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Involvement of COX-2/PGE_2 signalling in hypoxia-induced angiogenic response in endothelial cells

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Abstract

To evaluate the impact of hypoxia on the angiogenic capability of endothelial cells (ECs), and further investigate whether the cyclooxygenase-2 (COX-2)/prostaglandin E_2 (PGE_2) signalling is involved in the angiogenic response of ECs to hypoxia. We explored the impact of various periods (1, 3, 6, 12, 24 hrs) of hypoxia (2% O_2) on human umbilical vein endothelial cells (HUVECs) *in vitro*. We observed cell viability, migration, tube formation, analysed COX-2, vascular endothelial growth factor (VEGF), AQP1 mRNA transcription, protein expression and measured PGE_2, VEGF protein concentration in cell supernatants. Then we treated HUVECs with COX-2 selective inhibitor NS398, EP1/2 combined antagonist AH6809 and exogenous PGE_2 to investigate the role of COX-2/PGE_2 signalling in the angiogenic response of ECs to hypoxia. The results demonstrated that short-term hypoxic treatment enhanced HUVECs proliferation, migration, tube formation, significantly up-regulated COX-2, VEGF, AQP1 mRNA level, protein expression and promoted PGE_2, VEGF release. The pharmacological inhibition study revealed that exposure of HUVEC to NS398 and AH6809 under hypoxia impaired the biological responses of ECs to hypoxia. Exogenous PGE_2 augments the effects of hypoxia on HUVECs, and partially reversed the inhibitory effects of NS398 on HUVECs proliferation and angiogenic capability. Short-term hypoxic treatment enhanced angiogenic capability of ECs, and COX-2/PGE_2 signalling may play a critical role in the biological response of ECs to hypoxia.

Keywords: hypoxia • endothelial cells • angiogenesis • COX-2/PGE_2 signalling • VEGF • AQP1

Introduction

Angiogenesis, formation of new capillaries which enables delivery of oxygen and nutrients, is essential for tumour growth and metastasis [1], ischaemic disorder recovering [2] and periodontal tissue remodelling [3]. It is a highly organized process and involves a complex sequence of steps that include endothelial cells (ECs) proliferation, maturation and assembly [4]. As the main cell type involved in angiogenesis, ECs migrate from capillaries toward the pathological zone, undergoing various stress conditions including hypoxia and acidic pH [5]. Moreover, the endothelium plays a predominant role in modulating many aspects of vascular homeostasis. Dysfunction of ECs structure and function may contribute to the overall endothelial dysfunction in a range of clinic settings, including ischaemic diseases [6]. Understanding specific mechanisms underlying angiogenesis from ECs point of view offers the best approach to develop therapies for cancer, ischaemic diseases and modulate the periodontal remodelling process, where neovascularization is either impaired or activated [2].

Hypoxia is a characteristic feature of many physiological or pathological processes *in vivo*: driving angiogenesis in tumours [7], taking part in ischaemic diseases [8] and participating in bony or soft tissue injury [9]. It has been shown to be an important regulator of blood vessel tone, vessel structure and a potent stimulus of angiogenesis [10]. Hypoxic environment primarily affect the fundamental characteristics of ECs, which

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situates at the interface between blood and tissue and the first line to sense hypoxia [11]. Vascular ECs cope with hypoxia to maintain vascular homeostasis by expressing a number of genes, which are mediated by a variety of oxygen-sensitive signalling cascades [12]. Interestingly, the presence of hypoxia is always associated with and accompanied by inflammation [13]. For instance, the poorly vascularized regions of tumours with low oxygen tension is closely linked with inflammation in the form of tumour-associated macrophages accumulation [14]; injury in acute myocardial infarction is characterized by inflammatory event of neutrophils infiltration after a period of ischaemia [15]; the inflammatory mediators interleukin-1β and β-glucuronidase have been shown to present in the gingival crevicular fluid of children undergoing orthodontic treatment [16]. Reciprocity of inflammation, oxidative stress and neovascularization is emerging as an important mechanism underlying numerous processes from tissue healing and remodelling to cancer progression [5]. However, the mechanism of hypoxia-driven angiogenesis and the role of inflammatory mediators in the biological response of ECs to hypoxia remains unclear.

Previous study [17] revealed that short-term hypoxia can directly activate ECs towards a pro-inflammatory phenotype through the synthesis of lipid mediators. ECs produce several PGs, such as PGE_2, PGI_2 and thromboxane A_2 in response to various stimuli [18, 19] and these prostanoids, particularly PGE_2, have been implicated in angiogenesis [20]. These pro-inflammatory eicosanoids directly stimulate the synthesis of angiogenic factors, promote vascular sprouting, migration and tube formation, and also enhance endothelial survival [20, 21]. Specifically, it has been reported that PGE_2 stimulates vascular endothelial growth factor (VEGF) expression in osteoblasts [22], fibroblasts [23], as well as in rat ECs [24] and that COX-2–dependent VEGF induction enhances angiogenesis *in vivo* [25].

Cyclooxygenase (COX) is a rate-limiting enzyme in the prostaglandin (PG) biosynthetic pathway. Cyclooxygenase-1 is found constitutively expressed in a wide range of tissues, while COX-2 is an inducible enzyme that produces prostaglandins during inflammatory and tumorigenic settings [26]. Furthermore, it is one of the genes induced by hypoxia and acts as a mediator of both inflammation and angiogenesis. EC-derived COX-2 is suggested important in angiogenesis [27]. Although there have been many reports regarding the mechanism of angiogenesis, few studies have explored the direct effect of COX-2/PGE_2 signalling on angiogenesis.

Vascular endothelial growth factor (VEGF) is a major regulator of endothelial proliferation and migration [28], and is the most potent inducer of angiogenesis and capillary permeability [29]. It has been reported the key role of oxygen tension in regulating the expression of a variety of genes, and VEGF mRNA is induced by exposure to low oxygen tension under various pathophysiological circumstances [30]. Moreover, the hypoxia-induced COX-2 activation may augment PGE_2 release, resulting in either an autocrine or paracrine action that enhances expression of VEGF through the generation of hypoxia inducible factor (HIF)-1α [31, 32].

The aquaporins (AQP) are a family of proteins which mediate water resorption in mammalian renal tubules and several other tissues [33]. Aquaporin-1 is one family member of AQP with a molecular weight of 28 kDa. Previous studies validated that AQP1 is responsible for the high vascular permeability and interstitial fluid pressure. AQP1 is reported to be required for hypoxia-inducible angiogenesis in human retinal vascular ECs [34]. However, the role of COX-2/PGE_2 signalling in the VEGF and AQP1 expression of ECs are rarely reported. Therefore, the mechanisms involved in the impact of hypoxia on VEGF and AQP1 expression of ECs remains to be further elucidated from the perspective of inflammation.

In order to better understand how ECs adapt to hypoxic environment, we explored the impact of hypoxia on the human umbilical vein endothelial cells (HUVECs) by observing cell viability, migration, tube formation, analysing COX-2, VEGF and AQP1 expression and measuring PGE_2, VEGF concentration in supernatants of HUVECs with different periods of hypoxic treatment. Then to further investigate into the potential role of COX-2/PGE_2 signalling in the biological response of ECs to hypoxia, we used COX-2 selective inhibitor NS398, E-prostanoid receptor 1/2 (EP1/2) combined antagonist AH6809 and exogenous PGE_2 to treat HUVEC under hypoxia. The findings of present study may elucidate the relationship between inflammation and angiogenesis in human ECs response to hypoxia, and provide theoretical basis for further understanding of the mechanisms involved in cancer, ischaemic disorder and periodontal tissue remodelling.

Materials and methods

Culture and characterization of HUVECs

Human umbilical vein endothelial cells were purchased from American Typical Culture Collection (ATCC code: CRL-1730). Cells were cultured in minimal essential α medium (α-MEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) foetal bovine serum (FBS; CS, Hyclone, Auckland, New Zealand), 100 units/ml penicillin and 100 mg/ml streptomycin. All cells were cultured in 25 cm^2 flasks (Corning Glass Works, Corning, NY, USA) and maintained in an incubator supplied with 95% air, 5% CO_2 and 100% humidity at 37°C. Every three days the media was exchanged with fresh α-MEM (plus 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin). Cells were washed twice with phosphate buffered solution (PBS) and detached with 0.25% trypsin plus 0.05% ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, MO, USA) for passage when they reached a confluence of 80% approximately [35].

Cell characterization was evaluated by their morphology on reaching confluence. Besides, immunostaining was performed in order to detect factor VIII-related antigen and CD31 in HUVECs raised to confluence. Briefly, HUVECs were fixed in 4% (w/v) paraformaldehyde for 20–30 min., washed with PBS three times and air-dried at 4°C. Then treated with 3% hydrogen peroxide for 15 min. at room temperature to block intrinsic peroxidase and washed with PBS three times. Fixed cells were incubated for 1 hr at 37°C with primary antibody anti-factor VIII-related antigen or

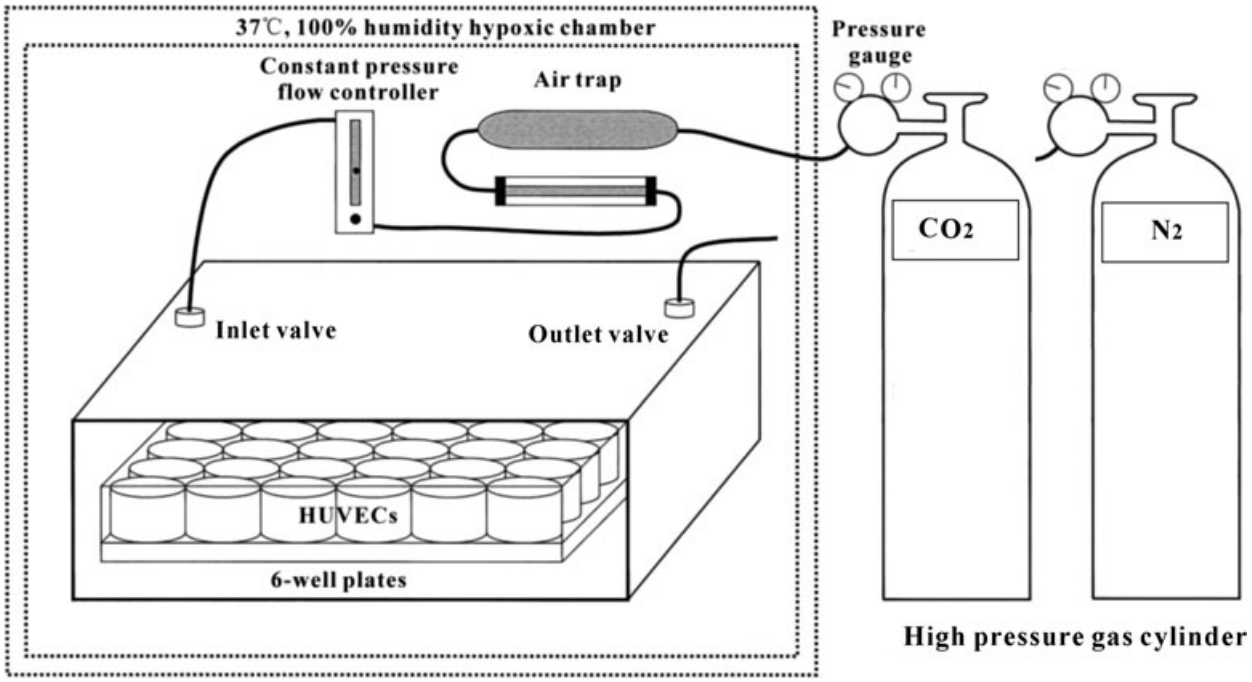


Fig. 1 Schematic diagram of the Binder, a three gas modular hypoxic incubator used to simulate hypoxic conditions *in vivo*. Ambient oxygen concentrations of 2% were maintained using this device with CO_2/O_2 monitoring and CO_2/N_2 gas sources. A pure N_2 entrance is set for the nitrogen replacement of air in incubator to control the oxygen concentration and achieve the purpose of hypoxia.

anti-CD31 (mouse anti-human; Abcam, Cambridge, UK). Subsequently, cells were washed in PBS and incubated with biotin-conjugated rabbit anti-mouse IgG (Santa Cruz, CA, USA) for 1 hr at 37°C. Finally, they were incubated with horseradish peroxidase labelled streptavidin for 20 min. at 37°C. At least five independent experiments were performed and cultures were examined with a microscope (Nikon, Japan).

Hypoxic treatment of HUVECs

At 80% confluence, HUVECs were placed in 6 well plates at $1 \times 10^4/cm^2$ and incubated with 5% CO_2, 100% humidity at 37°C for two days. The cells were assigned to two groups as hypoxic group (2% O_2) and normoxic control group (20% O_2), while the former divided into five subgroups (1, 3, 6, 12, 24 hrs) according to different periods of hypoxic exposure. Then, the cells were subjected to hypoxia maintained using a three gas modular hypoxic incubator with CO_2/O_2 monitoring and CO_2/N_2 gas sources (Binder, Camarillo, CA, USA). Figure 1 is schematical diagram of the device. Culture medium was pre-equilibrated overnight prior to cell exposure. Cell culture plates were placed in the incubator and saturated with a gas mixture containing 2% oxygen, 5% CO_2 and 93% nitrogen for the generation of hypoxia at 37°C for defined time periods (1, 3, 6, 12, 24 hrs), each with three replicates and the experiments were done for at least three times. In some experiments, the combined EP1/2 antagonist AH6809 (Sigma-Aldrich), or exogenous PGE_2 (Sigma-Aldrich) and/or NS398 (Sigma-Aldrich), a selective inhibitor of COX-2, were also added into the HUVECs medium 4 hrs before hypoxic treatment.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assays

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to determine HUVECs proliferation and viability. Human umbilical vein endothelial cells were seeded at 1×10^4/well in the 96 well plates and incubated with normal condition for 48 hrs, then were subjected to hypoxic treatment as mentioned above, each with five replicates. At each set point, the cells were supplemented with MTT (5 mg/ml; Sigma-Aldrich) and incubated for a further 3.5 hrs. The blue formazan thus produced was solubilized with 200 μl/well dimethyl sulfoxide (DMSO) and absorbance was measured at 570 nm by HTS 7000 Plus high efficient analyser (Perkin Elmer, Norwalk, CT, USA).

Transwell migration assay

Human umbilical vein endothelial cell motility was measured using 24-multiwell insert system (Millipore, CA, USA) with 8 μm pore size polycarbonate filter insert that divides the chamber into upper and lower portions. The filters were coated with human recombinant fibronectin. Briefly, HUVECs that were previously starved overnight in 5% foetal calf aerum (FCS; Sigma-Aldrich), were trypsinized and resuspended in 0.1% FCS medium at a density of 4×10^5 cells/ml. Two hundred and fifty microlitres of this suspension were then added to the upper chamber of the insert while 750 μl of 0.1% FCS was added to the lower chamber. After 3 hrs of adhesion, cells were allowed to migrate across the 8 μm pore size

Table 1 Primers used for real-time PCR analysis

Gene	GeneBank	Primers sequences (5′-3′)	Fragment size (bp)
Runx2	NM_001015051	R5′-GTGAAGACGGTTATGGTCAAGG-3′; F5′-CAGATGGGACTGTGGTTACTGT-3′	169
Osterix	NM_152860	R5′-CCACTATTTCCCACTGCCTTG-3′; F5′-ACCTACCCATCTGACTTTTGCTC-3′	125
COX-2	NM_000963	R5′-CTACCAGAAGGGCAGGATACAG-3′; F5′-GCAGGCAGATGAAATACCAGTC-3′	165
GAPDH	NM_002046	R5′-GTAGAGGCAGGGATGATGTTCT-3′; F5′-CTTTGGTATCGTGGAAGGACTC-3′	132

GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

polycarbonate membrane under normoxia or hypoxia for 1, 3, 6, 12 and 24 hrs. Cells on the top of the filter were removed by gentle swabbing and the remaining cells on the bottom side of the filter were stained using 0.25% cresyl violet, and counted under a light microscope with an eyepiece grid to visualize set fields. At least four fields in duplicate wells were counted for each condition.

Tube formation assay

Matrigel (BD Biosciences, New Bedford, MA, USA) was added to wells of a cold 96-well plate (45 μl/well), and then incubated at 37°C for 1 hr to allow gelling. Human umbilical vein endothelial cells were removed from confluent cultures by treatment with trypsin and 0.05% EDTA. The cells were washed in serum-containing medium and then resuspended to 10^5 cells/ml. Into each culture well was added 100 μl cell suspension. Cell culture was carried out at 37°C for 48 hrs in a humidified 5% CO_2 atmosphere for complete penetration of cells into the Matrigel. Then after 12 and 24 hrs of normoxic or hypoxic treatment, cells were observed directly and scored on a scale of 0–5 for tube formation using dark field illumination on a light microscope. Based on quality and number of the tubes, they were assigned numeric values: 0, no real tubes; 1, some poorly formed tubes; 2, some formed tubes; 3, network of tubes both formed and poorly formed; 4, network of formed tubes; and 5, network of well formed tubes. Score results from four random fields in duplicate wells were averaged.

Pharmacological COX-2 inhibition

This pharmacological inhibition study was employed to further investigate the biological response of HUVECs to hypoxia. NS398 was added into the HUVECs medium 4 hrs before hypoxic treatment. Minimum effective concentration (MEC) of inhibitor necessary for significant inhibition of COX-2 protein expression was determined by a concentration gradient test, in which NS398 was used at final concentrations of 0, 5, 10 and 20 μM, respectively.

RNA isolation and real-time quantitative PCR

After hypoxic treatment, cells were washed twice with PBS. The total RNA was extracted using TRIzol regent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA was quantified, in a spectrophotometer, at an absorbance (*A*) of 260 nm. The RNA samples had an A260:A280 ratio of 2.0 to guarantee high purity. Two micrograms of total RNA from each sample were subjected to reverse transcription using the SYBR PrimeScript™ RT-PCR Kit (TaKaRa Biotechnology, Dalian, Liaoning, China) according to the manufacturer's protocol. Each real-time PCR was carried out in triplicate in a total of 20 μl reaction mixture (6.8 μl of cDNA, 10 μl of SYBR1 Premix Ex TaqTM, 0.4 μl of ROX Reference Dye II, 0.4 μl of each 10 mM forward and reverse primers and 2 μl of H_2O) in an Applied Biosystem (ABI) Prism 7300 Real-time PCR System [Applied Biosystems (ABI), Foster City, CA, USA]. Primers used for real-time PCR analysis are presented in Table 1. The PCR program was initiated by 10 sec. at 95°C for pre-degeneration before 40 thermal cycles, each of 5 s at 95°C (degeneration), 31 s at 60°C (annealing) and 30 s at 72°C (elongation). The starting copy numbers of unknown samples were calculated by the 7300 System SDS Software from the standard curve. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was concurrently amplified in each sample as control and was used for normalization. The cDNA of control HUVECs untreated of hypoxia normalized to the level of GAPDH mRNA have been ascribed a fold induction of 1. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product. Data analysis was performed using the $2^{-\Delta\Delta Ct}$ method described previously, where GAPDH was used as the reference gene [36].

Western blotting

To obtain whole-cell extracts, cells that were treated with or without hypoxia were washed twice with ice-cold PBS and then lysed and sonicated in a lysis buffer (Keygen total protein extraction kit; Keygen Biotech., Nanjing, China). The cytosolic fraction was collected as the supernatant after centrifugation at 14,000 × *g* at 4°C for 15 min. and assayed it quantitatively with the BCA method. After boiling for 5 min., 20–25 ml of the lysate (50 mg of protein) was applied to SDS–12% PAGE at 80 V for 30 min. and 120 V for 1 hr. The proteins in the gel were then transferred to a PVDF membrane (Millipore). After blocking, the membranes were probed with 1:1000 dilutions of the anti-AQP1 and 1:100 dilutions of the anti-VEGF (Abcam, Hong Kong), followed by the addition of horseradish peroxidase (HRP)–conjugated secondary antibody (diluted 1:5000) at 37°C for 1 hr. Immunoreactive proteins were visualized using a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate; Millipore). Band intensities were determined using the ChemiDoc XRS Gel documentation system and Quantity One software (Bio-Rad, CA, USA).

Enzyme-linked immunosorbent assay assays

The supernatant media of HUVECs, stored in aliquots at −70°C, were thawed on ice before the measurement of VEGF and PGE_2 using specific

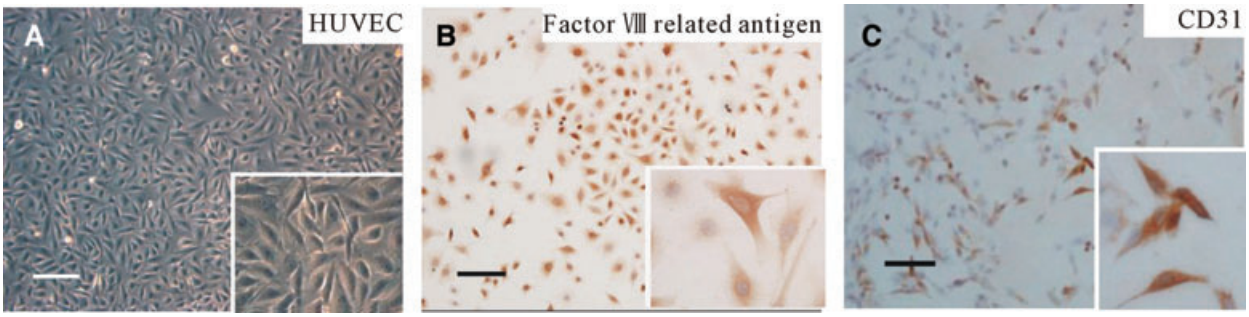


Fig. 2 Characterization of HUVECs by morphological observation and immunocytochemical staining. (**A**) The cells exhibited typical cobblestone morphology on reaching confluence, insert with higher magnification of ×200. (**B** and **C**) Immunocytochemical stained with factor VIII-related antigen and CD31 antibody, respectively, the immunoreactive positive cells were stained brownish-yellow, insert with higher magnification of ×400 confirming brownish-yellow stained cytoplasm. Scale bars: 100 μm.

enzyme-linked immunosorbent assay (ELISA) kits obtained from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions. Results were expressed after normalization to total protein contents of the supernatant media and in pg of VEGF or PGE_2 per mg of total protein. α-MEM–10% FBS was used as control.

Statistical analysis

All experiments were performed at a minimum of three times. Measurements are expressed as mean ± S.D. Statistical comparisons were made using factorial analysis of variance (ANOVA), for comparing treatments from controls. In MTT, cell migration and tube formation assay, Student's *t*-test was used to compare between the two groups at same time point. A value of $P < 0.05$ was statistically considered significant.

Results

Characterization of HUVECs

The cells exhibited typical cobblestone morphology on reaching confluence (Fig. 2A), and immunocytochemical staining showed that they expressed factor VIII-related antigen and CD31 (Fig. 2B and C), which initially suggested that the cells are ECs.

Hypoxia enhances HUVECs proliferation, migration and tube formation

To ensure that hypoxia used in this study (2% oxygen, 5% CO_2 and 93% nitrogen in a modular hypoxic incubator) induces typical cell responses to hypoxic stress, we initially verified HUVECs proliferation and viability following hypoxic exposure by MTT assay. As shown in Figure 3A, an up-regulation of HUVECs viability occurs after temporary exposure to hypoxia (1, 3 and 6 hrs), the cells viability was increased gradually with increasing time of hypoxia. At 3 and 6 hrs, it was significantly augmented by hypoxia compared with normoxia control ($P < 0.05$) and reached maximum at 6 hrs. With increasing time of hypoxia (12 and 24 hrs), it was decreased, less than those of the normoxia control group ($P < 0.05$).

Then, we assessed *in vitro* migration of HUVECs using fibronectin-coated 8 μm filters under either normoxic or hypoxic conditions for 1, 3, 6, 12 and 24 hrs. We found that hypoxia induced migration of HUVECs in a time-dependent manner. As compared with the migration value under normoxia of 12 ± 0.8, 63 ± 4.9, 96 ± 7.3, 147 ± 9.4 and 264 ± 17.7 cells/field at 1, 3, 6, 12 and 24 hrs, respectively, and the amount of cell migration induced by various periods of hypoxia were 67 ± 4.2, 118 ± 8.3, 173 ± 12.6, 295 ± 19.5, 347 ± 21.6, which were significantly higher than that of normoxia at each time point ($P < 0.05$) (Fig. 3B).

Finally, to study the effect of hypoxia on capacity of HUVECs to form functional capillaries *in vitro*, wells containing Matrigel were used in a tubeforming assay. Human umbilical vein endothelial cells were observed after 12, 24 hrs of normoxic or hypoxic treatment and analysed for the quality and number of the tubes formed in the gel. Overall, hypoxia led to a significantly higher level of tube formation than that of normoxia ($P < 0.05$) (Fig. 3C).

Hypoxia increases angiogenesis-related gene transcription and protein expression after temporary exposure

To find out the angiogenic capability in ECs under hypoxia, we proceeded to demonstrate the VEGF, AQP1 mRNA synthesis, protein expression and VEGF release of HUVECs after hypoxic treatment. As shown in Figure 4A, the VEGF mRNA was significantly increased by hypoxia at all time points ($P < 0.05$). During cultivation, it transiently increased about 2.3 folds, then peaked at 3 hrs and decreased after 6 hrs of the culture, still higher than those of

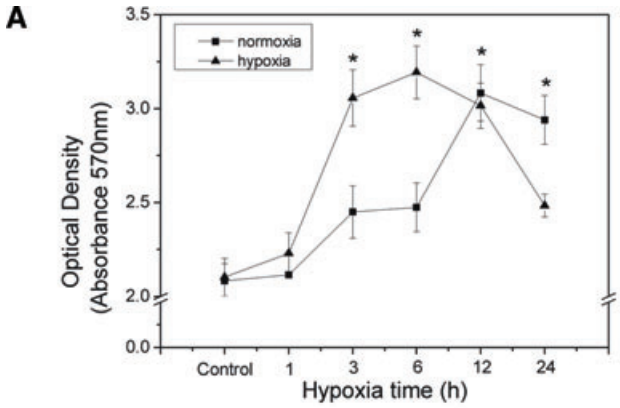
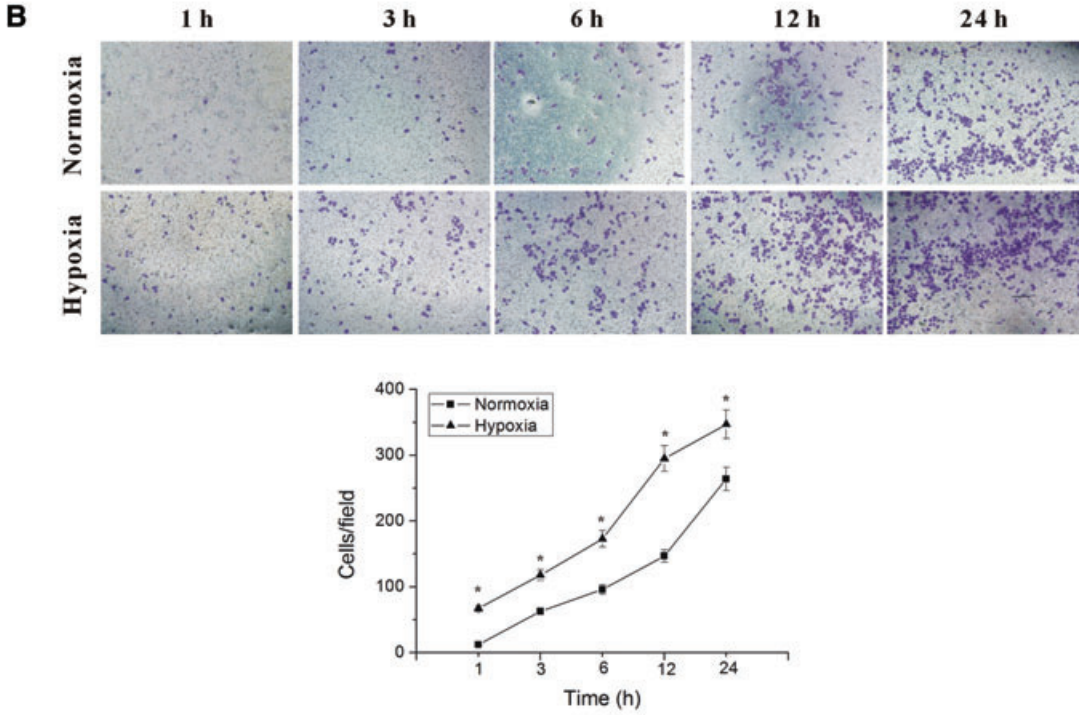
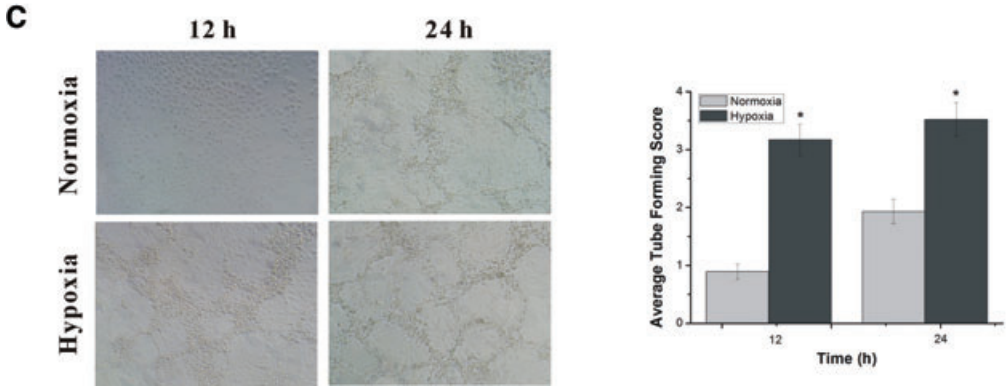


Fig. 3 HUVECs proliferation, migration and tube formation under hypoxic exposure. (**A**) HUVECs proliferation and viability following hypoxic exposure by MTT assay. $*P < 0.05$ *versus* control group. (**B**) HUVECs were assayed for migration under normoxia or hypoxia for indicated periods of time. The cells migrate across the membrane were counted under a light microscope with an eyepiece grid to visualize set fields. At least four fields in duplicate wells were counted for each group to generate the bar chart. (**C**) The tube formation of HUVECs after 12 and 24 hrs of normoxia or hypoxia was viewed by phase-contrast microscopy at ×100 magnification, tube formation of each group was scored on a scale of 0–5 based on quality and number of the tubes, score results from four random fields in duplicate wells were averaged to generate the bar chart. $*P < 0.05$ *versus* normoxia group.





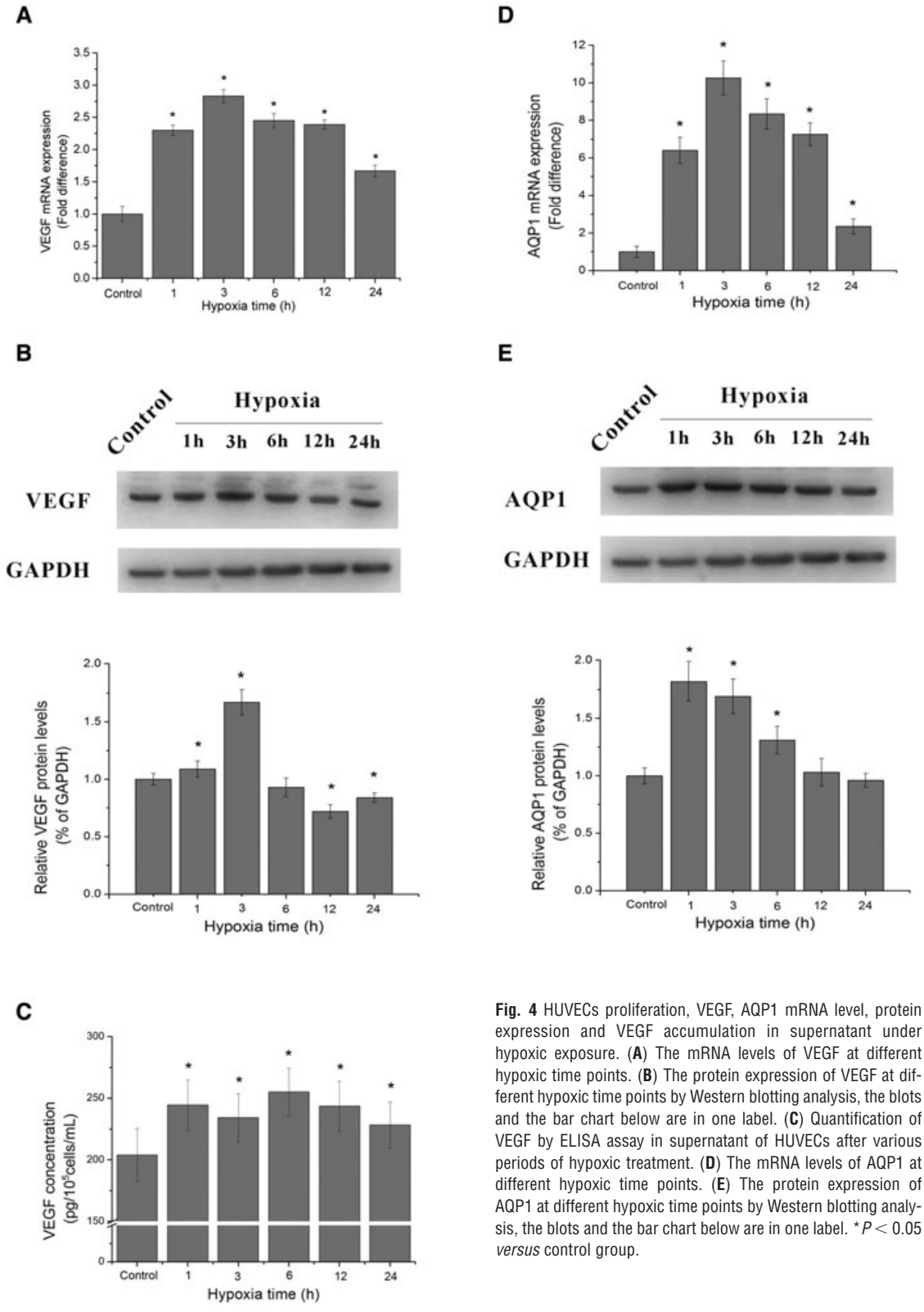


Fig. 4 HUVECs proliferation, VEGF, AQP1 mRNA level, protein expression and VEGF accumulation in supernatant under hypoxic exposure. (**A**) The mRNA levels of VEGF at different hypoxic time points. (**B**) The protein expression of VEGF at different hypoxic time points by Western blotting analysis, the blots and the bar chart below are in one label. (**C**) Quantification of VEGF by ELISA assay in supernatant of HUVECs after various periods of hypoxic treatment. (**D**) The mRNA levels of AQP1 at different hypoxic time points. (**E**) The protein expression of AQP1 at different hypoxic time points by Western blotting analysis, the blots and the bar chart below are in one label. $^{*}P < 0.05$ *versus* control group.

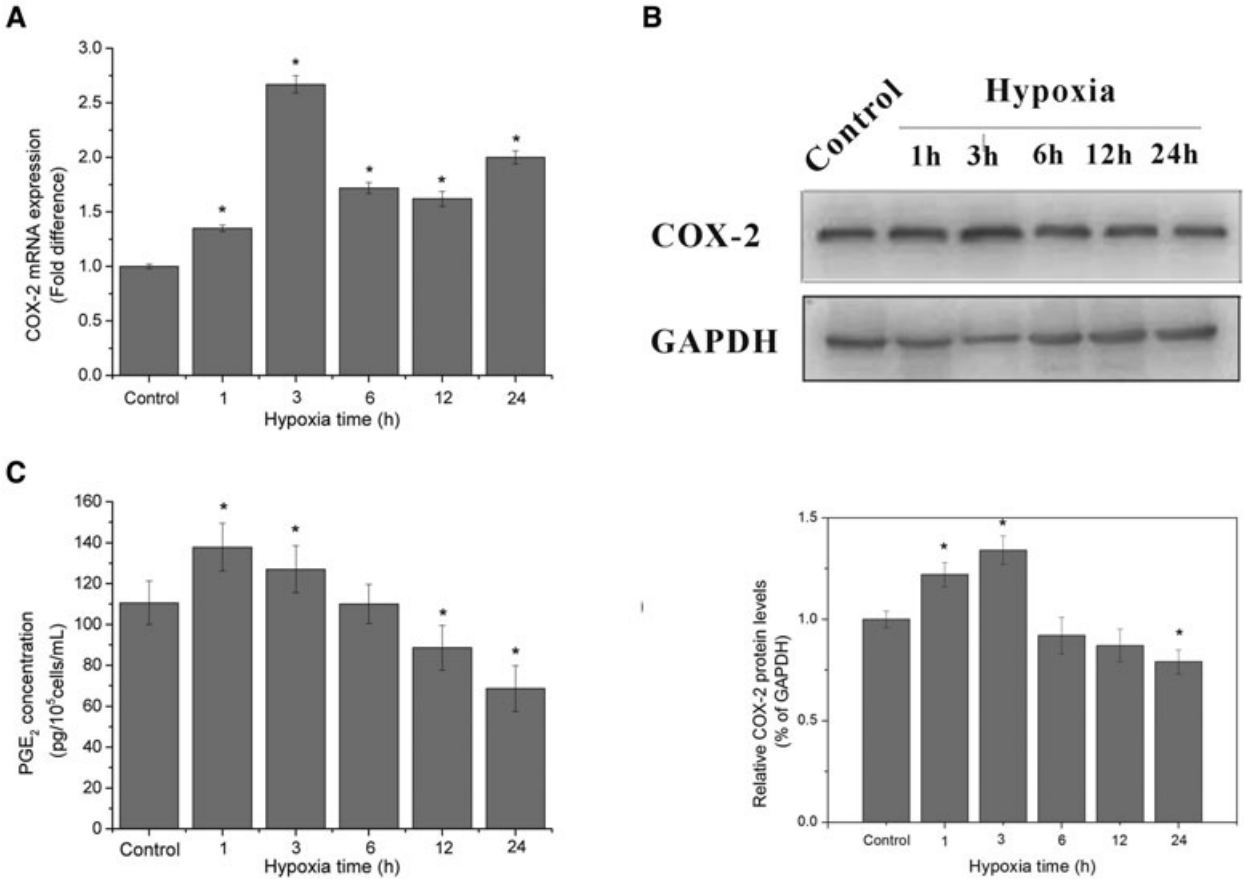


Fig. 5 COX-2 mRNA level, protein expression and PGE_2 accumulation in supernatant under hypoxic exposure. (**A**) The mRNA levels of COX-2 at different hypoxic time points. $2^{-\Delta\Delta Ct}$ values were obtained by real-time RT-PCR analysis using GAPDH transcripts for the normalization. (**B**) The protein expression of COX-2 at different hypoxic time points by Western blotting analysis, the blots and the bar chart below are in one label. (**C**) Quantification of PGE_2 by ELISA assay in supernatant of HUVECs after various periods of hypoxic treatment, the data are expressed in concentration of PGE_2. $^*P < 0.05$ *versus* control group.

the control group. Vascular endothelial growth factor protein expression increased significantly at 1 hr after hypoxic exposure and reached summit at 3 hrs ($P < 0.05$), at 6 hrs, it decended and there was no significant difference between 6 hrs group and control ($P > 0.05$), then declined to the level lower than control at 12 and 24 hrs ($P < 0.05$) (Fig. 4B). The accumulation of VEGF protein in ECs supernatants treated by hypoxia was also measured. Data showed that VEGF secretion was significantly increased by hypoxia at all time points. ($P < 0.05$), and reached the summit at 6 hrs, with the concentration of 255.29 pg/10^5cells/ml (Fig. 4C).

Aquaporin-1 mRNA level was transiently increased about 6.4 folds and peaked at 3 hrs by 10.26 folds. After 6 hrs, it decreased and remained higher than the control ($P < 0.05$) (Fig. 4D). AQP1 protein expression was significantly up-regulated by hypoxia in short periods (1, 3, 6 hrs, $P < 0.05$), and declined to the level lower than the control at 12 and 24 hrs, while the differences were not significant ($P < 0.05$) (Fig. 4E).

Hypoxia up-regulates COX-2 mRNA level, protein expression and promotes PGE_2 release in short periods

In order to better understand the effect of hypoxia on the function of HUVECs, we carried out real-time quantitative PCR and Western blot experiments to measure COX-2 mRNA level and protein expression of HUVECs with different periods of hypoxic exposure. The results showed that COX-2 mRNA was at very low levels in unstimulated cells, early minutely induced by 1.35 folds at 1 hr after hypoxic treatment, and reached top at 3 hrs by 2.67 folds, then afterwards declined to level below 2 folds and remained higher than the untreated control group ($P < 0.05$) (Fig. 5A). Similarly, COX-2 protein expression also increased significantly at 1 hr and maximized by 1.34 folds at 3 hrs, thereafter declined to levels significantly lower than control ($P < 0.05$) (Fig. 5B).

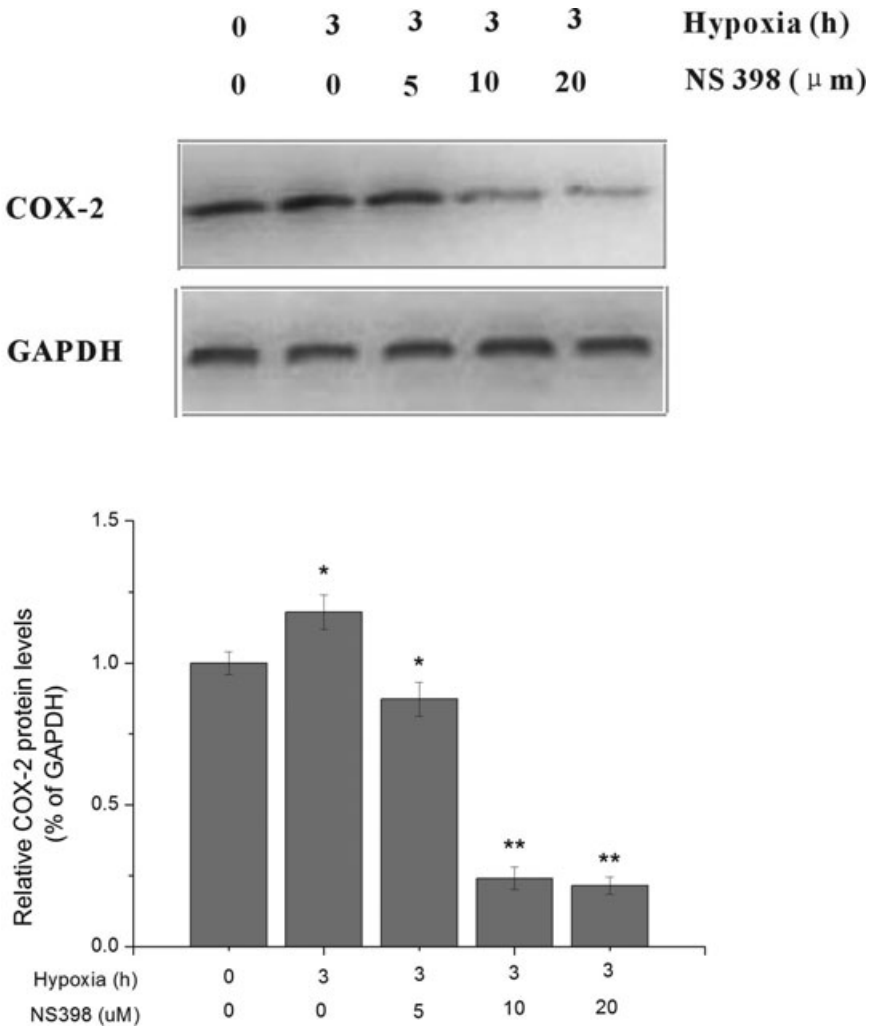


Fig. 6 Effect of the selective COX-2 inhibitor NS398 on COX-2 activity by concentration gradient test. HUVECs were exposed to hypoxia and supplemented with NS398 at indicated concentrations of 0, 5, 10 and 20 μM. Cells without hypoxia or NS398 treatment served as control. The COX-2 activity was determined by Western blot analysis. $*P < 0.05$, $**P < 0.01$ *versus* control.

Having proved that hypoxia regulates COX-2 at transcription and expression level, we further verified whether it induces COX-2 expression by the production of prostaglandin, determined as PGE_2 accumulation in the supernatants of hypoxia treated ECs by ELISA assay. The results indicated that hypoxia stimulated a significant short-term increase in PGE_2 release that peaked at 3 hrs, with a concentration of 137.83 ± 11.66 pg/10^5 cells/ml. Then the amount of PGE_2 in supernatants went down, possibly following the pattern of COX-2 expression. Late PGE_2 release was decreased, making the quantity of it accumulated in the culture medium significantly lower than the control ($P < 0.05$), which was consistent with the progression pattern of COX-2 protein expression (Fig. 5C).

Concentration-dependent effect of NS398 on COX-2 expression

The data show that NS398 regulated COX-2 expression in a concentration-dependent manner (Fig. 6). When NS398 was used at 5 μM, COX-2 protein expression was decreased to level about that of control. And when NS398 was used at concentrations of 10 and 20 μM, COX-2 protein expression was decreased to 24.1% and 21.5% of control group. This indicated that 10 μM of NS398 was more effective to inhibit COX-2 activity than 5 μM, but had similar effects to 20 μM under current experimental conditions. To avoid inflicting excessive injury to the cells and unwanted effects, we selected 10 μM as final concentration in the following pharmacological inhibition study.

Selectively inhibition of COX-2 activity by NS398 impaires the biological response of HUVECs to hypoxia

In order to verify whether the endogenous COX-2/PGE_2 signalling contributes to the biological response of ECs to hypoxia, we pretreated cell cultures with 10 μM of COX-2-selective inhibitor, NS398 for 4 hrs prior to indicate periods of hypoxic treatment.

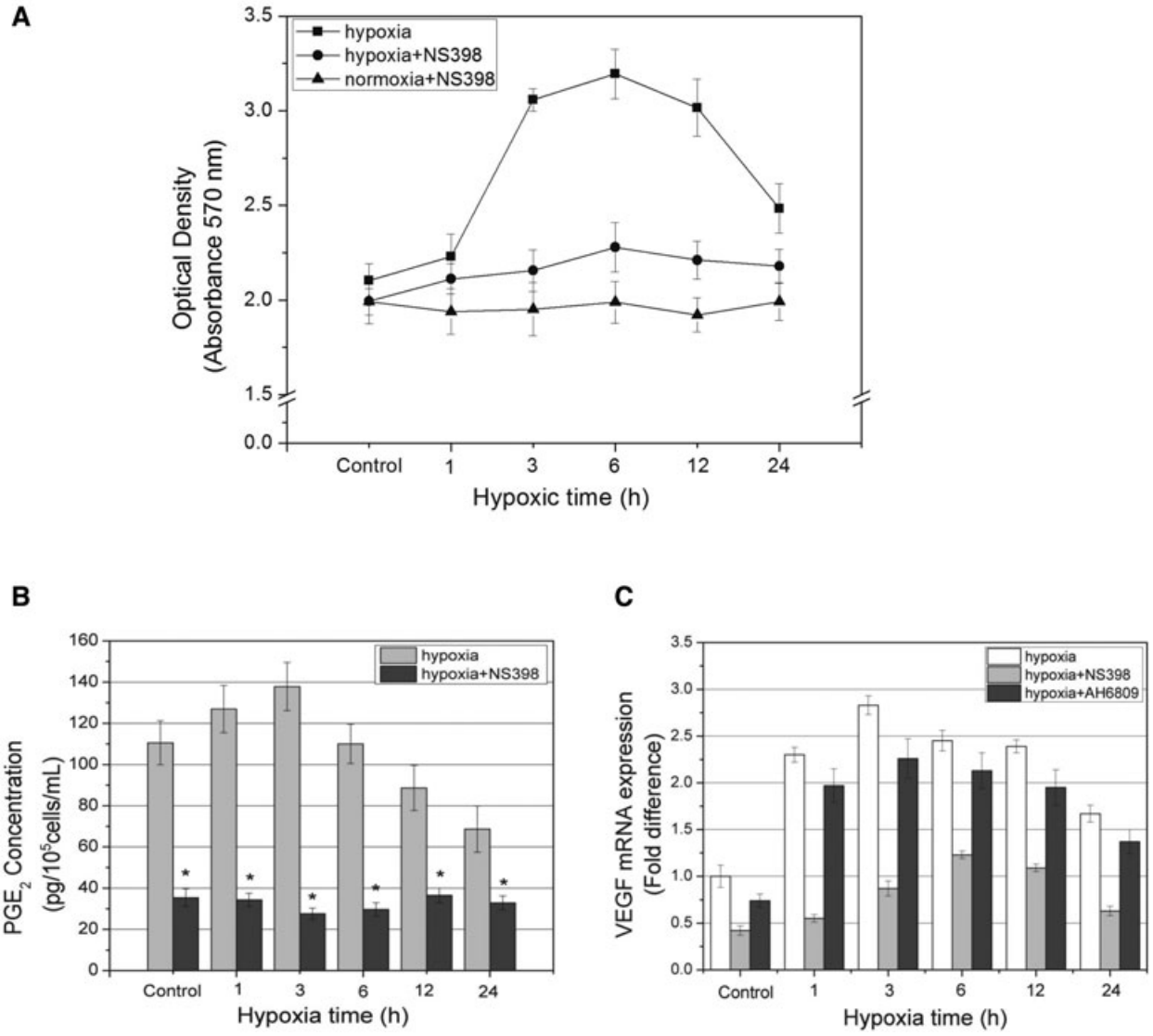


Fig. 7 Selectively inhibition of COX-2 activity by NS398 and blockade of EP1/2 by AH6809 impair the biological response of HUVECs to hypoxia. (**A**) HUVECs proliferation and viability following normoxic, hypoxic or hypoxic + NS398 treatment by MTT assay. (**B**) Quantification of PGE_2 release by ELISA assay in supernatant of HUVECs after various periods of hypoxic treatment with or without 10 μM NS398. (**C**) VEGF mRNA level assessed by real-time RT-PCR experiment, HUVECs were exposed to hypoxia in the presence or absence of NS398 (10 μM) for indicated periods of time. (**D**) VEGF concentration in supernatant of HUVECs measured by ELISA assay. (**E**) AQP1 mRNA level assessed by real-time RT-PCR experiment. * $P < 0.05$ *versus* control.

First, MTT assay was employed to examine whether NS398 has any effects on HUVECs proliferation. We found that NS398 had an inhibitory effect on HUVECs proliferation under normoxic culture conditions, and the increased proliferation of HUVECs by temporary hypoxia exposure was completely blocked by NS398 ($P < 0.05$) (Fig. 7A). Moreover, the difference between the cell viability of NS398 + hypoxia-treated group and the hypoxia-treated group was small especially at earlier hypoxic time points of 1 hr ($P > 0.05$), with prolongation of hypoxic time (3, 6, 12 and 24 hrs), the cell viability of NS398-treated groups significantly decreased compared to the untreated groups.

To find out whether NS398 attenuated prostanoid production induced by hypoxia, supernatants were collected and PGE_2 concentrations were measured. Results showed that PGE_2 synthesis and release were strongly inhibited by NS398 in hypoxia-treated cultures ($P < 0.05$) (Fig. 7B). NS398 at the concentration of

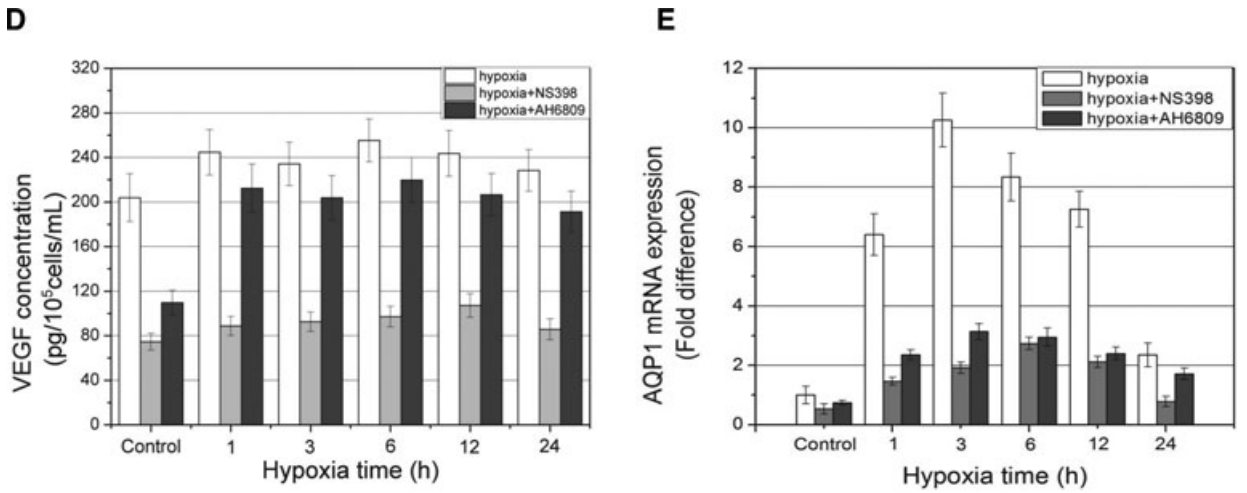


Fig. 7 Continued

10 μM completely abolished the inductive effect of PGE_2 production by hypoxia, suggesting that the enhancing effect of hypoxia on PGE_2 release may be COX-2-dependent.

In order to study the effect of COX-2/PGE_2 signalling on angiogenic capability of HUVECs, we examined the effects of NS398 on HUVECs VEGF mRNA transcription, secretion and AQP1 mRNA level. The findings demonstrated that VEGF mRNA transcription was obviously blocked by NS398 ($P < 0.05$), and its transcription levels gradually rose to a summit (6 hrs) before declination ($P < 0.05$) (Fig. 7C), and VEGF release was also significantly inhibited ($P < 0.05$), indicating that NS398 abrogated the inductive effect of hypoxia on VEGF synthesis and secretion (Fig. 7D). Moreover, NS398 significantly down-regulated AQP1 mRNA transcription under normoxic or hypoxic condition ($P < 0.05$) (Fig. 7E).

Combined blockade of EP1/2 by AH6809 attenuates the biological response of HUVECs to hypoxia

Then, we proceeded to evaluate the effects of AH6809 (combined EP1/2 antogonist) on HUVECs VEGF mRNA transcription, secretion and AQP1 mRNA level to confirm whether PGE_2 receptor EP1/2 is involved in pro-angiogenic reaction of ECs to hypoxia. The data demonstrated that VEGF mRNA transcription and secretion were obviously blocked by AH6809 ($P < 0.05$), but the inhibitory effect was much smaller than that of NS398 ($P < 0.05$) and hypoxia stimuli could still promote VEGF mRNA transcription and secretion (Fig. 7C and D). In addition, AH6809 significantly decreased AQP1 mRNA transcription to a value which was still higher than that of NS398-treated group under normoxia or hypoxia ($P < 0.05$) (Fig. 7E).

Exogenous PGE_2 enhances and partially reversed the inhibitory effects of NS398 on COX-2 expression, cell viability and VEGF, AQP1 mRNA transcription of HUVECs

From the above mentioned results, we could find that the inhibitory effects of COX-2 inhibitor NS398 on VEGF, AQP1 transcription and VEGF release were stronger than that of combined EP1/2 antagonist AH6809. To better understand the effect of PGE_2 as a COX-2 downstream effector, we examined the combinational treatment of NS398 (10 μM) and exogenous PGE_2 (10 μM) on COX-2 expression, comparing with that of NS398 or exogenous PGE_2 treatment alone. Specifically, HUVECs were treated with 10 μM of NS398 and/or 10 μM of exogenous PGE_2 under normoxia for 4 hrs. Results demonstrated that 10 μM of NS398 significantly blocked COX-2 expression, similar to the situation observed in hypoxic condition, while exogenous PGE_2 prominently augments the protein expression of COX-2. Interestingly, when treated together with NS398, exogenous PGE_2 partially reversed the inhibitory effects of NS398 on COX-2 expression of HUVECs (Fig. 8).

For cell viability, the MTT value of HUVECs under hypoxia was significantly higher than that under normoxia. NS398 exerted a significantly inhibitory effect on HUVECs proliferation both under hypoxia and normoxia, while exogenous PGE_2 stimulated them, and combinational treatment of NS398 and PGE_2 resulted in a value between the NS398 and PGE_2, with hypoxic group significantly higher than the normoxic group ($P < 0.05$) (Fig. 9A). Similarly, for VEGF and AQP1 mRNA transcription, NS398 alone also significantly blocked while exogenous PGE_2 significantly augmented them, and exogenous PGE_2 (10 μM) partially reversed the inhibitory effect of NS398 (Fig. 9B and C).

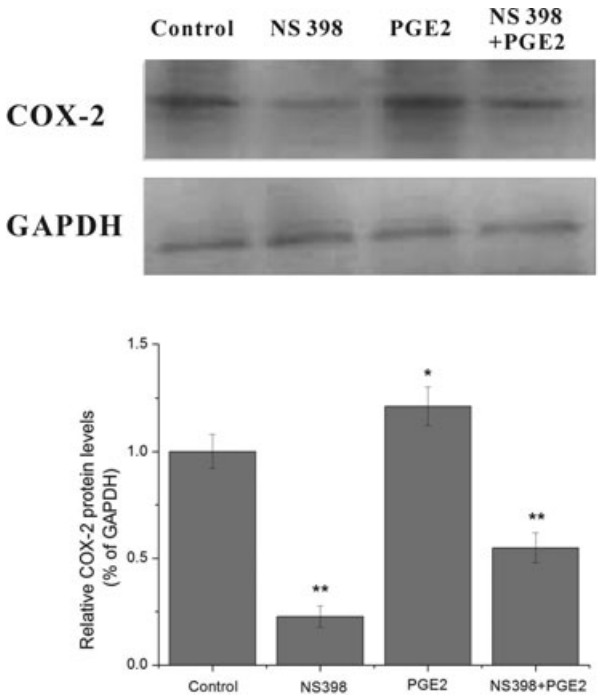


Fig. 8 Effect of NS398, exogenous PGE_2 and combinational treatment of NS398 and exogenous PGE_2 on COX-2 protein expression of HUVECs under normoxia. HUVECs were treated with 10 μM of NS398 and/or 10 μM of exogenous PGE_2 under normoxia for 3 hrs. Cells without NS398 or exogenous PGE_2 treatment served as control. The COX-2 protein expression was determined by Western blot analysis. $*P < 0.05$ and $**P < 0.01$ *versus* control.

Discussion

The endothelium plays a predominant role in modulating many aspects of vascular homeostasis. When facing a stimulus like hypoxia, this barrier is able to orchestrate a protective cellular response, including angiogenesis, cell proliferation and apoptosis [37, 38]. In the present study, we explored how hypoxia exerts its angiogenic activities on ECs by measuring HUVECs viability, migration, tube formation and angiogenesis related genes/protein (VEGF, AQP1) expression. Vascular endothelial growth factor is a potent angiogenic stimulator with the ability to promote ECs growth [39], enhance ECs survival [40] and induce vasodilatation [41]. Aquaporin-1 is abundantly presented in endothelia of non-fenestrated capillaries [42] and maybe responsible for the vascular hyper-permeability and increases in the hydrostatic interstitial pressure of tissue [43]. Study showed that high degree of AQP1 expression was preferentially associated with enhanced angiogenesis and ECs migration [44, 45]. It has been reported that up-regulation of AQP1 by hypoxia may worked as an O_2 transporter in ECs and accelerate intracellular hypoxia directly and induce pro-angiogenic molecules in response to hypoxia, thereby leading to hypoxia inducible angiogenesis [46]. In the present

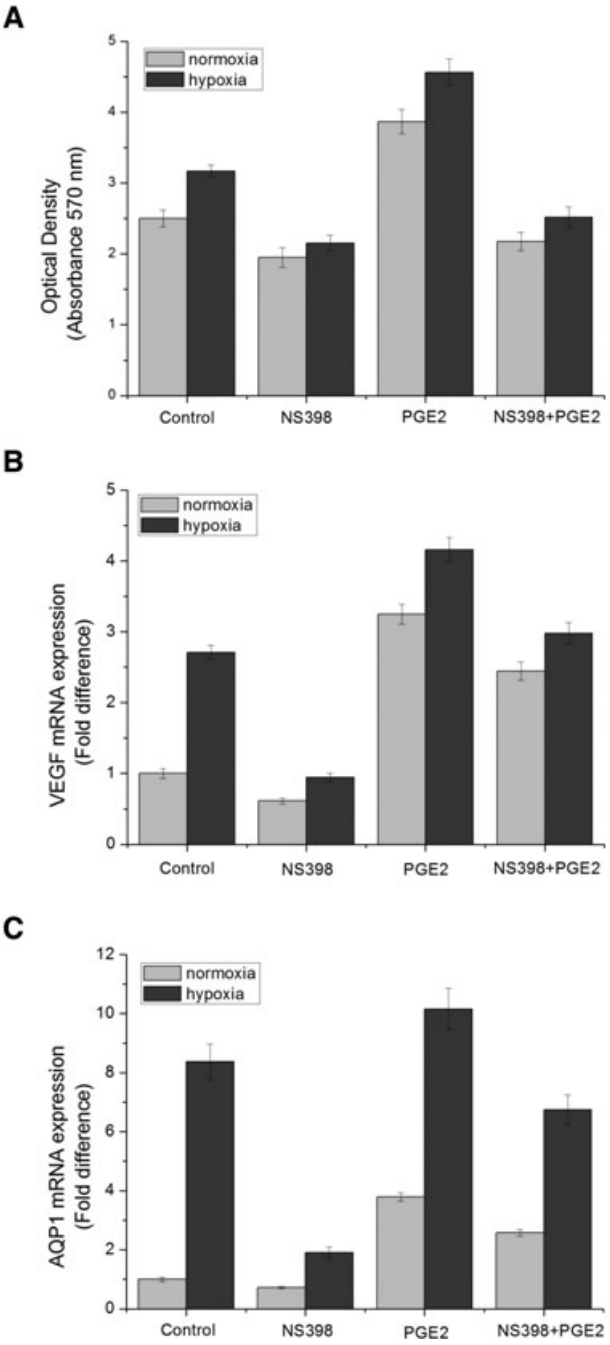


Fig. 9 Effect of NS398, exogenous PGE_2 and combinational treatment of NS398 and exogenous PGE_2 on cell viability and VEGF, AQP1 mRNA transcription of HUVECs under normoxia or hypoxia. HUVECs were treated with 10 μM of NS398 and/or 10 μM of exogenous PGE_2 under normoxia or hypoxia for 3 hrs. Cells without NS398 or exogenous PGE_2 treatment served as control. (**A**) HUVECs proliferation and viability was measured by MTT assay. (**B**) VEGF mRNA level assessed by real-time RT-PCR experiment. (**C**) AQP1 mRNA level assessed by real-time RT-PCR experiment. $*P < 0.05$ and $**P < 0.01$ *versus* control.

study, we demonstrated specifically that short-term hypoxic treatment: (i) enhanced HUVECs proliferation, motility, tube formation capability; (ii) increased VEGF mRNA level, protein expression and promoted VEGF secretion; (iii) augmented AQP1 mRNA transcription and protein expression. The findings have suggested that temporary hypoxia may promote ECs proliferation and migration, facilitate the angiogenic capability of ECs, and high expression of AQP1 may be associated with the pro-inflammatory phenotype. The adaptive responses of ECs to hypoxia could increase regional blood vessel number, collateral circulation formation and vascular permeability, thus ameliorate local hypoxic microenvironment in ischaemic and remodelling tissue.

Understanding of the pathways involved in ECs hypoxic response and identifying strategies to activate or inhibit this process in tissue remodelling promotion, ischaemic diseases and cancer therapy would have important clinical implications. The important master regulator of angiogenesis under hypoxic conditions, hypoxia-inducible factor-1 (HIF-1), is a dimeric transcription factor composed of HIF-1α and HIF-1β [47]. It promotes the synthesis of protein that increases the cellular supply with O_2 and initiates the defence against hypoxia at different levels [48]. Various traditional studies have focused their efforts on unravelling the importance of HIF-1α pathway in tumour progression and angiogenesis, as well as in the adaptive response of ECs to low oxygen availability [49]. Recent data [50, 51] also suggest that ECs are highly specialized in acquiring a pro-inflammatory phenotype when encountering stressors. They cope with hypoxia by expressing a number of inflammation-related genes, which are mediated by a variety of signalling cascades [52, 53]. However, the reports from the perspective of inflammatory mediators on the relationship between angiogenesis and hypoxia are rare.

Numerous molecules have been implicated in ECs proliferation and angiogenesis. COX-2/PGE_2 signalling may be a key regulator of angiogenesis and mediate angiogenesis through multiple mechanisms, including an increase in VEGF transcription and expression [54, 55]. Previous investigations validated that amplification of the COX-2/PGE_2 pathway in hypoxic colorectal cancer cells might have important implications for stimulating angiogenesis [56]. There is now ample evidence that COX-2 is involved in controlling cell growth and implicated as a key regulator of angiogenesis. The hypoxia-induced COX-2 activation may augment PGE_2 release, resulting in either an autocrine or paracrine action that enhances expression of VEGF [57, 58]. Stimulation of VEGF production by PGE_2 has also been demonstrated in previous studies in various cellular systems [59–61]. Our data documented that short-term hypoxia significantly up-regulated COX-2 mRNA transcription, protein expression and promoted PGE_2 release (the production of secreted PGE_2 is an appropriate measure of COX activity). Accompanied with COX-2 activation and PGE_2 secretion, the VEGF and AQP1 expression also increased. Hence, based on the data herein, we demonstrated a potential parallel in the activation way of COX-2 and VEGF to hypoxic stress, and elucidated a positive correlation between COX-2 and VEGF expressions in angiogenesis. The present data also indicated that hypoxia signalling may transfer to COX-2/PGE_2 and VEGF signalling, playing cooperative role in the angiogenic action of ECs. Furthermore, we could deduce a possible parallel relationship between inflammation and angiogenesis under hypoxia from the results above, and they might have a positive interaction with each other when faced with hypoxia.

To further study the function of COX-2/PGE_2 in the biological response of ECs to hypoxia, we used NS398 (a selective COX-2 inhibitor to inhibit the activity of COX-2) and AH6809 (a combined antogonist of EP1/2) to pre-treat HUVECs prior to indicated periods of hypoxic exposure. Overexpression of COX-2 has been described among various human malignancies [62–64] and blood vessels in pathological conditions [65, 66], suggesting a critical role of COX-2 in tumorigenesis and inflammation. By contrast, selective pharmacological inhibition or genetic depletion of COX-2, which abolishes the eicosanoid synthesis in stimulated ECs, may abrogate an important homeostatic response to hypoxic stress, leading to inhibition of angiogenesis and induction of apoptosis [67]. Previous study has demonstrated that inhibition of COX-2 activity and thus resulting in significantly down regulation of VEGF expression and angiogenesis inhibition *in vivo* [68]. The present study demonstrated that exposure of HUVECs to NS398: (i) impaired proliferative response to hypoxia; (ii) strongly inhibited PGE_2 release; (iii) attenuated VEGF mRNA and AQP1 mRNA transcription, and diminished VEGF secretion. Furthermore, AH6809 modestly abrogated the expression of hypoxia-induced angiogenesis related factors (VEGF and AQP1). Comparing to NS398, the blockade of the PGE_2 receptors EP1/2 with a selective antagonist AH6809 received a much smaller effect on angiogenic capability of HUVECs under hypoxia. These results indicate that COX-2 induction ought to be a critical determinant of the angiogenic response of ECs to hypoxia and hypoxia probably exerts its effects in a COX-2-dependent manner. Moreover, the function of EP1/2 is not as crucial as that of COX-2 in the angiogenic capability of HUVECs, but a study has reported that the EP1/2 receptor contributed to growth factor-mediated tubular formation in angiogenesis [69].

To better understand the role of PGE_2 in angiogenic response of ECs to hypoxia, we compared NS398, exogenous PGE_2 and the combinational treatment of NS398 (10 μM) and exogenous PGE_2 (10 μM) on cell viability, COX-2 expression and VEGF, AQP1 mRNA level. The results indicated that PGE_2 augmented cell viability and angiogenic capability of HUVECs, and it could partially reversed the inhibitory effect of NS398. From the findings that inhibition of COX-2 activity resulted in attenuated PGE_2 secretion, and exogenous PGE_2 enhanced COX-2 expression, we described a positive feedback loop of COX-2-PGE_2-COX-2. This was consistent with the over-production of PGE_2 observed in COX-1 and COX-2 knockout cell lines, and also supported by previous studies using human synovial fibroblasts, prostatic carcinoma cell line (PC-3) and mouse lung fibroblast cells [70–72]. We speculated that PGE_2 may lead to increased cell viability and angiogenic capability of HUVECs through its own action and/or the signal amplification of COX-2.

To sum up, the aforementioned results indicated that COX-2 products produced endogenously may serve to facilitate hypoxia mediated cell proliferation and angiogenic capability, and the involvement of endogenously produced prostanoids in promoting ECs proliferation and angiogenic capability occurs dependently of changes in COX-2 activity, and exogenous PGE_2 augments the angiogenic effect of hypoxia on HUVECs. Therefore, the hypoxia-induced functional responses and expression of VEGF and AQP1 in ECs is dependent of PGE_2 mediated by COX-2 activation. In conclusion, (i) hypoxia could significantly enhance the angiogenic capability of HUVECs; (ii) COX-2 may be a potential therapeutic target for ischaemic disorder and cancer management, and also the critical factor in tissue remodelling as the COX-2/PGE_2 pathway influences most, if not all, of the hallmarks of these events; (iii) inflammation and angiogenesis maybe two respects of one response of HUVECs to hypoxic exposure, and they might have a positive interaction with each other.

Acknowledgements

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