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Cardiac-specific knockout and pharmacological inhibition of *Endothelin receptor type B* lead to cardiac resistance to extreme hypoxia

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Abstract

Oxygen plays a central role in cardiac energy metabolism. At high-altitude where the ambient oxygen level is low, we found *EDNRB* is associated with human hypoxia adaptation. Our subsequent study in global heterozygous knockout mice (*Ednrb*$^{+/-}$) revealed that cardiac function was conserved in these mice when exposed to extreme hypoxia. The major goal of this study was i) to determine the functional role of cardiomyocyte *EdnrB* in maintaining cardiac function under hypoxic stress and ii) to validate the phenotypes we detected in *Ednrb*$^{+/-}$ mice using EDNRB blockers. Unlike the global knockouts, cardiac specific heterozygote (*EdnrB*$^{flox/+}$) and homozygote (*EdnrB*$^{flox/flox}$) *EdnrB* knockout mice were phenotypically normal. When treated with graded low levels of oxygen (10% and 5% O_2), both *EdnrB*$^{flox/+}$ and *EdnrB*$^{flox/flox}$ were hypoxia tolerant. The cardiac-index at 10% and 5% O_2 for *EdnrB*$^{flox/+}$ were significantly higher and lactate levels were significantly lower when compared to the cre-negative controls ($P<0.05$). Simultaneously, mice treated with BQ-788 (EDNRB specific) had a significantly higher cardiac-index ($P<0.005$) and significantly lower lactate levels ($P<0.0001$) than in control mice. A similar result was obtained with mice treated with Bosentan (non-specific). These data indicate that a lower level or complete lack of *EdnrB* in the cardiomyocytes significantly improves cardiac performance under extreme hypoxia, a novel role of cardiomyocyte *EdnrB* in the regulation of cardiac function. Furthermore, this rescue under extreme hypoxia can also be achieved using EDNRB specific pharmacological agents e.g., BQ-788. This systematically confirms, both genetically and pharmacologically, the protective role of a lower EDNRB under extreme hypoxia stress.

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Disclosures

None.

Keywords

EdnrB; Bosentan; BQ-788; high-altitude; hypoxia; cardiac index

Introduction

Humans adapted to high-altitude (HA) environment are anticipated to have certain favorable variants, accumulated in their genome due to their long habitation in harsh hypoxic environments. Through whole genome sequencing, we were able to identify DNA regions and candidate genes, under 'selection' [1, 2]. Since better therapeutic modalities for diseases involving hypoxia and ischemia in their pathogenesis can be developed by understanding mechanisms of HA adaptation, we have identified *Endothelin receptor type B* (*EDNRB*), as one of the candidate genes from the Ethiopian HA adapted population [1]. More recently, we have focused on validating the role of *EDNRB* in hypoxia tolerance in *EdnrB* knockout (KO) mice [3]. We found that a lower level of *EdnrB* contributes to the sustenance of a steady cardiovascular response to various degrees of hypoxia, even severe hypoxia of 5% O_2. The heterozygote *EdnrB* (*EdnrB*$^{-/+}$) mice were maintaining higher cardiac output (CO), peripheral perfusion and better O_2 delivery to vital organs [3]. Together with the human study, *EDNRB* seems to play a key role in hypoxia tolerance.

In parallel, there has been considerable interest in the endothelin system because of its role in development of diseases and pathological conditions, and also because the receptors (*EdnrA* and *EdnrB*) are important target for developing drugs for treatment. Cardiac hypoxia tolerance in *EdnrB*$^{-/+}$ mice is interesting from several points of view. First, the HA exposure itself is recognized as a cardiac stress [4] and a conventional approach for adaptation to such environment would be to cope with such stress. And as we noticed in the *EdnrB*$^{-/+}$ mice, downregulating *EDNRB* is a good example. Second, endothelin-1 (Edn1), the ligand that binds to both receptors, increases during HA exposure [5] and lead to pulmonary hypertension. Third, the receptor antagonist Bosentan, although non-specific, is given to reduce pulmonary artery pressure [6]. Fourth, the improvements post receptor antagonist treatment likely occurs through blocking *EdnrB* because in spite of the higher ratio of EDNRA/EDNRB (4 to 1) in the heart [7], KO studies have ruled-out any important role of *EdnrA* in maintaining cardiac function [8]. And when Bosentan treatment during septic shock improves cardiac performance and microcirculatory blood flow [9, 10], the specific binding of this antagonist to *EdnrB* can be viewed as a major contributor. And lastly, the phenotype that describes *EdnrB*$^{-/+}$ mice as 'hypoxia tolerant' were directly related to cardiac function, e.g. blood pressure (BP), CO, contractility etc., or its downstream mechanism/s e.g. lactate levels, O_2 perfusion [3]. Based on this evidence, we hypothesized that by lowering *EdnrB* expression in the heart, either directly by tissue specific KO or using specific blockers; these mice would be able to demonstrate significant tolerance to acute hypoxia. In the current study, we systematically confirmed our hypothesis, first by generating cardiomyocyte-specific *EdnrB* KO mice (both heterozygote (*EdnrB*$^{+/flox}$) and homozygote (*EdnrB*$^{flox/flox}$)), exposing them to acute hypoxia and then measuring cardiac functions. Simultaneously, we also treated adult C57BL/6J mice with Bosentan (a dual

endothelin receptor blocker which blocks both EDNRA and EDNRB) and BQ-788 (EDNRB specific blocker) and measured their cardiovascular tolerance to hypoxia.

Materials and Methods:

Generation of *EdnrB* KO and its validation

All animal care and handling was performed according to the NIH guidelines and protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California San Diego (UCSD), US. Generation of *EdnrB-LoxP* (*EdnrB*$^{flox/flox}$) mice has been previously detailed [11]. Prof. David Webb, Univ. Edinburgh and Prof. Donald Kohan, Univ. Utah, kindly provided these mice. Briefly, the *LoxP* sequence was inserted within intron 2 and 4 (flanking exon 3 and 4) of *EdnrB* gene (Fig. 1a). Earlier reports suggested that exon 3 was crucial as there was an amino acid encoded across a splice junction [12]. Subsequent sequencing confirmed no mutations within *LoxP* sites and no known phenotype related to *EdnrB* disruption e.g., abnormality of pigmentation, gut development, litter size, or mortality rate. These mice were crossed with αMHC-MerCreMer (stock# 005650, Jackson Lab) and selected for cre'+' F1 progeny. Subsequently they are inbred to generate cardiac specific *EdnrB* homozygote and heterozygote KO mice. A schematic of the breeding strategy and its validation using PCR is provided in the Fig 1b and 1c. For the hypoxia experiments, we used cre'+' mice, both *EdnrB*$^{flox/+}$ and *EdnrB*$^{flox/flox}$ and compared with cre'-' *EdnrB*$^{flox/+}$ mice. All the mice were treated in advance with 3×30mg/kg of *tamoxifen*.

Pharmacological validation by EDNRB blockers

Studies were performed in adult (>3 months) male C57BL/6J mice (Stock# 000664, Jackson Lab). For the pharmacological inhibition studies, we used EDNRB specific blocker 'BQ-788' and endothelin receptor non-specific blocker 'Bosentan'. We believe that the drug BQ-788 would mimic *EdnrB* KO mice and the use of Bosentan would help decipher the involvement of other subtype i.e., *EdnrA*. A pilot study with different doses of BQ-788 (at 0.5 and 1.0 mg/kg) and Bosentan (at 25 and 50 mg/kg) was initially conducted to estimate the dose-response activity. For the final experiment, we used 0.5 mg/kg of BQ-788 and 50 mg/kg Bosentan. Animals were randomly assigned to three different groups. Group I (n=5) was injected with carrier, Group II (n=6) with Bosentan (50 mg/kg) and Group III (n=5) with BQ-788 (0.5mg/kg). In total, all groups were injected four times at 48-hour interval with respective dosages. The baseline measurements i.e., 21% O_2, was carried out after 2 hours of 4th dose injection.

Mice surgical preparation

The IACUC approved the experimental protocol (protocol S04052 and S11306). The animals were anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg). Mice were prepared for cardiac output (CO) measurements by first surgically implanting a jugular catheter (PE10 filled with heparin/saline solution 30 IU/mL) and a small thermocouple (IT18T75, Physitemp Instruments). A midline incision was made in the exposed area and the right jugular vein and carotid artery were exposed. The thermocouple probe was inserted approximately 0.7 cm into the carotid artery. The tip of the jugular vein catheter was inserted past the subclavian branch of the vein. The temperature probe and PE10 catheter were under

the skin on the animal's back. A short femoral catheter (PE10 filled with heparin/saline solution 30 IU/mL) was also implanted to monitor blood pressure and withdraw blood samples, which was secured underneath the skin. Mice recovered from catheter implantation for 24 hours before the hypoxia protocol. Mice were suitable for the experiments if: i) systemic parameters were within normal range, namely, heart rate (HR)>450 beat/min, mean arterial blood pressure (MAP)>90mmHg, systemic Hct>42%, and arterial O_2 partial pressure (p_aO_2)>80mmHg.

Cardiac Output Measurements

CO was measured by a modified thermodilution technique [13]. Briefly, a saline bolus indicator (0.1 mL at 25°C) was injected via the jugular vein catheter, and the change in temperature was detected at the aortic arch using the implanted thermocouple. This procedure was selected because in these small awake animals it is not feasible to inject the indicator directly into the right atrium. The conversion of the area under the indicator dilution temperature curve into flow was done with a fluid circuit that reproduced flow rates, volumes, and heat exchange properties of the mice circulation between the locations of saline injection and temperature measurements. Thermodilution curves peaked rapidly and decayed exponentially. The signal due to recirculation was eliminated by truncating the curve 30–50% after the maximum change in temperature. Thermodilution curves area is proportional to CO. Cardiac index (CI) was calculated as the CO divided by the animal body weight.

Hypoxia protocol

The awake animals were placed in a restraining tube within seal acrylic box (4" x 4" x 10"). The inlet of the box was connected to gas tanks with different O_2 concentrations: 1) compressed air; 2) 15% O_2 balance N_2; 3) 10% O_2 balance N_2; and 4) 5% O_2 balance N_2. The gas flow rate into the box was kept at 0.2 L/min using a gas flow meter. Air coming into the box was warmed to 25°C and diffused using a cotton filter. O_2 concentration in the box was measured continuously using an O_2 gas sensor (Vernier Corp. Beaverton, OR). Mice were first exposed to compress air and given 20 min to adjust to the experimental environment before baseline measurements were completed. Then, the compressed air was replaced by 15% O_2, and mice were given 15 min to adjust to the change in the gas environment before measurements. Similarly, the 15% O_2 gas was replaced for the 10% O_2 gas, and another 15 min were given to the mice before measurements. Lastly, the 10% O_2 gas was replaced for the 5% O_2 gas, and another 10–15 min were given to the mice before measurements. At each time point, systemic parameters and blood gas analysis were performed which also included lactate level measurements. Briefly, a capillary blood sample was taken from the femoral artery catheter ~15 mins after animals were exposed to specific oxygen concentrations. Blood samples were immediately analyzed in a blood gas analyzer (ABL90 FLEX analyzer Radiometer America Inc., Brea, CA). To prevent animal stress or discomfort, hypoxia was stopped if BP dropped below 40 mmHg, and the animal was excluded from the study.

Data analysis

Results are presented as means ± standard deviations. The Grubbs' method was used to assess closeness for all measured parameters values at baseline. Data comparison between groups was analyzed using two-way analysis of variance (ANOVA) with genotype as a between factor and hypoxia treatment as a repeated measure. Data within each group was analyzed using Kruskal-Wallis one-way ANOVA. When appropriate, post-hoc analyses were performed with the Dunn's multiple comparison test and Bonferroni post-tests comparison. All statistics were calculated using Analyse-it for Microsoft excel 4.18.1 (Analyse-it Software, Ltd.). Changes were considered statistically significant if $P<0.05$.

Results

Generation of cardiac specific *EdnrB* KO

We hypothesized that decreasing functional EDNRB specifically in the heart would be advantageous in hypoxia, and hence we generated cardiac-specific KO mice of this gene and then studied its phenotype in both normoxia and hypoxia. While the generation of *EdnrB-LoxP* (*EdnrB*$^{flox/flox}$) mice has been previously reported [11], a detailed schematic of the crosses to generate *EdnrB* KOs in the cardiomyocytes is presented in Fig 1B. Both homozygous *EdnrB*$^{flox/flox}$*;Myh6-cre'+'* (*EdnrB*$^{flox/flox}$) as well as the heterozygotes *EdnrB*$^{flox/+}$*;Myh6-cre'+'* (*EdnrB*$^{flox/+}$) mice were injected with *tamoxifen* (3×30mg/kg) 10 days prior to the hypoxia experiment. Simultaneously, the control *EdnrB*$^{flox/+}$*;Myh6-cre'-'* (cre negative) mice were also injected with equal dosages i.e., 3×30mg/kg of *tamoxifen* (Fig 1b and 1c). At the end of experiment brain, lung and heart were collected for DNA isolation and PCR confirmation of the cardiac-specific *EdnrB* KO (*EdnrB*$^{flox/+}$and *EdnrB*$^{flox/flox}$). Genotype of brain, lung and heart confirms exon 3–4 deletion in the heart and not in other tissues or the heart of *tamoxifen*-treated cre-negative mice (Fig 1C).

Hypoxia tolerance in cardiac specific *EdnrB*$^{flox/+}$ and *EdnrB*$^{flox/flox}$ KO

Three sizeable groups of mice i.e., *tamoxifen* injected *EdnrB*$^{flox/+}$ (n=12), *EdnrB*$^{flox/flox}$ (n=9), and the cre-negative controls (n=11), were analyzed utilizing modified thermodilution technique after exposing to acute hypoxia of different O_2 i.e., 21%, 15%, 10% and 5% (see Methods and Stobdan et al. 2015) [3]. Both *EdnrB*$^{flox/+}$ and *EdnrB*$^{flox/flox}$ mice, similar to *EdnrB*$^{-/+}$ (global heterozygote) [3], maintained a higher cardiac performance at various levels of hypoxia, including a severe one. Although the CI dropped as the O_2 decreased from room air (21% O_2) to 5% O_2 (Fig 2a), the CI in *EdnrB*$^{flox/+}$ and *EdnrB*$^{flox/flox}$ were significantly higher at 10% and 5% O_2 when compared to the control mice (Fig 2a, $P<0.05$). The CI at 10% and 5% O_2 were 0.33±0.02 and 0.22±0.02 mL/min/gm in *EdnrB*$^{flox/+}$ and 0.31±0.02 and 0.21±0.01 mL/min/gm in *EdnrB*$^{flox/flox}$, which is significantly higher than the cre-negative control group with 0.26±0.02 and 0.18±0.02 mL/min/gm (Fig 2a). When we analyzed the percentage drop in CI from its baseline values, the cre-negative CI drop at 5% O_2 was ~52% of the levels at 21% O_2. The drop at the same O_2 level was 42% in the *EdnrB*$^{flox/+}$. Similarly, no drop was detected at 15% O_2 in *EdnrB*$^{flox/flox}$ and 4.8% in *EdnrB*$^{flox/+}$ but a 7.3% drop in the controls. When we analyze CO, especially under extreme hypoxia (10% and 5% O_2), *EdnrB*$^{flox/+}$ mice maintained a higher CO (supplementary Fig 1S) and the relative drop from its 21% O_2 was significant when compared with the controls

(supplementary Fig 1S). Additionally, the lactate levels strongly correlated with the hypoxia tolerance observed in *EdnrB*$^{flox/+}$ and *EdnrB*$^{flox/flox}$ (Fig 2b). The lactate levels at 5% O_2 in both *EdnrB*$^{flox/+}$ and *EdnrB*$^{flox/flox}$ ($P<0.05$) and at 10% O_2 in *EdnrB*$^{flox/+}$ ($P<0.05$) were significantly lower than the cre-negative mice at respective O_2 levels. This is also supported by the trends between the three groups with levels in cre-negative > *EdnrB*$^{flox/flox}$ > *EdnrB*$^{flox/+}$ both at 10% and 5% O_2, which is inversely related to the CI and CO measurements (Fig 2b and supplementary Fig 1S).

EDNRB specific blocker depict maximum hypoxia tolerance

In order to systematically evaluate the protective role of relatively lower EDNRB in a hypoxic environment, we utilized both non-specific (Bosentan, blocks both EDNRA and EDNRB) and specific (BQ-788) blockers of EDNRB. Adult C57BL/6J mice were randomly assigned to three different groups. Group I (n=5) was treated with carrier, Group II (n=5) was treated with Bosentan (50 mg/kg) and Group III (n=5) was treated with BQ-788 (0.5mg/kg). Cardiovascular functions were measured utilizing modified thermodilution technique (materials and methods). Remarkably, both Bosentan and BQ-788 rescue mice by maintaining better cardiac functions under extreme hypoxia. Under ambient conditions i.e., at 21% O_2, and also at 15% O_2, the CI was comparable in all the three groups (Fig 3a). However, at 10% and 5% O_2 a clear trend was seen where the CI was in the ascending order from controls<Bosentan<BQ-788. Under extreme hypoxia of 5% O_2 the CI was highest for BQ-788 treated mice with P-value <0.005 when compared to the controls and P-value <0.05 when compared with Bosentan treated mice (Fig 3a). On the contrary and as anticipated, the lactate levels at 10% O_2 followed a reverse order with BQ-788<Bosentan<controls. The differences between the groups were further cumulated at 5% O_2 ($P<0.0001$, Fig 3b). At this O_2 the mice treated with BQ-788 had only 44.2% increase in the lactate levels from the level detected at 21% O_2. This level was lower than Bosentan treated mice with 62.3% increase and control mice with 77.4% increase. Of note, the BQ-788 treated mice had higher mean arterial pressure, higher heart rate and lower stroke volume (supplementary Fig 2S) and these trends are similar to our findings in *EdnrB*$^{-/+}$ mice.

Discussion

EDNRB was one of the top candidate genes we identified in our whole-genome sequence analysis for hypoxia adaptation in Ethiopian highlanders [1]. This was primarily marked by a strong signature of positive selection where a large block containing SNPs were fixed in the highlanders but were at a significantly lower frequency in nearby lowlander populations. Subsequently, our findings in *EdnrB*$^{-/+}$ mice exquisitely depicted that these mice were resistant to various levels of hypoxia, maintained higher CO, peripheral perfusion and better O_2 delivery to vital organs, even in severe hypoxia [3]. In other words, by downregulating *EdnrB,* mice were able to conserve cardiac function when exposed to extreme hypoxia. There is other evidence also demonstrating the advantages of lower *EDNRB* in the heart. For example, a lower *EDNRB* in patients with ischemic heart disease is thought to be beneficial [14, 15].

The EDNRB receptor is a G protein-coupled receptor that activates a phosphatidylinositol - calcium (Ca^{2+}) second messenger system [16]. The gene is express in most tissues (Fig.4A). Functionally, EDNRB is involved in vasodilatation, opposing EDNRA, which is involved in vasoconstriction [17]. However, the role of EDNRB is much more complex than was originally anticipated. During early development, it plays a critical role throughout neural crest cells migration, proliferation and differentiation [18]. Its disruption leads to phenotypes like Hirschsprung's disease and megacolon [19, 20]. Recent studies specifically involving spatiotemporal KO of *EdnrB* in mice have depicted a variety of roles it can play in different tissues (Fig. 4b). Interestingly, most of the phenotypes affected are directly or indirectly related to cardiovascular function or hypoxia response (Fig. 4b). For example, its direct involvement in blood pressure (BP) or sodium retention was depicted in the collecting duct-specific KO mice [21] and not in the endothelial cell-specific KO [11], unlike previously anticipated. The contribution of endothelial cells' *EdnrB* in BP regulation, however, is indirect as it mediates nitric oxide release and responsible for ET-1 clearance from the circulation [22, 23]. At the same time, the *EdnrB* in smooth muscle contributes to an increased BP but surprisingly had no role in vascular contraction [24]. Even in our previous study on $EdnrB^{-/+}$ mice the BP was consistently higher but we did not observe any increase in circulating ET-1 [3]. To summarize, an overview of whether *EDNRB* is expressed in the particular tissues and major phenotype observed when the gene is KO in that specific tissue is simplified in Fig 4.

EdnrBs' involvement in cardiac function, especially in extreme hypoxic stress, has been an important issue because of the maintenance of cardiac function in $EdnrB^{-/+}$. Available literature suggests that all the major components of the endothelin system, *Edn1*, *EdnrA* and *EdnrB* are expressed in cardiomyocytes. Although *Edn1* expression is reportedly necessary for cardiomyocyte's survival and maintenance of normal cardiac function, even during stressful conditions [25], we did not observe any difference in its level in the $EdnrB^{-/+}$ mice at baseline [3]. Of the two receptors, only *EdnrB* was reportedly present on the cardiomyocytes' nuclear membrane and was associated with regulation of nuclear Ca^{2+} signaling [26]. This may be an indication of the important role it plays in contractility. In addition, another study just confirmed the role of nuclear membranes' EDNRB in Ca^{2+} signaling [27], but the authors noted this in endocardial endothelial cells of human left ventricles. From our present study, it is clear that the *EdnrB* in cardiomyocyte has an important role in maintaining cardiac contractility especially under acute stressful condition i.e., severe hypoxia. We used inducible cardiomyocyte specific driver because our intention was to KO *EdnrB* only in the adult cardiomyocytes to avoid the spatiotemporal complexities related to its diverse role (as depicted in Fig. 4b). The cardiomyocyte's specific *EdnrB* KO is viable and therefore we could test hypoxia tolerance in both heterozygote ($EdnrB^{flox/+}$) as well as homozygote ($EdnrB^{flox/flox}$) KO mice. Remarkably, similar to $EdnrB^{-/+}$, cardiac specific *EdnrB* KO mice, both $EdnrB^{flox/+}$ and $EdnrB^{flox/flox}$, withstood various levels of hypoxia with a significant cardiac tolerance. For example, CI was better maintained at 10% and 5% O_2 by both $EdnrB^{flox/+}$ and $EdnrB^{flox/flox}$ (Fig. 2a) with lower lactate levels (Fig. 2b), and the differences were profoundly visible particularly at 5% O_2. Interestingly, the current study also indicates that, while the cardiomyocytes' *EdnrB* makes a significant contribution to hypoxia tolerance by protecting the heart during hypoxia by maintaining CI,

it does not fully account for the whole phenotype that we observed in the *EdnrB*$^{-/+}$. For example the higher MAP is seen in *EdnrB*$^{-/+}$ but not in the cardiomyocyte-specific EdnrB down-regulation. Only at 10% O_2 was the SPB and DBP differs between *EdnrB*$^{flox/+}$ and controls.

Finally, our current study using EDNRB blocker comprehensively illustrates the protective role of lower EDNRB under extreme hypoxia. Data from the literature show that both Bosentan and specific blockers of EDNRA prevent the increase in pulmonary artery pressure during hypoxia and help in the restoration of oxygen saturation [28–31]. Since CI is maintained during hypoxia and lactate levels are lower in the EDNRB specific blocker (BQ-788)-treated mice, we suggest that both EDNRA and EDNRB are involved in hypoxia tolerance. However, in this study, which focuses on cardiac measurements, we demonstrate that cardiac specific EDNRB (*EdnrB*$^{flox/+}$ and *EdnrB*$^{flox/flox}$) are involved in maintaining cardiac output.

Given that *EDNRB* was first identified in human HA dwellers from Ethiopia known to be best adapted to HA [32], it is interesting to note a recent study showing that the level of *EDNRB* in serum was significantly higher in chronic mountain sickness (CMS) patients [33]. Furthermore, the cerebrovascular reactivity (CRV), i.e., the change in cerebral blood flow in response to a stimulus, was reportedly lower in these patients pretty much similar to patients with cardiac failure [34]. This is an interesting observation because (i) we anticipated lower *EdnrB* to be protective at HA and higher as seen in CMS is deleterious. (ii) This was a study done in Tibetan HA population and we discovered *EDNRB* as a candidate gene in the Ethiopian HA natives where no case of CMS was ever reported. Therefore, it will be interesting to explore the differences in genetic variants at this locus between these two populations. (iii) A lower CRV, as depicted in cardiac failure patients, possibly indicates that the heart in these CMS patients is less efficient. And we know from our study, both global and cardiac specific *EdnrB* KO mice, that a lower *EdnrB* under hypoxic environment could lead to a better cardiac function. Furthermore, similar to our *EdnrB* study, the ET-1 levels were similar when CMS subjects were compared to controls. Alternatively, the higher EDNRB in CMS can also be correlated with the inhibitory role of EDNRB in suicidal erythrocyte deaths and therefore preventing ‘eryptosis’ [35]. In other words, a higher EDNRB in CMS may be inducing polycythemia while a lower level plausibly promoting eryptosis and lowering the abnormally high hematocrit levels.

In summary, it is reasonable to conclude that by lowering *EdnrB*, genetically or pharmacologically, plays an important role in hypoxia tolerance. Specifically, the cardiomyocyte *EdnrB* plays the major role in hypoxia tolerance in the *EdnrB*$^{-/+}$ mice model. And when we connect to a comprehensive role of EDNRB at HA, it appears that the protective role of a lower *EDNRB* is potentiated through its two-pronged hypoxia-tolerance-strategy, i.e., by (i) sustaining cardiac function, as seen in *EdnrB* KO and BQ-788 treated mice under extreme hypoxia and (ii) preventing hypoxia-induced excessive erythrocytosis. This may indicate better adaptation in the HA Ethiopians or lack of CMS thereof. An important question that remains unclear is related to the molecular mechanisms leading to protection against hypoxic injury. Since we now have the crystal structures of *EDNRB* in

both ligand-free form and in complex with *EDN1* [36], progress towards novel therapies is possible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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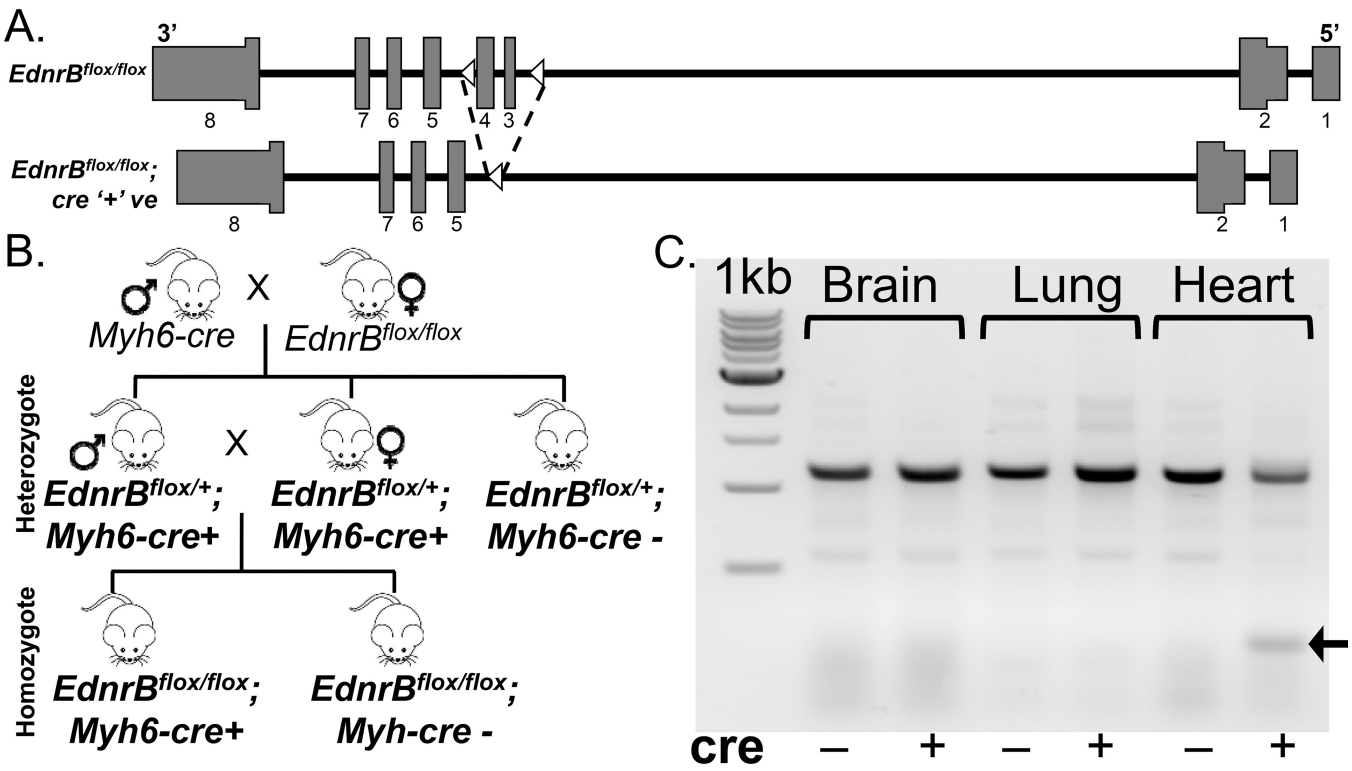

Fig 1:

Generation of cardiac specific *EdnrB* KO. a) *EdnrB* gene in mice before and after Cre-LoxP recombination. B) Schematic of the crosses between $EdnrB^{flox/flox}$ and *Myh6-cre* to generate heterozygote ($EdnrB^{flox/+}$) and homozygote ($EdnrB^{flox/flox}$) KO mice. b) Gel picture depicting tamoxifen induced exon 3–4 deletion in the Heart.

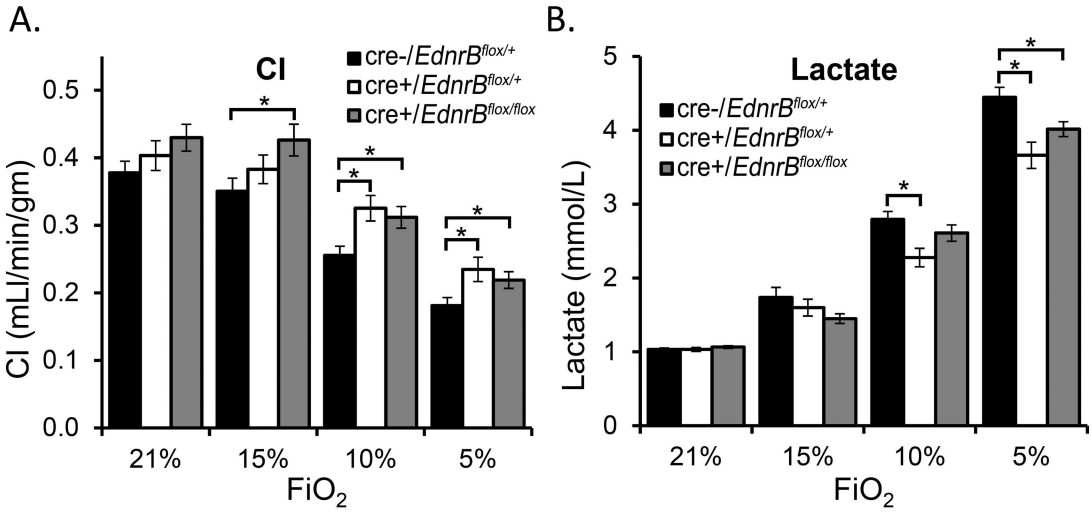

Fig 2:

Cardiac index and blood lactate level comparisons between cardiac specific *EdnrB* homozygous (*EdnrB*$^{flox/flox}$), heterozygous (*EdnrB*$^{flox/+}$) and cre-negative control mice. a) Both *EdnrB*$^{flox/flox}$ and *EdnrB*$^{flox/+}$ mice maintains a higher CI under extreme hypoxia of 10% and 5% O_2. b) Lactate levels were significantly lower in *EdnrB*$^{flox/+}$ mice at 10% and 5% O_2 when compared with the cre-negative group and in *EdnrB*$^{flox/flox}$ at 5% O_2. The trends were inversely related to the CI where it is high in the cre-negative controls, medium in the *EdnrB*$^{flox/flox}$ group and low in the *EdnrB*$^{flox/+}$ group. Error bar indicates ± standard error.

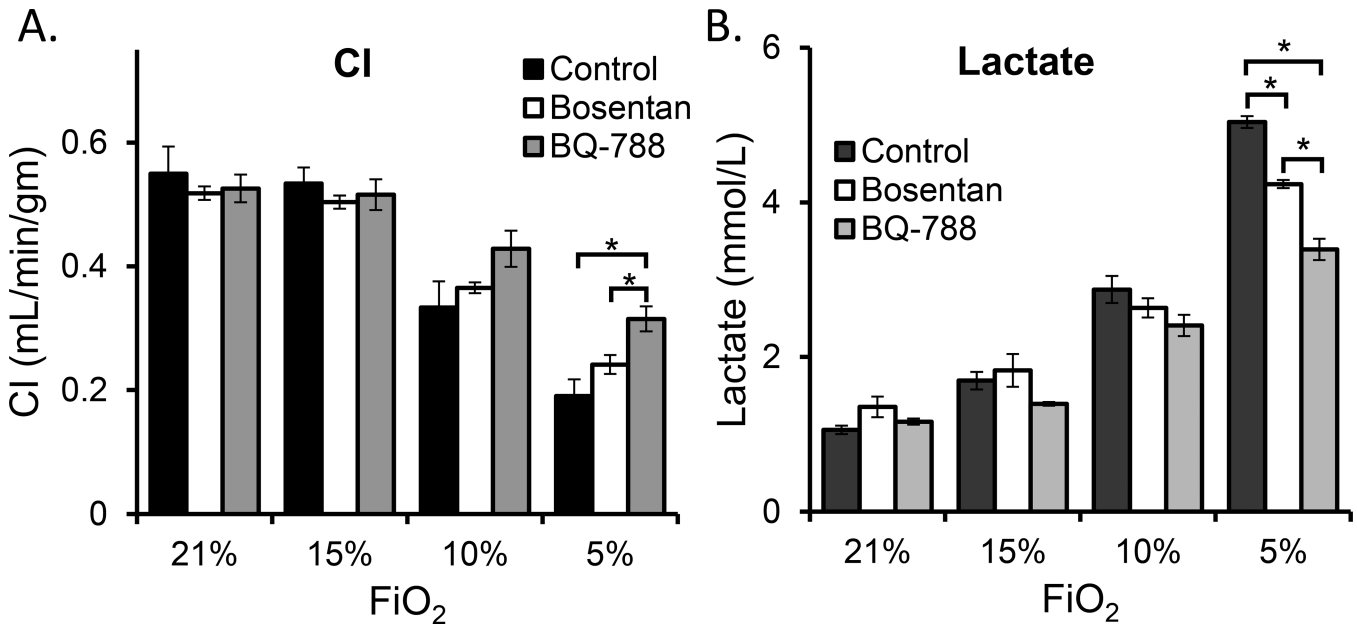

Fig 3:

Cardiac index and blood lactate level comparisons between BQ-788, Bosentan treated and control mice. a) Both Bosentan and BQ-788 treated mice maintains a relative higher CI under extreme hypoxia of 10% and 5% O_2. However, the level in BQ-788 treated mice was significantly higher at 5% O_2 when compared to both Bosentan and control mice. b) Lactate levels at 10% O_2 show a trend towards where it is high in the controls, medium in the Bosentan treated mice and low in the BQ-788 treated mice. The difference is significant ($P<0.005$) between all the three groups. The error bar indicates ± standard error.

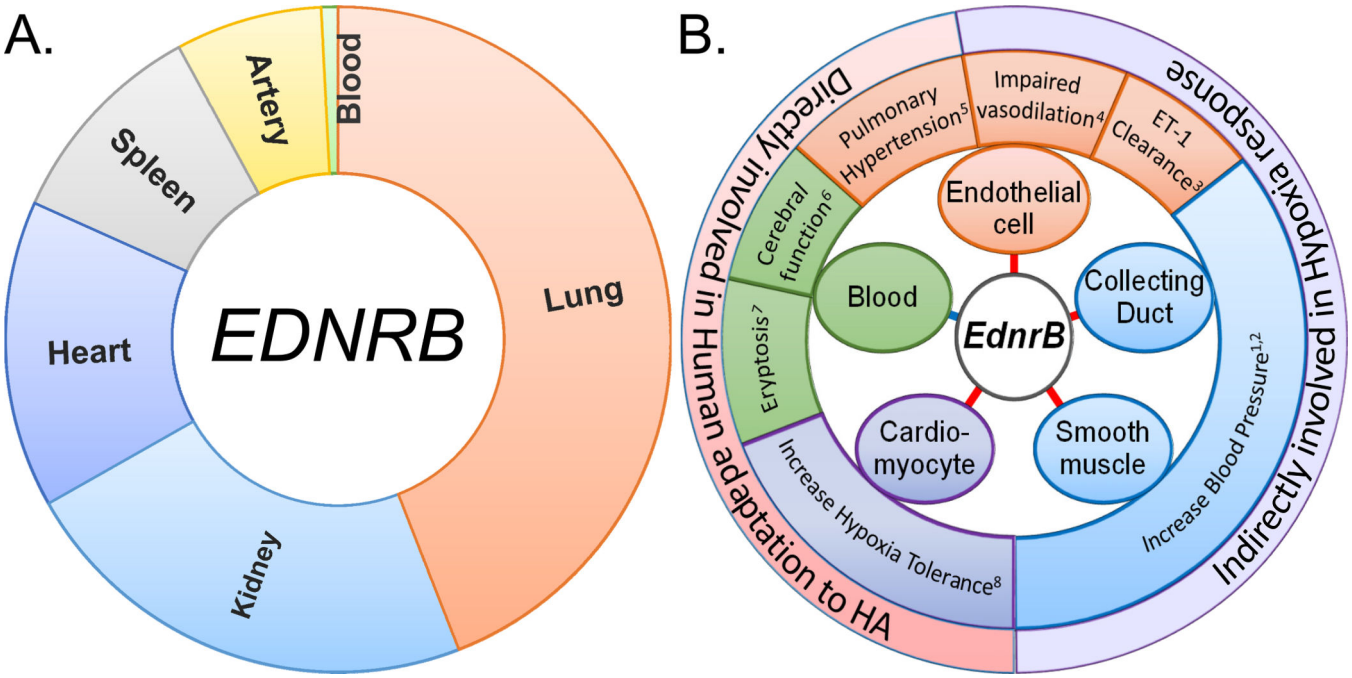

Fig 4:

EDNRB expression and phenotype associated with tissue specific KO of this gene. a) Tissues wise *EDNRB* expression in humans. RPKM value for *EDNRB* were obtained from GTEx portal (https://gtexportal.org/)). b) Diverse role of *EdnrB* identified using tissue specific KO mice (red line) and levels measured in the whole blood/serum (blue line). Middle zone depicts the phenotype related to the tissue specific KO study (color-coded). For the role of *EdnrB* in blood, 'eryptosis' was retrieved from rescued global *EdnrB* KO mice. The 'cerebral function' was indicated by 'cerebral vasoconstriction reactions' and the EDNRB level measured in the blood serum of CMS patients. All indicate a direct or indirect involvement in hypoxia adaptation. The respective numbers indicate the related references: 1, *Ge et al. (2006)*; 2, *Miller et al. (2017)*; 3, *Kelland et al. (2010a)*; 4, *Bagnall et al. (2006)*; 5, *Kelland et al. (2010b)*; 6, *Wu et al. (2016)*; 7, *Foller et al. (2010)*; 8, *Present study*.