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Hypoxia increases Nrf2-induced HO-1 expression via the PI3K/Akt pathway

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1. ABSTRACT

Accumulating evidence indicates that transient hypoxic preconditioning improves the resistance of endothelial progenitor cells (EPCs) to severe hypoxia and enhances the therapeutic potential of EPC-based therapies used in vascular repair and ischemic disease. However, the mechanisms underlying these processes remain unknown. This study tested the hypothesis that hypoxic preconditioning activates nuclear factor E2-related factor 2 (Nrf2) and the expression of its target genes, which improves biological functioning and resistance to hypoxia. Exposure to hypoxia after small interfering RNA (siRNA)-mediated knockdown of Nrf2 resulted in increased apoptosis, impaired proliferation, and angiogenesis *in vitro*. These changes were due to the activation of Nrf2 nuclear translocation via the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway and subsequent increase in the expression of the Nrf2 target gene heme oxygenase 1 (*HO-1*). Moreover, the hypoxia-induced secretion of hypoxia-inducible factor 1-α (HIF-1α) in EPCs was inhibited by Nrf2 siRNA. In conclusion, the increased resistance to hypoxia and improved therapeutic potential of EPCs, as a result of hypoxia preconditioning, are mediated by the PI3K/Akt-Nrf2-HO-1 signaling pathway and the secretion of HIF-1α after Nrf2 activation.

2. INTRODUCTION

Endothelial progenitor cells (EPCs) compose a subtype of progenitor cells isolated from the bone marrow, umbilical vessels, and peripheral blood. EPCs proliferate, self-renew, and differentiate into mature endothelial cells that line the blood vessel lumen (1-3). EPCs circulate in the blood and appear to be recruited preferentially to sites of vascular or tissue injury, thereby contributing significantly to re-endothelialization and angiogenesis (4-6). Moreover, EPCs are considered optimal candidates for cell-based therapies for myocardial infarction (7) and limb ischemia (8). However, EPCs undergo apoptosis in ischemic tissues with O_2 concentrations of 0.4–2.3% (9). Therefore, EPCs and other stem cells, such

as mesenchymal stem cells (MSCs), are preconditioned under severe hypoxic conditions (1–3% O_2) before transplantation to overcome apoptotic induction. Although this preconditioning approach improves the efficacy of cell-based therapies (10), its mechanisms are poorly understood.

Recent reports have shown that hypoxia induces the formation of reactive oxygen species (ROS) (11,12). ROS induce oxidative stress and activate the transcription factor nuclear factor-E2-related factor 2 (Nrf2). Nrf2 belongs to the cap 'n' collar/basic region leucine zipper family of transcription factors and controls the induction of genes that regulate the redox state of cells. Under basal conditions, Nrf2 is predominantly located in the cytoplasm where it is repressed by Kelch-like associating protein 1 (Keap1) and degraded via the Cul3-based E3 ligase pathway. However, when cells are exposed to oxidative stress or electrophiles, Nrf2 dissociates from Keap1 and translocates to the nucleus where it binds to antioxidant response elements in the promoters of its target genes to drive their expression.

Given these previous data, we designed the current study to test the hypothesis that hypoxia-induced expression of Nrf2 and its target genes contributes to the enhanced therapeutic potential of EPCs after hypoxia preconditioning. Hypoxia induced the translocation of Nrf2 into the nucleus in EPCs and increased both heme oxygenase 1 (HO-1) expression and hypoxia-inducible factor 1-α (HIF-1α) concentration via the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. As a result, apoptosis was reduced and both proliferation and angiogenesis were improved in response to severe hypoxia in natural EPCs. These responses to hypoxia were reduced in Nrf2-knockdown EPCs.

3. MATERIALS AND METHODS

3.1. Materials

EBM-2 medium was obtained from Lonza (Walkersville, MD, USA; catalog number CC-3156). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). 1, 1′-Dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (Dil-ac-LDL), fluorescein isothiocyanate (FITC)-*Ulex europaeus* agglutinin-1 (UEA-1), and trypsin were obtained from Life Technologies Corporation (Invitrogen, Waltham, MA, USA). A rat enzyme-linked immunosorbent assay (ELISA) kit for HIF-1α was purchased from Westang Biotech Corporation (Shanghai, China). Nrf2 small interfering RNA (siRNA) and control siRNA were obtained from Santa Cruz Biotechnology (Dallas, TX, USA; catalog numbers sc-156128 and sc-37007, respectively). Polyclonal anti-Nrf2 antibody, polyclonal anti-mouse IgG1-FITC secondary antibody, and monoclonal antibody to HO-1, vascular endothelial growth factor (VEGF) receptor, CD31, and CD34 were purchased from Abcam Inc. (Cambridge, UK), and CD133 was obtained from US Biological (Swampscott, MA, USA). Fibronectin was obtained from Sigma-Aldrich (St. Louis, MO, USA). Ficoll and 3-(4,5-dimethylthylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Solarbio Corporation (Tianjin, China). An annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit was obtained from Beyotime (Shanghai, China). A BCA Protein Assay Kit was obtained from Thermo Scientific (Waltham, MA, USA). Matrigel and calcein AM fluorescent dye were obtained from BD Biosciences (East Rutherford, NJ, USA; catalog numbers 354149 and 354216, respectively).

3.2. Cell culture and characterization

This study was approved by the ethics committee of the Third Military Medical University (Chongqing City, China). Bone-marrow-derived EPCs were obtained from male Sprague-Dawley rats as described previously (13). Briefly, mononuclear cells were isolated from bone marrow aspirates via density-gradient-based Ficoll centrifugation and cultured on 6-well plates coated with rat-derived fibronectin (10 μg/ml) with EBM-2 medium supplemented with 5% (v/v) FBS. Non-adherent cells were harvested after 3 days and cultured in EBM-2 medium containing 5% (v/v) FBS, (VEGF), human fibroblast growth factor-β, R3-insulin-like growth factor-1, human epidermal growth factor, ascorbic acid, hydrocortisone, gentamicin, and amphotericin B. Spindle-shaped cells were observed after 3 days and detached by using 0.25% (w/v) trypsin while growing cell populations reached 70–80% confluence. Third-passage cells were characterized by uptake of Dil-ac-LDL and binding to FITC-conjugated UEA-1. These events were assayed with laser scanning confocal microscopy (LSCM) and analyzed with fluorescence-activated cell sorting as described previously (14). For cell analysis, a total of 2×10^5 cells were incubated for 30 min at 4°C with monoclonal FITC-conjugated mouse anti-rabbit antibodies against CD34, CD31, flk-1, and CD133. FITC-positive cells were detected with a FACSCalibur flow cytometer (BD Biosciences). All of the EPCs used in this study were harvested at passage 3.

Cells were washed with EGM-2 medium, incubated with Dil-ac-LDL for 4 h, fixed in 2% (w/v) paraformaldehyde, and counterstained with 5 μg/ml FITC-UEA-1 for 30 min. Cells exhibiting Dil-ac-LDL uptake and FITC-UEA-1 staining were identified with LSCM by an investigator blinded to the treatment groups. Cells positive for both antigens were identified as EPCs (15).

3.3. Hypoxia preconditioning

Hypoxic conditions were generated in an incubator (Thermo Fisher Scientific, USA) with 5% CO_2, 1.5% O_2, and an N_2 balance. Hypoxic conditions were maintained at 1.5% O_2 for all experiments.

3.4. Detection of nuclear Nrf2 translocation

To detect nuclear Nrf2 translocation, we seeded 5×10^6 EPCs per well into a 6-well plate containing

4 small coverslips. The cells were synchronized via overnight incubation in EBM-2 medium supplemented with 0.1% (v/v) FBS and were then introduced to the hypoxic incubator after replacement of the medium with fresh EBM-2 containing 5% (v/v) FBS. Hypoxic treatment was stopped after 0.5, 1, 4, or 8 h, and normoxic cells, either untreated or treated with H_2O_2 (100 nM) for 2 h, served as negative and positive controls, respectively. All cells were stained as described previously (16). Briefly, cells grown on fibronectin-coated coverslips were fixed for 15 min in 4% (w/v) paraformaldehyde and permeabilized for 10 min with 0.2% (v/v) Triton X-100. After the blocking of non-specific protein-protein interactions for 1 h with 0.2% (w/v) bovine serum albumin in phosphate-buffered saline (PBS), the cells were incubated with a mouse anti-rat Nrf2 polyclonal antibody (1 μg/ml) for 2 h at room temperature. The secondary antibody (excitation λ 488 nm and emission λ 520 nm) was a goat anti-mouse IgG1 (heavy chain) used at a 1:1000 dilution for 1 h. Cell nuclei were stained with 4′, 6-diamidino-2-phenylindole at a concentration of 50 μg/ml (excitation λ 350 nm and emission λ 470 nm). The cells on the coverslips were washed 3 times for 5 min in PBS and then analyzed with LSCM.

3.5. Detection of HIF-1α with ELISA

Levels of HIF-1α protein in the supernatant were determined by using a rat HIF-1α ELISA kit for EPCs after varying the duration of hypoxic treatment with or without transfection with Nrf2 siRNA or control siRNA. The assay was performed in accordance with the manufacturer's protocol. The amount of HIF-1α was calculated by comparing the absorbance of each sample to a standard concentration curve with CurveExpert 1.3 software. Each sample was analyzed 3 times.

3.6. Apoptosis assay

Apoptotic EPCs transfected with Nrf2 siRNA or control siRNA under hypoxic conditions were quantified with an annexin V-FITC/PI Apoptosis Detection Kit in accordance with the manufacturer's protocol. Briefly, cells were washed in PBS and incubated in the dark at room temperature for 10 min in binding buffer containing annexin V-FITC and PI. The cells were then analyzed with a FACSCalibur flow cytometer (Becton Dickinson) within 1 h of staining. Annexin V-FITC staining was detected in the FL1 channel, and PI staining was monitored in the FL2 channel. Unstained cells and cells stained with annexin V-FITC or PI alone were used as controls. The results were analyzed with WinMDI 2.8 software.

3.7. Cell proliferation assay

Cell proliferation was analyzed with the MTT method. Briefly, untransfected EPCs and EPCs transfected with Nrf2 siRNA or control siRNA were cultured in 96-well plates at a concentration of approximately 2×10^3 cells per well. After overnight incubation, the cells were exposed to hypoxic conditions for 24, 48, or 72 h. The medium was removed at various time points post-hypoxia. Then, 10 μl MTT (5 mg/ml) was added to each well, and the cells were incubated at 37 °C for 4 h. After the addition of 100 μl formazan solution, the cells were incubated for an additional 4 h until the purple formazan precipitate dissolved. EPCs in each group were arranged in 6 duplicate wells, and the absorbance was measured at 570 nm in a microplate reader.

3.8. Tube-formation assay

Matrigel assays were performed as described previously (17). Briefly, untransfected EPCs and EPCs transfected with Nrf2 siRNA or control siRNA (1.5×10^4 cells in 600 μl EBM-2 medium) were seeded into 96-well plates coated with Matrigel and incubated for 18 h at 37 °C in 5% CO_2. The cell cultures were supplemented with 50 μl of 6 μM calcein AM solution and then incubated at 37 °C in 5% CO_2 for 30 min. Calcein AM-labeled cells were observed and photographed under a fluorescence microscope. The degree of tube formation was quantified by counting the number of tube-like structures in 6 fields (×100 magnification) chosen at random among the triplicate wells.

3.9. Western blotting

EPCs treated with or without the PI3K inhibitor LY294002 (20 μM) were exposed to hypoxia for 0.5, 1, 2, 4, or 8 h to examine Nrf2 activation and signaling in hypoxia. Furthermore, EPCs transfected with Nrf2 siRNA or control siRNA were exposed to hypoxia for 1, 2, 8, or 24 h to analyze the effect of Nrf2 on HO-1 expression as a function of hypoxia. For protein expression examination, EPCs were collected, washed with PBS, and lysed in lysis buffer. The protein concentration of each lysate was determined with a Pierce BCA Protein Assay Kit. An appropriate amount of protein from each sample (50 μg) was separated on 10% (v/v) sodium dodecyl sulfate-polyacrylamide gels and transferred overnight (90 mA, 4 °C) onto nitrocellulose membranes. The membranes were blocked with 5% (w/v) skim milk reconstituted in PBS and probed with the following primary antibodies: p-Akt (1:1000), Akt (1:1000), Nrf2 (1:200), HO-1 (1:200), and glyceraldehyde 3-phosphate dehydrogenase (1:2000). Horseradish peroxidase-conjugated secondary antibodies were used at 1:2000 dilution in accordance with the manufacturer's instructions. Protein bands were detected with an enhanced chemiluminescence kit (Vazyme Biotech Co., Ltd., Nanjing City, China) and quantified with an automated digitizing system.

3.10. Statistical analyses

All data were expressed as Mean (SD). Differences between 2 groups were analyzed with unpaired Student's *t*-tests. Data for more than 2 groups were analyzed with 1-way ANOVA. Differences were considered significant at p values of <0.05.

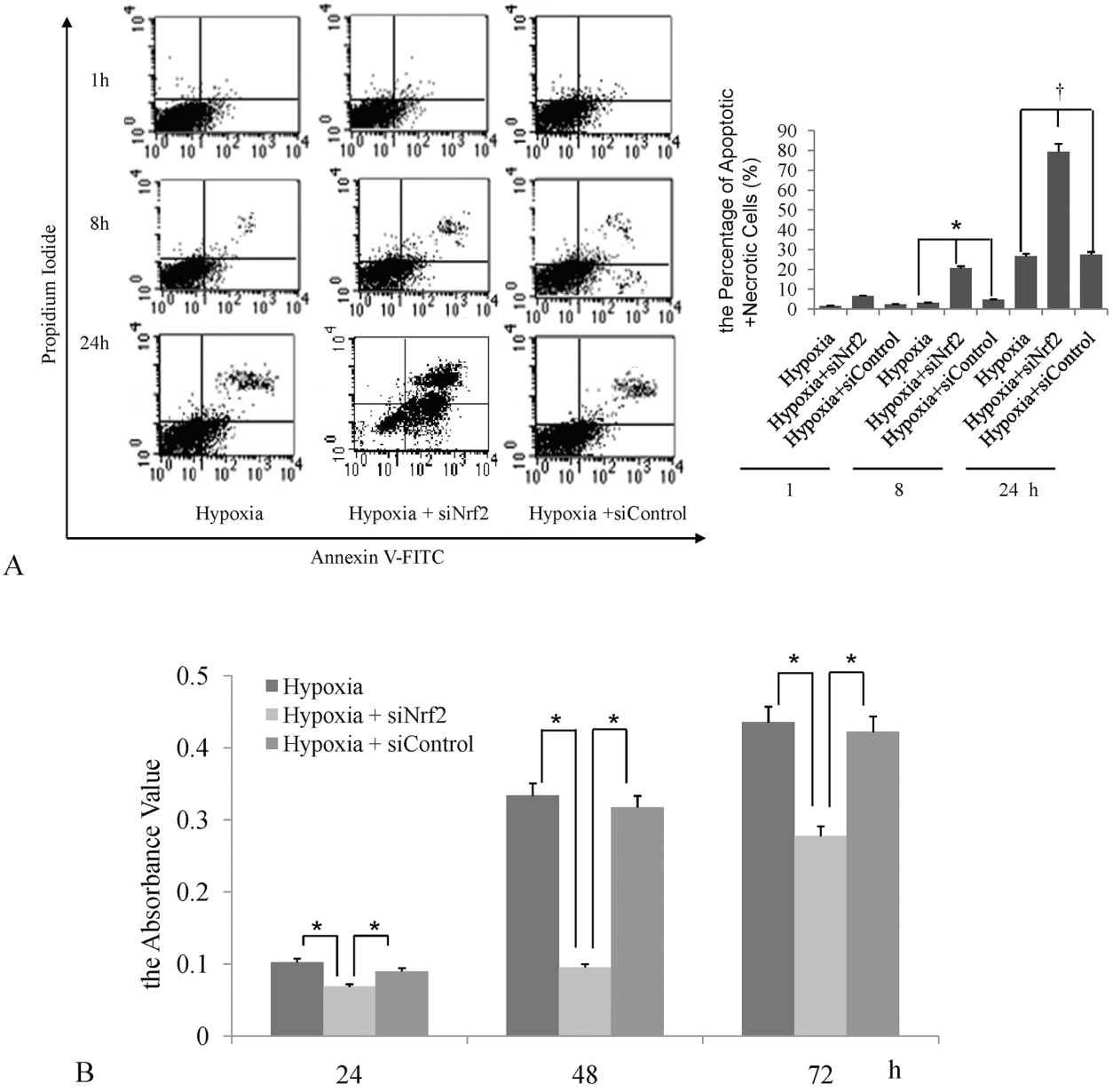

Figure 1. (A) siRNA-mediated knockdown of Nrf2 expression increased hypoxia-induced apoptosis of EPCs. Untransfected EPCs and EPCs transfected with Nrf2 siRNA or control siRNA were maintained in hypoxic conditions (1.5% O_2) for 1, 8 or 24 h. After washing in PBS, the cells were incubated in binding buffer, to which annexin V-FITC was subsequently added. The cells were then incubated for 10 min in the dark and analyzed immediately by flow cytometry. The data are represented as mean ± SD from triplicate samples and represent the results of three separate experiments ($^*p$ <0.0.5, $^\dagger p$ <0.0.1). (B) siRNA-mediated knockdown of Nrf2 inhibited the proliferation of EPCs exposed to hypoxia. Untransfected EPCs or EPCs transfected with Nrf2 siRNA or control siRNA were seeded in 96-well plates at a concentration of 2×10^3 cells per well and incubated overnight prior to exposure to hypoxia (1.5% O_2) for 24, 48 or 72 h. The medium was subsequently removed and 10 μl MTT (5 mg/ml) was added to each well. The cells were then incubated at 37°C for 4 h. Following an additional 4-h incubation with 100 μl formazan solution, the absorbance in each well was measured with a microplate reader. Six duplicate wells were analyzed for each sample. The results are represented as mean ± SD ($^*p$ <0.0.5).

4. RESULTS

4.1. Knockdown of Nrf2 expression by siRNA enhanced apoptosis and inhibited the proliferation of EPCs after exposure to hypoxia

Although provisional hypoxic conditioning of EPCs increases the tolerance to severe hypoxia of these cells and improves their therapeutic potential, sustained hypoxia increases apoptosis and decreases the proliferation of cells *in vitro* (18). We therefore examined the effect of silencing Nrf2 on apoptosis and proliferation of EPCs after exposure to hypoxia. The results of annexin V/PI staining and flow cytometry showed that apoptosis increased nearly 3.8-fold in EPCs after transfection with

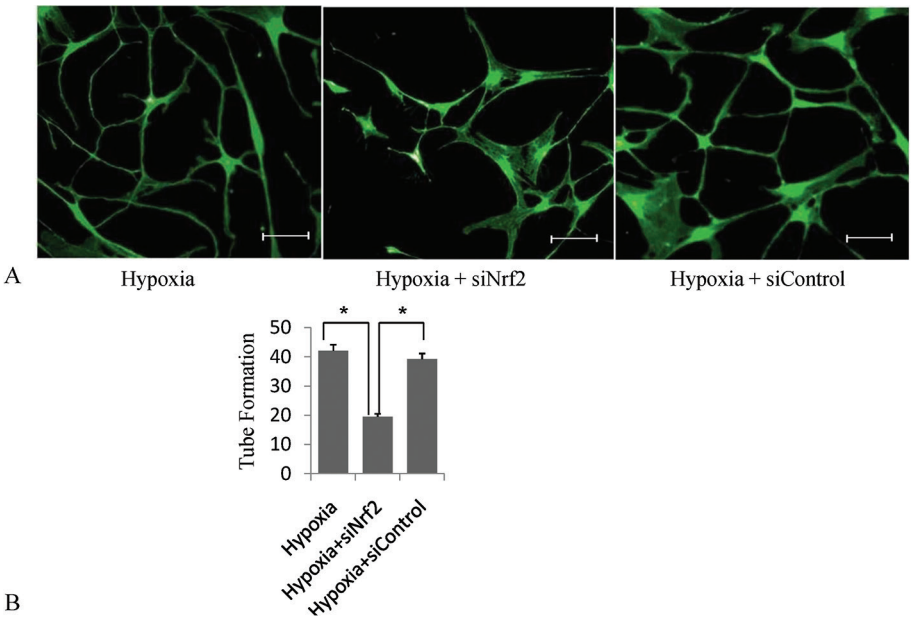

Figure 2. siRNA-mediated knockdown of Nrf2 impaired hypoxia-induced angiogenesis of EPCs in vitro. Untransfected EPCs and EPCs transfected with Nrf2 siRNA or control siRNA were seeded in 96-well plates coated with Matrigel and incubated for 18 h at 37°C in a mixture of 5% CO_2 and 1.5% O_2. The cells received 50 µl of 6 µM calcein AM followed by incubation at 37°C for 30 min and were then photographed under a fluorescence microscope. Tube formation was quantified by counting the number of tube-like structures in six randomly chosen fields (×100) from triplicate wells. The data are represented as mean ± SD ($^*p$ <0.0.5). Bars =100 µm.

Nrf2 siRNA and exposure to hypoxia for 24 h (Figure 1A). By contrast, compared to hypoxia-treated cells with or without control siRNA transfection, EPCs transfected with control siRNA and exposed to hypoxia and those treated with hypoxia alone showed no increase in apoptosis. Furthermore, the results of MTT analysis showed that compared with hypoxia-treated cells with or without control siRNA, EPCs treated with Nrf2 siRNA and exposed to hypoxia for 24, 48, or 72 h showed inhibited proliferation (Figure 1B). Collectively, these results demonstrate that Nrf2 protected EPCs by reducing apoptosis and increasing proliferation after exposure to hypoxic conditions.

4.2. Knockdown of Nrf2 impaired *in vitro* angiogenesis of EPCs after exposure to hypoxia

EPCs play crucial roles during angiogenesis induced by tissue ischemia or tumor growth. We used a Matrigel-based tube-formation assay to examine the effect of Nrf2 on angiogenesis in EPCs exposed to hypoxia. Compared with EPCs exposed to hypoxia for 24 h with or without control siRNA transfection, EPCs transfected with Nrf2 siRNA showed significantly fewer tube-like structures after hypoxic exposure (Figure 2). This result indicates that reduced Nrf2 expression impaired hypoxia-induced angiogenesis in EPCs.

4.3. Hypoxia induced nuclear translocation of Nrf2 in EPCs

Oxidative stress induces the translocation of Nrf2 into the nucleus where it promotes the transcription of cytoprotective antioxidant target genes. We therefore investigated whether hypoxia influenced the translocation of Nrf2 from the cytoplasm to the nucleus in EPCs. The results of fluorescence immunostaining showed that Nrf2 had a predominantly perinuclear localization within the cytoplasm in normoxic EPCs (Figure 3). However, exposure to 1.5% O_2 for 4 h (but not for 30 min) induced the activation and translocation of Nrf2 into the nucleus, as evidenced by the results of fluorescence staining in both the nucleus and the cytoplasm. These translocation was also observed in cells treated with 100 nM H_2O_2 for 2 h. These results demonstrate that Nrf2 accumulated in the nucleus in response to hypoxia in EPCs.

4.4. PI3K/Akt signaling mediated hypoxia-induced Nrf2 activation

Previous reports have shown that PI3K/Akt signaling contributes to the activation of simvastatin-induced Nrf2 (19). Additional evidence suggests that hypoxia activates the PI3K/Akt pathway (20). We therefore investigated the role of the PI3K/Akt pathway in the activation of hypoxia-induced Nrf2. We quantified activated PI3K levels via immunoblotting for the downstream target phosphorylated Akt (p-Akt; Ser 473) in EPCs exposed to hypoxia and determined the corresponding Nrf2 expression levels. p-Akt was significantly increased after 2 h of hypoxic treatment and peaked at 4 h, whereas total Akt levels were unchanged at these time points (Figure 4). Accordingly, Nrf2 expression increased after 2 h of hypoxic treatment and peaked after 8 h. To determine whether the PI3K/

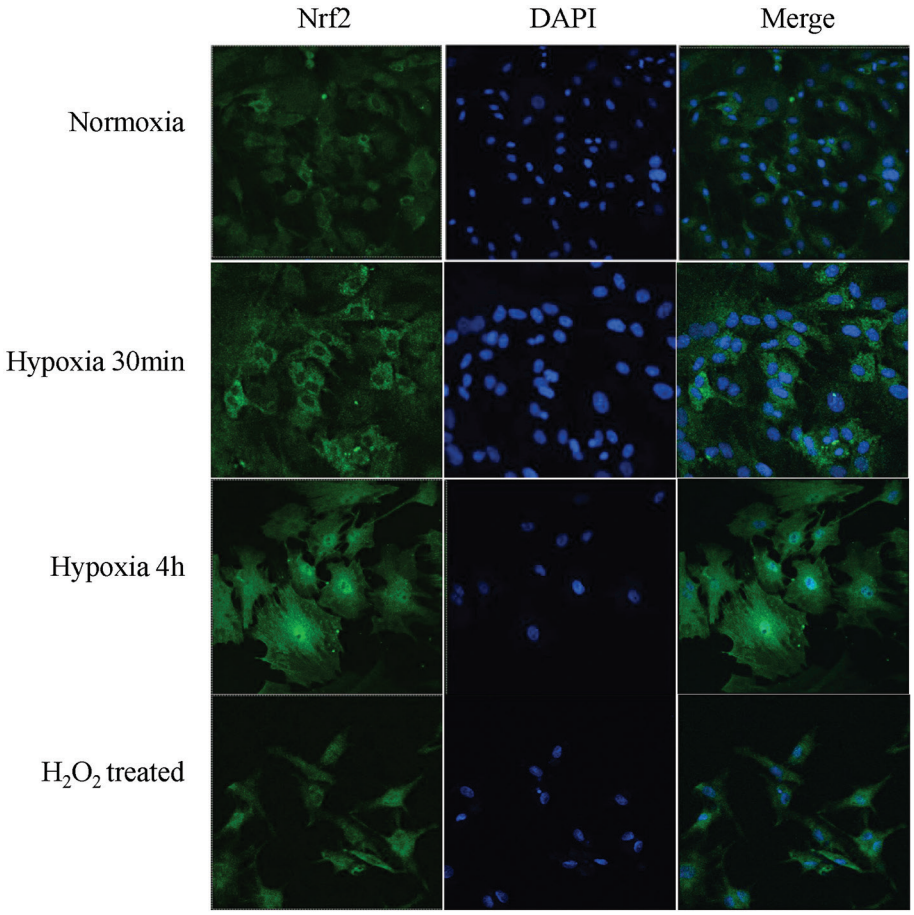

Figure 3. Hypoxia induced the translocation of cytoplasmic Nrf2 to the nucleus in EPCs. EPCs grown on glass coverslips were exposed to normoxia or hypoxia (1.5% O_2) for 0.5, 4 or 8 h or were treated with H_2O_2 (100 nM) for 2 h under normoxic conditions (positive control). At the conclusion of each treatment, the cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and probed with an anti-Nrf2 antibody (1:1000). DAPI was used at a concentration of 50 μg/ml to label the nuclei (blue). The cells were analyzed by LSCM. The Figure shows representative immunofluorescence images from three independent experiments in which Nrf2 translocated from the cytoplasm to the nucleus in response to 4-h exposure to hypoxic conditions.

Akt signaling pathway was involved in hypoxia-induced Nrf2 activation, we blocked PI3K activity with the inhibitor LY294002. As expected, p-Akt was significantly inhibited by LY294002 (20 μM), and Nrf2 expression was markedly decreased between 2 and 8 h of hypoxic treatment, demonstrating that LY294002 largely abolished the stimulatory effect of hypoxia on Nrf2 expression. These results indicate that hypoxia activated Nrf2 expression via the PI3K/Akt pathway.

4.5. Hypoxia induced HO-1 expression by activating Nrf2

Nrf2 protein is activated in response to numerous types of stress and accumulates in the nucleus where it drives the expression of target genes, including the HO-1, glutathione S-transferase, and NADPH: quinine oxidoreductase 1 genes. HO-1 is one of the most important Nrf2 target genes because it plays a role in anti-atherogenesis, vascular protection, angiogenesis, and re-endothelialization after arterial injury (14). We therefore examined HO-1 protein expression in EPCs exposed to hypoxia. HO-1 expression increased in EPCs exposed to hypoxia for 8 or 24 h, augmented by Nrf2 protein. Compared with control siRNA, Nrf2 siRNA transfection significantly inhibited Nrf2 expression (Figure 5). The results of densitometric analysis showed that nuclear Nrf2 protein levels in EPCs transfected with Nrf2 siRNA and treated with hypoxia for 8 and 24 h were decreased by nearly 85% and 90%, respectively, relative to those in control-siRNA transfected, hypoxia-treated cells. Moreover, compared with cells exposed to hypoxia alone for 8 or 24 h, EPCs transfected with Nrf2 siRNA showed significantly reduced HO-1 expression, but no change was observed in cells transfected with control siRNA. Although the results of the current study provide no evidence for the direct binding of activated Nrf2 to the antioxidant response element of the HO-1 promoter, this possibility is supported by other studies (21). Together, these results demonstrate that

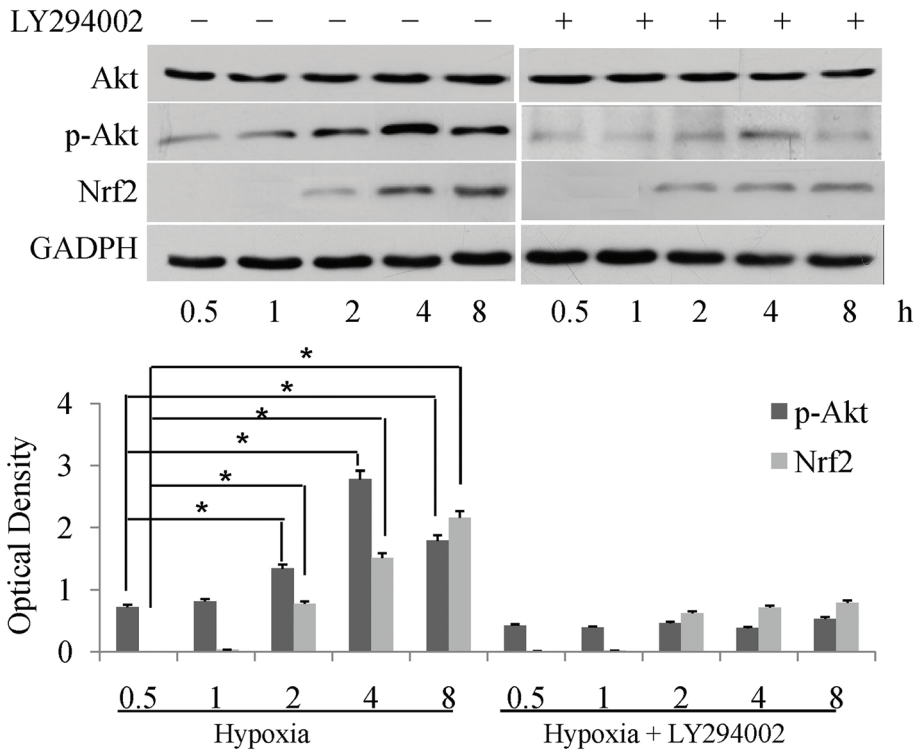

Figure 4. Hypoxia induced the activation of Nrf2 via the PI3K/Akt signaling pathway. EPCs were treated with or without 20 μM LY294002 and exposed to hypoxic conditions for the indicated times. Total protein was extracted from the cells using a Pierce BCA Protein Assay Kit, and total Akt, p-Akt Ser 473 and Nrf2 expression levels were assayed by western blotting. Data from three independent experiments are represented as mean ± SD ($^{*}p < 0.05$, compared with expression at 30 min).

hypoxia induced the expression of HO-1 protein after the activation of Nrf2.

4.6. Nrf2 knockdown decreases the secretion of hypoxia-induced HIF-1α in EPCs

Similar to many somatic progenitor and stem cells, EPCs exhibit paracrine activity that contributes to their therapeutic effects via the secretion of growth factors and cytokines such as VEGF and HIF-1α (22) under hypoxic conditions. The regulation of HIF-1α secretion and activity is tightly linked to cellular O_2 concentration (23,24). Additionally, HIF-1 regulates other angiogenic growth factors, including angiopoietin 2, stem cell factor, stromal-derived factor 1, and platelet-derived growth factor B (25,26). Accordingly, we evaluated the paracrine role of Nrf2 in hypoxic EPCs by determining the HIF-1α levels in cell supernatants. As shown in Figure 6, the results of ELISA showed that HIF-1α concentrations increased gradually in normal EPCs exposed to hypoxic conditions and peaked between 16 and 24 h, with expression levels approximately 3-fold greater at 24 h than at 2 h. However, HIF-1α concentrations were reduced in the supernatants of EPCs transfected with Nrf2 siRNA after 8, 16, and 24 h of hypoxia—a nearly 61% reduction relative to concentrations in normal cells at 24 h. By contrast, compared to normal cells, cells transfected with control siRNA showed no apparent change in HIF-1α concentration at any time point. These results suggest that Nrf2 influenced the secretion of HIF-1α in EPCs exposed to hypoxia.

5. DISCUSSION

The present study provides the first evidence that Nrf2, a key transcriptional regulator of antioxidant genes, plays a key role in determining the therapeutic potential of EPCs after hypoxia. The results demonstrate that hypoxia induces the translocation of cytoplasmic Nrf2 into the nucleus, where it stimulates the expression of HO-1, an important Nrf2 target gene that enhances cytoprotection by regulating the proliferation, apoptosis, and angiogenesis of EPCs exposed to hypoxic conditions. Moreover, activated Nrf2 promotes the secretion of HIF-1α in hypoxia-induced EPCs. It is therefore plausible that Nrf2 has a critical role in improving the biological function of EPCs after exposure to hypoxia, especially in the case of hypoxic preconditioning before cell-based therapy for ischemic disease.

Hypoxia has been proposed to stimulate NAD(P)H oxidase-dependent ROS in adipose-derived stem cells (12), pulmonary vasculature (27), and fetal rat heart tissue (28). Moreover, many studies have shown that

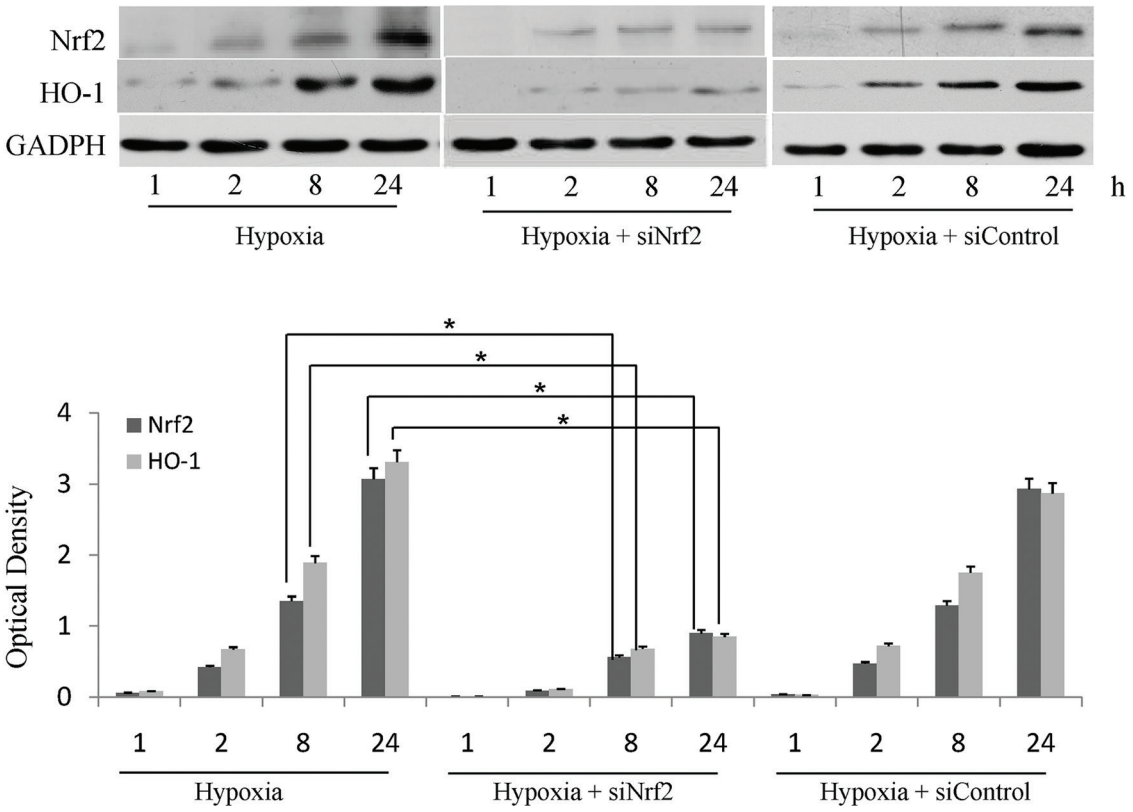

Figure 5. Hypoxia induced the expression of HO-1 protein via the Nrf2 pathway. Untransfected EPCs and EPCs transfected with Nrf2 siRNA or control siRNA were exposed to hypoxia for the indicated times. Total protein was extracted from the cells and prepared using a Pierce BCA Protein Assay Kit. HO-1 and Nrf2 expression levels were then determined by western blotting. The images are representative of three independent experiments. The results are presented as mean ± SD (*p <0.0.5, compared with hypoxia alone and hypoxia + control siRNA at the corresponding time point).

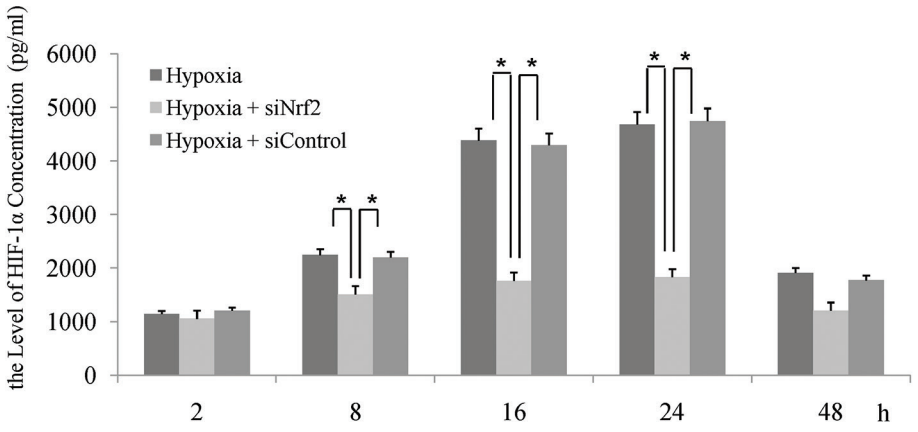

Figure 6. siRNA-mediated knockdown of Nrf2 reduced HIF-1α concentrations in the supernatant of cultured EPCs. Untransfected EPCs and EPCs transfected with Nrf2 siRNA or control siRNA were exposed to hypoxia for 2, 8, 16, 24 or 48 h, after which the supernatant was collected and assayed using an HIF-1α ELISA kit. Data were obtained from six duplicate wells over three independent experiments and are shown as mean ± SD (*p <0.0.5, compared to hypoxia alone and hypoxia + control siRNA at the corresponding time point).

hypoxia activates the PI3K/Akt pathway. PI3K/Akt signaling is also involved in the activation of Nrf2 by simvastatin (19). The results of the present study provide new insights into the mechanisms through which hypoxic pretreatment improves cellular resistance to hazardous microenvironments such as hypoxia or ischemia in myocardial infarction and stroke. Exposing EPCs to hypoxia induces the activation of cytoplasmic Nrf2 via PI3K/Akt, leading to its dissociation from Keap1, an inhibitor of Nrf2, and its translocation to the nucleus, where it induces HO-1 expression.

Considerable evidence suggests that HO-1 plays a central role in the involvement of activated Nrf2 in cytoprotection and cardiovascular protection (29,30). In the present study, transfection with Nrf2 siRNA abolished HO-1 expression, exacerbated apoptosis and angiogenesis, and further reduced proliferation in cultured EPCs exposed to hypoxia. In accordance with the results of Lin and co-workers (31), Wu and colleagues (14) showed that pharmacological induction of HO-1 increased circulating bone-marrow-derived EPCs and contributed to vascular repair in a rabbit model of aortic balloon injury. Other studies have also reported a role for HO-1 in angiogenesis (32,33). For example, an adenoviral vector encoding HO-1 facilitates angiogenesis in ischemic rat hindlimbs, an effect that is abolished by HO-1 inhibition (34). Collectively, the results of these previous reports together with our data suggest that activated Nrf2 promotes angiogenesis at least in part via the expression of HO-1. However, a report by Ichihara and colleagues (35) demonstrated that ablation of Nrf2 ($Nrf2^{-/-}$) promotes ischemia-induced neovascularization in ischemic mouse hindlimbs via an enhanced inflammatory response. Recently, Berenice *et al*. (36) reported that Nrf2 has unfavorable effects on plasma lipoprotein and cholesterol transport that overshadow its antioxidant protection properties and subsequently promote the formation of atherosclerotic lesions. However, HO-1 expression was not evaluated in these studies, and a more rigorous analysis of the role of activated Nrf2-driven HO-1 expression in angiogenesis is necessary.

In the present study, the siRNA-mediated knockdown of Nrf2 was associated with reduced HIF-1α levels in the supernatant of cultured EPCs. Reduced levels of HIF-1α may have a detrimental effect on cell proliferation, apoptosis, and angiogenesis during hypoxia. Increased HIF-1α in hypoxic EPCs contributed to cell proliferation and angiogenesis and protected against apoptosis. Malec and colleagues (37) reported that activated Nrf2 enhances HIF-1α signaling during intermittent hypoxia in lung adenocarcinoma A549 cells expressing high levels of the NADPH oxidase subunit NOX1. A study by Yun *et al*. (38) demonstrated that 17β-estradiol partially stimulates the proliferation of human MSCs through HIF-1α activation and VEGF expression via the PI3K/Akt pathway. Liu *et al*. (39) also reported that hypoxic preconditioning enhances the migration, adhesion, and survival of MSCs via PI3K/Akt-HIF-1α- and CXCR4/CXCR7-dependent pathways. Furthermore, hypoxia activates HIF-1α, thereby promoting the expansion of CD133-positive glioma stem cells (40). Increasing evidence indicates that hypoxia-induced increases in HIF-1α protein may be largely attributable to the regulation of HIF-1α stability (41,42). The knockdown of Nrf2 by siRNA disturbs the regulation of hypoxia-induced HIF-1α expression (43) and accelerates HIF-1α degradation via hydroxylation by activated prolyl hydroxylase domain proteins. Notably, hypoxia-induced increases in HIF-1α are unaffected by treatment with either a pharmacological inhibitor or an inducer of HO-1, which suggests that the role of Nrf2 in regulating hypoxia-induced HIF-1α is independent of HO-1 expression. However, these data are specific to cancer cells, and their applicability to EPCs or other non-neoplastic cell types remains to be determined.

In conclusion, the results of the present study verify the vital roles of the PI3K/Akt-Nrf2-HO-1 signaling pathway and HIF-1α in hypoxic EPC preconditioning and provide novel insights into the mechanisms underlying the improved therapeutic potential of EPCs exposed to hypoxia.

6. ACKNOWLEDGEMENTS

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Abbreviations: EPCs: endothelial progenitor cells; Nrf2: nuclear factor E2-related factor 2; HO-1: heme oxygenase 1; MSCs: mesenchymal stem cells; ROS: reactive oxygen species; Keap1: Kelch-like associating protein 1; AREs: anti-oxidant response elements; FACS: fluorescence-activated cell sorting; BSA: bovine serum albumin; MTT: 3-(4,5-dimethylthylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; GST: glutathione S-transferase; SDF-1: stromal-derived factor 1; SCF: stem cell factor; PHD: prolyl hydroxylase domain proteins

Key Words: Hypoxia Preconditioning, Endothelial Progenitor Cells, Nuclear Factor-E2-related factor 2, Heme Oxygenase-1

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