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Angiotensin-(1–7) inhibits autophagy in the brain of spontaneously hypertensive rats

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ABSTRACT

Autophagy is an important cellular process that mediates lysosomal degradation of damaged organelles, which is activated in response to a variety of stress-related diseases, including hypertension. The basal level of autophagy plays an important role in the maintenance of cellular homeostasis, whereas excessive autophagic activity leads to cell death and is considered as a contributing factor to several disorders. Recent works have demonstrated that Angiotensin-(1–7) [Ang-(1–7)] exerted its neuroprotective effects by modulating classic components of renin–angiotensin system associated with reducing oxidative stress and apoptosis in brains of spontaneously hypertensive rats (SHRs). However, the effect of Ang-(1–7) on autophagic activity in brain of hypertensive individual remains unclear. In this study, Wistar–Kyoto rats received intracerebroventricular (I.C.V.) infusion of artificial cerebrospinal fluid (aCSF) while SHRs received I.C.V. infusion of aCSF, Ang-(1–7), Mas receptor antagonist A-779, or angiotensin II type 2 receptor antagonist PD123319 for 4 weeks. Brain tissues were collected and analyzed by western blotting analysis, immunofluorescence assay, and transmission electron microscopic examination. Our study showed that infusion of Ang-(1–7) for 4 weeks inhibited the increase of microtubule-associated protein 1 light chain 3 (LC3)-II and Beclin-1 levels, as well as the autophagosome formation in SHR brain. Meanwhile, the reduction of p62 expression in SHR brain was also reversed by Ang-(1–7). Of note, the anti-autophagic effects of Ang-(1–7) were independent of blood pressure reduction and can be inhibited by A-779 and PD123319. These findings suggest that treatment with Ang-(1–7) may be useful to prevent hypertension-induced excessive autophagic activation in brain.

1. Introduction

Autophagy is a conserved cellular process that allows cells to recycle cytoplasmic components, remove defective organelles, and maintain cellular functions during development, differentiation, and tissue remodeling [1]. Autophagy involves sequestration of proteins and cell organelles in autophagosomes, which directs them to lysosomes for degradation and reuse. The formation of autophagosomes is dependent on several factors including microtubule-associated protein 1 light chain 3 (LC3) and Beclin-1 [2]. As a double-edged sword in processes of physiology and pathology, the basal level of autophagy plays an important role in the maintenance of cellular homeostasis. Conversely, unrestrained and excessive autophagic activity leads to apoptosis or non-apoptotic programmed cell death, and is considered as a contributing factor to several disorders [3]. Autophagic activity is commonly increased under stress conditions, such as ischemia and hypertension [4,5]. In brain, we have revealed that autophagy was markedly induced in peri-infarction regions after ischemic insult, which aggravated the neuronal damage [6]. To date, little is currently known about the autophagic activity in the brain of hypertensive individual.

Besides, the renin–angiotensin system (RAS) is usually up-regulated in stress-related disorders, including hypertension [7]. Overactivation of brain Angiotensin-converting enzyme (ACE) – Angiotensin II (Ang II) – Angiotensin II type 1 receptor (AT_1R) axis, the classic pathway of RAS, has been found to play a vital

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role in the development and maintenance of hypertension through elevating oxidative stress in brain and increasing the activity of sympathetic nervous system [8]. Recently, accumulating evidence suggested a potential mechanistic link between RAS overactivation and autophagic induction. In 2009, Porrello et al. first bridged the gap between RAS and autophagy by demonstrating that the autophagy was induced by Ang II via the AT_1R in cardiomyocytes, which contributed to the progression of heart failure [9]. Later study extended the observation to kidney by showing that Ang II promoted autophagic activity in podocyte and led to renal injury [10].

Angiotensin 1–7 [Ang-(1–7)] is a newly established bioactive peptide of RAS, which is formed from Ang II by ACE2 and has been shown to oppose the deleterious effects of ACE-Ang II-AT_1R axis in peripheral organs [11–13]. Indeed, Ang-(1–7) and its receptor Mas are present as endogenous constituents of the brain [14,15]. Elevation of brain Ang-(1–7) levels was found to play a protective role against diabetes-induced lesions in the central nervous system [16]. In addition, we have recently demonstrated that chronic treatment with Ang-(1–7) alleviated hypertension-related physiopathologic changes in brain by down-regulation of Ang II and AT_1R expression and by reduction of oxidative stress levels [17]. Based on the above evidences, we adopted spontaneously hypertensive rats (SHRs), a genetic model of hypertension, to investigate: (1) the levels of autophagic activity in brain of hypertensive individual; (2) the effects of Ang-(1–7) on the brain autophagic activity under hypertensive states; (3) which receptors mediate these effects of Ang-(1–7).

2. Materials and methods

2.1. Reagents

Ang-(1–7) and PD123319 were purchased from Sigma–Aldrich Inc. A-779 was purchased from Abbiotec Inc. They were dissolved in an artificial cerebrospinal fluid (aCSF, composition in mmol/L: NaCl 130, KCl 2.99, $CaCl_2$ 0.98, $MgCl_2{\cdot}6H_2O$ 0.80, $NaHCO_3$ 25, $Na_2HPO_4{\cdot}12H_2O$ 0.039, $NaH_2PO_4{\cdot}2H_2O$ 0.46).

2.2. Animals and drug treatment

Sixteen-week-old male normotensive Wistar–Kyoto (WKY) rats and SHRs were used in this study. The rats were housed in a standard room with a 12-h light/dark cycle and given free access to food and water. The experimental protocol was approved by the Nanjing Medical University Experimental Animal Care and Use Committee and all efforts were made to minimize the number of animals used and their sufferings.

Two experiments were carried out throughout the whole study. In the dose response curve experiment, five treatment groups were established. Group 1: WKY rats + aCSF (0.25 μL/h); Group 2: SHRs + aCSF (0.25 μL/h); Group 3: SHRs + low-dose Ang-(1–7) (0.1 nmol/0.25 μL/h); Group 4: SHRs + medium-dose Ang-(1–7) (1.1 nmol/0.25 μL/h); Group 5: SHRs + high-dose Ang-(1–7) (11.1 nmol/0.25 μL/h). In the second experiment, another four treatment groups were established. Group 1: SHR + Ang-(1–7) (1.1 nmol/0.25 μL/h) + A-779 (1.1 nmol/0.25 μL/h); Group 2: SHR + A-779 (1.1 nmol/0.25 μL/h). Group 3: SHR + Ang-(1–7) (1.1 nmol/0.25 μL/h) + PD123319 (6.5 nmol/0.25 μL/h); Group 4: SHR + PD123319 (6.5 nmol/0.25 μL/h). The dose of Ang-(1–7) was chosen according to the results of the first experiment. The doses of A-779 and PD123319 were selected based on the previous studies of our group [17,18]. Implantation of osmotic pumps (Model 2004; ALZET Inc., USA) was carried out according to a well-established procedure as described previously [19], and aCSF, Ang-(1–7), A-779 or PD123319 were continuously infused into the right lateral cerebral ventricle for 4 weeks by osmotic pumps.

2.3. Blood pressure measurements

Mean arterial pressure (MAP) of conscious rats was recorded by the tail-cuff method (BP-2000; Visitech Systems Inc. USA). In brief, unanesthetized rats ($n = 6$) were placed in a holding device mounted on a thermostatically controlled warming plate to make the pulsations of the tail artery detectable. Tail cuffs were fixed on animals, and animals were allowed to acclimate to cuffs for 10 min prior to each pressure recording session. MAP was measured at baseline (16 weeks of age) and then weekly until the end of the treatment period.

2.4. Brain tissue preparation

Rats were sacrificed under deep anesthesia at the end of the 4-week treatment period and handled as follows:

(1) For western blot analysis, rats ($n = 6$) were perfused transcardially with 0.9% saline (pH 7.4) only. The brains were removed rapidly and stored in liquid nitrogen until use.
(2) For immunofluorescence, rats ($n = 6$) were perfused transcardially with 0.9% saline (pH 7.4), followed by a fixative solution containing 4% paraformaldehyde in 0.9% saline (pH 7.4). The brains were removed and fixed in the same fixative at 4 °C until use.
(3) For transmission electron microscopic examination, rats ($n = 3$) were transcardially perfused with 0.9% saline (pH 7.4), followed by a fixative solution containing 4% PFA and 0.25% glutaraldehyde. The brains were removed and fixed in another solution containing 2% PFA and 2.5% glutaraldehyde at 4 °C until use.

2.5. Western blotting analysis

The brain tissues were homogenized and the total proteins were extracted by RIPA lysis buffer (Beyotime Inc., China). The protein concentrations were determined using a BCA kit (Beyotime Inc., China). Different samples with an equal amount of protein (60 μg) were separated on 10–15% SDS polyacrylamide gels, transferred to nitrocellulose membranes, and blocked in 5% bovine serum albumin powder in 1× tris-buffered saline with 0.1% Tween 20 (1× TBST) at room temperature for 2 h. Membranes were incubated overnight at 4 °C with the primary antibodies against LC3 (1:1000, L7543; Sigma–Aldrich Inc., USA), Beclin-1 (1:1000, #3738; Cell Signaling Technology, USA), p62 (1:1000, BML-PW9860; Enzo Life Science, USA), and β-actin (1:500, sc-47778; Santa Cruz Biotechnology, USA). After rinsing with 1× TBST, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000, ZB-2301; Zhongshan goldenbridge, China) for 2 h at room temperature. After washing, protein bands were detected with chemiluminescent HRP substrate (SuperSignal West Pico; Thermo scientific Inc., USA) for 5 min at room temperature and exposed to X-ray film (Fujifilm Inc., Japan). The signal intensity was analyzed using Quantity One software 4.6.2 (Bio-Rad Laboratories Inc., USA) and normalized to β-actin.

2.6. Immunofluorescence assay

After dehydrated in alcohol, the brains were embedded in paraffin and cut into 4–5 μm sections. The brain sections were deparaffinized, hydrated in distilled water, treated with 3% H_2O_2 for 10 min to remove residual peroxidase activity, and rinsed again with phosphate buffer solution (PBS). Then, the sections were permeabilized with 0.1% TritonX-100 for 10 min, blocked with 10% normal goat serum for 2 h at room temperature, and incubated in the primary antibodies against LC3 (1:600, #2775; Cell Signaling Technology, USA) at 4 °C for 24 h. After washing, the sections