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Thrombin-induced autophagy: a potential role in intracerebral hemorrhage

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Abstract

Autophagy occurs in the brain after intracerebral hemorrhage (ICH) and thrombin contributes to ICH-induced brain injury and cell death. In this study, we investigated whether thrombin may activate autophagy (in vivo and in cultured astrocytes) and its potential role in ICH. Autophagy was examined using electron microscopy, conversion of light chain 3(LC3) from the LC3-I form to LC3-II, cathepsin D Western blotting and monodansylcadaverine (MDC) staining to detect autophagic vacuoles. 3-Methyladenine (3-MA) was used as an autophagy inhibitor. In vivo, we found that intracaudate injection of thrombin increased conversion of LC3-I to LC3-II, cathepsin D levels, and formation of autophagic vacuoles in the ipsilateral basal ganglia. ICH-induced upregulation of LC3-I to LC3-II conversion and cathepsin D levels was reduced by a thrombin inhibitor, hirudin. In cultured astrocytes, thrombin enhanced the conversion of LC3-I to LC3-II and increased MDC-labeled autophagic vacuoles. 3-MA inhibited thrombin-induced autophagic vacuole formation and exacerbated thrombin-induced cell death. These results indicate that thrombin activates autophagy in the brain and that thrombin has a role in ICH-induced autophagy.

Keywords

autophagy; cell death; cerebral hemorrhage; thrombin

1. Introduction

Thrombin, a serine protease generated by the cleavage of prothrombin, is an essential component of the coagulation cascade. As such, it is produced in the brain immediately after a cerebral hemorrhage to induce hemostasis. However, thrombin has multiple effects in brain injury. Thus, evidence indicates that thrombin contributes to early brain injury following intracerebral hemorrhage (ICH) and cerebral ischemia (Xi et al., 2003; Xi et al., 2006). In contrast to these early effects, thrombin is also associated with brain recovery after ICH (Yang et al., 2008).

Autophagy is a cellular degradation process in which cellular proteins and organelles are sequestered in double membrane vesicles known as autophagosomes, delivered to lysosomes, and digested by lysosomal hydrolases (Wang and Klionsky, 2003). Autophagy plays an important role in cellular homeostasis, and it is involved in a number of human

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diseases (Adhami et al., 2006; Komatsu et al., 2006; Rubinsztein et al., 2005). We have demonstrated that autophagy occurs after ICH and iron has a role (Gong et al., 2011; He et al., 2008) and most autophagic brain cells are astrocytes (He et al., 2008). It is known that thrombin and iron are two major factors causing brain injury after ICH (Xi et al., 2006). However, it is unclear whether thrombin also causes autophagic cell death after ICH and whether modifying thrombin-induced autophagy might affect brain injury or recovery after ICH. The purpose of the current study was, therefore, to investigate whether thrombin causes autophagy in brain (in vivo) and astrocytes (in vitro). This was examined using electron microscopy and three markers of autophagy. Light chain 3 is a marker for the detection of autophagosomes. Light chain 3 has two forms: type I (LC3-I) is cytosolic and type II (LC3-II) is membrane-bound. During autophagy, LC3-II is increased by conversion from LC3-I (Kabeya et al., 2000). Cathepsin D is a protein known to mediate autophagy (Araki et al., 2006; Klionsky et al., 2008) and monodanysylcadaverine (MDC) staining is a marker of autophagic vacuoles.

2. Results

Thrombin increased the conversion of LC3-I to LC3-II and upregulated cathepsin D in brain

The time-course study showed that ratio of LC3-II to LC3-I (Western blotting) in the ipsilateral basal ganglia was increased by thrombin injection (Figure 1). The conversion of LC3-I to LC3-II in the ipsilateral basal ganglia was significantly higher in the thrombin-treated group at day 1 (ratio: 0.8 ± 0.1 vs. 0.2 ± 0.1 in the saline group, $p<0.01$, Figure 1) or day 3 (ratio: 0.9 ± 0.1 vs. 0.4 ± 0.2 in the saline group, $p<0.05$, Figure 1). Thrombin also induced upregulation of cathepsin D (Figure 2). The levels of cathepsin in the ipsilateral basal ganglia were significantly higher at day 1 and day 3 after thrombin injection compared with the saline control ($p < 0.01$; Figure 2B).

Thrombin induced vacuole formation in brain

Electron microscopy demonstrated normal nuclei, mitochondria, synapses, endoplasmic reticulum, and myelinated axons in the ipsilateral basal ganglia of saline-injected rats. No autophagic vacuoles were observed. In contrast, numerous cytoplasmic vacuoles containing membranous structures and parts of the cytoplasm were found in the ipsilateral basal ganglia after thrombin injection. These structures resembled autophagic vacuoles described in previous studies (Adhami et al., 2006; Nitatori et al., 1995; Xue et al., 1999). According to the ultrastructure, most dying cells containing numerous autophagic vacuoles were glia-like cells (Figure 3).

Hirudin blocked ICH-induced autophagy activation

In a previous study, we showed that the peak in autophagy activation after ICH is at day 7 (He et al., 2008). To determine whether ICH-induced autophagic activation is related with thrombin, we treated rats with hirudin or saline by the co-injection with blood into the right caudate. The ratio of LC3-II to LC3-I in the ipsilateral basal ganglia of rats at 7 days after ICH was markedly decreased by hirudin co-injection (1.3 ± 0.2 vs. 1.7 ± 0.3 in saline co-injection group, $p<0.05$). Hirudin also reduced ICH-induced upregulation of cathepsin D in the ipsilateral basal ganglia (ratio: 0.6 ± 0.1 vs. 1.1 ± 0.3 in saline co-injection group, $p<0.05$).

Thrombin induced the conversion of LC3-I to LC3-II and accumulation of MDC-labeled vacuoles in astrocytes

Thrombin at 5U/ml significantly increased the conversion of LC3-II to LC3-I in cultured astrocytes at 24 hours (0.8 ± 0.1 vs. 0.5 ± 0.1 in the control group, $p<0.05$; Figure 4). A

time-course showed that the number of MDC-labeled vacuoles increased at 6 hours, peaked at 24 hours and decreased at 48 hours in astrocytes incubated with 5U/ml thrombin (Figure 5). The increased number of MDC-labeled vacuoles with thrombin was attenuated by 3-MA, a specific inhibitor of autophagy (268 ± 43 vs. 376 ± 61/mm^2, $p<0.01$; Figure 5). 3-MA also caused a small decrease in the number of MDC-labeled vacuoles in vehicle treated astrocytes (Figure 5).

3-MA aggravated thrombin-induced cell death

To examine the effects of autophagy inhibition on thrombin-induced cell death, cultured astrocytes were treated with thrombin plus 3-MA or vehicle. We found that 3-MA alone did not induce astrocyte death. Thrombin (5U/ml) caused moderate cell death (e.g. lactate dehydrogenase (LDH): 293 ± 20 vs. 105 ± 3 mU/ml in the control group, $p<0.05$; Figure 6) and 3-MA exacerbated cell death induced by thrombin (e.g. dead cell rate: 10.4 ± 2.6 vs. 3.8 ± 1.2% in the vehicle-treated group, $p<0.01$; Figure 6).

3. Discussion

In the current study, we found: 1) thrombin causes autophagy in brain and cultured astrocytes; 2) hirudin, an inhibitor of thrombin, reduces ICH-induced autophagy; and 3) 3-MA, an inhibitor of autophagy, reduces MDC-labeled vacuoles accumulation after thrombin exposure and aggravates thrombin-induced cell death. The results suggest that thrombin has a role in autophagy after ICH.

Thrombin is a serine protease and an essential component in the coagulation cascade. It is produced immediately in the brain after an ICH to stop the bleeding. Direct infusion of large doses of thrombin into brain causes inflammatory cell infiltration, brain edema formation, and cell death. Thrombin at high concentrations also kills neurons and astrocytes in vitro. We have demonstrated that thrombin and iron are two major factors causing brain injury after ICH (Xi et al., 2006). Our previous studies have indicated that iron plays an important role in autophagy after ICH, and we also suggest that factors other than iron in ICH may also have impact on autophagy (He et al., 2008). This study showed the role of thrombin in autophagy after ICH.

Autophagy is a cellular degradation process in which cellular proteins and organelles are sequestered in double-membrane vesicles known as autophagosomes, delivered to lysosomes and, digested by lysosmal hydrolases (Wang and Klionsky, 2003). Autophagy plays an important role in cellular homeostasis and has been implicated to play a role in cancer, neurodegeneration and myopathology (Komatsu et al., 2006; Kondo et al., 2005). Recent studies indicate that autophagy occurs in cerebral ischemia, trauma, subarachnoid hemorrhage and ICH (Adhami et al., 2006; He et al., 2008; Lai et al., 2008; Lee et al., 2009). Whether enhancing autophagy provides a protective mechanism against brain injury has not been confirmed. Our current study showed that inhibition of autophagy exacerbates thrombin-induced cell death.

Light chain 3 has been used as a marker of autophagy because it was identified as the first mammalian protein localized in the autophagosome membrane (Kabeya et al., 2000). LC3 has two forms: type I is cytosolic and type II is membrane-bound. During autophagy, LC3 type II is increased by conversion from types I and the ratio of LC3-II to LC3-I is correlated with the extent of autophagosome formation. In the present study, the ratio of LC3-II to LC3-I in the ipsilateral basal ganglia was increased by day 3 after thrombin infusion, indicating the occurrence of autophagy. There was a decrease in LC3-II to LC3-I ratio by day 7, which may indicate a decrease in the rate of autophagy. However, it is known that LC3-II can be rapidly degraded by lysosomal proteases and this result might also be

explained by increased lysosomal activity. Cathepsin D is a hydrolytic enzyme in lysosomes that degrades damaged proteins. A recent study showed that cathepsin D can act as an autophagic mediator, and inhibition of cathepsin D prevents the formation of vacuoles, suggesting that cathepsin D plays an important role in the execution of autophagic cell death (Araki et al., 2006). In this study, cathepsin D levels increased at day-3 and decreased at day-7 after thrombin infusion, a similar time course to the LC3-II to LC3-I conversion ratio. The greater expression of cathepsin D after thrombin infusion might indicate increased lysosomal activity and autophagy. However, it should be noted that cathepsin D is not a specific marker for autophagy. It may also be involved in apoptotic cell death. Previous studies have shown the existence apoptosis with thrombin. Therefore, it is possible that increased activity of cathepsin D may be involved in both apoptotic and autophagic cell death.

Electron microscopy is currently considered as the most sensitive and accurate way to determine whether cells are undergoing autophagy (Klionsky et al., 2008). Numerous autophagic vacuoles were found particularly 3 days after thrombin infusion. These autophagic vacuoles contain multi-vesicular bodies and organelles surrounded by a sequestering membrane. According to ultrastructural morphology, most of the damaged cells containing numerous autophagic vacuoles were glia. Cells in the contralateral basal ganglia of thrombin injection possessed normal nucleus, mitochondria, synapses, endoplasmic reticulum, myelinated axons, and no autophagic vacuoles.

MDC is a selective marker for autophagic vacuoles. In the present study, primary cultured astrocytes exposed to thrombin showed the accumulation of MDC-labeled vacuoles indicating thrombin-induced autophagy.

It is still controversial whether autophagy is harmful or beneficial. Evidence from some studies shows that in certain pathological situations autophagy can trigger and mediate programmed cell death (Adhami et al., 2007; Klionsky et al., 2008). However, some other researchers consider that autophagy has an important role for cell survival (Levine and Yuan, 2005; Tolkovsky et al., 2002; Uchiyama et al., 2008). In the current study, autophagy modification with 3-MA decreased the number of MDC-labeled vacuoles and increased cell death after thrombin exposure suggesting that autophagy was protective. However, future studies should continue to explore whether thrombin-induced autophagy is protective or detrimental because a recent study has shown that the effects of 3-MA on autophagy are complex and condition dependent (Wu et al., 2010).

In summary, the present study showed that thrombin induces autophagy both in vivo and vitro.

4. Experimental Procedures

Animal Preparation and Intracerebral Infusion

The University of Michigan Committee on the Use and Care of Animals approved the protocols for these studies. Male Sprague–Dawley rats (weighing 275 to 350 g, Charles River Laboratories, Portage, MI, USA) were anesthetized with pentobarbital (45 mg/kg, i.p.). A polyethylene catheter (PE-50) was then inserted into the right femoral artery to monitor arterial blood pressure and blood gases, and to obtain blood for intracerebral blood infusion. Rectal temperature was maintained at 37.5 ± 0.5°C using a feedback-controlled heating pad. The animals were positioned in a stereotactic frame (Kopf Instruments, Tujunga, CA, USA) and a cranial burr hole (1 mm) was drilled. Thrombin (Sigma, USA), blood or saline was infused into the right caudate nucleus through a 26-gauge needle for 10 minutes using a micro infusion pump (Harvard Apparatus Inc., South Natick, MA, USA).

The coordinates were 0.2 mm anterior and 3.5 mm lateral to the bregma and a depth of 5.5 mm. After intracerebral infusion, the needle was removed and the skin incision closed with suture (He et al., 2008).

Cell culture

Astrocyte cultures were prepared from the brains of neonatal (1 to 3 days–old) Sprague-Dawley rats with some modifications (McCarthy and de Vellis, 1980). Cerebral cortex was isolated, meninges were removed and the tissue was incubated in 0.5% trypsin for 20 minutes at 37°C. After digestion, the tissue was rinsed twice in Hank's buffered salt solution, followed by a mechanical dissociation in Dulbecco's modified Eagle's medium (DMEM, 10% fetal calf serum, 0.5 mmol/L glutamine and 2% Antibiotic–Antimycotic). The cells were plated on precoated poly-L-lysine plates in DMEM medium. The cells were incubated at 37°C with 5% CO2 and growth medium was changed twice a week.

Experimental Groups

This study was divided into two parts, *in vivo* and *in vitro* studies. In the studies *in vivo*, rats received an infusion of either 50 μl saline (n=12) or thrombin (3U in 50 μl saline, n=12) into right caudate and were euthanized 1, 3 and 7 days later for Western blot analysis and electron microscopy examination. Some rats (n=5 for each group) had 100 μl autologous blood injection with or without 5 U hirudin, an inhibitor of thrombin, and the rats were euthanized at day 7 for Western blot analysis. In the studies *in vitro*, primary cultured rat astrocytes (7 to 10 of culture days) were used in the experiments. Astrocytes were treated with either vehicle control or thrombin (3U/ml or 5U/ml) and the cells were used for the measurements of the conversion of LC3-I to LC3-II and monodansylcadaverine (MDC) staining. Some astrocytes were treated with thrombin (5U/ml) ± 3-methyladenine (3-MA; 10mM) and the cells were used for MDC staining. Cell death was determined using LDH assay and live/dead cell staining (Hu et al., 2010).

Western Blot Analysis

Rats were anesthetized and underwent intracardiac perfusion with 0.1mol/L phosphate-buffered saline (pH7.4). The brains were removed and a 3-mm-thick coronal brain slice was cut approximately 4 mm from the frontal pole. The slice was separated into ipsi- and contralateral basal ganglia. Western blot analysis was performed as previously described (Xi et al., 1999). Briefly, brain samples were sonicated with Western blot lysis buffer. Protein concentration was determined using a Bio-Rad Laboratories (Hercules, CA, USA), protein assay kit. A 50 μg portion of protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a hybond-C pure nitrocellulose membrane (Amersham, Piscataway, NJ, USA). The membranes were blocked in Carnation nonfat milk and probed with primary and secondary antibodies. The primary antibodies were mouse anti-cathepsin D antibody (Sigma, St Louis, MO, USA; 1:1,000 dilution) and rabbit anti-MAPLC3 antibody (Abgent Inc., San Diego, CA, USA; 1:400 dilution). The secondary antibodies were goat anti mouse and goat anti-rabbit IgG (Bio-Rad; 1:2,500 dilution). The antigen–antibody complexes were 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).

Electron Microscopy

Rats were anesthetized and subjected to intracardiac perfusion with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1mol/L Sorensen's buffer (pH 7.4). The brains were removed and a 1-mm-thick coronal brain slice was cut with a blade approximately 4-mm from the frontal pole. The slices were separated into 4 parts: (1) contralateral basal ganglion; (2)

ipsilateral basal ganglion near the needle track; (3) ipsilateral basal ganglion further from the thrombin injection site; (4) ipsilateral cortex and basal ganglion border. They were immersed in the same fixative overnight at 4°C. The samples were then post fixed with 1.0% OsO4 and dehydrated in graded ethylalcohol. After complete dehydration, samples were infiltrated with propylene oxide, embedded in Epon, and sectioned. The ultra-thin sections 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.

MDC labeling

Thrombin (5U/ml) or vehicle was added in astrocytes with or without 3-MA (10mM) for 1, 6, 12, 24 and 48 hours. Cells were incubated with 0.05 mM MDC (Sigma, USA) in PBS for 30 min at 37 °C and were washed 3 times with PBS and immediately imaged by a fluorescence microscope (Olympus America Inc., Melville, NY).

LDH activity measurement and live/dead assay

Astrocytes were treated with thrombin (5U/ml) or vehicle for 24 hours in the present of 3-MA or vehicle. Cell medium was collected. LDH activity in cell culture was measured using a commercially available kit (Roche Phamaceuticals, Germany) according to manufacturer's instruction. Remaining cells were assessed using the fluorescent probes calcein AM and ethidium homodimer (LIVE/DEAD Viability kit; Molecular Probes, Eugene, OR). Viable cells took up and retain the green calcein dye while excluding the orange ethidium dye. Cells incubated with PBS containing 4 μM ethidium homodimer and 2 μM calcein AM for 30 min at room temperature. These cells were then rinsed with PBS and viewed under fluorescence microscopy. Quantification of dead cells (percent of red cells/ red+ green cells) was performed by NIH ImageJ software.

Statistical Analysis

All data in this study are presented as mean ± S.D. Data were analyzed with Student's t-test and analysis of variance. Statistical significance was set at $p<0.05$.

Research Highlights

- Thrombin causes autophagy in brain and cultured astrocytes.
- Hirudin, an inhibitor of thrombin, reduces intracerebral hemorrhage-induced autophagy.
- 3-Methyladenine, an inhibitor of autophagy, attenuates autophagic vacuoles accumulation after thrombin exposure.
- 3-Methyladenine aggravates thrombin-induced cell death.

Abbreviations

ICH	intracerebral hemorrhage
LC3-I	light chain 3 type I
LC3-II	light chain 3 type II
LDH	lactate dehydrogenase
MDC	monodansylcadaverine

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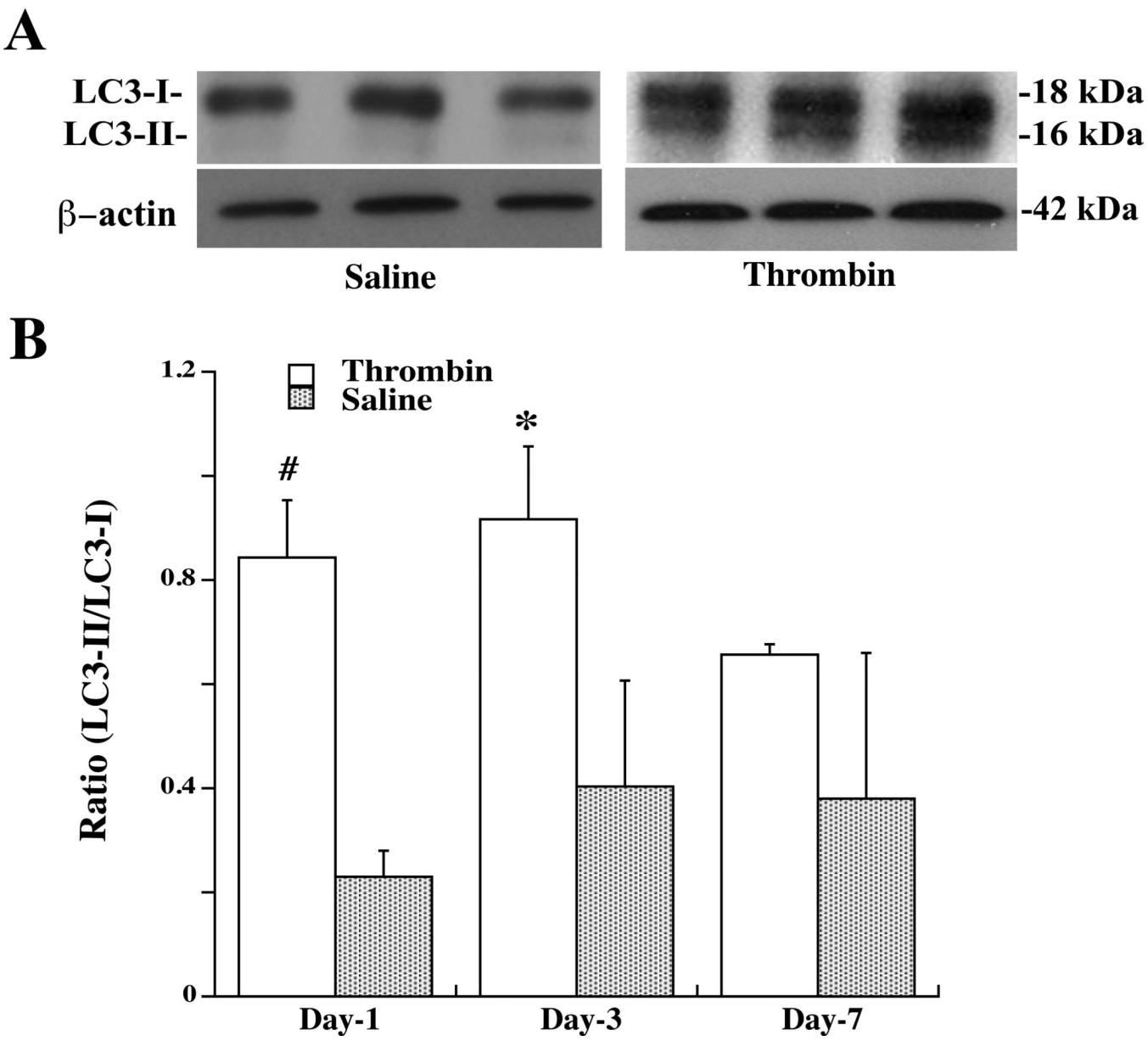

Figure 1.

(A) Western blot analysis showing LC3-I (18 kDa) and LC3-II (16 kDa) levels in the ipsilateral basal ganglia one day after thrombin (3 U) or saline injection. (B) Western blot analysis showing the ratio of LC3-I to LC3-II in the ipsilateral basal ganglia at days 1, 3 and 7 after an intracaudate injection of either saline or thrombin. Values are mean ± S.D., #$p<0.01$, *$p<0.05$ vs. saline group.

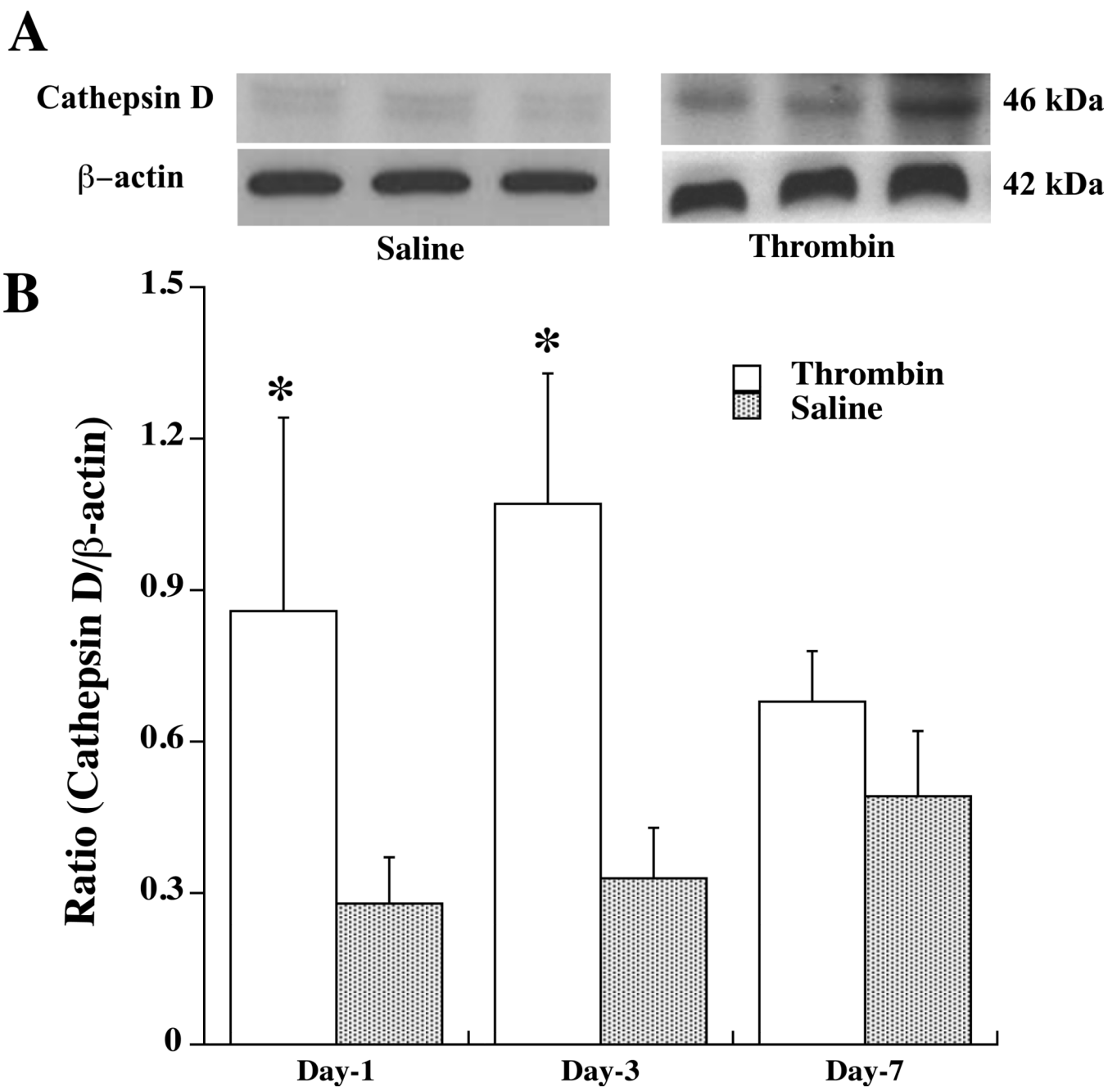

Figure 2.

(A) Western blot analysis showing cathepsin D levels in the ipsilateral basal ganglia one day after intracaudate saline or thrombin injection. (B) Western blotting showing levels of cathepsin D in the ipsilateral basal ganglia at days 1, 3 and 7 after an injection of saline or thrombin. Values are mean ± S.D., *$p<0.05$ vs. saline.

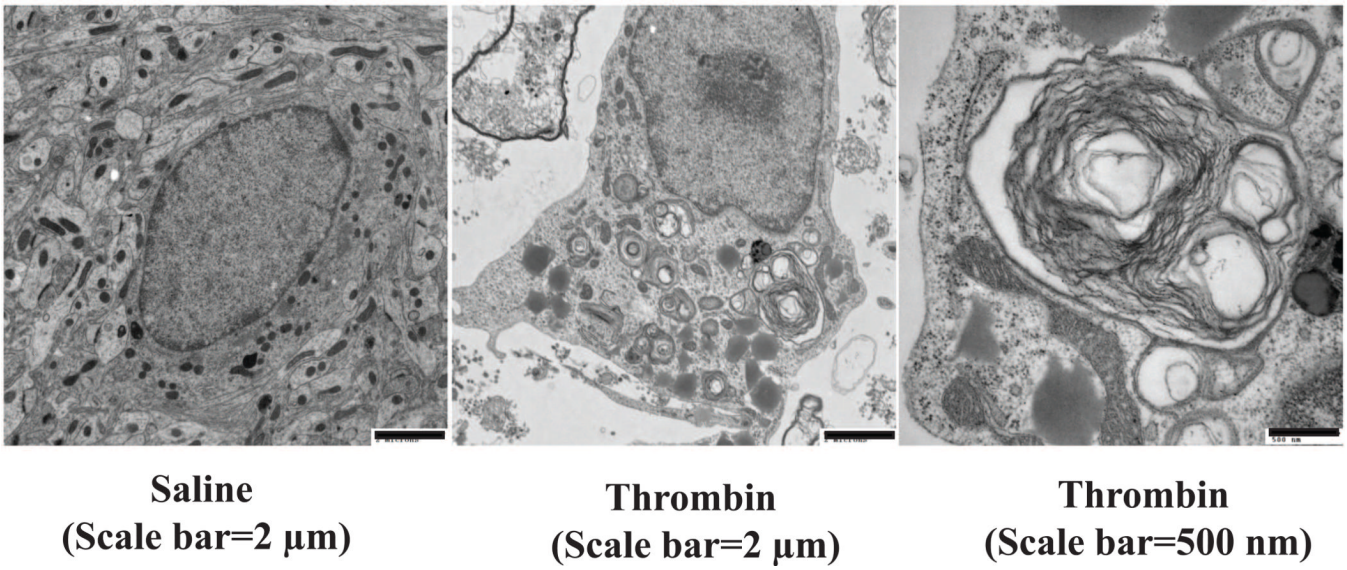

Figure 3.
Electron micrographs showing the ultrastructure of cells of the ipsilateral basal ganglia 3 days after intracaudate injection of either saline or thrombin. Note the presence of autophagosomes in the thrombin treated animals. Scale bar = 2 µm or 500 nm.

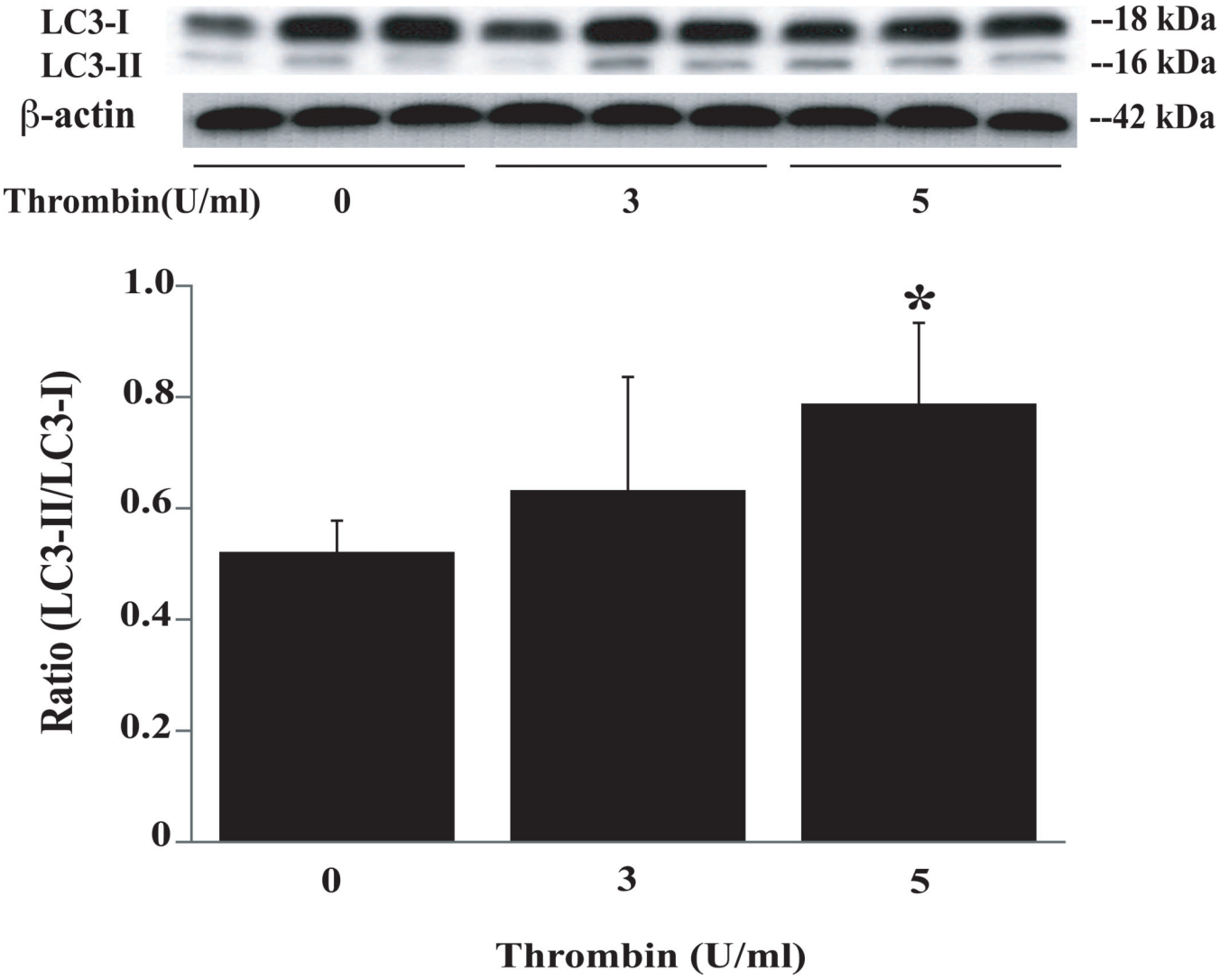

Figure 4.

Western blot analysis showing LC3-I (18 kDa) and LC3-II (16 kDa) levels in astrocytes 24 hours after vehicle or thrombin (3U/ml or 5U/ml) treatment. Values are mean ± S.D., *p<0.05 vs. vehicle-treated group (thrombin=0).

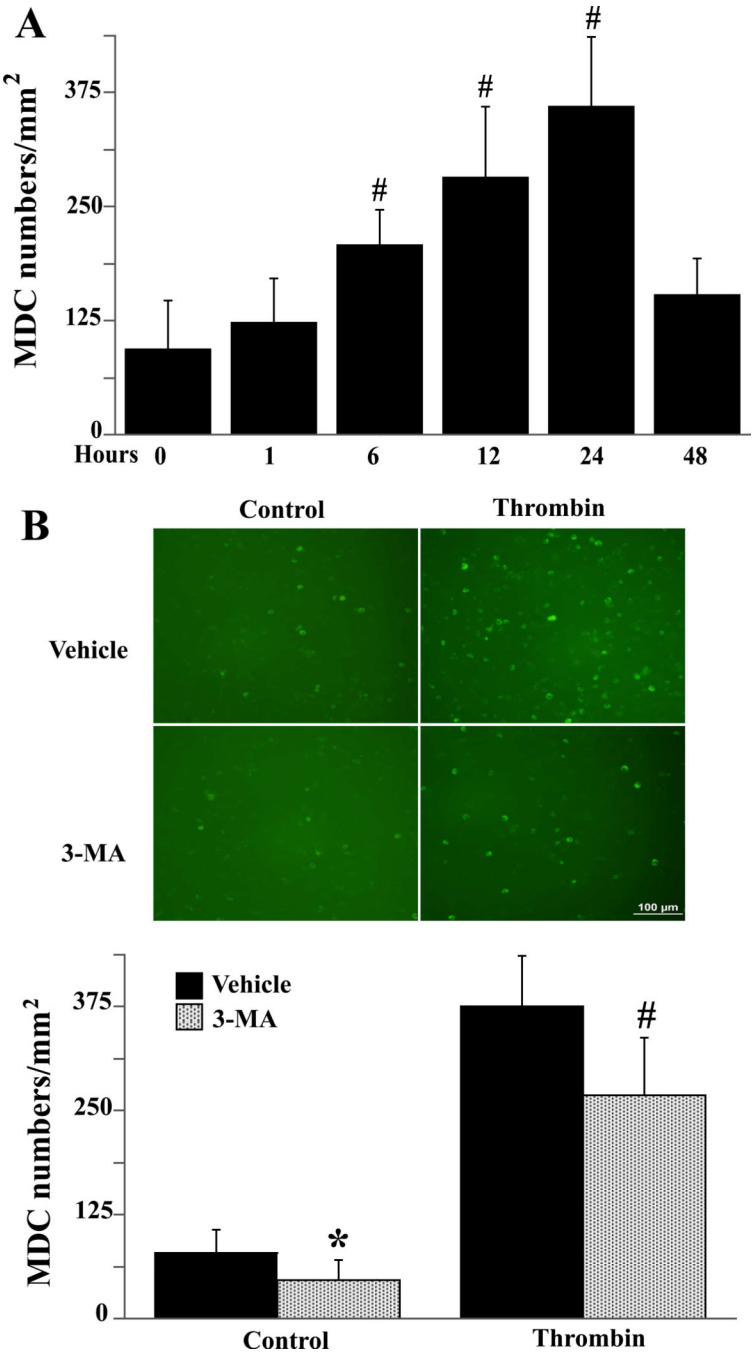

Figure 5.

(A) The number of MDC-positive vacuoles in astrocytes at different times after thrombin (5U/ml) treatment. Values are mean ± S.D., #p<0.01 vs. at hour 0. (B) The number of MDC-labeled vacuoles in astrocytes 24 hours after thrombin (5 U/ml) treatment with or without 3-MA (10 mM). Scale bar=100 μm. Values are mean ± S.D., #p<0.01, *p<0.05 vs. the vehicle-treated group.

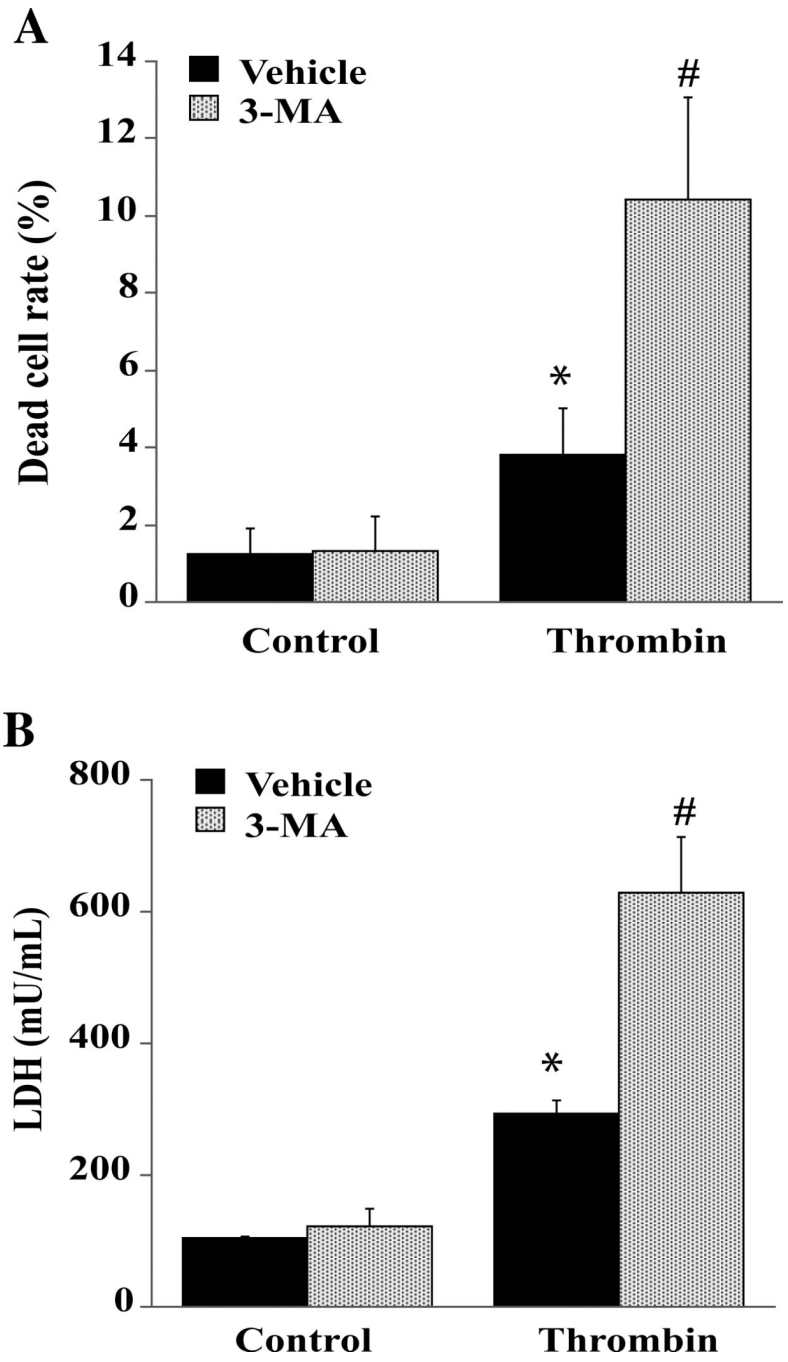

Figure 6.
Bar graphs showing dead cell rate (A) and LDH release (B) at 24 hours after thrombin treatment with or without 3-MA. Values are mean ± S.D., *p<0.05 vs. control, #p<0.01 vs. control and vehicle.