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Review Article

Hemoglobin α in the blood vessel wall

Joshua T. Butcher [a], Tyler Johnson [a], Jody Beers [b], Linda Columbus [c], Brant E. Isakson [a,d,*]

[a] Robert M. Berne Cardiovascular Research Center, University of Virginia, Charlottesville, VA 22908, USA
[b] Hopkins Marine Station, Stanford University, Pacific Grove, CA 93950, USA
[c] Department of Chemistry, University of Virginia, Charlottesville, VA 22908, USA
[d] Department of Molecular Physiology and Biophysics, School of Medicine, University of Virginia, Charlottesville, VA 22908, USA

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ABSTRACT

Hemoglobin has been studied and well characterized in red blood cells for over 100 years. However, new work has indicated that the hemoglobin α subunit (Hbα) is also found within the blood vessel wall, where it appears to localize at the myoendothelial junction (MEJ) and plays a role in regulating nitric oxide (NO) signaling between endothelium and smooth muscle. This discovery has created a new paradigm for the control of endothelial nitric oxide synthase activity, nitric oxide diffusion, and, ultimately, vascular tone and blood pressure. This review discusses the current knowledge of hemoglobin's properties as a gas exchange molecule in the bloodstream and extrapolates the properties of Hbα biology to the MEJ signaling domain. Specifically, we propose that Hbα is present at the MEJ to regulate NO release and diffusion in a restricted physical space, which would have powerful implications for the regulation of blood flow in peripheral resistance arteries.

Contents

Introduction

For over 100 years [1], hemoglobin has been known as the gas exchange molecule found within red blood cells (RBCs) that is responsible for delivering oxygen to tissues and subsequently removing carbon dioxide [2]. The structure of normal functional adult hemoglobin (HbA; Fig. 1A) is composed of twin α and β globin subunits ($\alpha_2\beta_2$), each of which contains a heme, or iron ion, within a heterocyclic ring of four pyrrole (C_4H_4NH) molecules known as a porphyrin. The assembled tetramer can interact with the aforementioned gases, as well as carbon monoxide and nitric oxide (NO). Today, this paradigm in RBCs remains unchanged. However we [3], and others [4,5], have demonstrated that Hbα is expressed in the blood vessel wall but, in contrast to other globins in the blood vessel wall [3,6,7], Hbα localization and physiological effects are concentrated at myoendothelial junctions (MEJs) in the endothelial cells (ECs) lining the lumen of blood vessels.

Although the discovery of Hbα in the blood vessel wall is unique, there have been clues from human diseases of Hbα deletion, possibly independent of RBC function, that already indicated its possible presence. The Hbα gene is located on chromosome 16 and has two identical but duplicated coding sequences, HBA-1 and HBA-2. Genetic deletion of or loss-of-function mutations in the Hbα alleles are known

* Corresponding author at: University of Virginia School of Medicine, Robert M. Berne Cardiovascular Research Center, P.O. Box 801394, Charlottesville, VA 22908, United States. Fax: +1 434 924 2828.
E-mail address: brant@virginia.edu (B.E. Isakson).

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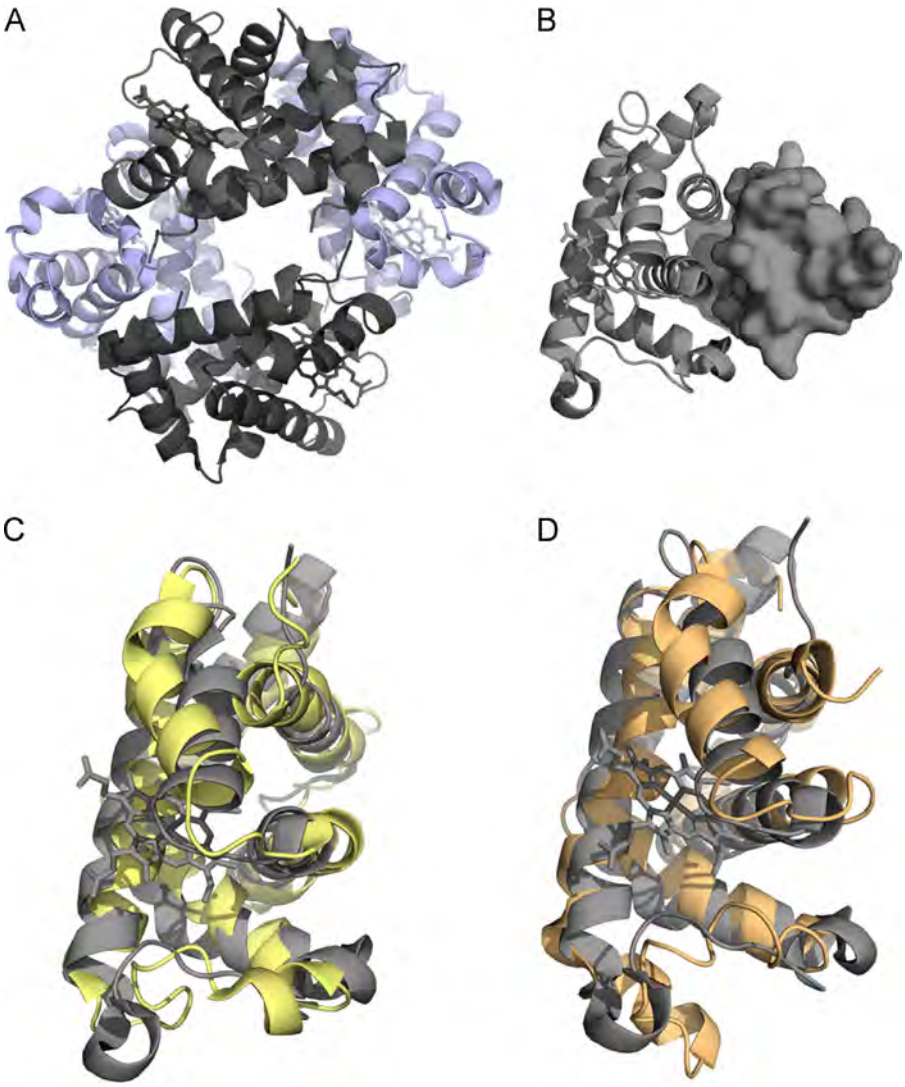

Fig. 1. Structure of Hbα. (A) HbA is a heterotetramer with two α subunits (gray) and two β subunits (purple). (B) Monomeric Hbα (rendered in cartoon) is stabilized by AHSP (surface representation). The overall backbone fold of Hbα bound to AHSP (C and D, gray) is similar to Hbα (C, yellow) and Hbβ (D, orange) of HbA.

as α-thalassemia, a condition that is defined by the severity of changes to red blood cell indices [8,9]. Patients with α^{+}-thalassemia (silent carriers) will have deletion of or loss-of-function mutation in one of the HBA-1 or HBA-2 alleles. However, compensation enables them to remain asymptomatic and many go undiagnosed. Deletion of two of the HBA-1 and HBA-2 alleles causes α^{0}-thalassemia, which presents with moderate or severe RBC indices, depending on the deletion type or mutation [9]. In terms of the vascular phenotype, this frequently includes moderate hypotension. Deletion of three of the HBA-1 and HBA-2 alleles is known as HbH disease and is characterized by hemolytic anemia, hepatosplenomegaly, and the formation of a tetramer of β chains in vivo. Owing to the severity of RBC indices, it remains unclear if the vascular phenotype remains consistent with α^{0}-thalassemia or is hidden by development of other pathologies. Hydrops fetalis syndrome (Hb Bart syndrome) occurs with deletion of all of the Hbα alleles and results in death in utero [10]. The vascular phenotype of dilated cerebral arteries is observed via ultrasound and also is used to assist in the diagnosis of the disease although difficult to delineate from the accompanying severe anemia (Table 1) [11–15]. Regardless, this would correlate with observations from patients with different degrees of Hgα deletion presenting with differences in capillary diameter [16]. In each of these cases, a role for Hbα in RBCs is challenging to fully explain the vascular phenotype presented. However, reduction or deletion of Hbα in ECs of the resistance arteries, where it is hypothesized to regulate NO delivery to vascular smooth muscle cells (VSMCs), could be a reason. Future research in this area could lead to important new insights into the pathology as well as diagnosis related to these diseases.

Hemoglobin α, *certae sedis*

The first evidence of localization and function for Hbα in the blood vessel wall was seen in MEJs from resistance arteries in the systemic vasculature [3]. Resistance arteries are the small arteries that contribute the greatest amount to peripheral resistance and thus overall blood pressure regulation [17,18]. In resistance vasculature heterocellular communication is critical to the maintenance of vascular tone and blood pressure (for review, see [19,20]). Crucial to heterocellular communication in resistance arteries is the presence of MEJs. The MEJ is the physical link between ECs and VSMCs, characterized as a small protrusion of mostly ECs (approximately 0.5 μm wide and long) through the internal elastic lamina, linking with VSMCs through gap junctions [12]. Myoendothelial junctions are found throughout the vasculature; however, a gradient is observed such that the junctions are more prevalent as the diameter of the vascular tree decreases [21]. Thus, MEJs represent the closest physical location of ECs to VSMCs in the

Table 1
α-Thalassemias.

Chromosome 16 (both copies shown)			Genotype	RBC indices	Vascular phenotype	Classification
HBZ	HBA-2	HBA-1				
ζ	α α	α α	αα/αα	Normal	Normal	Normal
ζ	α α	α	α−/αα	Normal	Moderate hypotension with potential compensation	α-Thalassemia minima, also known as heterozygosity for α^{+}-thalassemia, silent carrier of α-thalassemia, and α-thalassemia-2 trait
ζ	α	α α				
ζ	α	α	α−/α− or −−/αα	Minimal anemia, decreased MCV and MCH	Moderate hypotension	α-Thalassemia minor, also known as α-thalassemia-1 trait (due to homozygosity for α^{+}-thalassemia (α−/α−) or heterozygosity for α^{0}-thalassemia (−−/αα))
ζ		α α				
ζ		α	α−/−−	Hemolytic anemia with the formation of β-chain tetramers	None documented	Hemoglobin H (HbH) disease
ζ			−−/−−	Severe anemia due to formation of γ4 tetramers (hemoglobin Bart's)	Hydrops fetalis with increased cerebral blood flow	Hydrops fetalis syndrome with hemoglobin Bart's

Chromosome 16 contains two identical coding sequences for hemoglobin α, hemoglobin α2 and hemoglobin α1 (shown here in reference to the ζ-globin gene, which is also located on chromosome 16). Deletions/nonfunctional mutations are depicted by the absence of a corresponding box. Genotype, red blood cell (RBC) indices, vascular phenotype with relation to blood pressure, and medical classification of the various α-thalassemia's are also listed [8,10,44,74].

small resistance arteries. The MEJ is acknowledged as an important signaling microdomain (for review, see [22]) not only because of its spatially limited structure, but also because the MEJ serves as the gateway between EC signal transduction to the VSMC [22]. For instance, S-nitrosylation/denitrosylation of connexin 43 is controlled by specific proteins localized to the MEJ, including *S*-nitrosoglutathione reductase (GSNOR), endothelial nitric oxide synthase (eNOS), and IP_3 receptor type 1 [23]. Together, these proteins participate in localized signaling that promotes negative feedback on vasoconstriction induced by α_1-adrenergic receptor activation. Importantly, whereas eNOS is localized to intracellular regions including plasma membrane caveolae and the Golgi complex, it is also localized to the MEJ [24,25]. Endothelial cells exhibit a strict and varied control over the production of NO (reviewed in [26]) and the predominant enzyme responsible for the generation of NO in these cells is eNOS. This localization of eNOS at the MEJ presumably occurs because NO is a highly reactive gaseous free radical, enabling targeted release of NO at proximal sites to the overlying VSMCs, where it functions as a potent vasodilator. Subsequent work demonstrated that eNOS and Hbα reside in close proximity to each other and potentially form a macromolecular complex at the MEJ [3], where they provide a regulatory mechanism for NO-mediated vasodilation (Fig. 2).

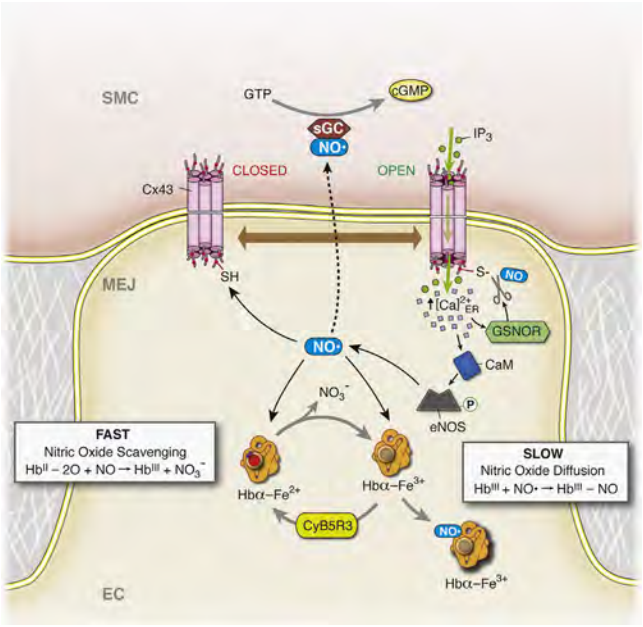

Fig. 2. Schematic of NO regulation at the MEJ: negative feedback after α_1-adrenergic receptor-induced vasoconstriction at the myoendothelial junction (MEJ) of an endothelial cell (EC). α_1-Adrenergic receptor agonists cause an increase in smooth muscle cell (SMC) cytosolic inositol 1,4,5-trisphosphate (IP_3), which travels down its concentration gradient through open (i.e., S-nitrosylated) connexin 43 (Cx43) channels. IP_3 binds to localized IP_3-receptor 1 on the endoplasmic reticulum (not shown), which causes localized calcium (Ca^{2+}) release and an increase in cytosolic Ca^{2+} concentration at the MEJ. This increase in cytosolic Ca^{2+} facilitates activation of *S*-nitrosoglutathione reductase (GSNOR), which denitrosylates and closes Cx43 channels, making them impermeable to IP_3. The increase in cytosolic Ca^{2+} also activates calmodulin (CaM) through binding-induced conformational changes. The Ca^{2+}/CaM complex binds to endothelial nitric oxide synthase (eNOS), facilitating its phosphorylation, activation, and production of nitric oxide (NO). Some of this newly produced NO will diffuse into SMCs, where it binds to and activates soluble guanylyl cyclase (sGC), facilitating the conversion of GTP to cGMP, which ultimately leads to a reduction in constriction. The newly produced NO may also facilitate opening of Cx43 channels through S-nitrosylation. Finally, excess NO may be scavenged by free hemoglobin α (Hbα) at the MEJ through reaction with oxyhemoglobin α (fast), or NO may be chelated through reaction with methemoglobin α (slow). Cytochrome b_5 reductase (CyB5R3) converts methemoglobin α to Hbα, which readily binds oxygen [3,6,21–23].

Three general outcomes await NO once it is produced in EC: it can (1) diffuse into the bloodstream where it is rapidly scavenged, first by cell-free hemoglobin in the plasma and even further by hemoglobin in RBCs [27]; (2) diffuse to neighboring VSMCs and cause vasorelaxation; or (3) be scavenged by any number of molecules, such as reactive oxygen species (ROS) (for review, see [28]). In the bloodstream, most NO diffuses across its concentration gradient into RBCs, where it is either scavenged or stored. NO scavenging proceeds via oxidation by oxyhemoglobin, which produces methemoglobin and nitrate. This is the favored reaction; however, NO can also bind directly to deoxygenated hemoglobin in a simple addition reaction to form iron–nitrosyl–hemoglobin [29]. Furthermore, it has been proposed that nitric oxide reacts in an oxygen-sensitive mechanism with the conserved Cys93 residue of the hemoglobin β-chain [30]; however, these findings have been intensely debated [31–33]. Finally, data have suggested that eNOS is present in RBCs [34], which possibly contributes to vasoprotectivity [35–37]. Once in the VSMC, NO activates soluble guanylyl cyclase by binding to its heme moiety, allowing a several hundredfold increase in the catalysis of GTP to cGMP [38] and causing vasodilation through several mechanisms [39]. Scavenging of NO occurs in pathologies that result in, produce, or maintain elevated oxidant stress, especially when accompanied by an inability to be counterbalanced by antioxidants. Increased ROS can scavenge NO directly or alter pathways that mediate NO production. The multiple fates of NO in the vasculature are further complicated by its very short half-life (<5 s) [40]. Conceivably, in the ECs at the MEJ, any one of the above mechanisms may be in play [41].

An important regulator in the binding of NO (and oxygen) to hemoglobin is the oxidation state of the heme iron: ferrous (Fe^{2+}) or ferric (Fe^{3+}). The ferric state of hemoglobin is also frequently known as methemoglobin (MHb) [42,43]. However, the HbA in the bloodstream contains less than 1% MHb because it possesses a higher affinity for oxygen and excess MHb can result in tissue hypoxia and even death [44]. The oxidation state of iron also controls the sensitivity of Hbα to NO; the Fe^{2+} state rapidly scavenges NO and the resulting production of nitrate and methemoglobin decreases NO bioavailability, whereas the heme moiety in the Fe^{3+} state reacts slowly and transiently with NO, allowing for increased diffusion of NO into smooth muscle [45–47]. This reaction would presumably hold true for the Hbα found in the MEJ. In RBCs, a methemoglobin reductase, cytochrome b_5 reductase 3 (CytB5R3, also known as diaphorase 1), is present to recycle the Fe^{3+} state and prevent the accumulation of MHb. When CytB5R3 is absent because of genetic defect or ingestion of oxidizing toxins the resulting methemoglobinemia [48] (famously characterized as a bluish tint to the skin) causes decreased tissue oxygenation, hypoxia, and cyanosis [49]. Not surprisingly, the enzyme CytB5R3 found in RBCs is also found in ECs and at the MEJ (although not as strictly localized as Hbα). However, in contrast to the RBC in which the Fe^{2+} oxidation state predominates, the MEJ heme moieties in resistance arteries exist in both states (Fe^{3+} approximately 58%). Knockdown of CytB5R3 reveals altered reactivity in ex vivo vessels to adrenergic and endothelial-dependent NO stimulus, revealing a permissive effect of CytB5R3 on NO bioavailability [3]. Interestingly, the CytB5R3 inhibitor and anti-thyroid drug propylthiouracil (PTU) also presents with a reduction in blood pressure in rats [50]. This could be explained by PTU preventing the CytB5R3 enzyme from reducing the Fe^{3+} heme moieties in Hbα at the MEJ, thus maintaining the slower NO-scavenging reaction and allowing for increased NO diffusion into the VSMC. The evidence increasingly indicates that Hbα could play an important role in regulating NO bioavailability through control of the heme oxidation state.

Lastly, it is possible that Hbα is found in resistance arteries to chelate excessive NO. This rationale is based on the fact that conduit arteries have been previously characterized as largely dependent on NO for vasodilator activity, whereas smaller resistance

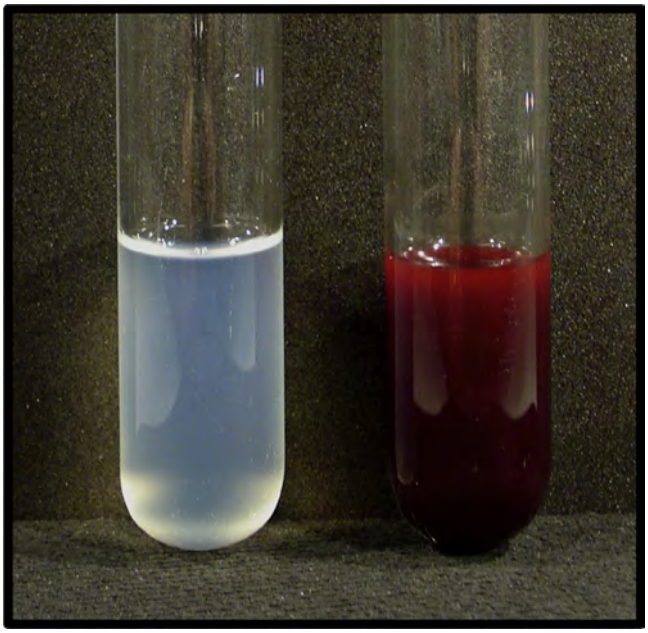

Fig. 3. Blood from HbA and HbA-null fish. Blood samples from two Antarctic notothenioid fishes illustrate a striking contrast in the level of hemoglobin expression. The test tube on the right contains blood from an HbA-expressing species, *Notothenia coriiceps*, and the tube on the left depicts the completely HbA-null phenotype of the "crocodile" icefish, *Chaenocephalus aceratus*.

vessels rely on the action of endothelium-derived hyperpolarizing factor (EDHF) [22,51]. However, there is no observable difference in the total protein expression of eNOS in carotid and third-order mesenteric arteries (i.e., resistance arteries; Fig. 3). Thus, Hbα at the MEJ in resistance arteries may be chelating the NO generated by eNOS, allowing for EDHF to remain dominant for increased endothelial-dependent vasodilation, and maintaining strict control over NO for other cellular functions (e.g., negative feedback after vasoconstriction). It remains unclear how Hbα scavenges the excessive NO and whether this action results in a permanent loss of NO or simply a reservoir of NO that can be released upon appropriate stimulus. It is also possible that the amount of eNOS between resistance arteries and conduit arteries is inconsequential because the eNOS that is present in resistance arteries is uncoupled compared to that in conduit arteries, negating eNOS generation (e.g., [52,53]). It is unclear why such a potent enzyme would still be present if this were the case; however, the possibility exists and it is clear more work needs to be done in this regard.

The importance of NO scavenging by Hbα at the MEJ is predominantly derived from its location. Nitric oxide scavenging by RBC hemoglobin requires diffusion through the EC monolayer, the glycocalyx, and the plasma, and then NO interacts with cell-free hemoglobin or must cross the RBC membrane before it reaches HbA. Based on these assumptions, it is tempting to speculate that the likelihood of locally produced NO at the MEJ interacting with Hbα is greatly increased because of its proximity to the NO source. However, NO produced on the luminal side of the endothelium would favor scavenging by RBC hemoglobin. This implies that although the relative abundance of Hb in RBCs may be greater, its importance for the regulation of diffusion of NO into VSMCs may be reduced, although this may change with deviation from basal conditions or pathological states.

Why hemoglobin α in the endothelium?

One of the interesting questions that arises from the discovery of Hbα at MEJs of ECs is, why Hbα, and not Hbβ? Wouldn't both subunits be better than one for microcirculation where MEJs are prevalent? As of yet there is no clear answer to this question.

Expression of both hemoglobin subunits has been reported in many cell types beyond erythrocytes [54–59], which has sparked hypotheses of functions in addition to oxygen transport and delivery. The oxygen transport by Hb is specific to vertebrates, with globins in other walks of life having a variety of functions such as NO dioxygenase and peroxidase activity [60]. Individually, the Hb subunits have damaging effects; thus, the assembly of HbA is regulated. Only Hbα has a chaperone, α hemoglobin stabilizing protein (AHSP), which maintains Hbα solubility and reduces reactive oxygen species production by stabilizing the Fe^{3+} state [61]. AHSP binds to Hbα in proximity to the Hbβ binding site (Fig. 1B) and is displaced during the assembly of Hb. Therefore, Hbα subunits may be considered the less toxic of the two subunits for additional physiological roles owing to the added protection of AHSP.

The Hbα and Hbβ subunits have very similar structures (Fig. 1C and D) yet very different amino acid sequences (43% identity). There are some structural rearrangements of Hbα upon binding AHSP; however, the overall structure is very similar to both Hbα and Hbβ in the heterotetramer. Both subunits similarly bind heme; thus, in terms of oxygen binding and reactivity the subunits are similar. However, the sequence differences between the subunits could certainly provide differences in signaling. For instance, Hbα molecular interactions that regulate NO signaling at the MEJ [3] may be mediated by distinct amino acid regions of Hbα that are different from Hbβ such that Hbβ would not elicit the same signaling cascade. More work on this fundamental question will certainly be required.

Vasculature effects of genetic hemoglobin deletion

In mice, similar to humans, deletion of all of the Hbα alleles is lethal, making genetic studies on animals in vivo difficult. However, there are other animal models found in nature in which Hbα and Hbβ have been reported to be deleted, yet the animals still prove viable, with very interesting effects on the vasculature [62,63]. These animals, icefishes (family Channichthyidae), dominate the fish fauna of the Southern Ocean surrounding Antarctica [64]. Icefishes are exceptional in that they are the only known vertebrate animals to completely lack the oxygen-carrier HbA in their blood [65]. Loss of this key respiratory protein has produced a phenotypically pale, translucent white blood and has earned these animals the common names of "white-blooded fishes" and "icefishes" (Fig. 4) [66]. Absent a gaseous transporter protein, oxygen is carried unassisted in solution in icefish blood and results in an oxygen-carrying capacity that is $<10\%$ of that exhibited by red-blooded notothenioid fishes [67]. Given this consequence and the fact that the HbA-null condition proves lethal in all other examples, both laboratory-based and natural, how has this

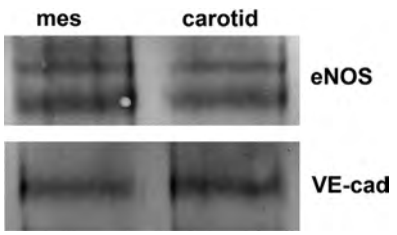

Fig. 4. Expression of eNOS between resistance and conduit arteries is equal. Representative Western blot analysis of endothelial nitric oxide synthase (eNOS) protein level in third-order mesenteric arteries compared to carotid. Samples were equalized according to the amount of VE-cadherin, an endothelial cell marker.

condition persisted in icefishes? Although more complex and beyond the scope of what we can present here (for an excellent review, see [68]), there are several basic considerations that help to answer this question. First, icefishes possess cardiovascular systems with unusually enhanced features compared to their red-blooded notothenioid relatives; large hearts, large-diameter capillaries, and large blood volumes collectively enable icefishes to maintain a high-throughput circulatory design without excessive pressure development [69,70]. Combined with the abundantly high oxygen content of Antarctic waters and their relatively low metabolic rates, these cardiovascular traits permit icefishes to sufficiently oxygenate their tissues and support their aerobic mode of metabolism [71]. As one might expect, however, the loss of expression of HbA has implications for the metabolism of NO. It was proposed that the loss of NO-oxygenase activity with genetic deletion of HbA may have led to subsequent elevation of NO levels that could explain many, if not all, of the unique cardiovascular and physiological traits that evolved in icefishes [68]. Substantiating at least part of that hypothesis, Beers et al. [72] established that NO concentrations in blood plasma appear to be greater in icefishes than in HbA-expressing species. They also reported that the high NO levels in icefishes were not the result of greater synthesis but, rather, appeared to be due primarily to the loss of the degradative pathway for NO [72].

Building upon the above work, Borley and collaborators [73] subsequently conducted a study in which they induced severe anemia in *Notothenia coriiceps,* an HbA-expressing notothenioid with a normal hematocrit of 35–40%. Surgically implanted osmotic pumps were used to treat individuals with a powerful hemolytic agent that resulted in a drastic reduction in hematocrit ($>90\%$) and HbA concentration ($>70\%$). Levels of NO were significantly higher in anemic animals compared to the full HbA-expressing controls and were similar to the levels of NO reported for white-blooded icefishes [72,73]. Although MEJs have not been identified in these fish, it would be surprising if these anatomical structures were not present. The regulation of NO in the arteries of icefish could be one potential avenue for understanding how Hbα regulates NO delivery in arteries with an in vivo model capable of withstanding severe hemoglobin depletion.

Summary

This review describes a new paradigm of localized NO regulation by Hbα in MEJs of resistance arteries. This unique microdomain has the potential for pharmacological targeting and serves as an explanation for several different pathologies associated with Hbα deletion. Although some investigation into this observation has been done, there is still much to do and we look forward to extending this review in the future.

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