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Botulinum toxin type A activates protective autophagy by modulating endoplasmic reticulum stress in hypoxia/reoxygenation-treated endothelial cells

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ABSTRACT

Botulinum toxin type A (BTXA) previously protected endothelial cells in free skin flaps from ischemia/reperfusion injury by inducing autophagy. Endoplasmic reticulum (ER) stress-autophagy activation may have a role in BTXA-antagonized ischemia/reperfusion damage in human dermal microvascular endothelial cells (HDMECs), however, this has yet to be proven. HDMECs were pretreated with BTXA at various concentrations for 12 h before being subjected to hypoxia and reoxygenation (H/R). Cell Count Kit 8 (CCK8) and Western blot (WB) data showed that H/R treatment significantly increased the expression of ER stress (GRP78, CHOP) and autophagy (LC3II/I, Beclin-1) proteins. The optimal BTXA pretreatment concentration was 1.6 U/mL, which resulted in the highest levels of cell survival and expression of ER stress and autophagy. Following pretreatment with 1.6 U/mL BTXA and the addition of the ER stress inducer Thapsigargin (Tg), the ER stress inhibitor 4-phenylbutyrate (4-PBA), and the inhibitor of autophagy Bafilomycin A1 (Baf A1), respectively, HDMECs were then subjected to H/R treatment. Cell survival and the percentage of ethynyldeoxyuridine-labeled cells in the BTXA pretreatment groups were reduced by the addition of Tg, 4-PBA, and Baf A1. While the expression of GRP78, CHOP, and LC3 was upregulated by Tg and Baf A1, it was downregulated by 4-PBA. The findings showed that ER stress produced by BTXA pretreatment triggers protective autophagy and protects HDMECs from H/R damage. There were no cytoprotective effects from either excessive activation or reduction of ER stress. Our results are consistent with the notion that autophagy and moderate ER stress are critical for cellular longevity and, consequently, functional integrity and may represent a potential therapeutic target.

1. Introduction

Free skin flap transplantation is a kind of effective strategy to treat skin defects, which can achieve the purpose of eliminating wounds and repairing deformities. However, the damage due to skin flap procedures has not been well solved, mainly due to flap ischemia/reperfusion (I/R) injury and subsequent flap necrosis and failure [1].

I/R injury is mainly mediated by oxidative stress, intracellular and mitochondrial calcium overload, and inflammatory cell accumulation in damaged flaps [1]. For reducing I/R injury of flap, therapeutic strategies such as ischemia preconditioning, hyperbaric

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oxygen, hydrogen, sulfur dioxide, and pharmacological interventions have been intensively investigated [2–4]. However, there are only a few effective therapeutic strategies applied in clinical practice to reduce I/R injury.

Botulinum toxin type A (BTXA) is a polypeptide secreted by Clostridium botulinum that contains a protease and inhibits muscle contraction by blocking acetylcholine release at the neuromuscular junction and limiting norepinephrine release [5]. Recently, the clinical practice of BTXA has been expanded for hypertrophic scar, keloid, facial spasms, blepharospasm, primary facial hyperhidrosis, and facial rejuvenation [6–8]. Furthermore, there is growing evidence that BTXA can be used to treat I/R injury in a variety of animal flap and cell models [9–12]. For example, pre-surgical BTXA treatment increased angiogenesis and blood perfusion and obtained a greater proportion of the survival area of skin flaps. Several studies demonstrated that BTXA pretreatment prevented apoptosis of vascular endothelial cells and promoted flap survival in vitro [5,12].

The endoplasmic reticulum (ER) is a crucial organelle for the synthesis, modification, and processing of proteins, and its typical operations are crucial for maintaining intracellular homeostasis. But adverse stimuli such as ischemia and hypoxia can cause unfolded protein accumulation in ER and ER stress [13]. When these mechanisms fail to restore normal ER function as a result of excessive stress reactions, other stress-response pathways are activated in order to remove the damaged cells or cellular component.

It has been reported that autophagy is closely interconnected with ER stress to counteract the possible injurious effects related to the impairment of protein folding [14]. As a lysosomal-dependent catabolic pathway, autophagy significantly contributes to maintaining cellular hemostasis. Our research group previously demonstrated that BTXA pretreatment reduced the apoptosis of skin flaps after I/R treatment by activating protective autophagy [12,15]. However, the mechanism by which BTXA pretreatment activates protective autophagy remains unclear. Additionally, the effect of BTXA on I/R-induced ER stress remains unclear.

The purpose of this study was to investigate the role of ER stress-autophagy activation in BTXA antagonizing hypoxia/reoxygenation (H/R) in human dermal microvascular endothelial cells (HDMECs).

2. Materials and methods

2.1. Cell extraction and culture

HDMECs were extracted from excised flaps of 25 female patients with blepharoplasty (ages 18–40) from September 2019 to September 2022. The experimental protocols were approved by the Ethics Committee of Beijing Anzhen Hospital, Capital Medical University (Beijing, China; approval no. 2015021X). As previously described [16], flaps were washed with cold phosphate-buffered saline (PBS), trimmed to small pieces (0.5 × 0.5 × 0.1 cm), and digested with the neutral protease, dispase II (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), to remove the epidermal layer from the dermis. Then the dermis was digested using collagenase I (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to obtain a cell suspension containing HDMECs. After a week of cultivation, cells were digested with trypsin to prepare single-cell suspension and purified using the CD31 microbead kit (Miltenyi Biotec GmbH; Bergisch Gladbach, Germany). Then the cells were directly transferred into culture and subjected to a second purification. The present study was authorized by the Ethics Committee of Beijing Anzhen Hospital (approval no. 2015021X), and all patients provided signed, informed consent. HDMECs were cultured in endothelial cell medium (ECM; cat. no. 1001, ScienCell) containing 5% fetal bovine serum (FBS), 1% endothelial cell growth supplement, and 1% penicillin/streptomycin. All experiments were repeated at least three times.

2.2. H/R treatment

To mimic I/R injury in vitro, HDMECs were transferred to fresh serum-free ECM and cultured in a hypoxic incubator containing a gas mixture comprising 94% N_2, 1% O_2, and 5% CO_2 for 8 h (also called hypoxia). Then cell medium was replaced with fresh ECM containing 5% FBS, and HDMECs were incubated under normoxic oxygen (also called reoxygenation) [16].

2.3. Reagents preparation

BTXA (Botox, 100 Units, America) is available as freeze-dried power that must be stored at 2–8 °C. The powder was dissolved in ECM, then stored at 4 °C and used within 2 h. The solution was adjusted to a final concentration of 6.4 U/ml by the addition of ECM. When the cell density reaches 60%, the cells were pretreated for 12 h with BTXA. Bafilomycin A1 (Baf A1; cat. no. HY-100558, MedChemExpress) [17], as an autophagy inhibitor, primarily inhibits by suppressing the fusion of autophagosomes and lysosomes. ER stress inducer thapsigargin (Tg; cat. no. HY-13433, MedChemExpress) [18] and inhibitor 4-phenylbutyrate (4-PBA; cat. no. HY-A0281, MedChemExpress) [19] and Baf A1 were dissolved in DMSO, respectively, and then stored at −20 °C. In the last 1 and 2 h of BTXA pretreatment, Tg and Baf A1 were added at concentrations of 1 μM and 10 nM, respectively. At the beginning of hypoxia, 4-PBA with a concentration of 5 mM was added for 8 h.

2.4. Cell growth and proliferation assay

Cell Counting Kit 8 (CCK8; cat. no. C0038, Beyotime, China) was used to test the viability of HDMECs following the manufacturer's instructions. Briefly, cells were cultured in 96-well dishes at a density of 1600 cells per well. After H/R treatment, HDMECs were washed twice with PBS. 10 μL of CCK8 solution and 100 μL of ECM were completely mixed and then added to each well and incubated for 2 h at 37 °C. For the blank control, 10 μL of CCK8 solution and 100 μL of ECM were added to cell-free wells. A microplate reader

(Multiskan GO, Thermo Fisher Scientific) was used to measure optical density (OD) at 450 nm, which was then converted to cell viability rate.

The ethynyldeoxyuridine (EdU) assay was performed using the EdU Cell Proliferation Kit with Alexa Fluor 555 (cat. no. CX003, Cellor Lab, China) according to the manufacturer's instructions. Cells were cultured at a density of 1×10^4 per well in 24-well dishes. After H/R treatment, cell medium was removed, and EdU-labeled (also called EdU+) medium was added to 24-well dishes and incubated at 37 °C for 2 h. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed with PBS three times for 5 min. Then cells were permeabilized in 0.3% Triton X-100 at room temperature and washed with PBS three times for 5 min. Later, 200 μL of click-working solution was added to each well and incubated for 30 min in a dark place. After washing three times with PBS, the nuclei were counterstained with Hoechst 33,342 for 10 min at room temperature. Finally, EdU-labeled cells were shown as orange fluorescence and observed by a fluorescence microscope (Nikon DS-Ri2, China). Image processing and analysis were performed using Image J (version 1.51).

2.5. Western blot

Cells were cultured at a density of 5×10^4 cells per well in 6-well dishes. After H/R treatment, HDMECs were washed twice with cold PBS and exposed to RIPA lysis buffer (cat. no. R0020, Solarbio, China) containing a protease inhibitor for 20 min, followed by centrifugation at 16,000×g for 10 min at 4 °C. The protein concentrations were determined by the bicinchoninic acid assay kit (BCA; cat. no. 23227, Thermo Fisher Scientific, America), and the protein content was adjusted to achieve equal concentrations and volumes. Next, protein samples were analyzed by 10% or 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. Samples were then incubated with primary antibodies at 4 °C for 12 h, including anti-glucose-regulated protein 78 (GRP78; 1:1,000, cat. no. A0241, ABclonal, China), anti-C/EBP homologous protein (CHOP; 1:1,000, cat. no. A11346, ABclonal, China), anti-MAP1LC3B (LC3; 1:1,000, cat. no. A17424, ABclonal, China), anti-Beclin-1 (1:1,000, cat. no. GB112053, Servicebio, China), and anti-β-actin (1:50,000, cat. no. AC026, ABclonal, China). Secondary antibody incubation was then performed using IRDye 680 Goat anti-Rabbit IgG (H + L) (1:10,000, cat. no. C80605-15, LiCOR, America) and Donkey anti-mouse IgG
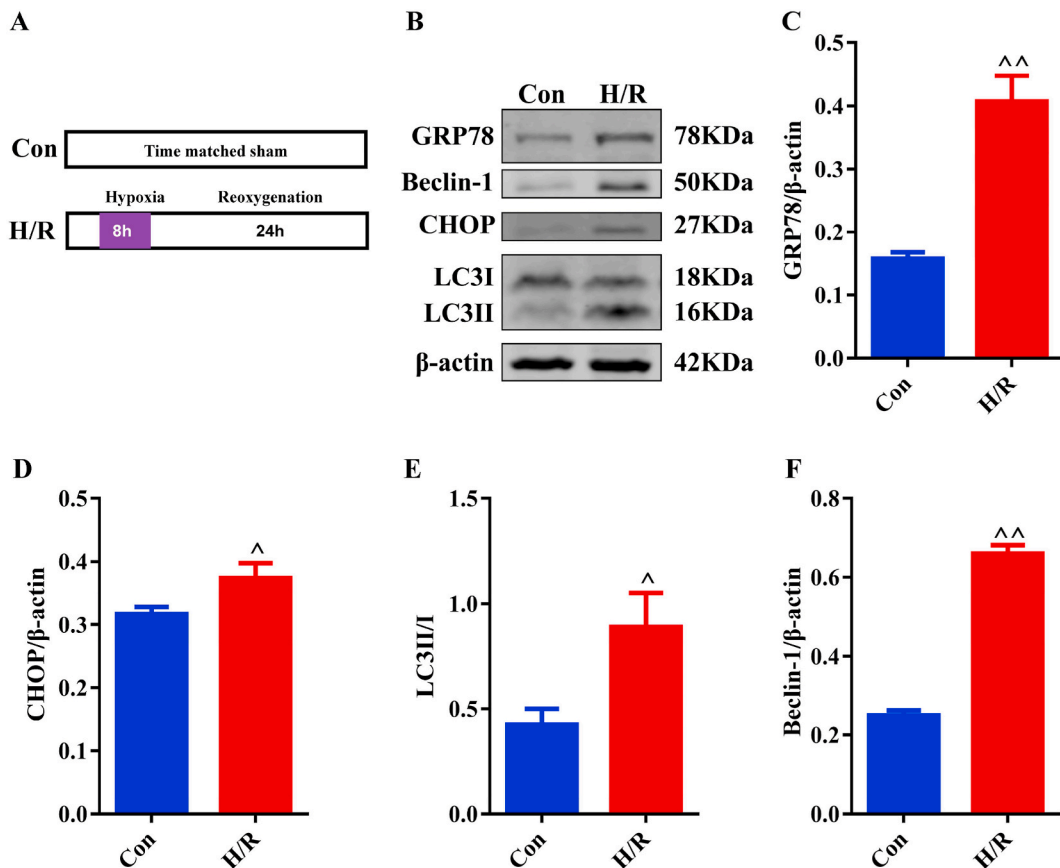

Fig. 1. Hypoxia/reoxygenation (H/R) treatment activated endoplasmic reticulum (ER) stress and autophagy in human dermal microvascular endothelial cells (HDMECs). (A) Experimental protocol: HDMECs were divided into control (Con) and H/R groups. (B) Proteins were measured by Western blot, and the relative expression results of related proteins are shown in panels C, D, E, and F. All data are presented as mean ± standard derivation. ˆP < 0.05 vs Control, ˆˆP < 0.01 vs Control.

(H + L) (1:10,000, cat. no. ab175738, Abcam, Shanghai). Protein bands were visualized and quantified using an Odyssey infrared imaging system (Gene Company, Ltd., Beijing, China).

2.6. Statistical analysis

All statistical analyses were performed with SPSS 26.0 version (IBM, State of California, America). Differences among groups were analyzed by Student's t-test or one-way analysis of variance. The normality of the data distribution was confirmed using the Shapiro–Wilk test, while the homogeneity of variance was confirmed using the F-test. Pairwise comparisons within groups were conducted using the student–Newman–Keuls q test. For all comparisons, $P < 0.05$ was considered statistically significant. All data are presented as mean $\pm$ standard derivation.
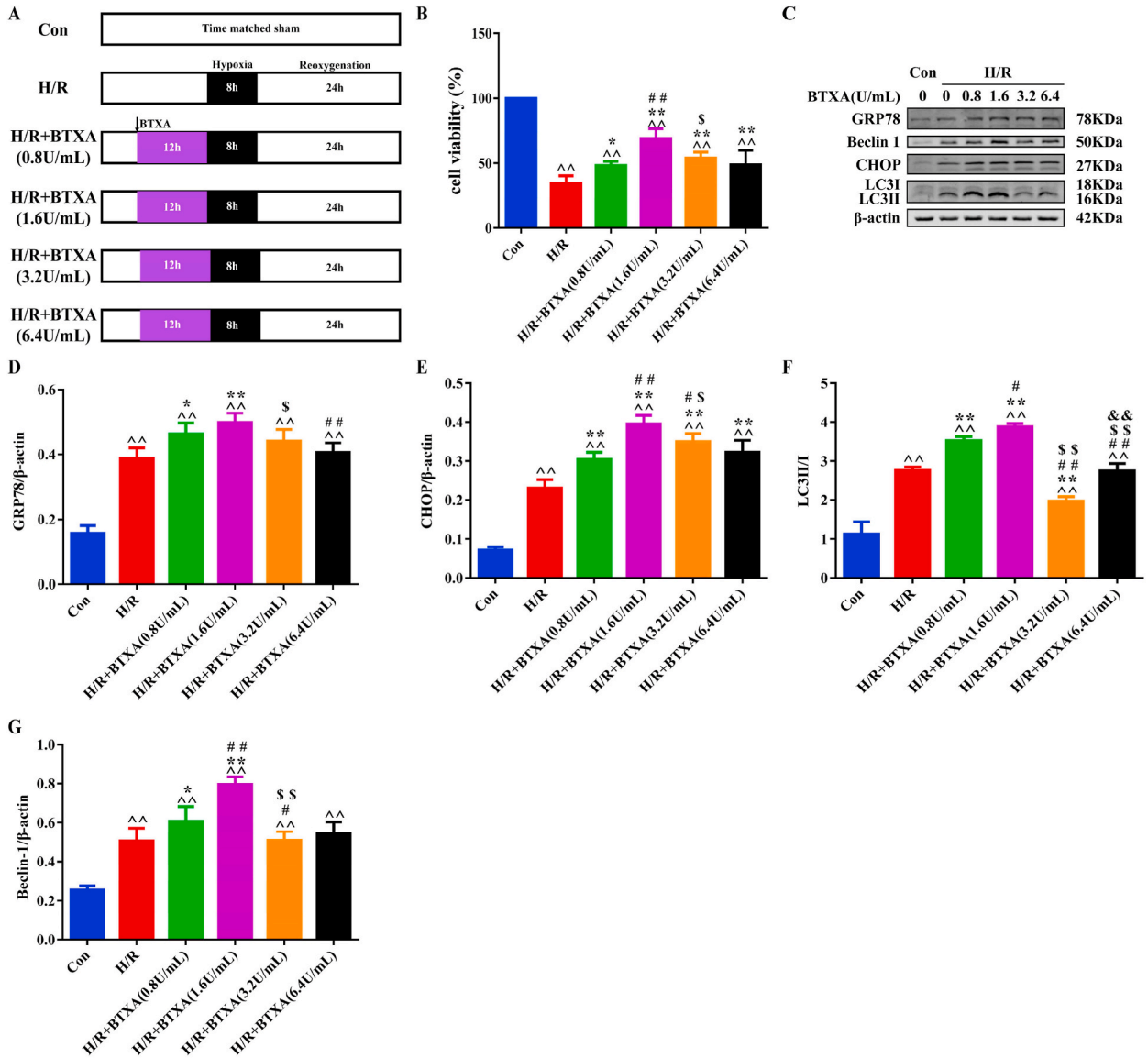

Fig. 2. The cell viability and protein expressions of endoplasmic reticulum (ER) stress and autophagy in human dermal microvascular endothelial cells (HDMECs). (A) Experimental protocol. The down arrow (↓) indicates the administration of BTXA before hypoxia. (B) Optical density (OD) values were tested using the CCK8 assay and transformed into cell viability rates. (C) Proteins were measured by Western blot, and the relative expression results of related proteins are shown in panels D, E, F, and G. All data are presented as mean $\pm$ standard derivation. ^P < 0.05 vs Control, ~P < 0.01 vs Control. *P < 0.05 vs H/R, **P < 0.01 vs H/R. #P < 0.05 vs H/R + BTXA (0.8U/mL), ##P < 0.01 vs H/R + BTXA (0.8U/mL). $P < 0.05 vs H/R + BTXA (1.6U/mL), $$P < 0.01 vs H/R + BTXA (1.6U/mL). &&P < 0.01 vs H/R + BTXA (3.2U/mL).

3. Results

3.1. H/R treatment activated ER stress and autophagy

To evaluate the effects of H/R on HDMECs in vitro, we compared the expression of marker proteins of ER stress (GRP78 and CHOP) and autophagy (LC3 and Beclin-1) in HDMECs with or without H/R. Fig. 1A depicts the cell treatment method. As shown in Fig. 1B–D, the expression of GRP78 and CHOP in the H/R group were both higher than that in the Control group (P < 0.05) and were 1.25 and 2.5 times that in the Control group, respectively. Meanwhile, the expression of Beclin-1 and the ratio of LC3II/I in the H/R group were also higher than those in the Control group (P < 0.05) and were 2.5 and 2.2 times higher in the Control group (Fig. 1E and F) respectively. The results demonstrated that H/R treatment activated ER stress and autophagy in HDMECs.

3.2. Pretreatment with BTXA promoted cell viability

To identify the optimal concentration of BTXA pretreatment, HDMECs were pretreated with different concentrations of BTXA (0.8,
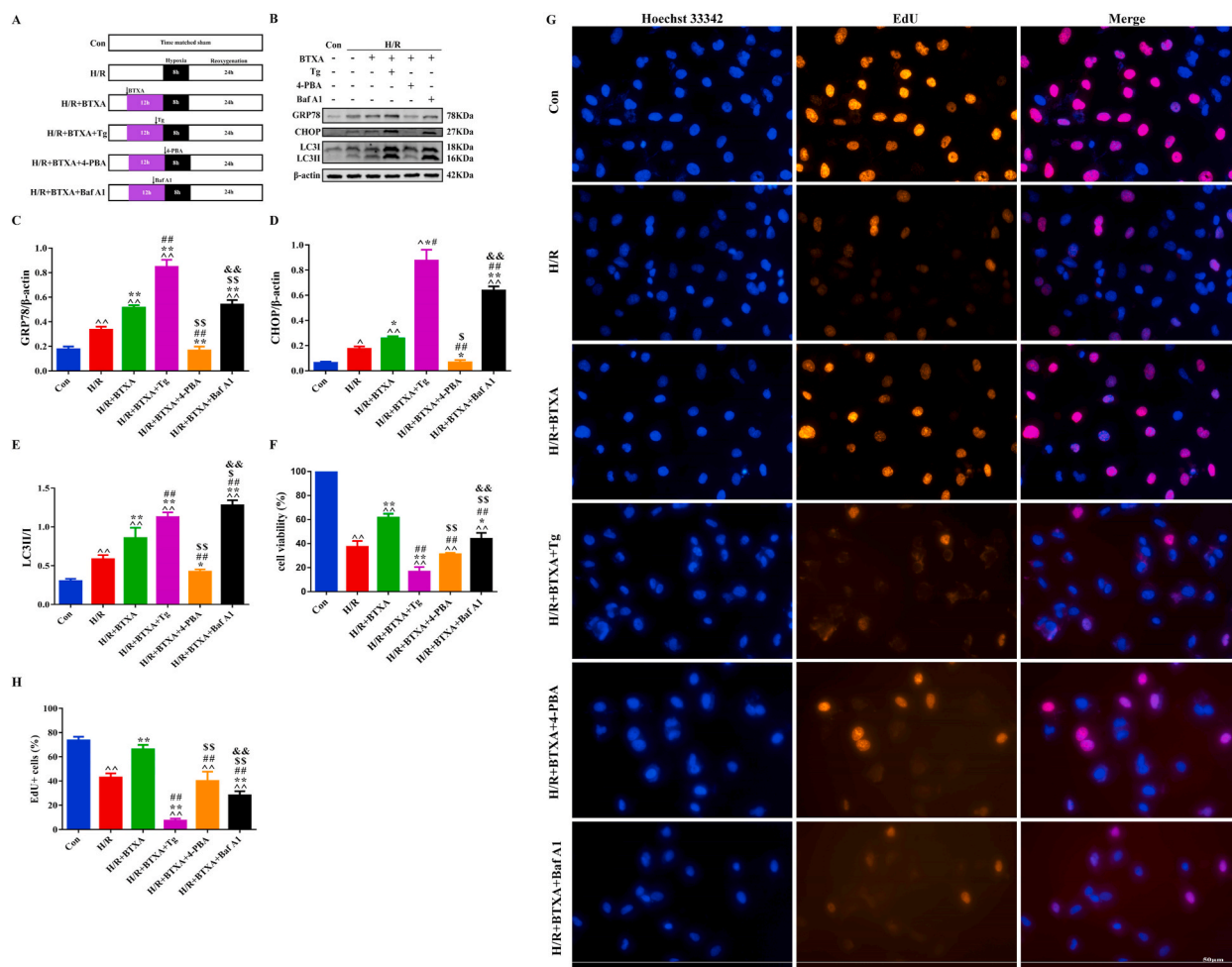

Fig. 3. Botulinum toxin type A (BTXA) activated protective autophagy by initiating endoplasmic reticulum (ER) stress and promoted human dermal microvascular endothelial cells (HDMECs) proliferation. (A) Experimental protocol. Thapsigargin (Tg; 1 μM) and bafilomycin A1 (Baf A1; 10 nM) were added in the first and second hours of BTXA pretreatment, respectively. 4-Phenylbutyric acid (4-PBA; 5 mM) was added at the beginning of hypoxia for 8 h. The administration of BTXA, Tg, or 4-PBA is indicated by the down arrow (↓). (B) Optical density (OD) values were tested using the Cell Counting Kit 8 assay and transformed into cell viability rates. (C) To assess DNA replication activity, the ethynyldeoxyuridine (EdU) test was used. The rate of EDU + cells was determined by dividing the number of EdU + cells (orange fluorescence) by the number of nuclei (Hoechst 33,342; blue fluorescence), as shown in D. (E) Western blot was used to assess proteins, and the relative expression levels of associated proteins are shown in panels F, G, and H. All data are presented as mean ± standard derivation. ˆP < 0.05 vs Control, ˆˆP < 0.01 vs Control. *P < 0.05 vs H/R, **P < 0.01 vs H/R. #P < 0.05 vs H/R + BTXA, ##P < 0.01 vs H/R + BTXA. $P < 0.05 vs H/R + BTXA + Tg, $$P < 0.01 vs H/R + BTXA + Tg. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

1.6, 3.2, or 6.4 U/ml) for 12 h prior to exposure to hypoxia (Fig. 2A). The CCK8 assay was conducted to test the viability of HDMECs in all the groups (Fig. 2B). The results showed that, compared with the Control group, the viability of HDMECs in the H/R group was obviously decreased (P < 0.01). Cell viability in the H/R + BTXA groups was higher than in the H/R group (P < 0.05), but it was not dose dependent. The cell viability gradually increased with increasing concentrations of BTXA and was highest at a concentration of 1.6 U/mL. However, further increases in BTXA concentration did not increase cell viability. Therefore, the results demonstrated that the optimal concentration of BTXA was 1.6 U/ml, which exhibited the best cytoprotective effect on HDMECs.

3.3. BTXA increased the expression of ER stress and autophagy

The impact of BTXA pretreatment on the expression of ER stress in HDMECs is still unclear. Here, we treated HDMECs with different concentrations of BTXA (0.8, 1.6, 3.2, and 6.4 U/ml) for 12 h prior to exposure to hypoxia (Fig. 2A). As shown in Fig. 2C–E, GRP78 and CHOP expression were higher in the H/R + BTXA groups than in the H/R group. GRP78 and CHOP levels did not increase with increasing BTXA concentration but did peak at 1.6 U/mL.

As shown in Fig. 2C–G, the expression of Beclin-1 and the ratio of LC3II/I in HDEMCs after pretreatment with different concentrations of BTXA were higher than those in the H/R group, except for 3.2 U/mL, and were highest at 1.6 U/mL. The results showed that ER stress and autophagy were activated differently in different concentrations of BTXA pretreatment groups, and the expression levels of ER stress and autophagy were the highest at 1.6 U/mL (P < 0.05).

3.4. 4-PBA and Baf A1 weakened the proliferative effect of BTXA

Both CCK8 and EdU were used to detect the proliferation of HDMECs. The cells were divided into six groups and treated as shown in Fig. 3A. As shown in Fig. 3F, the viability of HDMECs in the H/R + BTXA group was higher than that in the H/R group (P < 0.01). However, compared with the H/R + BTXA group, both the addition of 4-PBA (5 mM) and Baf A1 (10 nM) reduced cell viability in the H/R + BTXA+4-PBA and H/R + BTXA + Baf A1 groups (P < 0.01). The EdU assay was used to detect the replication activity of DNA in HDMECs (Fig. 3G and H). EdU + cells were shown as having orange fluorescence, and the nuclei were counter-stained with Hoechst 33,342 and shown as having blue fluorescence. The rate of EdU + cells showed the number of EdU + cells divided by the number of nuclei. The rate of EdU + cells in the H/R group was significantly lower than in the Control group (P < 0.01). When compared to the H/R group, BTXA pretreatment clearly increased the EdU + cell rate (P < 0.01). Unfortunately, the promoting proliferative effect of BTXA in the H/R + BTXA group was weakened after the addition of 4-PBA and Baf A1, as shown by the lower EdU + cell rate both in the H/R + BTXA + 4-PBA and H/R + BTXA + Baf A1 groups (P < 0.01). The results showed that the proliferative effect of BTXA pretreatment was weakened after applying the ER stress inhibitor 4-PBA and the autophagy inhibitor Baf A1.

3.5. BTXA pretreatment activated protective autophagy by initiating ER stress

We previously confirmed that the BTXA inhibits apoptosis through inducing autophagy in HDMECs exposed to an in vitro model of I/R injury [15]. ER stress, as one of the earliest responses to stress, may be involved in autophagy activation. To investigate whether the BTXA-induced ER stress activated autophagy, 4-PBA and Baf A1 were used on the basis of BTXA pretreatment. The cells were divided into six groups and treated as shown in Fig. 3A. The results showed that the expression of GRP78, CHOP, and LC3 in the H/R + BTXA group was higher than that in the H/R group (P < 0.05) (Fig. 3B, C–E). However, GRP78 and CHOP expression in the H/R + BTXA+4-PBA group was significantly lower than in the H/R + BTXA group (P < 0.01). Meanwhile, the ratio of LC3II/I was lower than that in the H/R + BTXA group (P < 0.01) (Fig. 3B and E). Compared with the H/R + BTXA group, the application of the autophagy inhibitor Baf A1 inhibited the degradation of LC3II and caused an increase in the LC3II/I ratio (P < 0.01) and abnormal accumulation of CHOP (P < 0.01) in the H/R + BTXA + Baf A1 group. Taken together, the results of the present study demonstrated that BTXA pretreatment activated protective autophagy by initiating ER stress.

3.6. Tg weakened the proliferative effect of BTXA

We had demonstrated that BTXA pretreatment reduced the H/R-induced damage of HDMECs through initiating ER stress. However, whether further activation of ER stress based on BTXA pretreatment can reduce the H/R injury of HDMECs remains unclear. Then the ER stress inducer Tg was added during the last hour of BTXA pretreatment. The expression of GRP78 and CHOP and the ratio of LC3II/I of HDMECs in the H/R + BTXA + Tg group were higher than those in the H/R + BTXA group (P < 0.05, Fig. 3E–H), indicating that pretreatment with Tg succeeded in inducing ER stress and then activating autophagy.

CCK8 and EdU, on the other hand, revealed that the cell viability and EdU + cell rate in the H/R + BTXA + Tg group were lower than in the H/R + BTXA group (P < 0.01, Fig. 3B–D). The CCK8 and EdU assays, however, revealed that the cell viability and EdU + cells rate in H/R + BTXA + Tg group were lower than that in the H/R + BTXA group (P < 0.01, Fig. 3B–D). The results indicated that ER stress was overactivated in the H/R + BTXA + Tg group after the addition of Tg, which in turn aggravated cell damage. Taken together, the results of the present study demonstrated that neither inhibition nor excessive activation of ER stress played a cytoprotective role.

4. Discussion

In this study, we demonstrated that BTXA pretreatment activated protective autophagy through initiating ER stress to protect HDMECs from H/R damage. Neither inhibition nor excessive activation of ER stress played a cytoprotective role. Our results supported the idea that moderate ER stress and autophagy are important in maintaining cellular longevity and the consequent functional integrity, and therefore could be a potential target of pharmaceutical management.

In the present study, HDMECs were used to illuminate the role of ER stress-autophagy activation in BTXA pretreatment antagonizing H/R injury. It is generally accepted that endotheliocyte damage stands at the center of the I/R injury of the free skin flap and contributes to damage initiation and progression [20]. The I/R injury reduced the number of microvessels, destroyed the structure of microvessels in free skin flaps, caused disordered and irregular arrangements of CD34^{+} endothelial cells, and caused necrosis of the rat free flap [21]. Therefore, it is suitable for using HDMECs to illuminate the role of ER stress-autophagy activation in BTXA pretreatment antagonizing H/R injury in vitro.

As a member of the heat shock protein 70 (Hsp70) family, GRP78 is a calcium-binding protein that acts as a molecular chaperone to mediate the correct folding and assembly of new proteins and assist protein transport across the ER membrane [22]. When ER stress is activated, GRP78 preferentially binds to misfolded or unfolded proteins accumulated in the ER, resulting in the separation of GRP78 from protein kinase R-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE1α), and activating transcription factor 6 (ATF6). Then it promotes the degradation of abnormal accumulation proteins in ER to alleviate ER stress and activates the expression of autophagy initiator Beclin-1 and ubiquitin-like protein systems [including LC3, autophagy related 5 (ATG5), ATG7, ATG12, and ATG16L] through ATF4 [23], thus maintaining cells' survival under stress [24,25]. A knockdown of GRP78 can result in ER dysfunction, and ER stress and the formation of autophagosomes induced by nutrient deficiency would be inhibited [26]. CHOP, as another marker protein of ER stress, is a downstream component of ER stress at the convergence of the PERK and ATF6 signaling pathways, and its expression could be regulated by ATF4. Studies have shown that CHOP plays an important role in ER stress-triggered autophagy. For example, ATF4 and CHOP in the PERK pathway were activated in a severe hypoxic environment. Then ATF4 directly binds to the LC3 promoter region to up-regulate LC3 expression, and CHOP up-regulates the expression of the autophagosome-formation marker ATG5 gene and promotes the formation of autophagy under hypoxia [27]. Besides, previous studies also found that autophagosome formation took place at the ER–mitochondria contact site during starvation [28].

Moderate ER stress allows cells to restore the homeostasis of ER and maintain normal physiological activities as an intracellular response to environmental disturbances like hypoxia and oxidative damage. The findings of the current investigation demonstrated that BTXA pretreatment induced modest ER stress, which in turn triggered protective autophagy. On the one hand, the cytoprotective impact of BTXA was lessened by the administration of the ER stress inhibitor 4-PBA because it reduced the expression of the proteins GRP78, CHOP, and LC3II/I. The ER stress inducer Tg induced excessive accumulation of GRP78, CHOP, and LC3II, and reduced the cytoprotective impact of BTXA. A prior study revealed that CHOP upregulated the expression of the pro-apoptotic protein Bax/Bcl-2 to induce apoptosis while downregulating the expression of anti-apoptotic proteins of the Bcl-2 family [29]. Additionally, CHOP can induce protein translation by activating the DNA damage-inducible gene 34 (GADD34) to promote dephosphorylation of the elF2 protein, which can then lead to an increase in intracellular protein burden and cell apoptosis [30].

The occurrence of ER stress can induce autophagy, which plays a complementary mechanism like UPR to further degrade abnormal proteins and damaged ER and other organelles and inhibit ER stress through a negative feedback mechanism to weaken the influence of stress on cells and maintain cell homeostasis. The application of Baf A1 inhibited the fusion of autophagosome and lysosome, causing protein accumulation of GRP78, CHOP, and LC3II in BTXA-pretreated cells. The results showed that the application of the autophagy inhibitor Baf A1 blocked the negative feedback mechanism and caused overactivated ER stress and cell damage.

But under strong forms of stress, autophagy overactivation can lead to excess cytoplasmic degradation, resulting in the autophagic death of cells, in which the specific suppression of autophagy prevents cell death and the final cell death process is mediated by enhanced autophagic flux rather than by apoptosis or necroptosis [31]. In a mouse model of ischemia, for example, LC3II levels were significantly increased, and many hippocampal neurons died, whereas the death of hippocampal neurons in mice lacking ATG7 was reduced [32].

Our previous study showed that BTXA pretreatment protected rat skin free flaps from I/R injury by enhancing autophagy [21]. In the present study, we demonstrated that BTXA activated protective autophagy by initiating ER stress in advance to protect HDMECs from H/R damage. However, Akiko Sekiguchi et al. reported that botulinum toxin type B (BTXB) suppressed the I/R-induced ER stress signal in a mouse pressure ulcer model [33]. Although the two experiments differed in mechanism, both show that botulinum toxin can reduce vascular damage and prevent cutaneous I/R injury, while abnormally activated ER stress can cause damage to endotheliocytes.

The study has a few limitations. First, the relationship of BTXA activation of ER stress-autophagy on endothelial cells in vitro was investigated. The pathways that BTXA triggers the autophagy mechanism by activating one or more endoplasmic reticulum stress signaling have not been studied. Our next step will be to use small interfering RNAs for further investigation. Further research is required on the target of ER stress-inducing autophagy. Macroautophagy, microautophagy, and chaperon-mediated autophagy are the three distinct types of autophagy, as is common knowledge. This experiment focuses on macroautophagy. However, it is unknown whether the ER stress caused by BTXA affects other autophagy pathways. Third, although BTXA promotes autophagy by inducing ER stress to protect skin flaps in cell experiments, it still requires further validation in animal studies. Our subsequent studies can also focus on the effect of the optimum dose for using BTXA in flap surgery and its dose-response.

In conclusion, we showed that the induced ER stress was related to BTXA pretreatment. Furthermore, autophagy was simultaneously enhanced, and it mediated to salvage the injuries caused by ER stress in the short term. BTXA pretreatment increased the LC3 conversion and exhibited a protective effect on H/R-treated HDMECs via ER stress-related autophagy. We proposed that adequate, but

not excessive, ER stress and autophagy are crucial to help maintain the cell survival and viability of HDMECs as an important cell in flap tissues.

Author contribution statement

Yinhua Zhao: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Huang Lin: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jiaxing Ma; Yue Cui: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17907.

References

[1] A. Ballestín, J. Casado, E. Abellán, et al., Ischemia-reperfusion injury in a rat microvascular skin free flap model: a histological, genetic, and blood flow study, PLoS One 13 (2018), e0209624, https://doi.org/10.1371/journal.pone.0209624.

[2] J. Lee, H. You, T. Lee, H. Kang, Current status of experimental animal skin flap models: ischemic preconditioning and molecular factors, Int. J. Mol. Sci. 23 (2022), https://doi.org/10.3390/ijms23095234.

[3] S. Kleban, R. Baynosa, The effect of hyperbaric oxygen on compromised grafts and flaps, Undersea Hyperb. Med.: J. Undersea Hyperb. Med. Soc. 47 (2020) 635–648.

[4] Y. Tian, Y. Zhang, Y. Wang, et al., Hydrogen, a novel therapeutic molecule, regulates oxidative stress, inflammation, and apoptosis, Front. Physiol. 12 (2021), 789507, https://doi.org/10.3389/fphys.2021.789507.

[5] A. Uchiyama, K. Yamada, B. Perera, et al., Protective effect of botulinum toxin A after cutaneous ischemia-reperfusion injury, Sci. Rep. 5 (2015) 9072, https://doi.org/10.1038/srep09072.

[6] A. Samarth, L. Budamakuntla, K. Shilpa, The use of botulinum toxin-A as an adjunctive treatment for facial atrophic and hypertrophic scars after surgical correction, J. Cutan. Aesthetic Surg. 15 (2022) 131–134, https://doi.org/10.4103/jcas.jcas_8_21.

[7] V. Maneksha, S. Chakrabarty, M. Tanwar, M. Pillai, Outcomes of a regional variant of botulinum toxin type A in the treatment of essential blepharospasm and hemifacial spasms: a retrospective study, Ind. J. Ophthalmol. 69 (2021) 2777–2781, https://doi.org/10.4103/ijo.IJO_3656_20.

[8] E. Nofal, S. Salem, S. Khashaba, Intradermal botulinum toxin A injection versus topical 2% glycopyrrolate for the treatment of primary facial hyperhidrosis: a pilot study and review of literature, Dermatol. Surg. 48 (2022) 843–848, https://doi.org/10.1097/dss.0000000000003490.

[9] S. Shi, R. Jin, C. Huang, J. Zhou, Effect of botulinum toxin type A on flap surgery in animal models: a systematic review and meta-analysis, J. Plast. Surg. Hand Surg. 56 (2022) 198–207, https://doi.org/10.1080/2000656x.2021.1953044.

[10] T. Roh, B. Jung, I. Yun, D. Lew, Y. Kim, Effect of botulinum toxin on vasoconstriction and sympathetic neurotransmitters in a murine random pattern skin flap model, Wound Repair Regen. 25 (2017) 75–85, https://doi.org/10.1111/wrr.12501.

[11] M. Iranpour, A. Khodarahmi, M. Beigi, O. Azari, S. Shahrokh Shahraki, Effects of botulinum toxin type A on the axial skin flap survival, World J. Plast. Surg. 11 (2022) 30–37, https://doi.org/10.52547/wjps.11.1.30.

[12] Y. Shi, H. Lin, J. Cao, C. Cui, Botulinum toxin type A attenuates apoptosis in human dermal microvascular endothelial cells exposed to an in vitro model of ischemia/reperfusion injury, Transplant. Proc. 51 (2019) 966–971, https://doi.org/10.1016/j.transproceed.2018.11.006.

[13] B. Yan, S. Liu, X. Li, Y. Zhong, F. Tong, S. Yang, Preconditioning with endoplasmic reticulum stress alleviated heart ischemia/reperfusion injury via modulating IRE1/ATF6/RACK1/PERK and PGC-1α in diabetes mellitus, Biomed. Pharmacother. = Biomed. Pharmacother. 118 (2019), 109407, https://doi.org/10.1016/j.biopha.2019.109407.

[14] Y. Cheng, J. Chang, C. Chen, H. Chen, Autophagy modulates endoplasmic reticulum stress-induced cell death in podocytes: a protective role, Exp. Biol. Med. 240 (2015) 467–476, https://doi.org/10.1177/1535370214553772.

[15] Y. Shi, H. Lin, J. Cao, C. Cui, Botulinum toxin type A induces protective autophagy in human dermal microvascular endothelial cells exposed to an in vitro model of ischemia/reperfusion injury, Exp. Ther. Med. 16 (2018) 4379–4386, https://doi.org/10.3892/etm.2018.6741.

[16] Kun WY. Lu, Haihong Wang, Guan Xiao, Lvya Wang, Xin Yi, Xiaolin Chu, Lin Huang, Primary cultured and establishment of hypoxic/reoxygenation model of human dermal microvascular endothelial cells, J. Cardiovasc. Pulmonary Dis. 34 (2015) 132–136, https://doi.org/10.3969/j.issn.1007-5062.2015.02.015.

[17] Z. Li, S. Liu, Y. Cao, T. Fu, L. Jiang, J. Zhang, High concentration of sodium metasilicate impairs autophagic flux and induces apoptosis in human umbilical vein endothelial cells, Biol. Trace Elem. Res. 191 (2019) 88–97, https://doi.org/10.1007/s12011-018-1608-3.

[18] K. Dibdiakova, S. Saksonova, I. Pilchova, K. Klacanova, Z. Tatarkova, P. Racay, Both thapsigargin- and tunicamycin-induced endoplasmic reticulum stress increases expression of Hrd1 in IRE1-dependent fashion, Neurol. Res. 41 (2019) 177–188, https://doi.org/10.1080/01616412.2018.1547856.

[19] H. Li, D. Li, Z. Ma, et al., Defective autophagy in osteoblasts induces endoplasmic reticulum stress and causes remarkable bone loss, Autophagy 14 (2018) 1726–1741, https://doi.org/10.1080/15548627.2018.1483807.

[20] J. Cao, H. Lin, W. Li, et al., Ischemia postconditioning protects dermal microvascular endothelial cells of rabbit epigastric skin flaps against apoptosis via adenosine A2a receptors, J. Plast. Surg. Hand Surg. 53 (2019) 76–82, https://doi.org/10.1080/2000656x.2018.1550417.

[21] Z.S. Cui Chao, Lin Huang, Study on the protective effect of botulinum toxin A on rat skin flap ischemiareperfusion injury through autophagy pathway, Chin. J. Appl. Mech. 31 (2022) 117–121.

[22] M. Smith, S. Wilkinson, ER homeostasis and autophagy, Essays Biochem. 61 (2017) 625–635, https://doi.org/10.1042/ebc20170092.

[23] H. Harding, Y. Zhang, A. Bertolotti, H. Zeng, D. Ron, Perk is essential for translational regulation and cell survival during the unfolded protein response, Mol. Cell 5 (2000) 897–904, https://doi.org/10.1016/s1097-2765(00)80330-5.

[24] W. B'chir, A. Maurin, V. Carraro, et al., The eIF2α/ATF4 pathway is essential for stress-induced autophagy gene expression, Nucleic Acids Res. 41 (2013) 7683–7699, https://doi.org/10.1093/nar/gkt563.

[25] Y. Cai, J. Arikkath, L. Yang, M. Guo, P. Periyasamy, S. Buch, Interplay of endoplasmic reticulum stress and autophagy in neurodegenerative disorders, Autophagy 12 (2016) 225–244, https://doi.org/10.1080/15548627.2015.1121360.

[26] P. Pyrko, A. Schönthal, F. Hofman, T. Chen, A. Lee, The unfolded protein response regulator GRP78/BiP as a novel target for increasing chemosensitivity in malignant gliomas, Cancer Res. 67 (2007) 9809–9816, https://doi.org/10.1158/0008-5472.can-07-0625.

[27] K. Rouschop, T. van den Beucken, L. Dubois, et al., The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5, J. Clin. Investig. 120 (2010) 127–141, https://doi.org/10.1172/jci40027.

[28] M. Hamasaki, N. Furuta, A. Matsuda, et al., Autophagosomes form at ER-mitochondria contact sites, Nature 495 (2013) 389–393, https://doi.org/10.1038/nature11910.

[29] T. Tamaki, K. Kamatsuka, T. Sato, et al., A novel transmembrane protein defines the endoplasmic reticulum stress-induced cell death pathway, Biochem. Biophys. Res. Commun. 486 (2017) 149–155, https://doi.org/10.1016/j.bbrc.2017.03.017.

[30] I. Novoa, H. Zeng, H. Harding, D. Ron, Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha, J. Cell Biol. 153 (2001) 1011–1022, https://doi.org/10.1083/jcb.153.5.1011.

[31] G. Mariño, M. Niso-Santano, E. Baehrecke, G. Kroemer, Self-consumption: the interplay of autophagy and apoptosis, Nat. Rev. Mol. Cell Biol. 15 (2014) 81–94, https://doi.org/10.1038/nrm3735.

[32] M. Koike, M. Shibata, M. Tadakoshi, et al., Inhibition of autophagy prevents hippocampal pyramidal neuron death after hypoxic-ischemic injury, Am. J. Pathol. 172 (2008) 454–469, https://doi.org/10.2353/ajpath.2008.070876.

[33] A. Sekiguchi, S. Motegi, A. Uchiyama, et al., Botulinum toxin B suppresses the pressure ulcer formation in cutaneous ischemia-reperfusion injury mouse model: possible regulation of oxidative and endoplasmic reticulum stress, J. Dermatol. Sci. 90 (2018) 144–153, https://doi.org/10.1016/j.jdermsci.2018.01.006.