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Endoplasmic reticulum stress is associated with neuroprotection against apoptosis via autophagy activation in a rat model of subarachnoid hemorrhage

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HIGHLIGHTS

- We explored the effect of ER stress on rats subjected to subarachnoid hemorrhage.
- Activation of ER stress improved neurological deficits and reduced cell apoptosis.
- ER stress inducer Tm promoted autophagy, while ER stress inhibitor TUDCA reduced autophagic activity.
- Suppression of autophagic activity with 3-MA resulted in the inhibition of ER stress-induced protective effect.

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ABSTRACT

Endoplasmic reticulum (ER) stress might play an important role in a range of neurological diseases; however, this phenomenon's role in subarachnoid hemorrhage (SAH) remains unclear. In this study, we explored the potential role of endoplasmic reticulum stress in early brain injury following SAH.84 rats were used for an endovascular perforation-induced subarachnoid hemorrhage model. The rats were intraperitoneally pretreated with the ER stress inducer tunicamycin (Tm) or with the inhibitor tauroursodeoxycholic acid (TUDCA) before SAH onset. An intracerebral ventricular infusion of autophagy inhibitor 3-methyladenine (3-MA) was also used to determine the relation between autophagy and ER stress in early brain injury following SAH. At 24 h, rats were neurologically evaluated, and their brains were extracted for molecular biological and histological studies. ER stress was activated in rats after 24 h of SAH. Enhanced ER stress via Tm pretreatment significantly improved neurological deficits, attenuated the expression of pro-apoptotic molecules of caspase-3 and reduced the number of TUNEL-positive cells. In contrast, the ER stress inhibitor TUDCA aggravated neurological deficits and apoptotic cell death. Western blot analysis revealed that levels of the autophagic protein Beclin 1 and the ratio of LC3-II to LC3-I were both increased by Tm infusion and reduced by TUDCA administration. The suppression of autophagic activity with 3-MA attenuated Tm-induced anti-apoptotic effects. Our study indicates that ER stress alleviates early brain injury following SAH via inhibiting apoptosis. This neuroprotective effect is most likely exerted by autophagy activation

Introduction

Subarachnoid hemorrhage (SAH) is a devastating disease with high mortality and significant morbidity among survivors. Past research efforts have traditionally focused on vasospasm, which is considered the most important determinant of brain injuries and outcomes after SAH. However, several studies indicated that anti-vasospastic treatments fail to improve outcomes in SAH patients [1–3]. These findings have finally clarified the influence of early brain injury on the outcome of SAH. Recently, the possible process of early brain injury, including increased intracranial pressure, decreased cerebral blood flow, blood–brain barrier disruption, inflammation, oxidative stress and cell death, have been systematically studied [4,5]. However, definitive mechanisms of early brain injury after SAH remain unclear, and cell apoptosis has been reported to be an important factor in this mechanism [6].

The endoplasmic reticulum(ER) is necessary for synthesizing and folding all secreted and membrane proteins. Many pathophysiological conditions that impair its function result in cell apoptosis [7,8]. The accumulation of incorrectly folded proteins in the ER lumen induces a state that is known as ER stress. To restore a stable cellular environment, ER stress triggers a series of cellular

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responses. The ability of cells to respond to ER stress is critical for cell survival. ER stress has been implicated in a range of neurological diseases, including cerebral ischemia, Alzheimer's disease and multiple sclerosis. Researchers suggest that ER stress is induced in cerebral ischemia and that ER stress-mediated responses inhibit neuronal apoptosis [7]. The overproduction of the amyloid β protein is considered to be the major cause of Alzheimer's disease, and the activation of ER stress helps to eliminate the unwanted amyloid β protein and protect against cell death [9]. Mild ER stress activates autophagy and inhibits neuronal death in mouse models of Parkinson disease [10]. However, severe ER stress might violate Ca^{2+} homeostasis and contribute to neuronal cell death [11]. ER stress seems to play dual roles in cell fate, and the mechanism that elicits ER stress-mediated protection remains to be explored. In this study, we used pharmacological approaches to modulate ER stress in a rat model of SAH and to determine whether ER stress confers neuroprotection in SAH. We investigated whether autophagy activation participates in ER stress-induced protection in early brain injury following SAH.

Materials and methods

Study protocol

Male Sprague–Dawley rats (280–300 g) were purchased from the SLAC Laboratory Animal Co. Ltd (Shanghai, China). All experimental procedures were approved by the ethics committee of Zhejiang University and followed the NIH guidelines for the Care and Use of Laboratory Animals. The study used two sets of rats. The first set of 24 rats was randomly assigned to 4 groups: the sham-operated group, the SAH untreated(vehicle) group, the SAH treated with ER stress inducer tunicamycin (Tm) group, and the SAH treated with ER stress inhibitor tauroursodeoxycholic acid (TUDCA) group. At 24 h after SAH, these rats were first evaluated for SAH grades and neurological deficits, then euthanized and used for ER stress-related/autophagy-related protein immunoblotting experiments. The second set of 60 rats was randomly divided into 5 groups: the sham-operated group, the SAH untreated(vehicle)group, the SAH treated with ER stress inducer Tm group, the SAH treated with TUDCA group, and the SAH treated with Tm+ autophagy inhibitor 3-methyladenine(3-MA) group. At 24 h, these rats were first evaluated for SAH grades and neurological deficits, then euthanized and used for apoptosis-related experiments. Animals that died after surgery were replaced until the final group size reached the expected number in each group.

Rat SAH model

The rat SAH monofilament puncture model was performed as previously described [12]. Briefly, rats were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg). The carotid artery and its branches were dissected, and then a blunted 4-0 monofilament nylon suture was advanced via the external carotid artery into the internal carotid artery until resistance was felt. The filament perforated 3 mm to create a SAH. In the sham surgery group, a similar procedure was performed without perforation. SAH was confirmed by autopsy in each rat.

Evaluation of SAH grades and neurological deficits

We used a previously published grading scale to confirm the equivalent level of SAH severity across groups [13]. The scale was based on the amount of subarachnoid blood in 6 segments of the basal cistern as follows: grade 0, no subarachnoid blood; grade 1, minimal subarachnoid blood; grade 2, moderate blood with visible arteries; grade 3, blood clot covering all arteries within the segment. A total score that ranged from 0 to 18 was obtained after adding the scores from all 6 segments. Neurological testing was performed with an 18-point scoring system in a blinded fashion [14]. Briefly, spontaneous activity, symmetry in the movement of four limbs, forepaw outstretching, climbing, body proprioception, and response to vibrissae touch were tested. These six tests were each scored from 0 to 3, and behavioral deficits were graded on a total score from 0 to 18.

Drug administration

Tunicamycin (Tm, Sigma-Aldrich Inc, St. Louis, MO, USA) was dissolved in DMSO (1 mg/ml) and administered by intraperitoneal (I/P) injection (4.5 mg/kg) 18 h and at 1 h before SAH. The rats in the TUDCA intervention group were administered 250 mg/kg TUDCA (Sigma-Aldrich Inc, St. Louis, MO, USA) saline solution (100 mg/ml) intraperitoneally (i.p.) at1 h before the operation. The dose and method of Tm and TUDCA infusion was chosen according to previous studies [10,15].The autophagy-inhibited rats were positioned in a stereotactic frame and injected with 3 μl 3-MA (0.1 mol/l, Sigma-Aldrich Inc, St. Louis, MO, USA) intraventricularly at 30 min before SAH onset. The vehicle group rats were injected identically with the same volume of the vehicle (DMSO) relative to body weight.

Western blot analysis

Rats were sacrificed 24 h after SAH induction, and cerebral cortical samples facing blood clots were extracted. The Western blot was performed as described previously [16]. Briefly, a cortical sample was homogenized in buffer and centrifuged at 1000*g* for 10 min at 4 °C. The resulting supernatants were further centrifuged, and protein concentrations were measured using a BCA kit (Pierce, 23227, Rockford, US). An equal amount of protein (60 μg) was resuspended in loading buffer and loaded onto sodium dodecyl sulfate–polyacrylamide gels. The proteins were electrophoresed at 80 V for 3 h and transferred to polyvinylidene fluoride membranes at 100 V for 2 h. The membranes were incubated overnight at 4 °C with primary antibodies for GRP78 (1:1000, sc-1051, Santa Cruz Biotechnology), CHOP (1:800, sc-793, Santa Cruz Biotechnology), Caspase-3 (1:1000, sc-22140, Santa Cruz Biotechnology), LC3 (1:1000, Cell Signaling Technology), Beclin 1(1:1000, Abcam, ab16998) and β-actin (1:2000, Santa Cruz Biotechnology). The membranes were processed with horseradish-peroxidase-conjugated secondary antibodies at room temperature for 1 h. The protein band densities were detected using X-ray film and quantified using the Image J software (NIH). The results were normalized to the sham-operated group.

TUNEL staining and quantitative cell count

At 24 h after SAH, rats ($n = 6$) from each group were anesthetized and transcardially perfused with 0.1 mmol PBS followed by 4% paraformaldehyde (pH 7.4). Brains were collected and placed at 4 °C in the same fixative for 2 days. Coronal frozen sections (10 μm) were placed onto slides. TUNEL staining was performed to detect apoptotic cell death according to the manufacturer's protocol (Roche Inc., Basel, Switzerland) and examined under a laser scanning confocal microscope (LSM-710; Zeiss). TUNEL-positive cells were calculated in a blind manner; the total number of cells (DAPI+) and the TUNEL-positive cells were counted in five separate fields in four different slices.

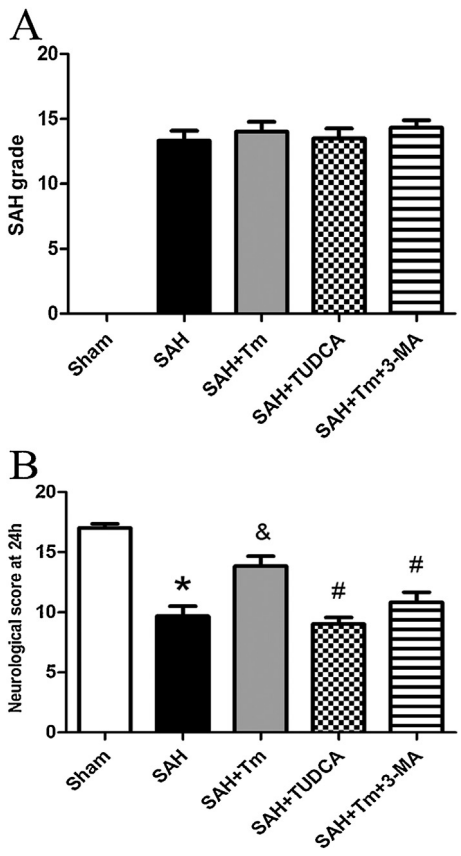

Fig. 1. SAH grade and neurological scores in a rat model of SAH after drug treatments. (A) Quantification of SAH severity. Bars represent the mean ± SD, $n = 18$; (B) Quantification of neurologic scores. Bars represent the mean ± SD, $n = 18$, * $P < 0.05$ vs. the shamgroup, & $P < 0.05$ vs. the SAH + vehicle group, # P< 0.05 vs. the SAH + Tm group.

Statistical analysis

The data were presented as the means ± the standard error of the mean. Statistical significance was verified by a one-way analysis of variance (ANOVA), which was followed by Dunnett's test for multiple comparisons. The probability level $P < 0.05$ was considered statistically significant.

Results

Physiological data and SAH severity

All physiological parameters were monitored during the surgical procedure. The mean arterial pressure (85–125 mmHg), arterial pH (7.35–7.45), PO_2 (80–95 mmHg), PCO_2 (35–45 mmHg) and blood glucose levels (95–125 mg/dl) remained with normal ranges and were not significantly different between the groups (data not shown). At the end, rats were sacrificed, and brains were collected. Subarachnoid blood clots were found on the ipsilateral side, around the circle of Willis and on the ventral brainstem. The mean SAH scores were 0 in the sham group, 13.3 ± 3.5 in the SAH + vehicle group, 14.0 ± 3.6 in the SAH + Tm group, 13.5 ± 3.5 in the SAH ± TUDCA group, and 14.3 ± 1.9 in the SAH + Tm + 3-MA group (Fig. 1A). There was no significant difference in the severity scores across the groups ($P > 0.05$).

Mortality and neurological scores

None of the sham-operated rats died. In total, 33.3% (9/27) mortality was recorded in the SAH + vehicle group, whereas Tm treatment significantly reduced the mortality to 18.1% (4/22). Tm + 3-MA treatment markedly increased the mortality to 29.4% (5/17), and there was a significant difference in the mortality between the SAH + Tm + 3-MA group and the SAH + Tm group ($P < 0.05$). The mortality in the TUDCA treatment group was 35.7% (10/28), which was higher than that of the SAH + Tm group and had no difference compared with the SAH + vehicle group. Neurological scores of survival rats were recorded at the end. Neurological scores in the SAH + vehicle group were significantly lower than that in the sham group ($n = 18$, $P < 0.05$, Fig. 1B). Tm-treated rats ($n = 18$) exhibited an improved neurological status compared with those rats in the SAH + vehicle group ($n = 18$, $P < 0.05$, Fig. 1B). Tm + 3-MA treatment markedly reduced the neurological scores to 11($n = 12$), and there was a significant difference between the SAH + Tm + 3-MA group and the SAH + Tm group ($n = 18$, $P < 0.05$, Fig. 1B). The neurological score in the TUDCA treatment group was 9 ($n = 18$), which was not different when compared with the SAH + vehicle group but was significantly lower than that in the Tm-treated group ($n = 18$, $P < 0.05$, Fig. 1B).

ER stress is activated 24 h after SAH

GRP78 and CHOP are biomarkers of ER stress in mammalian cells. The Western blot revealed that the protein levels of GRP78 and CHOP both increased in the SAH + vehicle group ($n = 6$, $P < 0.05$ vs. the sham group, Fig. 2A and B). Tm treatment further upregulated the expression levels of GRP78 and CHOP ($n = 6$, $P < 0.05$ vs. the SAH + vehicle group, Fig. 3A and B), whereas TUDCA treatment significantly reduced their levels ($n = 6$, $P < 0.05$ vs. the SAH + vehicle group, Fig. 2A and B).

ER stress inducer Tm ameliorated apoptotic cell death following SAH

Apoptotic cell death was detected by Western blotting of cleaved caspase-3 and by TUNEL staining at 24 h post-SAH. Caspase-3 activation triggers the cleavage of many proteins and ultimately leads to DNA fragmentation and apoptosis [17]. Cleaved caspase-3 levels in the cortex were markedly increased in the SAH + vehicle group compared with the sham-operated group ($P < 0.05$, Fig. 3G). Tm treatment significantly reduced the protein levels of cleaved caspase-3 ($P < 0.05$ vs. the SAH + vehicle group, Fig. 3G). TUNEL staining was used to detect apoptotic cells in the injured brain (Fig. 3). In sham-operated rats, no TUNEL-positive cells were detected. Numerous TUNEL-positive cells were observed in the cortex of SAH + vehicle rats, and its apoptotic index was significantly increased compared with the sham-operated group ($P < 0.05$, Fig. 3F). Tm treatment grossly reduced the number of TUNEL-positive cells ($P < 0.05$ vs. the SAH + vehicle group, Fig. 3F).

ER stress inhibitor TUDCA attenuates Tm's anti-apoptosis effect in SAH

Western blot analysis showed that the expression levels of cleaved caspase-3 in the TUDCA treatment group was significantly upregulated compared with the SAH + Tm group ($P < 0.05$, Fig. 3G). Many TUNEL-positive cells were detected in the cortex of TUDCA-treated rats, and quantitative analysis demonstrated that TUDCA treatment markedly increased the apoptotic index compared with the SAH + Tm group ($P < 0.05$, Fig. 3F).

ER stress-mediated autophagy activation in SAH

Beclin 1and LC3 are two important biomarkers for autophagy activation in mammalian cells. The Western blot analysis

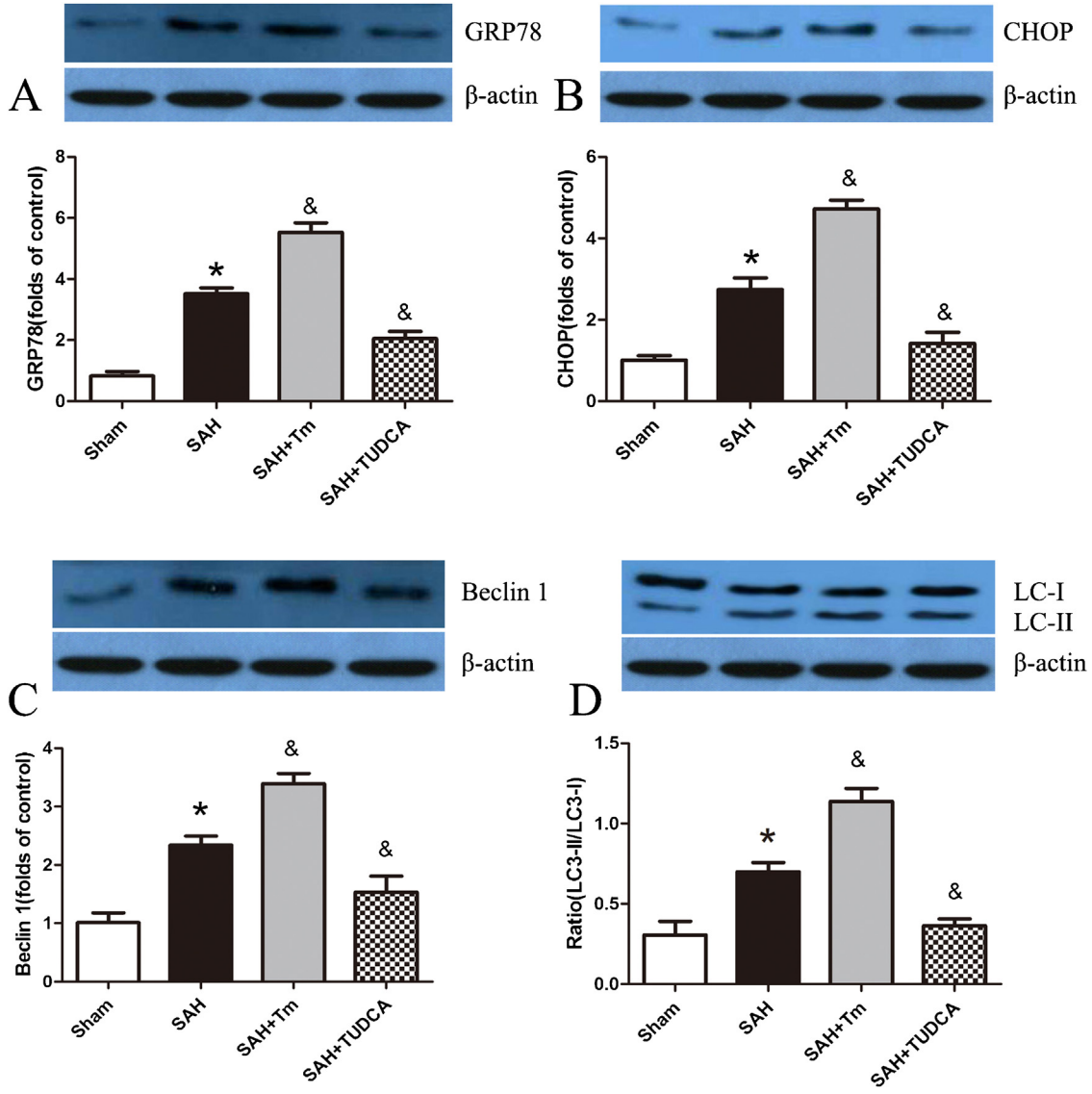

Fig. 2. ER stress levels and autophagic activity in rats after 24 h of SAH. (A-D) Protein levels of GRP78, CHOP, Beclin 1 and LC3 in the ipsilateral cortex 24 h after SAH. Densities of protein bands were analyzed and normalized to β-actin. The data are expressed as a percentage of the sham control. Bars represent the mean ± SD, $n = 6$. * $P < 0.05$ vs. sham group, & $P < 0.05$ vs. the SAH + vehicle group.

demonstrated that the protein level of Beclin 1 and LC3-II were both increased at 24 h after SAH ($P < 0.05$ vs. the sham-operated group). The pretreatment of Tm significantly upregulated Beclin 1 and LC3-II expression compared with the SAH group ($P < 0.05$, Fig. 2C and D). However, the increase in Beclin 1 and LC3-II in the cortex were inhibited by TUDCA treatment ($P < 0.05$ vs. the SAH + vehicle group, Fig. 2C and D).

Autophagy activation is required for ER stress-induced neuroprotection

Autophagy and cell death are both highly conserved cellular processes [18]. We chose to study the contribution of autophagy to the ER stress-induced protection against apoptotic cell death in SAH, because previous results have shown that inhibiting autophagy with 3-MA markedly increased mortality and neurological deficits in Tm-treated rats ($P < 0.05$ vs. the SAH + Tm group, Fig. 1B).The Western blot analysis and TUNEL staining demonstrated that Tm +3-MA-treated rats underwent dramatic apoptotic cell death ($P < 0.05$, Fig. 3F) and have an increased expression level of cleaved caspase-3 compared with rats that were treated with Tm alone ($P < 0.05$, Fig. 3G).

Discussion

The results of this study indicated that enhancing ER stress provides neuroprotection in the rat model of subarachnoid hemorrhage, and autophagy was a downstream mediator of the ER stress-induced protective effect. The activation of ER stress improved neurological deficits in rats that were subjected to SAH, attenuated the expression of pro-apoptotic molecules of caspase-3 and reduced cell apoptosis. The ER stress inducer Tm promoted autophagy, upregulated the autophagic protein Beclin 1, and converted LC3-I to LC3-II, whereas the ER stress inhibitor TUDCA reduced autophagic activity. The suppression of autophagic activity with 3-MA resulted in the inhibition of the ER stress-induced protective effect.

The endoplasmic reticulum(ER) is an organelle in which proteins are synthesized and folded. Insults that impair its function result in the accumulation of unfolded or misfolded proteins in

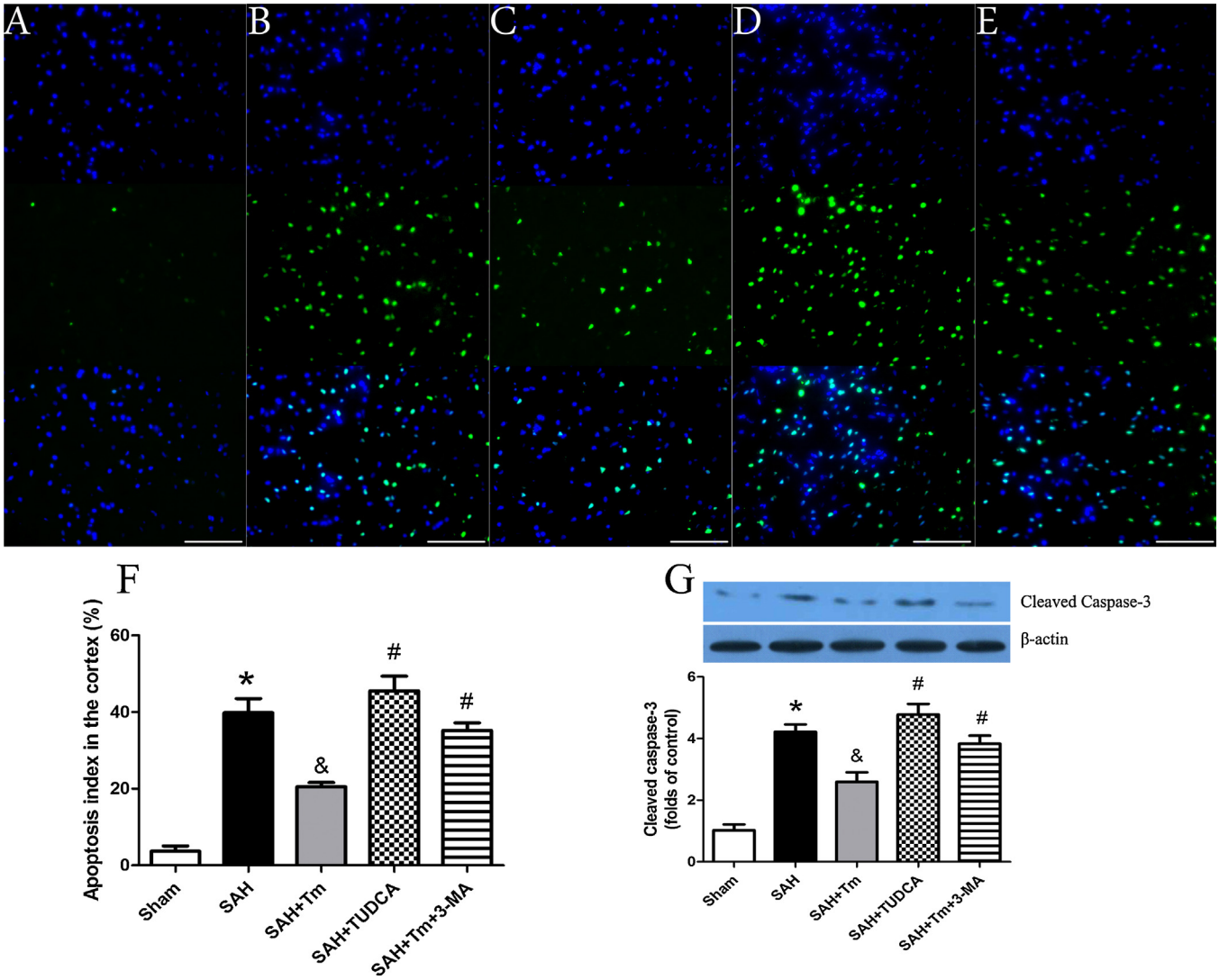

Fig. 3. Effects of Tm, TUDCA or 3-MA on apoptosis in rats 24 h after SAH. (A–E) Representative TUNEL photomicrographs of the ipsilateral cortex in different groups (scale bar = 100 μm). (F) Quantification of TUNEL results, which are expressed as the percentage of total (DAPI+) cells. Bars represent the mean ± SD, $n = 6$. * $P < 0.05$ vs. the sham group, $^{\&}$ $P < 0.05$ vs. the SAH + vehicle group, and $^{\#}$ $P < 0.05$ vs. the SAH + Tm group. (G) Protein levels of cleaved caspase-3 in the ipsilateral cortex 24 h after SAH. The data are expressed as the percentage of the sham control. Bars represent the mean ± SD, $n = 6$. * $P < 0.05$ vs. the group, $^{\&}$ $P < 0.05$ vs. the SAH + vehicle group, and $^{\#}$ $P < 0.05$ vs. the SAH + Tm group.

the ER lumen, which activates ER stress. ER stress is a regulatory mechanism that allows cells to adapt to a series of metabolic, redox, and other environmental changes. This mechanism, which is called the unfolded protein response (UPR), is critical for cell survival [19]. The UPR involves three distinct major pathways through three ER-localized transmembrane proteins, namely, pancreatic ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). In the absence of stress, the above three proteins associate with the 78 kDa glucose-regulated protein (GRP78) and remain in an inactive state. Under pathophysiological conditions, GRP78 dissociated from these receptors and resulted in the activation of these proteins. The translational and transcriptional components of UPR protect cells from apoptosis [7]. In Drosophila and mouse models of Parkinson's disease, ER stress reduced neuron apoptosis and promoted neurological improvement [10]. The enhanced activation of ER stress in oligodendrocytes significantly attenuated the severity of experimental autoimmune encephalomyelitis disease and was associated with reduced oligodendrocyte apoptosis, demyelination, and axonal degeneration [20]. In human melanoma cells, increased ER stress up-regulated anti-apoptotic Bcl-2 protein level and limit the induction of apoptosis [21]. Our study indicated that cells underwent apoptosis at an early stage of SAH, and enhanced ER stress alleviated early brain injury following SAH via the inhibition of apoptosis. Mortality and neurological deficits after SAH have been ascribed to this excessive cell apoptosis. In this study, the neuroprotection of ER stress was accompanied by neurological improvement in the treated rats. This study expands the protective effect of ER stress in rat model of SAH.

This study showed that SAH alone induced an increase level of GRP78 and CHOP, and ER stress activation have existed in SAH + vehicle rats. However, aggravated mortality and neurological scores were still observed in vehicle-treated SAH group. The explanation might be that other toxic mechanism of SAH was so strong that the cells still underwent apoptosis. And the present study demonstrated that mild ER stress activation might existed little impact on SAH rats, as ER stress inhibitor TUDCA did not aggravated SAH rats mortality and neurological scores. However, when treated with Tm, ER stress was further enhanced and cell apoptosis

was inhibited. These results implied that only enhanced ER stress conferred neuroprotection in rats model of SAH. Further studies are needed to detect the role of ER stress in SAH.

A candidate mechanism for this ER stress-mediated neuroprotection is autophagy. Autophagy is a self-digesting process that has an important role in maintaining cellular homeostasis and survival under stress. Many studies have reported that ER stress stimulates autophagy, which removes unwanted components in the cytoplasm and is believed to be beneficial for cell survival [22]. In an earlier study, we also demonstrated that autophagy activation is associated with neuroprotection against apoptosis in a rat model of SAH [23]. This study showed that an ER stress inducer also increased autophagic activity in rats that were subjected to SAH, whereas an ER stress inhibitor suppressed this effect. In the current study, specifically inhibiting autophagy with 3-MA activated apoptotic cascades, aggravated neurological deficits and attenuated the ER stress-induced protective effect. These findings support the hypothesis that enhanced ER stress protects against cell death via autophagy activation in this rat model of SAH.

Several previous studies investigated the link between ER stress and autophagy. However, much remains to be explored regarding how ER stress activates autophagy and promotes neuroprotection. Many studies have reported that both ER stress and autophagy are primarily pro-survival mechanisms [10,24]. However, some studies have argued that excessive ER stress and autophagy might promote cell apoptosis under certain circumstances [25,26]. Both ER stress and autophagy seem to play dual roles in cell fate, depending on specific pathophysiological conditions. Further exploration is required for better understanding the role of ER stress and autophagy in early brain injury following SAH.

In conclusion, our present results strongly suggest that enhanced ER stress plays a protective role in early brain injury following SAH. This neuroprotective effect was associated with autophagy activation. Targeting ER stress and autophagy seems to be a promising therapeutic strategy for SAH and deserves further investigation.

Acknowledgments

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