Lumbrokinase regulates endoplasmic reticulum stress through IRE1 signaling to improve neurological deficits in ischemic stroke

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Abstract

Background

Ischemic stroke is characterized by the loss of cerebral blood flow, which frequently leads to neurological deficits. Therefore, minimizing post-stroke functional disability is an important research topic. The fibrinogen-depleting agent lumbrokinase has been used to improve myocardial perfusion in symptomatic stable angina and to prevent secondary ischemic stroke. In this study, we aimed to clarify the neuroprotection of lumbrokinase on ischemic stroke and whether improve neurological dysfunction. We explored the neuroprotection and the underlying mechanisms of lumbrokinase in C57BL/6 mice subjected to permanent middle cerebral artery occlusion.

Results

Lumbrokinase at 1 mg/kg significantly attenuated the infarct volume and improved the neurological dysfunction. Lumbrokinase dramatically decreased the expressions of the endoplasmic reticulum (ER) transmembrane receptor protein inositol-requiring enzyme-1 (IRE1) and its downstream transcription factor, X-box binding protein-1, caspase-12, and nuclear factor kappa B activity. Moreover, lumbrokinase significantly inhibited apoptosis and autophagy and decreased the expression levels of the NOD-like receptor 3 inflammasome, caspase-1, and interleukin-1 β compared with the vehicle treatment.

Conclusions

We suggest that post-stroke treatment with lumbrokinase protects against ischemic stroke by regulating ER stress through the IRE1 signaling pathways to inhibit apoptosis, autophagy, and inflammatory responses.

1. Introduction

Stroke is the second leading cause of death, morbidity, and severe long-term disability in adults worldwide [1]. With the ever-increasing life expectancies in countries throughout the developed world, the absolute numbers of individuals suffering from stroke will continue to increase [2]. Approximately 80% of strokes in adults are ischemic [3]. The mortality rate in the acute phase of ischemic stroke is as high as 20% and remains high for several years after the acute event [4]. Therefore, clinically effective and safe interventions that reduce ischemic neuronal death and improve functional recovery are urgently needed.

Endoplasmic reticulum (ER) stress plays a crucial role during stroke-induced injury in the brain. Protein misfolding in the ER induces the unfolded protein response (UPR) to restore ER functions in the energy-deprived neurons of the ischemic brain by activating three ER transmembrane receptors, namely protein kinase RNA-like endoplasmic reticulum kinase [5], inositol-requiring enzyme 1 (IRE1), and activating

transcription factor 6 (ATF6), all of which are regulated by the ER chaperone and signaling regulator glucose-regulated protein 78/immunoglobulin heavy-chain-binding protein (GRP78/BiP) [6]. The UPR is initially a protective response that aims to restore ER functions, but cellular dysfunction and cell death occur under conditions of prolonged ER stress or when folding capacity is exceeded and proteins accumulate in the ER lumen [7]. Evidence shows that ER stress and cell death are pivotal to the pathophysiology of cerebral ischemia: the phosphodiesterase type 3 inhibitor, cilostazol, ameliorates cerebral ischemia injury-induced blood-brain barrier disruption by inhibiting ER stress and is thus neuroprotective in ischemic stroke [8]. Recently, it has been reported that IRE1, which is the most conserved ER stress sensor, and the expression of X-box binding protein-1 (XBP1) and GRP78/Bip markedly increased in the striatum and cortex of injured brain regions after ischemic stroke [9, 10]. In addition, research shows that a flavonol glycoside, icariin, suppresses IRE1-XBP1 signaling to protect neurons from ER stress-induced apoptosis after oxygen-glucose deprivation/re-oxygenation injury [11]. Recent research suggests that the IRE1-XBP1 pathway can activate the NOD-like receptor 3 (NLRP3) inflammasome-mediated inflammatory response [12], initiate caspase cascade reaction, and then activate caspase-3, which leads to apoptosis [13]. Clearly, therapeutic interventions that inhibit ER stress-induced cell death provide neuroprotective effects and are promising strategies in the treatment of ischemic stroke.

Lumbrokinase, an extract of *Lumbricus rubellus*, contains plasminogen activators and plasmin. Lumbrokinase thrombolytic activity can only occur in the presence of fibrin; this indicates that treatment with lumbrokinase greatly reduces hemorrhage due to hyperfibrinolysis, in contrast with either streptokinase or urokinase [14]. Lumbrokinase has been used to treat patients with cardiovascular disease [15] to prevent secondary ischemic stroke [16] and to improve myocardial perfusion in patients with stable angina [17]. Previous research suggests that the anti-ischemic activity of lumbrokinase is explained by its antiplatelet and antithrombotic activity, anti-apoptotic effect [18], and ability to significantly reduce P-selectin and E-selectin immunoreactivity in the ischemic lesion [19] in rodent models of cerebral ischemia. However, no studies have examined the role of ER stress in how lumbrokinase mediates neuroprotection in rodent models of permanent middle cerebral artery occlusion (MCAO). Therefore, our major goal was to investigate the mechanisms involved in the neuroprotective effect of lumbrokinase against ischemic stroke in mice. We specifically examined how lumbrokinase mediates the IRE1 signaling pathway to regulate ER stress-induced cell apoptosis, autophagy, and inflammation response in mice after ischemic stroke.

2. Materials And Methods

2.1 Animals

Adult male C57BL/6 mice (8–10 weeks old, BioLasco Taiwan Co., Ltd, Taiwan) were used in this study. All animals were housed in the Animal Center of Chung Shan Medical University, Taichung, Taiwan, at an ambient temperature of $24 \pm 1^\circ\text{C}$ and humidity of $55 \pm 5\%$ under a 12 h light-dark cycle. They received normal chow and water *ad libitum*. All surgical procedures for permanent MCAO and experimental

protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University (IACUC 1637). All animal handling conformed to Directive 2010/63/EU, and all efforts were made to minimize animal suffering and to reduce the number of animals to be sacrificed. This investigation also conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85 – 23, revised 1996).

2.2 Permanent MCAO model

The method was modified from previous studies [20]. All mice were anesthetized with intraperitoneal pentobarbital (50 mg/kg in normal saline). Body temperature was maintained during surgery at $37 \pm 0.5^{\circ}\text{C}$ with a heating pad. Focal ischemic infarcts were made in the right lateral cerebral cortex in the territory of the middle cerebral artery. The bilateral common carotid arteries were exposed by midline anterior cervical incision. The mice were placed in a lateral position, and a skin incision was made at the midpoint between the right lateral canthus and the anterior pinna. The temporal muscle was retracted, and a small (3 mm in diameter) craniectomy was made at the junction of the zygoma and squamosal bone using a drill (Dremel Multipro + 5395, Dremel Company, USA) cooled with saline solution. The dura was opened with fine forceps using a dissecting microscope (OPMI-1, ZEISS®, Germany), and permanently cauterized the distal MCA along with simultaneous occlusion of the bilateral common carotid arteries with microaneurysm clips for 20 min to paralyze the dominant forelimb. After 20 min of ischemia, the CCAs were unclamped, and the restoration of blood flow was visualized.

2.3 Drug administration

The selection of experimental dosage regimen was referred Zhang et al and Ji et al [18, 19]. In our preliminary experiment, we investigated that the effectiveness of various doses of lumbrokinase (from 0.1 mg/kg to 10 mg/kg) in mice subjected to ischemic stroke. We found that 1 mg/kg lumbrokinase significantly reduced infarcted volume and improved functional recovery after ischemic stroke. In order to reduce the number of animals used in the present study, we examine the neuroprotective effect of lumbrokinase at the dose of 0.1 mg/kg and 1 mg/kg for follow-up experiments. All mice underwent MCAO surgery and were randomly assigned to three groups: (1) vehicle: normal saline, (2) LK-0.1: lumbrokinase 0.1 mg/kg, and (3) LK-1: lumbrokinase 1 mg/kg. Intraperitoneal injection with lumbrokinase or vehicle was performed once a day for six days. The first treatment was 20 min after MCAO. Lumbrokinase (Canada RNA Biochemical Inc., Richmond, BC, Canada) solution was freshly prepared before administration.

2.4 Infarct volume analysis

Brain infarct size was measured seven days after the onset of MCAO-induced ischemia after decapitating each animal after the induction of anesthesia by intraperitoneal urethane injection (1.25 g/kg, Sigma-Aldrich, St. Louis, MO, USA). The brains were removed, inspected visually for MCA anatomy and signs of hemorrhage or infection, immersed in cold saline solution for 10 min, and then sectioned into standard coronal 1 mm slices using a Jacobowitz brain matrix slicer (Zivic-Miller Laboratories, Inc., Allison Park, PA, USA). The slices were immersed in vital dye 2,3,5-triphenyltetrazolium chloride (TTC, 2%, Sigma-

Aldrich) for 30 min at 37°C in the dark and fixed overnight in a 10% formalin solution at room temperature. The TTC staining clearly distinguished infarcted from non-infarcted tissue. The cerebral infarction volume was measured using digital imaging and image analysis software (National Institutes of Health ImageJ software 1.42), which subtracted the ipsilateral regional volume from the contralateral regional volume. The person who analyzed the images was blinded to the treatment of each animal. The methods were modified from the method of Huang et al [21].

2.5 Neurological evaluation

Neurological evaluations were performed in all animals before MCAO surgery, and the first and third days after ischemic stroke.

Rotarod test. The accelerating rotarod assesses motor deficits [22]. The mice were placed on the rungs of the accelerating rotarod cylinder, and the duration (in seconds) of the animals remaining on the rotarod was measured. The speed was slowly increased from 4 to 40 rev/min within 4 min. A trial would end if the animal fell off the rungs. Each animal underwent three consecutive trials.

Grip test. Motor function and coordination were evaluated by the grip test [23]. Each mouse was placed on a taut, 43 cm-long string suspended 34 cm high from the bracket between both sides of the string. The experimenters ensured that both front paws came in contact with the string, allowing the mice an equal chance to grasp the string if they could. The tail was then released, at which time the mice either fell immediately or remained on the string. Neurological status was evaluated according to a grip test, which measured the length of time the mice could remain on the string in some manner (i.e., using 1–4 paws, tail, or paws plus tail) within a 30-s time period. Each mouse was placed on a string midway between two supports and evaluated according to the following scale: 0, fell off; 1, held on in some way for 30 s; 2, held on with four paws for at least 5 s; 3, held on with four paws and placed the tail on the string for at least 5 s; 4, held on with four paws, placed the tail on the string, and traveled along the string in either direction for at least 5 s; and 5, traveled to one of the vertical sides within the 30-s test period.

Adhesive removal test. The adhesive removal test was modified from a previous experiment [24]. Patches of adhesive tape (4 mm × 4 mm) were attached to the animal's paws in alternating sequence and with equal pressure. The animals were individually released into the testing cage, and the latency times of the contact and removal of the adhesive patch were recorded. Contact was recorded when either shaking of the paw or mouth contact occurred. The trial ended after the removal of the adhesive patch or after 2 min had elapsed. Preoperative training was conducted for three days for one trial per day prior to pretesting. The animals were tested for three trials postoperatively. The best two trials from each mouse were averaged for statistical analysis.

2.6 Western blotting

Brains were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Scientific, USA) containing the Protease Inhibitor Cocktail (Sigma, USA). The homogenates were centrifuged at 12,000 g for 10 min at 4°C. Protein concentration was determined with protein assay kits (BioRad, USA) using bovine serum

albumin (BSA) as the standard. The samples were mixed with an equal volume of loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% v/v glycerol, 2% SDS, 5% v/v 2-mercaptoethanol, and 0.05% w/v bromophenol blue) and heated for 10 min at 95°C. The mixture was subjected to SDS-PAGE and transferred electrophoretically to nitrocellulose membranes at a constant voltage of 30 V at 4°C overnight. The membranes were blocked with 5% w/v non-fat milk in PBS containing 0.1% v/v Tween 20 (PBST) for 90 min at room temperature. The membranes were reacted with primary antibodies (1:3,000 dilutions) at 4°C overnight and washed three times with PBST. HRP-conjugated secondary antibody (1:20,000 dilutions) was added, and the membranes were incubated at room temperature for 40 min to detect the primary bound antibody. Reactive proteins were detected by enhanced chemiluminescence (Amersham, UK), and the density of specific immunoreactive bands was quantified by densitometric scanning.

2.7 Primary antibodies

The primary antibodies used were as follows: mouse anti- β -actin monoclonal antibody, rabbit anti-LC3 polyclonal antibody, rabbit anti-p62 polyclonal antibody, rabbit anti-interleukin (IL)-1 β polyclonal antibody, mouse anti-caspase-1 monoclonal antibody, mouse anti-cytochrome C monoclonal antibody (Novus, USA), rabbit anti-p-IRE1 polyclonal antibody, rabbit anti-IRE1 polyclonal antibody, rabbit anti-Bax monoclonal antibody, rabbit anti-Bcl-2 monoclonal antibody, rabbit anti-Beclin-1 monoclonal antibody, rabbit anti-XBP1 polyclonal antibody, rabbit anti-NLRP3 polyclonal antibody, rabbit anti-COX-2 polyclonal antibody (Abcam, UK), rabbit anti-caspase-12 polyclonal antibody, mouse anti-GRP78 polyclonal antibody (BD Biosciences, USA), rabbit anti-caspase-3 polyclonal antibody, rabbit anti-cleaved-caspase-3 polyclonal antibody, rabbit anti-p-nuclear factor kappa B (NF- κ B) polyclonal antibody, and rabbit anti-NF- κ B polyclonal antibody (Cell Signaling, USA).

2.8 Secondary antibodies

HRP-conjugated goat anti-mouse IgG (H + L) antibody, HRP-conjugated bovine anti-goat IgG (H + L) antibody, and HRP-conjugated goat anti-rabbit IgG (H + L) antibody were all purchased from Jackson ImmunoResearch Laboratories, Inc. (USA).

2.9 Gelatin zymography

Stroke-induced proteinase in mouse brain was analyzed by gelatin zymography, as described previously [25], with slight modifications. In brief, heart homogenates were loaded onto SDS-PAGE [7.5% (w/v) polyacrylamide gel copolymerized with 0.1% w/v gelatin (Sigma, USA)]. The stacking gels were 4% w/v polyacrylamide and did not contain a gelatin substrate. Electrophoresis was performed in a running buffer (25 mM Tris, 250 mM glycine, 1% SDS) at room temperature. The gel was washed twice in double-distilled water containing 2.5% TritonX-100 for 30 min each time and then incubated in a reaction buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM CaCl₂, 0.02% w/v Brij® – 35, 0.01% w/v NaN₃) at 37°C for 18 h. The gel was stained with 0.25% w/v Coomassie Brilliant Blue R-250 (Sigma, USA) for 1 h and destained in 15% v/v methanol and 7.5% v/v acetic acid. Gelatinase activity was detected as unstained bands on a blue background. Quantitative analysis was performed with a computer-assisted imaging densitometer system (UN-SCAN-ITTM gel version 5.1, Silk Scientific, UT, USA).

2.10 Immunofluorescence assay

Mouse brain was removed as fresh and fixed directly with 4% paraformaldehyde. All the samples were embedded in an optimal cutting temperature compound, and sections were cut at a 25- μ m thickness. For immunostaining, frozen brain sections were fixed with methanol for 20 min at -20°C and washed repeatedly. The tissue sections were blocked with 5% BSA in PBS for 1 h and incubated with 2 N HCl at 37°C for 30 min, followed by blocking the non-specific binding sites in 0.1 M PBS containing 0.3% Triton X-100 and 2% fetal bovine serum (FBS). The sections were incubated with a rabbit anti-p-IRE1 monoclonal antibody and doubly stained with another antibody against various cellular marker proteins, including mouse anti-neuronal nuclei (NeuN) and rabbit anti-glial fibrillary acidic protein (GFAP), all diluted in PBS containing 0.3% Triton X-100 and 2% FBS. The secondary antibodies were Alexa Fluor 488-conjugated goat anti-rabbit and TRITC-conjugated donkey anti-mouse antibody. Nuclei were stained with antifade reagent (Molecular Probes Inc., UK). The slides were examined under a confocal microscope (Zeiss LSM 510 META; Carl Zeiss, Oberkochen, Germany) and a fluorescence microscope (ZEISS Axio Imager A2).

2.11 Statistical analysis

Data analysis was performed using SigmaPlot Version 11.0. All data are expressed as the mean $\pm$ standard error of the mean. Between-group differences were assessed using one-way analysis of variance, followed by Student's *t* tests with the post hoc Bonferroni's comparison tests. The threshold for statistical significance was set to $p < 0.05$ using a two-tailed hypothesis test.

3. Results

3.1 Post-stroke lumbrokinase treatment attenuated cerebral infarction and improved functional recovery in mice subjected to ischemic stroke

The neuroprotective effects of lumbrokinase were determined by intraperitoneal injections of lumbrokinase (0.1 mg/kg or 1 mg/kg) or vehicle 20 min after MCAO. As shown in Fig. 1A, lumbrokinase dose-dependently reduced the infarct volumes. As indicated by TTC staining, the post-stroke treatment with 1 mg/kg lumbrokinase significantly attenuated the infarct volume compared with the vehicle treatment (25.5 ± 2.2 vs. 36.4 ± 4.0 ; $p < 0.05$). In addition, standardized neurological testing was conducted before the permanent MCAO and on days 1 and 3 after the permanent MCAO. The significantly worse rotarod performance, grip score, and contact and removal times of the adhesive removal task on day 1 after MCAO were compared with the pretest, as reflected by the sensorimotor dysfunction due to ischemic stroke. The post-stroke treatment with 1 mg/kg lumbrokinase was associated with significant improvements compared with the vehicle treatment in the rotarod performance and grip score, as well as with significant recoveries in the adhesive contact and removal times of the adhesive patch on day 3 (Fig. 1B–E). These results indicate that post-stroke treatment with 1 mg/kg lumbrokinase provides neuroprotection in mice subjected to ischemic stroke.

3.2 Post-stroke lumbrokinase treatment mediated ER stress in mice subjected to ischemic stroke

Ischemic stroke is known to disrupt ER homeostasis, which leads to ER stress, and to facilitate neuronal injury [26]. As shown in Fig. 2, GRP78/BiP protein expression was markedly attenuated following lumbrokinase, unlike in the vehicle treatment. IRE1 is the most conserved ER stress sensor, and it exerts neuroprotective effects in rats after ischemic stroke [10]. Immunoblotting revealed that post-stroke treatment with lumbrokinase significantly decreased the levels of IRE1 phosphorylation, the unspliced form of X-box binding protein 1 (XBP1) protein (XBP1u), and the active/spliced form of XBP1 protein (XBP1s) compared with the vehicle treatment (Fig. 2A). Immunofluorescence microscopy showed that the relative fluorescence intensity of p-IRE1 was significantly decreased in the peri-infarct area following the post-stroke lumbrokinase administration compared with the vehicle treatment (Fig. 2B). This suggests that the neuroprotective effect of post-stroke lumbrokinase treatment may occur through the mediation of stroke-induced ER stress through the IRE1 signaling pathway.

3.3 Post-stroke lumbrokinase treatment reduced apoptosis and autophagy in mice subjected to ischemic stroke

Apoptosis is the main cause of neuronal death following acute brain ischemia [27]. We investigated whether the neuroprotective effects of post-stroke lumbrokinase treatment are regulated by an anti-apoptosis response and whether the mechanism is mediated by ER stress-induced apoptosis or mitochondria-associated apoptosis. We found similar levels of expression of the pro-form of caspase-12 (an ER stress-induced apoptosis-related protein) between the vehicle-treated and lumbrokinase-treated groups, whereas the cleaved form of caspase-12 was significantly decreased following the post-stroke lumbrokinase administration compared with the vehicle treatment (Fig. 2A). Conversely, the anti-apoptotic protein expression of Bcl-2 and the ratio of Bcl-2/Bax were significantly increased, whereas the pro-form of caspase-3, the active form of caspase-3, cleaved-caspase-3, and Cyt c were all significantly decreased in the lumbrokinase-treated mice compared with the vehicle-treated mice (Fig. 3A). Furthermore, evidence indicates that ER stress is linked to autophagy in ischemic stroke [28, 29]. The levels of autophagy-related protein expression of Beclin-1, p62, and LC3-II were significantly reduced with the post-stroke lumbrokinase treatment compared with the vehicle treatment (Fig. 3B). These results suggest that post-stroke lumbrokinase treatment significantly attenuates mitochondria-associated apoptosis and ER stress-induced apoptosis and autophagy in ischemic stroke.

3.4 Post-stroke lumbrokinase treatment reduced inflammasome formation and inflammation in MCAO mice

The p-NF- κ B and COX-2 expression levels were significantly decreased by lumbrokinase treatment compared with the vehicle treatment (Fig. 4A). The results of gelatin zymography revealed no change in MMP-2 activity following lumbrokinase or vehicle treatment, whereas MMP-9 activity was significantly

decreased in the lumbrokinase-treated mice (Fig. 4A). Studies have shown that the NLRP3 inflammasome mediates inflammatory responses following cerebral ischemia [30, 31]. Thus, we examined the role of the NLRP3 inflammasome in the neuroprotective effect of post-stroke lumbrokinase treatment on ischemic stroke. Immunoblotting revealed that post-stroke treatment with lumbrokinase significantly decreased the levels of the NLRP3 inflammasome formation, cleaved-caspase-1, and IL-1 β , and significantly increased the levels of caspase-1 compared with the vehicle treatment (Fig. 4B). GFAP is a biomarker for the activation of astrocytes and is also a brain-specific protein that is released following injury or stress in the central nervous system [32]. As shown in Fig. 4C, the relative fluorescence intensity of GFAP was markedly attenuated following lumbrokinase compared with the vehicle treatment. These results demonstrate that post-stroke treatment with lumbrokinase attenuates stroke-induced the NLRP3 inflammasome formation and neuroinflammatory responses.

3.5 Post-stroke lumbrokinase treatment reduced the phosphorylation of IRE1 in neurons and astrocytes

We confirmed that the cellular localization of p-IRE1 in the neuroprotective effects of post-stroke lumbrokinase treatment and the immunofluorescence assay occurred in the vehicle group and the LK-1 group. Post-stroke lumbrokinase treatment significantly decreased p-IRE1 co-localized with not only the neuronal marker NeuN but also the astrocytic marker GFAP in the peri-infarct cortex compared with the vehicle group (Fig. 5). This indicates that the downregulation of the p-IRE1 expression in neurons and astrocytes may be involved in the neuroprotective effects of post-stroke lumbrokinase treatment.

4. Discussion And Conclusion

This study shows the neuroprotective effects of lumbrokinase in mice subjected to ischemic stroke. Post-stroke treatment with 1 mg/kg lumbrokinase significantly reduced the infarct volume and significantly improved the neurologic function, as determined by the rotarod performance, grip score, and contact and removal times of the adhesive removal test in mice after permanent MCAO. Post-stroke lumbrokinase treatment also significantly reduced mitochondria-associated apoptosis, ER stress-induced apoptosis, autophagy, NLRP3 inflammasome formation, and inflammatory effects through the IRE1 signaling pathway in cerebral tissue among ischemic stroke mice. The data in Fig. 6 summarize the effect of post-stroke treatment with lumbrokinase in mice subjected to ischemic stroke. This study is the first to suggest that the IRE1 signaling pathway plays a critical role in the neuroprotective effects of lumbrokinase.

Ischemic stroke-induced damage is a complex pathology involving a cascade of cellular mechanisms that deregulate proteostasis and lead to neuronal death. ER stress contributes to the development of cerebral ischemic injury. Under ER stress conditions, three pivotal transmembrane proteins (IRE1, PERK, and ATF6) are isolated from the GRP78/BiP to become functional, initiating ER stress through their respective signal transduction pathways [33]. In the present study, lumbrokinase significantly reduced stroke-induced ER stress, as evidenced by the decrease in GRP78/BiP and the phosphorylation of IRE1. IRE1 can trigger the acceleration of cell apoptosis-promoting pathways regulated by the caspase-12

pathway [34]. Once caspase-12 is activated, it transfers from the ER to the cytoplasm and indirectly activates cytoplasmic caspase-3, eventually mediating cell apoptosis. In the present study, post-stroke treatment with lumbrokinase markedly decreased the stroke-induced activation of caspase-12 and caspase-3. Our results reveal that post-stroke lumbrokinase treatment antagonizes stroke-induced injury by attenuating ER stress-induced apoptosis through the IRE1-caspase-12 axis.

Recent research has demonstrated that ER stress is closely related to the activation of autophagy, which is a critical mechanism for maintaining cellular homeostasis [35]. Feng et al. observed that the expression of ER stress markers increased in association with an increase in autophagy markers in a mouse model of transient MCAO, indicating that the activation of autophagy is associated with ER stress [9]. Other studies have shown that stroke-induced autophagy is significantly blocked by an ER stress inhibitor (4-phenyl butyric acid) and ER-related signaling inhibitors (GSK2656157 and 3, 5-dibromosalicylaldehyde), suggesting that the activation of autophagy depends on ER stress by detecting the expression of autophagy markers, such as LC3-II, Beclin-1, and p62 [36, 37]. Similarly, we found that lumbrokinase significantly decreased the downstream IRE1 pathway XBP1 expressions and autophagy markers (LC3-II, Beclin-1, and p62). During ER stress, *XBP1* mRNA is subjected to unconventional splicing in an *IRE1*-dependent manner and binds directly to the *Beclin-1* promoter, triggering autophagy [38]. Thus, we suggest that post-stroke lumbrokinase treatment regulates the IRE1-XBP1 axis to decrease ER stress-mediated autophagy against stroke-induced injury.

Several studies have reported that inflammation is a crucial factor in the pathologies of several vascular diseases, including cerebral ischemia [39, 40]. Inflammasomes are the key mediators and master switches of inflammation. Increasing evidence has reported that the NLRP3 inflammasome plays a well-characterized role in mediating neuroinflammatory responses in cerebral ischemia [41]. The NLRP3 inflammasome mediates inflammation and innate immunity responses by triggering the activation of caspase-1 and subsequently the maturation of the proinflammatory cytokine IL-1 β [42]. ER stress is an endogenous trigger for NLRP3 inflammasome activation [43] and that it is frequently accompanied by the activation of NF- κ B [44]. The IRE1 pathway reportedly regulates NF- κ B [45]. It is an important downstream molecule of the IRE1 pathway, XBP1, and also controls the NLRP3 inflammasome-mediated maturation and secretion of IL-1 β [46]. As expected, the post-stroke treatment with lumbrokinase significantly decreased the XBP1 and p-NF- κ B levels, significantly decreasing the NLRP3 inflammasome, cleaved-caspase-1, and IL-1 β compared with the vehicle treatment. These results indicate that post-stroke lumbrokinase treatment effectively ameliorates the ER stress-associated NLRP3 inflammasome activation and inflammation reaction through the IRE1-NF- κ B and IRE1-XBP1 axes.

In conclusion, our results indicate that post-stroke treatment with lumbrokinase exerts neuroprotection by antagonizing stroke-induced injury, significantly reducing the cerebral infarcted zone and improving neurologic function in mice subjected to permanent MCAO. Our findings support the argument that IRE1 plays a central role in stroke-induced injury by interacting with multiple factors, such as caspase-12, XBP1, and NF- κ B, to modulate apoptosis, autophagy, inflammatory responses, and NLRP3 inflammasome formation. Reducing disability is a major therapeutic goal after ischemic stroke.

According to our evidence, lumbrokinase shows good therapeutic potential for restoring neurologic function after ischemic stroke. The neuroprotection of lumbrokinase may be mediated by ER stress through the IRE1 signaling pathways to decrease apoptosis, autophagy, and inflammatory responses.

Abbreviations

ATF6, activating transcription factor 6

ER, endoplasmic reticulum

GRP78/BiP, glucose-regulated protein 78/immunoglobulin heavy-chain-binding protein

IRE1, inositol-requiring enzyme-1

LK, lumbrokinase

MCAO, middle cerebral artery occlusion

NF- κ B, nuclear factor kappa B

NLRP3, NOD-like receptor 3

PERK, protein kinase RNA-like endoplasmic reticulum kinase

UPR, unfolded protein response

XBP1, X-box binding protein-1

Declarations

Ethics approval and consent to participate

All surgical procedures for permanent MCAO and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University (IACUC 1637).

Consent for publication

The manuscript has neither been published nor is currently under consideration for publication by any other journal. All authors have read and approved the final version of the manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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Author Contributions

Y.H. Wang designed and performed the experiments, analyzed the data, and wrote and reviewed the manuscript; S.S. Huang, H.H. Su and P.H. Liu assisted with the animal experiments; J.M. Liao and K.M. Chen and Y.H. Chen contributed new reagents or analytic tools, analyzed data, helped with data interpretation, and reviewed the manuscript; C.F. Tsai and S.S. Huang assisted with the design of the project and edited the manuscript.

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Figures

Figure 1

Post-stroke treatment with lumbrokinase attenuates cerebral infarction and improved neurological functioning after ischemic stroke in mice. Mice were subjected to permanent MCAO followed by treatment with vehicle or lumbrokinase (0.1 mg/kg or 1 mg/kg) for 7 consecutive days. (A) TTC staining was performed to quantify infarct volumes (n=8–11). Behavioral function tests (B) rotarod, (C) grip, and (D-E) adhesive-removal task before and after MCAO on days 1 and day 3 (n=5–18). Results are expressed as the mean $\pm$ SEM. *p<0.05 compared with vehicle treatment.

Figure 2

Post-stroke treatment with lumbrokinase 1 mg/kg reduced endoplasmic reticulum stress in mice subjected to ischemic stroke. (A) Representative images of the Western blot results and quantitative densitometric analysis of GRP78/BiP, IRE1, p-IRE1, XBP1u, XBP1s, pro-caspase-12 and cleaved-caspase-12 protein expression in brain tissue after ischemic stroke. (B) Representative images of the immunofluorescence stain results and quantitative relative fluorescence intensity of p-IRE1 in the peri-infarct cortex following ischemic stroke. Each value is represented as the mean $\pm$ SEM (n=6). *p<0.05 compared with vehicle treatment.

Figure 3

Post-stroke lumbrokinase treatment reduced apoptosis and autophagy in mice subjected to ischemic stroke. (A) Representative images of Western blot results and quantitative densitometric analysis of Bcl-2, Bax, Cyt c, pro-caspase-3, and cleaved-caspase-3 protein expression. (B) Representative images of Western blot results and quantitative densitometric analysis of Beclin-1, p62, and LC3-I/II protein expression in mice brain after ischemic stroke. Each value is represented as the mean $\pm$ SEM (n=6). *p<0.05 compared with vehicle treatment.

Figure 4

Post-stroke lumbrokinase treatment reduced inflammation in mice subjected to ischemic stroke. (A) Representative images of the immunoblot results and quantitative densitometric analysis of NF-κB, p-NF-κB and COX-2 protein expression, and MMP-2, MMP-9 activity. (B) Representative images of the immunoblot results and quantitative densitometric analysis of NLRP3, pro-caspase-1, cleaved-caspase-1 and IL-1β protein expression in mouse brain after ischemic stroke. (C) Representative images of the immunofluorescence stain results and quantitative relative fluorescence intensity of GFAP in the peri-infarct cortex following ischemic stroke. Each value is represented as the mean ± SEM (n=6). *p<0.05 compared with the vehicle group.

Figure 5

Post-stroke lumbrokinase treatment reduced phosphorylation of IRE1 in neurons after ischemic stroke. Representative images of the immunofluorescence stain results and quantification images of the number of p-IRE1 and (A) NeuN (B) GFAP double positive cells per low power field (200X). Scale bar: 50 μ m. Each value is represented as the mean $\pm$ SEM (n=6). *p<0.05 compared with vehicle treatment.

Lumbrokinase — ischemic stroke

Figure 6

Schematic diagram of the effects of post-stroke lumbrokinase treatment in ischemic stroke. Post-stroke lumbrokinase treatment attenuates ischemic stroke induced-injury by decreasing ER stress and thereby reduces apoptosis, autophagy, NLRP3 inflammasome formation and inflammation.