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Apelin-36, a potent peptide, protects against ischemic brain injury by activating the PI3K/Akt pathway

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ABSTRACT

Apelin is an endogenous ligand of G protein-coupled receptor-apelin and angiotensin-1-like receptor (APJ). The biological effects of apelin–APJ system are reported in multiple systems including cardiovascular, endocrinal, and gastrointestinal system. Previous studies had shown that apelin-13 is a potential protective agent on cardiac ischemia; however, the role of apelin in the central nervous system remained unknown. In this study, we investigated therapeutic effects of apelin-36, a long form of apelin, in ischemic brain injury models. We found that apelin-36 reduced cerebral infarct volume in the middle cerebral artery occlusion (MCAO) model and the neonatal hypoxic/ischemic (H/I) injury model. Apelin-36 improved neurological deficits in the MCAO model and promoted long-term functional recovery after H/I brain injury. We further explored the protective mechanisms of apelin-36 on H/I brain injury. We clearly demonstrated that apelin-36 significantly reduced the levels of cleaved caspase-3 and Bax, two well-established apoptotic markers after H/I injury, indicating the anti-apoptotic activity of apelin-36 in ischemic injury. Since apelin-36 increased the level of phosphorylated Akt after H/I injury, we treated neonates with a specific PI3K inhibitor LY294002. We found that LY294002 decreased the phosphorylated Akt level and attenuated protective effects of apelin-36 on apoptosis. These suggested that the PI3K/Akt pathway was at least in part involved in the anti-apoptotic mechanisms of apelin-36. Our findings demonstrated that apelin-36 was a promising therapeutic agent on the treatment of ischemic brain injury.

1. Introduction

Neonatal hypoxia/ischemia encephalopathy (HIE) is a common central nervous system disease in perinatal patients. It is a serious threat to newborn health for its poor prognosis, high mortality, and disability. Cerebral palsy, epilepsy, learning disability, memory retardation, or other abnormal behaviors are often observed in most survivors of HIE (Arteni et al., 2003; Hagberg et al., 2005; Jin et al., 2007). Importantly, these neurological complications seriously affect their life quality and increase social burdens. Therefore, it is very important to study the pathogenesis of HIE and find out therapeutic targets to maximally reduce neurological complications (Cull-Candy et al., 2001). Previous studies have demonstrated that mitochondrial damage, free radicals production, inflammation, glutamate excitatory toxicity, endothelial cell dysfunction, caspase-3 activation, and apoptosis are involved in pathological mechanisms of HIE (Bickler and Fahlman, 2006; Jantas and Lason, 2009). Neuroprotective agents, which target these different pathological mechanisms, may offset or reduce harmful biochemical activities, or strengthen protective signaling pathways, providing a promising strategy to treat HIE.

Apelin is an endogenous ligand of apelin and angiotensin-1-like receptor (APJ), a G protein-coupled receptor. Human *APELIN* gene encodes a pre-proprotein of 77 amino acids (Tatemoto et al., 1998). After translation and cleavage, the proprotein of apelin generates several active fragments, including a 36-amino acid peptide corresponding to the sequence 42–77 (apelin-36), a 17-amino acid peptide corresponding to the sequence 61–77 (apelin-17), and a 13-amino acid peptide corresponding to the sequence 65–77

Abbreviations: CCA, common carotid artery; ECA, external carotid artery; H/I, hypoxic/ischemic; HIE, hypoxia/ischemia encephalopathy; I/R, ischemia/reperfusion; MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride.

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(apelin-13). The active forms of apelin are widely distributed in different tissues including the brain and the heart (O'Carroll et al., 2000). The biological effects of apelin–APJ system are involved in the multiple systems (Falcao-Pires et al., 2009; Tatemoto et al., 1998). In the cardiovascular system, apelin is found to have cardiovascular protection by activating phosphokinase C, protein kinase C, and endogenous Na^+–H^+ converting enzyme, inhibiting the production of angiotensin II, and increasing myocardial contractility (Barnes et al., 2010; Lee et al., 2005; Zeng et al., 2009). In the endocrinal system, apelin inhibits insulin secretion induced by high blood glucose (Wei et al., 2005); also, distribution of APJ mRNA in the hypothalamus is similar to that of anti-diuretic hormone, implying that apelin–APJ is involved in the hypothalamus–adrenal–pituitary axis to regulate water balance and feeding activities (O'Carroll et al., 2003). The role of apelin in the central nervous system remains unclear and needs further investigations.

A recent result showed that apelin-13, had neuroprotective effects by reducing excitotoxicity of excitatory receptor NMDA and ROS production and activating ERK1/2 and the PI3K/Akt pathway in cultured cortical neurons (Cheng et al., 2012; Zeng et al., 2010). In addition, apelin-13 at the doses of 50 and 100 μg (intracerebroventricularly, i.c.v.) protects against cerebral ischemia injury in a cerebral middle artery occlusion (MCAO) model (Khaksari et al., 2012). Although these studies demonstrated neuroprotective effects of apelin-13 on ischemia injury *in vitro* and *in vivo*, it is unclear whether apelin has protective effect on HIE. In this study, we demonstrated low dose (0.1 μg) of apelin-36, a long form of apelin, had a high potent protection in a MCAO model. Further, we examined the neuroprotection of apelin-36 in a HIE animal model. We confirmed its protection on neonatal hypoxia/ischemia (H/I) injury and explored its protective mechanisms.

2. Materials and methods

2.1. Experimental animals

Male ICR mice (23–28 g, about 5-week old) and Sprague–Dawley rats (postnatal day 5) were purchased from SLAC Company (Shanghai, China). Mice were used for the MCAO model and rat pups were used for the H/I injury model. All animal procedures were approved by the University Committee on Animal Care of Soochow University and performed according to the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals.

2.2. The MCAO model

MCAO was carried out by using a nylon monofilament as described previously (Xu et al., 2006). Briefly, a mouse was anesthetized by 7.2% chloral hydrate (400 mg/kg body weight). When the right common carotid artery (CCA) and the right external carotid artery (ECA) were isolated, the ECA was ligated with a silk suture at 2 mm distal from the ECA–CCA branch. A 6-0 nylon filament (Ethilon, Ethicon Inc., USA) coated with silicon resin was inserted through the incision near the ECA–CCA branch about 1.5 mm into the right internal carotid artery and advanced about 9–11 mm. Reperfusion was achieved by withdrawing the monofilament after 75 min of MCAO. Sham mice underwent the same experimental procedures but the nylon filament was advanced only about 5 mm. Body temperature was maintained at 36.5–37.5 °C between the beginning of surgery and the recovery from anesthesia after surgery. The local cerebral blood flow was measured by a laser-Doppler blood flow meter. Apelin-36 (0.1 μg in 10 μl saline, Peptide International, Louisville, Kentucky, USA) or saline was injected (i.c.v.) into the left lateral ventricle at 30 min before MCAO.

2.3. The cerebral H/I injury model

The newborn rat model with cerebral H/I injury was established as described previously (Rodrigues et al., 2004; Ten et al., 2003; Vannucci et al., 1999). Newborn rats at the age of day 7 were anesthetized with diethyl ether and the left CCA was ligated. The duration of the procedure was less than 5 min, followed by 1 h break. During 1 h recovery, the body temperature of the pups was maintained with a heating carpet. The mice were then placed into a hypoxic chamber flushed with the mixed gas of 8% O_2 and 92% N_2. The hypoxic chamber was partially submerged in a water bath at 37 °C to maintain a constant internal temperature. After 2.5 h of ischemia, the rats were returned the cages. Apelin-36 (1 μg in 100 μl saline) was administrated intraperitoneally (i.p.) at the beginning of recovery.

2.4. Experimental groups

For the MCAO model, mice were randomly divided into four groups (n = 6–8): vehicle-treated group (sham group); apelin-treated group (apelin group); vehicle-treated ischemia/reperfusion group (I/R group); apelin-treated I/R group (I/R + apelin group).

For the H/I injury model, newborn rats were randomly assigned into 4 individual groups (n = 6–8 for each group): sham surgical group (sham group): the left CCA was exposed but not ligated, and then placed into the hypoxic compartment; hypoxia/ischemia group (H/I group): as described above; apelin-treated H/I group (apelin group): 1 μg apelin-36 was injected (i.p.) 1 h before ischemia and other conditions were same as H/I group; apelin/LY294002-treated H/I group (LY294002 group): after the neonates received 1 μg apelin-36 and recovered for 1 h, PI3K inhibitor LY294002 (0.1 ml 1 mg/ml in 1% dimethylsulfoxide, Sigma–Aldrich, St. Louis, Missouri, USA) was then administrated (i.p.) before ischemia; other conditions were same as apelin group.

2.5. Intracerebroventricular administration of apelin-36

A small burr hole was drilled in the parietal region of mice (0.5 mm posterior and 1.0 mm lateral to the Bregma). Apelin-36 or saline was injected into the left lateral ventricle via the hole with a depth of 2.5 mm.

2.6. Neurological deficit evaluation

Neurological deficits were evaluated at 24 h after the MCAO. The modified neurological deficit evaluation system was based on a 5-point scale system described previously (Xu et al., 2010): no deficit, 0; flexion of the contralateral torso and forelimb, 1; turning to the ipsilateral side when held by tail, 2; leaning to affected side, 3; no spontaneous locomotor activity, 4.

2.7. The measurement of infarct volume

After indicated time points, the animals were anesthetized and the brains were removed for the measurement of infarct volume. The brains were sliced into 1-mm slices and incubated with 0.1% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma–Aldrich, St. Louis, Missouri, USA) for 30 min at 37 °C. After fixing in 4% paraformaldehyde for 24 h, the slices were photographed. The cross-sectional area of infarction in each brain slice was determined with AlphaEase Image Analysis Software V 3.1.2 (Alpha Innotech Corp., San Leandro, CA). The percentage of hemispheric infarction volume was calculated as described in our previous study (Xu et al., 2008).