Biomedicine & Pharmacotherapy 89 (2017) 124–134

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Original article

Synergism and mechanism of Astragaloside IV combined with Ginsenoside Rg1 against autophagic injury of PC12 cells induced by oxygen glucose deprivation/reoxygenation

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ARTICLE INFO

Article history:
Received 29 September 2016
Received in revised form 3 February 2017
Accepted 7 February 2017

Keywords:
Astragaloside IV
Ginsenoside Rg1
Synergism
PC12 cells OGD/R
Autophagic injury
PI3K I/Akt/mTOR
PI3K III/Beclin-1/Bcl-2

ABSTRACT

The aim of this study was to explore the effect by which the combination of Astragaloside IV (AST IV) and Ginsenoside Rg1 (Rg1) resisted autophagic injury in PC12 cells induced by oxygen glucose deprivation/reoxygenation (OGD/R). We studied the nature of the interaction between AST IV and Rg1 that inhibited autophagy through the Isobologram method, and investigated the synergistic mechanism via the PI3K I/Akt/mTOR and PI3K III/Becline-1/Bcl-2 signaling pathways. Our results showed that, based on the 50% inhibiting concentration (IC50), AST IV combined with Rg1 at a 1:1 ratio resulted in a synergistic effect, whereas the combination of the two had an antagonistic effect on autophagy at ratios of 1:2 and 2:1. Meanwhile, AST IV and Rg1 alone increased cell survival and decreased lactate dehydrogenase (LDH) leakage induced by OGD/R, reduced autophagosomes and the LC3 II positive patch, down-regulated the LC3 II/LC3 I ratio and up-regulated the p62 protein; the 1:1 combination enhanced these effects. Mechanistic study showed that Rg1 and the 1:1 combination increased the phosphorylation of PI3K I, Akt and mTOR; the effects of the combination were greater than those of the drugs alone. AST IV and the 1:1 combination suppressed the expression of PI3K III and Becline-1, and the combination elevated Bcl-2 protein expression; the effects of the combination were better than those of the drugs alone. These results suggest that after 2 h-OGD followed by reoxygenation for 24 h, PC12 cells suffer excessive autophagy and damage, which are blocked by AST IV or Rg1; moreover, the combination of AST IV and Rg1 at a 1:1 ratio of their IC50 concentrations has a synergistic inhibition on autophagic injury. The synergistic mechanism may be associated with the PI3K I/Akt/mTOR and PI3K III/Becline-1/Bcl-2 signaling pathways.

1. Introduction

Ischemic stroke is a serious human health risk, and reperfusion plays an important role in cerebral ischemic injury. Cerebral ischemia/reperfusion (I/R) injury refers to severe brain dysfunction when blood supply returns to the tissue after a period of brain tissue ischemia, which has a very complex pathophysiology. In addition to apoptosis and necrosis, two vital types of cell death in ischemic brain injury [1], autophagic cell death has recently been proposed as the third type of cell death in ischemic tissue [2,3]. Autophagy is a cellular process that degrades intracellular substances via the lysosomal pathway to balance the metabolic needs of a cell and its organelles. It has been described as a physiological and dynamic process that is essential for cellular health and survival [4]. Autophagy not only functions as a defense mechanism by removing damaged organelles and metabolites from the cytoplasm, but it also triggers the cell death program to induce cell death [5,6]. Recently, autophagy in cerebral ischemic injury has been increasingly reported [7]. With new focus on the mechanisms of autophagy, progress has been made in the use of traditional Chinese medicine (TCM) in adjusting autophagy [8].

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http://dx.doi.org/10.1016/j.biopha.2017.02.015
0753-3322/

A key aspect of TCM is the prevention and treatment of disease by combining Chinese medicinal drugs of different properties on the basis of Chinese medical theory to exploit synergistic drug interactions and enable comprehensive regulation of the body. That is, the effect of the TCM combination is mainly achieved by the interaction of their active components. *Astragalus membranaceus* (Fisch.) Bge. var. mongholicus (Bge.) Hsiao(family Fabaceae) and *Panax notoginseng* (Burk.) F.H. Chen (family Araliaceae) have been used clinically in TCM for prevention and treatment cardio-cerebrovascular diseases and are often combined to improve the curative effect [9]. Astragalosides (AST) are the main bioactive ingredients with a cardio-cerebrovascular pharmacological effect in *Astragalus membranaceus*, the major component of which is Astragaloside IV (AST IV); P. notoginseng saponins (PNS) is one of the main active ingredients of *Panax notoginseng* with a cardio-cerebrovascular pharmacological effect, containing mainly Ginsenoside Rg1 (Rg1), Ginsenoside Rb1 (Rb1), and Notoginsenoside R1 (R1). In *Panax notoginseng*, the content and absolute bioavailability of Rg1 after peroral (po) administration are greater than those of the other components [10,11]. Previously, we observed that AST IV in combination with Rg1, Rb1 and R1 conferred greater protection against cerebral I/R injury. This neuroprotective effect may arise from the interaction between AST IV and Rg1 [12].

A key finding was that deprivation/reoxygenation (OGD/R) decreased cell viability, increased lactate dehydrogenase (LDH), increased autophagy in PC12 cells, all of which were reversed by AST [13]. Luo et al. [14] revealed that Rb1 inhibited both OGD and transient ischemia-induced neuronal death and mitigated OGD-induced autophagic vacuoles in SH-SY5Y cells. Guo et al. [15] demonstrated that SMXZF (a combination of Rb1, Rg1, schizandrin, and DT-13) significantly inhibited autophagosome formation and significantly decreased the expression levels of Beclin1 and microtubule-associated protein 1 light chain 3 (LC3). Yet, it remains unclear whether AST IV or Rg1 has the antagonistic effect on autophagy, and whether the effect of AST IV combined with Rg1 against ischemic injury results from the inhibition of autophagy in nerve cells. Additionally, the nature and underlying mechanism of the interaction remains to be elucidated. The PC12 cell lines, which can be differentiated into nerve cells with the characteristics of sympathetic neurons, is widely used in the study of neural physiology and pharmacology. OGD/R of PC12 cells is commonly employed as a model for cerebral I/R and has been used in the study of autophagy-mediated injury and drug intervention [13,16,17]. Therefore, using OGD/R PC12 cells as a model for cerebral I/R, we sought to characterize the effect and mechanism of AST IV combined with Rg1 antagonizing autophagic injury, thus providing a scientific basis for further study, clinical application and the development of a new drug.

2. Materials and methods

2.1. Cells

PC12 cell lines, derived from rat adrenal pheochromocytoma, were purchased from China Center for Type Culture Collection (CCTCC, Wuhan, China); the number in American Type Culture Collection (ATCC) is CRL-1721.

2.2. Drugs

AST IV and Rg1 were provided by Chengdu MUST Bio-technology Co. Ltd. AST IV was extracted from the root of *Astragalus membranaceus* (Fisch.) Bge. var. mongholicus (Bge.) Hsiao(family Fabaceae), purity ≥98%; the voucher specimen number was A0070. Rg1 was extracted from the dried roots of *Panax notoginseng* (Burk.) F.H. Chen (family Araliaceae), purity ≥98%; the voucher specimen number was A0237. AST IV was dissolved in phosphate-buffered saline (PBS) containing 0.1% DMSO, and Rg1 was dissolved in PBS.

2.3. Cell culture and the establishment of autophagic injury model

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA, NZM1290) containing 4.5 g/l glucose, 10% heat-inactivated bovine serum albumin (BSA) and 1% mycillin in an incubator at 37 °C, 20% O_2,75% N_2, 5% CO_2 and saturated humidity. The cells were washed with PBS every two days and subcultured. Subsequently, the cells were plated at a density of 1×10^4 cells/ml for 24 h and supplemented with nerve growth factor (NGF) (Sigma, Saint Louis, US, N2513) at a final concentration of 50 μg/l for 48 h after attachment [18]. For G_0 phase synchronization of cells, the original medium was discarded and replaced with serum-free medium for 24 h.

PC12 cells exposed to 2 h-OGD followed by 24 h-reoxygenation were reported to have an increased calcium concentration ($[Ca^{2+}]$ i), increased mitochondrial membrane potential (MMP), Beclin-1 dependent autophagy and decreased cell viability [17]. In addition, the results from our preliminary experiments suggest that PC12 cell injury following OGD/R (2 h-OGD and 24 h-36 h-reoxygenation) is mediated by excessive autophagy. Thus, exposure to 2 h-OGD followed by 24 h-reoxygenation was used as a model for autophagic injury in PC12 cells. For OGD/R exposure, cells were first incubated in 200 μl of glucose-free Earle's medium under hypoxic conditions (gas mixture of 1% O_2, 94% N_2, 5% CO_2) for 2 h, cultured in DMEM and incubated at 20% O_2, 75% N_2, 5% CO_2 for 24 h.

2.4. Detection of AST IV and Rg1 alone on autophagy inhibition

PC12 cells were divided into three culture conditions: normal, model (ODG/R) and OGD/R with component-treatment. In the normal condition, cells were maintained under standard culture conditions. In the OGD/R condition, cells were exposed to OGD/R as stated above. In the OGD/R with component-treatment condition, AST IV (0, 9.81, 19.63, 39.25, or 78.5 μg/ml) and Rg1 (0, 10, 20, 40, or 80 μg/ml) [19] were added 30 min prior to OGD/R exposure. PC12 cells in the normal and OGD/R conditions were administered PBS. Each treatment group was repeated three times ($n = 3$).

LC3 protein is usually distributed in the cytoplasm in the LC3 I form, which appears diffuse when visualized by laser confocal microscopy. Upon induction of autophagy, LC3 accumulates in the cytoplasm as LC3II, which appears puncta when visualized by laser confocal microscopy. The number of puncta represents the relative expression of LC3 II and reflects the number of autophagosomes or the activity of autophagy to some extent. For LC3 immunofluorescence, PC12 cells were washed three times with PBS and were fixed for 15 min in 4% paraformaldehyde. The fixed cells were then permeabilized using PBS containing 0.5% Triton X-100 for 10 min. After the cells were blocked with 1% BSA for 1 h, the PC12 cells were hybridized with a rabbit anti-mouse LC3 antibody (1:500; PM036, MBL, Japan) overnight at 4 °C. The primary antibody-hybridized cells were washed with PBS three times and incubated for 30 min with TRITC-conjugated anti-rabbit IgG (1:100; BA1090, Boster, China) and for 10 min with Hochest 33258 (1:200; COO20-10, Solarbio, China); then, they were washed three times with PBS and sealed with glycerol. A laser confocal microscope ($A_1$130309, NiKon, Japan) was used for image acquisition. Five different visual fields were randomly selected to measure the cell area and number of LC3 II-positive puncta. Image Pro-Plug6.0 analysis software was used to calculate the LC3 II-positive puncta density ($10^{-1}/\mu m^2$). The autophagy inhibition rate of a component was determined as follows: