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

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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.

Key words

intracerebral hemorrhage; autophagy; thrombin; astrocyte; neuron

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 interesting 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.

Materials and methods

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 (3U 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).

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 .

Electron Microscopy

Rats were anesthetized and subjected to intracardiac perfusion with 4% paraformaldehyde and 2.5% glutaraldehyde in 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 4mm 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.

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, 0.5mM glutamine and 1% Antibiotic-Antimycotic and maintained in a humidified incubator at 37°C with 5% CO₂. 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 The dissociated cells were plated in 6-well dishes (cell density: 1×10^6 /ml per well)

or 24-well dishes (5x10⁵/ml per well), kept at 37°C with 5% CO₂ and growth medium was changed twice a week.

Cells (7-10 days old cultures) were treated with either vehicle control or thrombin (3 or 5U/ml). After 24h, cells were used for the measurements of the conversion of LC3-I to 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 5U/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 5U/ml treated cultures. Experiments were triplicated for statistical analysis).

Western blotting

Western blots were performed as described elsewhere [14]. Briefly, cells were lysed in cell lysis buffer (350mmol/L sodium chloride, 20mmol/L HEPES, 1mmol/L magnesium chloride, 0.5mmol/L edetic acid, 0.1mmol/L ethyleneglycoltetracetic acid, 0.5mmol/L chlorophenothane, 0.2mmol/L phenylmethylsulfonyl fluoride, 2U/mL aprotinin, 1% NP40) and sonicated for 5 seconds. The insoluble materials were removed by centrifugation at 12,000 g for 10 minutes at 4°C. An aliquot of the sample was taken for protein assay (Bio-Rad). Samples containing 50g 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 (Amersham) and exposed to a Kodak X-OMAT film. Relative densities of bands were analyzed with NIH Image program (Version 1.61).

MDC labeling

Thrombin was added in astrocytes or neurons for 24 hours. Cells were incubated with 0.05 mM MDC in PBS for 30 min at 37 °C. They were washed 3 times with PBS and immediately imaged using a fluorescence microscope (Olympus America Inc., Melville, NY). Vacuole numbers was blindly counted in individual wells (24 well dish, neurons or astrocytes were pooled from 30 or 26 brains, respectively, each well 2 cm²) for 0, 3 or 5U/ml treated neuronal or astrocytic cultures using a cytometer. Mean vacuole numbers per 6 dish were then averaged between 3 triplicates for each condition.

Statistical Analysis

For all quantification, error bars represent for S.E.M. For immunofluorescent co-localization ratio, co-localized cells in 7 sections of the basal ganglion from thrombin or saline injected animals were counted blindly by a technician. Data were firstly averaged per animal and then between 4 animals of thrombin or saline injection, respectively. **, $p < 0.01$, student's t test. For quantification of relative ratio of LC3-I/LC3-II and LC3-II levels at 0, 3U or 5U/ml thrombin conditioned neuronal or astrocytic cultures, 2 wells from pooled brain tissues (30 and 26 brains for neuronal and astrocytic cultures, respectively) in a 6-well dish were loaded in each well in SDS-PAGE. Data were averaged between triplicated experiments. **, $p < 0.01$, one-way ANOVA followed by Bonferroni post hoc correction. For quantification of MDC⁺, 6 wells from pooled brain

tissue (30 and 26 brains for neuronal and astrocytic cultures, respectively) in a 24-well dish were used for 0, 3U and 5U/ml thrombin conditioned neuronal or astrocytic cultures, respectively. Data were first averaged between 6 wells and then averaged between triplicated experiments. **, $p < 0.01$, one-way ANOVA followed by Bonferroni post hoc correction.

Results

Thrombin activated significant beclin 1 & LC3 expression in astrocytes, but not neurons in vivo.

To investigate whether thrombin-induced autophagy occurred in neurons or glia cells, we co-immunostained brain sections from thrombin infused animals with beclin 1, a member of the autophagy-initiation complex [15], and a glia marker glial fibrillary acid protein (GFAP) or a neuron marker neuron-specific enolase (NSE). Around the thrombin injection site, the beclin1 staining showed a high rate (78.8 ± 8.93 %) of co-localization with GFAP⁺ glia cells (Fig. 1A1-A3, E). In contrast, beclin1⁺ cells rarely co-localized with NSE⁺ neurons (8.33 ± 1.37 %) (Fig. 1C1-C3, E). Therefore, beclin1 induction preferentially occurred in local glia cells, rather than in neurons after intracerebral thrombin infusion. To further confirm that autophagy favored astrocytic cells, we stained brain sections from thrombin infused animals with LC3, a marker to monitor autophagy [16]. Along with the results of beclin1 staining, GFAP (63.67 ± 8.14 %), but not NeuN (4.34 ± 0.96 %), a specific neuronal marker, co-localized well with the LC3 immunofluorescence (Fig. 1B1-B3, 1D1-D3, F).

Thrombin induced astrocytic, but not neuronal, vacuole formation in vivo.

To confirm that cerebral thrombin infusion caused astrocytic autophagy in vivo, we examined brain tissues using electron microscope. We identified cells containing cytoplasmic vacuoles formed by membranous structures and parts of the cytoplasm, which resembled autophagic vacuoles described in previous studies [14, 19]. Interestingly, cytoplasmic vacuoles were only identified in glia like cells (Fig. 2D-F). We detected no vacuole formation in neurons (Fig. 2A-C). Instead, we observed neuronal necrosis in some cases (Fig. 2B).

Thrombin specifically induced the conversion of LC3-I to LC3-II in cultured astrocytes, but not neurons.

We then tested whether thrombin had a cell autonomous effect to induce astrocytic autophagy. During autophagy, LC3-II is increased by conversion from LC3-I [16]. In cultured astrocytes, the ratio of LC3-II to LC3-I was significantly increased, in a dose-dependent manner, at 24 hour after thrombin addition, when compared to that of the control group (Fig. 3A,B). In contrast, the ratio of LC3-II/LC3-I remained unchanged in thrombin added neurons (Fig. 3C,D). To further confirm the occurrence of autophagy, we also directly compared the LC3-II levels when vehicle, 3U/ml thrombin or 5U/ml thrombin were added [17]. Consistent with the LC3-II/LC3-I ratio, LC3-II levels were specifically raised in astrocytic, but not neuronal cultures after thrombin treatment. These results indicated that the induction of autophagy in astrocytes is dependent on its internal cellular characteristics.

Thrombin caused accumulation of MDC-labeled vacuoles in cultured astrocytes, but not neurons.

To validate the observation of astrocytic autophagy caused by thrombin, we stained the primarily cultured astrocytes or neurons with monodansylcadaverine (MDC), a specific marker for autophagy vacuoles. In cultured astrocytes, the number of MDC⁺ vacuoles significantly increased after thrombin incubation (Fig. 4A1-3, B), indicating the occurrence of autophagy. In cultured neurons, however, the number of MDC⁺ vacuoles remained at a very low level after thrombin incubation (Fig. 4C1-3, D), indicating that thrombin had no autophagic effects on neurons.

Discussion

To investigate the extent of autophagy in different cell types after thrombin infusion, we examined the cellular and anatomical changes of both neurons and glia cells around the injury site. Our results indicated that thrombin induced autophagy preferentially in glia cells, but not neurons in vivo. Consistent with this, the ratio of LC3-II/ LC3-I and the number of MDC⁺ cells, two autophagy indicators, were significantly higher in cultured astrocytes than those in cultured neurons. We concluded that thrombin induces cell autonomous, astrocytic autophagy.

Immediately after ICH, thrombin is released to alleviate the bleeding. Low dose of thrombin protects both neurons and astrocytes after oxygen-glucose deprivation [19,20]. However, high concentration of thrombin infusion, which occurs after ICH, leads to inflammatory cell infiltration, brain edema formation, and neuronal cell death [19-21]. It is unclear whether astrocytes and neurons respond the same way to local thrombin infusion after ICH. One interesting finding is that astrocytes that are surrounding capillaries express a much higher level of protease nexin-1, a serine protease that inhibits thrombin activation [22,23]. Therefore, it is likely that the relatively high level thrombin

released after injury is lethal to local neurons, but not astrocytes. In our case, we did observe cases of neuronal, but not astrocytic, cell death (Fig. 2B).

Autophagy, primarily evolved for cellular adaptation to extreme conditions, is generally considered a protective response [24, 25]. By degradation and recycling cytoplasmic materials, autophagy generates new building blocks and energy that are required for cellular renovation and homeostasis. However, in some cases autophagy can also result in autophagic cell death or apoptosis [25]. Therefore, the role of autophagy after ICH could be highly condition dependent [26]. In our previous study [14], we demonstrated that addition of 3-MA, an autophagy inhibitor, exacerbated cell death. We then hypothesized that the astrocytic induction of autophagy after thrombin infusion plays a protective role in glia cell survival.

The fact that autophagy occurs in surrounding glia cells but not neurons in the injury site after ICH indicated that neurons are more vulnerable upon injury. Our findings therefore provided new insights into the pathophysiology of ICH and brain trauma. Emerging evidences indicate that astrocytes in the brain play a key role in maintain normal function of neural circuits [27,28]. Therefore, it would be highly interesting to investigate whether the astrocytic autophagy induction benefits the functional outcome after ICH in future.

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Figure legends

Fig. 1 Significant up-regulation of beclin1 & LC3 in glia cells after cerebral thrombin infusion.

(A1-D3) Fluorescent immunostaining of beclin 1 (A1, C1), LC3 (B1, D1), GFAP (A2,B2), NSE (C2) and NeuN (D2) with the merged image (A3 for beclin & GFAP, B3 for LC3 and GFAP, C3 for beclin & NSE, and D3 for LC3 and NeuN, respectively) on brain sections at ipsilateral basal ganglion after thrombin infusion. (E-F) Statistical analysis of co-localization ratio of beclin 1⁺ cells relative to GFAP⁺ or NSE⁺ cells (C) and LC3⁺ cells relatively to GFAP⁺ or NeuN⁺ cells (D), respectively. Scale bar in A1 (50 μ m) applies to other panels. Co-localized cells in 7 sections of the basal ganglion from 4 animals received thrombin or saline injection, respectively, were used for quantification. **, $p < 0.01$, Student's t test.

Fig. 2 Autophagic vacuole formation in glia, but not neurons in vivo.

(A-C) Electron microscope images showing the ultrastructure of neurons at the thrombin injected site. (D-F) Electron microscope images showing the ultrastructure of glia cells at the thrombin inject site. F is a zoom-in image of the area marked by rectangle in E. Arrows indicate the autophagic vacuoles. Scale bars represent 2 μ m.

Fig. 3 Induction of LC3-II in cultured astrocytes, but not neurons after thrombin incubation.

(A, C) Western blotting showing LC3-I and LC3-II expression in cultured astrocytes (A) and neurons (C) at 24 hour after vehicle, 3U/ml thrombin, and 5U/ml thrombin incubation, respectively. Each lane represents an independent measurement. β -actin was used as loading control. (B, D) Statistical analysis of relative band intensity (LC3-II/LC3-I) and LC3-II expression (ratio of LC3-II/ β -actin) at vehicle, 3U/ml thrombin, and 5U/ml thrombin treated, astrocytic (B) or neuronal (D) cultures. For quantification of relative ratio of LC3-I/LC3-II and LC3-II levels at 0, 3U and 5U/ml thrombin conditioned neuronal or astrocytic cultures, 2 wells of pooled cultures (30 and 26 brains for neuronal and astrocytic cultures, respectively) in a 6-well dish were loaded in each well in SDS-PAGE for neuronal and astrocytic cultures, respectively. Data were averaged between triplicates per each condition. **, $p < 0.01$, one-way ANOVA followed by Bonferroni post hoc correction. N.S. no statistical significance, one-way ANOVA.

Fig. 4 Increase of MDC-labeled vacuoles in cultured astrocytes, but not neurons after thrombin incubation.

(A, C) Staining of MDC⁺ vacuoles in cultured astrocytes (A1-A3) and neurons (C1-C3) at 24 hour after vehicle, 3U/ml thrombin, and 5U/ml thrombin incubation, respectively. Scale bar in A2 (100 μ m) applies to other images. (B, D) Statistical analysis of MDC⁺ cell intensity (number of MDC⁺ cell/ mm²) at vehicle, 3U/ml thrombin, and 5U/ml thrombin treated astrocytic (B) or neuronal (D) cultures. For quantification of MDC⁺, 6 wells in the 24-well dish from pooled cultures (30 and 26 brains for neuronal and astrocytic cultures, respectively) were used for 0, 3U and 5U/ml thrombin conditioned neuronal or astrocytic

cultures, respectively. Data were first averaged between 6 wells obtained from each experiment and then averaged between triplicates per condition. **, $p < 0.01$, one-way ANOVA followed by Bonferroni post hoc correction. N.S. no statistical significance, one-way ANOVA.

Fig. 1

Fig. 2

Fig. 3

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