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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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marker for astrocytes) or HMGB1. Briefly, the slides were incubated with rabbit polyclonal anti-APJ (1:200, Biorbyt), rabbit polyclonal anti-Iba1 (1:1000, WAKO), rabbit polyclonal anti-GFAP (1:1000, Abcam), or rabbit polyclonal anti-HMGB1 (1:200, Cell Signaling) overnight at 4 °C, followed by incubation in the secondary goat anti-rabbit IgG for 30 min, and an avidin–biotin horseradish peroxidase (HRP) complex (Zhongshan Golden Bridge Inc., Beijing, China) for 15 min at 37 °C. The sections were then incubated in diaminobenzidine to visualize the HRP activity. The reactions were terminated once an optimal contrast between specific cellular and nonspecific background labeling was reached. The sections from each treatment group were processed simultaneously. For negative controls, the primary antibodies were replaced by PBS. We randomly obtained five visual field images from every section to evaluate total numbers of APJ, Iba1, GFAP and HMGB1-positive cells in the ischemic penumbra using an Olympus IX 71 inverted microscope (Olympus, Tokyo, Japan).

Western blot

Total proteins were extracted from ischemic penumbra after 24 h of reperfusion using a kit from the Keygen Institute of Biotechnology (Keygen, Nanjing, China). Equal amounts of protein per lane (50 μg) were electrophoresed on 10% SDS-PAGE gel. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Roche). The membrane was blocked with 5% skimmed milk/0.1% Tween-20 in Tris-buffered saline for 2 h at 37 °C. Thereafter, the membrane was incubated with different primary antibodies, including rabbit anti-APJ (1:1000, Biorbyt), rabbit anti-Iba1 (1:1000, WAKO), rabbit anti-GFAP (1:5000, Abcam) and rabbit anti-HMGB1 (1:1000, Cell Signaling) overnight at 4 °C. Subsequently, the membrane was treated with horseradish peroxidase-labeled secondary antibody (1:5000, Zhongshan Golden Bridge Inc., Beijing, China) for 1 h at 37 °C. A rabbit anti-β-actin antibody (1:5000, Zhongshan Golden Bridge Inc., Beijing, China) was used as a loading control. The blots were visualized using ECL reagent kits (Multi-Sciences, Hangzhou, China) and the optical density of the lanes was quantified using ImageJ software.

Statistical analysis

All Gaussian data are presented as mean ± standard deviation (*SD*) and analyzed by one-way analysis of variance (ANOVA) test with Bonferroni test for individual comparisons between group means or the independent-samples *T*-test. All non-Gaussian data are presented as median with 25% and 75% quartiles and analyzed by non-parametric Kruskal–Wallis test or Mann–Whitney *U* test. Statistical significance was assumed when $P < 0.05$.

Table 1
Effect of apelin-13 on the neurological scores in rats subjected to 2 h focal cerebral ischemia followed by 24 h reperfusion.

Group	Neurological scores	Statistical method	F/x^2	*P*
I/R	3 (2, 3)	Mann–Whitney *U*	−3.135	0.002
Apelin-13	1 (1, 2)			

Data are presented as median with 25% and 75% quartiles ($n = 10$).

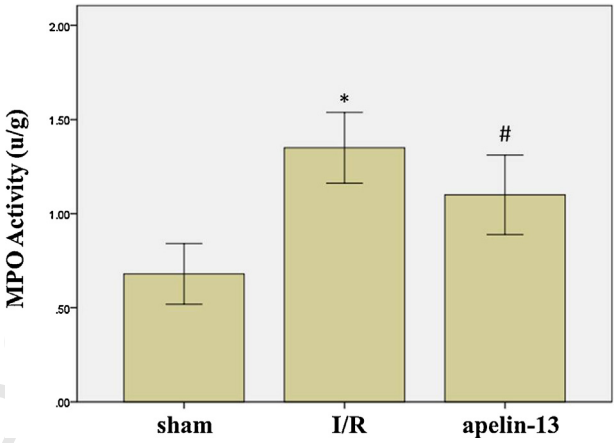

Fig. 2. Apelin-13 decreased myeloperoxidase (MPO) activity induced by cerebral ischemia/reperfusion (I/R) injury. MPO activity was significantly increased in I/R rats compared with sham-operated rats. Apelin-13 significantly reduced the level of MPO activity in I/R. Data are presented as mean ± SD ($n = 10$). $^{*}P < 0.001$ vs. sham group; $^{\#}P = 0.018$ vs. I/R group.

Results

Apelin-13 reduced neurological dysfunction after cerebral I/R

Focal cerebral I/R caused a prominent impairment of motor performance. The rats subjected to I/R injury exhibited severe neurological deficit compared with sham operated animals. However, administration of apelin-13 significantly prevented I/R induced

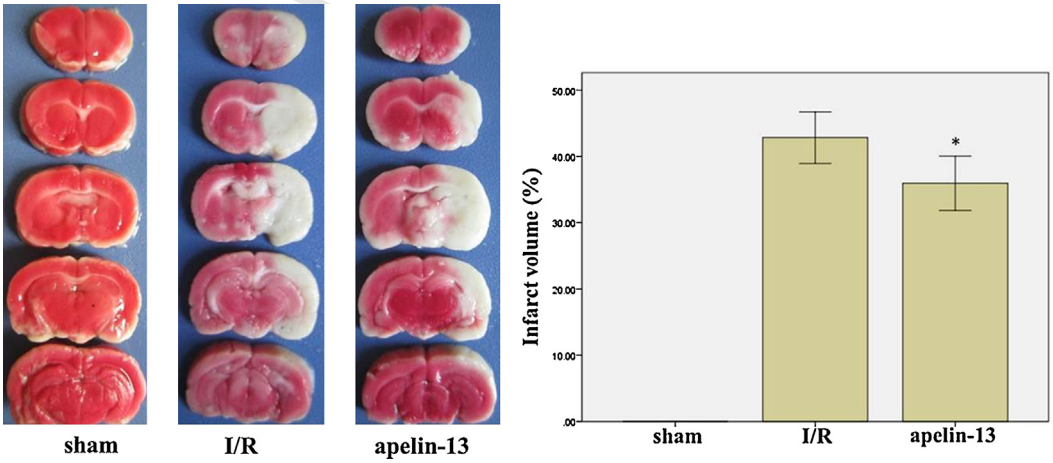

Fig. 1. Reduction in cerebral infarct volume following middle cerebral artery occlusion (MCAO) in rats treated with apelin-13. (a) Representative images in sham-operated rats and MCAO rats with or without apelin-13 after 24 h reperfusion. (b) The columnar diagram for the infarct volume of brains in each group. Each column represented the infarct area as a percentage of the total area. Data are expressed as the mean ± SD ($n = 10$). $^{*}P = 0.001$ vs. I/R group.

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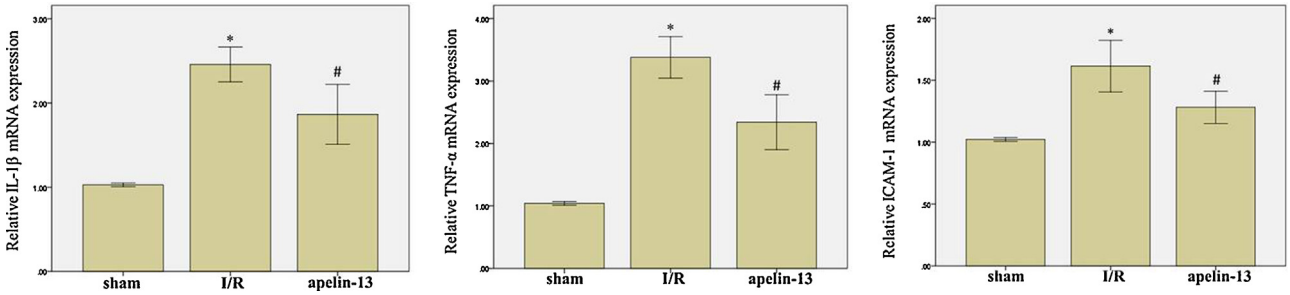

Fig. 3. Apelin-13 downregulated gene expression of inflammatory cytokines following ischemia/reperfusion (I/R) injury. Real-time PCR was performed to detect the mRNA levels of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and intercellular adhesion molecule-1 (ICAM-1). All three were increased significantly in gene expression in I/R group, but there was a dramatic decrease in apelin-13 treated group. Data are presented as median with 25% and 75% quartiles ($n = 10$). $^{*}P < 0.001$ vs. sham group; $^{\#}P < 0.001$ vs. I/R group.

impairment ($P = 0.002$). The neurological scores are shown in Table 1.

Apelin-13 decreased infarct volume after cerebral I/R

Rats after I/R insult exhibited marked infarct volume as is shown in Fig. 1, TTC staining of the serial coronal brain section showed 42.833 ± 3.884% infarct area in I/R group. In contrast, administration of apelin-13 at 50 ng/kg significantly reduced infarct volume by 35.945 ± 4.116% ($P = 0.001$) (Tables 2–4).

Apelin-13 reduced MPO activity after cerebral I/R

In this present work, we performed a MPO activity assay to explore the role of apelin-13 in attenuating neutrophils infiltration in the ischemic penumbra (Fig. 2). MPO activity was significantly higher in the I/R group than that in the sham group. The increased MPO activity was significantly reduced by treatment with apelin-13 after I/R injury ($P < 0.001$).

Apelin-13 inhibited the expression of inflammatory cytokines after cerebral I/R

As shown in Fig. 3, the levels of these inflammatory mediators including IL-1β, TNF-α and ICAM-1 were markedly elevated in I/R group compared with sham group, while administration of apelin-13 after the onset of reperfusion significantly decreased the induction of these cytokines ($P < 0.001$), indicating that the neuroprotective role of apelin-13 on cerebral I/R injury might be attributed to its suppressive effects on neuroinflammation.

Table 2
Analysis of variance of for inflammatory agents among groups.

		M ± SD/quartile	Statistical method	F/x^2	P
MPO	sham	0.680 ± 0.161 A	ANOVA	32.443	<0.001
	I/R	1.350 ± 0.188 B			
	Apelin-13	1.100 ± 0.211 C			
IL1-β	sham	1.02 (1.01, 1.04) A	Kruskal–Wallis test	24.405	<0.001
	I/R	2.460 (2.278, 2.580) B			
	Apelin-13	1.815 (1.635, 2.105) C			
TNF-α	sham	1.018 (1.035, 1.073) A	Kruskal–Wallis test	25.089	<0.001
	I/R	3.390 (3.158, 3.730) B			
	Apelin-13	2.380 (2.138, 2.460) C			
ICAM-1	sham	1.030 (1.018, 1.065) A	Kruskal–Wallis test	23.842	<0.001
	I/R	1.610 (1.465, 1.777) B			
	Apelin-13	1.280 (1.160, 1.375) C			
APJ-IHC	sham	26.50 (23.00, 31.25) A	Kruskal–Wallis test	17.647	<0.001
	I/R	33.5 (30.0, 35.0) B			
	APE	42.00 (36.25, 45.50) C			
Iba1-IHC	sham	8.00 (7.75, 9.00) A	Kruskal–Wallis test	22.887	<0.001
	I/R	14.00 (12.75, 16.00) B			
	APE	12 (10, 13) C			
GFAP-IHC	sham	23 (20, 28) A	Kruskal–Wallis test	21.518	<0.001
	I/R	37.50 (32.75, 40.25) B			
	APE	30.50 (30.00, 34.25) C			
HMGB1-IHC	sham	13.50 (12.75, 15.25) A	Kruskal–Wallis test	22.027	<0.001
	I/R	25.50 (24.25, 27.00) B			
	APE	17.5 (15.75, 20.50) C			
APJ-WB	sham	0.177 (0.109, 0.206) A	Kruskal–Wallis test	20.937	<0.001
	I/R	0.297 (0.223, 0.325) B			
	APE	0.400 (0.306, 0.521) C			
Iba1-WB	sham	0.146 (0.119, 0.319) A	Kruskal–Wallis test	19.654	<0.001
	I/R	0.617 (0.434, 0.749) B			
	APE	0.344 (0.319, 0.475) C			
GFAP-WB	sham	0.262 (0.118, 0.422) A	Kruskal–Wallis test	13.020	0.001
	I/R	0.517 (0.451, 0.777) B			
	APE	0.418 (0.315, 0.571) C			
HMGB1-WB	sham	0.146 (0.119, 0.319) A	Kruskal–Wallis test	16.516	<0.001
	I/R	0.597 (0.433, 0.811) B			
	APE	0.346 (0.224, 0.559) C			

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Table 3
Multiple linear regression analysis between apelin and the other inflammatory agents.

Dependent variable	Independent variable	Non-standardization coefficient		Standardization coefficient	*t*	*P*	95% Confidence interval	
		Non-standardization coefficient	Standard error				Lower	Upper
MPO	Constant	0.680	0.059		11.440	0.000	0.558	0.802
	*X*1	0.670	0.084	0.960	7.970	0.000	0.498	0.842
	*X*2	0.420	0.084	0.602	4.996	0.000	0.248	0.592
IL-1β	Constant	1.028	0.075		13.671	0.000	0.874	1.182
	*X*1	1.430	0.106	1.073	13.447	0.000	1.212	1.648
	*X*2	0.837	0.106	0.628	7.871	0.000	0.619	1.055
TNF-α	Constant	1.041	0.101		10.305	0.000	0.834	1.248
	*X*1	2.338	0.143	1.098	16.366	0.000	2.045	2.631
	*X*2	1.300	0.143	0.611	9.100	0.000	1.007	1.593
ICAM-1	Constant	1.038	0.045		22.908	0.000	0.945	1.131
	*X*1	0.577	0.064	0.997	9.004	0.000	0.446	0.708
	*X*2	0.244	0.064	0.422	3.8080	0.001	0.113	0.375
AP-IHC	Constant	26.500	1.616		16.401	0.000	23.185	29.815
	*X*1	6.500	2.285	0.410	2.845	0.008	1.811	11.189
	*X*2	13.900	2.285	0.878	6.083	0.000	9.211	18.589
Iba1-IHC	Constant	8.100	0.476		17.027	0.000	7.124	9.076
	*X*1	6.200	0.673	1.003	9.216	0.000	4.820	7.580
	*X*2	3.600	0.673	0.582	5.351	0.000	2.220	4.980
GFAP-IHC	Constant	23.800	1.325		17.968	0.000	21.082	26.518
	*X*1	13.400	1.873	0.932	7.154	0.000	9.557	17.243
	*X*2	7.700	1.873	0.536	4.111	0.000	3.857	11.543
HMGB1-IHC	Constant	13.800	0.920		15.001	0.000	11.912	15.688
	*X*1	11.600	1.301	0.993	8.916	0.000	8.931	14.269
	*X*2	4.700	1.301	0.402	3.613	0.001	2.031	7.369
APJ-WB	Constant	0.164	0.024		6.719	0.000	0.114	0.214
	*X*1	0.126	0.035	0.476	3.646	0.001	0.055	0.197
	*X*2	0.248	0.035	0.935	7.165	0.000	0.177	0.319
Iba1-WB	Constant	0.207	0.044		4.671	0.000	0.116	0.298
	*X*1	0.404	0.063	0.898	6.438	0.000	0.275	0.532
	*X*2	0.186	0.063	0.413	2.963	0.006	0.057	0.314
GFAP-WB	Constant	0.270	0.047		5.715	0.000	0.173	0.367
	*X*1	0.322	0.067	0.785	4.817	0.000	0.185	0.459
	*X*2	0.170	0.067	0.414	2.540	0.017	0.033	0.307
HMGB1-WB	Constant	0.207	0.052		3.979	0.000	0.100	0.314
	*X*1	0.407	0.074	0.841	5.529	0.000	0.256	0.558
	*X*2	0.187	0.074	0.386	2.538	0.017	0.036	0.338

Apelin-13 up-regulated the expression of APJ, down-regulated the expression of Iba1, GFAP and HMGB1

In order to examine the effect of I/R on the apelin/APJ system, we examined the expression of APJ in the ischemic penumbra. Interestingly, we found that APJ$^+$ cells were significantly higher in the ischemic penumbra of I/R rats than those in sham-operated rats, while administration of apelin-13 could further increase numbers of APJ$^+$ cells (Fig. 4a and e). To further investigate the anti-inflammatory effects of apelin-13 in the post-ischemic brain, the activation of microglia and astrocytes and the expression of HMGB1 were examined by immunohistochemistry staining. The number of both Iba1$^+$ cells and GFAP$^+$ cells were significantly higher in the I/R group but they were significantly decreased in the apelin-13 group (Fig. 4b, c, f and g). The expression of HMGB1 in the cytoplasm was counted. As shown in Fig. 4d and h, the HMGB1$^+$ cells increased obviously after cerebral I/R injury, which could be significantly inhibited by apelin-13 treatment ($P < 0.001$).

We further analyzed the protein levels of APJ, Iba1, GFAP and HMGB1 in ischemic penumbra by western blot (Fig. 5). As shown in Fig. 5, the level of APJ protein in I/R group was higher than that in sham group in cytosolic fractions. Treatment with apelin-13 could further increase the protein expression of APJ. In addition, the over-expression of Iba1, GFAP and HMGB1 induced by I/R injury was also decreased by apelin-13 ($P \leq 0.001$).

Table 4
Effect of apelin-13 on cerebral ischemia infarction in rats subjected to 2 h focal cerebral ischemia followed by 24 h reperfusion.

Group	Infarct volume (%)	Statistical method	F/x^2	*P*
I/R Apelin-13	42.833 ± 3.884 35.945 ± 4.116	Independent-samples *T*-test	3.489	0.001

Data are presented as mean ± SD ($n = 10$).

Discussion

Recently, the apelin/APJ system has been investigated as a neuroprotective regulator for various neurological disorders. It has been reported that central application of apelin-13 exerts protective effects against cerebral damage and brain edema via preventing apoptosis in a transient model of focal stroke in rats [16]. There is clear evidence suggests that inflammation plays an important role in the evolution of brain injury after cerebral ischemia reperfusion [3,6].

In this study, we found apelin-13 elicited neuroprotective roles by alleviating the post-ischemic inflammation in sub-acute phase. We found that rats after I/R insult exhibited serious neurological dysfunction and large brain infarct. In contrast, administration of apelin-13 significantly decreased the neurological dysfunction and infarct size induced by I/R.

MPO activity has been used as a distinct indicator for the tissue infiltration of neutrophilic granulocytes [4]. The early accumulating neutrophils are reported to cause brain edema and to be one of the vital cellular targets to elicit neuroprotection during the early post-ischemic period [1]. In the present study, the activity of MPO was significantly increased in response to I/R insult, which was markedly inhibited by apelin-13 treatment. The reduction of

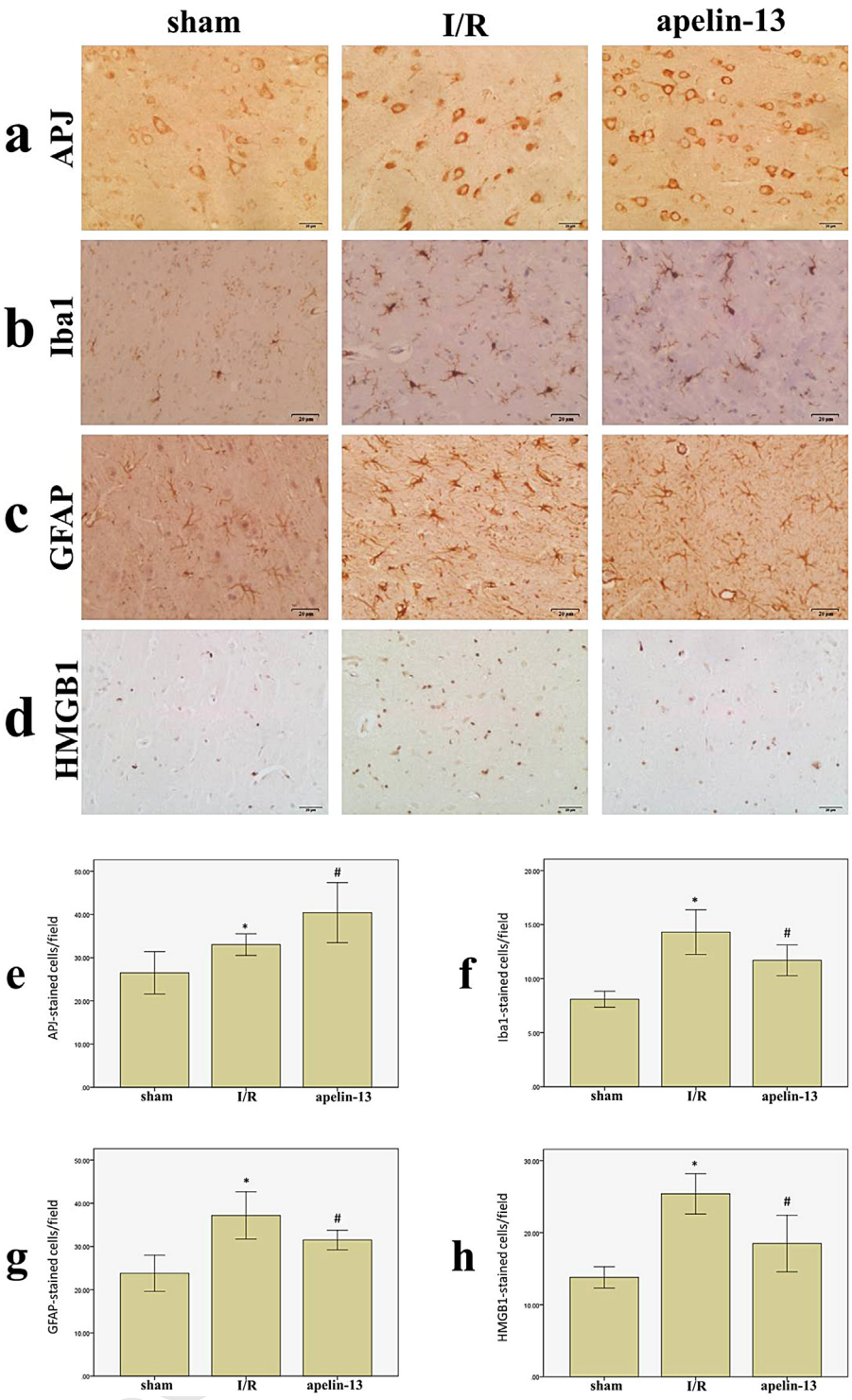

Fig. 4. Apelin-13 increased the numbers of APJ$^+$ cells and decreased the numbers of ionized calcium-binding adapter molecule 1-positive cells (Iba1$^+$), glial fibrillary acidic protein-positive cells (GFAP$^+$), and high mobility group box 1-positive cells (HMGB1$^+$) following ischemia/reperfusion (I/R) injury. Representative images for immunohistochemical staining of APJ (a), Iba1 (b), GFAP (c) and HMGB1 (d) in different groups. Scale bar, 20 μm. Quantitative analysis for immunohistochemical staining of APJ (e), Iba1 (f), GFAP (g) and HMGB1 (h). Data are presented as median with 25% and 75% quartiles ($n = 10$). For APJ, $^*P = 0.005$ vs. sham group; $^\#P = 0.017$ vs. I/R group; for Iba1, $^*P < 0.001$ vs. sham group; $^\#P = 0.002$ vs. I/R group; for GFAP, $^*P < 0.001$ vs. sham group; $^\#P = 0.005$ vs. I/R group; for HMGB1, $^*P < 0.001$ vs. sham group; $^\#P < 0.001$ vs. I/R group.

neutrophils infiltration might be one of the neuroprotective mechanisms of apelin-13.

It has been demonstrated that pro-inflammatory cytokines including TNF-α and IL-1β are markedly up-regulated in the ischemic region after cerebral ischemia. Subsequently, these mediators enhance the expression of cell adhesion molecules including ICAM-1 and endothelium selectin (E-selectin) on endothelial cells and leukocytes, and facilitate the adhesion and transendothelial migration of leukocytes [14,24]. The accumulation of inflammatory cytokines in stroke does not appear to be a mere consequence of the degenerative processes, but it appears to play a role in the cascade of events inducing neuronal death by stress-activated signal transduction pathways. Consistent with previous findings, we found that the production of IL-1β, TNF-α and ICAM-1 was significantly

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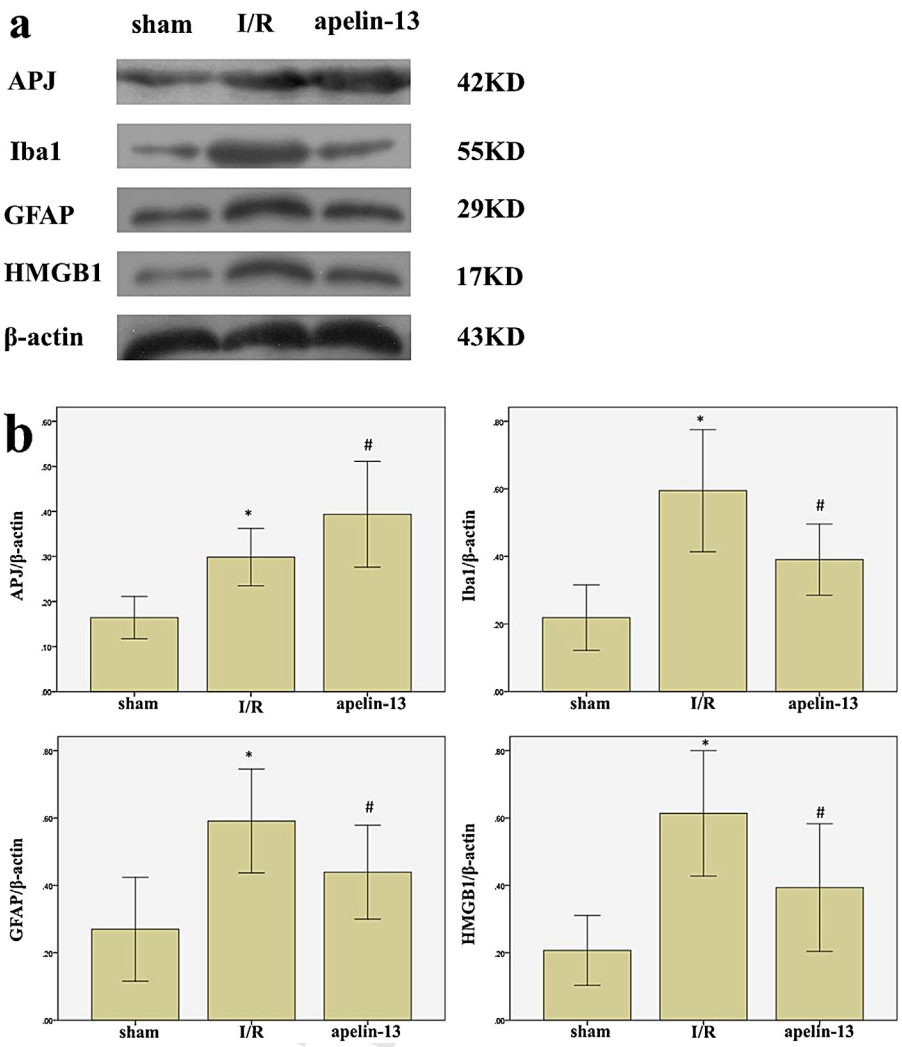

Fig. 5. Apelin-13 increased the protein expression of APJ and decreased the expression of ionized calcium-binding adapter molecule 1 (Iba1), glial fibrillary acidic protein (GFAP), and high mobility group box 1 (HMGB1) following ischemia/reperfusion (I/R) injury. Representative images of western blot for APJ, Iba1, GFAP and HMGB1 in different groups (a). Quantitative analysis of western blot for APJ, Iba1, GFAP and HMGB1 in different groups (b). Data are presented as median with 25% and 75% quartiles ($n = 10$). For APJ, $^{*}P < 0.001$ vs. sham group; $^{\#}P = 0.016$ vs. I/R group; for Iba1, $^{*}P < 0.001$ vs. sham group; $^{\#}P = 0.002$ vs. I/R group; for GFAP, $^{*}P < 0.001$ vs. sham group; $^{\#}P = 0.033$ vs. I/R group; for HMGB1, $^{*}P < 0.001$ vs. sham group; $^{\#}P = 0.018$ vs. I/R group.

increased in response to ischemic injury, which could be effectively suppressed by apelin-13. Therefore, we presume that the mechanism responsible for the anti-inflammatory effects of apelin-13 may be related to the suppression of pro-inflammatory mediators.

In the central nervous system (CNS), apelin and APJ are known to be expressed in the hippocampus, cerebellum, striatum and hypothalamus [8,25]. In order to examine the effect of I/R on the apelin/APJ system, we examined the expression of APJ in the ischemic penumbra. Interestingly, we found that APJ expression was significantly increased after cerebral I/R injury. Administration of apelin-13 could further induce APJ expression, thus we proposed that APJ overexpression induced by I/R may be one of the mechanisms to assess the role of apelin-13 as a post-conditioning mimetic.

A substantial body of literature supports the notion that microglia contribute to the evolution of brain injury following ischemia, serving as a principle source of inflammatory factors in acute phase injury [33,34]. Activated microglia are known to release pro-inflammatory cytokines and increase neuronal death by producing potentially neurotoxic substances like prostaglandin E2 (PGE2), nitric oxide, oxygen radicals, as well as proteolytic enzymes [15,36].

Astrocytes are the most numerous glia in the CNS, which play essential roles in the physiology and pathology of CNS. Like microglia, astrocytes are capable of secreting inflammatory factors such as cytokines and chemokines, which exacerbate brain injury. Reactive astrocytes, microglia, and endothelial cells can express matrix metalloproteinase (MMPs) after injury, which subsequently leads to the blood brain barrier (BBB) breakdown [18]. In the present study, cerebral I/R injury induced a notable increase in the expression of Iba1 and GFAP in the ischemic penumbra, based on immunostaining analysis and western blot. However, the expression of Iba1 and GFAP was significantly inhibited in the apelin-13-treated group. These results indicate apelin-13 can suppress the activation of microglia and astrocytes.

HMGB1 is an endogenous danger signal molecule, which can be actively released into the cytoplasm by activated microglia, myeloid dendritic cells, and natural killer cells [13,32]. HMGB1 has been characterized as a cytokine-like mediator that plays a critical role in the development of post-ischemic cerebral injury by the amplification of plural inflammatory response [29,35]. An important finding of the present study is that the expression of HMGB1 is significantly increased in the I/R group and treatment with apelin-13 can decrease its expression.

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In conclusion, this study provides evidence that the endogenous expression of apelin/APJ system and their interactions suggests neuroprotective signal transduction in the CNS. Apelin-13's inhibitory influence prevents the post-ischemic inflammation in sub-acute phase. These findings support the concept that cerebral apelin-13 could be explored as a possible target for novel therapeutic approaches to ameliorate inflammation, the first acute event in brain injury induced by I/R.

Conflict of interest

All authors have no conflict of interests.

Acknowledgments

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