Stroke

BASIC AND TRANSLATIONAL SCIENCES

CAPN1 (Calpain1)-Mediated Impairment of Autophagic Flux Contributes to Cerebral Ischemia-Induced Neuronal Damage

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BACKGROUND AND PURPOSE: CAPN1 (calpain1)—an intracellular Ca^{2+}-regulated cysteine protease—can be activated under cerebral ischemia. However, the mechanisms by which CAPN1 activation promotes cerebral ischemic injury are not defined.

METHODS: In the present study, we used adeno-associated virus-mediated genetic knockdown and pharmacological blockade (MDL-28170) of CAPN1 to investigate the role of CAPN1 in the regulation of the autophagy-lysosomal pathway and neuronal damage in 2 models, rat permanent middle cerebral occlusion in vivo model and oxygen-glucose–deprived primary neuron in vitro model.

RESULTS: CAPN1 was activated in the cortex of permanent middle cerebral occlusion–operated rats and oxygen-glucose deprivation–exposed neurons. Genetic and pharmacological inhibition of CAPN1 significantly attenuated ischemia-induced lysosomal membrane permeabilization and subsequent accumulation of autophagic substrates in vivo and in vitro. Moreover, inhibition of CAPN1 increased autophagosome formation by decreasing the cleavage of the autophagy regulators BECN1 (Beclin1) and ATG (autophagy-related gene) 5. Importantly, the neuron-protective effect of MDL-28170 on ischemic insult was reversed by cotreatment with either class III-PI3K (phosphatidylinositol 3-kinase) inhibitor 3-methyladenine or lysosomal inhibitor chloroquine (chloroquine), suggesting that CAPN1 activation-mediated impairment of autophagic flux is crucial for cerebral ischemia-induced neuronal damage.

CONCLUSIONS: The present study demonstrates for the first time that ischemia-induced CAPN1 activation impairs lysosomal function and suppresses autophagosome formation, which contribute to the accumulation of substrates and aggravate the ischemia-induced neuronal cell damage. Our work highlights the vital role of CAPN1 in the regulation of cerebral ischemia–mediated autophagy-lysosomal pathway defects and neuronal damage.

GRAPHIC ABSTRACT: An online graphic abstract is available for this article.

Key Words: autophagy ■ glucose ■ ischemia ■ lysosomes ■ neurons

Autophagy is a regulated process that is activated to digest and recycle obsolete cellular constituents and damaged organelles.[1] Cerebral ischemia is usually associated with autophagy-lysosomal pathway (ALP) dysfunction.[2–6] Recently, we have shown that ALP function is impaired during cerebral ischemia, which is associated with permanent middle cerebral artery occlusion (pMCAO)–induced ischemic damage in rats.[7]

CAPN (calpain) can be activated by Ca^{2+} influx during cerebral ischemia.[8–10] The most abundant and

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Nonstandard Abbreviations and Acronyms

α-FBDP	alpha-fodrin breakdown product
3-MA	3-methyladenine
ALP	autophagy-lysosomal pathway
ATG	autophagy-related gene
BECN1	Beclin1
CAPN	calpain
CQ	chloroquine
CTSB	cathepsin B
CTSD	cathepsin D
CTSL	cathepsin L
LAMP	lysosomal membrane protein
LC3	microtubule-associated protein 1 light chain 3
LDH	lactate dehydrogenase
LMP	lysosomal membrane permeabilization
OGD	oxygen-glucose deprived
pMCAO	permanent middle cerebral artery occlusion
shCapn1	calpain1 shRNA
SQSTM1	sequestosome1

best-characterized brain calpains are the 2 major isoforms, CAPN1 (μ-calpain) and CAPN2 (m-calpain).[11] CAPN1 is activated by micromolar concentrations of Ca^{2+}, while the activation of CAPN2 usually requires millimolar Ca^{2+} concentrations.[12] CAPN1 is usually identified as the major form of CAPN in cerebral ischemia.[13–16] Activated CAPN1 has been shown to break down various cytoskeleton proteins,[17] enzymes,[18] and mitochondrial membrane proteins,[19] which may participate in ischemia-induced neuronal death. In addition, it has been shown that CAPN1 can modulate ALP function by cleaving autophagy-related LAMPs (lysosomal membrane proteins)[13,20–22] and ATG (autophagy-related gene) proteins.[23–26] Evidence has shown that lysosomal and autophagy defects caused by CAPN1-induced lysosomal membrane permeabilization (LMP) are closely associated with cell death in retinitis pigmentosa,[27] and CAPN1-mediated lysosomal dysfunction contributes to autophagy defects, which are responsible for glucose deprivation/glucose reintroduction–induced neuronal death.[21] Moreover, Russo et al[25] showed that CAPN1-mediated proteolytic cleavage of BECN1 (Beclin1; homologous to yeast ATG6) led to the dysregulation of autophagy in ischemia/reperfusion-induced retinal injury. In addition, it has been reported that retinal injury–induced CAPN1 activation can cause cleavage of ATG5 and impairment of autophagic flux, subsequently promoting cell death in photoreceptor cells.[26]

In the present study, we investigated the role of CAPN1 in regulating ALP function and its contribution to the ischemic damage in pMCAO-operated rats and oxygen-glucose–deprived (OGD) neurons. Here, we demonstrate that genetic knockdown or pharmacological blockade of CAPN1 not only improves lysosomal function but also promotes the formation of autophagosomes. These changes accelerate the enclosure and degradation of substrates, thus contributing to the amelioration of ischemia-induced neuronal damage. The present study provides a mechanistic link between CAPN1 and impaired ALP function and identifies CAPN1 as a promising molecular target for ischemic stroke therapies.

METHODS

The present study adheres to the American Heart Association journals' implementation of the Transparency and Opennesss Promotion Guideline (available online at https://www.ahajournals.org/top-guidelines); the authors declare that all supporting data are available within the article and in the Data Supplement. Full descriptions of Materials and Methods are available online.

Animals

All animal procedures complied with the international standards stated in the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Shenyang Medical College.

pMCAO Animal Model

Male Sprague-Dawley rats were anesthetized with isoflurane-O_2. A 4-0 nylon suture (Φ0.26 mm) was inserted from the right common carotid artery incision to the circle of Willis to occlude the origin of the right middle cerebral artery. Sham rats underwent the same surgical procedure without artery occlusion.

Statistical Analysis

Statistical analysis was performed using the SPSS 19.0 software (SPSS, Inc, Chicago, IL). All experimental data were expressed as mean±SD. The data from Western blot and immune-staining experiments were analyzed with 1- or 2-way ANOVA, followed by Tukey test. The data for the brain water content and infarct area were analyzed using 1-way ANOVA with Tukey test. Modified Neurological Severity Scores were analyzed with Kruskal-Wallis test followed by the Dunn method. The values were considered significant when P was <0.05.

RESULTS

CAPN1 Is Activated in Cerebral Ischemia In Vivo and In Vitro

We measured cerebral blood flow in the ipsilateral brain of the rats before and at 30 minutes and 24 hours after pMCAO by laser speckle imaging system. As shown in Figure IA in the Data Supplement, the cerebral blood flow in the ipsilateral hemisphere of rats at 30 minutes and 24 hours after pMCAO reduced by ≈70% to 80% and 50% to 60%, respectively, confirming that all animals in

the pMCAO group were satisfied with the criteria of the pMCAO model. During CAPN1 activation, a 80-kDa subunit is converted to a 76-kDa enzymatically active form—a process known as autolysis. Activated CAPN1 can cleave α-fodrin to generate 2 α-FBDPs (alpha-fodrin breakdown products) molecules with 150/145 kDa, which are considered as markers for CAPN activation.[21,28] We generated an adeno-associated virus (AAV) delivery system encoding GFP (green fluorescent protein) and a CAPN1 shRNA (sh*Capn1*) to knock down CAPN1 expression (Figure IB in the Data Supplement). As shown in Figure IC in the Data Supplement, the autolysis of CAPN1 (76 kDa) and the generation of the α-FBDPs (150/145 kDa) were significantly increased at 24 hours in the cortex of sh*Scr*-injected pMCAO rats (all $P<0.001$) compared with sh*Scr*-injected sham rats. Furthermore, the expressions of autolyzed CAPN1 and α-FBDPs (150/145 kDa) were markedly reduced in sh*Capn1*-treated pMCAO rats compared with sh*Scr*-treated pMCAO rats (all $P<0.001$), indicating that sh*Capn1* can specifically target and inhibit pMCAO-induced CAPN1 activation. Next, we determined a time course of α-FBDP expression in the primary neurons exposed to OGD for 1 to 24 hours. As shown in Figure ID in the Data Supplement, the level of α-FBDPs was increased in OGD-exposed primary neurons in a time-dependent manner ($P<0.05$), and this increase was inhibited by MDL-28170 (MDL; 5 μM; $P<0.001$). MDL is a widely used CAPN1 inhibitor.[21,29–31] We performed a dose-response experiment to confirm that MDL at a dose of 1 mg/kg had a maximal inhibition of CAPN1 expression in the rats in vivo ($P<0.05$; Figure II in the Data Supplement). Consistent with the findings in sh*Capn1*-treated rats, MDL significantly inhibited CAPN1 autolysis ($P<0.001$) and α-fodrin cleavage products at 150/145 kDa ($P<0.001$; Figure IIIA through IIIC in the Data Supplement). It is worth noting that α-fodrin can also be cleaved by caspase to yield fragments of 150 kD and 120 kDa.[28] To exclude the caspase-specific cleavage of α-fodrin, the level of α-FBDP at 120 kDa was examined; neither cerebral ischemia in vivo (pMCAO-operated rats) nor in vitro (OGD-exposed neurons) affected the level of α-FBDP at 120 kDa ($P>0.05$; Figures IC, ID, and IIID in the Data Supplement). These results indicate that CAPN1 is activated in cerebral ischemia.[9,32,33]

Cerebral Ischemia–Induced CAPN1 Activation Disrupted Lysosomal Integrity and Induced LMP

To investigate the role of CAPN1 in the regulation of cerebral ischemia–induced LMP, lysosomes and cytosolic fractions were isolated from the cortex of Sh*Capn1*- and Sh*Scr*-treated pMCAO rats. The results showed that the levels of LAMP1, LAMP2, and the cysteine CTSB (cathepsin B), CTSD (cathepsin D), and CTSL (cathepsin L) were decreased in the lysosomes and increased in the cytosolic compartments of pMCAO rats (Figure 1A). Interestingly, genetic knockdown of CAPN1 by sh*Capn1* reversed the cerebral ischemia–induced changes in the protein expressions and the cellular distributions of all these lysosome-related proteins (Figure 1A). Immunofluorescence staining revealed that CTSB (red) mainly colocalized with LAMP1 (gray) in the GFP-positive cells from sham/Sh*Scr* rats, and this colocalization was markedly reduced in pMCAO-operated rats, as shown by a decreased Manders coefficient ($P<0.01$; Figure 1B and 1C). Knockdown of CAPN1 restored the colocalization of CTSB with LAMP1 in GFP-positive cells from pMCAO/sh*Capn1* rats (white arrows, $P<0.05$, Figure 1B and 1C). Likewise, the pharmacological inhibition of CAPN1 by MDL also prevented the decrease of LAMP1 expression ($P<0.01$) induced by pMCAO (Figure IVA through IVC in the Data Supplement). Consistent with the in vivo results, MDL significantly reduced CTSB leakage from lysosomes and increased its colocalization with LAMP1 (white arrowheads), as compared with the OGD-treated group (white arrows, all $P<0.001$, Figure VA and VB in the Data Supplement). In addition, we used acridine orange to further evaluate the role of CAPN1 in regulation of LMP.[34] As shown in Figure VI in the Data Supplement, there was intense red fluorescence but weak green fluorescence in the perinuclear regions of control neurons. In OGD-exposed neurons, the green fluorescence intensity was gradually increased, accompanied by a decreased red fluorescence intensity ($P<0.001$; Figure VIA through VIC in the Data Supplement). Interestingly, all of these phenomena were partially reversed by MDL treatment ($P<0.01$; Figure VIA through VIC in the Data Supplement). These results suggest that CAPN1 activation may be important for the destabilization of lysosome membrane and LMP during ischemic injury.

Cerebral Ischemia–Induced CAPN1 Activation Contributed to the Accumulation of Autophagic Substrates

We examined the expression of 2 autophagic substrates, SQSTM1 (sequestosome1; soluble and insoluble forms), and ubiquitinated proteins in vivo and in vitro. Cerebral ischemia significantly decreased the expression of soluble SQSTM1 ($P<0.001$) and increased the expression of insoluble SQSTM1 ($P<0.001$) and ubiquitinated proteins in pMCAO-operated rats ($P<0.05$; Figures 2A, 3A, and 3B). Inhibition of CAPN1 by genetic knockdown with Sh*Capn1* or pharmacological inhibitor MDL markedly decreased the accumulation of insoluble SQSTM1 ($P<0.001$) and ubiquitinated proteins ($P<0.001$) and increased the expression of soluble SQSTM1 ($P<0.01$; Figures 2A, 3A, and 3B). In OGD-exposed primary neurons, there were time-dependent decreases in the levels of soluble SQSTM1 ($P<0.01$) and increases in the levels of insoluble SQSTM1 ($P<0.05$) and ubiquitinated proteins ($P<0.001$; Figure 4A and 4B). Treatment with MDL significantly reversed the changes in the protein expressions of soluble and insoluble SQSTM1

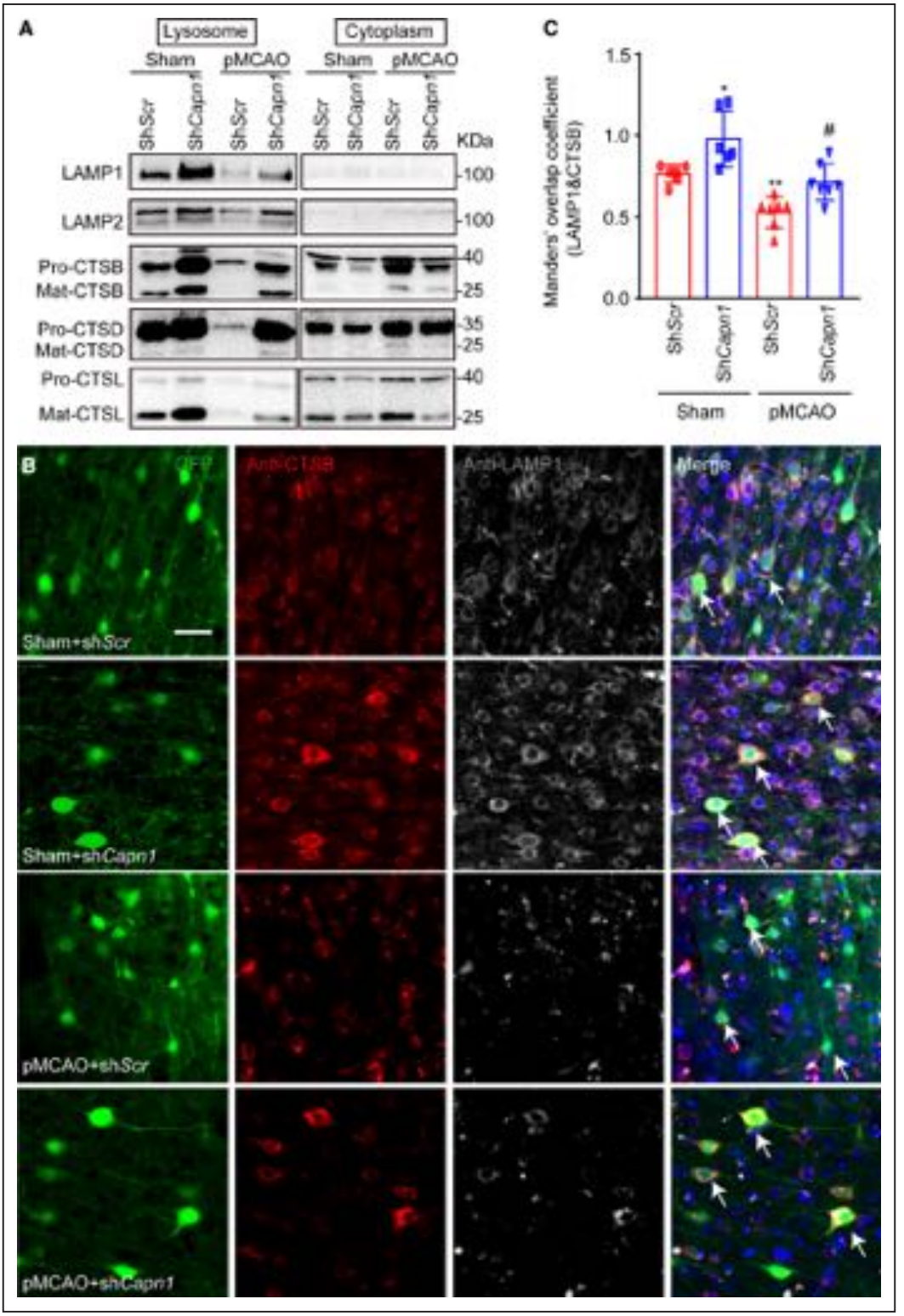

Figure 1. Knockdown of CAPN1 (calpain1) attenuated lysosomal membrane permeabilization and preserved lysosomal integrity in permanent middle cerebral artery occlusion (pMCAO)–operated rats.

The pMCAO- or sham-operated rats were euthanized at 24 h after surgery; brain tissues were collected. Lysosomes and cytosolic fractions were isolated from the ipsilateral cortex of rats injected with adeno-associated virus carrying sh*Scr* or sh*Capn1* vectors. **A**, Representative immunoblot images of LAMP (lysosomal membrane protein) 1 and LAMP2, and the precursor (Pro) and mature (Mat) forms of CTSB (cathepsin B), CTSD (cathepsin D), and CTSL (cathepsin L) in the lysosome and cytoplasm fractions (n=6). **B**, The representative immunofluorescence images of the colocalization of LAMP1 (gray) with CTSB (red) in the cortex of sham-operated or pMCAO-treated rats. The nuclei were stained with DAPI (blue). Scale bar=20 μm. **C**, Quantitative analysis of colocalization of LAMP1 and CTSB as indicated by Manders overlap coefficient. Data were presented as mean±SD. ***P*<0.01, **P*<0.05 versus sham+shScr group; #*P*<0.05 versus pMCAO+shScr group. n=6 to 7. GFP indicates green fluorescent protein.

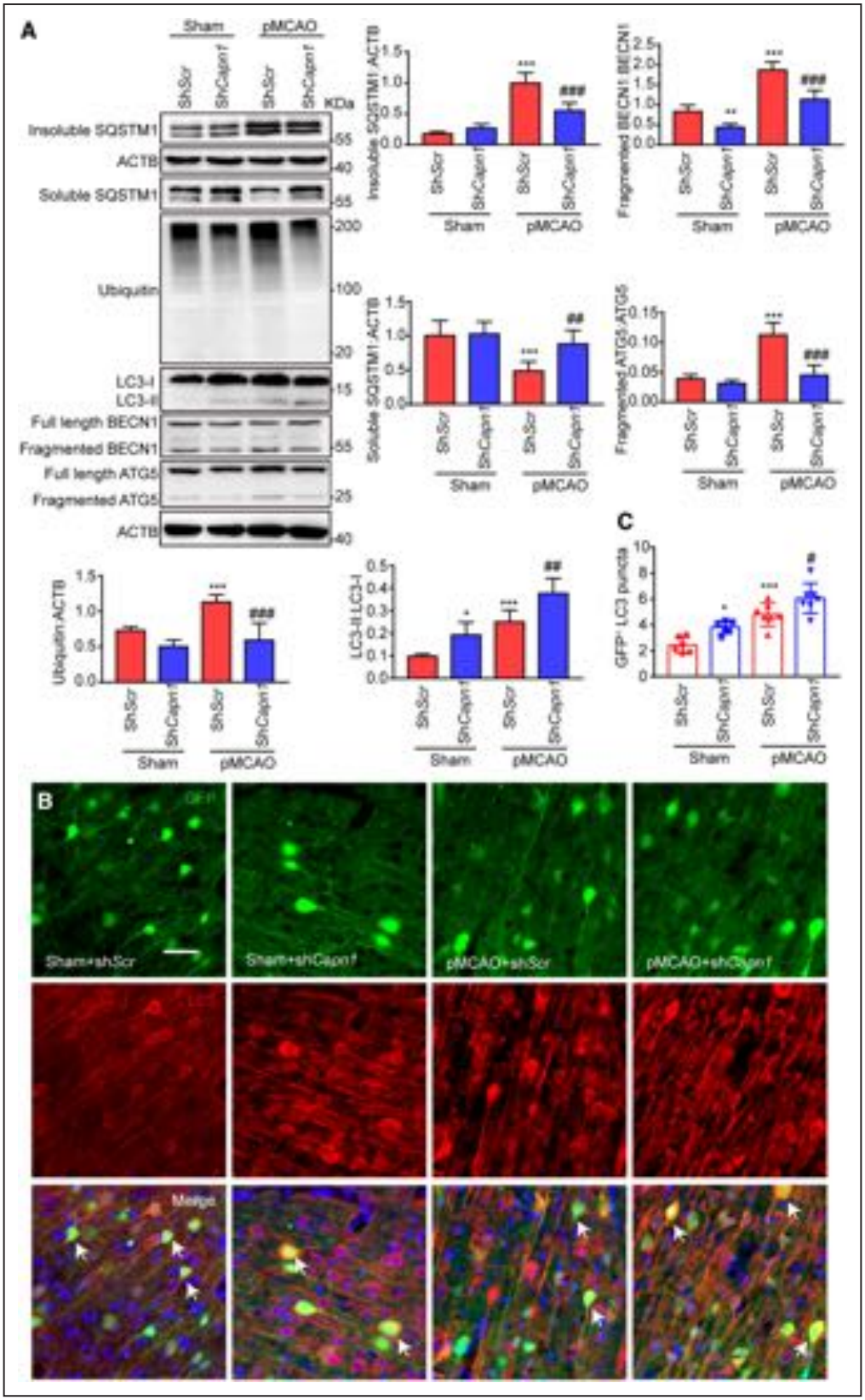

Figure 2. Knockdown of CAPN1 (calpain1) by sh*Capn1* increased autophagosome formation and accelerated substrate degradation in permanent middle cerebral artery occlusion (pMCAO)–operated rats.

The pMCAO- or sham-operated rats were euthanized at 24 h after surgery, and brain tissues were collected. **A**, Representative immunoblot and quantitative analysis of Triton-X 100–soluble SQSTM1 (sequestosome1), Triton-X100–insoluble SQSTM1, ubiquitinated proteins (ubiquitin), LC3 (microtubule-associated protein 1 light chain 3)-I (16 kDa), LC3-II (14 kDa), full-length BECN1 (Beclin1; 60 kDa), (*Continued*)

in OGD-treated primary neurons ($P<0.05$; Figure 4A and 4B). Immunostaining analysis showed that MDL could partially reverse the ischemia-induced downregulation of SQSTM1 fluorescence signals in OGD-exposed neurons ($P<0.05$; Figure VIIA and VIIB in the Data Supplement) and pMCAO-treated rats ($P<0.01$; Figure VIIC and VIID in the Data Supplement).

Next, we examined autophagosomes formation in vivo and in vitro under the condition of CAPN1 inhibition. Inhibition of CAPN1 by either sh*Capn1* ($P<0.01$; Figure 2A) or MDL ($P<0.001$; Figure 3A and 3B) further increased the ratio of LC3 (microtubule-associated protein 1 light chain 3)-II/ LC3-I in pMCAO-operated rats. Consistent with the findings from immunoblotting, fluorescence staining revealed that the LC3 signals were low and diffuse in the cytoplasm of GFP-positive cells from sham/ sh*Scr* rats but became punctate and enhanced in the cortex of pMCAO-operated rats ($P<0.001$; Figure 2B and 2C). Likewise, knockdown of CAPN1 by Sh*Capn1* further increased the punctate LC3 fluorescence signals in the GFP-positive cells from both sham- and pMCAO-operated rats (white arrows, $P<0.05$, Figure 2B and 2C). Similarly, OGD-exposed neurons also showed a time-dependent increase in the LC3-II/LC3-I ratio ($P<0.001$; Figure 4A and 4B), and this was further increased by MDL treatment ($P<0.05$; Figure 4A and 4B). These results suggest that the inhibition of CAPN1 activation can promote autophagosome accumulation.

CAPN1 has been demonstrated to cleave ATG proteins into fragments.[23,35] Therefore, we further examined the cleavage of BECN1 (homologous to yeast ATG6) and ATG5 in the pMCAO and OGD models. The results showed that the cleavage of BECN1 ($P<0.001$) and ATG5 ($P<0.001$) was significantly increased in the cerebral cortex of pMCAO-operated rats; this increase was ameliorated by Sh*Capn1* ($P<0.001$; Figure 2A) or MDL treatment ($P<0.001$; Figure 3A and 3B). In addition, a similar change in the cleavage of BECN1 and ATG5 was also observed in OGD-exposed primary neurons ($P<0.05$; Figure 4A and 4B). These results provide the evidence that cerebral ischemia–activated CAPN1 may participate in the suppression of autophagosome formation.

Cerebral Ischemia–Induced CAPN1 Activation Impaired Autophagic Flux

To further identify the role of ischemia-induced CAPN1 activation in regulating the autophagic flux, autophagosomes and substrates were examined in the presence of the lysosomal inhibitors bafilomycin A1 (BafA1; 200 nmol/L)[36] or CQ (chloroquine; 10 μM)[7] in OGD-exposed primary neurons. Immunoblot and immunofluorescence analyses showed that BafA1 or CQ treatment alone significantly upregulated the LC3-II/LC3-I ratio ($P<0.05$; Figure 5A and 5B) and punctate LC3 signals in the control neurons ($P<0.001$; Figure 5C and 5D), suggesting that autophagic flux is intact. We observed that MDL in the presence of BafA1 or CQ further upregulated the LC3-II/LC3-I ratio ($P<0.01$; Figure 5A and 5B); OGD exposure for 12 hours increased the punctate LC3 level ($P<0.01$), but this increased LC3 level was not further affected by BafA1 or CQ treatment (Figure 5C and 5D). These results suggest that inhibition of CAPN1 increased autophagosome formation under normal circumstances and that autophagic flux was significantly impaired in OGD-exposed neurons. Interestingly, treatment with MDL significantly increased the LC3-II/LC3-I ratio (all $P<0.05$; Figure 5A and 5B) and punctate LC3 level ($P<0.01$; Figure 5C and 5D) in the OGD-exposed neurons, and the LC3 level was further upregulated after cotreatment with CQ ($P<0.05$; Figure 5B through 5D). These results suggest that CAPN1 activation may contribute to an abnormal suppression of autophagosome formation in OGD-exposed neurons. Furthermore, MDL facilitated substrate digestion, as indicated by the downregulation of insoluble SQSTM1 and ubiquitinated proteins in OGD-exposed primary neurons (all $P<0.01$), and this effect was reversed by BafA1 treatment (all $P<0.05$; Figure 5A). These data suggest that inhibition of CPAN1 alleviates the autophagic flux, promoting the digestion of autophagic substrates.

CAPN1 Activation Promotes Ischemic Cerebral Injury via the Impairment of Autophagic Flux

It has been shown that CAPN1 inhibition reduces cerebral ischemic injury.[37,38] We investigated the effects of MDL on ischemic insult and neuron survival in pMCAO rats in the presence of 2 different autophagy inhibitors, CQ (60 mg/kg, intraperitoneally)[7,39] or 3-methyladenine (3-MA; 600 nmol, intracerebroventricularly).[3] As shown in Figure 6A, CQ significantly increased the ratio of LC3-II/LC3-I ($P<0.001$), indicating its inhibition of lysosome function, while 3-MA significantly decreased the ratio of LC3-II/LC3-I ($P<0.01$), supporting its inhibition of autophagosome formation. As shown in Figure 6B through 6D, pMCAO rats significantly increased the infarct volume (49.5±3.40%; $P<0.001$), brain

Figure 2 Continued. fragmented BECN1 (50 kDa), full-length ATG (autophagy-related gene) 5 (35 kDa), and fragmented ATG5 (24 kDa) levels in the cortex of rats injected with shScr or sh*Capn1* adeno-associated virus (AAV) vectors. ACTB (β-actin) was used as a loading control. Data were presented as mean±SD. ***$P<0.001$, **$P<0.01$, *$P<0.05$ versus sham+shScr group; ###$P<0.001$, ##$P<0.01$ versus pMCAO+shScr group. n=6. B, Representative immunofluorescence images of the colocalization of LC3-positive (red) and GFP (green fluorescent protein)-labeled shScr or shCapn1 (green) cells. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bar=20 μm. **C**, Quantitative analysis of GFP+ LC3 puncta in the cortex of sham- or pMCAO-operated rats injected with shScr or shCapn1 vectors. Data were presented as mean±SD. ***$P<0.001$, *$P<0.05$ versus sham+shScr group. #$P<0.05$ versus pMCAO+shScr group. n=6 to 7.

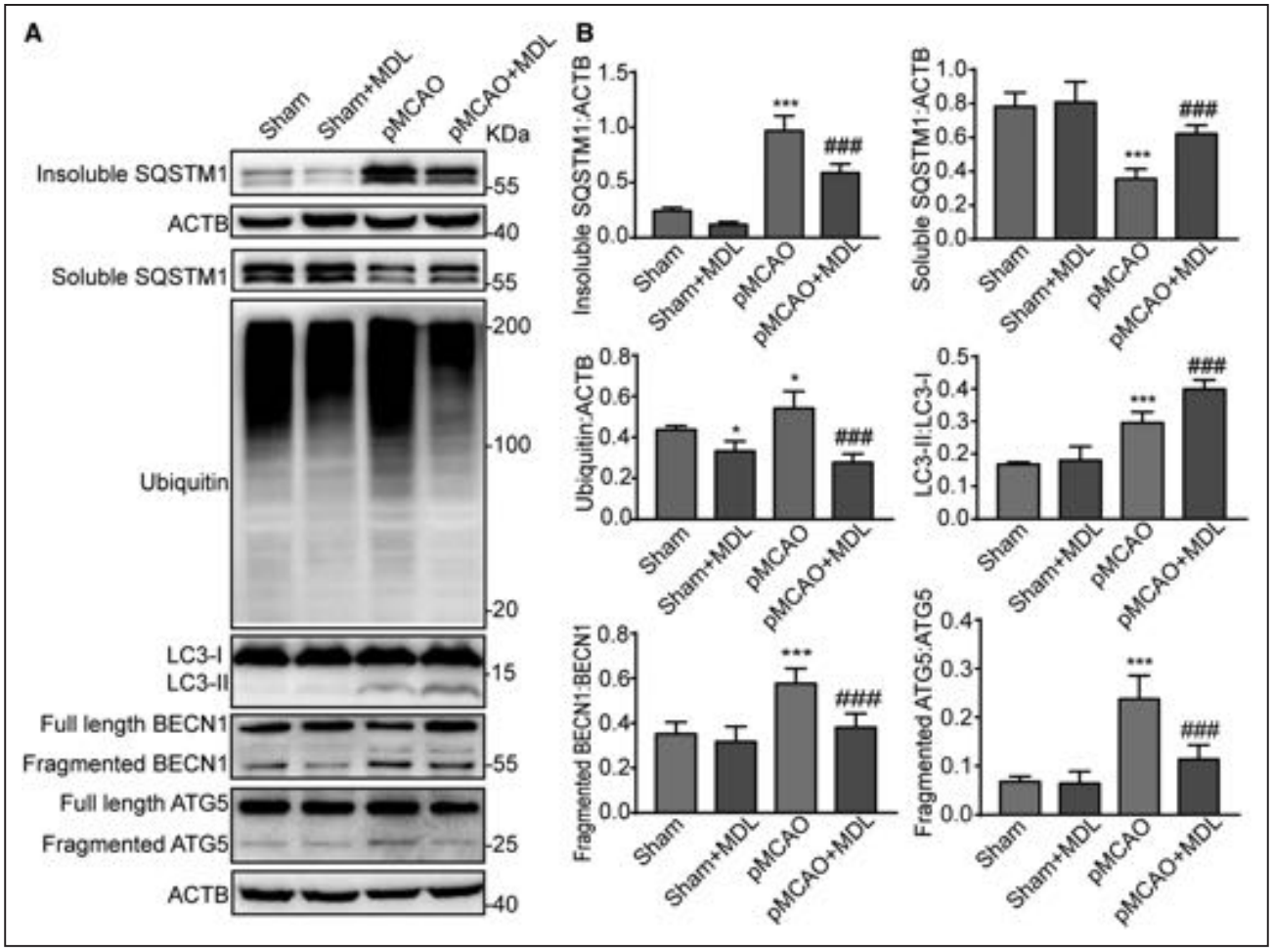

Figure 3. Inhibition of CAPN1 (calpain1) by MDL increased the formation of autophagosomes and the degradation of autophagy substrates in permanent middle cerebral artery occlusion (pMCAO)–operated rats.
The pMCAO- or sham-operated rats were euthanized at 24 h after surgery, and brain tissues were collected. **A**, Representative immunoblot of Triton-X 100–soluble SQSTM1 (sequestosome1), Triton-X100–insoluble SQSTM1, ubiquitinated proteins (ubiquitin), LC3 (microtubule-associated protein 1 light chain 3)-I (16 kDa), LC3-II (14 kDa), full-length BECN1 (Beclin1; 60 kDa), fragmented BECN1 (50 kDa), full-length ATG (autophagy-related gene) 5 (35 kDa), and fragmented ATG5 (24 kDa) in the cerebral cortex of pMCAO-operated rats in the absence and presence of MDL-28170 (MDL). **B**, Quantitative assessment of the levels of the abovementioned proteins. ACTB (β-actin) was used as a loading control. Data were expressed as mean±SD. ***P<0.001, *P<0.05 versus sham group; ###P<0.001 versus pMCAO group. n=6.

water content (82.2±0.58%; P<0.001), and neurological scores (12.0±0.89; P<0.001), and these effects were reversed by MDL treatment (infarct volume, 16.13±4.68%; brain water content, 78.99±0.67%; neurological scores, 3.286±0.49; all P<0.001). Furthermore, inhibition of autophagy-lysosomal function by either CQ or 3-MA abolished these beneficial effects of MDL on ischemic insult (all P<0.05; Figure 6B through 6D). We also used NeuN and Fluoro-Jade B to label the surviving and degenerating neurons, respectively. Confocal fluorescence analysis revealed that pMCAO caused a significant decrease in the number of NeuN-positive neurons and a significant increase in the number of Fluoro-Jade B–positive neurons in the ischemic cerebral tissues (P<0.001; Figure VIIIA through VIIID). These changes were ameliorated by MDL (P<0.05), but the beneficial effect of MDL was abolished by either cotreatment with CQ (P<0.01) or 3-MA (P<0.05; Figure VIIIA through VIIID). Moreover, effect of CQ or 3-MA on the ratio of LC3-II/LC3-I in the OGD-exposed neurons was similar to that in pMCAO rats (all P<0.01; Figure IXA and IXB in the Data Supplement). OGD exposure significantly decreased neuronal survival, as demonstrated by a reduction in neuron viability (MTT assay; P<0.001) and an increase in LDH (lactate dehydrogenase) leakage (LDH assays; P<0.001). Treatment with MDL prevented OGD-induced changes in neuron viability and LDH leakage, suggesting that MDL has a neuroprotective effect (P<0.05; Figure IXC and IXD in the Data Supplement). Likewise, the neuroprotective effects of MDL were abolished by CQ or 3-MA treatment during OGD exposure (P<0.05; Figure IXC and IXD in the Data Supplement). These results strongly suggest that CAPN1 activation–mediated impairment of autophagic flux may be responsible for cerebral ischemia–induced neuronal cell damage.

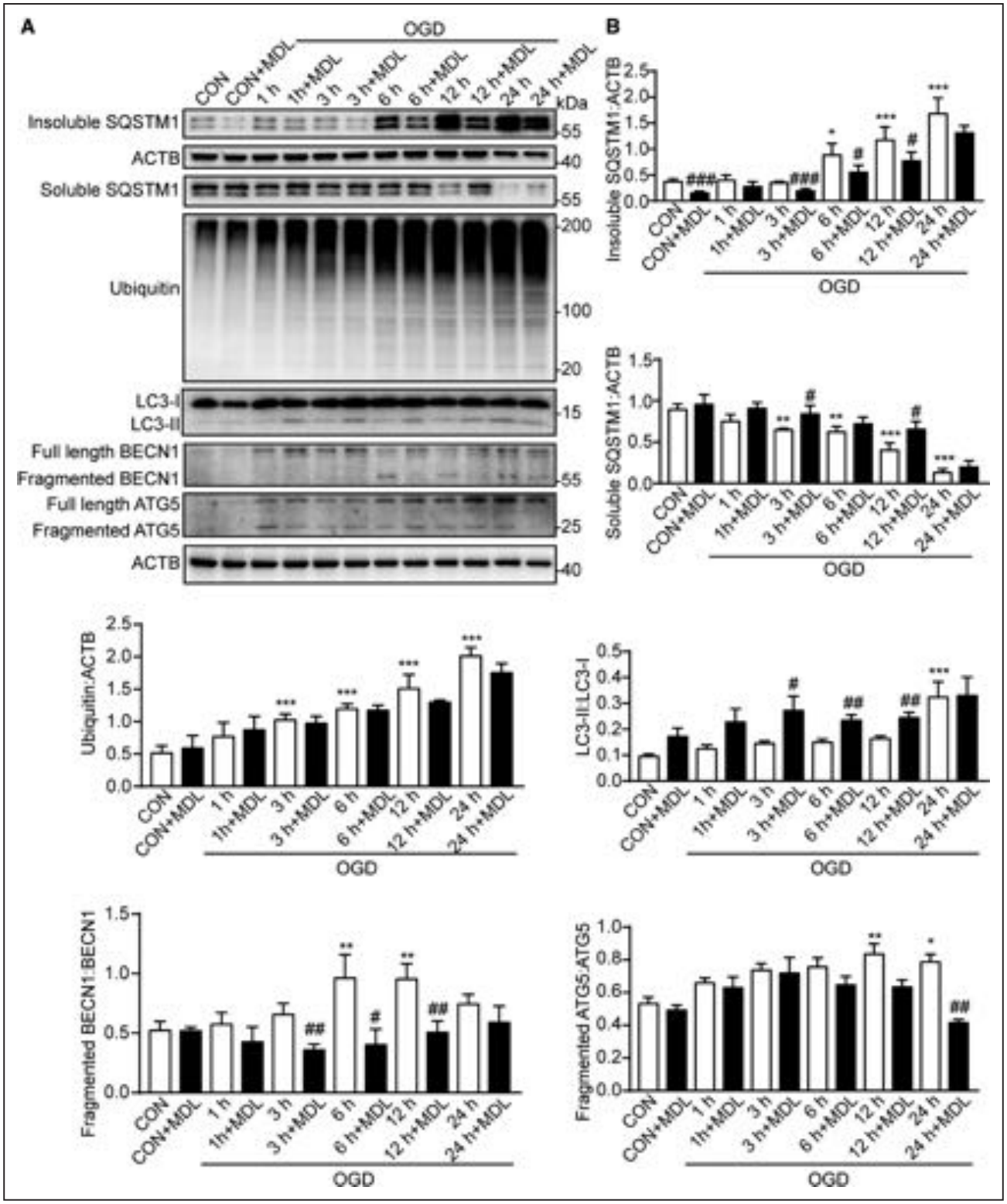

Figure 4. Inhibition of CAPN1 (calpain1) by MDL increased autophagosome formation and decreased the accumulation of autophagy substrates in oxygen-glucose deprivation (OGD)–exposed neurons.

Primary neurons were subjected to OGD for 1, 3, 6, 12, and 24 h. MDL (5 μM) was added to the culture media at the onset of OGD. **A**, Representative immunoblot of Triton-X 100–soluble SQSTM1 (sequestosome1), Triton-X 100–insoluble SQSTM1, ubiquitinated proteins (ubiquitin), LC3 (microtubule-associated protein 1 light chain 3)-I (16 kDa), LC3-II (14 kDa), full-length BECN1 (Beclin1; 60 kDa), fragmented BECN1 (50 kDa), full-length ATG (autophagy-related gene) 5 (35 kDa), and fragmented ATG5 (24 kDa) levels in control (CON) and OGD-exposed primary neurons in the absence and presence of MDL-28170 (MDL). **B**, Quantitative analysis of the levels of the abovementioned proteins. ACTB (β-actin) was used as a loading control. Data were presented as mean±SD. ***P<0.001, **P<0.01, *P<0.05 versus CON group; ##P<0.01, #P<0.05 versus OGD group at the corresponding time points. n=3.

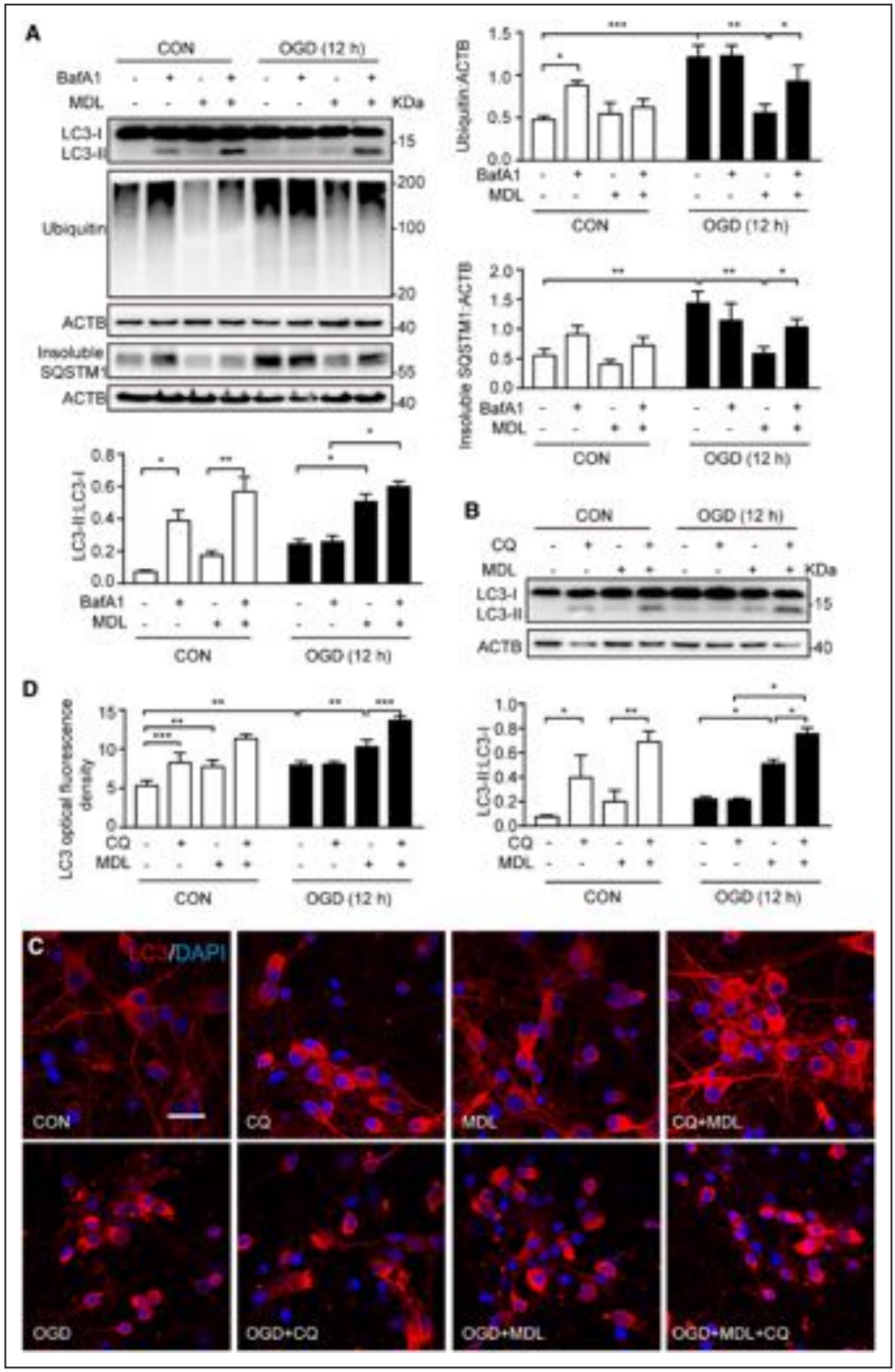

Figure 5. Inhibition of CAPN1 (calpain1) by MDL ameliorated cerebral ischemia–induced autophagic flux defects.
A, Representative immunoblot images and quantitative analysis of LC3 (microtubule-associated protein 1 light chain 3)-1 and LC3-II, insoluble SQSTM1 (sequestosome1), and ubiquitinated proteins (ubiquitin) in primary neurons subjected to oxygen-glucose deprivation (OGD) treatment for 12 h in the presence or absence of the lysosomal inhibitor bafilomycin A1 (BafA1; 200 nmol/L) with or without the CAPN1 inhibitor MDL-28170 (MDL; 5 μM). ACTB (β-actin) was used as a loading control. Data were expressed as mean±SD. ***P<0.001, **P<0.01, *P<0.05 versus the indicated group. n=3. B, Representative immunoblot images and quantitative analysis of LC3-I and LC3-II in primary neural cells subjected to OGD treatment for 12 h in the presence or absence of the lysosomal inhibitor CQ (chloroquine; 10 μM) with or without MDL (5 μM). Data were expressed as mean±SD. **P<0.01, *P<0.05 versus the indicated group. n=3. C, Representative immunofluorescence images of autophagosomes (LC3-positive cells, red). The primary neurons were subjected to OGD for 12 h in the presence of the lysosomal inhibitor CQ (10 μM) with or without MDL treatment. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bar=20 μm. D, Quantitative analysis of fluorescence intensity of LC3-positive puncta. Data were expressed as mean±SD. ***P<0.001, **P<0.01 versus the indicated group. n=6.

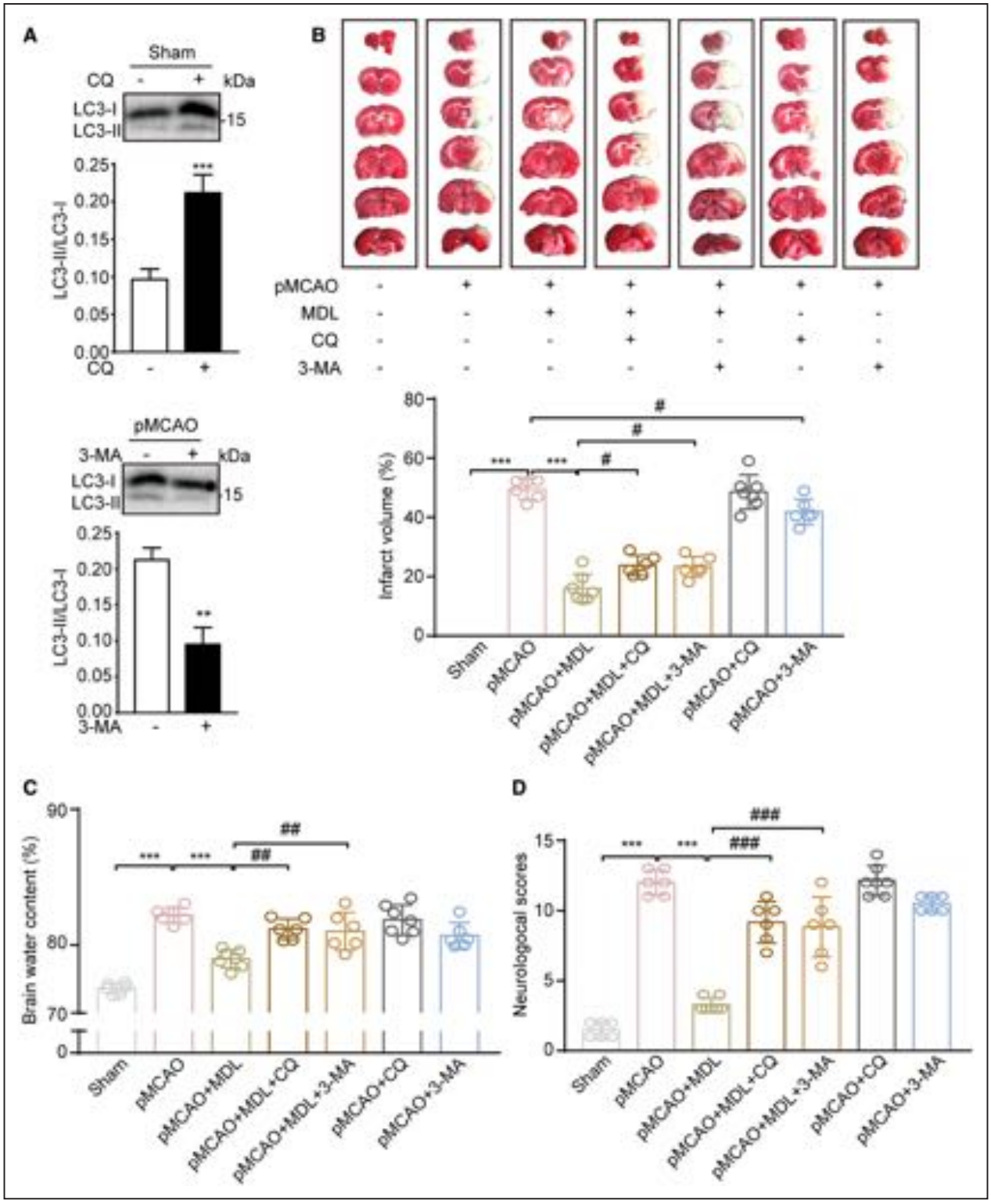

Figure 6. Inhibition of CAPN1 (calpain1) by MDL-28170 (MDL) alleviated ischemic injury by ameliorating autophagic flux defects in permanent middle cerebral artery occlusion (pMCAO)–operated rats.

The pMCAO- or sham-operated rats were euthanized at 24 h after surgery, and brain tissues were collected. **A**, The ratio of LC3 (microtubule-associated protein 1 light chain 3)-II/LC3-I in the cortex of sham rats treated with the lysosome inhibitor CQ (chloroquine; 60 mg/kg, intraperitoneally) and in the cortex of pMCAO rats treated with the class III PI3K (phosphatidylinositol 3-kinase) inhibitor 3-methyladenine (3-MA; 600 nmol, intracerebroventricularly). Data were expressed as mean±SD. ***P<0.001 versus non-CQ group; **P<0.01 versus non–3-MA group. n=4. B, Representative images of 2,3,5-triphenyltetrazolium chloride–stained brain slices for the evaluation of cerebral infarct volume in pMCAO rats treated with MDL plus autophagy inhibitors CQ or 3-MA. Quantification of infarct volume (B), brain water content (C), and neurological scores (D) in pMCAO rats treated with MDL in the presence of CQ or 3-MA. Data were expressed as mean±SD. ***P<0.001 versus pMCAO group; ###P<0.01, ##P<0.01, #P<0.05 versus pMCAO+MDL group. n=6 to 7.

DISCUSSION

It has been shown that CAPN activation can disrupt survival signals including cleavage of cytoskeletal, mitochondrial, and lysosomal membrane proteins, which may cause necrotic-like cell death and apoptosis. Yamashima et al[8,9,32,40] proposed that cerebral ischemia–induced activation of CAPN may promote LMP and the release of cathepsins from lysosomes into the cytoplasm, leading to cathepsin-dependent apoptosis. In addition, it has been reported that the majority of human ATG proteins can be cleaved by CAPN1.[23] Functional ATG proteins and intact lysosomal membranes are both essential for the function of ALP. In the present study, we present the first evidence that CAPN1 activation–mediated impairment of autophagic flux plays an important role in cerebral ischemia–induced neuronal damage.

Until now, the dynamic change of autophagic flux and its role in ischemic injury is still controversial.[3,41] Several studies indicate that ischemic stroke–induced autophagic flux is responsible for the cerebral ischemia–induced cell damage.[3,6] Recently, we have shown that both pMCAO and OGD could cause a dynamic change in autophagic flux during the first 24 hours of ischemia[7] and that CAPN1 activity was time dependently increased with a peak at 24 hours following cerebral ischemia.[42] Therefore, in the present study, we performed a time course (1–24 hours) of in vitro and 24 hours of in vivo experiments to further investigate the role of CAPN1 in regulating the function of ALP. We showed that CAPN1 activation after cerebral ischemia impaired lysosomal function, which reduced the ability to degrade the cargo delivered from damaged organelles. Moreover, activated CAPN1 directly cleaved autophagy-related proteins including ATG5 and BECN1, which may impair the enclosure of cargo within autophagic vesicles due to the suppression of autophagosome formation. The combination of suppressed autophagosome formation and impaired lysosomal degradation together led to the accumulation of substrates, which resulted in the ischemia-mediated neuronal damage.

Notably, although ubiquitinated proteins and insoluble forms of SQSTM1/p62 were accumulated following ischemic stroke, the opposite trend was observed for the soluble form of p62. MDL treatment reduced the level of insoluble p62 while increasing the level of soluble p62. Furthermore, we observed that the fluorescence signals of p62 (reflecting both soluble and insoluble p62 forms) were reduced after ischemic stroke; this reduction was reversed by MDL treatment. These results seem somewhat contradictory. To explain the results, we surmise that cerebral ischemia induces activation of CAPN1, which impairs autophagic flux, resulting in accumulation of insoluble p62. Meanwhile, activation of CAPN1 may also directly degrade p62, since it has been reported that p62 is also a substrate of CAPN1.[1,23] Therefore, it is possible that CAPN1 activation-induced p62 breakdown is more efficient than it impairs lysosome digestion, which can be reflected by the reduction in total p62 signals. Of course, this hypothesis has not been fully tested and warrants to be further investigated.

The present study further demonstrates that CAPN1 promotion of cerebral ischemic injury is mediated by the impairment of autophagy flux because inhibition of CAPN1 improved ischemic insult and neuron survival, and these effects were abolished by either autophagy inhibitor CQ or 3-MA. It must be pointed out that 3-MA may have dual roles in autophagy since it has been reported that 3-MA promotes autophagy flux under the nutrient-rich conditions, but it still suppresses the starvation-induced autophagy.[43] The present study showed that 3-MA decreased the ratio of LC3-II/LC3-I, indicating that 3-MA inhibits autophagy flux. These results strongly suggest that impairment of autophagy flux importantly contributes to CAPN1-mediated cerebral ischemic injury.

There are some limitations of this study need to be noted. Although MDL 28170 is a widely used calpain inhibitor, like other CAPN inhibitors, it may inhibit other cysteine proteases[11] and, therefore, leads to off-target effects and lacks of calpain sensitivity. Therefore, we constructed the AAV-mediated genetic knocking down of CAPN1 by encoding sh*Capn1*. The effects of specific blockade of CAPN1 with sh*Capn1* on ischemic stroke outcome were consistent with the findings in MDL-treated pMCAO rats. Moreover, in the present study, only male young rats were used. To further evaluate the role of CAPN1 in stroke, female and other stroke models and species should be considered in future studies.

In summary, we demonstrate for the first time that ischemia-induced CAPN1 activation disrupts lysosomal function and suppresses the formation of autophagosomes, leading to the impairment of autophagic flux and subsequent aggravation of ischemic insult. Our results provide scientific evidence that CAPN1-mediated defects of autophagic flux play an important role in ischemic stroke–induced cerebral injury.

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Disclosures

None.

Supplemental Materials

Expanded Materials & Methods
Online Figures I–IX

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