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Research article

Thrombin preferentially induces autophagy in glia cells in the rat central nervous system

Shukun Hu[a], Gang Wu[a], Xin Ding[b], Yi Zhang[a,*]

[a] Department of Neurosurgery, Affiliated Huashan Hospital, Fudan University, Shanghai 200042, China
[b] Division of Neonatology, Affiliated Children's Hospital, Soochow University, Suzhou 215003, China

HIGHLIGHTS

- Thrombin activates beclin1 expression preferentially in astrocytes.
- Thrombin induces astrocytic, but not neuronal, vacuole formation.
- Thrombin increases the level of LC3II and MDC$^+$ cells in cultured astrocytes.

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ABSTRACT

Autophagy widely occurs after intracerebral hemorrhage (ICH). In our previous study, we demonstrated that thrombin, a serine protease produced after hematoma, contributes to ICH-induced autophagy. However, whether thrombin plays a neuronal and/or astrocytic role in autophagy induction is largely unknown. Here, we examined the autophagic role of thrombin on neurons and glia cells, respectively. In vivo, we found that intracaudate injection of thrombin specifically elevated the astrocytic expression of beclin-1 and LC3, two autophagic markers, and promoted the formation of autophagic vacuoles within astrocytes rather than neurons in the ipsilateral basal ganglia. Consistent with this, thrombin enhanced the LC3-II level and increased the number of MDC-labeled autophagic vacuoles in cultured astrocytes. These results indicated that thrombin preferentially activated astrocytic autophagy after ICH, and therefore provided novel insights into the pathophysiological mechanisms and therapeutic targets for hemorrhage stroke and brain trauma.

1. Introduction

Thrombin, an essential component for blood coagulation, is acutely produced around the injury site after intracerebral haemorrhage (ICH) [1–3]. The injury-induced thrombin accumulation significantly affects the pathophysiology of haemorrhage stroke and brain trauma [4,5]. In the early phase post injury, thrombin accumulation induces oedema formation that leads to secondary brain damage [4–6]. In the late phase post injury, thrombin formation promotes neurogenesis after ICH [7], probably contributing to the functional recovery after brain damage. Therefore, thrombin might affect distinctive cell types in a time dependent manner after ICH.

Significant changes of cell death and survival occur after ICH, resulting in severe neurological deficits. Besides necrosis and apoptosis, autophagy is also involved in pathophysiology after ICH [3,8]. A major intracellular degradation process in which cytoplasmic materials are sequestered and degraded in the lysosome [9], autophagy occurs after cerebral ischemia, trauma, subarachnoid hemorrhage and ICH [8,10–12]. Whether autophagy plays a beneficial or deleterious role after brain injuries remains controversial. In a traumatic brain injury model, inhibition of autophagy by an antioxidant promoted both anatomical and functional recovery [11]. In contrast, application of 3-methyladenine (3-MA, autophagy inhibitor) exacerbated brain edema and later neurological deficits in the experimental subarachnoid hemorrhage model [13]. One possible explanation is that autophagy occurs in different cell types. It could help maintain neural circuits function by protecting injured neurons, whereas it could also prevent the elimination of harmful cells, for example, reactive astrocytes, and thereby result in more severe inflammatory responses. Therefore, it will be highly inter-

* Corresponding author. Department of Neurosurgery, Affiliated Huashan Hospital, Fudan University, 12 Wulumuqi Middle Road, Shanghai 200042, China.
E-mail address: zhangyi@huashan.org.cn (Y. Zhang).

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esting to investigate how autophagy affects different cell types after ICH.

Our previous findings revealed that thrombin induces autophagy after ICH [14]. In this study, we sought out to examine whether the thrombin-induced autophagy occurred in neurons and/or glia cells both in vivo and in vitro. Elucidating how autophagy affects neurons or glia cells after thrombin infusion will not only provide novel insights into the potential role of thrombin accumulation in ICH pathophysiology, but also shed lights on the discovery of therapeutic targets for haemorrhage stroke and brain trauma.

2. Materials and methods

2.1. Intracerebral infusion

The animal protocols were approved by Fudan University Institution Use and Care of Animals. Fourteen male Sprague-Dawley rats (275–350 g, Shanghai experimental animal center of Chinese Academy of Sciences, Shanghai, China) were used. The surgery was performed as described previously [14]. Briefly, rats were anesthetized (pentobarbital, 45 mg/kg, i.p.) and positioned under a stereotactic frame (Kopf Instrument, Tujunga, CA, U.S.A.). A cranial burr hole (1 mm) was drilled 4.0 mm lateral to the bregma. Thrombin (3 U in 50 μl saline, n = 7) or vehicle (saline 50 μl, n = 7) was then infused into the right basal ganglia stereotactically through a 26-gauge needle (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 4.0 mm lateral to the bregma) using a microinfusion pump (World Precision Instruments, Sarasota, FL, U.S.A.). Previous study showed thrombin-induced autophagy reached the peak at day 3 [14]. Therefore, animals were euthanized 3 days later for immunostaining (4 animals/group) and electron microscopy examination (3 animals/group).

2.2. Immunohistochemistry

For immunofluorescent staining, the primary antibodies used were mouse anti-beclin 1 (Sigma; 1:100 dilution), goat anti-glial fibrillary acid protein (GFAP, Santa Cruz, 1:100 dilution), rabbit anti neuron-specific enolase (NSE, Chemicon, 1:100 dilution), rabbit anti-microtubule-associated protein 1 light chain 3 (LC3, Cell Signaling Technology, 1:100 dilution), and mouse anti-neuron-specific nuclear protein (NeuN, Abcam, 1:200 dilution). Rhodamine- conjugated goat anti-rabbit, rabbit anti-goat antibodies (Boehringer Mannheim Bio, 1:100 dilution) and fluorescein isothiocyanate (FITC)-labeled horse anti-mouse antibody (Vector, 1:100 dilution) were used as secondary antibodies.

Cells from 4 animals (7 sections of the basal ganglion/mouse) received thrombin or saline injection, respectively, were blindly quantified for the immunofluorescent co-localization ratio. The percentages were calculated as beclin$^+$ &GFAP$^+$/GFAP$^+$, beclin$^+$ &NSE$^+$/NSE$^+$, LC3$^+$ &GFAP$^+$/GFAP$^+$, and LC3$^+$ &NeuN$^+$/NeuN$^+$ for beclin and GFAP, beclin and NSE, LC3 and GFAP, LC3 and NeuN colocalization ratio (mean ± S.E.M, Fig. 1), respectively.

2.3. Electron microscopy

Rats were anesthetized and subjected to intracardiac perfusion with 4% paraformaldehyde and 2.5% glutaraldehydein 0.1 mol/L Sorensen's buffer (pH 7.4). The brains were removed and a 1-mm-thick coronal brain slice was prepared with a blade approximately 4 mm from the frontal pole. We used slices from both ipsilateral and contralateral basal ganglions from 6 animals received thrombin (3) or vehicle (3) injection, respectively. The samples were post-fixed in perfusion solution overnight and then immersed with 1.0% OsO4 and dehydrated in graded ethylalcohol. After dehydration, samples were infiltrated with propylene oxide, embedded in Epon, and sectioned. The ultra-thin sections (16 & 14 from thrombin or vehicle injected animals, respectively) were then stained with uranyl acetate and Reynold's lead citrate, and evaluated using Philips CM 100 TEM and digitally imaged using a Hamamatsu (Hamamatsu City, Shizuoka, Japan), ORCA-HR camera.

Neurons and glia cells were distinguished by their intracellular morphological characteristics revealed by the electron microscope: astrocytes were characterized by the enrichment of GFAP fibers and glycogen particles, whereas neurons were characterized by well-aligned microtubules and synaptic structures.

2.4. Primary neuronal and astrocytic cultures

Primary neuronal cultures were obtained from embryonic day-17 Sprague-Dawley rats (Shanghai experimental animal center of Chinese Academy of Sciences, Shanghai, China). Briefly, cerebral cortex from 30 E-17 rats was dissected, stripped of meninges, pooled and dissociated by a combination of 0.5% trypsin digestion and mechanical trituration. The dissociated cells were plated in 6-well dishes (cell density: 1×10^6/ml per well) or 24-well dishes (5×10^5/ml per well) using neurobasal medium with 2% B27, 0.5 mM glutamine and 1% Antibiotic-Antimycotic and maintained in a humidified incubator at 37 °C with 5% CO_2. Culture media was changed every 3–4 days.

Primary cultures of astrocytes were prepared from the brains of 26 1–3 day old Sprague-Dawley rat pups. Cerebral cortex was isolated, meninges were removed and the pooled tissue was dissociated by trypsinization in glial medium (Dulbecco's modified Eagle medium with 10% fetal calf serum, 0.5 mM glutamine and 2% Antibiotic-Antimycotic). The dissociated cells were plated in 6-well dishes (cell density: 1×10^6/ml per well) or 24-well dishes (5×10^5/ml per well), kept at 37 °C with 5% CO_2 and growth medium was changed twice a week.

Cells (7–10 days old cultures) were treated with either vehicle control or thrombin (3 or 5 U/ml). After 24 h, cells were used for the measurements of the conversion of LC3-I–LC3-II (For one experiment, 2 wells of neurons or astrocytes, respectively, in a 6-well dish culture were collected to load one well of SDS-PAGE gel from 0, 3 or 5 U/ml treated cultures. Experiments were triplicated for statistical analysis) and monodansylcadaverine (MDC) staining (For one experiment, 6 wells of neurons or astrocytes, respectively, in a 24-well dish were collected from 0, 3 or 5 U/ml treated cultures. Experiments were triplicated for statistical analysis).

2.5. Western blotting

Western blots were performed as described elsewhere [14]. Briefly, cells were lysed in cell lysis buffer (350 mmol/L sodium chloride, 20 mmol/L HEPES, 1 mmol/L magnesium chloride, 0.5 mmol/L edetic acid, 0.1 mmol/L ethyleneglycoltetracetic acid, 0.5 mmol/L chlorophenothane, 0.2 mmol/L phenylmethylsulfonyl fluoride, 2 U/mL aprotinin, 1% NP40) and sonicated for 5 s. The insoluble materials were removed by centrifugation at 12,000 × g for 10 min at 4 °C. An aliquot of the sample was taken for protein assay (Bio-Rad). Samples containing 50 g protein were applied to 10% sodium dodecyl sulfate-polyacrylamide gels with a 5% stacking gel. After electrophoretic transfer of the protein to a hybond-C nitrocellulose membrane (Amersham UK Ltd., Little Chalfont, Bucks, U.K.), the membranes were blocked in Carnation nonfat milk and probed with primary and secondary antibodies. The primary antibody used was rabbit anti-MAPLC3 (Abgent, 1:400 dilution), anti-beta-actin (Sigma, 1:1000 dilution). The secondary antibody was goat anti-rabbit IgG (Bio-Rad; 1:2500 dilution). The antigen–antibody complex was visualized with a chemiluminescence system (Amer-