

FGF21 attenuates hypoxia-induced dysfunction and apoptosis in HPAECs through alleviating endoplasmic reticulum stress

ALI CHEN¹, JINGJING LIU¹, JIANFENG ZHU¹, XUETAO WANG¹,
ZHAONA XU¹, ZHIMIN CUI¹, DAN YAO¹, ZHIFENG HUANG², MIN XU¹, MAYUN CHEN¹,
PEILIANG WU¹, MANXIANG LI³, LIANGXING WANG¹ and XIAOYING HUANG¹

¹Division of Pulmonary Medicine, The First Affiliated Hospital of Wenzhou Medical University, Key Laboratory of Heart and Lung; ²Key Laboratory of Biotechnology and Pharmaceutical Engineering of Zhejiang Province, Wenzhou Medical University, Wenzhou, Zhejiang 325000; ³Department of Respiratory Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shanxi 710061, P.R. China

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Abstract. Vascular endothelial apoptosis and dysfunction have a crucial role in triggering pathological vascular remodeling of hypoxia-induced pulmonary arterial hypertension (PAH). Fibroblast growth factor (FGF)21, an endocrine regulator, has recently been reported to protect cardiac endothelial cells from damage and suppress inflammatory responses. In addition, FGF21 is reported to be involved in endoplasmic reticulum stress (ERS). Previous studies have suggested that

ERS participates in the development of PAH, and attenuation of ERS could be an effective therapeutic strategy for the protection of pulmonary arteries. However, whether FGF21 has a protective function via suppression of ERS in pulmonary arterial endothelial cells in hypoxia remains unclear. The present study aimed to explore whether FGF21 could reduce the hypoxia-induced apoptosis of human pulmonary arterial endothelial cells (HPAECs) and prevent endothelial dysfunction via the inhibition of ERS. HPAECs were divided into six groups: Normoxia, hypoxia, hypoxia plus FGF21, hypoxia plus salubrinal (an ERS inhibitor), hypoxia plus tunicamycin (an ERS agonist), and hypoxia plus tunicamycin plus FGF21. The endoplasmic reticulum ultrastructure in HPAECs was assessed by transmission electron microscopy, and proliferation and apoptosis were examined by cell counting kit-8 and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling assays, respectively. The expression levels of ERS-related proteins, including binding immunoglobulin protein (BiP), protein kinase R-like endoplasmic reticulum kinase (PERK), phosphorylated (p-) PERK, transcription factor C/EBP homologous protein (CHOP), B-cell lymphoma-2 (Bcl-2) and caspase-4 were detected by western blotting. Transwell migration chamber assays were performed, and the concentration of nitric oxide (NO)/endothelin-1 (ET-1) in the culture medium was determined to examine endothelial function. The results revealed that hypoxia increased the % of apoptotic cells and diminished the viability of HPAECs, accompanied by an upregulation of ERS-dependent apoptosis by increasing the expression of the proapoptotic caspase-4 and decreasing the antiapoptotic Bcl-2. Additionally, hypoxia upregulated the expression of representative proteins in the PERK branch of ERS, including BiP, p-PERK and CHOP, while it downregulated the expression of PERK. Furthermore, the secretion of NO/ET-1 and the migration rate of HPAECs were downregulated under conditions of hypoxia. FGF21 significantly attenuated the hypoxia-induced apoptosis and dysfunction of HPAECs through alleviating the aforementioned changes in ERS-dependent signaling pathways. In conclusion, ERS may be a crucial mechanism in the hypoxia-induced

Correspondence to: Professor Xiaoying Huang or Professor Liangxing Wang, Division of Pulmonary Medicine, The First Affiliated Hospital of Wenzhou Medical University, Key Laboratory of Heart and Lung, 1 Nanbaixiang Street, Ouhai, Wenzhou, Zhejiang 325000, P.R. China
E-mail: zjwzhxy@126.com
E-mail: wzyxywlx@163.com

Abbreviations: PAH, pulmonary arterial hypertension; ERS, endoplasmic reticulum stress; FGF21, fibroblast growth factor 21; HPAECs, human pulmonary arterial endothelial cells; CCK-8, cell counting kit-8; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling assay; BiP, binding immunoglobulin protein; CHOP, transcription factor C/EBP homologous protein; PERK, protein kinase R-like endoplasmic reticulum kinase; Bcl-2, B cell lymphoma-2; NO, nitric oxide; ET-1, endothelin-1; COPD, chronic obstructive pulmonary disease; UPR, unfolded protein response; PASMCs, pulmonary artery smooth muscle cells; ECM, endothelial cell media; FBS, fetal bovine serum; ECGS, endothelial cell growth supplement; PBS, phosphate-buffered saline; eIF2 α , eukaryotic translation initiation factor 2 α ; IRE1, inositol-requiring enzyme 1; XBPI, X-box binding protein 1; ATF6, activating transcription factor 6; CerS6, ceramide synthase 6; 4-PBA, 4-phenylbutyric acid

Key words: pulmonary arterial hypertension, endoplasmic reticulum stress, fibroblast growth factor 21, human pulmonary arterial endothelial cells

apoptosis and endothelial dysfunction of HPAECs. FGF21 may attenuate the hypoxia-induced apoptosis and dysfunction of HPAECs through alleviating ERS, via the PERK/CHOP signaling pathway and inhibition of caspase-4 expression.

Introduction

Pulmonary arterial hypertension (PAH) is a vascular remodeling disease of the lungs that results in right ventricular failure and death within a few years of diagnosis (1). Chronic hypoxia-induced PAH, a common type of PAH, is predominantly secondary to disorders of the respiratory system, including chronic obstructive pulmonary disease (COPD) and obstructive sleep apnea (2). Although the precise pathogenesis of hypoxia-induced PAH remains unclear, it is thought that vascular endothelial damage and dysfunction serve a crucial role in triggering pathological vascular remodeling (3). Pulmonary endothelial cell dysfunction in hypoxia-induced PAH has been demonstrated to be associated with decreased production of nitric oxide (NO) and increased production of endothelin-1 (ET-1), which participate in the subsequent abnormal proliferation of pulmonary endothelial cells and pulmonary smooth muscle cells (4,5). In addition, experimental studies have suggested that endothelial cell apoptosis in the pulmonary microvasculature causes arteriolar occlusion and increases pulmonary vascular resistance, suggesting that vascular endothelial cell apoptosis is closely associated with the pathogenesis of PAH (6,7). The mechanisms responsible for endothelial cell apoptosis in the early stages of hypoxia-induced PAH are yet to be fully elucidated.

In recent studies, endoplasmic reticulum stress (ERS) has been demonstrated to have an important role in the development of PAH. Hypoxia, toxicity, infection and perturbation of Ca^{2+} homeostasis lead to the accumulation of unfolded or misfolded proteins, which results in ERS, activating the adaptive cellular response termed the unfolded protein response (UPR) (8,9). Under mildly stressful conditions, these pathways activate several transcription factors involved in cell survival. However, under conditions of severe acute or chronic stress, the ER UPR shifts from survival to cell death signaling pathways (10). In previous studies, it was identified that ERS participates in the development of PAH *in vitro* and *in vivo*. Sutendra and Michelakis (1) reported that, in response to hypoxia-induced stress, the Nogo B protein from mice pulmonary artery smooth muscle cells (PASMCs), which regulates ER structure and has been implicated in vascular remodeling, serves a role in the development of PAH. Koyama *et al* (11) demonstrated that activation of all branches of the UPR and accompanying inflammation in human pulmonary smooth muscle cells have a major role in the pathogenesis of PAH, and that the chemical chaperones that inhibit ERS may be potential therapeutic agents for PAH. Although studies have indicated that ERS participates in the process of PASMC proliferation in hypoxia-induced PAH, the connection between endothelial dysfunction and ERS in the development of hypoxia-induced PAH remains unclear.

Fan *et al* (12) demonstrated that ERS-associated proteins and the ERS-dependent apoptotic protein caspase-12 were upregulated and the number of pulmonary apoptotic cells was markedly increased in hypoxia-induced PAH in rats,

indicating that ERS-induced apoptosis may be one of the mechanisms underlying hypoxic pulmonary hypertension and pulmonary vascular wall remodeling. The present study aimed to investigate whether ERS participates in hypoxia-induced apoptosis and damage in human pulmonary arterial endothelial cells (HPAECs) through *in vitro* experiments, and to provide additional evidence on the role of ERS in hypoxia-induced PAH.

Fibroblast growth factor (FGF)21, a novel member of the FGF superfamily, is a crucial endogenous regulator of lipid metabolism and systemic glucose. Circulating endogenous FGF21 is elevated in obesity, type 2 diabetes and coronary artery disease. Therefore, FGF21 is considered a stress hormone, mediating the adaptive metabolic response to various stress conditions (13). In addition, FGF21 is reported to be involved in various pathological conditions, including fatty liver disease, ERS, and chronic inflammation (14). Recent studies have indicated that both triglycerides and tunicamycin-induced ERS stimulate FGF21 expression in hepatocytes and increase serum levels of FGF21, indicating that FGF21 has a crucial role in ERS (15,16). Guo *et al* (17) demonstrated that administration of FGF21 restores insulin signaling via inhibiting ERS in the adipose tissue of high-fat diet-induced obese mice. In addition, FGF21 has been reported to protect cardiac endothelial cells from damage and to suppress inflammatory responses (18). FGF21 may be a signal of injured target tissue and may have physiological roles in improving endothelial function at an early stage of atherosclerosis and in halting the development of coronary heart disease (18). It is well known that there are many similarities between the cardiovascular system and the pulmonary vasculature. However, research regarding the associations between FGF21 and PAH is limited. Whether FGF21 could protect endothelial cells from damage, as is the case in the cardiovascular system, remains poorly delineated. Considering the aforementioned relationship between FGF21 and ERS, the present study hypothesized that FGF21 could decrease hypoxia-induced apoptosis and repair damage to HPAECs to improve endothelial function through alleviating ERS.

Materials and methods

Reagents. Salubrinal (an ERS inhibitor; cat. no. SML0951) and tunicamycin (an ERS agonist; cat. no. T7765) were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Rabbit antibodies against binding immunoglobulin protein (BiP), protein kinase R-like endoplasmic reticulum kinase (PERK), B-cell lymphoma-2 (Bcl-2), caspase-4 and GAPDH were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit antibodies against phosphorylated (p-) PERK were purchased from Biorbyt (Cambridge, UK). Mouse antibodies against cluster of differentiation 31 (CD31) and transcription factor C/EBP homologous protein (CHOP) were purchased from Abcam (Cambridge, UK) and Cell Signaling Technology, Inc., respectively. An anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRP)-linked antibody and an anti-rabbit IgG HRP-linked antibody were purchased from Cell Signaling Technology, Inc. FGF21 was obtained from the Wenzhou Medical College Pharmacy School (Wenzhou, China). Endothelial

cell medium (ECM; 1001), fetal bovine serum (FBS) and an endothelial cell growth supplement (ECGS) were purchased from ScienCell Research Laboratories, Inc. (San Diego, CA, USA). Phosphate-buffered saline (PBS) was purchased from Hyclone; GE Healthcare Life Sciences (Logan, UT, USA). The cell counting kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). The ELISA kit was purchased from BoyunBiotechnology Company (Shanghai, China). The terminal deoxyribonucleotide transferase-mediated dUTP nick end-labelling (TUNEL) kit was purchased from Roche Diagnostics (Basel, Switzerland).

Cell lines and cell culture. HPAECs (cat. no. 3100) were purchased from ScienCell Research Laboratories, Inc., and used as previously described (19). The HPAECs were cultured in ECM, supplemented with 5% FBS, 1% ECGS, 100 U/ml penicillin and 100 µg/ml streptomycin. The medium was changed every 2 days. When the cells reached 80-90% confluence, they were washed with PBS and treated with 0.05% trypsin-EDTA for passaging. The cells were identified through microscopy and by positive staining for CD31 (Fig. 1A). For experimental use, HPAECs from passages 3-5 were subcultured and randomly divided into 6 groups: Normoxia group (N); hypoxia group (H); hypoxia+FGF21 group (H+F); hypoxia+salubrinal (ERS inhibitor; 20 µM) group (H+S), with the concentrations used determined from a previous study (20); hypoxia+tunicamycin (ERS agonist; 2.5 ng/ml) group (H+T) with the concentrations used determined from a previous study (21); and hypoxia+tunicamycin+FGF21 group (H+T+F). The H+F group was subdivided into 5 groups with different concentrations of FGF21, as follows: H+F (100 ng/ml), H+F (200 ng/ml), H+F (400 ng/ml), H+F (800 ng/ml) and H+F (1,600 ng/ml), to obtain the optimum concentration of FGF21. Hypoxia-treated HPAECs were treated as aforementioned for 24 h. All hypoxia groups were kept for 24 h in the hypoxia incubator at 37°C with 5% CO₂, 5% O₂ and 90% N₂, with these conditions based on the results of previous studies (22,23), whereas the normoxia group was maintained in a normal incubator at 37°C with 21% O₂, 5% CO₂ and 74% N₂.

Cell viability assay. Cell viability was quantified through CCK-8 assays. HPAECs were seeded at a density of 1x10⁴ cells/well in flat-bottomed 96-well plates. Following pre-incubation in complete medium at 37°C and in 21% O₂ and 5% CO₂ for 24 h, HPAECs were pretreated with FGF21, salubrinal and tunicamycin in prior to exposure to hypoxia. Cell viability was observed under a microscope before the CCK-8 assay. Cell viability was observed under a light microscope (magnification, x100) prior to the CCK-8 assays. After 24 h of hypoxia, the CCK-8 reagent (10 µl/well) was added to each well. Following incubation at 37°C for 2 h, the absorbance of each well was determined using a micro plate reader at 450 nm. Cell viability was determined based on the absorbance of each well.

Detection of apoptosis. Cell apoptosis was evaluated using the TUNEL assay (*In Situ* Cell Death Detection Kit, POD), according to the manufacturer's protocol. HPAECs were exposed to hypoxia and treated with or without the reagents indicated above. Following the treatment, the cells adhered

to cover slips and were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 h at 25°C. The cells were then subjected to TUNEL assays, according to the manufacturer's protocol. DAB was employed as the chromogen and hematoxylin as the counterstain. Finally, the cover slips were observed via light microscopy, and the % of TUNEL-positive cells was assessed in six randomly selected fields from each cover slip.

Ultrastructural examination of the endoplasmic reticulum of HPAECs. HPAECs were cultured in 25 cm² flasks. At the end of the hypoxia exposure period, the HPAECs were washed with PBS and treated with 0.05% trypsin-EDTA to collect cells. Cell aggregates were fixed with 2.5% glutaraldehyde and 1% osmic acid for 1 h at 37°C, prior to being dehydrated with acetone and embedded in epoxy resin 812. The fixed cell aggregates were subsequently cut into ultrathin sections (the thickness of the sections was ~50 nm) with an ultramicrotome and examined with a Hitachi H-7500 transmission electron microscope (Hitachi, Ltd., Tokyo, Japan) following staining with uranyl acetate and lead citrate for 3-6 h at 45°C and for 48 h at 65°C, respectively. Three fields were obtained randomly.

Western blot analysis. Following treatment, HPAECs were lysed with ice-cold RIPA lysis buffer containing PMSF for 30 min. Then, the lysates were centrifuged at 16,000 x g for 30 min at 4°C, and the supernatant was collected. The protein concentrations were determined using a Pierce bicinchoninic acid protein assay kit. Equal amounts of protein (20 µg) were then separated by 12% SDS-PAGE, transferred onto polyvinylidene fluoride membranes, blocked with 5% milk for 1 h at room temperature, and incubated overnight with specific primary rabbit antibodies against BiP (dilution, 1:1,000; 3183), PERK (dilution, 1:1,000; 3192), p-PERK (dilution, 1:100; orb6693), Bcl-2 (dilution, 1:1,000; 2870), caspase-4 (dilution, 1:1,000; ab22687) and GAPDH (dilution, 1:1,000; 5174S) and a mouse antibody against CHOP (dilution, 1:1,000; 2895) at 4°C. The membranes were subsequently washed with TBS/0.1% Tween-20 (TBST) buffer and membranes that had been incubated with anti-BiP, anti-PERK, anti-p-PERK, anti-Bcl-2, anti-caspase-4 and anti-GAPDH antibodies were incubated with anti-rabbit IgG HRP-linked antibody (dilution, 1:10,000; 7074), while membranes that had been incubated with the anti-CHOP antibody were incubated with an anti-mouse IgG HRP-linked antibody (dilution, 1:10,000; 7076) for 1 h at room temperature. Super Signal West Fem to Maximum Sensitivity substrate (cat. no. 3409; Shanghai Boyun BioTech Co., Ltd., Shanghai, China) was used as the visualization reagent. GAPDH was used as an internal control. The optical density of the immune blots was quantified with Quantityone-4.6.2 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Transwell migration chamber assay. The 24-well Transwell system (5 µm; Corning Incorporated, Corning, NY, USA) was used to perform cell migration assays. A total of 600 µl ECM, containing 5% FBS was added to the lower chamber with the experimental treatments. Then, HPAECs were suspended in ECM without serum, and 200 µl cell suspension (at a density of 1.5x10⁵/ml) was added to the upper chambers of the Transwell plates. Following incubation at 37°C for 24 h, the non-migrated cells were removed using a cotton swab. The

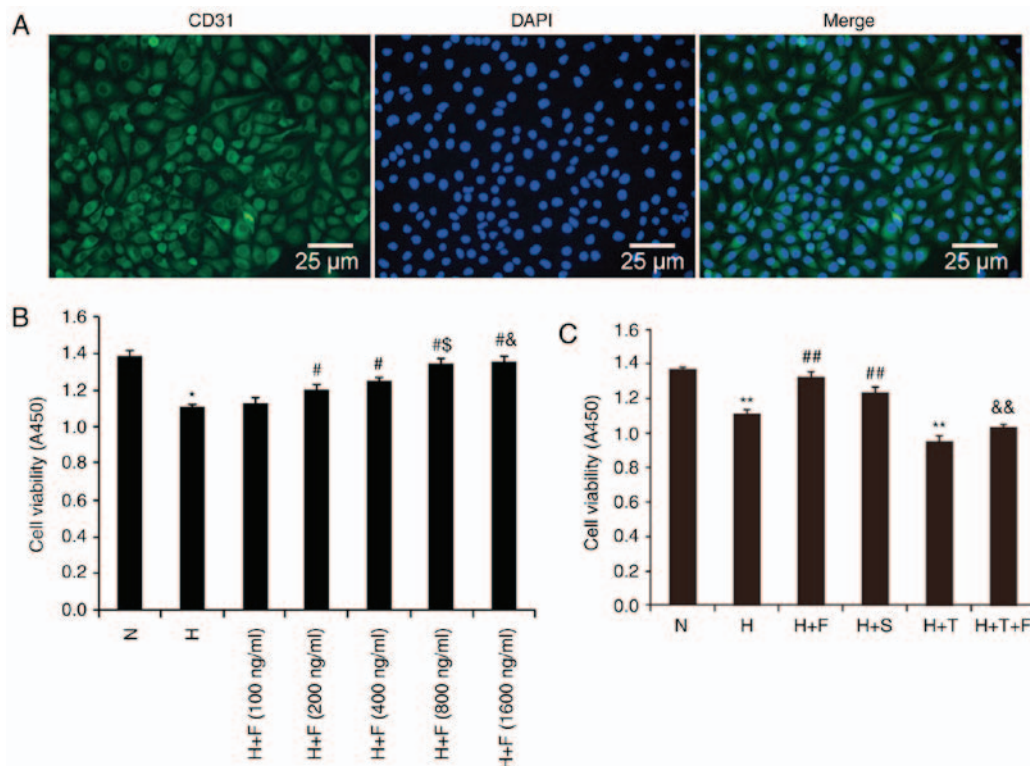


Figure 1. Characterization of HPAECs and cell viability in different experimental groups. (A) HPAECs were identified by microscopy, based on positive staining for CD31 (magnification, $\times 100$; scale bar, $25\ \mu\text{m}$). CD31 staining appears green in the images, while nuclei are counterstained with DAPI and appear blue. (B) HPAECs were exposed to hypoxia and treated with different concentrations of FGF21 (100, 200, 400, 800 and 1,600 ng/ml). Absorbance of cell counting kit-8 at 450 nm in HPAECs was used to determine the relative cell number and viability. (C) HPAECs were exposed to hypoxia and treated with FGF21 (800 ng/ml), ERS inhibitor salubrinal (20 μM), ERS agonist tunicamycin (2.5 ng/ml) or tunicamycin plus FGF21. Cell viability was analyzed via CCK-8 assays. The absorbance of CCK-8 at 450 nm was used to determine the relative cell numbers and viability. Data are expressed as the mean \pm standard deviation ($n=6$). * $P<0.05$ vs. the N group; ** $P<0.01$ vs. the N group; # $P<0.05$ vs. the H group; ## $P<0.01$ vs. the H group; \$ $P<0.05$ vs. the H+F (400 ng/ml); & $P>0.05$ vs. the H+T group (800 ng/ml); && $P<0.01$ vs. the H+T group. HPAECs, human pulmonary arterial endothelial cells; FGF21, fibroblast growth factor 21; ERS, endoplasmic reticulum stress; CCK-8, cell counting kit-8; N, normoxia; H, hypoxia; F, FGF21; S, salubrinal; T, tunicamycin.

migrated cells were then fixed with 4% paraformaldehyde for 10 min, followed by staining with crystal violet for 30 min. Finally, the number of migrated cells was counted under a microscope (Nikon Corporation, Tokyo, Japan), six fields were obtained randomly.

ELISA of NO and ET-1. The accumulated NO and ET-1 levels in the culture medium were determined using ELISA kits (NO, cat. no. 30417H; ET-1, cat. no. 30538H), according to the manufacturer's protocol.

Statistical analysis. All of the experiments were repeated at least three times. The results are expressed as the mean \pm standard deviation. Statistical significance was determined by one-way analysis of variance, followed by the least significant difference (LSD) test (equal variances assumed) or Dunnett's T3 test (equal variances not assumed). $P<0.05$ was considered to indicate a statistically significant difference. All calculations were performed using SPSS version 20.0 (IBM Corporation, Armonk, NY, USA).

Results

Hypoxia and the ERS agonist tunicamycin reduce the viability of HPAECs, and this effect is improved by FGF21 and the ERS inhibitor salubrinal. The absorbance measured at 450 nm in

HPAECs subjected to the CCK-8 assay was used to determine relative cell numbers and viability. As indicated in Fig. 1B, the absorbance was reduced in the H group compared with the N group ($P<0.05$). Under cotreatment with FGF21, the absorbance was significantly enhanced in the H+F (200 ng/ml), H+F (400 ng/ml), H+F (800 ng/ml) and H+F (1,600 ng/ml) compared with the H group ($P<0.05$; Fig. 1B). The absorbance was significantly increased in the H+F (800 ng/ml) group compared with the H+F (400 ng/ml) group ($P<0.05$; Fig. 1B). Meanwhile, compared with the H+F (800 ng/ml) group, there was no significance in the increase of absorbance in the H+F (1,600 ng/ml) ($P>0.05$; Fig. 1B). We chose 800 ng/ml as the optimum concentration of FGF21 in subsequent experiments.

As indicated in Fig. 1C, the absorbance was reduced in the H group and the H+T group compared with that of the N group ($P<0.01$). Under co-treatment with FGF21 or salubrinal, the absorbance was significantly enhanced in the H+F and H+S groups compared with that of the H group ($P<0.01$). The absorbance was increased in the H+T+F group compared with that of the H+T group ($P<0.01$). The results indicated that FGF21, as well as the ERS inhibitor salubrinal, improved the viability of HPAECs under conditions of hypoxia.

Hypoxia and the ERS agonist tunicamycin increase apoptosis, and this effect is reversed by FGF21 and the ERS inhibitor salubrinal. The effects of FGF21 on the apoptosis of HPAECs

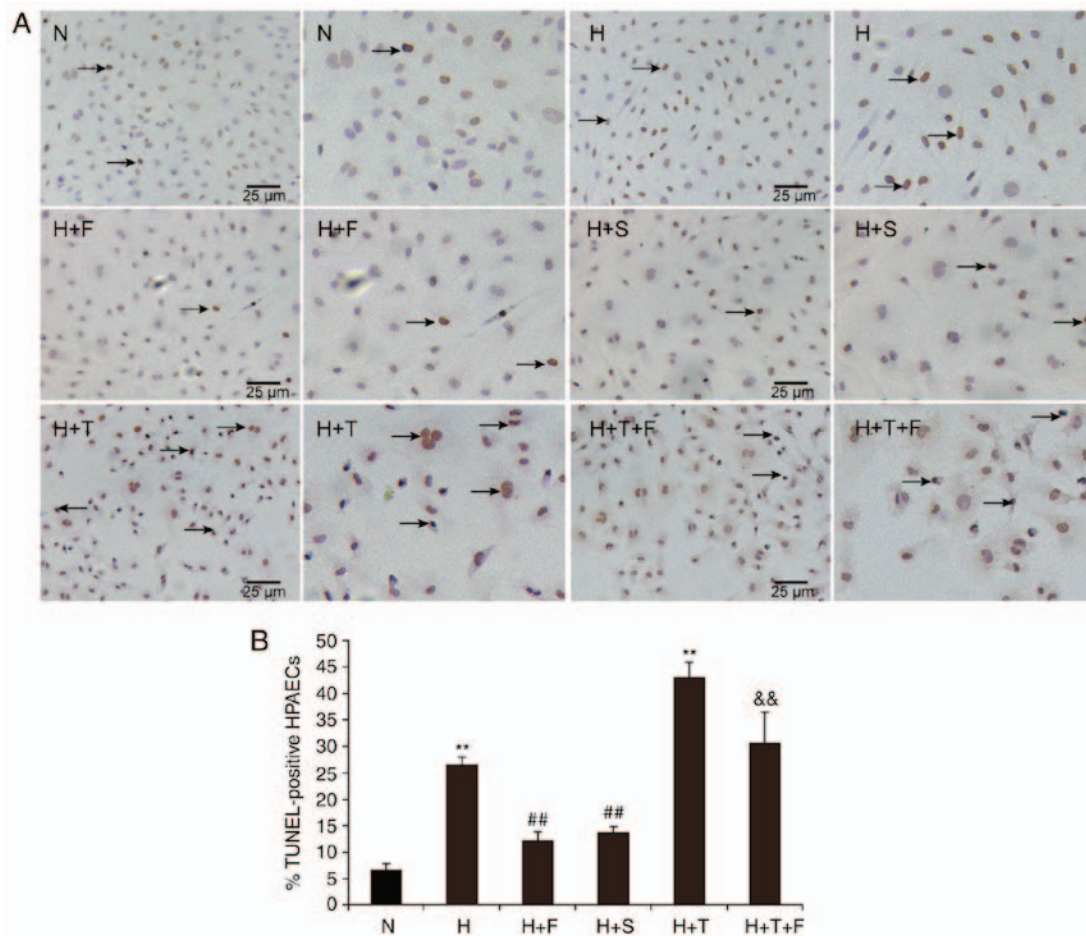


Figure 2. (A and B) Hypoxia increases the apoptosis of HPAECs, and this is reversed by FGF21. The HPAECs from each experimental group were stained with the TUNEL reagent and analyzed by microscopy (magnification, x100; scale bar, 25 μm). The arrows denote TUNEL-positive apoptotic HPAECs (brown staining). Data are expressed as the mean ± standard deviation (n=6). **P<0.01 vs. the N group; ##P<0.01 vs. the H group; &&P<0.01 vs. the H+T group. HPAECs, human pulmonary arterial endothelial cells; FGF21, fibroblast growth factor 21; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling assay; N, normoxia; H, hypoxia; F, FGF21; S, salubrinal; T, tunicamycin.

were evaluated by TUNEL assays. As illustrated in Fig. 2, the % of apoptotic HPAECs (indicated by brown-stained cell nuclei) was significantly increased in the H group compared with the N group (P<0.01). FGF21 or salubrinal treatment decreased the % of apoptotic cells in the H+F and H+S groups compared with the H group (P<0.01; Fig. 2). There was a significant increase in TUNEL-positive cells in the H+T group compared with the N group (P<0.01; Fig. 2), but cotreatment with FGF21 attenuated cell apoptosis in the H+T+F group (P<0.01; Fig. 2). These findings were consistent with the protective function of FGF21 in cardiac endothelial cells (18), suggesting that FGF21 could decrease the rate of apoptosis induced by hypoxia and protect HPAECs from damage or stress.

FGF21 reverses the hypoxia-induced endoplasmic reticulum dilation of HPAECs. To determine whether FGF21 could protect HPAECs against hypoxia-induced ERS, the cells were examined at the ultrastructural level. Compared with the N group, the rough endoplasmic reticulum lumens of the H group and the H+T group were notably expanded, and ribosomes had become detached from the endoplasmic reticulum (Fig. 3). In the H+F and the H+S groups, the expansion of the endoplasmic reticulum lumen was reversed by FGF21 or salubrinal, similar to the normoxia group (Fig. 3). The endoplasmic reticulum

lumen of the H+T group was considerably expanded, while the endoplasmic reticulum lumen of the H+T+F group was not repaired well, and mild expansion of the endoplasmic reticulum lumen continued to be observed in the H+T+F group (Fig. 3). These results indicated that FGF21 is a protective factor that could reverse the hypoxia-induced endoplasmic reticulum dilation of HPAECs.

FGF21 alleviates hypoxia-induced ERS via the PERK/CHOP signaling pathway and via inhibition of caspase-4 expression. In the present study, the roles of the PERK branch and the ERS-dependent apoptosis factor caspase-4 were examined, as regulators of ERS in hypoxia-induced HPAEC damage. To this effect, the protein expression levels of these proteins were measured by western blotting.

The expression of representative proteins in the PERK branch of ERS, including BiP, p-PERK and CHOP, was significantly increased by hypoxia in the H group compared with the N group (P<0.01; Fig. 4A, B and D). This result indicated that the PERK branch of ERS was activated under hypoxic conditions in HPAECs. FGF21 or salubrinal treatment reduced the expression of BiP, p-PERK and CHOP in the H+F and H+S groups, respectively, compared the H group (P<0.01; Fig. 4A, B and D). The expression of BiP, p-PERK and CHOP

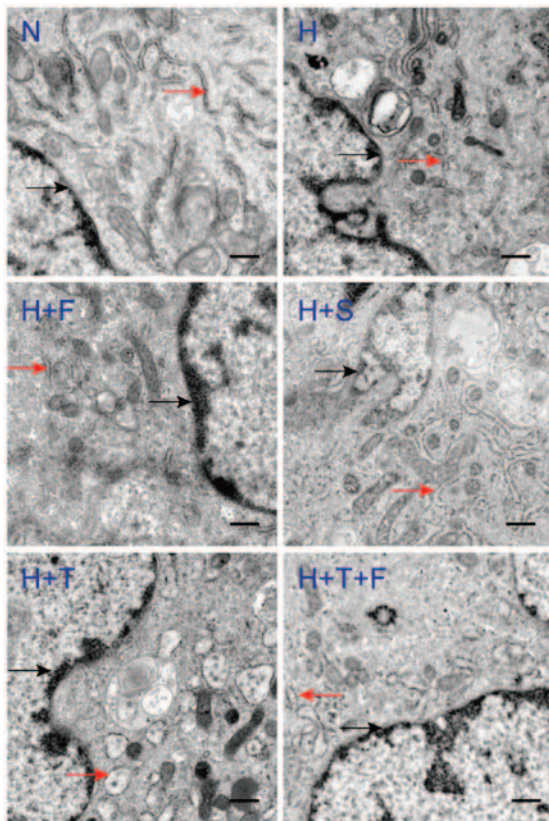


Figure 3. FGF21 reverses the hypoxia-induced endoplasmic reticulum dilation in HPAECs. Ultrathin sections of HPAECs from each experimental group were observed under a Hitachi H-7500 transmission electron microscope. Representative images are shown from three random fields per group. The red arrow denotes the endoplasmic reticulum, and the black arrow denotes the nuclear membrane. Scale bar, 0.5 μ m. FGF21, fibroblast growth factor 21; HPAECs, human pulmonary arterial endothelial cells; N, normoxia; H, hypoxia; F, FGF21; S, salubrinal; T, tunicamycin.

was significantly increased in the H+T group compared with the N group ($P<0.01$; Fig. 4A, B and D), but cotreatment with FGF21 attenuated their expression in the H+T+F group ($P<0.01$; Fig. 4A, B and D). By contrast, the expression of PERK exhibited the opposite trend to the expression of p-PERK (Fig. 4B). The ratio of p-PERK/PERK exhibited a consistent trend with the expression of p-PERK (Fig. 4B and C).

The antiapoptotic mediator Bcl-2 is a downstream target of CHOP, which can downregulate the expression of Bcl-2, thereby increasing the rate of apoptosis. In the present study, the expression of Bcl-2 was downregulated in the H group and the H+T group compared with the N group ($P<0.01$; Fig. 4E). FGF21 or salubrinal treatment increased the expression of Bcl-2 in the H+F and H+S groups compared with the H group ($P<0.01$; Fig. 4E).

The expression of the ERS-induced apoptosis protein caspase-4 was significantly elevated in the H group and the H+T group compared with the N group ($P<0.01$; Fig. 4F). Under cotreatment with FGF21 or salubrinal, the expression of caspase-4 was reduced in the H+F and H+S groups, respectively, compared with the H group ($P<0.01$; Fig. 4F). The expression of caspase-4 was decreased in the H+T+F group compared with the H+T group ($P<0.01$; Fig. 4F). In summary, the present findings indicate, for the first time, that ERS is one of the crucial mechanisms in hypoxia-induced HPAEC

apoptosis, which was reversed by FGF21 through modulation of the PERK/CHOP signaling pathway and inhibition of caspase-4 expression.

Hypoxia and the ERS agonist tunicamycin inhibit the migratory ability of HPAECs, and this effect is improved by FGF21 and the ERS inhibitor salubrinal. As indicated in Fig. 5, the migratory ability of HPAECs was decreased in the H group compared with the N group ($P<0.01$). The migratory ability of HPAECs was significantly increased in the H+F and H+S groups compared with the H group ($P<0.01$; Fig. 5). There was a significant reduction in the number of migrated cells in the H+T group compared with the N group ($P<0.01$; Fig. 5), but cotreatment with FGF21 increased the number of migrated cells in the H+T+F group ($P<0.01$; Fig. 5).

Hypoxia and the ERS agonist tunicamycin reduce the secretion of NO and increase the secretion of ET-1, and these effects are reversed by FGF21 and the ERS inhibitor salubrinal. As presented in Fig. 6A, the secretion of NO was decreased in the H group and the H+T group compared with the N group ($P<0.01$). NO secretion was enhanced under FGF21 or salubrinal treatment in the H+F and H+S groups, respectively, compared with the H group ($P<0.05$; Fig. 6A). NO secretion was increased in the H+T+F group compared with the H+T group ($P<0.01$; Fig. 6A). As far as ET-1 secretion is concerned, ET-1 levels were upregulated by hypoxia in the H group compared with the N group ($P<0.01$; Fig. 6B). FGF21 or salubrinal treatment decreased the secretion of ET-1 in the H+F and H+S groups, respectively, compared with the H group ($P<0.01$; Fig. 6B). The secretion of ET-1 was significantly increased in the H+T group compared with the N group ($P<0.01$; Fig. 6B), but cotreatment with FGF21 reduced the secretion of ET-1 in the H+T+F group ($P<0.01$; Fig. 6B). The balance of NO and ET-1 production has a key role in the function of endothelial cells. Hence, the present findings suggested that FGF21 could improve endothelial function and protect HPAECs from damage by regulating the secretion of NO and ET-1.

Discussion

Abnormal growth of PASMCs, endothelial cell, fibroblasts, and myofibroblasts is involved in the development of vascular remodeling in hypoxia-induced PAH. The pathology of PAH is also characterized by infiltration of activated inflammatory and immune circulating cells. Among the cells types found within remodeling pulmonary arteries, PASMCs have been the predominant subject of scientific research, but pulmonary artery endothelial cells (PAECs) are also increasingly studied (24). None of the drugs currently available for treating PAH have been demonstrated to effectively reverse the disease or significantly improve long-term survival (1). The present study demonstrated that hypoxia-stimulated ERS contributes towards endothelial apoptosis and dysfunction, which is one of the etiologies for hypoxia-induced PAH.

The data from the current study indicated that the expression of ERS-associated proteins BiP, p-PERK, CHOP and caspase-4 was significantly increased in HPAECs exposed to hypoxia. This was accompanied by an increased rate of

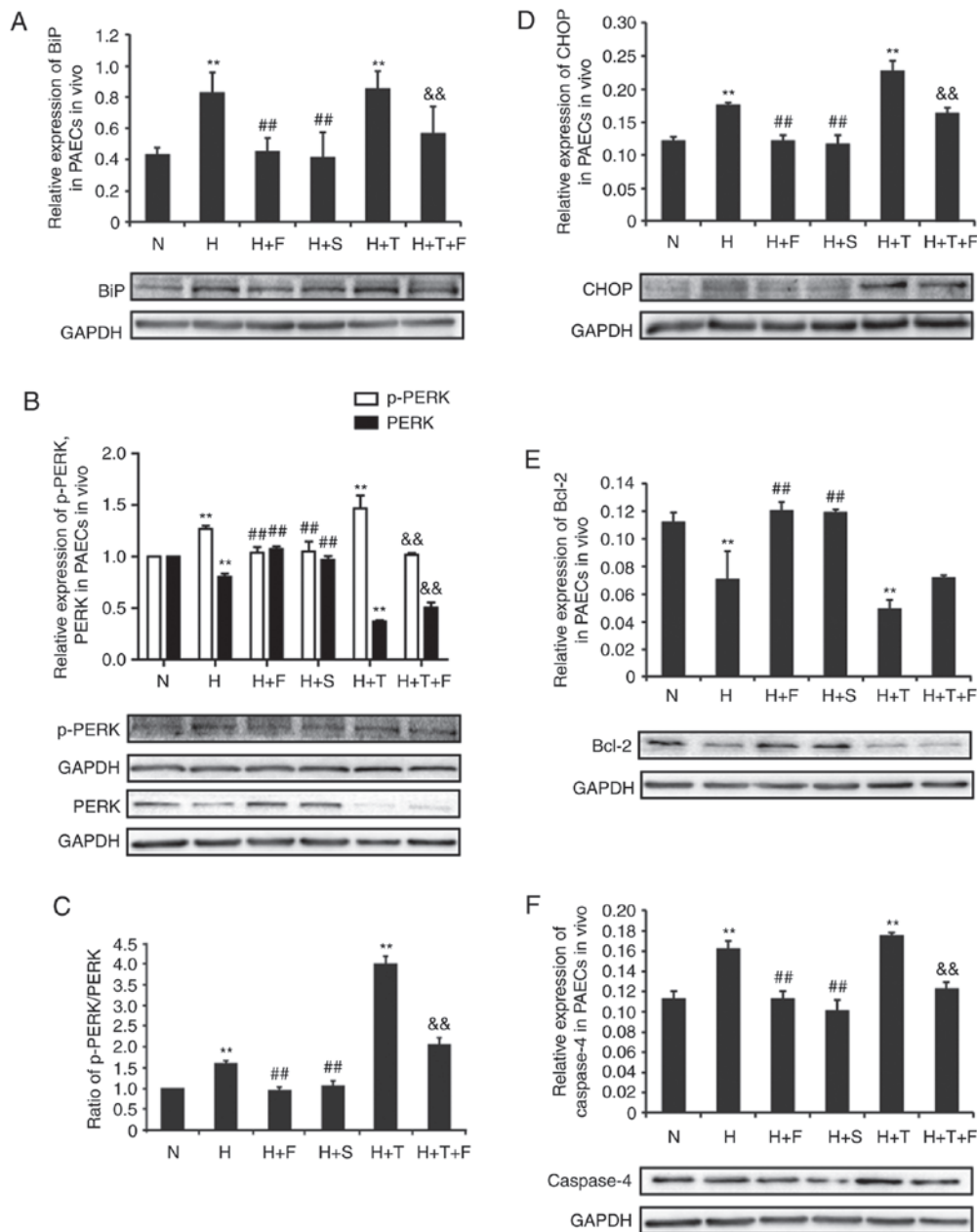


Figure 4. FGF21 alleviates the hypoxia-induced ERS by modulating the expression of ERS-related proteins in HPAECs. HPAECs were treated as indicated and protein expression levels were examined by western blotting. Representative blots and quantification are shown for (A) BiP, (B) p-PERK and PERK, (C) the ratio of p-PERK/PERK, (D) CHOP, (E) Bcl-2, (F) Relative expression of the ERS-dependent apoptotic protein caspase-4 in HPAECs was analysed by western blotting. GAPDH was used as an internal control. All experiments were performed in triplicate, and data are expressed as the mean \pm standard deviation (n=3). **P<0.01 vs. N group; ##P<0.01 vs. the H group; &&P<0.01 vs. the H+T group. FGF21, fibroblast growth factor 21; ERS, endoplasmic reticulum stress; HPAECs, human pulmonary arterial endothelial cells; BiP, binding immunoglobulin protein; p-, phosphorylated; PERK, protein kinase R-like endoplasmic reticulum kinase; CHOP, transcription factor C/EBP homologous protein; Bcl-2, B cell lymphoma-2; N, normoxia; H, hypoxia; F, FGF21; S, salubrinal; T, tunicamycin.

apoptosis and damaged migratory and secretory abilities, indicating that hypoxic conditions stimulate ERS, which in turn may induce endothelial apoptosis and dysfunction in HPAECs. These results are consistent with those of previous reports (12,25), where expression of the ERS marker proteins glucose-regulated protein (GRP)78, GRP94 and caspase-12 were demonstrated to be upregulated in hypoxia-induced PAH in rats, additionally, the number of pulmonary apoptotic cells in the hypoxia group was markedly increased compared with the control group (12,25), suggesting that ERS-induced apoptosis may be one of the mechanisms underlying hypoxic pulmonary hypertension and pulmonary vascular wall remodeling.

Under cellular stress, the ERS activates three branches of the UPR: The double-stranded RNA-activated PERK/eukaryotic translation initiation factor 2 α (eIF2 α) pathway; the inositol-requiring enzyme 1 (IRE1)/X-box binding protein 1 (XBP1) pathway; and the activating transcription factor 6 (ATF6) pathway (26).

PERK is a key ER resident transmembrane molecule whose activation is thought to be the first event in sensing the UPR (10). In resting cells under physiological conditions, as one of the most highly expressed ER chaperones, BiP (GRP78) binds to PERK. Under the effects of harmful stress, PERK disassociates from BiP and is activated by

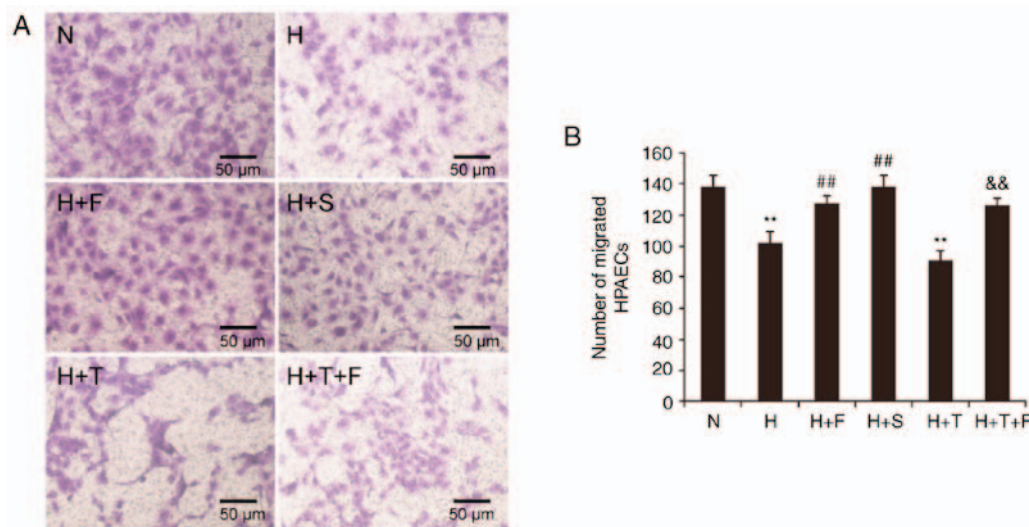


Figure 5. (A and B) Effect of FGF21 on the migratory ability of HPAECs. HPAEC migration was assessed using Transwell migration chambers. Representative images are shown for the migrated cells in each experimental group (magnification, $\times 100$; scale bar, $50 \mu\text{m}$), as well as quantification of the numbers of migrated HPAECs. Data are expressed as the mean \pm standard deviation ($n=6$). ** $P<0.01$ vs. the N group; ## $P<0.01$ vs. the H group; && $P<0.01$ vs. the H+T group. FGF21, fibroblast growth factor 21; HPAECs, human pulmonary arterial endothelial cells; N, normoxia; H, hypoxia; F, FGF21; S, salubrinal; T, tunicamycin.

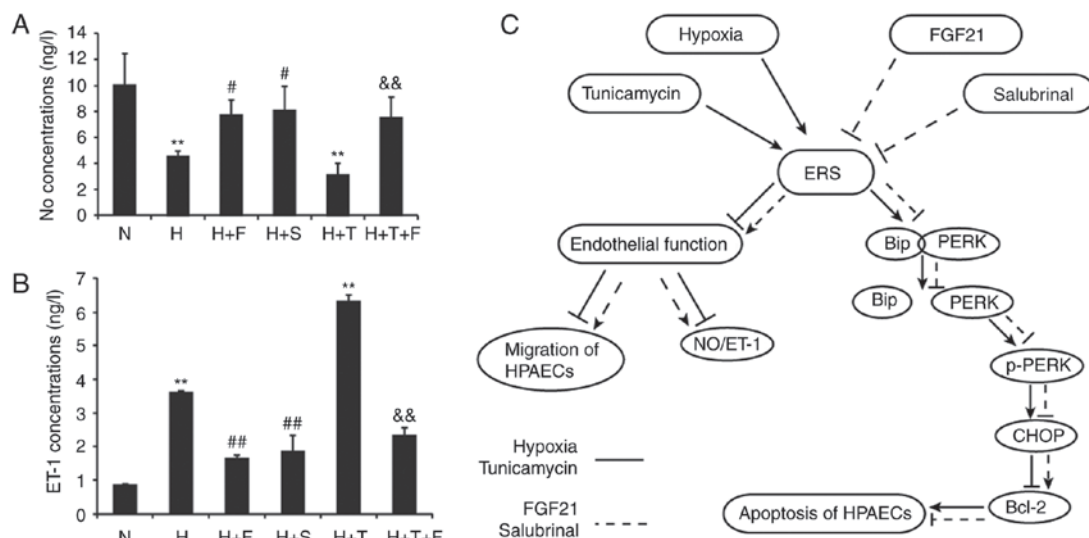


Figure 6. Effect of FGF21 on NO and ET-1 secretion in HPAECs. (A) HPAECs were treated as indicated and at the end of treatment, the cell culture medium was collected and assayed by ELISA for the levels of secreted NO and (B) the levels of secreted ET-1. Data are expressed as the mean \pm standard deviation ($n=3$). ** $P<0.01$ vs. the N group; # $P<0.05$ and ## $P<0.01$ vs. the H group; && $P<0.01$ vs. the H+T group. (C) Schematic of the proposed mechanism by which FGF21 attenuates hypoxia-induced apoptosis and dysfunction by alleviating ERS in HPAECs. FGF21, fibroblast growth factor 21; NO, nitric oxide; ET-1, endothelin-1; HPAECs, human pulmonary arterial endothelial cells; ERS, endoplasmic reticulum stress; N, normoxia; H, hypoxia; F, FGF21; S, salubrinal; T, tunicamycin; BiP, binding immunoglobulin protein; p-, phosphorylated; PERK, protein kinase R-like endoplasmic reticulum kinase; CHOP, transcription factor C/EBP homologous protein; Bcl-2, B cell lymphoma-2.

self-phosphorylation to initiate ERS (27-29). Chronic or severe ERS would activate ERS-dependent apoptosis, including the CHOP and caspase-mediated apoptotic pathways. The CHOP-mediated pathway is associated with PERK/eIF2 α mediated transcriptional activation of the C/EBP-homologous protein CHOP, which then downregulates the expression of the antiapoptotic mediator Bcl-2. Activation of the caspase cascade is a well-known proapoptotic event that is also involved in ERS-induced apoptosis (28). Several caspases are believed to serve roles in this process, including caspase-2, caspase-4 (human), caspase-12 (rodents), caspase-7, caspase-3 and caspase-9. It has been suggested that caspase-12 is specific

to death signals in ERS rather than to other cell death mechanisms (29,30). The present results demonstrated that hypoxic conditions activated PERK/CHOP signaling and increased the expression of human caspase-4, which may participate in the apoptosis and damage of HPAECs.

Both in yeast and in mammalian cells, UPR signaling can be initiated by the use of chemical agents that induce ERS, such as the N-linked glycosylation inhibitor tunicamycin, the calcium pump inhibitor thapsigargin, and the reducing agent dithiothreitol (31). A known ERS inducer tunicamycin mediates the degradation of ceramide synthase 6 (CerS6) and subsequent ATF-6 and caspase-3 activation. Senkal *et al* (32)

reported that downregulation of CerS6 increased ATF-6 activation, as determined by increased expression of its downstream target CHOP in UM-SCC-22A cells. CerS6 knockdown also activated CHOP in human squamous lung cancer H1650 cells, leading to cell death (32).

An increasing number of studies have reported that ERS inhibitors protect cells under adverse stimuli. In the current study, salubrinal was used as an ERS inhibitor. Salubrinal, a selective inhibitor of cellular complexes, dephosphorylates eIF2 α . During ERS, PERK, an ER-resident transmembrane protein, oligomerizes and phosphorylates eIF2 α at serine 51 (33). Salubrinal induces rapid and robust eIF2 α phosphorylation and its downstream effects, including downregulation of cyclin D1 and upregulation of growth arrest and DNA damage inducible protein 34 and CHOP (34). The phosphorylation of eIF2 α is cytoprotective during ERS. In recent years, another ERS inhibitor, 4-phenylbutyric acid (4-PBA), has also been employed. 4-PBA can inhibit ERS as a chemical chaperone by stabilizing peptide structures, improving luminal folding capacity, and attenuating cell damage. Wu *et al* (35) demonstrated that 4-PBA improved pulmonary arterial remodeling and suppressed the expression of ERS indicators, including GRP78, GRP94, ATF6, IRE-1, PERK, CHOP and Bcl-2, in a monocrotaline-induced PAH rat model.

FGF21 is a member of the FGF family. In the adult organism, FGFs are homeostatic factors and exhibit functions in tissue repair and response to injury (36). FGF21 is an endocrine regulator that participates in lipid and glucose metabolism, and has recently been reported to protect cardiac endothelial cells from damage and suppress inflammatory responses. In the present study, the role of FGF21 in hypoxia-induced apoptosis and dysfunction was investigated in HPAECs. Studies by Schaap *et al* (37) suggest that FGF21 expression is regulated by ERS. The authors reported that FGF21 mRNA levels are increased by triglyceride-induced ERS in rat H4IIE cells and rat primary hepatocytes. Wan *et al* (8) demonstrated that FGF21 is the target gene of CHOP, and that transcription and mRNA stabilization are responsible for the CHOP-mediated induction of FGF21 expression in ERS, indicating that ERS is the key mechanism of FGF21 regulation in several metabolic diseases. In addition, Jiang *et al* (38) indicated that intraperitoneal administration of the ER stressor tunicamycin activates ERS, which results in liver steatosis associated with upregulated expression of hepatic FGF21 in the liver. Furthermore, administration of recombinant FGF21 in mice alleviates PERK/CHOP signaling in the liver, resulting in the reversal of ERS-induced liver steatosis. In addition to the evidence presented above, previous studies have indicated that FGF21 and ERS are closely associated in metabolism. FGF21 may act as an inhibitor, similar to salubrinal or 4-PBA, and attenuate ERS to protect cells from damage. In the present study, the results following treatment of HPAECs with hypoxia and the ERS agonist tunicamycin demonstrated that administration of FGF21 ameliorated the hypoxia-induced endothelial apoptosis and dysfunction, and improved the viability of HPAECs. These observations are consistent with previous studies on the cardiovascular system, indicating that FGF21 could prevent damage to cardiac endothelial cells (18). In the present study, the results revealed that FGF21 downregulated p-PERK

and subsequently increased the expression of CHOP, ultimately upregulating the expression of the antiapoptotic mediator Bcl-2, and downregulating the expression of caspase-4, which decreased the number of apoptotic cells and enhanced the viability of HPAECs. Hypoxia and tunicamycin resulted in an expanded shape of the ER in HPAECs, while this effect was notably normalized following treatment with FGF21 and the ERS inhibitor salubrinal. Furthermore, the results indicated that FGF21 alleviated hypoxia-induced endothelial dysfunction, by decreasing the secretion of ET-1, increasing the secretion of NO and repairing damage to the cell migration ability, processes that are involved in the pathogenesis of hypoxia-induced PAH (39).

The balance between NO and ET-1 has a significant role in the development of hypoxia-induced PAH. In patients with PAH, changes in the expression of various endothelial vasoactive mediators, including NO, ET-1, prostacyclin, serotonin and thromboxane, have been increasingly recognized. Since most of these mediators affect the growth of smooth muscle cells, this may facilitate the development of pulmonary vascular hypertrophy. The present study aimed to explore the mechanism of the apoptosis and dysfunction of HPAECs under conditions of hypoxia through observing the cell morphology and function, and protein level changes. Endothelial function was investigated by assaying for the cell migration ability, and the secretion levels of NO and ET-1. Overproduction of eNOS, which stimulates the conversion of L-arginine to citrulline, producing NO (40), prevents hypoxia-induced PAH in transgenic mice (41). Several previous studies suggest that NO protects against hypoxia-induced vasoconstriction in the lungs, inhibits smooth muscle proliferation and platelet aggregation, and decreases the secretion of ET-1 (39). ET-1 expression is elevated in animal models of PH and in patients with PH (39). ET-1 receptor antagonists, such as bosentan, improve the functional status of patients and other indices of PH-related morbidity (42). The present results revealed that hypoxia reduced the secretion of NO and increased the secretion of ET-1 in HPAECs. In addition, FGF21 and the ERS inhibitor salubrinal were demonstrated to improve the secretion of NO and ET-1 in HPAECs under conditions of hypoxia.

In summary, the results of the present study revealed that ERS is a key mechanism involved in the hypoxia-induced apoptosis and dysfunction of HPAECs, which are important processes in the initiation of hypoxia-induced PAH. FGF21 attenuated the hypoxia-induced dysfunction and apoptosis of HPAECs through diminishing ERS, which was mediated by the PERK/CHOP signaling pathway (as illustrated in Fig. 6C), and by the inhibition of caspase-4 expression. Although FGF21 was demonstrated to alleviate the main ERS-dependent death signaling pathway in HPAECs, the mechanism by which FGF21 is associated with ERS has yet to be fully elucidated. Additionally, the role of FGF21 in ERS and PAH *in vivo* remains unclear and requires further research. However, the present findings strongly support the notion that FGF21 may have promising therapeutic potential for the treatment of hypoxia-induced PAH.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

AC designed and performed the experiments, analysed the data and wrote the paper; XH and LW designed the experiments and helped to draft the manuscript; JL, JZ and XW performed the experiments and collected data. ZX and MX performed the experiments; ZC, ML, DY and ZH participated in the study design and supervised the study; MC and PW collected data and performed the analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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