Phytomedicine 25 (2017) 8–14

Contents lists available at ScienceDirect

Phytomedicine

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

Salvianolic acid A inhibits calpain activation and eNOS uncoupling during focal cerebral ischemia in mice

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ARTICLE INFO

Article history:
Received 8 June 2016
Revised 21 October 2016
Accepted 11 December 2016

Keywords:
Salvianolic acid A
Stroke
Oxidative stress
Neurovascular protection
eNOS uncoupling

ABSTRACT

Background: Salvianolic acid A (SAA) is obtained from Chinese herb Salviae Miltiorrhizae Bunge (Labiatae), has been reported to have the protective effects against cardiovascular and neurovascular diseases.
Hypothesis: The aim of present study was to investigate the relationship between the effectiveness of SAA against neurovascular injury and its effects on calpain activation and endothelial nitric oxide synthase (eNOS) uncoupling.
Study design: SAA or vehicle was given to C57BL/6 male mice for seven days before the occlusion of middle cerebral artery (MCAO) for 60 min.
Methods: High-resolution positron emission tomography scanner (micro-PET) was used for small animal imaging to examine glucose metabolism. Rota-rod time and neurological deficit scores were calculated after 24 h of reperfusion. The volume of infarction was determined by Nissl-staining. The calpain proteolytic activity and eNOS uncoupling were determined by western blot analysis.
Results: SAA administration increased glucose metabolism and ameliorated neuronal damage after brain ischemia, paralleled with decreased neurological deficit and volume of infarction. In addition, SAA pretreatment inhibited eNOS uncoupling and calpain proteolytic activity. Furthermore, SAA inhibited peroxynitrite ($ONOO^-$) generation and upregulates AKT, FKHR and ERK phosphorylation.
Conclusion: These findings strongly suggest that SAA elicits a neurovascular protective role through the inhibition of eNOS uncoupling and $ONOO^-$ formation. Moreover, SAA attenuates spectrin and calcineurin breakdown and therefore protects the brain against ischemic/reperfusion injury.

Introduction

Stroke is a devastating disease, a massive socio-economic burden in all societies. Multiple deleterious pathological events during stroke caused death and disability (Lopez et al., 2006). Ischemic stroke subjected to deprivation of glucose and oxygen, reduction in blood flow linked to excitotoxicity, energy loss, and ionic imbalances, rapid apoptosis to endothelial cells (Alfieri et al., 2013; Manevich et al., 2001). The cerebral ischemia caused an excessive influx of calcium (Ca^{2+}) by increased glutamate release and activation of N-methyl-D-aspartate receptors (NMDARs) (Du et al., 2010). Increased neuronal-free Ca^{2+} by activation of NMDA receptors and other Ca^{2+} channels participate in signal transduction pathways leading to cell survival or cell death (Tauskela and Morley, 2004). The Ca^{2+} induces the binding of calmodulin (CaM) to the enzyme nitric oxide synthases (NOS) and activated CaM is important for the regulation of endothelial nitric oxide synthase (eNOS) (Förstermann and Sessa, 2012). The cerebral ischemia caused loss

Abbreviations: BH4, tetrahydrobiopterin; Ca^{2+}, calcium; CaM, calmodulin; CaN, calcineurin; CCA, common carotid artery; CT, computed tomography; ECA, external carotid artery; eNOS, endothelial nitric oxide synthase; 18F-FDG, [18F]-fluoro-2-deoxy-D-glucose; ICA, internal carotid artery; MCI, Mitsubishi Chemical Industry; NF-kB, nuclear factor kappa B; NADPH, nicotinamide adenine dinucleotide phosphate; NIH, National Institutes of Health; NMDARs, N-methyl-D-aspartate receptors; NO, nitric oxide; $O_2^{\cdot-}$, superoxide; $ONOO^-$, peroxynitrite; PKB, protein kinase B; PET, positron emission tomography; RNS, reactive nitrogen species; ROS, reactive oxygen species; rtTPA, recombinant tissue plasminogen activator; SAA, Salvianolic acid A; SDS, sodium dodecyl sulfate; SUV, standardized uptake value; tMCAO, transit middle cerebral artery occlusion.

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http://dx.doi.org/10.1016/j.phymed.2016.12.004
0944-7113/

of tetrahydrobiopterin (BH4) and altered eNOS function, with increased superoxide ($O_2^{\cdot -}$) production due to activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase induced reactive oxygen species (ROS) (Kukaya et al., 2003). The nitric oxide (NO) imbalance and to react with $O_2^{\cdot -}$ generates peroxynitrite ($ONOO^-$) which plays a key role in ischemic injury and triggers numerous pathological events *via* induction of nitrosative damage to lipids, DNA, and proteins (Han et al., 2011).

The multiple pathophysiological cascades activated as a result of ischemia-like excitotoxicity, nitrosative stress, inflammation, and blood-brain barrier (BBB) leakage (Mitsios et al., 2006). The calpain belongs to proteases that play an important role in ischemic cell death. The overload of Ca^{2+} disrupt the membrane integrity following activation of Ca^{2+}-dependent calpains, in turn, triggers apoptosis *via* activation of caspases (Bano and Nicotera, 2007).

Salvianolic acid A (SAA) is a caffeic acid derivative, obtained from Chinese herb Salviae miltiorrhizae Bunge (Labiatae), also known as Chinese sage, or Danshen in Chinese literature (Xu, 1990). Traditional Chinese medicine has been used for the treatment of a wide range of vascular diseases. SAA is one of the major active components of Danshen with strong antioxidant properties, a multi-target agent, used for the treatment of cardiovascular and cerebrovascular diseases (Du and Zhang, 1997; Fan et al., 2015; Ji et al., 2000; Wang et al., 2012; Wang et al., 2009). SAA inhibits inflammatory/oxidative stress-mediated neuronal and vascular damage by impairing nuclear factor kappa B (NF-kB) signaling during ischemic brain injury (Chien et al., 2016). The distinct ischemic factors trigger multiple intracellular signaling that converges into several pathways (Chien et al., 2016). Based on the above description and multi-target agent, we investigated for the first time the effects of SAA on inhibition of calpain activity and eNOS uncoupling in mice subjected to transit middle cerebral artery occlusion (tMCAO).

Materials and methods

Experimental animals

Male C57BL/6 mice, weighing 20–28 g, were obtained from the Zhejiang Medical Animal Centre (Hangzhou, China). Mice were housed under climate-controlled conditions with a 12 h light/dark cycle and provided with standard food and water. Animals were acclimated before initiating the experimental procedure. All experimental protocols and animal handling procedures were performed in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and were approved by the Committee for Animal Experiments at the Zhejiang University in China.

Extraction and isolation of SAA

Slices of roots and rhizomes of *Salvia miltiorrhiza* (10 kg) was purchased from East China Pharmaceutical Group Co. Ltd. (Hangzhou, China). For chromatographic analysis column was prepared by D-101 macroporous resin (Chemical Plant of Nankai University, Tianjin), MCI gel (CHP20P, 75–150 μm, Mitsubishi Chemical Industries Ltd.), and C_{18} reversed-phase silica gel (20–45 μm, Fuji Silysia Chemical LTD). The material was extracted with water under reflux three times each for at least 2 h For chromatographic analysis the extracted solution was then subjected to macroporous resin D-101 column (φ10 cm × 50 cm), eluted with MeOH/H_2O (10%, 30%, 50%, 70%, 95%) to afford five fractions (Fr-A∼Fr-E). Fr-C was found to have a high SAA content (Monitored by TLC), which was subsequently subjected to MCI gel column (φ5 cm × 40 cm) eluted with MeOH/H_2O (15%, 50%, 75%) to obtain the crude product.

Drug administration and transit middle cerebral artery occlusion mice model

Animals were divided into four groups sham, vehicle and treatment groups. SAA (1 mg and 5 mg per kg body weight) or vehicle (saline) was given intragastric by *i.p.* to mice for seven days before the MCAO for 60 min. The tMCAO model which has resemblances to stroke in humans was used for the study (Braeuninger and Kleinschnitz, 2009; Carmichael, 2005) and the surgery was performed as previously described by our lab (Tao et al., 2014). Animal procedures were approved by the Committee on Animal Experiments at the Zhejiang University. Mice were weighed prior to surgery. Anesthesia was induced with 3% trichloroacetaldehyde hydrate. Rectal temperature was monitored throughout the surgery, and the body temperature was maintained at 37 °C ± 0.5 °C with a heating pad. Mice were subjected to tMCAO with the use of an operating microscope; the left common carotid artery (CCA), internal carotid artery (ICA), and external carotid artery (ECA) were surgically exposed. A 6–0 silicon-coated nylon surgical suture (Ethicon (XZW8305.032), Lot no. HJB388, Johnson and Johnson Intl., Livingston, Scotland) was inserted into the ICA through the CCA and gently advanced to the orifice of MCA. After 1 h of MCA occlusion, the suture was removed to restore blood flow, the neck incision was closed, and the mice were allowed to recover.

Neurologic evaluation

Neurological deficits, including neurological scores and rotarod test, were examined at 24 h after tMCAO. Neurological scores were determined using a previously described scoring system (Shioda et al., 2007), with slight modifications: 0 = Normal motor function, 1 = failure to extend left forepaw fully, 2 = Circling to the contralateral side but normal posture at rest, 3 = Leaning to the contralateral side at rest, 4 = No spontaneous motor activity. Rotarod test was performed to examine the motor coordination, started 3 days before the surgery five times every day (Tao et al., 2014). The persisted time (s) on the rotarod after ischemia was recorded; the data were expressed as the mean duration of five trials.

Nissl staining to assess infarct volume

The brain infarct was measured from the vehicle and SAA-treated groups. Twenty-four hours after reperfusion, mice were anesthetized and transcardially perfused with phosphate-buffered saline (PBS) followed by 10% paraformaldehyde as described previously (Tao et al., 2014). Sections of the brains 45 μm thick were cut with the aid of a cryostat (Leica VT1000S). For Nissl-staining, slices were hydrated in 0.1% cresyl violet for 3–5 min. Then they were dehydrated in ethanol and cleaned with xylene. The slides were next examined *via* light microscopy; pictures were taken with a digital camera (Leica MZ95 and Leica application suite). The brain infarct area was evaluated from digital images of Nissl-stained brain sections using Image J software (NIH).

Immunoblotting analysis

Immunoblotting was carried out using penumbra brain region of mice after determination of protein concentrations by the Brad-Ford's solution. The brain lysates containing equivalent amounts of protein were applied to 10%–12.5% acrylamide denaturing gels (SDS-polyacrylamide gel electrophoresis) (Wang et al., 2015a). Proteins were then transferred to an immobilon polyvinylidene difluoride membrane for 1 h at 50 V. Membranes were blocked in 20 mM Tris hCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 (TBS-T) containing 5% fat-free milk powder for 1 h and immunodetected with antibodies to spectrin (1:3000, monoclonal