Biomedicine & Aging Pathology 3 (2013) 14–19

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Original article

Potassium aspartate attenuates apoptotic cell death after focal cerebral ischemia in rats

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ARTICLE INFO

Article history:
Received 1st November 2012
Accepted 9 November 2012
Available online 13 February 2013

Keywords:
Potassium aspartate
MCAo
Apoptosis

ABSTRACT

Potassium aspartate (PA), as an electrolyte supplement, is used widely in clinical practice. To test the idea that high extracellular K^+ might attenuate neuronal apoptosis, male Sprague-Dawley rats were subjected to 2 h of middle cerebral artery occlusion (MCAo) and 22 h of reperfusion. Vehicle or PA was administrated intraperitoneally after 1 h MCAo. The neurological deficit and dose-responses of PA to MCAo were examined. Brain ATP and lactic acid levels, caspase-3 and caspase-9 activities and the apoptotic cell death were evaluated as well as western blot analysis for cytochrome c (cyt c) release and Poly(ADP-ribose) polymerase-1 (PARP-1) cleaved. Our results show that PA treatment at the dose of 62.5 mg/kg significantly improved neurological deficits ($P < 0.01$) and decreased the infarct volume compared with vehicle treatment ($P < 0.05$). PA treatment significantly reduced the loss of ATP ($P < 0.05$), the activity of caspase-3 ($P < 0.05$), the cleavage of PARP-1 ($P < 0.05$), and the apoptotic cell death($P < 0.01$) compared with vehicle treatment. Moreover, PA attenuated cyt c release to some extent. Our results indicate that PA has neuroprotective effects against apoptosis through mitochondrial apoptotic pathway after cerebral ischemia in rats. It will provide an experimental evidence for the clinical application of PA.

1. Introduction

Ischemic stroke is the leading cause of death in the industrial countries despite current advances in medicine and implementation of the management guidelines [1]. Lots of literature demonstrated that apoptosis is a common mode involved in ischemic cell death [2]. Apoptosis is characterized by a distinct series of morphological and biochemical changes that result in cell shrinkage, DNA breakdown, and, ultimately, phagocytic death. Diverse external and internal stimuli trigger apoptosis, and enhanced K^+ efflux has been shown to be an essential mediator of not only early apoptotic cell shrinkage, but also of downstream caspase activation and DNA fragmentation [3]. Potassium aspartate (PA), as an electrolyte supplement, is used widely in clinical practice. Potassium is an important intracellular cation involved in a variety of enzymatic reactions and the process of muscle contraction. Aspartic acid, which is a precursor of oxaloacetate in vivo, plays an important role in the citric acid cycle and also participates in ATP generation. Aspartic acid has a strong affinity with cells and can increase the K^+ influx [4]. To test the idea that high extracellular K^+ might attenuate neuronal apoptosis, we examined the effects and mechanisms of PA on focal cerebral ischemic injury through energy metabolism and apoptotic pathway of caspase-3 activation, providing an experimental evidence for the clinical application of PA.

2. Materials and methods

2.1. Part I

2.1.1. Animal model of focal cerebral ischemia

All animal experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (issued by State Scientific and Technological Commission, 1988). Fifty male Spraque-Dawley rats, weighing 300 to 330 g (Beijing Vital River Laboratory Animals Technology Co., Ltd. China), were

Abbreviations: PA, Potassium aspartate; MC, Aomiddle cerebral artery occlusion; ECA, external carotidartery; ICA, internal carotid artery; MCA, middle cerebral artery; TTC, 2, 3, 5-Triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling; LAl, actic acid; Ac-DEVD-PN, Aacetyl-Asp-Glu-Val-Asp P-nitroanilide; Ac-LEHD-PN, Aacetyl-Leu-Glu-His-Asp Pnitroanilide; HRP, horse radish peroxidase; DAB, 3, 3'-diaminobenzidine; HPF, shigh-power fields; BCA, bicinchoninic acid; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; cyt c, cytochrome c; PARP-1, Poly(ADP-ribose) polymerase-1; SEM, standard error of the mean; LD50, median lethal dose; Apaf-1, apoptotic protein-activating factor-1; KATP, ATP-sensitive potassium.

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2210-5220/$ – see front matter.
http://dx.doi.org/10.1016/j.biomag.2012.11.002

anesthetized with 10% chloral hydrate (400 mg/kg, intraperitoneal) and placed in a supine position on a warming pad at 37 °C. The right middle cerebral artery occlusion (MCAo) was induced by insertion of a silicone-coated nylon filament (φ = 0.28 mm) as described previously [5]. Under the operating microscope, the right external carotid artery (ECA) and internal carotid artery (ICA) were exposed through a midline incision. The nylon filament was inserted from the ECA to the ICA and gently advanced approximately 20 mm until resistance was felt, indicating that the tip of the filament had passed the middle cerebral artery (MCA) origin and blocked the origin of the MCA. After 2 h the filament was withdrawn into the stump of the ECA to allow reperfusion.

2.1.2. Dose-responses of potassium aspartate to focal cerebral ischemia

Rats were divided randomly into five groups of ten animals:

- PA (bulk drug, 5 g/vial, Batch N^{o} 100602, Liaoning Union Pharmaceutical Co., Ltd. China), 10 mg/kg;
- PA, 25 mg/kg;
- PA, 62.5 mg/kg;
- PA, 125 mg/kg;
- Vehicle, normal saline, 1 ml/kg.

Drugs were administrated intraperitoneally at a volume of 1 ml/kg after 1 h ischemia.

2.1.3. Neurological evaluation

Neurological deficits of the animals were evaluated at 24 h after MCAo by a blinder using a 6-point neurological function score described previously: 0: no spontaneous activity; 1: apontaneous circling; 2: circling if pulled by tail; 3: lowered resistance to lateral push without circling; 4: contralateral forelimb flexion; and 5: no apparent deficit [6].

2.1.4. Measurement of infarct volume

The animals were anesthetized with 400 mg/kg 10% chloral hydrate and decapitated after 24 h MCAo. The brains were quickly removed and inspected to confirm the absence of subarachnoid hemorrhage. The brains were sectioned coronally with a self-made blade at 2-mm intervals, incubated for 20 minutes in a 1% solution of 2, 3, 5-Triphenyltetrazolium chloride (TTC, T8877, SIGMA-ALDRICH, USA) at 37 °C in the dark for vital staining, and fixed by immersion in 10% formalin solution for 10 minutes. The six brain sections per animal, stained with TTC, were recorded with a color camera for measurement of lesion areas. Areas not stained red with TTC, which were considered injured, were calculated by the image analyzing system (Beijing Konghai Co., China). The total infarct volume (in cubic millimeters) was calculated by the use of numerical integration of the TTC-pale areas for all of the sections per animal and the distances between them.

2.2. Part II

2.2.1. Neurological evaluation

Based on the dose-responses study, 62.5 mg/kg of PA was used in subsequent study. Sixty-six animals were randomly assigned to three groups treated with normal saline or PA:

- sham group, normal saline (1 ml/kg);
- vehicle group, normal saline (1 ml/kg);
- PA group, PA (62.5 mg/kg).

The respective agent was administrated intraperitoneally 1 h after ischemia. Rats of vehicle and PA groups were subjected to the same operative procedure as in Part I. Others were sham-operated without MCAo. Neurological deficits of the animals were performed at 24 h MCAo before sample collection.

2.2.2. Sample collection and processing

At 24 h of MCAo, animals were anesthetized and decapitated. In each animal the brain was sectioned into a 4 mm-thick slice-beginning 3 mm from the anterior tip of the frontal lobe, and then made a longitudinal cut about 2 mm from the midline through right hemisphere [7]. The tissue, as the infarct area, was homogenized for respective measurement. Thirty rats were used to determine brain ATP and lactic acid levels as well as caspase activities. Eighteen samples were performed western blot analysis. For terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) staining, eighteen rats were decapitated after 24 h MCAo and the brains were fixed in 4% formaldehyde and embedded in paraffin.

2.2.3. Measurement of brain ATP and lactic acid levels

The ATP and LA levels were determined by ATP Assay Kit and Lactic acid Assay Kit (Nanjing Jiancheng Bioengineering Institute, China) according to the instructions.

2.2.4. Caspase-3 and caspase-9 activity assay

The activity of caspase-3 and caspase-9 was measured by cleaving selective substrates acetyl-Asp-Glu-Val-Asp P-nitroanilide (Ac-DEVD-PNA) and acetyl-Leu-Glu-His-Asp P- nitroanilide (Ac-LEHD-PNA) respectively (Beyotime Institute of Biotechnology, China).

2.2.5. TUNEL assay for cell death

For each rat (six rats per group), three sections within primary somatosensory cortex area were analyzed using in Situ Cell Death Detection Kit (Cat. No. 11684817910, Roche, Germany). The 8-μm coronal slices were dewaxed, rehydrated and incubated for 30 minutes with proteinase K (10 μg/ml) at room temperature. TUNEL reaction mixture was added on samples for 60 minutes at 37 °C in the dark. Sections then developed using converter-Peroxidase and 3, 3'-diaminobenzidine (DAB) substrate, after which they were counterstained by hematoxylin prior to analysis by light microscope. Ten randomly chosen high-power fields (HPFs) (400×) of three sections from the ipsilateral hemisphere were observed for counting TUNEL-positive cells for each animal.

2.2.6. Western blot analysis for cyt c release and poly(ADP-ribose) polymerase-1 cleaved

Cyt c levels in both mitochondrial and cytosolic fractions were analyzed by western blot. Mitochondrial and cytosolic fractions were isolated using the Tissue Mitochondrial Isolation Kit (Beyotime Institute of Biotechnology, China). Total protein content in the tissue extract was determined colorimetrically by bicinchoninic acid (BCA) protein assay kit according the manufacturer's protocol (Beyotime Institute of Biotechnology, China), using Bovine serum albumin (BSA) as a standard. Forty micrograms of protein was separated on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred by electroelution onto PVDF membranes (Immobilon^{TM}, Millipore, USA) and were blocked by 5% BSA solution for 2 h. The membranes were incubated overnight at 4 °C with the primary antibody (cyt c, 1:1000; HRP-conjugated β-actin, 1:5000; HRP-conjugated VDAC1, 1:8000; Abcam, UK). Horse radish peroxidase (HRP)-conjugated rabbit polyclonal secondary antibody to mouse IgG (1:8000, Abcam, UK) was used for 1 h at room temperature. For Poly(ADP-ribose) polymerase-1 (PARP-1) analysis, 60 μg of protein was loaded on 10% separation gel. The membranes were blocked in 5% non-fat dry milk solution. An antibody that recognizes both uncleaved (116 KD) PARP-1 as well as the cleaved large fragment (89 KD)