Heme relieves the inhibition of proliferation, migration and angiogenesis of HMEC-1 under hyperoxia by inhibiting BACH1

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Research article

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Abstract

Background

The relatively hyperoxia inhibited vascular endothelial growth factor (VEGF) in retina is the main cause of angiogenesis retardation in phase I retinopathy of prematurity (ROP). Human retinal angiogenesis is related to the proliferation, migration and angiogenesis of microvascular endothelial cells. Previous studies have confirmed that BTB and CNC homology I (BACH1) can inhibit VEGF and angiogenesis, while heme can specifically degrade BACH1. However, the effect of heme on endothelial cells and ROP remains unknown.

Methods

In this report, we established a model of human microvascular endothelial cells (HMEC-1) induced by 40% hyperoxia to simulate the relatively hyperoxia of phase I ROP. Meanwhile, heme was added to investigate the effects on the growth and viability of HMEC-1. Cell counting kit 8 (CCK8) and 5-ethynyl-2'-deox-yuridine (EDU) methods were used to detect the proliferation ability. Cell scratch test and matrigel matrix glue were used to detect the migration or angiogenesis ability. Western blot and immunofluorescence methods were used to detect the relative protein expression of BACH1 and VEGF.

Results

The proliferation, migration and angiogenesis of HMEC-1 were inhibited under hyperoxia. Moderate heme can promote endothelial cell proliferation, while excessive can inhibit, 20 μM heme could inhibit the expression of BACH1, promote the expression of VEGF, and relieve the inhibition of proliferation, migration and angiogenesis induced by hyperoxia in HMEC-1.

Conclusions

20 μM heme can relieve the inhibitory effects induced by hyperoxia, via the mechanism of promoting VEGF by inhibiting BACH1 in HMEC-1, and maybe a potential medicine for retinal angiogenesis retardation induced by relatively hyperoxia in phase I ROP.

Background

Retinopathy of prematurity (ROP) is a retinal vascular disease in premature and low birth weight infants, which is one of the main causes of blindness in children [1]. With the rapid development of perinatal medicine and neonatology, the establishment of neonatal intensive care unit (NICU), the survival rate of premature and low birth weight infants is gradually improved, and the incidence of ROP has an obvious upward trend. The main pathological features of ROP are retinal angiogenesis retardation in early stage

and pathological neovascularization in later stage. The main cause of angiogenesis retardation in early ROP is due to the relatively hyperoxia induced by premature infants leaving the uterus prematurely and oxygen therapy, which inhibits the expression of VEGF in retina. The early angiogenesis development determines the severity of retinal hypoxia in the later stage, and then determines the retinal neovascularization [2, 3]. In order to solve the problem of neonatal hypoxia, and reduce the mortality rate, oxygen therapy is sometimes necessary [4]. However, there is no effective intervention for retinopathy caused by relatively hyperoxia in early ROP.

BACH1 is an important transcription factor involved in the regulation of cellular reactive oxygen species, heme homeostasis, hematopoiesis and immunity [5]. It can inhibit VEGF expression and angiogenesis by inhibiting heme oxygenase-1 (HO-1), enhancing the production of mitochondrial reactive oxygen species in endothelial cells, and competitively inhibiting β-Catenin [6–8]. Previous studies have shown that heme can specifically inhibit the expression of BACH1 in mice without affecting the normal physiological functions, this function has also been confirmed in triple negative breast cancer (TNBC) cells and murine erythroleukemia (MEL) cells [9, 10].

While the effects of heme on endothelial cells and ROP have not been studied. In this report, we established a model of HMEC-1 induced by 40% hyperoxia to simulate the relatively hyperoxia of early ROP. The effects of different doses of heme on the proliferation, migration and angiogenesis of HMEC-1 were observed, the expression of BACH1 and VEGF proteins were quantified. The results showed that the proliferation, migration and angiogenesis of HMEC-1 were inhibited under hyperoxia, 20 μM heme could promote the expression of VEGF by inhibiting BACH1 of HMEC-1, and relieve the inhibitory effects induced by hyperoxia in HMEC-1. These findings provide a certain experimental basis for heme as a potential medicine for the treatment of retinal angiogenesis retardation induced by relatively hyperoxia in early ROP.

Methods

HMEC-1 cell culture

HMEC-1 were purchased from American Type Culture Collection (ATCC, Rockefeller of Maryland, the certificate and STR test result are supplemented in additional file 1,2). According to the previous experimental practice of our research group [11]. The cell culture medium was prepared in MCDB131 medium containing 10% fetal bovine serum (FBS, Gibco, USA), 1% antibiotics (including streptomycin 0.1mg/mL, and penicillin 100U/mL, Gibco), 2mM glutamine(25030, Gibco) and 1μg/ml hydrocortisone (M3451, Abmole, USA) [12]. 10mM heme (51280, Sigma, USA) was prepared in 20 mM NaOH (PBS dilution) for standby [9]. HMEC-1 were cultured in vitro and divided into normoxia and hyperoxia groups. Normoxia group maintained in an incubator containing 5% CO_2, 21% O_2 and 74% N_2, and hyperoxia group maintained in an incubator containing 5% CO_2, 40% O_2 and 55% N_2. Both of them were kept at 37 °C and 90% humidity [13,14]. Meanwhile, different concentrations of heme, blank (medium) or negative control

(vehicle:20mM NaOH) were added into the culture medium according to groups. The cells used in the experiment were from the 5 to 10th generations.

HMEC-1 proliferation CCK8 assay

Cell proliferation was measured using CCK8 (42830, MCE, USA) according to the supplier's protocol. Cells were seeded in 96-well plates (5×10^3/well) for 12 hours and then adhered to the wall under normoxia condition. In dose effect test, the culture medium was changed according to the heme concentration (9 groups：0, 5, 10, 20, 40, 80, 160, 320μM and negative control), and then cultured under normoxia or hyperoxia conditions for 48 hours (Change the medium every 24 hours). Subsequently, add 100μL medium mixed with CCK8 reagent (1:10) into each hole, set blank control, incubate at 37 ℃ for 1 hour. Finally, the cells were measured on the microplate reader of enzyme-linked immunosorbent assay (Gene Company Limited, ELX800ux, Hong Kong) at a wavelength of 450nm [15]. The Cell proliferation value $OD_{450} = OD_{group}－OD_{blank}$. In time effect test, 4 groups were set up: normoxia control group(21%O2+0μM heme), normoxia heme group (21%O2+20μM heme), hyperoxia control group (40%O2+0μM heme) and hyperoxia heme group (40%O2+20μM heme), OD_{450} values were measured by CCK8 method at 0, 12, 24, 48, 72 and 96 hour after changing culture medium respectively. Four multiple holes were measured each experiment, the experiment was repeated three times.

HMEC-1 proliferation activity EDU assay

Cell proliferation activity was analyzed by EDU assay (RiboBio, Guangzhou, China) according to the manufacturer's instructions. Cells were seeded in Millicell (1.5×10^4/well, 200μL) for 12 hours and then adhered to the wall under normoxia condition. According to the heme concentration group (0μM heme, 20μM heme or vehicle), the culture medium was changed and cultured under normoxia or hyperoxia conditions respectively for 48 hours (Change the medium every 24 hours). Then, the cell culture medium was added to 200 μL of reagent A (1:1000) for 2 hours at 37℃. Step by step, cells were fixed for 30 minutes with 4% paraformaldehyde, destained by 2 mg/mL glycine decolorization for 5 minutes, washed with PBS for 5 minutes, permeabilized with 0.5% Triton for 10 minutes. Other reagents, B to F, were mixed together according to the instructions. Then washed with PBS for 5 minutes, cell nucleus were stained with DAPI (1:1000, 4083, CST, USA) for 5 minutes, washed with PBS and sealed with anti fluorescence quenching [16]. Three regions in each well were randomly selected and imaged under a laser confocal microscope (Leica, SP8, Germany), the number of proliferative cells were counted by Image J software (National Institutes of Health, Bethesda, Germany). Each experiment was repeated at least three times.

HMEC-1 migration assay

HMEC-1 were seeded in 6-well plates (1×10^5/well) under normoxia condition, change the medium every 24 hours until cells filled with the bottom of the well. The cells were then wounded with 200μL pipette tips and washed with PBS. According to the heme concentration group (0μM heme, 20μM heme or vehicle), the culture medium was changed and cultured under normoxia or hyperoxia conditions respectively. Using an inverted phase contrast microscope (Zeiss, Primo Vert, Germany) equipped with a 5×objective and CCD cameras to take photos at 0, 12and 24 hours respectively, the central area of the scratch of the 6-well plate was selected [17]. After taking pictures, firstly, a solid line was drawn along both sides of the cell scratch on the photo at 0h, and a dotted line was drawn along the cell migration front at 12/24h by drawing software (Photoshop 8.0, adobe, USA), then the scratch area was calculated by Image J software. The area between solid lines is $S_{0h\ Scratch\ area}$, and the area between dotted lines is $S_{12/24h\ Scratch\ area}$, $S_{Migration\ area}=S_{0h\ Scratch\ area}-S_{12/24h\ Scratch\ area}$. The experiment was repeated independently for three times.

HMEC-1 capillary-like tube formation assay

Tube formation was assessed as previously described [18]. HMEC-1 were seeded in 6-well plates (1×10^5/well) for 12 hours and then adhered to the wall under normoxia condition. According to the heme concentration group (0μM heme, 20μM heme or vehicle), the culture medium was changed and cultured under normoxia or hyperoxia conditions respectively for 48 hours (change the medium every 24 hours). Matrigel (356234, Corning, USA) was added to the precooled millicell (200μL/well) and polymerized for 40 minutes at 37℃. Then, the cultured cells in each group were digested by trypsin, adjusting the cell concentration to $3.5x10^5$/mL with corresponding medium. Finally, adding the adjusted cells into the millicell contained polymerized Matrigel (200μL/well). Incubating for 4 hours under normoxia or hyperoxia condition. Wash once with Hank's Balanced Salt Solution (HBSS), adding 8μg/mL (HBSS dilution, 200μL/well) Calcein AM Fluorescent Dye (354216, Corning), incubating for 4 hours under normoxia or hyperoxia condition. Wash twice with the HBSS, using a laser confocal microscope (Leica, SP8) to take pictures, three regions in each well were randomly selected and imaged. The Angiotool Software was used to measure the number of junctions, length and area of the tubular-like structures [19]. Three independent experiments were performed.

BACH1 and VEGF protein expression by Western blot (WB)

Refered to previous method [20], HMEC-1 were seeded in 6-well plates (1×10^5/well) for 12 hours and then adhered to the wall under normoxia condition. According to the heme concentration group (0μM heme, 20μM heme or vehicle), the culture medium was changed and cultured under normoxia or hyperoxia conditions respectively for 48 hours (change the medium every 24 hours). Washing with PBS, and then incubating them in 100μL lysis buffer for 30 minutes, scrape the cells with 1ml sterile pipette. Finally, the

samples were collected, and completely disrupted by ultrasound for 5 minutes. The lysates were centrifuged at 12000g for 15 minutes at 4°C and total protein was extracted from the supernatant. The protein concentration was detected by the double octanoic acid protein quantitative kit (Beyotime, ShangHai, China) and equaled with loading buffer, boiled for 10 minutes. Gel electrophoresis, and transferred to a 0.45μm polyvinylidene fluoride membrane. After blocking with 5% skimmed milk for 2 hours, the membranes were incubated successively with primary antibodies overnight at 4°C and secondary antibodies for 2 hours at room temperature. The primary antibodies and the dilutions used were: anti- BACH1(1:1000, ab49657, Abcam, UK), anti-VEGF (1:1000, ab46154, Abcam), anti-β-actin (1:10000, BS6007M, Bioworld, Minnesota), and were diluted by Western blot-specific diluent (Beyotime). The secondary antibodies and the dilutions used were: goat anti-rabbit (1:5000, BS13278, Bioworld), goat anti-mouse (1:5000, BS12478, Bioworld), and were diluted by TBST. Immunoreactive bands were developed by Immobilon Western Chemiluminescent Horseradish Peroxidase Substrate (Vilber-Lourmat, France) according to the manufacturer 's instructions. All bands were quantified by Image J software, and normalized with respect to the β-actin values. Three independent experiments were performed.

PECAM-1, BACH1 and VEGF expressions were detected by immunofluorescence (IF)

Refered to previous method [11], HMEC-1 were seeded in Millicell (1.5×10^4/well, 200μL) for 12 hours and then adhered to the wall under normoxia condition. According to the heme concentration group (0μM heme, 20μM heme or vehicle), the culture medium was changed and cultured under normoxia or hyperoxia condition respectively for 48 hours (change the medium every 24 hours). Then, cells were washed with PBS three times for 5minutes, fixed with 4% paraformaldehyde for 30minutes, permeabilized with 0.3% Triton for 20minutes, sealed with 5% goat serum for 30 minutes. Next, the cells were treated with anti-BACH1 (1:100, ab49657, Abcam), anti-VEGF (1:250, ab1316, Abcam), or anti-PECAM-1 (1:100, sc-133091, SANTA, USA), overnight at 4°C. After washing with PBS three times, cells were incubated for 2 hours at 37°C with goat anti-rabbit IgG/Alexa Flour 488 (1:300, bs-0295G-AF488, CST) and goat anti-mouse IgG/Alexa Flour 594 (1:300, bs-0296G-AF594, CST). After incubation, cells were washed with PBS again and the nucleus were stained with DAPI (1:1000, 4038, CST) for 5 minutes. Finally, washed with PBS and sealed with anti fluorescence quenching. Three regions in each well were randomly selected and imaged under a laser confocal microscope, the fluorescence intensity was counted by Image J software, in order to reduce the error, we use the system automatic default value when setting the threshold value. Each experiment was repeated at least three times. Negative control was set in the experiment, including omission of the primary antibody and use of an irrelevant polyclonal or isotypematched monoclonal primary antibody. In all cases, negative controls showed only weak staining.

Statistical analysis

SPSS 20.0 (IBM, Armonk, USA) statistical software was used for statistical analysis. All experiments were performed at least three times. Results are expressed as the mean ± SD. One-way ANOVA or multivariate analysis of variance was used in cases with homogeneity of variance and normal distribution, and for all other cases, a nonparametric test was used. $P<0.05$ was considered to indicate statistical significance.

Results

Moderate heme can relieve the inhibition of hyperoxia on HMEC-1 proliferation

Under hyperoxia condition, the proliferation of HMEC-1 is inhibited, 5–40 μM heme promoted the proliferation ability of HMEC-1, more than or equal to 80 μM showed inhibition; Under normoxia condition, 10–40 μM heme promoted the proliferation ability of HMEC-1, more than or equal to 160 μM heme showed inhibition; 20 μM heme has the peak effect on HMEC-1 proliferation under normoxia and hyperoxia (P□0.001) (Fig. 1A). The time effect curve of HMEC-1 proliferation showed that the proliferation of HMEC-1 first increased with time and reached the peak at 72 h, then it began to decrease;Under hyperoxia or normoxia condition, the cell proliferation time effect curves of 20 μM heme were higher than those of control group (P□0.001), there was no significant difference between hyperoxia heme group and normoxia control group (P□0.05), which indicated that 20 μM heme can relieve the inhibitory effect of hyperoxia on HMEC-1 proliferation (Fig. 1B).

EDU detection showed that the cells with proliferative activity were stained with green fluorescence (Fig. 1C). Data analysis showed that the total number of cells, the number of cells with proliferative activity and the percentage of cells with proliferative activity decreased under hyperoxia condition (P□0.001), while in 20 μM heme group increased under hyperoxia or normoxia condition (P□0.05), there was no significant difference between hyperoxia heme group and normoxia control group (P□0.05), it was further verified that 20 μM heme could relieve the inhibitory effect of hyperoxia on HMEC-1 proliferation (Fig. 1D-F).

20 μM heme relieves the inhibitory effect of hyperoxia on HMEC-1 migration

In the cell scratch experiment, we drew solid lines to mark the edges of the wound at 0 h after scratch, and dotted lines marked the fronts of cell migration at 24 h after scratch (Fig. 2A). Image J software was used to measure the scratch area, the area between the two marking lines was the cell migration area ($S_{Migration\ area}=S_{0h\ Scratch\ area}-S_{24h\ Scratch\ area}$). Data analysis showed that hyperoxia inhibited HMEC-1 migration ability (P□0.001), and 20 μM heme could improve HMEC-1 cell migration ability under hyperoxia or normoxia condition (P□0.01). There was no significant difference between hyperoxia heme group and normoxia control group (P□0.05), indicating that 20 μM heme could relieve the inhibition of hyperoxia on HMEC-1 migration (Fig. 2B).

20 μM heme relieves the inhibition of hyperoxia on angiogenesis of HMEC-1

HMEC-1 were cultured with different concentrations of heme for 48 hours. The capillary-like tube formation experiment showed that the vascular network formed by endothelial cells was stained green, compared with normoxia condition, the vascular network was sparse and the pore size was larger under hyperoxia condition. After adding 20 μM heme, the vascular network became denser and finer (Fig. 3A). Data analysis showed that hyperoxia inhibited the number of junctions, length and area of angiogenesis in HMEC-1 (P□0.001), 20 μM heme can promote the number of junctions, length and area of angiogenesis in HMEC-1 under hyperoxia or normoxia condition (P□0.01). There was no significant difference between hyperoxia heme group and normoxia control group (P□0.05), which showed that 20 μM heme could relieve the inhibition of hyperoxia on the angiogenesis of HMEC-1 (Fig. 3B-D).

20 μM heme inhibits the expression of BACH1 and promotes the expression of VEGF in HMEC-1

Firstly, we identified the specific protein platelet/endothelial cell adhesion molecule-1 (PECAM-1, is also called CD31) on HMEC-1 cells by IF [21], we found that PECAM-1 was normally expressed in cytoplasm (Fig. 4D). Subsequently, we used IF to co-localize BACH1 and VEGF proteins in HMEC-1, the results showed that BACH1 was widely expressed in the nucleus and cytoplasm and labeled green, VEGF was expressed in the cytoplasm of HMEC-1 and labeled red (Fig. 4G). Meanwhile, WB (Fig. 4B,C) and IF analysis (Fig. 4E,F) showed that hyperoxia inhibited the expression of BACH1 and VEGF in HMEC-1 (P□0.001); Under hyperoxia or normoxia condition, 20 μM heme could inhibit the expression of BACH1 protein (P□0.01) and promote the expression of VEGF protein in HMEC-1 (P□0.05).

Discussion

ROP is divided into two phases: Phase I is mainly caused by the relatively hyperoxia induced by premature infants leaving the uterus, oxygen therapy and other factors, leading to the rapid down regulation of vascular promoting factors such as VEGF in the retina, delaying the normal development of retinal blood vessels to the periphery. Phase II is mainly due to the relatively hypoxia induced by the separation from the relatively hyperoxia environment, early angiogenesis stagnation, the maturation of neurons in the peripheral vascular free area of the retina, and the increase of energy demand, leading to the overexpression of vascular promoting factors such as VEGF, result in pathological neovascularization, and eventually retinal detachment or blindness in severe cases [1]. If the retinal blood vessels in Phase I formed normally, the malignant proliferation of blood vessels caused by peripheral retinal ischemia in phase II can be avoided [3], thus ROP children could obtain better visual function. Studies have shown that human retinal angiogenesis is related to the proliferation, migration and angiogenesis of microvascular endothelial cells [2, 22], the HMEC-1 is a recognized model of vascular endothelial cells [11, 23]. Therefore, we established a model of HMEC-1 induced by 40% hyperoxia in vitro to simulate the relatively hyperoxia environment of phase I ROP, and found that the biological behaviors of HMEC-1 such as cell proliferation, migration and angiogenesis were inhibited in 40% hyperoxia environment.

At present, the treatment strategy of ROP is mainly focused on phase II. Fundus laser photocoagulation and intravitreal injection of anti VEGF drugs were the main therapies, but both have certain side effects:

Fundus laser treatment can cause certain damage to retina and irreversible damage to children's peripheral vision. Intravitreal injection of anti VEGF drugs may lead to endophthalmitis and traumatic cataract, when anti VEGF drugs enter the blood circulation, it will affect the development of blood vessels in other organs, especially the brain, lung and kidney [3, 24]. Moreover, these two therapies are not effective in all ROP cases, and the search for therapeutic drugs for phase I ROP has become a hot spot in recent years. Heme is a complex of iron and the tetrapyrrole protoporphyrin IX with essential functions in aerobic organisms, participate in biological oxygen transport, respiratory chain electron transfer, etc. It can be degraded to iron, carbon monoxide and biliverdin by HO-1, which has anti-inflammatory, anti-oxidant and anti-apoptotic effects [25]. It has been used in the treatment of porphyria hepatica, it is a safe and reliable drug, which can be orally and intravenously injected [26], more convenient and safer than intravitreal injection. In this study, we observed that moderate heme can promote endothelial cell proliferation, while excessive heme can inhibit (Fig. 1A), which is consistent with previous reports that excess free heme is toxic via pro-oxidant, cytotoxic and proinflammatory effects [27]. In mammals, heme homeostasis can be maintained by hemopexin neutralization or HO-1 enzymatic hydrolysis of excess free heme, 30 μM heme can accumulate in mitochondria that synthesize heme [28], while in hemolytic diseases vascular endothelial cells may encounter up to 100 μM concentration of heme and open a self-protective mechanism [25], suggesting that 20 μM heme is safe in dose, it was reported in *Nature* that heme at a dose of 50 mg/kg could specifically degrade BACH1 in mice without affecting normal physiological functions [9]. Therefore, for the first time, we attempted to use 20 μM heme to specifically inhibit BACH1 expression in vascular endothelial cells, and observed that this effect could promote VEGF expression in vascular endothelial cells. These findings have a certain reference value for heme to treat angiogenesis stagnation caused by the decrease of VEGF expression induced by relatively hyperoxia in phase I ROP.

Our experimental results also showed that heme could relieve the inhibition of endothelial cell proliferation, migration and tube formation ability induced by hyperoxia, possibly through the mechanism of heme specifically inhibits BACH1 and then promotes VEGF. However, when we analyzed the expression of VEGF protein by WB and IF, we found that although under hyperoxia or normoxia condition, 20 μM heme could promote the expression of VEGF protein ($P < 0.05$), the increase was 16.06% (WB) and 21.67% (IF) in hyperoxia, 11.91% (WB) and 15.61% (IF) in normoxia, the difference in the expression of VEGF protein between the hyperoxia heme group and the normoxia control group was still statistically significant ($P < 0.05$), which indicated that 20 μM heme did not completely relieve the inhibitory effect of hyperoxia on the expression of VEGF protein in HMEC-1. That is, heme still did not restore the expression of VEGF protein in vascular endothelial cells from the simulated phase I ROP pathological environment to the normal level (Fig. 4C, F). Previous studies have shown that the occurrence and development of ROP are closely related to energy metabolism. The energy demand of retinal neurons is met by a tightly coupled vascular network, while neurons and peripheral rod cells develop earlier than blood vessels, thus generating energy demand, activating the physiological hypoxia channel of "hypoxia—HIF-1α—VEGF", which is the main inducing power of angiogenesis, but excessive nutrient deficiency and reduced oxygen supply will promote pathological neovascularization [6, 29]. On the contrary, damaged retinal ganglion

cells (RGC) and photoreceptors can reduce neovascularization, and retinal blood vessels will further atrophy to match the reduced energy demand with the degeneration of neurons [30, 31]. Therefore, improving the energy metabolism efficiency of retinal energy may also have the potential to treat ROP. Recent studies have shown that the inhibition of BACH1 can induce the expression of electron transfer chain (ETC) gene and promote glucose utilization in mitochondrial respiration and tricarboxylic acid cycle (TCA) [9]. This may be another mechanism that heme can relieve the inhibition of HMEC-1 in hyperoxia by inhibiting BACH1. In addition, inflammation and oxygen toxicity are also one of the pathological mechanisms of ROP [3]. Interestingly, heme has anti-inflammatory, anti-oxidative and anti-apoptotic effects [25], therefore, the anti-inflammatory and anti-oxidative effects of heme on endothelial cells and the pathological effects of ROP may also need to be further explored.

In conclusion, this study provides a new idea for early prevention and treatment of retinal vascular development retardation induced by relatively hyperoxia in phase I ROP. That is, to relieve the inhibition of hyperoxia on microvascular endothelial cells by the specific inhibitory effect of heme on BACH1. The mechanism of action is related to the promotion of VEGF expression in endothelial cells, and may also be related to the promotion of energy metabolism, anti-inflammatory or anti-oxidative effects of heme, or the result of combined actions. the specific mechanism needs to be further studied and further verified in animal experiments.

Conclusions

In summary, we have provided direct evidence of BACH1 expression in HMEC-1 and heme can inhibit the expression of BACH1 in HMEC-1. Moreover, we demonstrated that 40% hyperoxia could inhibit the proliferation, migration and angiogenesis of HMEC-1, and 20 μM heme could relieve the inhibitory effects induced by hyperoxia.

Abbreviations

ATCC
American Type Culture Collection; BACH1:BTB and CNC homology l; CCK8:Cell counting kit 8; DAPI:4',6-diamidino-2-phenylindole; EDU:5-ethynyl-2'-deox-yuridine; ETC:Electron transfer chain; FBS:Fetal bovine serum; HBSS:Hank's Balanced Salt Solution; HO-1:Heme degrading enzyme; HMEC-1:Human microvascular endothelial cells; IF:Immunofluorescence; MEL:Murine erythroleukemia; NICU:Neonatal intensive care unit; PBS:Phosphate buffered saline; PECAM-1:Platelet/endothelial cell adhesion molecule-1; RGC:Retinal ganglion cells; ROP:Retinopathy of prematurity; TCA:Tricarboxylic acid cycle; TNBC:Triple negative breast cancer; VEGF:Vascular endothelial growth factor; WB:Western blot.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

YRD, LJ and YM contributed to the conception of the study; LJ and YM contributed significantly to carried out experiments and manuscript preparation; LJ performed the data analyses and wrote the manuscript; YRD and YM helped perform the analysis with constructive discussions. All authors have read and approved the manuscript.

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Availability of data and materials

All data used to support the findings of this study are included in this article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

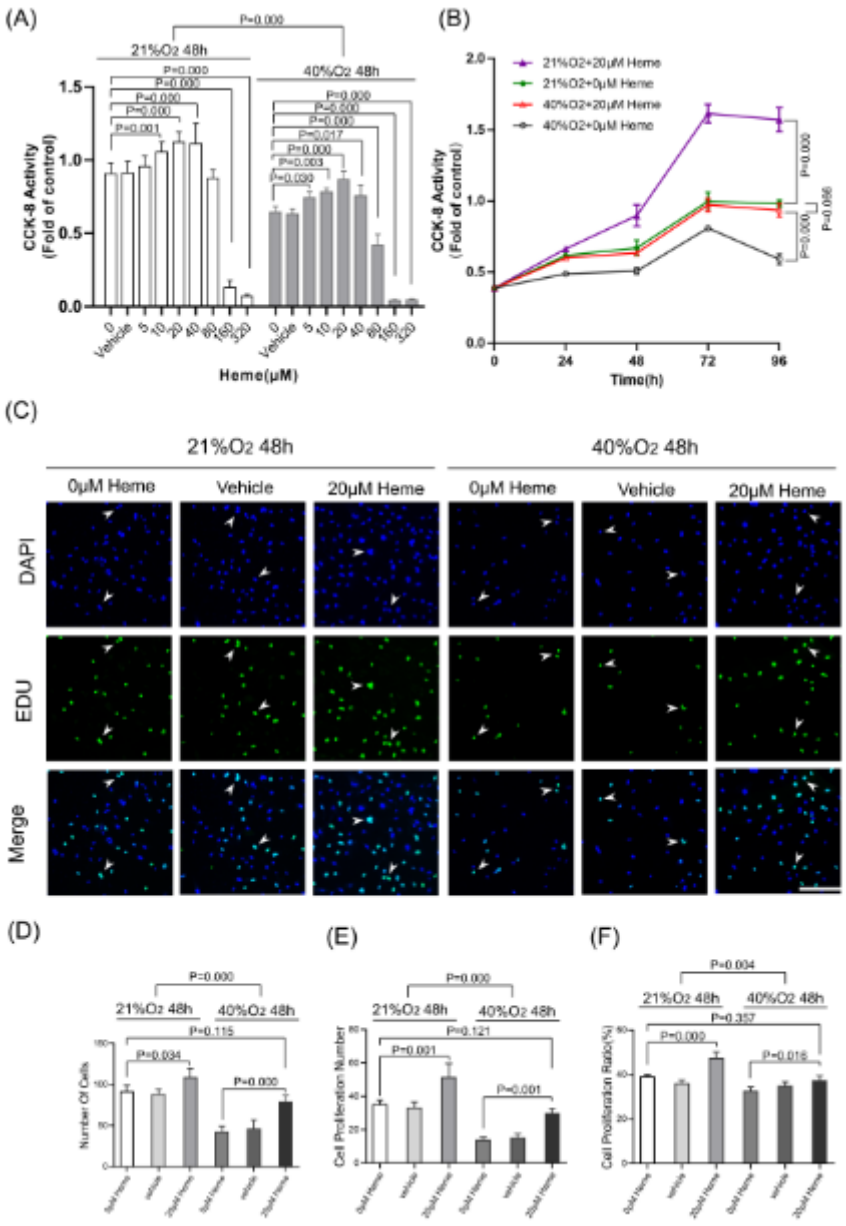

Figure 1

Effect of heme on HMEC-1 proliferation. A, Dose effect diagram of different concentrations heme on HMEC-1 cell proliferation detected by CCK8 method, all data are the mean ± SD, the experiment was repeated three times (n=4), One way ANOVA or multivariate ANOVA test. B, Time effect diagram of 20μM heme on HMEC-1 cell proliferation detected by CCK8 method, all data are the mean ± SD, the experiment was repeated three times (n=4), multivariate ANOVA with repeated measurements test. C, The pictures of proliferation activity of HMEC-1 by EDU assay, under the laser confocal microscope, the proliferative cells were stained green (488), DAPI-labeled nuclei blue, the arrow refers to the representative HMEC-1 cells with proliferative activity (scale bar=200μm). D-F, Quantitative analysis diagrams of the total number of cells, the number of cells with proliferative activity and the percentage of cells with proliferative activity of

HMEC-1, all data are the mean ± SD, the experiment was repeated three times (n=3), One way ANOVA or multivariate ANOVA test.

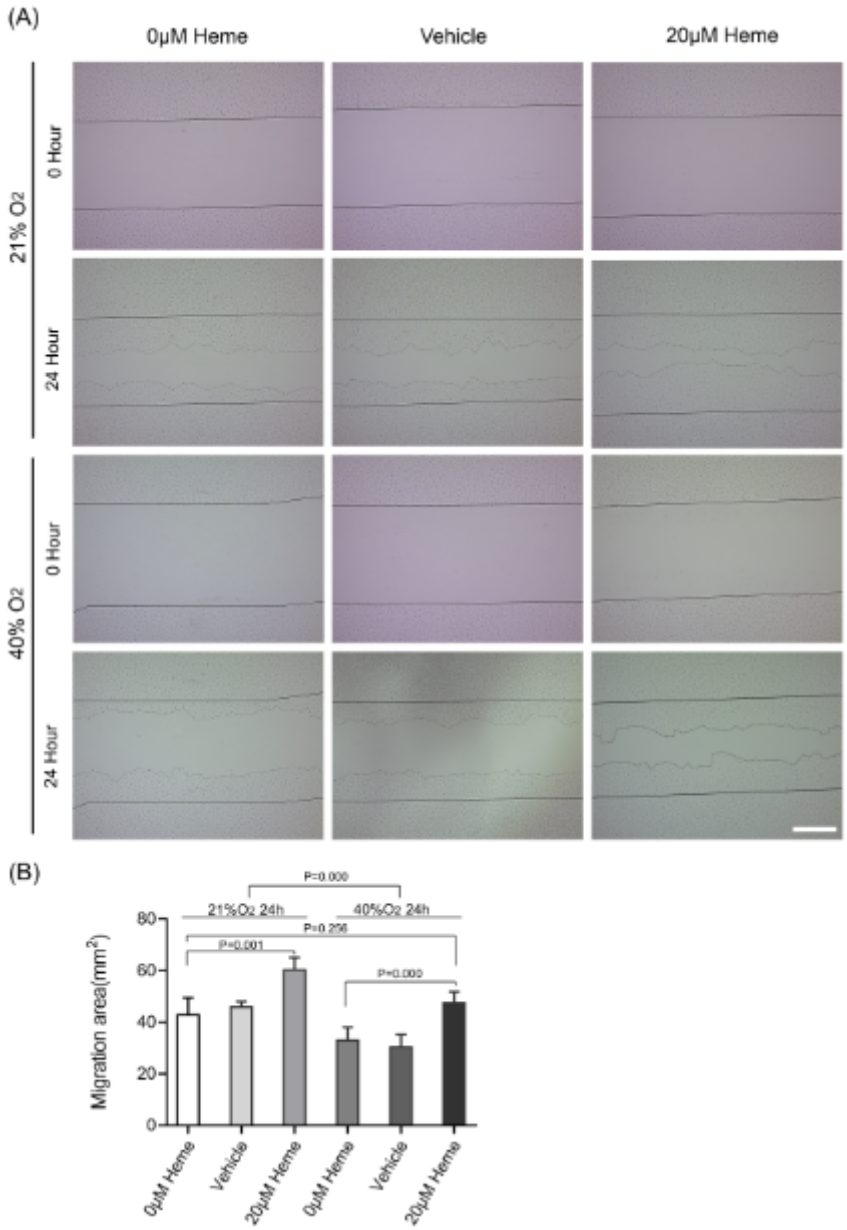

Figure 2

Effect of 20μM heme on HMEC-1 migration. A, The pictures of HMEC-1 cells were taken at 0h and 24h after scratch, the solid line was the edge of both sides of the wound at 0h after scratch, and the dotted line was the migration front of cells at 24h after scratch (scale bar=500μm). B, Quantitative analysis diagram of HMEC-1 cell migration area, all data are the mean ± SD, the experiment was repeated three times (n=3), One way ANOVA or multivariate ANOVA test.

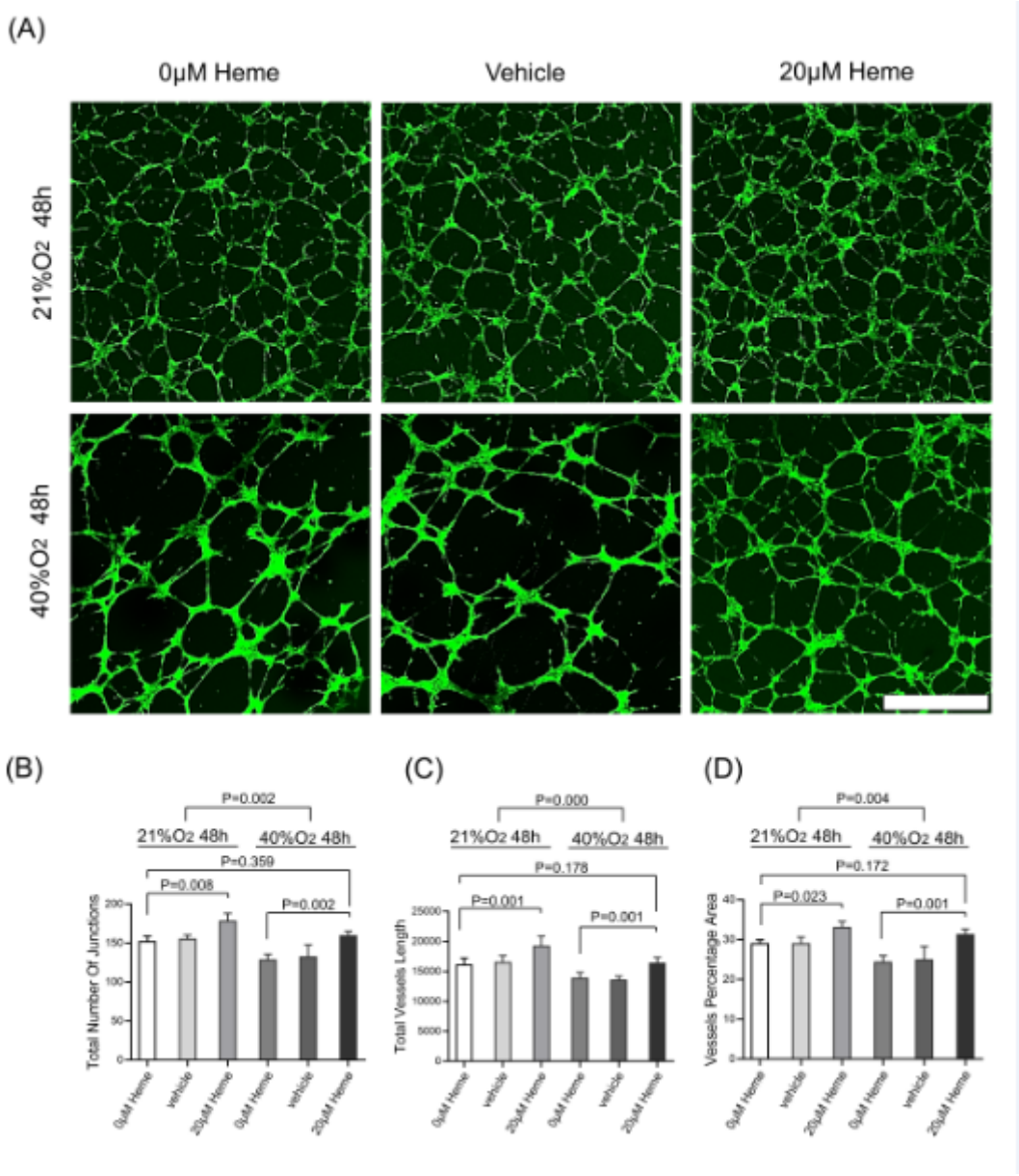

Figure 3

Effect of 20μM heme on angiogenesis of HMEC-1. A, The pictures of HMEC-1 capillary-like tube formation, the vascular network formed by HMEC-1 was stained green (488) after 48h cultured under different conditions. Under hyperoxia condition, the vascular network was sparse and the pore size was larger. After adding 20μM heme, the vascular network was denser and pore size was finer (scale bar=200μM). B-D, Quantitative analysis diagrams of the number of junctions, length and area of HMEC-1 capillary-like tube formation, all data are the mean ± SD, the experiment was repeated three times (n=3), One way ANOVA or multivariate ANOVA test.

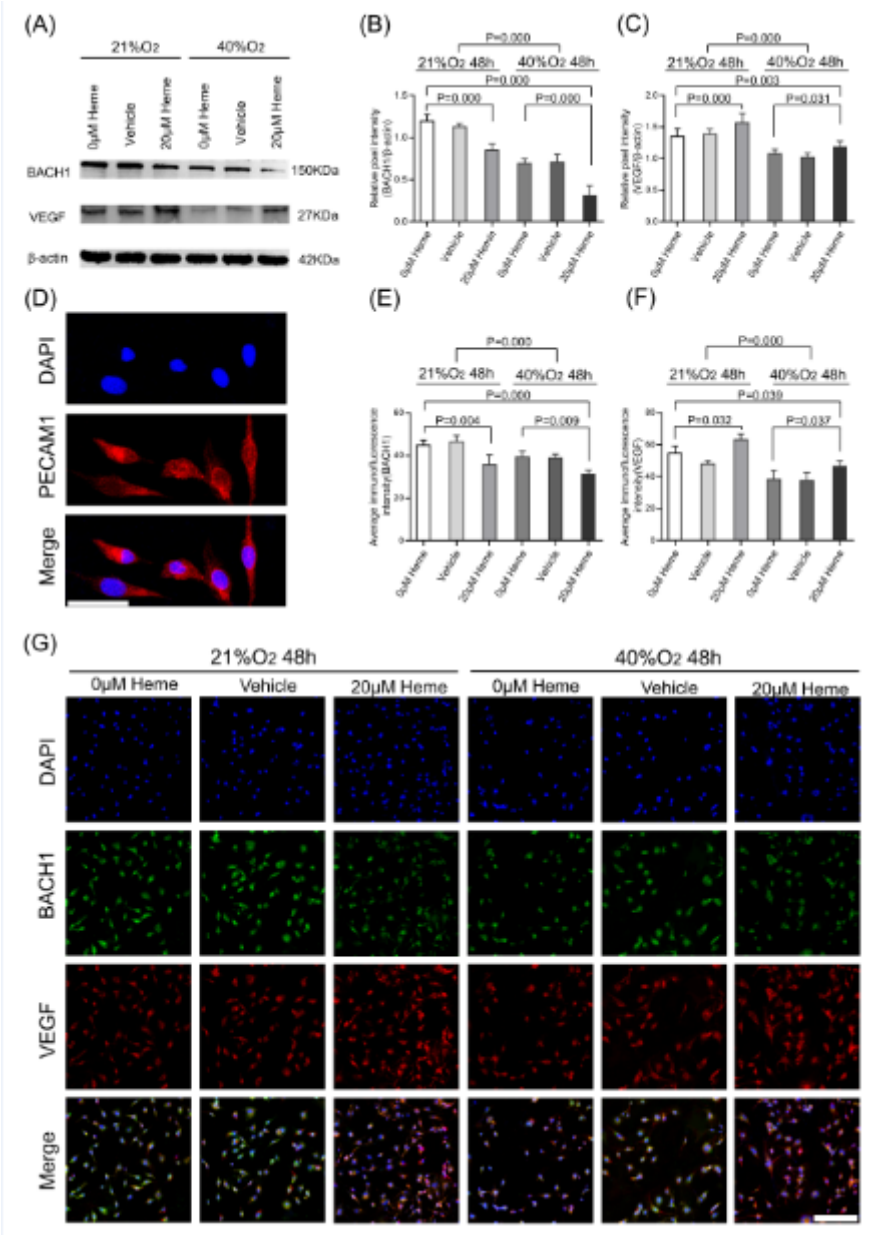

Figure 4

Effects of 20μM heme on expression of BACH1 and VEGF in HMEC-1. A, The pictures of Western blot of BACH1 and VEGF protein, under hyperoxia and normoxia conditions, after adding 20μM heme, the expression of BACH1 protein in HMEC-1 was lower than the control group, and the expression of VEGF protein in HMEC-1 was higher than the control group. B-C, Quantitative analysis diagrams of BACH1 and VEGF protein by Western blot assay, all data are the mean ± SD, the experiment was repeated three times (n=3), One way ANOVA or multivariate ANOVA test. D, The specific marker protein of endothelial cell PECAM-1 was stained red (594) in HMEC-1 cytoplasm, DAPI-labeled nuclei blue (scale bar=50μM). E-F, Quantitative analysis diagrams of fluorescence intensity per unit area of BACH1 and VEGF protein by immunofluorescence assay, all data are the mean ± SD, the experiment was repeated three times (n=3), One way ANOVA or multivariate ANOVA test. G, The pictures of immunofluorescence staining of HMEC-1,

BACH1 was widely expressed in the nucleus and cytoplasm and labeled green (488), VEGF was expressed in the cytoplasm and labeled red(594), DAPI-labeled nuclei blue (scale bar=200μM).

Supplementary Files

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