4-Phenylbutyrate protects rat skin flaps against ischemia-reperfusion injury and apoptosis by inhibiting endoplasmic reticulum stress

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Abstract. 4-phenylbutyrate (4-PBA) is a low molecular weight fatty acid, which has been demonstrated to regulate endoplasmic reticulum (ER) stress. ER stress-induced cell apoptosis has an important role in skin flap ischemia; however, a pharmacological approach for treating ischemia-induced ER dysfunction has yet to be reported. In the present study, the effects of 4-PBA-induced ER stress inhibition on ischemia-reperfusion injury were investigated in the skin flap of rats, and transcriptional regulation was examined. 4-PBA attenuated ischemia-reperfusion injury and inhibited cell apoptosis in the skin flap. Furthermore, 4-PBA reversed the increased expression levels of two ER stress markers: CCAAT/enhancer-binding protein-homologous protein and glucose-regulated protein 78. These results suggested that 4-PBA was able to protect rat skin flaps against ischemia-reperfusion injury and apoptosis by inhibiting ER stress marker expression and ER stress-mediated apoptosis. The beneficial effects of 4-PBA may prove useful in the treatment of skin flap ischemia-reperfusion injury.

Introduction

A skin flap is a common autograft procedure, which has been widely used in surgical practice. Skin flaps are often used to repair wounds, as well as in the field of plastic and reconstructive surgery (1). However, the clinical application of skin flaps for tissue repair and organ reconstruction is often accompanied by ischemia-reperfusion injury. Ischemia results in tissue reperfusion injury with the restoration of blood flow, which may lead to partial or complete skin flap necrosis,

and a decrease in the success rate of the surgical procedure. Therefore, reducing skin flap ischemia-reperfusion injury is of important clinical significance. Numerous compounds have been demonstrated to reduce ischemia-reperfusion injury; however, clinical trials have yet to be successful due to toxic side-effects (2). Therefore, the development of novel drugs and the identification of novel mechanisms for the treatment of skin flap ischemia are required.

Endoplasmic reticulum (ER) stress is defined as an accumulation of unfolded or misfolded proteins in the ER, a subcellular organelle predominantly involved in protein folding (3-6). The ER is also responsible for regulating protein synthesis, protein folding and trafficking, and intracellular calcium levels (7,8). Accumulation of unfolded or misfolded proteins in the ER may result in initiation of the unfolded protein response (UPR) or ER stress. Activation of the UPR may increase the expression levels of CCAAT/enhancer-binding protein-homologous protein (CHOP) and glucose-regulated protein 78 (GRP78). CHOP, which is considered a marker of ER stress, is an apoptotic transcription factor that is induced in response to ER stress (9). In addition, GRP78 is an ER chaperone, which stably interacts with unfolded or misfolded proteins; therefore, upregulation of GRP78 expression is commonly considered a marker of ER stress (3,10). It has previously been demonstrated that ER stress mediates cell apoptosis by generating endogenous reactive oxygen species or disturbing mitochondrial Ca^{2+} homeostasis, leading to the activation of caspase-3, which is a regulator of caspase-dependent apoptosis (11). Therefore, targeting the ER may provide a therapeutic approach for blocking the pathological progression induced by skin flap ischemia.

4-phenylbutyrate (4-PBA) has been demonstrated to contribute to the treatment of spinal muscular atrophy by altering gene expression patterns (12,13). Furthermore, 4-PBA is able to inhibit disease progression and neuroinflammation in multiple sclerosis (14). Numerous studies have described the use of 4-PBA as a chemical chaperone that reverses the mislocalization and/or aggregation of proteins associated with human disease (15-17). In addition, 4-PBA reduces the levels of mutant or dislocated proteins retained in the ER under conditions associated with cystic fibrosis and liver injury (18). Despite the fact that skin flaps have been shown to cause ER

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dysfunction, it remains unclear whether 4-PBA is able to protect against ischemia-reperfusion-induced damage, and regulate the protein expression of ER stress markers.

The present study used *in vivo* experimental systems to examine the effects of ER stress, as well as the underlying molecular mechanisms, on ischemia-reperfusion in rat skin flaps. The effects of 4-PBA may provide a novel therapeutic strategy for the treatment of skin flap ischemia-reperfusion injury.

Materials and methods

Induction of ischemia-reperfusion and 4-PBA treatment. Care of the laboratory animals and animal experimentation were performed in accordance with animal ethics guidelines and approved protocols. All animal studies were approved by the Animal Ethics Committee of the Xiaoshan Traditional Chinese Medical Hospital (Hangzhou, China). A total of 75 healthy male Wistar rats (age, 6 weeks; weight, 300-350 g; Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China) were randomly divided into three groups: The control group; the ischemia-reperfusion and 4-PBA administration group; and the ischemia-reperfusion and saline administration group, which served as a negative control (NC). Each group comprised 25 rats. The rats were maintained at room temperature, in a 12-h light/dark cycle with access to food and water *ad libitum*. Preparation of a skin flap was performed in all of the rats, as follows: The rats were anesthetized with 3% sodium pentobarbital (40 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) by intraperitoneal injection, their limbs were subsequently fixed, and the fur on their abdomens was sheared. The skin of the abdomen was sterilized with 75% alcohol. A right lower abdominal island skin flap (6x3 cm) was created, according to the method described by Shimamatsu and Wanless (19). In the control group, no other treatments were performed. In the 4-PBA and NC groups, the femoral artery located at the proximal end of the superficial epigastric artery was occluded using a vascular clamp. An OPMI 1 FR pro surgical microscope (Carl Zeiss AG, Oberkochen, Germany) was used to confirm the femoral artery and superficial epigastric artery were completely occluded. The flap was then sutured and the vascular clamp was removed after 8 h.

4-PBA was prepared by titrating equimolar amounts of 4-phenylbutyric acid (Wako Pure Chemicals Co., Ltd., Tokyo, Japan) and sodium hydroxide to pH 7.4. 4-PBA was administered intragastrically at a volume of 2 ml/kg 24 h and 30 min prior to the surgical procedure in the 4-PBA group. Saline was administered in the NC group. In the NC and 4-PBA groups, a 1x0.5 cm skin flap tissue sample was removed at the time the skin flap was created (0 h), and 1, 6, 12 and 24 h following ischemia-reperfusion. In the control group, a 1x0.5 cm skin flap tissue sample was removed at the time the skin flap was created (0 h), and 1, 6, 12 and 24 h following the surgical procedure.

Histology. The skin flap samples of the rats were removed and fixed with 10% (v/v) neutral-buffered formalin. The tissue samples were then dehydrated and embedded in paraffin. For histological examination, 4 μm sections of the fixed embedded tissue samples were cut using a Leica 2165 rotary microtome

(Leica Microsystem GmbH, Wetzlar, Germany). The sections were placed on glass slides, deparaffinized, and stained sequentially with hematoxylin and eosin (Richard-Allan Scientific Co., Kalamazoo, MI, USA). The stained tissue sections were analyzed using a light microscope (Axio Imager M1; Carl Zeiss AG) at x100 magnification.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. TUNEL staining was performed using the Roche *In Situ* Cell Death Detection kit for the detection of programmed cell death (Roche Applied Science, Pleasanton, CA, USA) (20). The tissue sections were then examined by microscopy (CX41RF; Olympus Corporation, Tokyo, Japan) and the number of TUNEL-positive cells was counted using National Institutes of Health (NIH) Image software version 1.61 (NIH, Bethesda, MA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the rat skin flap tissue samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), as previously described (21) and stored at -80°C. cDNA was reverse transcribed from RNA using a cDNA synthesis kit (Thermo Fisher Scientific, Inc.). The cDNA synthesis conditions were as follows: 37°C for 60 min, followed by 85°C for 5 min and 4°C for 5 min. A DyNAmo Flash SYBR® Green qPCR kit (Finnzymes Oy, Espoo, Finland) was used, according to the manufacturer's protocol, the thermal cycler used was an ABI 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.) in order to detect the mRNA expression levels of CHOP and GRP78. The gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (22). The following primers were used: CHOP, forward 5'-GGAGAAGGAGCAGGAGAATG-3', reverse 5'-GAGACAGACAGGAGGTGATG-3'; GRP78, forward 5'-TAATCAGCCCACCGTAAC-3', reverse 5'-GTTTCCTGTCCCTTTGTC-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward. 5'-GTCGGTGTGAACGGATTTG-3', and reverse 5'-TCCCATTCTCAGCCTTGAC-3' (Sangon Biotech Co., Ltd., Shanghai, China). Relative quantification of the signals was performed by normalizing the gene signals with those of GAPDH. The PCR cycling conditions were as follows: 95°C for 10 min; followed by 40 cycles at 95°C for 15 sec and 60°C for 45 sec; and a final extension step of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec.

Western blotting. The skin flaps were homogenized and total cell lysates were extracted using radioimmunoprecipitation assay buffer (JRDUN Biotechnology, Co., Ltd., Shanghai, China), containing 50 mmol/l Tris-HCl (pH 8.8), 150 mmol/l NaCl, 1% Triton X-100, 0.1% SDS and 1% deoxycholic acid sodium. Total protein concentration in each tissue sample was measured using a Lowry protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equivalent quantities (50 μg) of protein lysates were separated by 10 or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Sigma-Aldrich), followed by blocking in fat-free milk overnight at 4°C. The membranes were incubated with primary antibodies for 2 h with gentle agitation at room temperature, or overnight at 4°C, and then washed three times with Tris-buffered saline

with Tween 20 (Amresco, LLC, Solon, OH, USA). The following primary antibodies were used and diluted as per the manufacturer's recommendations: Rabbit monoclonal anti-GRP78 (1:1,000; Abcam, Cambridge, MA, USA; cat. no. ab108613), mouse monoclonal anti-CHOP (1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA; cat. no. 2895) and rabbit monoclonal anti-GAPDH (1:1,500; Cell Signaling Technology Inc.; cat. no. 5174) for 2 h at 4°C. The membranes were then incubated for 1 h at 37°C with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated immunoglobulin G secondary antibodies (1:1,000; Dako North America, Inc., Carpinteria, CA, USA; cat. nos. A0208 and A0216). Chemiluminescence detection was conducted using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Inc., Waltham, MA, USA) and signals were quantified by densitometry (Quantity One software, version 4.62; Bio-Rad Laboratories, Inc.).

Immunofluorescence staining for CHOP and GRP78. Paraffin-embedded skin flap tissue sections were deparaffinized and hydrated for histological assessment. For antigen retrieval, the tissues were put into the sodium citrate solution (JRDUN Biotechnology, Co., Ltd.) and heated using a microwave oven at 92-98°C for 10-30 min. Following antigen retrieval, the tissue sections were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS). Following blocking with 2% bovine serum albumin (Sigma-Aldrich, Shanghai, China) in PBS for 1 h, the tissue sections were incubated with the anti-GRP78 and anti-CHOP antibodies, prior to incubation with the corresponding fluorescein isothiocyanate-conjugated secondary antibody. The nuclei were then stained with 4',6-diamidino-2-phenylindole. The fluorescence signal was measured by CX41RF microscope at magnification x200.

Statistical analysis. The data are presented as the mean ± standard deviation. A paired, two-tailed Student's t-test was used to analyze the significance of statistical differences between the groups. Statistical analyses were performed using GraphPad Prism software, version 5 (GraphPad Software, Inc., La Jolla, CA, USA) and $P<0.05$ was considered to indicate a statistically significant difference.

Results

4-PBA attenuates ischemia-reperfusion injury in the skin flap of the rats. Histological analyses detected significant swelling and increased numbers of inflammatory cells in the tissue space of the NC group at 6 h, and the injury increased in severity 24 h following ischemia-reperfusion. Conversely, the morphological structure of the control group was normal, and no such changes were observed. The presence of abnormal cells was markedly increased in the NC group, as compared with in the 4-PBA group, in which damaged and inflammatory cells, and swelling in the tissue sections were rarely observed (Fig. 1).

4-PBA inhibits cell apoptosis in the skin flap. To determine whether 4-PBA was associated with antiapoptotic activity, apoptosis was evaluated using a TUNEL assay. The results demonstrated an increase in the number of TUNEL-positive cells to 28.6 and 48.4%, 6 and 24 h following ischemia-reperfusion injury, respectively. Treatment with 4-PBA significantly decreased the number of TUNEL-positive cells in the skin flap by ~19.4 and 26.1%, as compared with administration of saline, at 6 and 24 h following ischemia-reperfusion (Fig. 2). Furthermore, the number of TUNEL-positive cells in the NC group increased in a time-dependent manner, with a statistically significant increase 6 h following ischemia-reperfusion.

4-PBA suppresses the mRNA expression of CHOP and GRP78 in the skin flap. To evaluate whether ER stress was involved in ischemia-reperfusion injury in the skin flap, the mRNA expression levels of CHOP and GRP78 were quantified in the skin flap tissue samples of the rats. The mRNA expression levels of CHOP were increased by ~61.1, 88.9 and 161% at 6, 12, and 24 h following ischemia-reperfusion, respectively, as compared with the control group at 14, 20 and 32 h following skin flap creation (Fig. 3A). The mRNA expression levels of GRP78 were increased by ~34.0, 64.7 and 126% at 6, 12, and 24 h following ischemia-reperfusion, respectively, as compared with the control group at 6, 12 and 24 h following skin flap creation (Fig. 3B).

The increases in CHOP and GRP78 mRNA expression levels were significantly reduced following administration of 4-PBA from 6-24 h (~16.8, 17.4 and 32.9% reduction in CHOP expression levels, and ~14.2, 19.2 and 29.9% reduction in GRP78 expression levels), as compared with the NC group (Fig. 3).

4-PBA inhibits the upregulation of CHOP and GRP78 protein expression in skin flap tissue samples. The protein expression levels of CHOP and GRP78 were examined by western blot analysis. The results demonstrated that the protein expression levels of CHOP and GRP78 were similar to the mRNA expression levels, and were significantly increased 24 h following ischemia-reperfusion (Fig. 4A). Pretreatment with 4-PBA significantly inhibited the increases in protein expression levels of CHOP and GRP78 in the skin flap tissue samples at 6 and 24 h following ischemia-reperfusion.

To further investigate the hypothesis that CHOP and GRP78 expression levels were increased in the rat skin flap tissue samples following ischemia-reperfusion injury, these proteins were visualized by immunofluorescence staining (Fig. 4B). Fluorescence microscopy revealed that the expression of CHOP and GRP78 were markedly increased at 24 h following ischemia-reperfusion, as compared with the control group. However, following pretreatment with 4-PBA, the increases in CHOP and GRP78 protein expression were significantly inhibited at 24 h in the skin flap tissue samples following ischemia-reperfusion.

Discussion

To the best of our knowledge, the results of the present study demonstrated for the first time that the intragastric administration of 4-PBA at therapeutic doses protected rat skin flaps against ischemia-reperfusion injury. The protective effects of 4-PBA were further demonstrated by an inhibition of ER stress, and a decrease in the number of apoptotic cells.
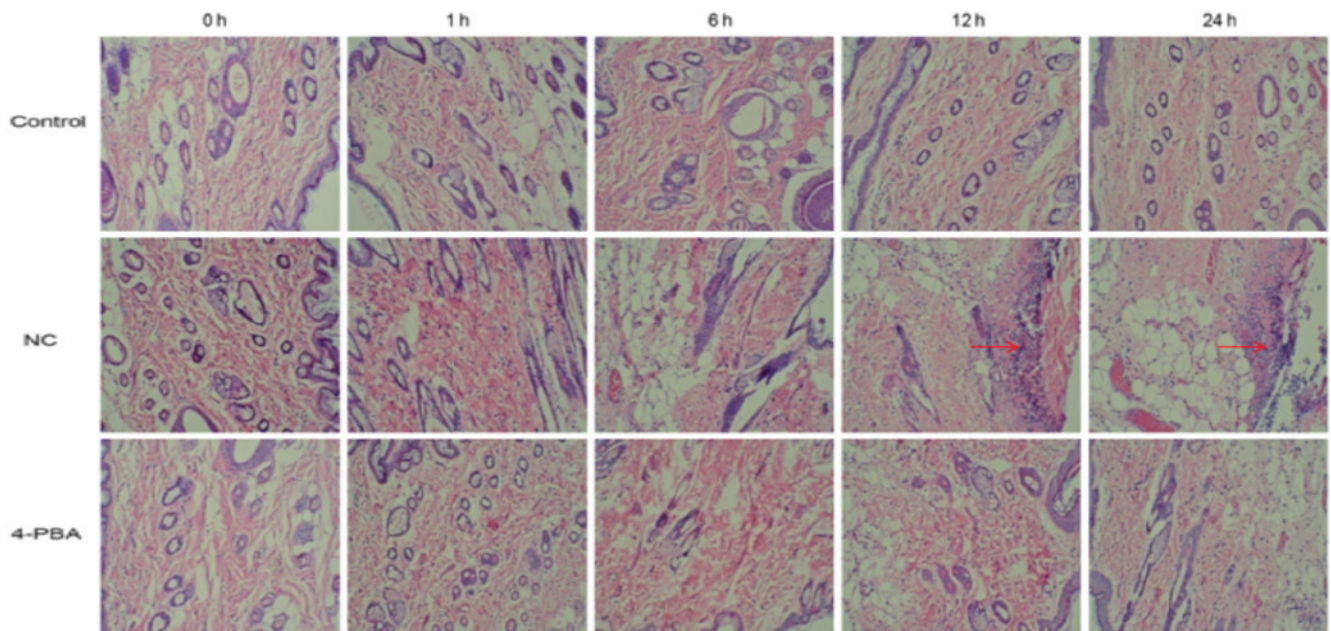
Figure 1. 4-PBA attenuates ischemia-reperfusion injury in the skin flap of rats. Skin flap tissue sections from rats undergoing ischemia-reperfusion were stained with hematoxylin and eosin (magnification, x100) . Significant swelling and increased numbers of inflammatory cells were detected in the tissue space of the NC group rats; however, these characteristics were absent from the control group, and attenuated in the 4-PBA group rats. Tissue sections were observed 0, 1, 6, 12, and 24 h following ischemia-reperfusion. NC, negative control; 4-PBA, 4-phenylbutyrate.
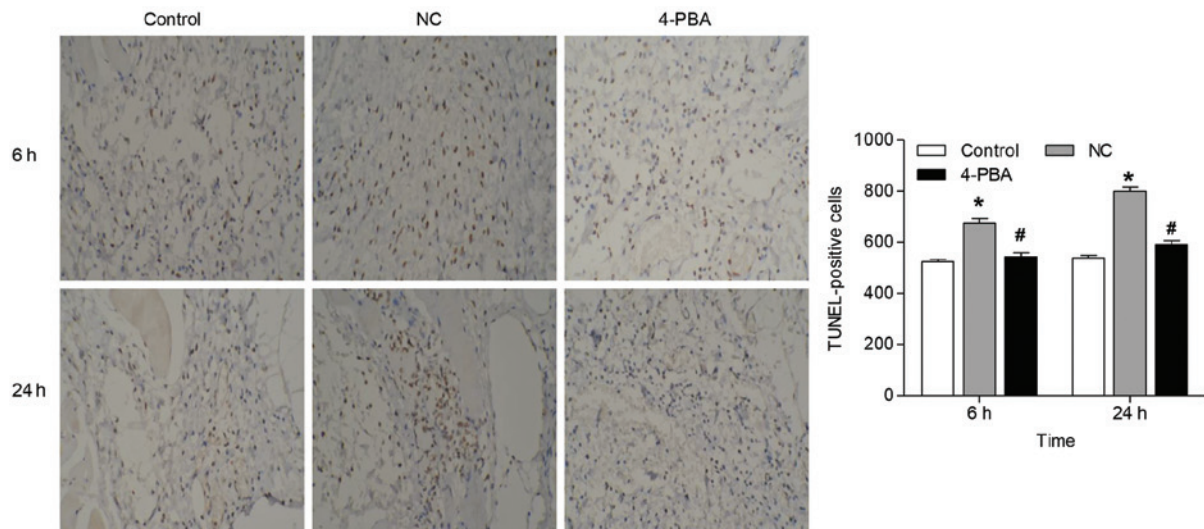

Figure 2. 4-PBA inhibits cell apoptosis in the skin flap of rats. Pretreatment with 4-PBA decreased the number of TUNEL-positive cells induced by ischemia-reperfusion injury. To estimate the number of TUNEL-positive cells, the TUNEL-positive area was determined using National Institutes of Health Image software. The error bars represent the mean ± standard deviation from five rats/group. $^*P<0.05$, vs. the control group; $^#P<0.05$, vs. the rats treated with saline (NC). NC, negative control; 4-PBA, 4-phenylbutyrate; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Notably, administration of 4-PBA was effective not only prior to but also following ischemia-reperfusion injury.

Ischemia-reperfusion injury is a severe limitation in the survival of tissues involved in reconstructive microsurgery, and skeletal muscles and skin flaps are particularly susceptible (23-26). 4-PBA is a potent ER stress inhibitor and numerous studies have reported the effect of 4-PBA on ischemia-reperfusion injury (27,28). Consistent with these observations, the results of the present study demonstrated that pretreatment with 4-PBA markedly attenuated

ischemia-reperfusion injury in the skin flap of rats, and damaged and inflammatory cells and tissue swelling were rarely observed. In addition, the data demonstrated that 4-PBA attenuated ER stress-mediated cell apoptosis in the rat skin flaps following ischemia-reperfusion injury. Therefore, targeting the ER-mediated apoptotic signaling pathway may be an effective strategy for the treatment of cellular injury.

ER stress is involved in the pathogenesis of various cardiovascular diseases, and promotes disease progression (29). CHOP is a downstream component of ER stress
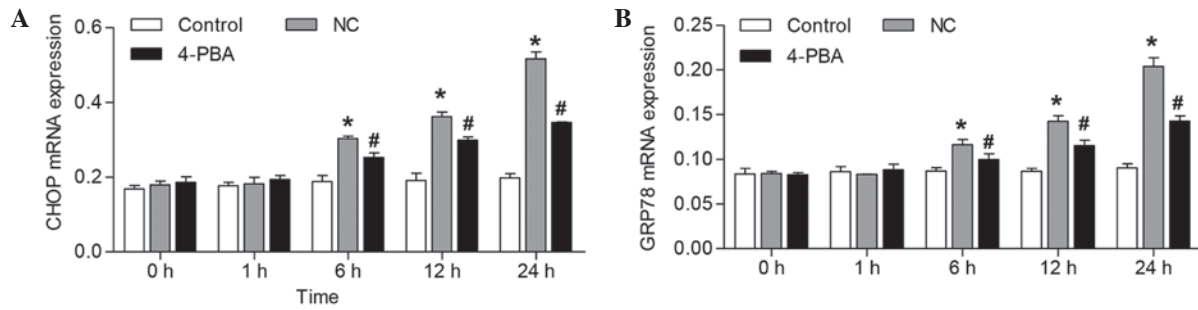
Figure 3. Effects of 4-PBA on the mRNA expression levels of CHOP and GRP78 in the skin flap tissue samples of rats following ischemia-reperfusion. Reverse transcription-quantitative polymerase chain reaction analysis of (A) CHOP and (B) GRP78 in the skin flap tissue samples of the rats. Expression levels were detected 0, 1, 6, 12, and 24 h following ischemia-reperfusion. The error bars represent the mean ± standard deviation from five rats per group. *P<0.05, vs. the control group; #P<0.05, vs. the rats treated with saline (NC). NC, negative control; 4-PBA, 4-phenylbutyrate; CHOP, CCAAT/enhancer-binding protein-homologous protein; GRP78, glucose-regulated protein 78.
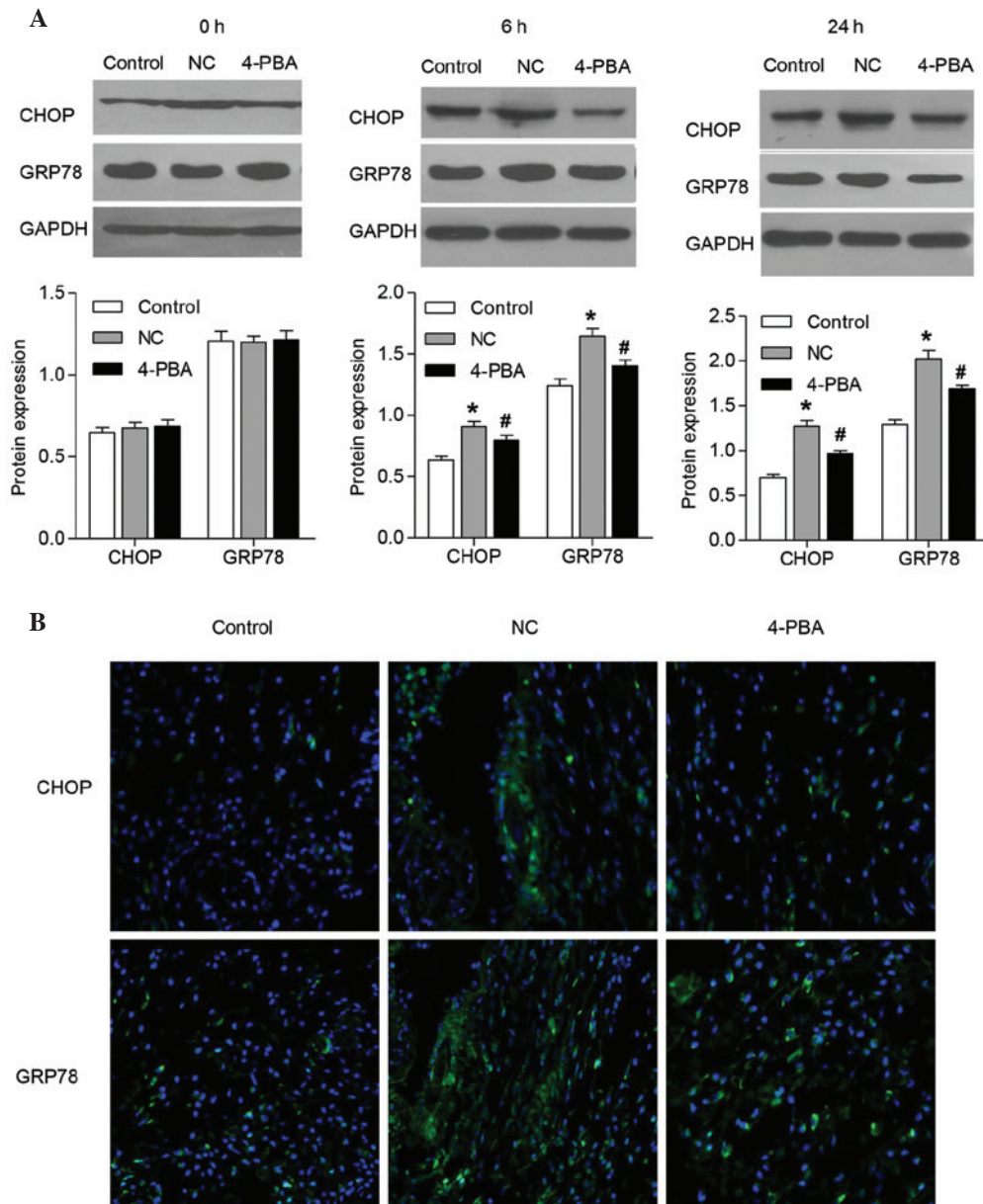

Figure 4. Effects of 4-PBA on the protein expression of CHOP and GRP78 in rat skin flaps following ischemia-reperfusion. CHOP and GRP78 protein expression levels were detected in the skin flap tissue samples (A) 6 and 24 h following ischemia-reperfusion by western blotting, and (B) 24 h following ischemia-reperfusion by immunofluorescence staining. Error bars represent the mean ± standard deviation from five rats/group. *P<0.05, vs. the control group; #P<0.05, vs. the rats treated with saline (NC). Green, CHOP and GRP78 staining; blue, nuclei staining with 4',6-diamidino-2-phenylindole. NC, negative control; 4-PBA, 4-phenylbutyrate; CHOP, CCAAT/enhancer-binding protein-homologous protein; GRP78, glucose-regulated protein 78; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

at the convergence of the endoplasmic reticulum-to-nucleus signaling 1, protein kinase RNA-like endoplasmic reticulum kinase and activating transcription factor-6 signaling pathways (30-33). In the early stages of ER stress, GRP78 expression is upregulated, and in prolonged ER stress over-expression of CHOP is observed. In the present study, the mRNA and protein expression levels of CHOP and GRP78 were significantly increased in a time-dependent manner 6 h following ischemia-reperfusion injury in the skin flap tissue samples of the rats. These findings suggested that CHOP and GRP78 are two important targets for therapeutic intervention, which may allow the inhibition of ischemia-reperfusion injury progression in the skin flap of rats.

Previous studies have suggested that 4-PBA is implicated in the inhibition of ER stress-mediated ischemic injury via the transcriptional regulation of CHOP and GRP78 (27,33,34). Concordant with these observations, the results of the present study demonstrated that pretreatment with 4-PBA significantly reduced the upregulation of CHOP and GRP78 in the skin flaps of the rats following ischemia-reperfusion. These alterations in CHOP and GRP78 expression levels were further supported by immunofluorescence staining. These results suggested that ER stress is important for the induction and maintenance of ischemia-reperfusion injury, and 4-PBA attenuates ER stress in this pathological condition.

In conclusion, the results of the present study suggested that 4-PBA protects against skin flap ischemia-reperfusion injury, and the mechanism underlying this process involves the inhibition of ER stress-mediated apoptosis and expression of ER stress markers, CHOP and GRP78. In addition, the study presented evidence that targeting the ER may provide a therapeutic approach for blocking the apoptotic process induced by skin flap ischemia. Finally, we proposed that the therapeutic potential of 4-PBA may extend to other ER stress-associated diseases.

Acknowledgements

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