Polysaccharides from *Enteromorpha Prolifera* Ameliorate Acute Myocardial Infarction *in Vitro* and *in Vivo* via Up-Regulating HIF-1 α

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Summary

Acute myocardial infarction (AMI) is a serious heart disease and the main reason for heart failure and sudden death worldwide. This study investigated the effects of polysaccharides from *Enteromorpha prolifera* (PEP) on AMI *in vitro* and *in vivo*, as well as the underlying mechanisms.

Human cardiac microvascular endothelial cells (HCMVEC) were cultured *in vitro* in an oxygen-glucose deprivation (OGD) environment to induce injury. The viability and apoptosis of HCMVEC were then detected using CCK-8 assay and Annexin V-FITC/PI staining, respectively. ELISA was performed to measure the concentrations of inflammatory cytokines. Cell transfection was conducted to reduce the expression of HIF-1 α . Expression of key factors involving in cell proliferation, apoptosis, autophagy, MEK/ERK, and the NF- κ B and mTOR pathways were evaluated using Western blotting. *In vivo*, Wistar rats were pre-treated by PEP and AMI was induced. The infarct size and cardiac functions (LVEDD, LVEF and LVFS) were measured.

In vitro, PEP treatment significantly protected HCMVEC from OGD-induced viability loss, proliferation inhibition, apoptosis, inflammatory cytokine expression, and autophagy. Moreover, PEP enhanced the expression of HIF-1 α in HCMVEC via the MEK/ERK pathway. HIF-1 α participated in the protective effects of PEP on OGD-treated HCMVEC. Furthermore, PEP attenuated OGD-induced NF- κ B pathway activation and promoted the mTOR pathway in HCMVEC. *In vivo*, PEP pre-treatment reduced the infarct size and enhanced the LVEDD, LVEF and LVFS of rats via up-regulation of HIF-1 α .

PEP ameliorated AMI *in vitro* and *in vivo* through up-regulation of HIF-1 α . *In vitro*, PEP could activate the MEK/ERK and mTOR pathways, but inactivate the NF- κ B pathway in OGD-treated HCMVEC.

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Key words: Oxygen-glucose deprivation, MEK/ERK pathway, NF- κ B pathway

Acute myocardial infarction (AMI) is a common and serious heart disease and the main reason for heart failure and sudden death worldwide.^{1,2} The clinical symptoms of AMI include severe persistent chest pain, shortness of breath, abnormal heart beating, sweating, and weakness.^{3,4} Even though the diagnosis and treatment methods for AMI have been improved in recent years, many patients still die of this disease.⁵⁻⁷ Pathological research has proved that acute and persistent ischemia or hypoxia of coronary microvasculature is closely related to the occurrence of AMI.^{8,9} Therefore, it is conceivable that searching for more effective novel medicines for alleviating coronary microvasculature damage will be helpful for AMI prevention and treatment.

Enteromorpha prolifera belongs to the phylum *Chlorophyta*, class *Chlorophyceae*, order *Ulinales*, and genes

Enteromorpha.¹⁰ As one of the main algae genera that causes green tide, the over-growth of *Enteromorpha prolifera* has a noticeable negative impact on the environment and aquaculture.¹¹ Researchers have been making great efforts to assess the value of *Enteromorpha prolifera* in bio-oil extraction, marine aquaculture, food processing and drug development.¹²⁻¹⁶ Polysaccharides are the major active components isolated from *Enteromorpha prolifera*,¹⁷ which account for over 50% of the dry weight of *Enteromorpha prolifera* and exert multiple beneficial activities, such as anti-oxidation, anti-microbial, anti-hyperlipidemia, immunomodulation and glucose metabolism.^{10,17-20} However, no information is currently available concerning the effects of polysaccharides from *Enteromorpha prolifera* (PEP) on AMI. More experimental and clinical research is still needed to further explore the potential protective roles

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of PEP in AMI.

Hypoxia inducible factor 1 α (HIF-1 α) is a transcription factor that is only stabilized in a hypoxia environment, which can control the expression of a number of genes in response to low oxygen conditions.^{21,22} In a normoxic environment, HIF-1 α is rapidly degraded by the ubiquitin-proteasome pathway.²³ Studies have reported that up-regulation of HIF-1 α can reduce infarction and attenuate the progression of cardiac dysfunction in AMI.^{24,25}

In this research, we explored the effects of PEP on AMI *in vitro* and *in vivo*. *In vitro*, human cardiac microvascular endothelial cells (HCMVEC) were cultured under oxygen-glucose deprivation (OGD) environment to simulate cardiac microvasculature cell damage. The effects of PEP on OGD-induced cell viability loss, proliferation inhibition, apoptosis, inflammatory cytokine expression and autophagy were investigated. *In vivo*, Wistar rats were pre-treated by PEP and AMI was induced. The infarct size and cardiac functions (LVEDD, LVEF and LVFS) were assessed. Moreover, the possible internal molecular mechanisms related to up-regulation of HIF-1 α were also analyzed *in vitro* and *in vivo*. Our findings will be helpful for understanding the protective effects of PEP on AMI.

Methods

Preparation of PEP solution: Fresh *Enteromorpha prolifera* was collected and dried in a drying cabinet (Thermo Fisher Scientific, MA, USA). Next, dried *Enteromorpha prolifera* powder was homogenized in 95% ethanol at 60°C for 2 hours to remove any pigments and low-molecular-weight substances. Subsequently, the residue was extracted 3 times with a 20-fold hot volume of distilled water at 90°C for 2 hours, and then centrifuged to remove water-insoluble materials. The suspension was concentrated and precipitated with 95% ethanol at 4°C for 24 hours to yield the crude PEP. The crude PEP was dissolved in distilled water, freeze-thawed, centrifuged at 4°C until no insoluble substance was visible, and deproteinized using the Sevag method.²⁶ After this, the deproteinized PEP was dried, weighed, and dissolved in distilled water to 1 mg/mL. Cells were pre-treated with PEP at the concentration of 0-100 μ g/mL for 1 hour before OGD treatment.

Cell culture and treatment: HCMVEC were obtained from Lonza (Basel, Switzerland) and cultured in EGM2-MV full medium (containing endothelial cell growth factors such as VEGF and bFGF, obtained from Cambrex BioScience, MD, USA) in 75 cm² cell culture flasks. Flasks were maintained at 37°C in a humidity incubator (Thermo Fisher Scientific) with 5% CO₂.

Culture medium was replaced by glucose-free medium with or without PEP treatment before OGD. Cultured cells were placed in an anaerobic, temperature controlled (37 $\pm$ 0.5°C) chamber which was flushed with 95% N₂ and 5% CO₂ for 6 hours to stimulate injury. HCMVEC cultured in normal medium under normoxia served as the control.

CH5126766 and rapamycin were both purchased from ApexBio Technology (TX, USA) and used as an MEK/ERK pathway inhibitor and mTOR pathway inhibi-

tor, respectively.

Cell transfection: si-HIF-1 α and its negative control (siNC) were designed and synthesized by GenePharma Corporation (Shanghai, China). Cell transfection was conducted using Lipofectamine 3000 reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Transfection efficiency was assessed by Western blotting.

Cell counting kit-8 (CCK-8) assay: CCK-8 assay (Beyotime Biotechnology, Shanghai, China) was used to detect the viability of HCMVEC. Briefly, HCMVEC were seeded onto 96-well plates (Thermo Fisher Scientific) with 5 $\times$ 10³ cells per well. After the relevant treatment or transfection, 10 μ L CCK-8 kit solution was added into each well of the plates and the plates were placed in a 37°C humidified incubator for 1 hour. Next, the absorbance of each well at 450 nm was recorded using a Microplate Reader (Bio-Tek Instruments, VT, USA). Cell viability (%) was calculated by determining the average absorbance of the treatment (transfection) group/average absorbance of the control group $\times$ 100%.

Cell apoptosis assay: Apoptosis of HCMVEC was measured using an Annexin V-FITC/PI Apoptosis Detection kit (Solarbio, Beijing, China). Briefly, HCMVEC were seeded onto 6-well plates (Thermo Fisher Scientific) with 5 $\times$ 10⁴ cells per well. After the relevant treatment or transfection, cells in each group were collected, washed with phosphate buffered saline (PBS) for twice, and stained using 5 μ L Annexin V-FITC and 5 μ L PI solution for 15 minutes at room temperature in the dark. The percentage of apoptotic cells in each group was recorded using a flow cytometer (Beckman Coulter, CA, USA). Data were analyzed using FlowJo software (Tree Star Instrument, OR, USA).

Enzyme-linked immunosorbent assay (ELISA): ELISA was performed to measure the concentrations of tumor necrosis factor α (TNF- α) and interleukin 6 (IL-6) in culture supernatant of HCMVEC. Briefly, HCMVEC were seeded onto 6-well plates with 5 $\times$ 10⁴ cells per well. After the relevant treatment or transfection, culture supernatant was collected from each group. The concentrations of TNF- α and IL-6 in culture supernatant were measured using a Human TNF- α ELISA kit and Human IL-6 ELISA kit (ab 181421, ab46027, Abcam Biotechnology, MA, USA), respectively.

Western blotting assay: After the relevant treatment or transfection, total proteins in HCMVEC were isolated using RIPA Lysis buffer (Beyotime Biotechnology) containing protease inhibitors (Roche, Basel, Switzerland). Total proteins in the cytoplasm of HCMVEC were isolated using a Cytoplasmic Protein Extraction kit (Boster Biology Corporation, Wuhan, China). The concentration of total proteins was quantified using a BCA Protein Assay kit (Beyotime Biotechnology) according to the manufacturer's protocol. The Bio-Rad Bis-Tris Gel system (Bio-Rad Laboratories, Hercules, CA, USA) was conducted to establish the Western blotting system. Then, equal concentrations of total proteins were electrophoresed in polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking with 5% bovine serum albumin (BSA, Sigma-Aldrich, MO, USA) at room temperature for 1 hour, the PVDF membranes were incubated with pri-

Figure 1. PEP protected HCMVEC from OGD-induced injury. **A:** After 0–100 μg/mL of PEP treatment, the viability of HCMVEC was detected by CCK-8 assay. **B–G:** After OGD incubation and/or PEP (60 μg/mL) treatment, the viability of HCMVEC (**B**), the protein levels of p53 and cyclin D1 in HCMVEC (**C**), the apoptosis of HCMVEC (**D**), the protein levels of Bcl-2, Bax, cytochrome C, pro-caspase 9, cleaved-caspase 9, pro-caspase 3 and cleaved-caspase 3 in HCMVEC (**E**), the concentrations of TNF-α and IL-6 in culture supernatant of HCMVEC (**F**), and the protein levels of LC3-I, LC3-II and p62 in HCMVEC (**G**) were assessed using CCK-8 assay, Western blotting, Annexin V-FITC/PI Apoptosis Detection kits, and ELISA, respectively. PEP indicates polysaccharides from *Enteromorpha prolifera*; OGD, oxygen-glucose deprivation; HCMVEC, human cardiac microvascular endothelial cells; TNF-α, tumor necrosis factor α; and IL-6, interleukin 6. All experiments were repeated 3 times and results are presented as the mean ± standard deviation (SD). For Western blotting, the most representative results are shown. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ versus control group; # $P < 0.05$ or ## $P < 0.01$ versus OGD group.

primary antibodies at 4°C for 12 hours. All primary antibodies were dissolved in 1% solution with a dilution of 1:1000. Anti-p53 antibody (ab131442), Anti-Cyclin D1 antibody (ab226977), Anti-Bcl-2 antibody (ab59348), Anti-Bax antibody (ab53154), Anti-Cytochrome C antibody (ab90529), Anti-Pro-caspase 9 antibody (ab2013), Anti-Cleaved-caspase 9 antibody (ab2324), Anti-Pro-caspase 3 antibody (ab32150), Anti-Cleaved-caspase 3 antibody (ab2302), Anti-LC3-I/II antibody (ab128025), Anti-p62 antibody (ab155686), Anti-HIF-1α antibody (ab82832), Anti-t-MEK antibody (ab32091), Anti-p-MEK antibody (ab129457), Anti-t-ERK antibody (ab54230), Anti-p-ERK antibody (ab201015), Anti-t-p65 antibody (ab16502), Anti-p-p65 antibody (ab131100), Anti-t-IκBα antibody (ab7217), Anti-p-IκBα antibody (ab133462), Anti-t-mTOR antibody (ab2732), Anti-p-mTOR antibody (ab84400) and Anti-β-

actin antibody (ab8226) were purchased from Abcam Biotechnology. Anti-p70S6K antibody (#9202) and Anti-p70S6K antibody (#9234) were purchased from Cell Signaling Technology (Beverly, MA, USA). Subsequently, the PVDF membranes were incubated with IRDye 680RD Goat anti-Rabbit (or anti-Mouse) (H+L) secondary antibodies (925-68070, 925-68071, Odyssey, Li-cor, NE, USA) at room temperature for 1 hour. The signals of proteins were recorded using an OdysseyClx Infrared Imaging system (Odyssey, Li-cor) and the intensities of the bands were analyzed using Li-cor Odyssey software (Odyssey, Li-cor).

AMI model in rats: Forty Wistar rats (male, 8–10 weeks, 254–308 g) were obtained from Shandong University Laboratory Animal Center (Jinan, China). After being fed in our facility for 1 week, 8 rats were used as controls

Figure 2. PEP up-regulated the expression of HIF-1 α in HCMVEC. The protein level of HIF-1 α in HCMVEC after OGD incubation and/or PEP (60 μ g/mL) treatment was measured using Western blotting. PEP indicates polysaccharides from *Enteromorpha prolifera*; OGD, oxygen-glucose deprivation; HCMVEC, human cardiac microvascular endothelial cells; and HIF-1 α , hypoxia inducible factor 1 α . Experiments were repeated 3 times and the results are presented as the mean $\pm$ standard deviation (SD). The most representative results are shown. * P < 0.05 or ** P < 0.01 versus control group; ## P < 0.01 versus OGD group.

(Sham group), while AMI was induced in all others by transient myocardial ischemia for 40 minutes followed by reperfusion²⁷⁾ and then the rats were randomly divided into an AMI group, AMI+PEP group, AMI+PEP+siNC group, and AMI+PEP+si-HIF-1 α group with 8 rats in each group. Rats in the Sham group were orally administered an equal concentration of saline. Rats in the AMI+PEP group were pre-treated by oral administration of PEP (300 mg/kg) every day for 4 weeks.¹⁰⁾ Rats in the AMI+PEP+siNC group were pre-treated by oral administration of PEP (300 mg/kg) every day for 4 weeks and the infarcted myocardium was injected with siNC. Rats in the AMI+PEP+si-HIF-1 α group were pre-treated by oral administration of PEP (300 mg/kg) every day for 4 weeks and the infarcted myocardium was injected with si-HIF-1 α .²⁸⁾ Four rats were sacrificed after a 2-hour reperfusion period to determine the infarct size. Four rats were anesthetized, fixed in a supine position, and then the skin was prepared to measure the left ventricular (LV) end diastolic diameter (LVEDD), LV ejection fraction (LVEF) and LV fractional shortening (LVFS) via M-mode ultrasound images using an L14-5WU probe (Mindray Bio-Medical Electronics Corporation, Shenzhen, China).

Experiments using rats were performed in according with the US National Institute of Health's Guide for the Care and Use of Laboratory Animal and were approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (Qingdao, China).

Infarct size determination: After reperfusion, the heart was removed and the left anterior descending (LAD)

coronary artery was re-occluded at the same site. Then, 1% Evans blue dye solution (0.3 mL) was injected into the abdominal vein to identify the area at risk (AAR, unstained). Non-ischemic parts of the myocardium were stained blue. Subsequently, the heart was cut transversely into 1 mm thick sections after storing at -20°C for 20 minutes. The heart slices were stained in 2% 2, 3, 5-triphenyltetrazolium chloride solution (TCC, Sigma-Aldrich) for 20 minutes at 37°C and fixed in 4% paraformaldehyde solution overnight. Viable myocardium was stained red while the infarcted tissues remained pale. The extent of the area was quantified by computerized planimetry (ImageJ software, National Institutes of Health, Bethesda, MA, USA).

Statistical analysis: All experiments in this research were repeated 3 times in triplicate. The results of multiple experiments are presented as the mean $\pm$ standard deviation (SD). Graphpad 6.0 software was used for statistical analysis. Statistical comparisons (P -values) were calculated using one-way analysis of variance (ANOVA). A P < 0.05 was considered to indicate a significant difference.

Results

PEP protected HCMVEC from OGD-induced injury:

The viability of HCMVEC after 20, 40, 60, 80 or 100 μ g/mL PEP treatment was detected by CCK-8 assay. The results in Figure 1A show that 20, 40 or 60 μ g/mL PEP treatment had no significant effects on HCMVEC viability, while 80 or 100 μ g/mL PEP treatment enhanced the viability of HCMVEC (P < 0.05). These results suggested that high concentrations of PEP could improve the viability of HCMVEC. Subsequently, we analyzed the effects of 60 μ g/mL PEP treatment on OGD-induced HCMVEC injury. Figure 1B shows that OGD incubation significantly reduced the viability of HCMVEC (P < 0.01), while 60 μ g/mL PEP treatment notably ameliorated the OGD-induced HCMVEC viability loss (P < 0.05). Figure 1C shows that OGD incubation markedly increased the protein level of p53 (P < 0.01) and decreased the protein level of cyclin D1 in HCMVEC (P < 0.05). Compared to the OGD group, the protein level of p53 was reduced and the protein level of cyclin D1 was enhanced in the OGD+PEP group (P < 0.05). Moreover, the results of Figure 1D show that OGD incubation distinctly induced HCMVEC apoptosis (P < 0.01), while 60 μ g/mL PEP treatment attenuated the OGD-induced HCMVEC apoptosis (P < 0.05). The OGD-induced Bcl-2 protein level decrease, as well as the increases in Bax, cytochrome C, C/P-caspase 9 and C/P-caspase 3 protein levels (rates) in HCMVEC were all alleviated by 60 μ g/mL PEP treatment (Figure 1E, P < 0.05 or P < 0.01). Furthermore, OGD incubation dramatically up-regulated the concentrations of TNF- α and IL-6 in culture supernatant of HCMVEC (P < 0.01 or P < 0.001), while 60 μ g/mL PEP treatment markedly alleviated the OGD-induced TNF- α and IL-6 concentration increases in culture supernatant of HCMVEC (P < 0.05) (Figure 1F). OGD incubation enhanced the expression rate of LC3-II/LC3-I and reduced the expression level of p62 in HCMVEC (P < 0.05 or P < 0.01) (Figure 1G). Compared to the OGD group, the expression rate of LC3-II/

Figure 3. HIF-1 α was involved in the protective effects of PEP on OGD-induced HCMVEC injury. **A:** After si-HIF-1 α transfection, the protein level of HIF-1 α in HCMVEC was detected using Western blotting. **B-E:** After OGD incubation and/or PEP (60 μ g/mL) treatment or si-HIF-1 α transfection, the viability of HCMVEC (**B**), the protein levels of p53 and cyclin D1 in HCMVEC (**C**), the apoptosis of HCMVEC (**D**) and the protein levels of Bcl-2, Bax, cytochrome C, pro-caspase 9, cleaved-caspase 9, pro-caspase 3 and cleaved-caspase 3 in HCMVEC (**E**) were evaluated using CCK-8 assay, Western blotting, and an Annexin V-FITC/PI Apoptosis Detection kit, respectively. PEP: Polysaccharides from *Enteromorpha prolifera*; OGD indicates oxygen-glucose deprivation; HCMVEC, human cardiac microvascular endothelial cells; and HIF-1 α : hypoxia inducible factor 1 α . All experiments were repeated 3 times and the results are presented as the mean $\pm$ standard deviation (SD). For Western blotting, the most representative results are shown. * P < 0.05, ** P < 0.01 or *** P < 0.001 versus control group; # P < 0.05 or ## P < 0.01 versus OGD or OGD + PEP + siNC group.

LC3-I was decreased and the expression level of p62 was increased in the OGD+PEP group (P < 0.05 or P < 0.01). Taken together, these results indicate that PEP could protect HCMVEC from OGD-induced injury by alleviating cell viability loss, proliferation inhibition, apoptosis, pro-inflammatory factors expression, and autophagy.

PEP up-regulated the expression of HIF-1 α in HCMVEC under OGD: The protein levels of HIF-1 α in HCMVEC after OGD incubation and/or 60 μ g/mL PEP treatment were measured using Western blotting. As shown in Figure 2, OGD incubation enhanced the protein level of HIF-1 α in HCMVEC (P < 0.05). In addition, compared to the OGD group, the protein level of HIF-1 α was significantly increased in the OGD+PEP group (P < 0.01). These findings suggest that PEP up-regulated the expression level of HIF-1 α in HCMVEC under OGD and imply that HIF-1 α might participate in the protective effects of PEP on OGD-induced HCMVEC injury.

HIF-1 α was involved in the protective effects of PEP on OGD-induced HCMVEC proliferation inhibition and apoptosis: To explore the roles of HIF-1 α in the protective effects of PEP, si-HIF-1 α was transfected into HCMVEC. si-HIF-1 α transfection reduced the protein level of HIF-1 α in HCMVEC (P < 0.05) (Figure 3A). Compared to the OGD+PEP+siNC group, the viability of

HCMVEC was decreased in the OGD+PEP+si-HIF-1 α group (P < 0.01) (Figure 3B). The protein level of p53 was increased and the protein level of cyclin D1 was decreased in the OGD+PEP+si-HIF-1 α group, compared to the OGD+PEP+siNC group (P < 0.05) (Figure 3C). Moreover, compared to the OGD+PEP+siNC group, the apoptosis of HCMVEC was increased in the OGD+PEP+si-HIF-1 α group (P < 0.05) (Figure 3D). The protein level of Bcl-2 was decreased and the protein levels (rates) of Bax, cytochrome C, C/P-caspase 9 and C/P-caspase 3 were increased in the OGD+PEP+si-HIF-1 α group compared to the OGD+PEP+siNC group (P < 0.05 or P < 0.01) (Figure 3E). Taken together, these results suggest that PEP protected HCMVEC from OGD-induced proliferation inhibition and from apoptosis perhaps via up-regulating HIF-1 α .

PEP up-regulated HIF-1 α expression in HCMVEC via the MEK/ERK pathway: CH5126766 was used as an inhibitor of the MEK/ERK pathway in this research.²⁹⁾ CH 5126766 treatment decreased the expression levels of p-MEK and p-ERK in a dose-dependent manner, which suggests that CH5126766 could inactivate the MEK/ERK pathway in HCMVEC (Figure 4A). CH5126766 treatment markedly reversed the PEP treatment-induced HIF-1 α protein level increase in HCMVEC under OGD (P < 0.05)

Figure 4. PEP up-regulated HIF-1α expression in HCMVEC via MEK/ERK pathway. **A:** After 0-50 nM CH5126766 treatment, the expression levels of t-MEK, p-MEK, t-ERK and p-ERK in HCMVEC were detected using Western blotting. **B:** After OGD incubation and/or PEP (60 μg/mL) or CH5126766 (30 nM) treatment, the protein levels of HIF-1α in HCMVEC were measured using Western blotting. PEP indicates polysaccharides from *Enteromorpha prolifera*; OGD, oxygen-glucose deprivation; HCMVEC, human cardiac microvascular endothelial cells; HIF-1α, hypoxia inducible factor 1α; and CH, CH5126766. Experiments were repeated 3 times and the results are presented as the mean ± standard deviation (SD). The most representative results are shown. * $P < 0.05$ versus control group; # $P < 0.05$ versus OGD or OGD + PEP group.

(Figure 4B). These findings indicate that PEP up-regulated the protein level of HIF-1α in HCMVEC via the MEK/ERK pathway.

PEP attenuated OGD-induced NF-κB pathway activation in HCMVEC by up-regulating HIF-1α: After OGD incubation and/or 60 μg/mL PEP treatment or si-HIF-1α transfection, activation of the NF-κB pathway in HCMVEC was evaluated using Western blotting. OGD incubation significantly activated the NF-κB pathway in HCMVEC by enhancing the expression rates of p/t-p65 and p/t-IκBα ($P < 0.05$ or $P < 0.01$) (Figure 5). PEP treatment slightly ameliorated the OGD-induced NF-κB

Figure 5. PEP attenuated OGD-induced NF-κB pathway activation in HCMVEC by up-regulating HIF-1α. After OGD incubation and/or PEP (60 μg/mL) treatment or si-HIF-1α transfection, the expression levels of t-p65, p-p65, t-IκBα and p-IκBα in HCMVEC were assessed using Western blotting. PEP indicates polysaccharides from *Enteromorpha prolifera*; OGD, oxygen-glucose deprivation; HCMVEC, human cardiac microvascular endothelial cells; HIF-1α, hypoxia inducible factor 1α; and IκBα, inhibitor of nuclear factor-kappa B. Experiments were repeated 3 times and the results are presented as the mean ± standard deviation (SD). The most representative results are shown. * $P < 0.05$ or ** $P < 0.01$ versus control group; # $P < 0.05$ versus OGD or OGD + PEP + siNC group.

pathway activation in HCMVEC by reducing the expression rates of p/t-p65 and p/t-IκBα ($P < 0.05$). In addition, si-HIF-1α transfection markedly reversed the effects of PEP on the NF-κB pathway in HCMVEC under OGD ($P < 0.05$). These findings suggest that PEP attenuated OGD-induced NF-κB pathway activation in HCMVEC possibly through up-regulating HIF-1α.

PEP alleviated OGD-induced HCMVEC autophagy by promoting mTOR pathway: Rapamycin was used as an inhibitor of the mTOR pathway in this research.³⁰ Rapamycin treatment reduced the expression levels of p-mTOR and p-p70S6K in HCMVEC in a dose-dependent manner, suggesting that rapamycin could inactivate the mTOR pathway in HCMVEC (Figure 6A). PEP treatment markedly activated the mTOR pathway in HCMVEC under OGD by up-regulating the expression rates of p/t-mTOR and p/t-p70S6K ($P < 0.05$) (Figure 6B). Rapamycin also

Figure 6. PEP alleviated OGD-induced HCMVEC autophagy by promoting mTOR pathway. **A:** After 0–50 nM rapamycin treatment, the expression levels of t-mTOR, p-mTOR, t-p70S6K and p-p70S6K in HCMVEC were detected using Western blotting. **B:** After OGD incubation and/or PEP (60 µg/mL) or rapamycin (30 nM) treatment, the expression levels of t-mTOR, p-mTOR, t-p70S6K, p-p70S6K, LC3-I, LC3-II and p62 in HCMVEC were evaluated using Western blotting. PEP indicates polysaccharides from *Enteromorpha prolifera*; OGD, oxygen-glucose deprivation; HCMVEC, human cardiac microvascular endothelial cells; and mTOR, mammalian target of rapamycin. Experiments were repeated 3 times and the results are presented as the mean ± standard deviation (SD). The most representative results are shown. * $P < 0.05$ or ** $P < 0.01$ versus control group; # $P < 0.05$ or ## $P < 0.01$ versus OGD or OGD + PEP group.

reversed the PEP treatment-induced mTOR pathway activation in HCMVEC under OGD ($P < 0.05$ or $P < 0.01$). In addition, compared to the OGD+PEP group, the protein rate of LC3-II/LC3-I was increased and the protein level of p62 was decreased in the OGD+PEP+rapamycin group ($P < 0.05$ or $P < 0.01$). These findings indicate that PEP may ameliorate OGD-induced HCMVEC autophagy via promoting the mTOR pathway.

PEP reduced infarct size and protected cardiac function in adult rats subjected to AMI via up-regulating HIF-1α: Finally, to explore the effects of PEP on AMI *in vivo*, AMI was induced in Wistar rats. PEP pre-treatment significantly reduced the infarct size of hearts ($P < 0.001$) (Figure 7A). Compared to the AMI+PEP+siNC group, the infarct size of hearts was markedly increased in the AMI+PEP+si-HIF-1α group ($P < 0.01$). AMI enhanced the LVEDD of rats ($P < 0.05$), while PEP pre-treatment alleviated the AMI-induced enhancement of LVEDD of rats ($P < 0.05$) (Figure 7B). The LVEDD was increased in the AMI+PEP+si-HIF-1α group, relative to the AMI+PEP+siNC group ($P < 0.05$). Moreover, AMI markedly decreased the LVEF and LVFS of the rats ($P < 0.01$), while PEP pre-treatment attenuated the AMI-induced decreases of LVEF and LVFS of the rats ($P < 0.05$) (Figure 7C and D). Compared to the AMI+PEP+siNC group, the LVEF and LVFS of the rats were both decreased in the AMI+PEP+si-HIF-1α group ($P < 0.05$ or $P < 0.01$). These above findings suggest that PEP also could relieve AMI *in vivo*, perhaps via up-regulating HIF-1α.

Discussion

As a common and serious heart disease, AMI has aroused increasing attention worldwide in recent years.^{9,31} Even though great efforts have been made to improve the prognosis of AMI,³² the outcome of therapy for this disease is still unsatisfactory. In the present study, we analyzed the effects of PEP on AMI *in vitro* and *in vivo*. *In vitro*, we observed that PEP treatment significantly alleviated OGD-induced HCMVEC viability loss, proliferation inhibition, apoptosis, inflammatory cytokine expression, and autophagy. Mechanistically, we found that PEP up-regulated the expression of HIF-1α in HCMVEC via the MEK/ERK pathway. HIF-1α participated in the protective effects of PEP on OGD-induced HCMVEC proliferation inhibition and apoptosis. Meanwhile, we found that PEP attenuated OGD-induced NF-κB pathway activation and promoted the mTOR pathway in HCMVEC. *In vivo*, we observed that PEP also ameliorated AMI in rats via a reduction in infarct size and enhancement of cardiac function (LVEDD, LVEF and LVFS). HIF-1α was also involved in the effects of PEP on AMI *in vivo*.

Acute and persistent ischemia or hypoxia of the cardiac microvascular plays a critical role in the occurrence of AMI.^{9,33} HCMVEC is an important ingredient of the cardiac microvascular, which participates in the release of inflammatory cytokines after AMI.³⁴ Wang, *et al.* reported that OGD-induced HCMVEC injury could mimic cardiac microvascular damage in AMI.³⁵ Thus, in the current research, OGD-induced HCMVEC damage was chosen for exploring the effects of PEP on AMI *in vitro*. We found

Figure 7. PEP reduced infarct size and protected cardiac function in adult rats via up-regulating HIF-1 α . After the relevant treatment, the heart infarct size of rats in each group was assessed (A); the LVEDD, LVEF and LVFS were detected via the M-mode ultrasound images using the L14-5WU probe (B-D). PEP indicates polysaccharides from *Enteromorpha prolifera*; HIF-1 α , hypoxia inducible factor 1 α ; AMI, acute myocardial infarction; LVEDD, left ventricular end diastolic diameter; LVEF, left ventricular ejection fraction; and LVFS, left ventricular fractional shortening. All indexes were measured 3 times and the averages were determined. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ versus Sham group; # $P < 0.05$ or ## $P < 0.01$ versus AMI or AMI + PEP + siNC group.

Figure 8. Proposed pathway of protective effects of PEP on OGD-induced HCMVEC damage *in vitro*.

that OGD stimulation inhibited HCMVEC viability and proliferation, but induced cell apoptosis, inflammatory cytokine expression, and autophagy, which suggests that the HCMVEC damage model induced by OGD stimulation was established successfully and could be used to test the effects of PEP on AMI *in vitro*.

Polysaccharides are a class of essential organic compounds that form the base of all living creatures.³⁶⁾ Marine algae are rich in polysaccharides.¹⁰⁾ Therefore, identifying the beneficial activities of marine algal polysaccharides

will not only be helpful for reducing the economic losses due to green tide, but also provide novel material sources for chemical engineering, aquaculture, food manufacture, and pharmaceutical development.^{11,14-16,37)} In this research, PEP treatment protected HCMVEC from OGD-induced injury, indicating that PEP may be able to prevent and treat cardiac microvasculature damage in AMI.

HIF-1 α plays critical roles in the response to cellular low oxygen levels by activating genes related to angiogenesis, glycolysis, modulation of vascular tone, and

erythropoiesis.³⁸⁾ Up-regulation of HIF-1 α has been found to contribute to reduction of infarction and to attenuate progression of cardiac dysfunction in AMI.^{24,25)} A number of compounds, including astragaloside, ω -Alkynyl arachidonic acid, and cobalt chloride have been reported to alleviate AMI via up-regulating HIF-1 α .³⁹⁻⁴¹⁾ Consistent with previous studies, we found that PEP treatment enhanced the expression of HIF-1 α in HCMVEC under OGD. More importantly, knockdown of HIF-1 α reversed the protective effects of PEP on OGD-induced HCMVEC viability loss, proliferation inhibition and apoptosis. These findings suggest that PEP exerted protective effects on OGD-induced cardiac microvasculature in our *in vitro* cell damage model, at least in part through up-regulating HIF-1 α .

Previous studies have shown that the MEK/ERK pathway participates in the regulation of HIF-1 α expression under low oxygen conditions.^{42,43)} In the present research, CH5126766 was used as an inhibitor of the MEK/ERK pathway. We found that CH5126766 treatment significantly reversed the PEP-induced HIF-1 α expression up-regulation in HCMVEC under OGD. These results suggest that PEP up-regulated the expression of HIF-1 α in HCMVEC under OGD perhaps via the MEK/ERK pathway.

Ischemia or hypoxia of coronary microvasculature induces an inflammatory response of microvascular endothelial cells.⁸⁾ In the current study, we revealed that PEP distinctly attenuated OGD-induced expression up-regulation of TNF- α and IL-6 in HCMVEC. The NF- κ B pathway has been demonstrated to play key roles in the regulation of cellular inflammatory responses, including endothelial cells.^{44,45)} Therefore, we also analyzed activation of the NF- κ B pathway in HCMVEC after PEP treatment and/or HIF-1 α knockdown under OGD. We found that OGD incubation dramatically activated the NF- κ B pathway in HCMVEC and PEP treatment alleviated the OGD-induced NF- κ B pathway activation in HCMVEC. Furthermore, knockdown of HIF-1 α alleviated the effects of PEP on OGD-induced NF- κ B pathway activation. These findings suggest that PEP exerted protective effects on the OGD-induced cardiac microvasculature *in vitro* cell damage model, also via inactivating the NF- κ B pathway.

The mTOR pathway has been confirmed to exert critical roles in the regulation of vascular endothelial cell autophagy.^{46,47)} In the present study, rapamycin was used as an mTOR pathway inhibitor. We found that PEP treatment significantly activated the mTOR pathway in HCMVEC. More importantly, rapamycin treatment reversed the protective effects of PEP on OGD-induced HCMVEC autophagy. These results suggest that PEP alleviated OGD-induced HCMVEC autophagy perhaps via activation of the mTOR pathway.

Finally, we also assessed the effects of PEP on AMI *in vivo*. We found that PEP exhibited a protective effect against AMI *in vivo*. Pre-treatment of PEP reduced the infarct size of hearts in rats subjected to transient myocardial ischemia and reperfusion. LVEDD, LVEF and LVFS are the main indexes of cardiac function.^{48,49)} PEP pre-treatment protected the cardiac function of rats by enhancing LVEDD, LVEF and LVFS. More importantly, we found that HIF-1 α is also related to the protective effects

of PEP on AMI *in vivo*.

In summary, our research verified the protective effects of PEP on AMI *in vitro* and *in vivo*. HIF-1 α played key roles in the protective effects of PEP. Moreover, *in vitro*, PEP could activate the MEK/ERK and mTOR pathways, but inactivated the NF- κ B pathway in OGD-treated HCMVEC (Figure 8). This study provides evidence for understanding the protective effects of PEP on AMI and offers a theoretic basis for further exploring the prevention and treatment of AMI by using PEP.

Disclosures

Conflicts of interest: The authors declare that they have no conflicts of interest with the contents of this article.

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