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Neuroprotective effects of apelin-13 on experimental ischemic stroke through suppression of inflammation

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ABSTRACT

Acute inflammation plays an important role in the pathogenic progression of post-ischemic neuronal damage. Apelin-13 has been investigated as a neuropeptide for various neurological disorders. The present study was performed to evaluate the effects of apelin-13 on the inflammation of cerebral ischemia/reperfusion (I/R) injury. Transient focal I/R model in male Wistar rats were induced by 2 h middle cerebral artery occlusion (MCAO) followed by 24 h reperfusion. Rats then received treatment with apelin-13 or vehicle after ischemia at the onset of reperfusion. The neurological deficit was evaluated and the infarct volume was measured by TTC staining. The activity of myeloperoxidase (MPO) was measured. The expression of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and intercellular adhesion molecule-1 (ICAM-1) were measured using real-time PCR. And the expression of apelin receptor (APJ), ionized calcium-binding adapter molecule-1 (Iba1), glial fibrillary acidic protein (GFAP) and high mobility group box 1 (HMGB1) were measured by immunohistochemistry and western blot. Our results demonstrated that treatment with apelin-13 in I/R rats markedly reduced neurological deficits and the infarct volume. The increase of MPO activity induced by I/R was inhibited by apelin-13 treatment. The real-time PCR showed that apelin-13 decreased the expression of inflammatory cytokines such as IL-1β, TNF-α and ICAM-1 in I/R rats. The expression of APJ in I/R rats was increased. And the expression of Iba1, GFAP and HMGB1 in I/R rats was decreased by apelin-13 treatment indicating the inhibition of microglia, astrocytes and other inflammatory cells. In conclusion, apelin-13 is neuroprotective for neurons against I/R through inhibiting the neuroinflammation.

Introduction

Stroke is the second most common cause of death in developed countries and the most major cause of permanent disability worldwide. Ischemic stroke, which accounts for approximately 87% of stroke, often results from the occlusion of a cerebral artery caused by a thrombus or embolus [10,11]. A growing body of data implicates that inflammatory mechanisms play important roles in the pathogenic progression of post-ischemic neuronal damage [9,26,31].

Apelin is an endogenous ligand for the G protein-coupled receptor APJ [27], which is synthesized as a 77-amino acid prepropeptide. Apelin is cleaved by proteases to yield significantly shorter, biologically active forms including apelin-36, apelin-17 and apelin-13. Among fragments, apelin-13 and apelin-36 are associated with cytoprotection. Apelin-13 is shown to specifically bind to APJ receptor with a high affinity and elicits greater degrees of biological potency than apelin-36 or apelin-17. The C-terminal 13 amino acids are completely conserved across all species [20,30].

The established biological effects of apelin have been shown to be involved in major cardiovascular actions, neoangiogenesis, immunologic modulation and body fluid homeostasis. Recently, the apelin/APJ system has been investigated as a neuroprotective regulator for various neurological disorders [5]. Recently, it has been shown that apelin-36 has protective effects on cerebral ischemia reperfusion (I/R) injury by decreasing neurological deficits and

Abbreviations: I/R, ischemia/reperfusion; MCAO, middle cerebral artery occlusion; MPO, myeloperoxidase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; ICAM-1, intercellular adhesion molecule-1; Iba1, ionized calcium-binding adapter molecule-1; GFAP, glial fibrillary acidic protein; HMGB1, high mobility group box 1.

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suppressing apoptosis [12]. In addition, pretreatment with apelin-13 reduces brain injuries and post-ischemic cerebral edema through blocking programmed cell death [16]. Moreover, there is a positive correlation between tumor necrosis factor-α (TNF-α) and apelin in human and mouse adipose tissue [7]. Apelin prevents aortic aneurysm formation by decreasing macrophage burden, as well as inhibiting proinflammatory cytokine and chemokine activation [22]. This evidence suggests the involvement of this peptide in inflammation. The effects of apelin-13 on the neuroinflammation in ischemic brain need to be further explored.

Materials and methods

Animal model

The experimental procedures of animal were approved by the Commission of Jining Medical University for ethics of experiments on animals and were conducted in accordance with international standards. All animals were housed conventionally in a constant temperature (22–26 °C) and humidity (50–60%) animal room with a 12-h-light–dark cycle and allowed free access to food and water. Male Wistar rats (280–320 g) were purchased from LuKang Company (Jining, Shangdong, PR China). Rats were randomly divided into 3 groups: (1) sham-operated group: rats were obtained by inserting the filament into the common carotid artery, but without advancing it to the middle cerebral artery; (2) I/R group: rats were subjected to 2-h middle cerebral artery occlusion (MCAO) followed by 24-h reperfusion by the intraluminal filament technique as described. Rats received saline (0.9% NaCl, 10 μl per rat) after ischemia at the onset of reperfusion; (3) apelin-13 group: rats were operated in the same way as rats in I/R group, Rats then received treatment with apelin-13 (50 ng/kg, 10 μl per rat) after ischemia at the onset of reperfusion. Rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (300 mg/kg) [17,19]. During and after the surgery, the perianal temperature was maintained at 37 °C with a heating pad until the complete recovery of the animals from the anesthesia. All efforts were made to minimize animal suffering and reduce the numbers of animals used.

Intracerebroventricular administration of apelin-13

Apelin-13 was obtained from Phoenix Pharmaceuticals, Inc. and dissolved in 0.9% saline. Apelin-13 (50 ng/kg), or the same volume of saline was administered intracerebroventricularly to rats in a volume of 10 μl per rat at the onset of reperfusion after ischemia. The stereotaxic coordinates from the bregma were: 0.8 mm posterior to bregma, 1.5 mm lateral to the midline and 3.8 mm ventral to the skull surface [28]. Apelin-13 or 0.9% saline was injected at 2 μl/min for 5 min. Needle was stayed for further 5 min after the end of injection to minimize leakage of drug.

Neurological scoring

Neurological test was performed by investigators blinded to group assignment at 24 h after reperfusion using the Longa Score Scale [23]: 0 = no neurologic deficit, 1 = failure to extend left forepaw fully, 2 = circling to the left, 3 = falling to the left, and 4 = being unable to walk spontaneously and having a depressed level of consciousness. The animals, received a score in the range 1–3, were used for further treatment.

Infarct volume measurement

Rats were subjected to 2 h MCAO and killed 24 h after reperfusion and the brains chilled at −20 °C for 20 min to harden the tissue. Using a rat brain matrix, each brain was cut into five 2 mm coronal slices starting at 1 mm from the frontal pole, which were immediately incubated for 30 min in a freshly prepared PBS (pH 7.4) solution containing 2% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma, St Louis, MO) at 37 °C in the dark. Stained sections were then fixed with 4% paraformaldehyde. Both hemispheres of each stained coronal section were scanned using a high-resolution scanner and analyzed by ImageJ software (National Institutes of Health). Briefly, infarct areas of all sections were calculated to get total infarct area which was multiplied by thickness of brain sections to obtain the volume of infarction. To eliminate the effect of brain edema, the infarct volume was calculated with the following formula: infarct volume = (red area of contralateral side − red area of ipsilateral side)/total area × 100% [21].

Measurement of cerebral myeloperoxidase (MPO) activity

MPO activity was evaluated after 24 h of reperfusion. MPO activity is used as an indicator of neutrophils infiltration in cerebral ischemia process [2]. MPO activity was determined by a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, China). The quantitative procedure was carried out according to the recommendations of detection kit. Briefly, the ischemic penumbra obtained 24 h after reperfusion were homogenized in cool normal saline (ischemic penumbra:normal saline = 1:10). The absorbance of the reaction product was recorded at 460 nm spectrophotometrically and the enzyme activity was expressed in units, where 1 unit represents the amount of enzyme degrading 1 μmol/L H_2O_2 per minute. Units of activity were normalized to 1 g of protein.

Real-time polymerase chain reaction

Total RNA was extracted from ischemic penumbra after 24 h of reperfusion using TRIzol reagent (Tiangen Biotechnology, Beijing, China) according to the manufacturer's instructions. Reverse transcription was performed using the Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. The expression of the mRNA was analyzed using the SYBR Green real-time PCR Master Mix (TOYOBO) according to the manufacturer's instructions. Real-time PCR was carried out in 96-well plates using the Roche LightCycler 480 (LC480). The primers were synthesized by Shanghai Biotechnology Co., Ltd., China. The sequences of rat interleukin-1β (IL-1β), TNF-α, intercellular adhesion molecule-1 (ICAM-1) and β-actin primers were as following: IL-1β, forward: 5′-aggacccaagcaccttcttt-3′ and reverse: 5′-agacagcacgaggcattttt-3′; TNF-α, forward: 5′-tgcctcagcctcttctcatt-3′ and reverse: 5′-cccatttgggaacttctcct-3′; ICAM-1, forward: 5′-tggggttggagactaactgg-3′ and reverse: 5′-gtgccacagttctcaaagca-3′; β-actin, forward: 5′-ctcagttgctgaggagtccc-3′ and reverse: 5′-attcgagagaagggagggct-3′. β-actin was amplified in parallel as the internal control. Each tissue sample of rats was run in triplicate and each reaction volume was 20 μl. PCR cycling conditions were: 95 °C for 10 min, 40 cycles of amplification at 95 °C for 15 s, and 60 °C for 1 min. Subsequently, a dissociation program was applied with one cycle at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Gene expression data were initially expressed as C_P values, the number of cycles required for the quantity of DNA to reach some preset value. $\Delta\Delta C_P$ was calculated for every sample, and the expression levels were indicated with $2^{-\Delta\Delta C_P}$.

Immunohistochemistry

Immediately after 24 h of reperfusion, the animals were deeply anesthetized and transcardially perfused with saline, followed by 4% paraformaldehyde. The brains were removed and post-fixed in the same fixative at 4 °C for 24 h. The sections were immunolabeled for APJ, Iba-1 (a marker for microglia/macrophages), GFAP (a

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