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Ligusticum chuanxiong prevents rat pheochromocytoma cells from serum deprivation-induced apoptosis through a protein kinase A-dependent pathway

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Abstract

Ligusticum chuanxiong (LC) is a traditional Chinese herbal medicine used to treat various cardiovascular diseases. In this study, the butanol extract of LC was found to protect neuronal-like pheochromocytoma cells from serum deprivation-induced apoptosis. Both a serine/threonine kinase inhibitor and a specific protein kinase A (PKA) inhibitor blocked the protective effect of LC. A transcription inhibitor (actinomycin D) and a protein synthesis inhibitor (cyclohexamide) also attenuated the protective effect of LC, suggesting the requirement of gene expression for the protection of LC. On the other hand, LC increased both the formation of cyclic-AMP and the phosphorylation of the cyclic-AMP response element-binding protein (CREB), a downstream target of PKA and a nuclear transcription factor known for neuroprotective mechanism. Furthermore, LC-induced CREB phosphorylation and protective effect could be blocked by a PKA inhibitor and overexpression of the dominant negative CREB, respectively. Taken together, the protective mechanism of LC in antagonizing serum deprivation-induced PC12 cell apoptosis might be mediated through a PKA/CREB-dependent pathway.

Keywords: *Ligusticum chuanxiong*; Pheochromocytoma cells; Apoptosis; Serum deprivation

1. Introduction

Ligusticum chuanxiong (LC) is a well-known traditional Chinese medicinal herb with hemodynamic and analgesic effects (Yao, 1995). This herb is commonly prescribed for the treatment of various cardiovascular diseases, such as angina pectoris, ischemic stroke, migraines, menstrual disorders, amenorrhea, dysmenorrheal, abdominal pain with mass formation, headaches, and rheumatic arthralgia. Because of its diverse therapeutic effects, several pure compounds, such as ferulic acid, senkyunolide, ligustilide, tetramethylpyrazine, etc., have been purified (Yan et al., 2005), and their biological functions studied. However, most of these studies were limited on peripheral functions instead of neuronal functions. Until recently, among these compounds, tetramethylpyrazine is perhaps the most intensively studied compound whose biological functions involve vasodilatation and antiplatelet activity (Sheu et al., 2000; Hintz and Ren, 2003). It also reduces ischemic brain injury (Liao et al., 2004) and prevents excitoxicity (Shih et al., 2002), suggesting a neuroprotective effect.

Neuronal death induced by apoptosis is a normal aspect of development, and it seems that the death program is triggered by the failure of a given neuron to receive limited supplies of target-derived neurotrophic factors. In the post-development period, neurons also undergo apoptotic death when deprived of appropriate trophic factors or when subjected to a variety of stresses and injuries. Recent findings indicated that several transcription factors such as cyclic AMP (cAMP) response element binding protein (CREB), nuclear factor-κB and hypoxia-inducible

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factor 1 are developmentally regulated in the neural system and are necessary for the induction of preconditioning against hypoxic-ischemia (Tanaka, 2001; Lee et al., 2004; Chang and Huang, 2006). Besides, CREB might also be involved in an active process of neuroprotection (Walton and Dragunow, 2000) or that its disruption in the brain might lead to neurodegeneration (Mantamadiotis et al., 2002). Further, nitric oxide has been shown to mediate through CREB activation in preventing neuroblastoma cells from serum deprivation-induced apoptosis (Ciani et al., 2002), suggesting a pivotal role of CREB in preventing neuronal cell death (Finkbeiner et al., 1997).

On the other hand, the rat pheochromocytoma (PC12) cell line is a commonly used model for studying neuronal differentiation and cell death. Apoptosis may occur when triggered by deprivation of either serum (Rukenstein et al., 1991) or trophic factors (Batistatou and Greene, 1991; Lindenboim et al., 1995). Thus, serum deprivation-induced PC12 cell death was used as an apoptotic model to investigate the therapeutic potential of LC as a neuroprotectant in this study. Besides, since LC could prevent ischemic injuries and CREB could not only play an important role in mediating ischemic injuries but also in serum-deprived injuries, the involvement of CREB signaling underlying the protective mechanism of LC was also examined and discussed.

2. Materials and methods

2.1. Reagents and cell culture

All reagents were purchased from Sigma Chemical (St. Louis, MO, USA) except where specified. Actinomycin D (Act D), cyclohexamide (CHX), dibutyl-cyclic AMP (db-cAMP), LY294002, and genistein were purchased from Tocris Cookson (Avonmouth, UK). H-89, K-252a, and a cAMP EIA kit were purchased from Biomol (Plymouth Meeting, PA, USA). Plasmid Midiprep Kit and lipofectamine^{TM} 2000 were purchased from Invitrogen (Carisbad, CA, USA). Plasmids were purchased from Clontech (Palo Alto, CA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and horse serum were purchased from HyClone (Logan, UT, USA). Antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). PC12 cells were maintained in DMEM supplemented with 10% (v/v) horse serum and 5% (v/v) fetal bovine serum and were incubated in a CO_2 incubator (5%) at 37 °C.

2.2. Extraction and isolation of LC

The rhizome of LC was purchased from a local herbal medicine shop in Taipei. Slices of LC were extracted in ethanol at 60 °C overnight three times. The extract was concentrated in a vacuum rotary evaporator under reduced pressure and dried in a vacuum oven to produce the crude ethanolic extract. The crude ethanolic extract of LC was then partitioned into ethyl acetate (EA)-, *n*-butanol (BuOH)- and water (H_2O)-soluble fractions. Since extracts of LC derived from EA and H_2O were not as effective as that of LC-BuOH in preventing serum deprivation-induced apoptosis (data not shown), the BuOH extract of LC was used in this study.

2.3. MTT assay

Survival was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Mosmann (1983). Cells growing on 150-mm plates were washed three times with PBS and resuspended in DMEM. Suspended cells were plated on 96-well plates (1×10^4 cells/well) and treated with the indicated reagent(s). After incubation for 24 h, MTT was added to the medium (0.5 mg/ml) and incubated at 37 °C for 3 h. After discarding the medium, DMSO (100 μl) was then applied to the well to dissolve the formazan crystals derived from the mitochondrial cleavage of the tetrazolium ring. The absorbances at 570 and 630 nm in each well were measured on a micro-ELISA reader.

2.4. Annexin V-FITC staining

An annexin V (FITC-conjugated) apoptosis kit (K101-400; BioVision, Mountain View, CA, USA) was used to analyze apoptotic cells. The experimental protocol followed the manufacturer's instructions. In brief, after treatment for 24 h, cells growing on 12-well plates at 3×10^5 cells/well were loaded with 0.5 ml binding buffer and 5 μl annexin V-FITC. After incubation for 5 min in the dark, cells were washed once with 1 ml culture medium (without phenol red) for taking fluorescent micrographs or resuspended for flow cytometric analysis (Beckton Dickinson, Franklin Lakes, NJ, USA). The mean values of the fluorescent intensities of FITC were collected using an FL-1 channel ($488/530^{Ex/Em}$ nm). Five thousand live cells were analyzed per sample.

2.5. Western blot analysis

Cells were rinsed with ice-cold PBS and lysed in ice-cold lysis buffer (20 mM HEPES, 1 mM DTT, 20 mM EGTA, 10% glycerol, 50 mM β-glycerophosphate, 10 mM NaF, 1% Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4, 2 μM aprotinin, 100 μM leupeptin, 2 μM pepstatin and 0.5 μM OKA). After sonication, cell debris was removed by centrifugation at 14,000 rpm for 10 min, and the supernatant was utilized for Western blot analysis. Equal amounts of sample were separated by 10% polyacrylamide gel electrophoresis. The resolved proteins (25 μg/lane) were then electroblotted onto Immobilon PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skim milk and then sequentially incubated with the first and second antibodies for 1 h at room temperature. After washing, blots were processed for visualization using an enhanced chemiluminescence system (Pierce). Blots were then exposed to Kodak XAR-5 film to obtain the fluorographic images.

2.6. Measurement of cAMP

The formation of intracellular cAMP was measured by following the instructions of the cAMP EIA kit (AK-205). In brief, after different period time of incubation with the indicated