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Adenosine as an active component of *Antrodia cinnamomea* that prevents rat PC12 cells from serum deprivation-induced apoptosis through the activation of adenosine A_{2A} receptors

Mei-Kuang Lu [1], Jing-Jy Cheng [1], Wen-Lin Lai, Yen-Ru Lin, Nai-Kuei Huang *

National Research Institute of Chinese Medicine, Taipei, Taiwan, No 155-1, Section 2, Li-Nung Street., Shipai, Pei-tou District (112), Taipei, Taiwan, ROC

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Abstract

Antrodia cinnamomea (formerly named *Antrodia camphorata*) is a rare medicinal fungus. We previously reported that it exhibits antioxidative, vasorelaxative, anti-inflammatory, and anti-angiogenic effects. When serum deprivation-induced apoptosis in neuronal-like PC12 cells was used as a stress model, the extract of *A. cinnamomea* displayed effectiveness in preventing serum-deprived apoptosis. Since our previous data show that the extract of *A. cinnamomea* contains adenosine (ADO), we attempt to investigate if the active component is ADO and to identify its targeting site in this study. After pre-incubation with ADO deaminase, neither ADO nor the extract of *A. cinnamomea* exerted any protection, demonstrating that the active component of *A. cinnamomea* is ADO. Furthermore, an ADO A_{2A} receptor (A_{2A}-R) antagonist was used and was able to block the protective effects of ADO and the extract of *A. cinnamomea*, demonstrating that the ADO targeting site in this model is A_{2A}-R. Taken together, the protective effect of *A. cinnamomea* is owed to its active component, ADO, which acts through activation of A_{2A}-R to prevent serum deprivation-induced PC12 cell apoptosis.

Keywords: *Antrodia cinnamomea*; Pheochromocytoma cells; Apoptosis; Serum deprivation; Neuroprotection

Introduction

Antrodia cinnamomea, also known as “niu-chang-chin” in Taiwan, is a medicinal fungus of the family Polyporaceae that grows slowly on the inner cavity of the camphor tree, *Cinnamomum kanehirai*. *A. cinnamomea* was named *Antrodia camphorata* until recently, when the detailed description and taxonomic position of *A. camphorata* was reconsidered and the fungus was renamed *A. cinnamomea* (Chang and Chou, 2004). It is an indigenous and rare species in Taiwan. *A. cinnamomea* has not only been utilized to treat a wide variety of diseases but has also drawn the attention of the pharmaceutical industry. Traditionally, it has been used to treat intoxication caused by food, alcohol, and drugs as well as to treat diarrhea, abdominal pain, hypertension, itchy skin, and tumorigenic diseases (Tsai and Liaw, 1985). Chemical compounds found in *A. cinnamomea* include sesquiterpene lactone, steroids, and triterpenoids (Chen et al., 1995; Cherng and Chiang, 1995; Chiang et al., 1995; Cherng et al., 1996; Yang et al., 1996). Its biological effects have rarely been studied. Recently, differential extracts of *A. cinnamomea* have been shown to exert antioxidative (Hseu et al., 2002; Song and Yen, 2002; Hsiao et al., 2003), vasorelaxatory (Wang et al., 2003), anti-inflammatory (Shen et al., 2004), and antihepatitis effects (Lee et al., 2002). Recently, we have shown that *A. cinnamomea* is effective in preventing serum-deprived PC12 cell apoptosis (Huang et al., 2005), suggesting a neuroprotective role. However, the major active compound(s) remain unknown. Our previous data showed that the extract of *A. cinnamomea* contains adenosine (ADO) (Wang et al., 2003; Huang et al., 2005); therefore the role of ADO on the mechanism of neural protection is investigated in this study.

ADO, which is released from metabolically active cells by facilitated diffusion and/or is generated extracellularly by

* Corresponding author. Tel.: +886 2 28201999x4212; fax: +886 2 28201999x4213.
E-mail address: andrew@mail.nricm.edu.tw (N.-K. Huang).
[1] These authors contributed equally to this paper.

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degradation of released ATP, is a potent biological mediator (Dava et al., 1991), especially in exerting neuroprotective effects (Fredholm, 1997). Its inactivation is mediated by ADO deaminase (ADOase) that subsequently generates inosine (INO). It is also well known that adenosine modulates the activity of numerous cell types including various neuronal populations, platelets, neutrophils, and smooth muscle cells (Dava et al., 1991). To date, four adenosine receptors (ADO-Rs) (A_1, A_{2A}, A_{2B}, and A_3) have been identified (Olah and Stiles, 1995). Importantly, A_{2A}-R has been regarded as a potential therapeutic target in protecting against neuronal trauma, such as hypoxia and ischemia (Kobayashi and Millhorn, 1999). Furthermore, it has been reported that A_{2A}-R activation delays apoptosis in human neutrophils (Walker et al., 1997) and we also reported that A_{2A}-R activation prevented serum-deprived apoptosis in PC12 cells (Huang et al., 2001). It is interesting and worthwhile to investigate whether ADO would also act on A_{2A}-R to prevent serum-deprived apoptosis in PC12 cells.

Neuronal death induced by apoptosis is a normal aspect of development in which it seems that the death program is triggered by failure of a given neuron to receive sufficient amounts of the target-derived neurotrophic factors, of which the supply is limited. In the post-developmental period, neurons also undergo apoptotic death when deprived of appropriate trophic factors or subjected to a variety of stresses and injuries. The rat PC12 cell line is a commonly used model for studies of neuronal differentiation and cell death. Apoptosis may occur when triggered by deprivation of either serum (Moskowitz et al., 2001) or trophic factor/nerve growth factor (NGF) (Ratan et al., 1994; Ferrari et al., 1995). In this study, serum deprivation-induced PC12 cell death was used as an apoptotic model to investigate the mechanism of action of the extract of *A. cinnamomea*. Unraveling the apoptotic and antiapoptotic mechanisms may offer a basis and new application for developing more effective drugs in preventing neuronal diseases.

Taken together, the protective role of ADO as the active component of *A. cinnamomea* B85 that acts on A_{2A}-R in preventing PC12 cells from serum deprivation-induced apoptosis was investigated.

Materials and methods

Reagents and cell culture

All reagents were purchased from Sigma Chemical (St. Louis, MO, USA) except where specified. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and horse serum were purchased from HyClone (Logan, UT, USA). PC12 cells were maintained in DMEM supplemented with 10% (vol./vol.) horse serum and 5% (vol./vol.) fetal bovine serum and incubated in a CO_2 incubator (5%) at 37 °C.

Liquid culture of A. cinnamomea

An *A. cinnamomea* isolate, strain B85, from Taitung, Taiwan, was a generous gift from fungi specialist Dr. T.T. Chang (Division of Forest Protection, Taiwan Forest Research Institute, Taipei, Taiwan). *A. cinnamomea* was maintained on potato dextrose agar (Sigma) and transferred to fresh medium at 3-week intervals. For liquid culture, 19-day-old seeding mycelium of *A. cinnamomea* on the surface of medium was cut into pieces (approximately 0.7×0.7 cm^2) before being transferred to 30 ml of potato dextrose broth (Sigma) in 125-ml flasks. Flasks were maintained in a stationary condition at 28 °C under 90 rpm shaking in the dark for 10 days. Thereafter, 300 ml of the shaking flask culture was inoculated into a 5-l fermentation tank containing 3 l of culture medium (PDB 24 g/l, agar 2 g/l, and glucose 20 g/l, pH 5.6) and then cultured at 28 °C for 10 days with an aeration rate of 1 vvm (aeration volume/medium (l)/min) by shaking at 50 rpm to obtain a mucilaginous medium containing mycelia. At the end of the incubation, mycelia were rapidly washed with 1 l of NaCl (250 mM) by an aspirator-suction system to remove the contaminating culture medium. Samples were then lyophilized and resuspended in milli-Q water to achieve a stock concentration of 50 mg/ml. Serial dilution in milli-Q water to adequate concentrations was performed from the stock immediately before use for the following experiments.

Preparation of mycelial extracts from liquid culture of A. cinnamomea

Lyophilized mycelia were extracted with 80 °C water twice in a 1 : 100 (w/w) ratio for 6 h. Supernatants were collected after centrifugation, whereupon 4 volumes of 95% ethanol was added then precipitated at 4 °C overnight. The dilute ethanolic supernatants were then lyophilized following centrifugation.

MTT metabolism assay

Survival was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) metabolism assay as described by Mosmann (1983). Cells growing on 150-mm plates were washed 3 times with PBS and resuspended in DMEM. Suspended cells were plated on 96-well plates and treated with the indicated reagent(s) (1×10^4 cells/200 μl/well). After incubation for 24 h, 20 μl of MTT stock (5 mg/ml) was added to the medium 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 by live cells. The absorbance at 570/630 nm, which highly correlates with the cell numbers, in each well was measured on a micro-ELISA reader. Cell viability was expressed as a percentage of the results of the MTT metabolism assay (OD 570/630 nm) measured in the serum-containing group.

Annexin V–FITC staining

An Annexin *V* (FITC-conjugated) apoptosis kit (K101-400; BioVision, Mountain View, CA, USA) was used to analyze the apoptotic cells. The experimental protocol followed the manufacturer's instructions. In brief, after 24-h treatment with