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ORIGINAL PAPER

Ethyl 3,4-dihydroxybenzoate (EDHB): a prolyl hydroxylase inhibitor attenuates acute hypobaric hypoxia mediated vascular leakage in brain

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Abstract Sudden exposure to altitude hypoxia is responsible for acute mountain sickness (AMS) in un-acclimatized persons. If not treated in time, AMS can worsen and leads to high altitude cerebral edema, which can be fatal. Present study explores the efficacy of ethyl 3,4-dihydroxybenzoate (EDHB), a prolyl hydroxylase enzyme inhibitor, in modulating adaptive responses to hypobaric hypoxia (HH) in rat brain. Male Sprague–Dawley rats treated with EDHB (75 mg/kg for 3 days), were subjected to acute HH exposure at 9144 m (30,000 ft) for 5 h. Animals were assessed for transvascular leakage and edema formation in brain and role of key inflammatory markers along with hypoxia responsive genes. HH stress increased transvascular permeability and edema formation in conjunction with upregulation of nuclear factor-κB (NF-κB) and its regulated proteins. There was surge in pro-inflammatory cytokines tumor necrosis factor-α, interleukin-6, interferon-γ, monocyte chemoattractant protein-1 and decrement in anti-inflammatory cytokine interleukin-10. Further, upregulation of vascular endothelial growth factor (VEGF), a vascular permeability marker and down-regulation of antioxidant and anti-inflammatory proteins hemoxygenase (HO-1) and metallothionein (MT-1) was also observed under hypoxia. EDHB supplementation effectively scaled down HH induced cerebral edema with concomitant downregulation of brain NF-κB expression. There was significant curtailment of pro-inflammatory cytokines and cell adhesion molecules. There was significant downregulation of permeability factor VEGF by EDHB with concomitant increment in hypoxia inducible factor (HIF1α) and anti-inflammatory proteins HO-1 and MT-1 compared to HH control thus accentuating the potential of EDHB as effective hypoxic preconditioning agent in ameliorating HH mediated injury in brain.

Keywords High altitude cerebral edema · EDHB · Preconditioning · PHD inhibitor · Inflammation · Vascular leakage

Introduction

According to WHO estimate, more than 35 million people including soldiers, sojourners and pilgrims travel to high altitude areas for recreational/military activities [1]. Hypobaric hypoxia or reduction in partial pressure of oxygen with altitude presents a significant challenge to individuals residing at that altitude and those who travel to high altitude locations and can have serious patho-physiological effects on human health. There is decrease in physical and mental performance which sometimes precipitates into high altitude illness viz: AMS, HAPE, high altitude cerebral edema (HACE) that develop in unacclimatized persons shortly after ascent to high altitude [2, 3]. Symptoms encountered by ascendants to high altitude include headache, anorexia, nausea, vomiting, fatigue, dizziness and sleep disturbance. These symptoms of mountain sickness may develop typically within 24 h of exposure but may be evident within first few hours of hypoxia exposure [4, 5]. HACE develops as a progression of AMS and remains a major problem due to lack of effective treatment [6, 7]. HACE is associated with osmotic

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cell swelling, vasogenic edema and alterations in structure and function of blood–brain barrier (BBB) [3]. BBB is physical barrier formed by the endothelial tight junctions and the transport barrier resulting from membrane transporters and vesicular mechanisms. It is a highly complex structure, separating the extracellular fluid of the central nervous system (CNS) from the blood of CNS vessels. Dysfunction of this barrier results in increased permeability, leading to extravasation of plasma constituents and vasogenic brain edema [7]. VEGF increases capillary permeability and stimulates vascular endothelial cell proliferation to promote angiogenesis and vascular permeability [8], therefore, it plays a special role in pathogenesis of many diseases including HACE. Earlier reports suggest the role of cytokine activation in damaging barrier function as well as vascular injury leading to fluid accumulation in brain [9].

Oxygen inhalation, immediate descent or bed rest is beneficial but when these facilities are not available, several deaths continue to occur. These high altitude maladies thus pose a public health problem and have severe economic consequences. Gradual ascent, allowing time for acclimatization, can reduce high altitude illness [10]. However, acclimatization is a slow process, taking place over a period of days to week. Therefore, the best way to acclimatize the humans to high altitude hypoxia is to induce necessary physiological and genetic changes in the body before they are inducted to high altitude and this can be achieved by hypoxia pre-conditioning. Preconditioning is a process by which a tissue is rendered more tolerant to a subsequent lethal insult such as hypoxia/ischemia resulting in intracellular adaptation and enhanced endogenous defense mechanism [11, 12]. Hypoxia inducible factors (HIFs) have been reported to play a central role in this adaptation process coordinated by HIF-prolyl hydroxylase domain containing enzymes (PHDs) including increased ventilation, erythropoiesis via erythropoietin (EPO) and neo-vascularization via VEGF to carry more oxygen, improved vascular tone (NOS), glycolysis [13–15]. HIF is a redox sensitive protein that binds to hypoxia responsive element in different hypoxia responsive genes, thus activating their transcription.

In cells under normoxia, HIF-1α is hydroxylated at prolyl 402 and/or 564 residues by HIF-prolyl hydroxylase Domain containing enzyme (PHDs). This hydroxylated HIF-1α is recognized by Von-Hippel–Lindau (VHL) protein of E3 ubiquitin ligase complex and is very rapidly degraded via ubiquitination/proteosomal degradation. PHDs are non-heme iron containing 2-oxoglutarate—dependent di-oxygenases which catalyze the incorporation of O_2 molecule into organic substrate. To be functionally active, PHDs require oxygen besides 2-oxoglutarate (2OG), iron (Fe^{2+}) and ascorbate, thus function as oxygen sensors [16, 17]. Under low oxygen availability, decreased activity of PHDs leads to accumulation of HIF-1α, which migrates from cytoplasm to nucleus, dimerizes with HIF-1β to form active transcription factor HIF and initiating transcription of downstream target genes, thus modulating the adaptive cellular response [18].

The inhibition or blocking of proteosomal degradation of HIF could enhance its activity and expression during normoxia and is likely to mimic, at least in part, the effects of hypoxia preconditioning [19], thus allowing rapid recovery from hypoxia, underscoring the modulation of both PHDs and HIF-1α as promising therapeutic targets. Prolyl hydroxylase inhibitors (PHI) interfere with PHD activity either non-selectively by replacing their essential co-substrates (iron and 2OG) or directly blocking the enzymes' catalytic site. The PHI EDHB, also known as protocetachuic acid ethyl ester/ethyl protocatachuate is a substrate analog of 2-oxoglutarate and competitive inhibitor of PHDs [20]. Beside its antioxidant and anti-inflammatory properties it is known to possess apoptotic and antitumor activity [21–25]. These multifold responses provided the rationale for the use of PHD inhibitor EDHB in stabilizing HIF under normoxia and to determine its preconditioning efficacy in attenuation of HH. Our earlier studies have shown cytoprotective efficacy of EDHB against hypoxia mediated oxidative damage in L6 myoblast cells [26]. We have also reported amelioration of exercise induced damages and improvement of physical performance by EDHB in Sprague–Dawley rats [27]. In continuation of our studies, here we have investigated the potential of EDHB in attenuating hypoxia induced cerebral injury.

Materials and methods

Materials

All chemicals were purchased from Sigma (St. Louis, USA) and SRL (Mumbai, India). Antibodies and ELISA kits were purchased from Santa Cruz Biotech (CA, USA) and BD Biosciences (USA).

Animals

Male Sprague–Dawley rats (180 ± 20 g) were used for the study. Animals were kept in cleaned cages with autoclaved husk in institute's animal house, maintained at temperature of 24 ± 2 °C with 12-h light–dark cycle and free access to food and water. All the animal procedures and experimental protocols were approved by Institution's Animal Ethical Committee and were in compliance with Committee for the Purpose of Control and Supervision of Experiments on Animals, India (CPCSEA).

Effective dose of EDHB and hypoxia tolerance

The assessment of effective dose of EDHB was based on hypoxia tolerance of rats which was determined by measuring hypoxia gasping time (HGT) and hypoxia survival time (HST) [28]. Different groups of rats ($n = 8$/group) were supplemented with varying doses of EDHB (50, 75, 100 mg/kg b.wt. dissolved in DMSO and diluted with sterile 0.9 % saline) i.p. for 3 and 5 days. Animals were exposed to an altitude of 9754 m (32,000 ft) one at a time in Animal decompression chamber (Seven Star instruments, India) at 32 °C. Time taken for appearance of first sign of gasp and survival time were recorded. On the basis of hypoxia tolerance, optimal dose of EDHB for hypoxia pre-conditioning was found to be 75 mg/kg b.wt. for 3 days and was used for all subsequent experiments.

Preconditioning with EDHB and hypoxia exposure

The whole experiment was designed to carryout to cover the two main aspects; firstly to study the extent of edema index and vascular leakage in brain under hypoxia and compare it to EDHB supplemented group. Secondly we wanted to access the role of inflammatory markers in vascular leakage and its amelioration by EDHB. Animals ($n = 8$/group) were divided into four groups (1) control group under normoxia C, (2) control exposed to hypoxia H, (3) EDHB supplemented group (75 mg/kg b.wt. EDHB for 3 days) under normoxia D, (4) EDHB supplemented group followed by exposure to hypoxia HD. Groups 2 (H) and 4 (HD) were exposed to an altitude of 9144 m for 5 h in a decompression chamber at 24 °C (similar to the temperature at which animals were housed in animal house) with an ascent rate of 300 m/min. Air flow was 2 L/min with relative humidity maintained at 50–55 %.

Determination of cerebral edema

Cerebral edema was determined by measuring edema index and vascular permeability in control and EDHB supplemented rats with/without hypoxia.

Edema index

Tissue wet/dry weight ratio was used as a measure of edema index or brain water content. After hypoxic exposure, animals were sacrificed under anesthesia ketamine/xylaxine (80:20 mg/kg b.wt.) mixture and brain was excised after perfusion with PBS (pH 7.4), blot dried and placed on empty pre-weighed glass plates. The wet weight of tissue was noted down immediately and the tissue was then dried in hot air oven at 55 °C for 72 h to obtain the constant dry weight.

Vascular permeability

The vascular permeability assay was performed using technique of Heike et al. (2001) with minor modifications using sodium fluorescein (Sigma Inc., USA) dye extravasation from tissue as an indicator of leakage [29]. Animals were taken out after 4 h 30 min of hypoxic exposure and injected with Sodium fluorescein (15 mg/kg b.wt.) dye intravenously through tail vain and again exposed to hypoxia for 30 min to complete 5 h of hypoxic exposure. Rats were sacrificed after hypoxic exposure under anesthesia and heart was perfused with PBS (pH 7.4) to remove fluorescein tracer and blood from vascular bed. The brain tissue was excised, washed with cold PBS and was kept in 3 % formamide for about 16–18 h in dark at room temperature. Later, the fluorescence in the formamide solution was measured using a spectrophotometer FLUOstar Omega (BMG Labtech, Germany) with excitation and emission at 485 and 530 nm. Results are presented as relative fluorescence units (rfu)/mg dry weight.

Determination of hematocrit, hemoglobin, EPO levels

To investigate the effect of EDHB on hematocrit (Hct), hemoglobin (Hb) and circulatory EPO levels, blood was collected by renal portal vein from control and EDHB supplemented groups under normoxia (75 mg/kg b.wt., 3 days). Hct and Hb content was measured using MS4 hematology analyzer (Melet Schloesing, France). Circulatory EPO was estimated in plasma using commercially available ELISA kit (R&D systems, USA) according to manufacturers' instructions.

Inflammatory markers by enzyme linked immunosorbent assay (ELISA)

After hypoxic exposure, animals were sacrificed, perfused with PBS (pH 7.4), brain was dissected out and carefully crushed in liquid nitrogen under sterile conditions. Powdered tissue thus obtained was aliquoted in 3 parts and stored at −80 °C. One part of powdered tissue was homogenized in cold buffer [PBS pH 7.4, 0.1 mM dithiothreitol (DTT), 100 ug/ml phenylmethylsulphonyl fluoride (PMSF)] fortified with 10 ul/ml protease inhibitor cocktail (Sigma Co., USA) to obtain 10 % homogenate (w/v) using hand homogenizer (Kinematica, Switzerland). Homogenate prepared was centrifuged at 12,000×*g* for 20 min to remove tissue/cell debris and supernatant obtained was used to determine cytokines. Levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-10 (IL-10), interferon-γ (IFN-γ), monocyte chemoattractant protein-1 (MCP-1) and TGF-β were

quantified using ELISA kits (BD Biosciences, USA) according to the manufacturer's protocol. Samples readings were measured in duplicates. Absorbance was read at 450 nm using spectrophotometer FLUOstar Omega (BMG Labtech, Germany).

Protein expression studies

Preparation of nuclear and cytosolic extracts

Nuclear and cytosolic extracts were prepared according to protocol mentioned by Basheer et al. [30]. Briefly, frozen powdered brain tissue as mentioned above was homogenized in ice cold buffer A (0.5 M sucrose, 10 mM HEPES, pH 7.9, 10 mM KCL, 1.5 mM MgCl2, 10 % Glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) fortified with protease inhibitor cocktail. Homogenate was kept on ice for 15 min and 0.6 % nonidet P-40 was added, centrifuged for 10 min at 2000×*g* at 4 °C. The supernatant with cytosolic fraction was collected and aliquots were stored. The pellet was dissolved in cold buffer B (20 mM HEPES, pH 7.9, 0.3 mM NaCl, 1.5 mM $MgCl_2$, 20 % Glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) and cocktail of protease inhibitors for nuclear fraction. It was allowed to incubate in ice for 30 min followed by centrifugation at 20,000×*g* for 15 min at 4 °C. The supernatant containing the nuclear fraction was aliquoted and stored at −80 °C for further analysis.

Western blotting

Protein expression of VEGF, ICAM-1, VCAM-1, P-selectin, HO-1 and MT-1 were quantified in cytosolic extract, whereas HIF-1α and NF-κB were analyzed in the nuclear extract by western blotting. Protein level in brain was estimated by Lowry method (Lowry et al. 1951). 50 µg of homogenate extract was subjected to 10 % SDS-PAGE and electro-blotted on nitrocellulose membrane (Millipore, USA). Membranes were then blocked with bovine albumin serum (3 % BSA in PBS), washed with Tris Buffer Saline with 0.1 % Tween-20 (TBST); and probed with respective mouse/rabbit/goat polyclonal primary antibodies of HIF-1α, PHD2, ICAM-1, VCAM-1, P-selectin, VEGF, HO-1, MT-1 and NF-κB and were incubated for 3 h at room temperature. After incubation with primary antibody, the membranes were washed 3–4 times with TBST and incubated with antimouse/antirabbit/antigoat—IgG-HRP conjugate (1:30,000 dilutions) for 1 h. Later the membranes were washed thoroughly with TBST, incubated with chemiluminescent peroxide substrate (Sigma) in dark and bands were visualized on X-ray film (Fujifilm, USA). Densitometry of bands was done by using Gel Doc system (UVP, Bioimaging Systems, USA).

Statistical analysis

All data are expressed as mean ± SD. For comparison between 2 groups, statistical significance was determined through an unpaired Student *t* test. For comparison among multiple groups, statistical significance was evaluated with two-way analysis of variance (ANOVA) for dose response studies and one way ANOVA for rest of the experiments followed by post hoc Bonferroni analysis using SPSS 16.0 for Windows. A minimum probability value of $p < 0.05$ was considered significant.

Results

EDHB supplementation boosts hypoxic tolerance

To investigate whether EDHB supplementation has effect on hypoxic tolerance, animals were subjected to hypobaric hypoxia (9754 m) in hypoxic decompression chamber. Effect of different concentrations of EDHB (for 3 and 5 days) on hypoxic tolerance was studied (Fig. 1). Control animals started gasping within 16–17 min after exposure to hypoxia and died after 2–3 min of onset of gasping. The average HST observed was 20 min. In the animals supplemented with 50 mg EDHB/kg b.wt. for 3 days, there was 1.5-fold increase in HST (32 ± 4 min) as compared to control which was further enhanced significantly (7.5-fold) when EDHB dose was increased to 75 mg/kg b.wt. for 3 days (158 ± 14 min, $p < 0.01$). No further boost in hypoxia gasping time (HGT) or HST was observed on increasing the concentration of EDHB to 100 mg/kg b.wt. for 3 days. Time dependent EDHB supplementation studies for 3 and 5 days showed optimum time to be 3 days at all the doses studied. There was no supplementary increase in HGT/HST on increasing the duration to 5 days. Thus the optimum dose of 75 mg EDHB/kg b.wt. for 3 days was taken for further studies. No adverse effect with regard to body weight, food and water intake, hematological parameters were observed in this group of rats supplemented with the dose of 75 mg EDHB/kg b.wt. for 3 days.

EDHB supplementation reduces hypoxia induced edema in brain

Brain water content and vascular leakage were determined to study the efficacy of EDHB in attenuating cerebral edema. Brain water content or edema index indicated by wet wt/dry wt ratio was 4.08 ± 0.060 in control group of animals which increased to 4.29 ± 0.28 on exposure to hypoxia (Fig. 2a). However, significant reduction (10 %) in edema index was observed in EDHB supplemented rats as compared to hypoxia group, showing the efficacy of EDHB in decreasing edema index when given before hypoxic exposure.

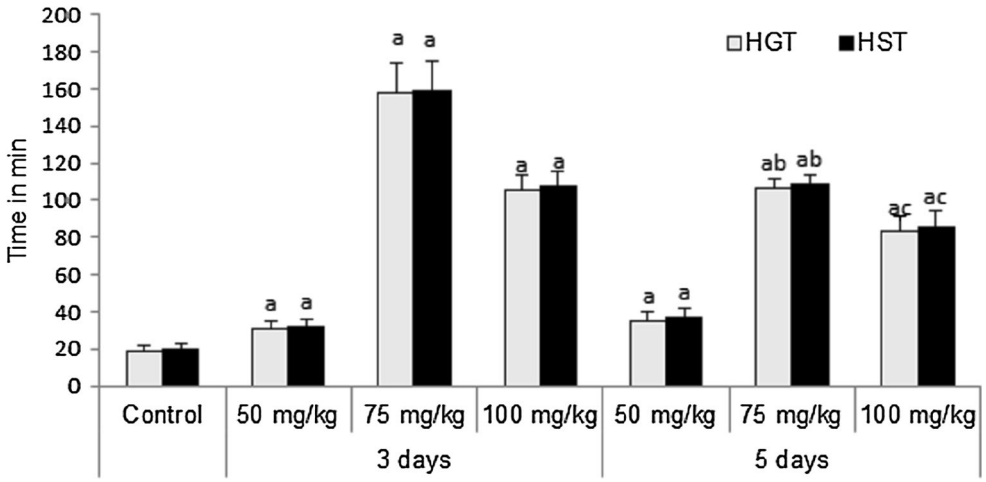

Fig. 1 Dose response study of EDHB based on hypoxia gasping time (HST) and hypoxia survival time (HST). EDHB supplemented animals (50, 75, 100 mg/kg b.wt. for 3 and 5 days) were subjected to an altitude of 9754 m (32,000 ft.) at 32 °C in animal decompression chamber. On the basis of hypoxia gasping and survival time recorded to measure hypoxia tolerance EDHB dose with 75 mg/kg b.wt for 3 days was selected for further studies. Values are mean ± SD ($n = 8$/group). (Control vs. EDHB $^{a}p < 0.01$; 3 vs. 5 days $^{b}p < 0.01$, $^{c}p < 0.05$)

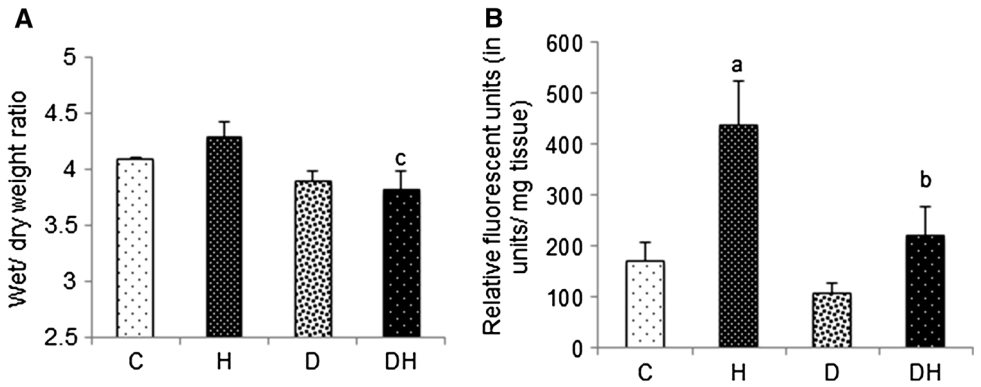

Fig. 2 Effect of EDHB supplementation on vascular leakage. **a** Edema index (wet/dry wt ratio). Animals were exposed to 9144 m hypoxia, excised brain was kept for drying at 55 °C for 72 h and weighed before and after drying. **b** Vascular permeability was determined by sodium fluorescein dye leakage. Results are expressed in relative fluorescence units (RFU). *C* control, *H* hypoxia, *D* drug, *DH* drug hypoxia. Values are mean ± SD ($n = 8$/group). (C vs. H $^{a}p < 0.01$; H vs. DH, $^{c}p < 0.05$, $^{b}p < 0.01$)

Vascular leakage, a more sensitive indicator of edema was determined by quantitation of sodium fluorescein dye leaked in brain tissue on exposure to hypoxia (Fig. 2b). There was >twofold increase in the fluorescein level in brain of rats exposed to hypoxia (435.54 ± 68.16 rfu./mg dry wt) as compared to control animals (171.53 ± 21.68 rfu./mg dry wt). Interestingly, EDHB supplementation halved the vascular leakage in brain (221.48 ± 39.96 rfu./mg dry wt, $p < 0.001$) even under hypoxia as compared to un-supplemented animals exposed to hypoxia.

Effect of EDHB on hematocrit, hemoglobin, EPO levels

Influence of EDHB supplementation (75 mg/kg b.wt. 3 days) on hemoglobin, hematocrit and EPO levels was studied under normoxia. The levels of hematocrit in control animals (47.5 %) and hemoglobin were found to be moderately increased in EDHB treated animals (49 %) under normoxia (Fig. 3). This increase in Hb and total volume percent of RBC i.e. hematocrit along with elevated levels of EPO by EDHB even under normoxia indicates better oxygen carrying capacity of blood.

EDHB preconditioning reduces inflammatory cytokines

To study the role of inflammatory markers in cerebral transvascular leakage, pro-inflammatory (IL-6, TNF-α, MCP-1, IFN-γ) and anti-inflammatory cytokines (IL-10 and TGF-β) were assayed by ELISA kits in the brain of different groups of animals. Exposure of animals to

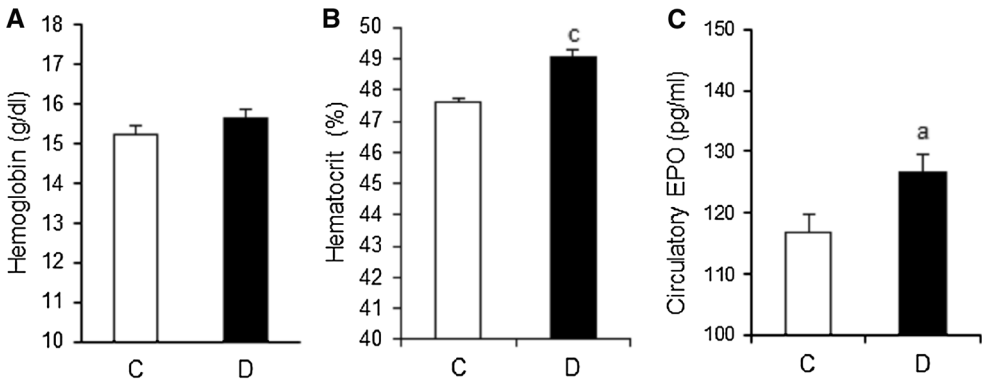

Fig. 3 Effect of EDHB supplementation on **a** hemoglobin (Hb), **b** hematocrit (Hct), and **c** erythropoietin (EPO) level. Experimental animals were administered with the optimal dose (75 mg/kg b.wt.) of EDHB for 3 days. Circulatory EPO was estimated by ELISA in plasma. Increased levels of Hb, Hct and EPO after EDHB supplementation even under normoxia indicates improved oxygen carrying capacity of blood. Values are mean ± SD ($n = 8$/group). *C* control, *D* drug, (C vs. D $^{c}p < 0.05$, $^{a}p < 0.01$)

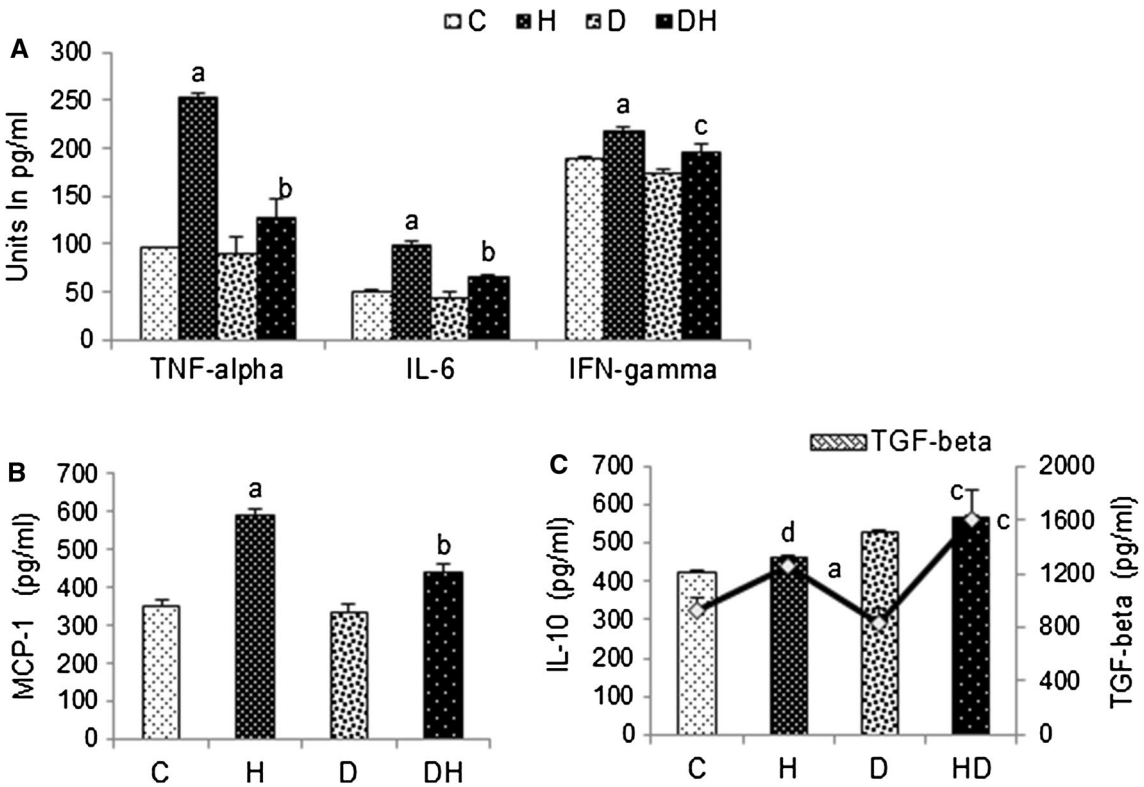

Fig. 4 Effect of EDHB on inflammatory markers in brain. **a** Pro-inflammatory cytokines (TNF-α, IL-6, IFN-γ) and **b** MCP-1. **c** Anti-inflammatory cytokines (IL-10, TGF-β). After hypoxic exposure of 5 h at 9144 m, levels of cytokines in whole brain homogenate were quantified by ELISA. EDHB preconditioning subdued hypoxia mediated proinflammatory cytokine level in brain compared with control. *C* control, *H* hypoxia, *D* drug, *DH* drug + hypoxia. Values are mean ± SD ($n = 8$/group). (C vs. H $^{a}p < 0.01$, $^{d}p < 0.05$; H vs. DH $^{b}p < 0.01$, $^{c}p < 0.05$)

hypoxia resulted in significant increase in IL-6 (94 %), TNF-α (161 %), MCP (67 %) and IFN-γ (15 %) in the hypoxic brain compared to control (Fig. 4a, b). This increase in pro-inflammatory cytokines was markedly attenuated IL-6 (34 %), TNF-α (49 %), MCP (25 %), IFN-γ (10 %) in rats preconditioned with EDHB as compared to the un-supplemented animals under hypoxia. There was hike in anti-inflammatory cytokines IL-10 and TGF-β in brain under hypoxia as compared to control animals (Fig. 4c). However, EDHB supplementation further boosted levels of IL-10(28 %) and TGF-β (22 %) under hypoxia compared with hypoxia control.

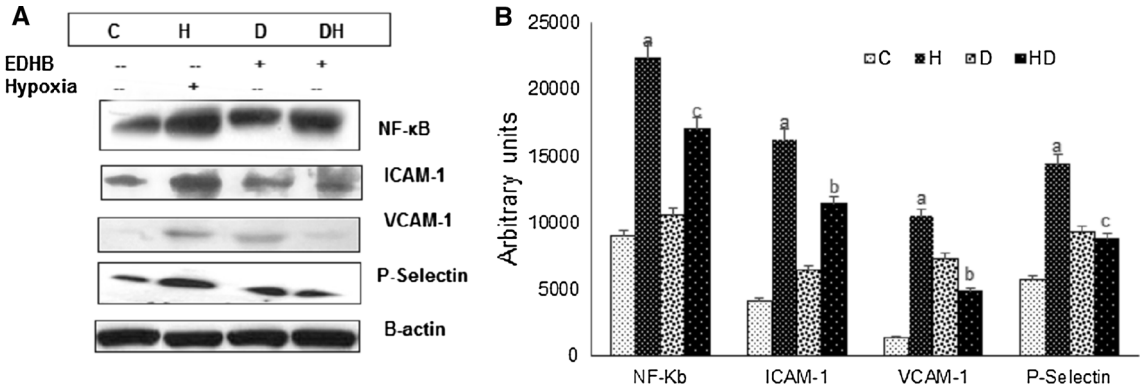

Fig. 5 Effect of EDHB preconditioning on expression of NF-κB and cell adhesion molecules in brain of rats subjected to normoxia and hypoxia (9144 m, 5 h). **a** Western blot of NF-κB, ICAM-1, VCAM-1, P-selectin. **b** *Bar graph* representation. Bands were analyzed with densitometry using Image-J software. β-actin was used to normalize the results. *C* control, *H* hypoxia, *D* drug, *DH* drug + hypoxia. Values for the blots are the mean ± SD ($n = 8$/group). (C vs. H $^{a}p < 0.01$, H vs. DH $^{b}p < 0.01$, $^{c}p < 0.05$)

NF-κB and cell adhesion molecules

NF-κB is expressed under hypoxia and is directly linked with inflammation. To illuminate the contribution of EDHB in reducing inflammation, we studied the expression of transcription factor NF-κB and its related cell adhesion molecules (ICAM-1, VCAM-1, P-selectin) in the brain of control and hypoxic animals supplemented with EDHB (Fig. 5a). NF-κB was found to be upregulated (twofold) under hypoxia. Low expression of ICAM-1, VCAM-1 and P-selectin observed in control animals was increased significantly under hypoxia (ICAM > 3.0-fold, VCAM-fivefold, P-selectin > twofold) (Fig. 5b). Amazingly, the escalated protein levels of NF-κB and cell adhesion molecules were markedly subdued by EDHB in normoxia as well hypoxia as compared to hypoxia control.

Effect of EDHB preconditioning on HIF-1α and its regulated genes

EDHB preconditioning stabilizes HIF-1α via downregulation of prolyl hydroxylase (PHD)

To explore the potential of EDHB in stabilizing HIF1α, we probed the expression of HIF-1α protein in the brain. Hypoxia resulted in escalation of HIF1α expression with or without EDHB supplementation. We were fascinated to observe profound increase in HIF-1α even under normoxia with EDHB. This increase indicates the preconditioning efficacy of EDHB in stabilization of HIF-1α even in the presence of normal oxygen levels, signaling the acclimation process at the basal level. Furthermore, EDHB mediated upregulation of HIF-1α under hypoxia was higher as compared to un-supplemented group exposed to hypoxia (Fig. 6). Further, we observed decreased PHD2 expression in brain on EDHB supplementation as compared to normoxia animals which was further lowered under hypoxia as compared to hypoxia controls.

Effect of EDHB preconditioning on VEGF and HO-1

VEGF, known as angiogenesis and permeability factor, was considerably upregulated in hypoxia compared with control group. Increase was observed in EDHB supplemented animals also but it was lower than hypoxia control (Fig. 6). HO-1, a potent antioxidant and anti-inflammatory protein, showed marked increase following EDHB supplementation even under normoxia. Significant escalation was observed when EDHB supplemented rats were exposed to hypoxia as compared to un-supplemented hypoxic group.

EDHB preconditioning upregulates MT-1 expression

Metallothioneins are effective free radical scavengers in various stress conditions. Exposure to acute hypoxia for 5 h downregulated MT-1 expression as compared to normoxic controls. However, EDHB preconditioned animals showed marked increase in MT-1 protein level even in normoxic conditions, and further increase was observed after exposure to hypoxia (Fig. 6).

Collectively, the present study demonstrates that hypoxia preconditioning with EDHB elevated expression of antioxidant proteins HO-1 and MT-1.

Discussion

The study herein demonstrates that hypoxic preconditioning with EDHB increased hypoxia tolerance accompanied by reduced vascular permeability in animals exposed to

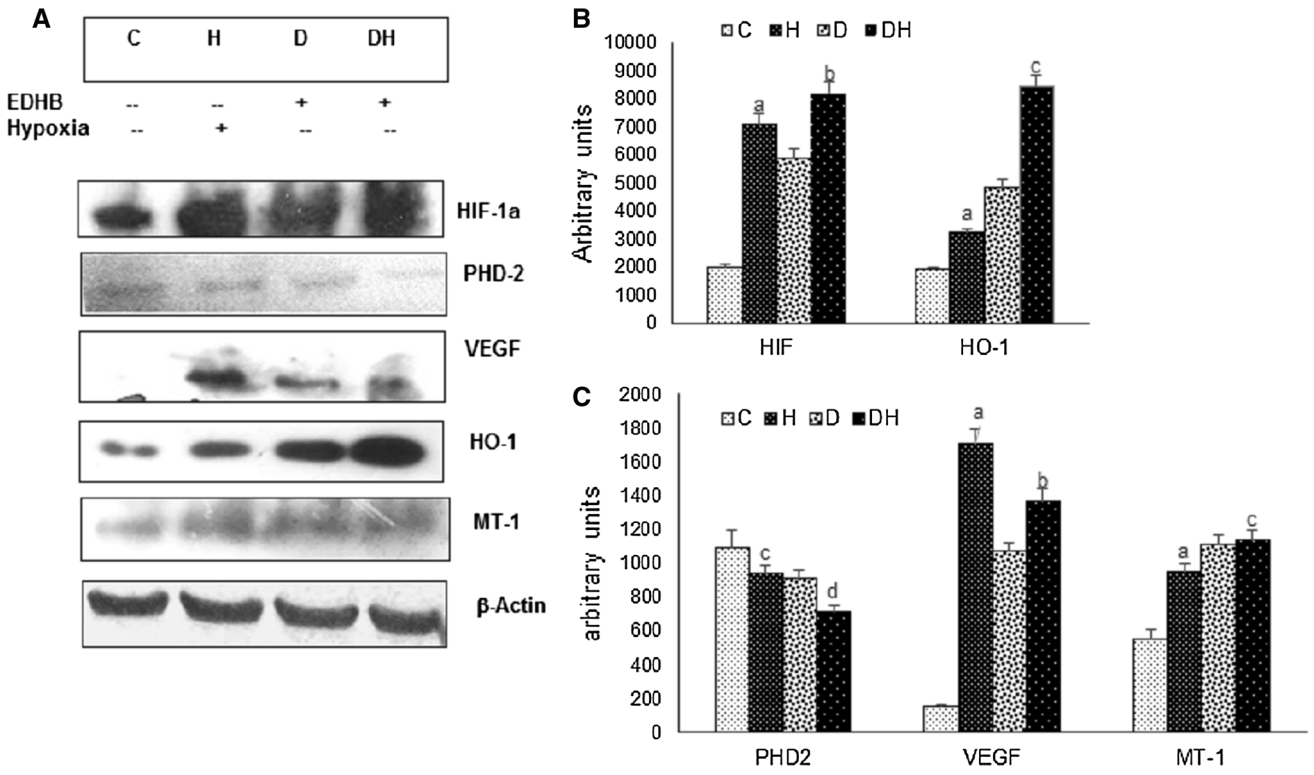

Fig. 6 Effect of EDHB preconditioning on HIF-1α, PHD2, VEGF and antioxidant proteins HO-1 and MT-1 in rat brain with/without EDHB supplementation exposed to normoxia and hypoxia (9144 m, 5 h). **a** Western blots of HIF-1α, PHD2, VEGF, HO-1 and MT-1. **b** *Bar graph* representation. Bands were analyzed with densitometry using Image-J software. Values are in relative optical density. *C* control, *H* hypoxia, *D* drug, *DH* drug + hypoxia. Data is the mean ± SD ($n = 8$). (C vs. H $^{a}p < 0.01$, $^{d}p < 0.05$; H vs. DH $^{b}p < 0.01$, $^{c}p < 0.05$)

hypoxia, evidenced by decreased edema index and fluorescein dye leakage in brain as compared with hypoxic animals. Further, there was a significant decrease in NF-κB levels resulting in decline in inflammatory cytokines and cell adhesion molecules in EDHB supplemented hypobaric hypoxia exposed rats. This was accompanied with upregulation of HIF-1α and anti-inflammatory proteins HO-1, MT-1, IL-10 and TGF-β. The cumulative effect of these modulations promotes hypoxic acclimatization, maintaining BBB integrity at high altitude, thereby preventing hypobaric hypoxia induced cerebral edema formation.

In the present study, we examined whether prolyl hydroxylase inhibitor EDHB would promote tolerance to hypoxia in rats. Animals were supplemented with increasing concentration of EDHB (50, 75, 100 mg/kg b.wt.) daily for 3 days before exposing to hypobaric hypoxia at 9754 m (32,000 feet). Animals were exposed to this altitude because small animals with higher capillary density in tissues make them more resistant to hypoxia than men [32]. Gasping time and hypoxia survival time (HST) are standard parameters to measure hypoxia tolerance in animals [32, 33]. Hypoxia tolerance was improved with the increase in EDHB concentration. Interestingly, we found that with 75 mg/kg b.wt. EDHB supplementation, there was tenfold increase in HST as compared with control animals (Fig. 1). This increased hypoxia tolerance could be result of better oxygen carrying capacity at low oxygen level which corresponds to hemoglobin level in blood. Therefore, we sought to determine the levels of hemoglobin, hematocrit of control and EDHB treated (during normoxia) rats. There was significant increase in hemoglobin (15.63 mg/dL) and hematocrit (49 %) level in EDHB supplemented animals compared with control (Fig. 3). This was further validated by measuring circulatory erythropoietin (EPO) which was found to be significantly elevated in this group, thereby, improving the oxygen transport in the blood at normal oxygen level thus having favorable impact on sudden exposure to high altitude. The increased EPO could be due to stabilization of HIF 1α by preconditioning with EDHB. Indeed HIF 1α expression was found to be significantly enhanced in animals pre-conditioned with EDHB even under normoxia.

Edema index and vascular leakage were studied to determine cerebral edema immediately after hypoxic exposure. No significant increase in brain water content (wet to dry weight ratio) was observed between control and

hypoxic group. This could be because the method is less sensitive to measure small increase in water content in tissues and also could be contributed to water loss in tissues caused by hyperventilation during hypoxia exposure. However, there was significant ($p < 0.001$) decrease in wet to dry weight ratio of brain tissue in EDHB treated animals both under normoxia as well as hypoxia compared with hypoxia control (Fig. 2a). Vascular leakage, a more sensitive indicator of edema, was therefore, studied using sodium fluorescein dye as probe. 2.5-fold increase in fluorescein level under hypoxia revealed vascular injury leading to fluid accumulation in brain (Fig. 2b) which is in concordance with previous studies [32]. Amazingly, EDHB preconditioning substantially attenuated the vascular leakage induced by hypoxia ($p < 0.001$) indicating that the permeability of BBB was minimally hampered under the influence of hypoxia showing the protective efficacy of EDHB in maintaining the integrity of BBB.

Hypoxia acts as an initial trigger for pathophysiological changes at the BBB such as altered distribution of water and ions, inflammatory events and oxidative stress, edema formation, infiltration of peripheral immune cells and leakage of blood proteins into the brain. Earlier in vivo and in vitro studies have demonstrated that hypoxia is a major stress factor inducing BBB disruption [29, 34–36].

In brain, cytokines are produced by the cells of the BBB, such as microglial cells, astrocytes and endothelial cells after injury or infection and contribute to the total inflammatory response which later, affect the function of the BBB. Here, in our study, exposure of animals to hypobaric hypoxia increased the level of pro-inflammatory cytokines such as IL-6, TNF-α, MCP-1 and IFN-γ in rat brain. Earlier in vitro studies revealed that administration of TNF-α and IL-6 to monolayers of endothelial cells leads to an increase in the permeability [37]. IFN-γ produced in response to viral or bacterial infection or their products is also induced by TNF-α and modulate inflammation [38]. Under normal physiological conditions, the basal level of TNF-α remains low but the concentration increases in acute inflammation, trauma and autoimmune diseases [39]. Protocatechuic acid has been reported to exhibit a slight inhibitory effect on NO production and TNF-α secretion in LPS-IFN-γinduced macrophages [40] and reduced the levels of IL-6 and TNF-α in heart and kidney in mice [41]. Earlier studies have shown that MCP-1 increases the paracellular permeability of endothelial monolayers via TJ redistribution mediated by Rho signaling and is involved in formation of edema in vivo [42, 43]. Our results indicated that EDHB supplementation prior to hypoxic exposure resulted in moderating inflammation by downregulating the production of these pro-inflammatory cytokines (Fig. 4). This might be the result of EDHB induced increase in anti-inflammatory cytokines such as IL-10 and TGF-β. IL-10 is a major immunosuppressive cytokine with its potent effects in reducing inflammation. TGF-β has both immune-regulatory and proinflammatory properties dependent on different microenvironments [44]. In an inflammatory situation, TGF-β is produced by macrophages upon their phagocytosis of apoptotic cells and exerts an anti-inflammatory effect, reflecting a negative regulation of inflammation processes [45].

Hypoxia has been shown to activate transcription factor NF-κB which plays a pivotal role in the regulation of immune system and inflammation [46]. NF-κB activation in response to pro-inflammatory stimuli involves the rapid phosphorylations of IκBs by the Iκ-B kinase (IKK) complex. The NF-κB subunits of p50/p65 leads to the transcription of inflammation-associated genes, such as IL-1β, TNF-α, IFN-γ, MCP-1 to favor cellular adaptation to hypoxia in tumor cells and is related to paracellular permeability promoting leukocyte adhesion [47]. In brain it regulates the expression of inflammatory cytokines such as TNF-α and IL-6. In our study (Fig. 5), this increased NF-κB activity, resulted in increase in the expression of cell adhesion molecules ICAM-1, VCAM-1, P-selectin which facilitates movement of leucocyte and trafficking into BBB. These results are in concordance with earlier studies who reported that the differential induction of cytokines involved in the atypical pattern of leukocyte recruitment induced in the brain [48, 49]. Interestingly, this hypoxia induced increased nuclear NF-κB expression was downregulated by EDHB preconditioning with a concomitant decrease in ICAM-1, VCAM-1 and P-selectin. As all of these inflammatory proteins are transcriptionally controlled by NF-κB, it was suggested that EDHB may exert a significant part of its anti-inflammatory properties by inhibiting this transcription factor through induction of IL-10. IL-10 inhibits I-kappa-B kinase (IKK) activity thereby blocking NF-κB nuclear translocation and blocks DNA binding of NF-κB already present in the nucleus [50]. In similar experiment, Siddiq et al. showed that prolylhydroxylase-4 inhibitors have capacity to dampen microglial activation via their ability to prevent oxidative induction of the MAPK and NF-κB signaling pathways, which in turn, reduces the synthesis and release of pro-inflammatory factors from activated microglia [51]. Protocatechuic acid has been shown to be protective against inflammation in different rat models of paw edema, granuloma exudates formation and arthritis index [41], however, to the best of our knowledge we have reported for the first time the anti-inflammatory effect of EDHB in hypobaric hypoxia mediated brain injury.

VEGF, although known for its neuroprotective and neurogenic properties has been reported to contribute to the inflammatory responses and blood–brain barrier breakdown in cerebral ischemia in a dose dependent manner

[52–55]. VEGF has been shown to result in edema and vascular leakage in almost every tissue to which it was applied including brain, lung, testis, bladder, skin, etc. and vascular leakage in the brain occurred rapidly, within 30 min of exposure to VEGF [56, 57]. The timing of VEGF upregulation corresponds closely to the peak of vasogenic edema in ischemic brain [58]. Therefore we measured this angiogenic and permeability factor in brain of rats exposed to hypobaric hypoxia. Our study (Fig. 6) revealed that VEGF was highly expressed in the brain under hypoxia in control animals. However, after EDHB preconditioning, its expression was subdued significantly ($p < 0.01$) in animals exposed to hypoxia as compared with only hypoxia exposed group. The downregulation of VEGF could be attributed to modulatory effect of EDHB via escalation of anti-inflammatory cytokine IL-10. Our results are in line with the studies of Silvestre et al., who reported that IL-10 negatively modulates ischemia-induced angiogenesis and associated this effect with the reduction of VEGF expression [59].

Hypoxia conditions the cell to stress to foster cellular adaptation by activating number of genes including EPO, HO-1 and VEGF. All these genes follow a common intracellular signaling pathway mediated by HIF-1α which is one of the essential transcription factors induced in hypoxia required for rapid acclimatization to the high altitude hypoxia [15]. In our study, nuclear HIF-1α protein was found to be increased in animals subjected to hypoxia as supported by earlier studies [32]. Interestingly, HIF-1α was significantly upregulated in animals supplemented with EDHB at normoxia also, accompanied with boost in its target gene HO-1 (Fig. 6). This robust increase in HIF-1α protein and HIF target gene expression strongly indicates the preconditioning effect of EDHB even before exposure to hypoxia. Stabilization of HIF under normoxia is also mediated by elevated levels of TGF-β which, in addition to its anti-inflammatory role, has been reported to increase normoxic HIF-1α protein stabilization, possibly through impaired prolyl hydroxylation, by inhibition of PHD2 expression level [60]. Indeed, we observed downregulation of prolyl hydroxylase 2 (PHD2) when EDHB supplemented rats were exposed to normoxia or hypoxia, thus resulting in stabilization of HIF1α.

To address whether the ability of EDHB to prevent cerebral edema is mediated via stimulation of anti-inflammatory proteins, we studied the protein expression levels of HO-1 and MT-1. HO-1 and MT-1 are the antioxidant proteins playing role in cell homeostasis and are expressed in response to stress [61]. HO-1, a potent anti-inflammatory protein is expressed in response to stimuli that are associated with oxidative stress and inflammation, including heme, hypoxia, ischemia, heavy metals, shear stress, pro-inflammatory cytokines etc. HO-1 plays an essential role in controlling tissue homeostasis in inflammation by inhibiting pro-inflammatory cytokine synthesis and inducing antiapoptotic processes [62]. HO-1 exerts protective effects via multiple pathways that involve direct cytoprotective and antiapoptotic effects of CO and antioxidant effects of biliverdin/bilirubin and ferritin [63, 64]. In our study, there was increase in HO-1 under hypoxia alone but profound increase in HO-1 protein expression was observed in rats with EDHB supplementation both under normoxia and hypoxia. Metallothioneins (MT) are low molecular weight cysteine rich proteins with multiple roles, such as detoxification of heavy metals, antioxidant and anti-inflammatory properties. We observed elevated MT-1 expression due to EDHB preconditioning independent of hypoxia, which was further increased under hypoxia. Murphy et al. reported that hypoxia induces the expression of MT-I through Metal responsive transcription factor (MTF-1) that also contributes to the stabilization and nuclear accumulation of the HIF-1α protein, which in turn is essential for induction of MT-1 through MTF1 [65–67]. Similarly in our study, EDHB mediated stabilization of HIF1 α via PHD enzyme inhibition seems to function as a modulator of MT-1 by interacting with MTF-1 during hypoxia.

Thus higher levels of anti-inflammatory interleukins (IL-10, TGF-β), HO-1 and MT-1 in EDHB preconditioned animals suggest the anti-inflammatory activity of EDHB under hypobaric hypoxic stress which, in part, might play some role in preventing hypoxia induced vascular leakage and BBB injury.

In conclusion, brain injury on exposure to hypobaric hypoxia is associated with altered vascular permeability and tissue swelling is of particular concern. This is further compounded by the lack of effective therapies. However, the inhibition of neurogenic inflammation with the help of preconditioning by prolyl hydroxylase inhibitors may provide a novel alternative therapy for the treatment of barrier dysfunction and tissue swelling in the setting of acute brain injury. Our study demonstrates that hypoxia induced cerebral edema, which involves disruption of blood brain permeability can be reduced significantly by diverse activity of EDHB, which exerts anti-inflammatory activity via downregulation of inflammatory mediators. EDHB mediated boost in HIF-1α and anti-inflammatory proteins, IL-10, TGF-β, HO-1 and MT-1 suggests its role in attenuating hypoxia induced cerebral edema. These findings underscore significance of hypoxic preconditioning with EDHB in the modulation of hypoxia mediated cerebral injuries.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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