Effect of glutamate on lysosomal membrane permeabilization in primary cultured cortical neurons

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Abstract. Glutamate is the principal neurotransmitter in the central nervous system. Glutamate-mediated excitotoxicity is the predominant cause of cerebral damage. Recent studies have shown that lysosomal membrane permeabilization (LMP) is involved in ischemia-associated neuronal death in non-human primates. This study was designed to investigate the effect of glutamate on lysosomal stability in primary cultured cortical neurons. Glutamate treatment for 30 min induced the permeabilization of lysosomal membranes as assessed by acridine orange redistribution and immunofluorescence of cathepsin B in the cytoplasm. Inhibition of glutamate excitotoxicity by the NMDA receptor antagonist MK-801 and the calcium chelator ethylene glycol-bis (2-aminoethylether)-N, N, N', N'-tetraacetic acid, rescued lysosomes from permeabilization. The role of calpain and reactive oxygen species (ROS) in inducing LMP was also investigated. Ca^{2+} overload following glutamate treatment induced the activation of calpain and the production of ROS, which are two major contributors to neuronal death. It has been reported that lysosomal-associated membrane protein 2 (LAMP2) and heat shock protein (HSP)70 are two calpain substrates that promote LMP in cancer cells; however, it was found that calpains were activated by glutamate, but only LAMP2 was subsequently degraded. Furthermore, LMP was not alleviated by treatment with the calpain inhibitors calpeptin and SJA6017, which blocked the cleavage of the calpain substrate α-fodrin. It was demonstrated that LMP was significantly alleviated by treatment with the antioxidant N-Acetyl-L-cysteine, indicating that LMP involvement in early glutamate excitotoxicity may be mediated partly by ROS rather than calpain activation. Overall, these data shed light on the role of ROS-mediated LMP in early glutamate excitotoxicity.

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Introduction

Glutamate, one of twenty essential amino acids, is well known to be the main physiological excitatory neurotransmitter in the mammalian central nervous system (CNS). It has been reported to regulate neurogenesis, neurite outgrowth, synaptogenesis and neuron survival (1). However, in various neurological disorders, including ischemia and stroke, a high concentration of glutamate is considered to lead to excitotoxicity and result in the death of neuronal cells. The concentration of glutamate is strictly maintained in the CNS; however, it is sharply and rapidly increased to 100-500 μM and sequentially induces excitotoxic damage during cerebral ischemia or trauma (2-4). Excessive glutamate production activates several types of pre- and post-synaptic glutamate receptor and then causes calcium overload. The accumulation of high levels of intracellular calcium triggers a range of downstream neurotoxic cascades, including energy deficiency, oxidative stress, mitochondrial dysfunction and calcium overload, leading to excitotoxic neuronal death (5).

Lysosomes, separated from the cytosol (which has a neutral pH) by a tough single membrane (6), are intracellular organelles characterized by an acidic environment with a pH of ~4.5. The pH value within lysosomes is precisely regulated by ATP-dependent proton pumps termed H^+-ATPases, which actively pump protons from the cytosol into the lysosomal lumen (7,8). H^+-ATPase is the key enzyme for physiologically maintaining the acidic microenvironment within lysosomes and regulating cellular pH. Thus, lysosomal destabilization not only influences normal cellular activities but also affects cell viability. Recently, it has been reported that partial rupture or permeabilization of the lysosomal membrane induces apoptosis through mitochondrial transmembrane potential loss or caspase activation, while severe lysosomal rupture induces necrosis in cancer cells (6,9). The leakage of cathepsins caused by lysosomal membrane permeabilization (LMP) has been

observed in the brain tissues of various rodents and non-human primate stroke models; for example, it was reported 1 h after global ischemia in monkey brain (10,11), after transient focal ischemia in mouse brain (12) and after a 5-min oxygen glucose deprivation in the rat hippocampal slices (13). However, it remains unclear whether LMP is involved in glutamate excitotoxic cascades, which are regarded as the leading cause of neuronal death.

To the best of our knowledge, the present study demonstrated for the first time that LMP is an early event in glutamate excitotoxic cascades. It was inhibited following treatment with the NMDA receptor antagonist MK-801 and the Ca^{2+} chelator ethylene glycol-bis (2-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA), and alleviated by treatment with the antioxidant N-Acetyl-L-cysteine (NAC), in primary cultured cortical neurons following glutamate treatment for 30 min.

Materials and methods

Materials. This study was approved by the ethics committee of the Sun Yat-sen University (Guangzhou, China). Newborn Sprague-Dawley rats were provided by the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). Neurobasal A medium, fetal bovine serum (FBS), B27 supplements, GlutaMAX, trypsin solution, penicillin-streptomycin (P/S) and Fluo-4AM were purchased from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The primary antibodies used in western blot analysis and immunohistochemistry were as follows: Goat anti-rat polyclonal cathepsin B (IgG; cat. no., sc-6493; dilution, 1:100) and goat anti-rat polyclonal heat shock protein (Hsp)70.1 (IgG; cat. no., sc-1060; dilution, 1:200) and goat anti-rat monoclonal anti-lysosomal-associated membrane protein 2 (LAMP2; IgG; cat. no., sc-8100; dilution, 1:100) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), and rabbit anti-rat monoclonal anti-α fodrin (IgG; cat. no., ab75755; dilution, 1:1,000) from Abcam (Cambridge, MA, USA). The secondary antibodies donkey anti-goat Alexa Fluor® 488 polyclonal IgG (cat. no., A-11055; dilution, 1:1,000) and goat anti-rabbit Alexa Fluor 488 polyclonal IgG (cat. no., A-11008; dilution, 1:1,000) were purchased from Invitrogen (Thermo Fisher Scientific, Inc.), and the goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody (cat. no. 7074; dilution, 1:1,000) was purchased from Cell Signaling Technology (Danvers, MA, USA) and the donkey anti-goat IgG-HRP antibody (dilution, 1:5,000) from Santa Cruz Biotechnology, Inc. (Stanta Cruz, CA, USA). Acridine orange (AO) EGTA and the calpain inhibitors calpeptin and SJA6017 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. Cortical neurons were prepared from newborn Sprague-Dawley rats as described previously with modifications (14). Briefly, the cortex was dissected and placed in ice-cold aseptic dissection solution (DS). After mincing, the tissue was placed in DS containing 0.25% trypsin (Thermo Fisher Scientific, Inc.) for 15 min at 37˚C. Digestion was terminated by DS containing 10% FBS followed by centrifuging for 5 min at 750 x g and the supernatant was discarded. The cell pellet was resuspended in DS containing DNase I (5 mg/ml; (Sigma-Aldrich), Mg^{2+} and FBS, and homogenized by pipetting up and down ~20 times. The supernatant was left for15 min and then centrifuged for 5 min at 750 x g. The cell pellet was then collected and resuspended in Neurobasal A medium supplemented with 10% FBS, 2% B27, 0.25% GlutaMAX and 1% P/S. Cells were then seeded at a density of $1x10^6$ cells/ml into poly-l-lysine (0.5 mg/ml; Sigma-Aldrich) coated dishes. Cells were incubated in a CO_2 chamber. The medium was replaced with non-serum formula after seeding for 4 h and half of the medium was replaced every 2-3 days. In the present study, the primary cultured cortical neurons were exposed to 100, 200 or 400 μM glutamate for 30 min

AO redistribution assay. Cells were treated with different concentrations of glutamate, glutamate and MK-801, or NAC. Cells were then incubated with 5 μg/ml AO in Neurobasal medium A for 15 min at 37˚C and washed twice with phosphate-buffered saline (PBS) (Invitrogen; Thermo Fisher Scientific, Inc.). Immunofluorescence images were captured from randomly selected microscopic fields using a fluorescent microscope equipped with a camera (IX71; magnification, x200; Olympus Corp., Tokyo, Japan). During exposure to blue light, AO-loaded cells showed red granular fluorescence and green diffuse fluorescence. Once rupture of lysosomes occurs, AO is relocated from lysosomes to the cytosol, this results in a decrease in the granular (lysosomal) red fluorescence in combination with the increased diffuse (cytosolic) green fluorescence.

Immunohistochemistry. Following glutamate exposure, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then washed three times with PBS for 5 min per wash. Permeabilization was performed with 0.3% Triton X-100 (Amresco LLC, OH, US) for 15 min. The cells were then washed three times with PBS and incubated in blocking buffer (ZSGB-BIO Company, Beijing, China) for 1 h, followed by overnight incubation at 4˚C with primary antibody anti-cathepsin B and anti-LAMP2. The following day, cells were washed with PBS three times, followed by 1 h incubation with secondary antibody Alexa Fluor 488-conjugated donkey anti-goat IgG for cathepsin B and goat anti-rabbit IgG for LAMP2 at room temperature, and three washes with PBS. Finally, cells were counterstained with DAPI (Sigma-Aldrich) for 3 min and rinsed with PBS. Digital images were collected using a Zeiss LSM 710 confocal microscope (Zeiss, Oberkochen, Germany).

Intracellular Ca^{2+} measurement. Intracellular calcium concentrations were measured by the fluorescent cell-permeant calcium indicator Fluo-4AM. Cells grown on black-walled 96-well plates were loaded with 5 μM Fluo-4AM for 45 min in Hank's balanced salt solution (Invitrogen; Thermo Fisher Scientific, Inc.) containing 0.02% Pluronic F-127 (Invitrogen; Thermo Fisher Scientific, Inc.) followed by 20 min of de-esterification. Following loading, neurons were treated with 200 μM glutamate, 200 μM glutamate and 5 μM MK-801, or 10 mM extracellular Ca^{2+} chelator EGTA for 30 min at 37˚C in the dark. Then, neurons were washed with Hank's balanced salt solution. Fluorescence with excitation at 488 nm and emission at 506 nm was then read on a microplate fluorescence reader (TECAN Group Ltd., Männedorf, Switzerland). Results are presented as a fold change of the control.

Western blotting. Following treatment with glutamate for 30 min, neurons were scraped and then resuspended in protein extraction reagent. The cell lysate was centrifuged at 12,000 x g for 10 min at 4˚C and the supernatant was collected for electrophoresis. Prior to electrophoresis, the concentration of protein was determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA) following the manufacturer's instructions. Equal quantities of protein (40 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (CWBIO, Beijing, China). Following electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (F. Hoffmann-La Roche AG, Basel, Switzerland), blocked with 5% skimmed milk in Tris-buffered saline for 2 h, and washed three times with Tris-buffered saline. Subsequently, the membranes were reacted with primary antibodies overnight. The membranes were then washed three times with Tris- buffered saline, followed by incubation with horseradish peroxidase-labeled secondary antibody for 1 h at room temperature. The immune complexes were visualized using enhanced chemiluminescence-detection reagents (Merck Millipore, Boston, MA, USA) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation and were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Lysosomal membrane permeabilization is involved in early glutamate excitotoxicity. It has been reported that treatment with high concentrations of glutamate (≥100 μM) for 24 h induces excitotoxic cell death with notable morphologic changes in rat primary cerebral cortical neurons. In the present study, the primary cultured cortical neurons were exposed to 100, 200 or 400 μM glutamate for 30 min, and then the cells displayed morphological body swelling and synapse injury in a concentration-dependent manner compared with the control group (Fig. 1A).

The permeabilization of the lysosomal membrane results in H^+-ATPase dysfunction and redistribution of cathepsins from lysosomes to the cytoplasm. As shown in Fig. 1B, following incubation with a lysosome-specific fluorescence probe, AO (15), the neurons in control group exhibited extensive lysosomal red fluorescence, while neurons treated with different concentrations of glutamate exhibited an increase in green fluorescence in the cytosol, indicating AO redistribution caused by LMP. LMP induced by glutamate was further confirmed by an immunofluorescence assay using antibodies against the lysosome specific enzyme, cathepsin B. As shown in Fig. 1C, the fluorescence intensity of cathepsin B in the cytoplasm decreased following glutamate treatment, which indicated leakage caused by LMP.

It is now unanimously accepted that NMDA receptors are important in excitotoxic neuronal death, predominantly owing to their Ca^{2+} permeability. Influx of extracellular Ca^{2+} is secondary to activation of the NMDA receptors by glutamate, and the accumulation of high levels of intracellular Ca^{2+} triggers a cascade of excitotoxic events. LMP of neurons was mostly inhibited by the NMDA receptor antagonist MK-801 using AO (Fig. 1B) and cathepsin B (Fig. 1C) staining. To further confirm the effect of glutamate on lysosomal stability, the neurons were switched to a Ca^{2+}-free medium containing 10 mM EGTA, which chelates extracellular Ca^{2+}. As expected, the glutamate-induced increase in intracellular Ca^{2+} was abolished (Fig. 1D) and LMP was markedly decreased (Fig. 1B), indicating that the Ca^{2+} influx is critical in glutamate induced-LMP. These results suggest that LMP is involved in early glutamate excitotoxicity.

Calpain activation is not required for LMP. It has been reported that calcium influx via the NMDA receptor results in the activation of cytoplasmic proteases (such as calpain), which hydrolyzes cytoskeletal and cellular proteins, and leads to LMP (16,17). Hsp70.1 and LAMP2, two substrates of calpain, have been reported to be involved in calpain-mediated LMP (16,17). Hsp70.1 is known to stabilize the lysosomal membrane by recycling damaged proteins, and protecting cells from oxidative stress and apoptotic stimuli (18). In the case of ischemia, Hsp70.1 is carbonylated by oxidative stressors and is more vulnerable to activated calpain (16,19). However, in a rat retinal light damage model, degradation of LAMP2 rather than Hsp70.1 by calpain is also hypothesized to promote LMP (17). Using western blot analysis (Fig. 2A) and immunofluorescence (Fig. 2B), it was found that in primary cortical neurons treated with different concentrations of glutamate, it was LAMP2 that was cleaved in different degrees. These results are consistent with a previous study of the model of light-induced retinal degeneration (17), suggesting that calpain-LAMP2 cascades are key in the induction of glutamate-mediated LMP.

It was further investigated whether the activation of calpain induced LMP following treatment with glutamate. Cleavage of α-fodrin leading to the formation of 145/150 kDa fragments is a well-recognized marker for calpain-generated protein breakdown (20-22). As shown in Fig. 2C, treatment with glutamate led to a significant increase of α-fodrin 145/150 kDa fragmentation. This effect was blocked by MK801, indicating that calpain was activated by overloaded intracellular Ca^{2+} via NMDA receptors in neurons following treatment with glutamate. Calpeptin and SJA6017 are two cell-permeable calpain inhibitors. It was found that calpeptin and SJA6017 abolished the 145/150 kDa fragmentation of α-fodrin (Fig. 2C), indicating that the glutamate-induced calpain activation was inhibited. However, AO staining suggested that the calpain inhibitors did not alleviate the permeabilization of lysosomal membrane induced by 200 μM glutamate (Fig. 2D). Therefore, these results suggest that calpain activation is not required for LMP caused by glutamate in primary cultured cortical neurons.

ROS are mediators of lysosomal permeabilization. It has been reported that the activation of calpain and the generation of free radicals markedly contribute to neuronal death that is induced by glutamate excitotoxicity. Free radicals, particularly ROS, damage cell function by oxidative modification of the proteins, lipids, carbohydrates and nucleic acids of intracellular organelles. To determine the role of ROS in LMP, the effect of

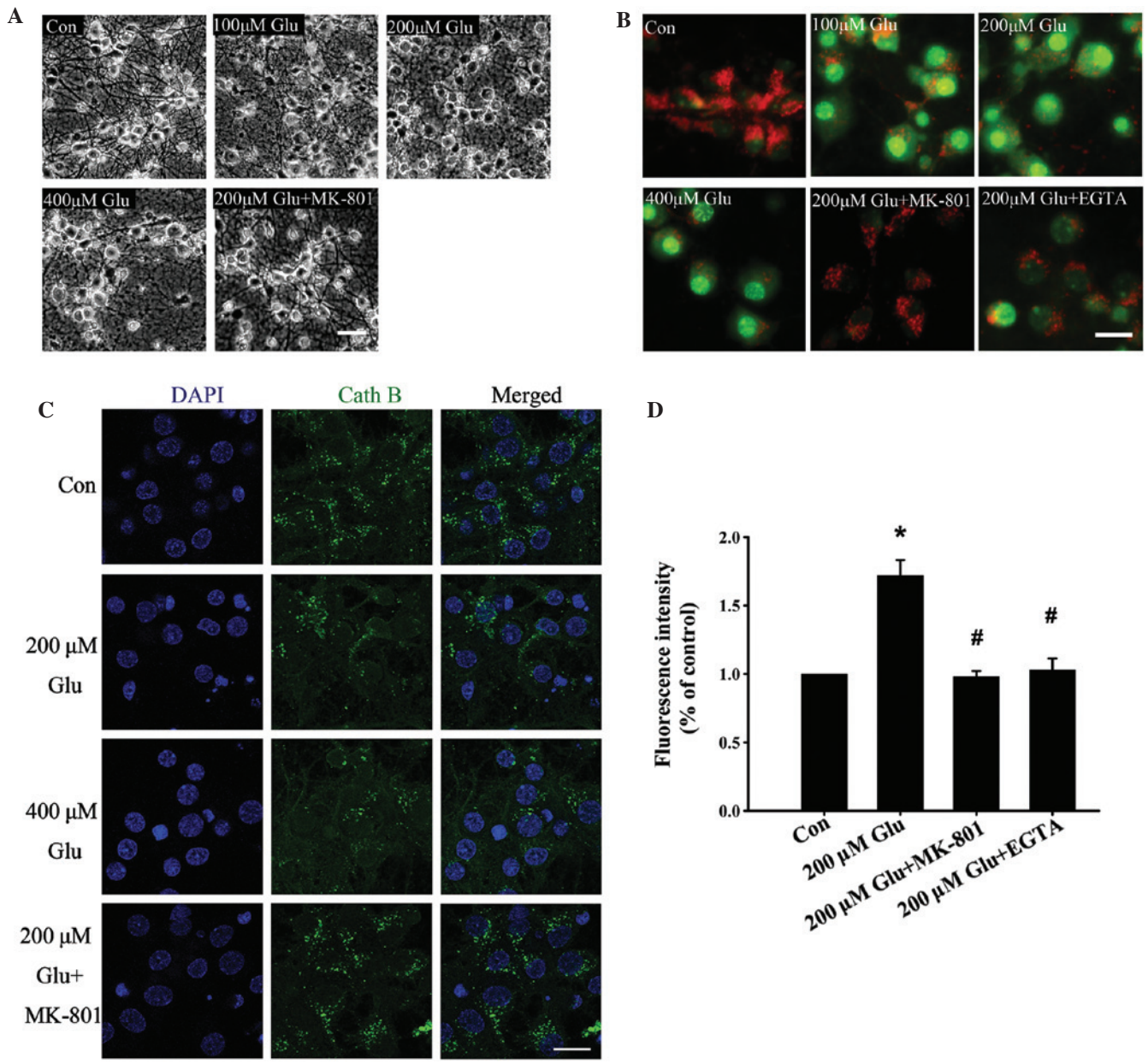

Figure 1. Effect of glutamate on lysosomal membrane permeabilization. (A) Representative phase contrast images of neurons left untreated, treated with different concentrations of glutamate after 30 min or treated with glutamate and MK-801. Scale bar, 50 μm. (B) Neurons were left untreated, treated with different concentrations of glutamate alone, or treated with 200 μM glutamate with MK-801 and EGTA for 30 min. Neurons were then stained with acridine orange and viewed under a microscope. Scale bar, 20 μm. (C) Immunohistochemical analysis of cathepsin B distribution following treatment with glutamate. In the control group, staining appeared punctate, while cytosolic cathepsin B increased following glutamate stimulation for 30 min. Scale bar, 20 μm. (D) The effect of glutamate (200 μM) on intracellular Ca^{2+} was monitored by Fluo-4 AM. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05, compared with the control group; $^{\#}$P<0.05, compared with the 200 μM glutamate group. Glu, glutamine.

the antioxidant NAC on glutamate-induced permeabilization of lysosomes was investigated. As shown in Fig. 3, LMP was significantly increased in neurons following treatment with 200 μM glutamate, which was markedly inhibited by NAC, as evidenced by decreased AO green signals and increased red fluorescence. These results indicate that ROS is a mediator of lysosomal permeabilization downstream of the NMDA receptor in glutamate excitotoxicity.

Discussion

Glutamate excitotoxicity is considered as one of the most important pathological mechanisms of neuron loss in acute and chronic CNS diseases, including acute ischemic stroke. Intra-arterial fibrinolytic therapy within a 3-6 h window following the onset of large artery cerebral thrombotic occlusions provides benefits for rescuing ischemic brain penumbra. This suggests that the irreversible biochemical processes activated by glutamate, for example abnormal proteolysis of calpain, contribute to neuronal death.

The results demonstrated that LMP was increased 30 min after treatment with glutamate, which was abolished by the NMDA receptor antagonist MK-801 or calcium chelator EGTA. Kubota *et al* (23) reported that the fluorescence signals of LysoSensor and AO dye, and the immunostaining signals of cathepsin D were unaltered following treatment with glutamate

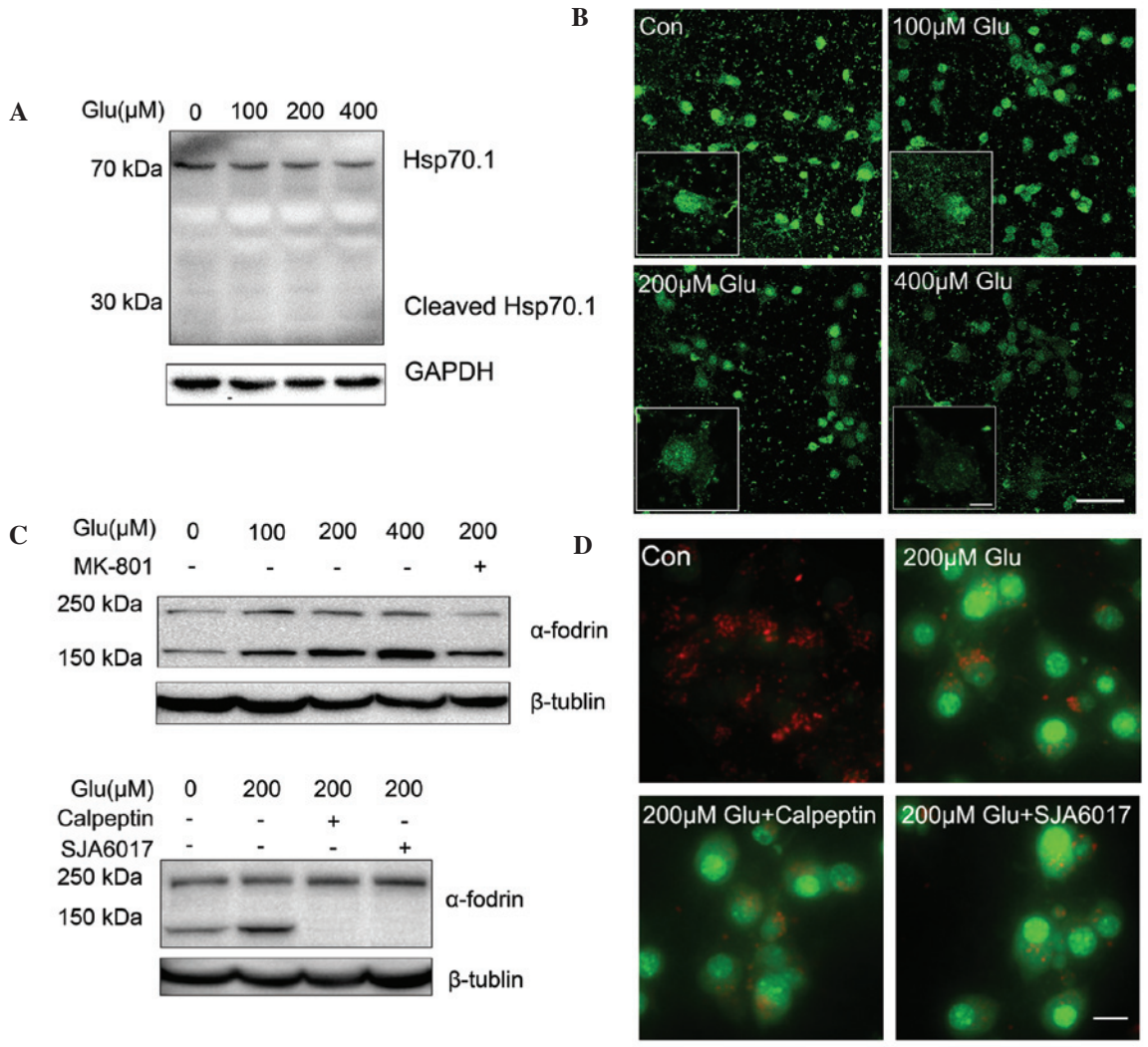

Figure 2. Calpain activation is not required for lysosomal membrane permeabilization. (A) Western blot analysis showed the Hsp70.1 level in neurons treated with different concentrations of glutamate for 30 min. (B) Immunofluorescence staining of lysosomal-associated membrane protein 2 following glutamate stimulation. The immunofluorescence intensity of LAMP2 in the glutamate-treated groups decreased by different degrees, indicating cleavage. Scale bar, 100 μm (scale bar for inset picture, 10 μm). (C) Top, effects of glutamate and MK-801 on calpain-specific α-fodrin cleavage. Bottom, effects of calpain inhibitors (calpeptin and SJA6017) on glutamate-induced α-fodrin cleavage. (D) Effects of calpain inhibitors (calpeptin and SJA6017) on glutamate-induced acridine orange redistribution. Scale bar, 20 μm. Hsp60.1, heat shock protein 60.1.

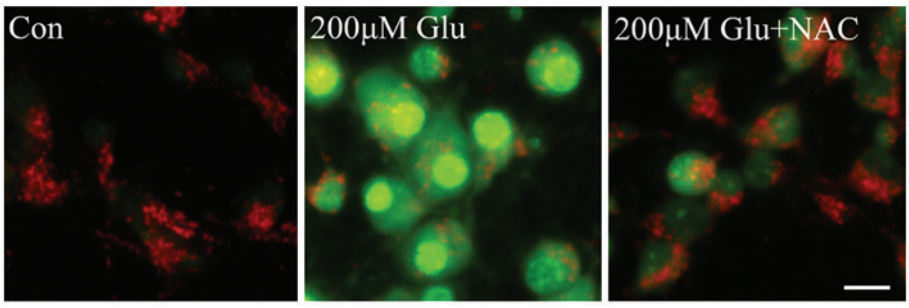

Figure 3. Inhibition of reactive oxygen species production rescues lysosomes from permeabilization. Acridine orange redistribution reflected the effect of NAC on 200 μM glutamate-induced lysosomal membrane permeabilization. Scale bar, 20 μm. NAC, N-Acetyl-L-cysteine; Glu, glutamine.

for 8 h in HT22 cells (an immortalized mouse hippocampal cell line) indicating that the organization and membrane integrity of lysosomes was stable. These inconsistencies may be due to the distinction between oxidative glutamate toxicity in HT22 and the excitotoxicity in primary cultured cortical neurons in which glutamate indirectly causes a depletion of intracellular glutathione through blocking the cystine uptake, which is mediated by cystine/glutamate antiporter (24).

Moreover, the present study demonstrated that glutamate-induced LMP was not dependent upon calpain activation.

Involvement in early cascade events of glutamate excitotoxicity strongly implied another key role of ROS-mediated LMP in irreversible neural injury. Brain tissue possesses a number of important endogenous defenses against ischemic injury, including glutathione, and the enzymatic superoxide dismutase. However, during injury, these natural antioxidant defenses can be quickly overwhelmed and followed by energy impairment, leading to increased production of superoxide radicals, nitric oxide and hydrogen peroxide. Development of oxidative stress can rapidly lead to serious disturbances in cerebral function via damage to proteins, lipids, carbohydrates and nucleic acids (25), while treatment with antioxidant NAC decreases the extent these injuries in different models of brain ischemia (26,27). Lysosomes contain a large number of acidic hydrolases and the pH inside lysosomes is ~4.5. Once rupture or permeabilization occurs, large quantities of acidic hydrolases, such as cathepsin B, are released into cytoplasm. This results in intracellular acidosis and promotes the irreversible degradation of proteins and lipids (8). Data from the present study demonstrated that inhibition of ROS by NAC significantly rescued lysosomes from permeabilization, 30 min after glutamate treatment. This suggested that the LMP-cathepsin activation mediated by ROS was an early irreversible injury to neural cells following glutamate excitotoxicity, which was consistent with the observations of a strong protective effect of cathepsin B inhibitors in monkey (10) and mouse (12) ischemia models. These findings, along with those of previous studies (10-12), provide further evidence that LMP may be a promising target for neuronal protection.

In order to determine the detailed molecular mechanisms underlying ROS-mediated LMP caused by glutamate further investigation is required. Increasing evidence suggests that LMP may be governed by several distinct mechanisms in a stimulus- and cell-type-dependent manner (6,9,10,12,13,16,18). Different from other studies which demonstrated that calpain promoted lysosomal membrane destabilization during neuronal death with different stimuli, such as transient focal ischemia ,oxygen glucose deprivation or global ischemia (10,13,28,29), the results of the present study revealed that glutamate-induced LMP was not mediated by calpain. It was hypothesized that activated calpain cleaves oxidative stress-induced carbonylated Hsp70.1 at the lysosomal membrane, which results in lysosomal rupture/permeabilization. In the present study, the degradation of Hsp70.1 was not detectable when the neurons had been subjected to glutamate treatment. Another substrate of calpain, LAMP2, was significantly degraded following glutamate treatment. This was consistent with a previous study, which demonstrated that degradation of LAMP2 mediated LMP in a model of light-induced retinal degeneration (17). However, inhibition of calpain activation had no effect on alleviating LMP, indicating that calpain is not required for LMP in early glutamate excitotoxicity.

It was hypothesized that ROS may compromise the integrity of lysosomes via intralysosomal iron-mediated peroxidation of membrane lipids (30). This may suggest a nonspecific mechanism for lysosomal permeabilization, such as pore formation or limited membrane damage, rather than selective transport across the membrane, which would likely be specific for a single group of biomolecules. Monomers of LAMP2, a type 1 transmembrane protein on the lysosomal membrane, can associate with other proteins to form a multi-protein channel required for delivery of proteins to lysosomes for degradation during chaperone-mediated autophagy (17). The present results suggest the possibility of cleavage of LAMP2 by other proteases (which are activated by free radical accumulation but not calpain) is involved in glutamate-mediated LMP in primary cultured cortical neurons.

In conclusion, LMP is a process that occurs at early stages of glutamate excitotoxicity in primary cultured cortical neurons, suggesting that LMP inhibitors may aid in minimizing ischemic neural injury.

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