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Commentary

The dual roles of red blood cells in tissue oxygen delivery: oxygen carriers and regulators of local blood flow

Frank B. Jensen
Institute of Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark
fbj@biology.sdu.dk

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Summary

Vertebrate red blood cells (RBCs) seem to serve tissue oxygen delivery in two distinct ways. Firstly, RBCs enable the adequate transport of O_2 between respiratory surfaces and metabolizing tissues by means of their high intracellular concentration of hemoglobin (Hb), appropriate allosteric interactions between Hb ligand-binding sites, and an adjustable intracellular chemical environment that allows fine-tuning of Hb O_2 affinity. Secondly, RBCs may sense tissue O_2 requirements *via* their degree of deoxygenation when they travel through the microcirculation and release vasodilatory compounds that enhance blood flow in hypoxic tissues. This latter function could be important in matching tissue O_2 delivery with local O_2 demand. Three main mechanisms by which RBCs can regulate their own distribution in the microcirculation have been proposed. These are: (1) deoxygenation-dependent release of ATP from RBCs, which stimulates production of nitric oxide (NO) and other vasodilators in the endothelium; (2) release of vasoactive NO from *S*-nitroso-Hb upon deoxygenation; and (3) reduction of naturally occurring nitrite to vasoactive NO by deoxygenated Hb. This Commentary inspects all three hypotheses with regard to their mechanisms, experimental evidence in their support and details that remain unresolved. The prime focus is on human/mammalian models, where most evidence for a role of erythrocyte ATP and NO release in blood flow regulation have accumulated. Information from other vertebrate groups is integrated in the analysis and used to discuss the evolutionary origin and general relevance of each hypothesis.

Key words: blood flow regulation, erythrocyte, vasodilation, ATP, nitric oxide, nitrite.

Introduction

It is generally recognized that red blood cells (RBCs) enable the transport of sufficient O_2 between respiratory surfaces (lungs, gills) and metabolizing tissues by means of their high intracellular concentration of hemoglobin (Hb) and appropriate allosteric interactions between ligand (O_2, CO_2, H^+) binding sites in the Hb molecule. In recent years, a new additional role of RBCs has been proposed; namely that RBCs – when they pass through the microcirculation – may sense tissue oxygen conditions *via* their degree of deoxygenation and couple this information to the release of vasodilatory compounds, such as ATP or nitric oxide (NO), that enhance blood flow to hypoxic tissues (Ellsworth et al., 1995; Jia et al., 1996; Cosby et al., 2003). This second function of RBCs could be essential, because tissue O_2 delivery to a large extent depends on blood flow. The emerging picture is that RBCs are not only vehicles that carry large amounts of O_2 to the tissues but that they are also direct effectors of local blood flow. This would contribute to hypoxic vasodilation, a mechanism that ensures fast matching of local O_2 supply with O_2 demand. This phenomenon has been known for more than a century, but its precise mechanisms are not yet fully understood (Gladwin et al., 2006).

Three different mechanisms have been proposed in the literature. It has been hypothesized that RBCs can: (1) release ATP that stimulates endothelial NO production (Ellsworth et al., 1995); (2) release nitric oxide from *S*-nitroso-Hb upon deoxygenation (Jia et al., 1996); and (3) reduce naturally occurring nitrite (NO_2^-) to vasoactive NO *via* the nitrite reductase activity of deoxyhemoglobin (Cosby et al., 2003). Controversial aspects of these hypotheses remain to be clarified, but evidence from human/mammalian models supports that NO and ATP release from RBCs is capable of increasing blood flow to hypoxic/exercising tissues.

Previous reviews have typically focused on one of the above-mentioned mechanisms and on mammalian models, where most studies have been performed. The present Commentary gives an overview of all three mechanisms, including their pros and cons, and applies a comparative approach, discussing the evolutionary origin of the mechanisms and their possible significance in lower vertebrates.

The 'classical role' of RBCs in O_2 transport

The red blood cells are highly adapted to serve their function in blood gas transport. They are densely packed with Hb molecules (~5 mmol tetramers per liter RBC), which secures an O_2 transporting capacity in the blood that amounts to some 9 mmol O_2 l^{-1} in endothermic mammals and birds (hematocrit ~45%) and some 5 mmol O_2 l^{-1} in ectothermic vertebrates (hematocrit ~25%); the difference reflecting the different metabolic rates and therefore O_2 transport requirements of endotherms and ectotherms. RBCs are also renowned for their deformability, allowing them to pass through capillaries that often have a smaller diameter than the RBCs (Nikinmaa, 1990). The O_2 binding and delivery properties of RBCs are guided by the allosteric properties of the Hb molecule inside the cells. The tetrameric Hb molecule is in equilibrium between two quaternary structures, the relaxed (R) structure with high O_2 affinity (characterizing oxygenated Hb, oxyHb) and the tense (T) structure with low O_2 affinity (characterizing deoxygenated Hb, deoxyHb). At the high oxygen tensions (P_{O_2}) prevailing at the respiratory surfaces, the blood will

normally become fully saturated with oxygen, and Hb will assume the R structure. As the blood enters the microcirculation, the P_{O_2} decrease will promote O_2 offloading from hemoglobin and a shift to the T structure. The homotropic interaction between Hb O_2 binding sites (cooperative O_2 binding) gives a sigmoid O_2 equilibrium curve. At rest, only 25% of the O_2 is extracted from the blood, and the venous point (i.e. Hb O_2 saturation at venous P_{O_2}) will be on the shoulder of the curve. Any further increase in O_2 unloading, as required in exercise, therefore occurs on the steep portion of the O_2 equilibrium curve *via* small decreases in capillary P_{O_2}. Heterotropic interaction between different ligand binding sites further facilitates O_2 offloading, as exemplified by the Bohr effect, which lowers O_2 affinity when pH decreases (Jensen, 2004). Thus, CO_2 (and lactic acid) produced in tissue metabolism leads to formation of H^+, which binds to specific amino acid residues (the Bohr groups) on the Hb molecule and stabilizes the T structure, whereby the O_2 affinity decreases and more O_2 can be offloaded by the Hb at any given P_{O_2} (Fig. 1). This right shift of the O_2 equilibrium curve, achieved by blood acidification during capillary transit, increases the steepness of the O_2 equilibrium curve *in vivo* and elevates capillary P_{O_2} (the O_2 diffusion pressure head) compared with the situation if incoming blood had maintained an unaltered O_2 affinity (Fig. 1).

The O_2 transporting properties of RBCs show considerable plasticity and can be adjusted to variable tissue O_2 needs and environmental constraints *via* changes in intraerythrocytic pH and organic phosphates (Nikinmaa, 1997; Jensen, 2004). Whereas a decreased O_2 affinity is advantageous for O_2 unloading to exercising muscles during activity, an increased O_2 affinity is beneficial for arterial O_2 loading in animals experiencing environmental hypoxia. Exposure to hypoxia induces an instantaneous hyperventilation that improves O_2 uptake across respiratory surfaces, and it additionally increases blood pH (by decreasing P_{CO_2}) and thereby O_2 affinity *via* the Bohr effect. Oxygen-sensitive ion-transport mechanisms in the RBC membrane may also adjust Hb O_2 affinity rapidly *via* pH. Teleost fishes, for instance, release catecholamines to the blood upon acute exposure to hypoxia, which promptly activates a β-adrenergic Na^+/H^+ exchange mechanism in the RBC membrane that selectively increases intracellular pH and O_2 affinity (Nikinmaa, 1997). On a longer time scale (hours), teleost fish reduce the nucleoside triphosphate (ATP and GTP) content of their RBCs, which increases O_2 affinity by diminishing the T-state-stabilizing binding of the phosphates to the Hb and by raising erythrocyte pH (Jensen, 2004). These responses collectively help maintain a high arterial O_2 saturation in the face of a globally lowered P_{O_2}, ensuring that appropriate amounts of Hb-bound O_2 continue to reach the microcirculation.

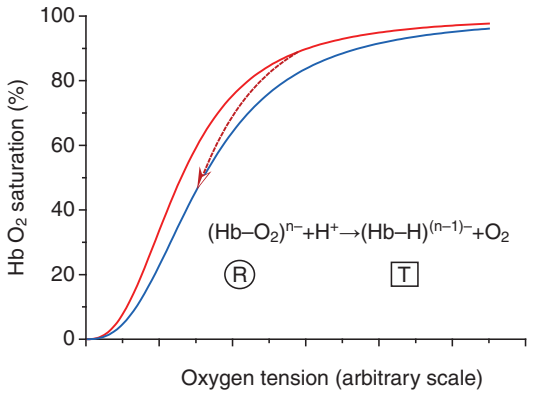

Fig. 1. Change in O_2 equilibria as the blood passes the capillary bed. Incoming blood has a relatively high O_2 affinity (red curve) and starts unloading O_2 from oxygenated R-state hemoglobin (Hb). The concomitant diffusion of metabolic CO_2 (and possibly lactic acid) into the capillary blood acidifies the red blood cells (RBCs) and causes a right shift of the O_2 equilibrium curve (blue curve). This increases the steepness of the *in vivo* curve (broken brown curve) and helps unload the O_2. The effect is primarily due to binding of H^+ to the Bohr groups of the Hb, which stabilizes the T structure of the Hb.

Local blood flow regulation

The delivery of a satisfactory O_2 supply to various tissues in the body not only requires the circulation of blood with appropriate O_2 transport properties at a sufficient bulk flow rate (i.e. cardiac output) but also demands mechanisms that can selectively distribute the blood among the numerous vascular beds according to variable O_2 needs.

The aerobic metabolism in a given tissue depends on matching O_2 delivery with O_2 demand. If tissue oxygenation is reduced – either by hypoxia or increase in O_2 usage (as in exercising muscle) – this should be met by an increase in O_2 delivery *via* the blood. This is achieved through vasodilation of precapillary resistance vessels (arterioles) and opening of precapillary sphincters, which increases the local blood flow and recruits more capillaries. Tissue blood perfusion is influenced by neural, humoral and local control mechanisms, but the precise mechanisms involved in different microvascular beds during exercise or hypoxia are not well resolved (Tune et al., 2002; Deussen et al., 2006; Saltin, 2007). The vascular endothelium produces different vasodilators such as NO, prostacyclin and EDHF (endothelium-derived relaxing factor) that induce relaxation of vascular smooth muscle (Vanhoutte, 2004) and are candidates for mediating hypoxic vasodilation. Another candidate is adenosine, which is produced by degradation of ATP during O_2 shortage. The specific blockage of these pathways individually and in combination, however, fails to completely inhibit hypoxic vasodilation (Tune et al., 2002; Saltin, 2007), suggesting other contributing mechanisms.

Local vasodilation in response to tissue hypoxia requires an O_2-sensing mechanism that is coupled to the generation of vasodilatory compounds. The O_2-sensing mechanism has been suggested to be located in the vessel wall or within the surrounding tissues, but the possibility that RBCs function as O_2 sensors (Ellsworth et al., 1995) has attracted increasing interest. Under hypoxic conditions, the oxygen content of the blood appears more important than its oxygen tension (P_{O_2}) in maintaining O_2 supply to skeletal muscles, which points to a role for the RBCs, because blood O_2 content reflects the degree of O_2 binding to Hb (Ellsworth et al., 1995). Indeed, blood flow to exercising muscles in humans is clearly increased in association with reduced Hb-bound O_2 rather than alterations in O_2 tension (González-Alonso et al., 2001). Taken together with the finding that RBCs can produce and/or release vasodilatory compounds in amounts that depend on the degree of deoxygenation of Hb (Ellsworth et al., 1995; Jia et al., 1996; Cosby et al., 2003), this has led to the paradigm that RBCs partake in both the sensing of O_2 conditions and control of local blood flow. The idea is that when the RBCs experience declining O_2 tensions in the microcirculation they will sense this *via* a decreased O_2 saturation (increased fraction of deoxyHb) and couple this to the production and release of vasodilatory compounds such as ATP or NO (Fig. 2).

In some microvascular beds, including mammalian skeletal muscles, the RBCs become significantly deoxygenated in arterioles before reaching the capillaries (Tsai et al., 2003), and vasodilation

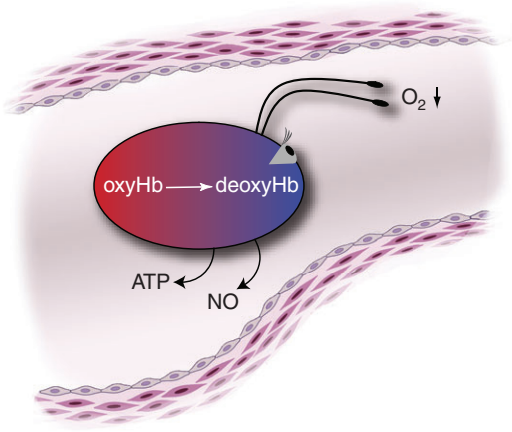

Fig. 2. Cartoon illustrating that red blood cells in the microcirculation see/sense local hypoxia though their degree of deoxygenation and couple this information to the release of vasodilators such as ATP and NO, which increases vessel diameter and local blood flow, to match O_2 demand and delivery.

can occur *via* relaxation of vascular smooth muscles close to the RBCs. If deoxygenation mainly occurs in capillaries (that are not surrounded by smooth muscle), an upstream propagation of the signal to the resistance arterioles seems necessary.

Vascular tone regulation by erythrocyte ATP release

Release of ATP was historically the first mechanism that assigned the erythrocyte as a regulator of vascular tone with Hb as the hypoxic sensor (Ellsworth et al., 1995; reviewed by Sprague et al., 2007; Ellsworth et al., 2009). At first sight it may seem surprising that RBCs should release ATP, because ATP is a valuable cellular energy reservoir. It is also a polyvalent anion (carrying 3–4 negative charges at physiological pH) that is normally considered impermeable to the membrane and, as such, plays a role in the Donnan-like passive distribution of Cl^-, HCO_3^- and H^+ across the RBC membrane (Jensen, 2004). However, a large variety of cells, including RBCs, are capable of releasing small amounts of ATP, and ATP is widely used as an extracellular physiological signaling molecule (Novak, 2003).

Mammalian RBCs release ATP when exposed to reduced oxygen tensions, and the amount released is linked to the decrease in Hb O_2 saturation (Ellsworth et al., 1995; Jagger et al., 2001). The extracellular ATP diffuses to the endothelium, where it binds to P_{2y} purinergic receptors, which activates the synthesis of vasodilators (including nitric oxide) that relax vascular smooth muscles and increase local blood flow and O_2 delivery (Ellsworth et al., 1995; Sprague et al., 2007). The local extracellular concentration of ATP in hypoxic tissue regions is in the 10^{-6} mol l^{-1} range, and ATP is clearly capable of inducing vasodilation at such concentrations (Ellsworth, 2000). An important finding is that the ATP signal can be propagated upstream, whereby ATP applied in capillaries and venules can lead to vasodilation of upstream arterioles in mammalian skeletal muscles (Ellsworth et al., 1995; Ellsworth, 2000). This signal transmission is most likely *via* the endothelial cells and may involve electrotonic spread through gap junctions (Ellsworth, 2000). Interestingly, ATP release from human RBCs may involve the gap junction protein pannexin 1, which forms an ATP-permeable channel (Locovei et al., 2006). Pannexin 1 channels could also be involved in calcium wave signal propagation in endothelial cells, enabling the signal to reach precapillary sphincters, where endothelial NO production would relax smooth muscles (Locovei et al., 2006).

ATP release is activated not only by P_{O_2} decrease but also by mechanical deformation (Sprague et al., 2001), as would occur when RBCs are squeezed through narrow vessels. The signal transduction seems to involve activation of G protein and adenylyl cyclase with the accumulation of cAMP (Sprague et al., 2001; Sprague et al., 2007). The exact membrane conduit for ATP release remains to be identified (Sprague et al., 2007), but, as mentioned above, a recent study points to the gap junction protein pannexin 1 that is expressed in human RBCs (even though they do not form gap junctions) and forms an ATP-permeable channel in the plasma membrane (Locovei et al., 2006).

The mechanistic link between Hb deoxygenation and ATP release has not been fully uncovered but it has been suggested to rely on the interaction of deoxyHb with the N-terminal cytoplasmic fragment of band 3 (Jagger et al., 2001). Band 3 (also known as the anion exchanger AE1) is the most abundant membrane protein (1 million copies per erythrocyte). Its membrane domain carries out anion (Cl^- and HCO_3^-) exchange whereas its N-terminal cytoplasmic domain is anchored to the cytoskeleton and additionally contains binding sites for Hb and glycolytic enzymes (Low, 1986). The binding of Hb is oxygenation dependent, with preferential binding of deoxyHb. When deoxyHb binds to band 3 at the membrane under low O_2 conditions, it displaces key regulatory glycolytic enzymes from shared binding sites (Campanella et al., 2005), which stimulates glycolysis (Messana et al., 1996). This deoxygenation-dependent stimulation of glycolysis and accumulation of ATP at the membrane is suggested to trigger the release of ATP from erythrocytes (Jagger et al., 2001).

In mammals, evidence for a role of erythrocyte ATP release in blood flow regulation has accumulated for more than a decade, and *in vivo* data support that ATP release contributes to the increased local blood flow during hypoxia and exercise in skeletal muscle and the coronary circulation of the heart (González-Alonso et al., 2002; Farias et al., 2005). Little is known, however, about the relative importance of the mechanism among species. So far, only few mammalian species have been investigated, and information on lower vertebrates is restricted to one recent paper on rainbow trout (Jensen et al., 2009). Rainbow trout RBCs and vascular cells in the coronary circulation release ATP, and both RBCs and vascular cells express ectonucleotidase activity. The latter is important for ATP signaling, because cellular ATP release needs to be balanced by controlled extracellular degradation of ATP *via* ectonucleotidases in order to limit the action of ATP to local autocrine or paracrine effects. In contrast to human RBCs, the release of ATP from rainbow trout RBCs was not influenced by lowered Hb O_2 saturation or elevated P_{CO_2}, and it therefore appears that the P_{O_2} and P_{CO_2}/pH changes that the RBCs experience in the microcirculation are not coupled to ATP release (Jensen et al., 2009). Unlike human Hb, rainbow trout Hb does not show deoxygenation-dependent binding to the N-terminal cytoplasmic domain of band 3 (Jensen et al., 1998). This may explain the absence of ATP release stimulated by deoxygenation in rainbow trout, assuming that the Hb–band 3 interaction and its influence on glycolysis trigger erythrocyte ATP release (cf. above). One may speculate that the deoxygenation-dependent ATP release evolved after the separation of teleost and tetrapod lineages and is important primarily in mammalian RBCs with anaerobic/glycolytic metabolism rather than in the aerobic RBCs of lower vertebrates, where ATP is produced primarily by oxidative phosphorylation in mitochondria (Jensen et al., 2009). It is evident, however, that much more information needs to be gathered before it can be firmly concluded

whether the mechanism has an ancient or more recent evolutionary origin.

The *S*-nitrosohemoglobin theory of vascular tone regulation

Nitric oxide produced in the vascular endothelium by endothelial nitric oxide synthase (eNOS) exerts its vasodilatory effect by diffusing to the underlying vascular smooth muscles, where NO activates soluble guanylyl cyclase and causes smooth muscle relaxation. Due to the free radical nature and high reactivity of NO, it has a short life time, and its effects are localized. NO entering the blood is subject to rapid inactivation *via* its reactions with oxyHb to form methemoglobin (metHb) and nitrate and *via* its tight binding to deoxygenated heme groups in Hb to form nitrosylhemoglobin (HbNO). However, it has been suggested that some NO-like vasoactivity can be preserved and carried in the blood through the formation of *S*-nitrosohemoglobin (SNO-Hb) (Jia et al., 1996).

The SNO-Hb theory was the first to directly link RBC O_2 sensing and vasoactivity with the allosteric properties of Hb. The idea is that when blood is oxygenated in the lungs and Hb assumes the R conformation, then some NO (with NO^+ character) from the small fraction of HbNO that is present in the blood will be transferred from the heme group to cysteine93 on the β chain of Hb (Cys β93) to form SNO-Hb. When the blood is subsequently deoxygenated in the microcirculation of systemic tissues, the Hb switches to the T structure, and this triggers the release of NO activity from SNO-Hb (Jia et al., 1996). The formation of SNO-Hb is facilitated in the R structure by an internal orientation of Cys β93, whereas Cys β93 points out towards the protein surface and the solvent in the T structure, facilitating the release of NO from SNO-Hb (Stamler et al., 1997). The export of NO activity from the RBCs is not *via* free NO (which would be scavenged by both oxyHb and deoxyHb) but is thought to involve NO group transfer from SNO-Hb to thiols on other proteins (transnitrosation), starting with the transnitrosation of a thiol in the cytoplasmic domain of band 3 (Pawloski et al., 2001). In human RBCs, SNO activity is associated primarily with the membrane. The preferential binding of deoxyHb to the N-terminal of the cytoplasmic domain of band 3 (cf. above) seems to allow the NO group transfer from SNO-Hb to a reactive cysteine residue in band 3 (Pawloski et al., 2001). The NO species transferred is formally analogous to NO^+ and is protected from heme scavenging and inactivation (Sonveaux et al., 2007). Further transnitrosation processes may subsequently be involved in transmitting the NO signal from the RBC to the vessel wall. This could involve low-molecular-mass nitrosothiols, but their exact nature is unknown (Sonveaux et al., 2007). It is documented that *S*-nitrosothiols are potent vasodilators (Jia et al., 1996; Palowski et al., 2001) and that human RBCs cause rapid and hypoxia-dependent vasodilation in aortic ring bioassays, as required if relevant in the microcirculation (McMahon et al., 2002).

According to the theory, NO will mostly be carried in the HbNO form in venous blood, whereas arterial blood contains significant amounts of SNO-Hb, with the likely presence of arterial–venous SNO-Hb gradients (Jia et al., 1996; Stamler et al., 1997). The SNO-Hb hypothesis has been challenged by research groups that cannot find an artery-to-vein gradient in SNO-Hb and that also record SNO-Hb levels that are much lower than originally reported and are deemed too low for a role in vasodilation (Gladwin et al., 2003). Some of these discrepancies arise from the different methodologies used to measure SNO-Hb levels, calling for independent validation of these (Gladwin et al., 2003; Robinson and Lancaster, 2005). Another controversial aspect of the SNO-Hb theory has been the one-electron oxidation that is required for the transfer of the NO group to Cys β93 to form SNO-Hb, because the electron acceptor has remained obscure (Gladwin et al., 2003; Robinson and Lancaster, 2005). Proposed candidates include O_2 (in which case superoxide is produced) or ferri(Fe^{3+})heme (Singel and Stamler, 2005; Sonveaux et al., 2007). In support of the latter, the reaction of nitrite with deoxyHb has been reported to form a Hb(Fe^{3+})NO intermediate with some Hb(Fe^{2+})NO^+ character, from which SNO-Hb can be formed (Angelo et al., 2006).

A further recent challenge to the SNO-Hb theory has been raised by a study on transgenic mice expressing either human wild-type Hb or human Hb where Cys β93 has been replaced with alanine. This study shows that loss of Cys β93 does not affect RBC-dependent hypoxic vasodilation in aortic ring bioassays, suggesting that SNO-Hb is not required for the response (Isbell et al., 2008). Thus, even though there are a number of studies that favor the SNO-Hb theory (reviewed in Singel and Stamler, 2005; Sonveaux et al., 2007), there are also many opposing arguments (e.g. Gladwin et al., 2003; Isbell et al., 2008), and no general consensus has been reached.

Whether or not the mechanism is applicable in lower vertebrates is of significant interest from a comparative perspective. As explained above, Cys β93 is an essential component of the SNO-Hb theory. This cysteine is also named Cys F9β (i.e. the ninth amino acid of the F helix in the β-chain of the Hb) and is situated right next to the proximal histidine (His F8β) that covalently links the heme group to the protein. Cys F9β is highly conserved in mammals and birds (Jia et al., 1996), which supports it having an important function. It has, however, also been noted that this particular cysteine is absent in fish Hb, which would preclude a similar function in fish (Jensen, 2008). Insight into the evolutionary origin of a cysteine residue at position F9β in vertebrate Hbs can be obtained by plotting the presence *versus* absence of Cys F9β on a phylogenetic tree (Fig. 3). This analysis suggests that absence of Cys F9β is the ancestral character for vertebrate Hb. Cys F9β is absent in the major fish groups and in frogs and toads, whereas it is present in some salamanders, most reptiles (including birds) and mammals (Fig. 3). The origin of Cys F9β is equivocal in amphibians, but the ancestors of reptiles and mammals had acquired Cys F9β. A secondary loss of this trait subsequently occurred in a few snake Hbs (e.g. Eguchi and Eguchi, 2003) and in caiman Hb (Fig. 3). The data suggest that Cys F9β evolved when vertebrates invaded the land and shifted from aquatic to terrestrial life. Due to the general presence of Cys F9β in ectothermic reptiles, Cys F9β does not seem to be related to the increased metabolic rate associated with the evolution of endothermy in birds and mammals.

The absence of Cys F9β in aquatic vertebrates precludes a heme-linked allosteric SNO mechanism similar to that proposed for mammals. It does, however, not exclude the finding of SNO groups in these Hbs, as reactive cysteines may be present at other positions in the Hb.

NO generation from nitrite and its role in hypoxic vasodilation

Nitrite (NO_2^-) is naturally present in animals at low concentrations, because it is formed as an oxidative metabolite of NO produced by NOS activity in the endothelium and other tissues. The constitutive NOS activity produces plasma nitrite levels of 0.1–0.6 μmol l^{-1} in mammals (Kleinbongard et al., 2003), and similar values apply to fish (Jensen, 2009). In recent years, it has become clear that endogenous nitrite is a physiological NO donor that can be activated by a number of cellular proteins under hypoxic conditions (Gladwin et al., 2005; Lundberg et al., 2008). Nitrite can accordingly be recycled to NO in situations where NOS-catalysed

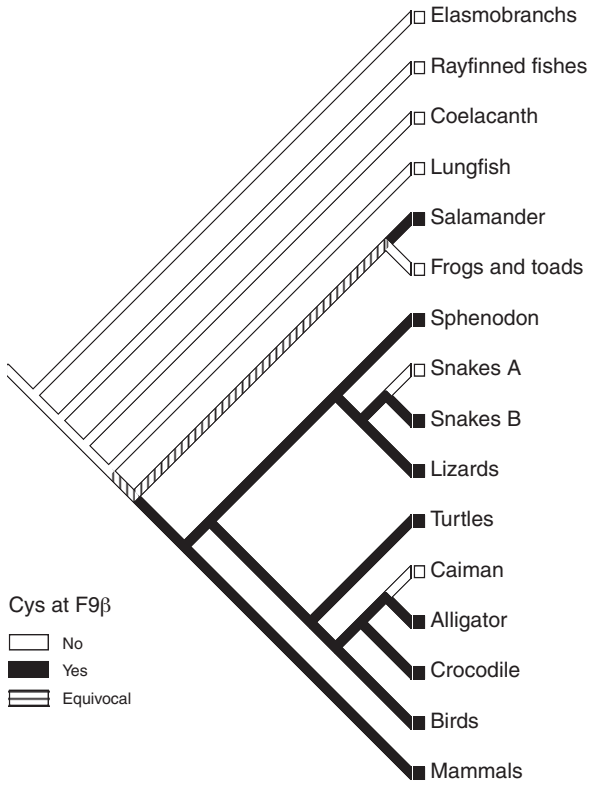

Fig. 3. Phylogenetic tree of jawed vertebrates illustrating the presence (black) *versus* absence (white) of a cysteine at position F9 of the hemoglobin β-chain (Cys F9β, also known as Cys β93 in human Hb). Presence of this cysteine residue is a critical element of the SNO-Hb theory (see text). Information was obtained from a search of available Hb sequences in the NCBI databases and published literature (F.B.J. and M. Berenbrink, unpublished data, 2008).

NO formation may be compromised by shortage of the substrate O_2. The reaction of nitrite with deoxyHb has attracted particular interest, because this reaction leads to NO production that is linked to the degree of Hb deoxygenation, which may supply a mechanism for matching blood flow to O_2 conditions, if the NO is capable of escaping the RBCs (Cosby et al., 2003; Nagababu et al., 2003):

$$Hb(Fe^{2+}) + NO_2^- + H^+ \rightarrow Hb(Fe^{3+}) + NO + OH^- .$$

The mechanism involves the following sequence of events: (1) transport of nitrite into the RBCs, (2) reaction of nitrite with Hb, (3) escape of NO activity from the RBCs and (4) induction of vasodilation.

Nitrite readily permeates the RBC membrane, and the mechanism probably involves both nitrite ion (NO_2^-) diffusion (possibly *via* AE1) and nitrous acid (HNO_2) diffusion (Jensen, 2003; Jensen, 2009). In some fish species – like carp – nitrite is preferentially transported into RBCs at low oxygen saturation, whereas in mammals the transport is similar in oxygenated and deoxygenated RBCs but increased at intermediate O_2 saturation (Jensen, 2003; Jensen, 2009; Vitturi et al., 2009). Inside the RBCs, nitrite reacts with Hb, which removes intracellular nitrite and establishes a continued diffusion gradient across the RBC membrane. The influx accordingly depends on the membrane permeability and the reaction rates between nitrite and Hb.

Nitrite reacts with both oxyHb and deoxyHb, but it is only the reaction with deoxyHb that produces NO (Fig. 4). Interestingly, at intermediate O_2 saturations (as prevails in the microcirculation *in vivo*), the reaction with deoxyHb is favored over that with oxyHb in both mammals and fish (Jensen, 2008), which directs entering nitrite towards NO formation. The nitrite reductase activity of deoxyHb is under allosteric control and regulated by the heme redox potential (tendency to transfer electrons). Deoxygenated heme groups have a lower redox potential (better ability to reduce nitrite) in the R structure than in the T structure, which results in the fastest Hb-mediated nitrite reduction at around 50% saturation in mammalian Hbs (Huang et al., 2005; Gladwin and Kim-Shapiro, 2008). This explains why nitrite influx into mammalian RBCs is elevated at intermediate O_2 saturations. Carp differs by showing an increased deoxyHb reaction rate with decreasing O_2 saturation (Jensen, 2008), which is paralleled by a gradually increased RBC nitrite influx with decreasing O_2 saturation (Jensen, 2009; S. Rohde and F.B.J., unpublished).

Species that are tolerant to environmental hypoxia typically have evolved Hb with high O_2 affinity (Jensen, 2004). This gives the Hb a large tendency to assume the R conformation, which can be predicted to promote deoxyHb-mediated nitrite reduction to NO. Indeed, the reaction of nitrite with the high O_2 affinity Hb of carp is faster and produces more NO at intermediate O_2 saturation than observed in species with lower Hb O_2 affinity (Jensen, 2008; Jensen, 2009). This suggests that the nitrite reductase function of Hb could be particularly important as a NO source for hypoxia-tolerant species with high O_2 affinity. A parallel can be drawn to the maternal–fetal situation in pregnant mammals, where the fetus lives in a low O_2 environment compared with the adult and where the high-affinity fetal Hb is a better nitrite reductase than the maternal/adult Hb with lower affinity (Blood et al., 2009).

One dilemma with NO formation inside RBCs is that both oxyHb and deoxyHb effectively scavenge NO (to produce nitrate and stable nitrosyl-Hb, respectively). This leads to the question: how can small amounts of NO escape the RBCs without

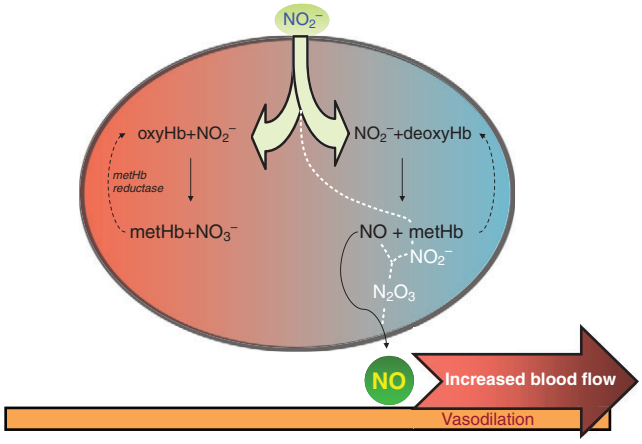

Fig. 4. Red blood cell (RBC) generation of NO by deoxyHb-mediated nitrite reduction. Nitrite entering RBCs reacts with both oxyHb (forming nitrate and metHb) and deoxyHb (forming NO and metHb), but the reaction with deoxyHb is favored at low O_2 saturations. The produced NO binds to deoxygenated heme groups, forming HbNO (or reacts with oxyHb, forming metHb and nitrate), but some NO may escape the RBCs and induce vasodilation. Escape of NO activity could be *via* the production of N_2O_3 from the reaction of NO with a nitrite–metHb intermediate, with subsequent homolysis of N_2O_3 to NO and NO_2 outside the cells. The small amounts of metHb generated in the reactions are easily converted into functional Hb by metHb reductase activity inside the RBCs. See text for further details.

becoming trapped by Hb? The mechanism is not fully clarified, but export may be eased *via* a localized reaction between deoxyHb and nitrite at the membrane (Gladwin et al., 2006). DeoxyHb bound to the cytoplasmic domain of band 3 would be ideally placed to reduce incoming nitrite (assuming that transport occurs *via* the anion exchanger) and to liberate NO activity directly at the membrane. Another possibility is that NO activity exits the RBCs as dinitrogen trioxide (N_2O_3) (Robinson and Lancaster, 2005; Basu et al., 2007). A nitrite–metHb intermediate with NO_2 radical properties is formed during the nitrite–Hb reaction, which can react with NO to produce N_2O_3 (Fig. 4), which may diffuse out to re-form NO (and NO_2) outside the RBCs (Basu et al., 2007). The export of NO activity may also be *via* formation of nitrosothiols (cf. above).

A number of experiments employing *in vivo* nitrite infusion in humans (Cosby et al., 2003; Maher et al., 2008) and *in vitro* bioassays with aortic rings (Cosby et al., 2003; Crawford et al., 2006) support the idea that sufficient NO activity can escape the RBCs to produce vasodilation. However, it has also been reported that nitrite has a high potency for relaxing aortic rings at low P_{O_2} even in the absence of RBCs or Hb, suggesting that the nitrite effect can originate in the vessel wall (Dalsgaard et al., 2007). Indeed, it is increasingly realized that nitrite reduction to NO within the vessel wall can be catalyzed by several proteins (myoglobin, xanthine oxidoreductase, eNOS and others) to contribute to nitrite-induced vasodilation (Webb et al., 2008; Alzawahra et al., 2008). Accordingly, there are alternative and competing routes of NO formation from nitrite in the circulation, and more research is needed to evaluate their relative importance.

Only a few studies have addressed the relevance of nitrite-derived NO in non-mammalian species. The potential role of RBCs in generating vasoactive NO from nitrite has been investigated in the coronary circulation of the isolated rainbow trout heart (Jensen and Agnisola, 2005). This study showed that NO of endothelial origin can induce vasodilation under hypoxia. It also reported NO production from nitrite when the coronary circulation was perfused with RBCs and nitrite, but failed to see vasodilation from this. Apparently, the nitrite-derived NO was produced in the capillaries after the RBC had passed through the resistance vessels, and the signal was not conducted to upstream arterioles (Jensen and Agnisola, 2005). It is possible that the effect of nitrite varies between different microvascular beds, and studies on other microcirculations are therefore required. Furthermore, the nitrite-reductase capacity of rainbow trout Hb is moderate compared with that of carp Hb (Jensen, 2009), and it may be more rewarding to use carp or other hypoxia-tolerant species in future work.

A role for NOS-catalyzed NO formation in RBCs?

In addition to the above-mentioned possibilities of RBC-mediated export of NO activity, it has also been reported that RBCs synthesize NO from L-arginine. Human and mice erythrocytes express an active and functional endothelial-type NOS enzyme that appears capable of exporting NO activity from the RBCs (Kleinbongard et al., 2006). It would be interesting to examine how this erythrocyte eNOS performs under different oxygenation conditions and whether its NO production contributes to blood flow regulation in the microcirculation. A search for NOS activity in RBCs from lower vertebrates also seems a relevant future endeavor to uncover its general occurrence. NO formation *via* erythrocyte NOS could play a role in the delicate balance between NO production and NO scavenging in the RBCs and thereby contribute to the regulation of vascular tone. NOS-derived NO may also modulate RBC deformability and thus ease RBC passage through the capillary bed (Kleinbongard et al., 2007).

Concluding remarks

The idea that RBCs are not only well-designed vehicles for transporting large amounts of O_2 to the tissues but also function as oxygen-sensing and vasoactive cells that regulate their own distribution in the microcirculation is appealing and has attracted much attention. The three major theories described in this article share the common feature that the R (oxyHb) → T (deoxyHb) allosteric change is essential in O_2 sensing and response induction, and they also collectively envisage that interactions between Hb and the membrane (through the anion exchanger, band 3) may be involved. Experimental evidence has accumulated in support of each individual theory and has provided an increased understanding of various biochemical and physiological aspects of the mechanisms. However, more research is needed to turn each of the attractive theories into well-established facts. There is no general consensus regarding the relative importance of each individual mechanism in mammals. Thus, it is not firmly established to what extent the mechanisms functions in parallel or whether one or another mechanism dominates or lacks physiological significance in the *in vivo* situation. It seems likely that RBCs have an important role in blood flow regulation in mammals, but continued research is needed to reach a full understanding of the phenomenon. In this process, the study of non-mammalian vertebrate species may help unravel the general importance of the mechanisms and further enlighten their evolutionary origin.

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Glossary

Allosteric interactions (homotropic and heterotropic)
: An allosteric effect in hemoglobin implies that binding of a ligand to one binding site induces conformational shifts that influence the binding of ligands at other binding sites on the same hemoglobin molecule. The homotropic interaction between O_2 binding sites means that binding of O_2 to one heme group facilitates the binding of O_2 to other heme groups in the same molecule (i.e. cooperative O_2 binding). Heterotropic effects arise from interactions between binding sites of different types of ligands. Thus, binding of non-heme ligands (e.g. H^+ and organic phosphates) to their binding sites leads to a decreased affinity of the heme groups for O_2.

Band 3/anion exchanger (AE1)
: The major membrane protein in red blood cells (approximately 1 million copies per cell). The membrane-spanning domain of band 3 mediates anion exchange (e.g. Cl^-/HCO_3^- exchange) across the membrane. The protein has a large N-terminal cytoplasmic domain that is anchored to the cytoskeleton and contains binding sites for hemoglobin and glycolytic enzymes. The name 'band 3' refers to the appearance of the protein as a major third band when erythrocyte membrane proteins are separated by SDS–PAGE.

Hemoglobin (Hb)
: A blood respiratory pigment composed of O_2 binding heme (iron protoporphyrin IX) and globin (protein). The molecule is a tetramer in most vertebrates, comprising four polypeptide chains (two α chains and two β chains) that each contain one heme group. Reversible O_2 binding requires that the heme iron is in the ferrous (Fe^{2+}) form. Hemoglobin undergoes a conformational change when it binds O_2: from the low O_2 affinity T (tense) structure (characterizing deoxygenated Hb) to the high O_2 affinity R (relaxed) structure (characterizing fully oxygenated Hb).

Methemoglobin (metHb)
: Methemoglobin is hemoglobin with the heme iron oxidized from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state. Oxidized heme cannot bind O_2.

Nitrosylhemoglobin (HbNO)

Nitrosylhemoglobin is hemoglobin with nitric oxide (NO) bound to ferrous heme.

S-nitrosohemoglobin (SNO-Hb)

S-nitrosohemoglobin is hemoglobin with NO bound to the sulfur of cysteine (Hb–S–N=O), specifically Cys β93 (also named Cys F9β).

Oxygen tension (P_{O_2})

The partial pressure of oxygen in solution in a liquid (e.g. blood).

Vasodilation

Increase in blood vessel diameter resulting from the relaxation of smooth muscle cells in the vessel wall. Dilation of blood vessels decreases vascular resistance and increases blood flow.

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