bajo et al., 2015; Lee et al., 2014; Moosavi et al., 2012; Pandareesh and Anand, 2013; Weinreb et al., 2012).

AD is the most common neurodegenerative disease. It is characterized by reduced neuronal activity and loss of neuronal cells, which results in memory impairment (Ghezzi et al., 2013). Memory impairment becomes more severe with age and has a major impact on daily activities, which negatively affects the quality of life of afflicted individuals and their family members (Brookmeyer et al., 2007). Neuronal activity can be controlled by metabolic support, extracellular ionic balance, synaptic transmission, and neurotrophic factors, all of which have been implicated in memory function (León et al., 2013; Pandareesh and Anand, 2013). AD is one of complex neurodegenerative disorders. Only neuron-oriented research can't overcome the Alzheimer's disease. Besides the degeneration of cholinergic neurons, it is essential to understand the role glial and microglia cells in the AD pathogenesis. Recently, Dzamba. D et al. reported that glial cells must be the key elements of Alzheimer's disease (Dzamba et al., 2016). Therefore, our attention towards glia would serve as important momentum to turn the significance of glia in the pathogenesis and progression of AD.

Memory impairment caused by neuronal cell loss can result from a variety of factors, such as beta amyloid (Aβ) (Blennow et al., 2015), free radicals, pesticides (Li et al., 2015; Yegambaram et al., 2015), and malnutrition (Ogawa, 2014). These factors are generally accepted as part of the etiology of AD, particularly when they affect the cerebral cortex and other areas of the brain (Kása et al., 1997). The extent of cholinergic neuron loss in the central nervous system (CNS) correlates with the severity of cognitive impairment. Scopolamine treatment generates reactive oxygen species (ROS) (Tao et al., 2014). Oxidative stress-induced cell death is an important cause of neurodegeneration in this AD model. Changes in the expression levels of synaptic proteins, neurotrophic factors, and antioxidant enzymes may be responsible for scopolamine-induced neurotoxicity, and modulation of such factors may be a way to protect cells or restore them to their original state (Xiong et al., 2015).

Recent studies showed that acetylcholine esterase (AChE) inhibitors suppress synaptic dysfunction, Aβ plaque formation, and other causes of neuronal damage, such as inflammatory reactions caused by T-cell activation, cytokines, and CNS inflammation (Kim et al., 2014b; León et al., 2013). AChE inhibitors, such as donepezil, rivastigmine, and galantamine, are approved for the treatment of AD patients (Murray et al., 2013). These inhibitors bind to and reversibly inactivate cholinesterase and inhibit hydrolysis of acetylcholine, resulting in increased acetylcholine concentration in cholinergic synapses, which improves synaptic function. However, synthetic AChE inhibitors are associated with a number of adverse events, such as nausea, diarrhea, and vomiting, which has contributed to discontinuation in Phase II/III clinical trials and extremely cautious use in patients with underlying conditions. The side effects of synthetic AChE inhibitors manifest as salivary gland dysfunction, arrhythmias, gastrointestinal disorders, hepatotoxicity, miosis, respiratory problems, bradycardia, bronchospasm, and cardiac diseases (Godyń et al., 2016; Haerter and Eikermann, 2016; Kim et al., 2014b). Many natural plant sources have been shown to have neuroprotective effects. Moreover, the side effects of natural compounds are often not as serious as those of synthetic drugs. Natural compounds also have strong antioxidant and neurotrophic-mimetic activities and many other properties that make them preferable to synthetic compounds (Kim et al., 2014b). In an effort to understand the mechanism of scopolamine-induced toxicity in astrocytes, we used a reversible AChE inhibitor, lactucopicrin (also known as intybin), a natural sesquiterpene lactone derived from *Lactuca virosa* (wild lettuce), *Cichorium intybus*, and dandelion coffee. Lactucopicrin-containing plants have been used as antimalarial agents, sedatives, and analgesics in humans (Rollinger et al., 2005; Wesołowska et al., 2006).

In the current study, we examined the neuroprotective effects of lactucopicrin against scopolamine-induced neurotoxicity in C6 glioma cells, with a focus on ROS production and mitochondrial dysfunction as assessed via cytochrome C release and mitochondrial membrane potential ($\Delta\psi_m$), free radical scavenging antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase, and NGF secretion. We also investigated the neurodifferentiation effect of scopolamine on N2a neuroblastoma cells. In addition, the molecular mechanisms underlying the effects of lactucopicrin were addressed. A recent study showed that nuclear factor (erythroid-derived 2)-like 2 (NRF2), a transcription factor that induces endogenous defense pathways and the generation of antioxidant enzymes (Ni et al., 2014), is involved in protection against oxidative stress in a model of AD. In the present study, we examined the ability of lactucopicrin to attenuate oxidative stress in a scopolamine-induced AD model via regulation of NRF2.

2. Materials and methods

2.1. Cell lines and reagents

C6 glioma and N2a neuroblastoma cells were obtained from the Korean Cell Line Bank (Seoul, South Korea). Hyclone, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], retinoic acid, scopolamine, and rhodamine 123 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lactucopicrin was purchased from Extrasynthese (Lyon Nord-69730, Genay, France) and galantamine hydrobromide was purchased from Tocris (Bristol, BS11 0QL, UK). All other chemicals were taken from our laboratory stocks.

2.2. Cell culture and treatments

C6 rat glioma cells were grown in high-glucose DMEM medium supplemented with 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS) in a humidified incubator under 5% CO_2 and 95% air at 37 °C and maintained by changing media every other day. Cells at 60% confluence were pre-treated with scopolamine (3 mM) in serum-free DMEM medium for 1 h. The medium was then replaced by DMEM medium containing 2% FBS and vehicle, lactucopicrin (0.5, 1, or 2 μM), or galantamine (2 μM) for 24 h, at which point cells were subjected to experimental assays.

2.3. Cell viability assay

An MTT assay was used to assess the viability of C6 cells. C6 cells grown to a density of 10×10^4 in 24-well plates were exposed to various concentrations (1, 3, 5, or 10 mM) of scopolamine and assessed for viability after 24 h. Because scopolamine induced cytotoxicity in a dose-dependent manner, we selected 3 mM as the dose of scopolamine for the cytoprotection assay with lactucopicrin and galantamine. Cells were pre-treated with 3 mM or 5 mM scopolamine for 1 h and then treated with various concentrations (0.5, 1, or 2 μM) of lactucopicrin or 2 μM galantamine for 24 h. The culture media was collected and stored at −20 °C. The cells were incubated with MTT reagent (0.5 mg/ml final concentration) for 1 h at 37 °C. The MTT solution was then removed and formazan was dissolved by addition of DMSO. The concentration of solubilized