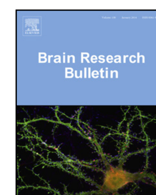
Brain Research Bulletin 121 (2016) 75–83

Contents lists available at ScienceDirect

Brain Research Bulletin

journal homepage: www.elsevier.com/locate/brainresbull



Research report

Protective effects of aloin on oxygen and glucose deprivation-induced injury in PC12 cells

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ARTICLE INFO

Article history:
Received 13 November 2015
Received in revised form
28 December 2015
Accepted 4 January 2016
Available online 6 January 2016

Keywords:
Aloin
PC12 cells
Ischemic injury
Oxygen and glucose deprivation
Apoptosis
Oxidative stress

ABSTRACT

The present study aims to determine whether aloin could protect cells from ischemic and reperfusion injury in vitro and to elucidate the related mechanisms. Oxygen and glucose deprivation model in PC12 cells was used in the present study. 2-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lactate dehydrogenase (LDH) assay and Hoechst 33342 nuclear staining were used to evaluate the protective effects of aloin, at concentrations of 10, 20, or 40 μg/mL in PC12 cells. PCR was applied to detect fluorescence caspase-3, Bax and Bcl-2 mRNA expression in PC12 cells. The contents of malondialdehyde (MDA), superoxide dismutase (SOD) activity were evaluated by biochemical method. The concentration of intracellular-free calcium $[Ca^{2+}]_i$, mitochondrial membrane potential (MMP) were determined to estimate the degree of neuronal damage. It was shown that aloin (10, 20, and 40 μg/mL) significantly attenuated PC12 cells damage with characteristics of an increased injured cells absorbance of MTT and releases of LDH, decreasing cell apoptosis, and antagonizing decreases in SOD activity and increase in MDA level induced by OGD-reoxygenation. Meanwhile pretreatment with aloin significantly reduced injury-induced intracellular ROS, increased MMP ($P < 0.01$), but it inhibited $[Ca^{2+}]_i$ ($P < 0.01$) elevation in a dose-dependent manner. Furthermore, pre-treatment with aloin significantly up-regulated Bcl-2 mRNA expression, down-regulated Bax mRNA expression and consequently activated caspase-3 mRNA expression in a dose-dependent manner. The results indicated that the protection of aloin on OGD-induced apoptosis in PC12 cells is associated with its suppression on OGD-induced oxidative stress and protection on mitochondrial function and inhibition of caspase activity. Alion could be a promising candidate in the development of a novel class of anti-ischemic agent.

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1. Introduction

Ischemic cerebrovascular disease is one of the most common and important causes of death all over the world (Shinotsuka et al., 2014). The interruption of blood flow in the brain can result in the deprivation of oxygen and nutrients (glucose) for covering the energy demands of the cells, a situation that constitutes a condition characteristic of ischemic stroke (Brassai et al., 2015). Cerebral ischemia/reperfusion can induce neuronal injury, which is a very complex process with multiple mechanisms, such as excitotoxicity, oxidative stress, apoptosis and variations in gene expression or the activation of kinase (Zhao et al., 2014a). Oxidative damage plays a pivotal role in the pathogenesis of cerebral ischemic stroke and may represent a target for treatment (Zhao et al., 2014b). Reactive oxygen species are mainly generated from abnormal mitochondria, which can disrupt antioxidant defense. The results of mitochondrial homeostasis, energy production and mediate mitochondrial-dependent apoptotic pathways, consequently show the death of neuronal cell (Fan et al., 2011). Because mechanism of ischemic brain injury is very complicated and there are extremely few therapeutic approaches available so far. More and more evidence indicate that Chinese herbal medicines are

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http://dx.doi.org/10.1016/j.brainresbull.2016.01.001
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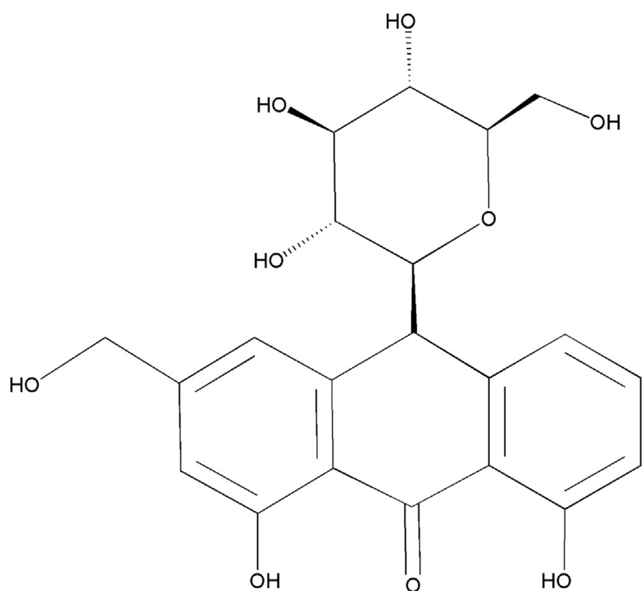

Fig. 1. The chemical structure of aloin. The molecular formula for aloin is $C_{21}H_{22}O_9$ and the molecular weight is 418.39 kD.

beneficial in the treatment of ischemic brain vascular diseases (Zhu et al., 2010).

The genus Aloe comprises more than 100 species of semitropical perennial flowering plants (Brown et al., 2014). Aloe vera is an herb that has been used as a constituent of many prescriptions in the traditional Chinese medicine as an anti-inflammatory, immunosuppress element. And it has also been used to promote blood circulation and to prevent the occurrence of stroke (Lucini et al., 2015). Aloe vera extracts, rich in polyphenols, have been shown to possess various pharmacological characteristics (Liu et al., 2015). Aloin is one of the main active ingredients extracted from Aloe vera (Esmat et al., 2015). However, there is no or little evidence to demonstrate if aloin can play a protective effect on cerebral ischemia and its possible mechanism. To understand the mechanisms of neuronal cell death after ischemic insult and to identify potential protective agents, in vitro cell culture model of ischemia rat pheochromocytoma PC12 cells has been widely used as a cellular model for studying neuronal diseases (Ryu et al., 2014). In this study, we demonstrated that aloin could protect cell death/injury caused by oxidative stress and apoptosis in PC12 cell model.

2. Materials and methods

2.1. Reagents

Aloin, yellowish with purity >97%, was purchased by Sigma (Fig. 1). Nimodipine injection (Nimo) (0.2 mg/mL) was obtained from the German Bayer Company (Leverkusen, Germany). The reagents used in this experiment are as follows: RPMI Medium 1640 (Gibco), fetal bovine serum (FBS, Gibco), 0.25% trypsin (Gibco), Earle's balanced salt solution (EBSS) (in mg/L: 6800 NaCl, 400 KCl, 264 $CaCl_2 \cdot H_2O$, 200 $MgCl_2 \cdot 7H_2O$, 2200 $NaHCO_3$, 140 $NaH_2PO_4 \cdot H_2O$, and 1000 glucose, pH 7.2), phosphate-buffered saline (PBS), and MTT (Solarbio, Beijing, China). The commercial kit for the detection of lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), reactive oxygen species (ROS) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Mitochondrial membrane potential (MMP), calcium fluorescence probe (Fluo-3 AM), and Hoechst 33342 were obtained from Beyotime Institute of Biotechnology (Jiangsu Province, China). Other commercial kits of the Transcript RT/RI Enzyme Mix and the TransStart Top Green qPCR SuperMix were purchased from the TransGen Biotech (Beijing, China).

2.2. Cell culture and OGD followed by reoxygenation

Rat adrenal pheochromocytoma cells (Shanghai Life Sciences, China) were grown as monolayers in tissue culture flasks at 37 °C in 5% CO_2 and 95% air in 90% 1640 (Gibico) containing 10% fetal bovine serum (Gibico), 1% penicillin/streptomycin. Experiments were carried out 72 h after the cells were seeded onto plates or dishes at an appropriate density according to each experimental protocol.

OGD followed by reoxygenation (OGD-reoxygenation) is used as an in vitro model of hypoxic–ischemic insult (He et al., 2011). Based on previous studies, PC12 cells are suitable as a model of oxygen and glucose deprivation and reoxygenation injury (Zhang et al., 2013). The culture medium was replaced with glucose-free Earl's balanced salt solution (EBSS) and exposed into the Hypoxia box with 95% N_2 and 5% CO_2 at 37 °C. After 4 h, PC12 cells were transferred to the normal incubator and refueled with normal culture medium to allow reoxygenation for 24 h. Cells in the experimental groups were treated with aloin (10, 20 or 40 μg/mL) and nimodipine (5 μg/mL) during the entire period of OGD-reoxygenation. Cells cultured in growth culture medium under normoxic condition were used as control.

2.3. MTT and lactate dehydrogenase (LDH) release assay

Cell viability was determined by MTT assay (Guo et al., 2014). Briefly, PC12Cells were cultured in 96-well plates (6×10^3 cells per well). MTT (5 mg/mL and 20 μL per well) was added to the media 12 h after OGD and the incubation lasted at 37 °C for 4 h. The medium with MTT was then removed and 150 μl DMSO was added to each well and pipetted up and down several times until the content is homogenized. The formation of formazan was measured by spectrophotometry at 490 nm using an ELISA reader (ELx800UV, Bio Tek Instruments, Winooski, VT). The results were expressed as the percentage of control (Zhou et al., 2013).

Cell injury was confirmed by measuring the amount of LDH released into the extracellular fluid from damaged or destroyed cells 24 h after OGD reoxygenation (Singh et al., 2009). The total activity of LDH and LDH activity in the medium were measured by LDH assay kit (Bio Vision Inc., Milpitas, CA) at 440 nm. The percentage of LDH leakage was expressed as (culture medium OD values/culture medium OD values + cells homogenate OD values) × 100% (Wang et al., 2014).

2.4. MDA content and SOD activity assay

For assay of free radicals, the cultures were washed with ice-cold PBS. The supernatant was used for the following analyses. The content of MDA was determined with the thiobarbituric acid method. The reagent was added to the sample supernatant and boiled for 40 min at 100 °C, and centrifuged at 3000 × *g* for 10 min after cooling. The absorbance of each supernatant was measured at 532 nm. SOD activity was measured by the xanthine–xanthine oxidase method. The supernatant was added to xanthine–xanthine oxidase reagent and incubated for 40 min at 37 °C.In order to stop the reaction, we added the SDS, and followed by measurement of absorbance at 550 nm.

2.5. ROS detection

Production of intracellular reactive oxygen species was determined using a fluorescent probe DCFH-DA which can cross cell membranes and can subsequently be hydrolyzed by intracellular

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