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Hemoglobin, nitric oxide and molecular mechanisms of hypoxic vasodilation

Barry W. Allen[1,2], **Jonathan S. Stamler**[3,4,5], and **Claude A. Piantadosi**[1,2,3]

[1]Center for Hyperbaric Medicine and Environmental Physiology, Duke University Medical Center, Durham, NC, USA

[2]Department of Anesthesiology, Duke University Medical Center, Durham, NC, USA

[3]Division of Pulmonary Medicine, Department of Medicine, Duke University Medical Center, Durham, NC, USA

[4]Division of Cardiovascular Medicine, Department of Medicine, Duke University Medical Center, Durham, NC, USA

[5]Department of Biochemistry, Duke University Medical Center, Durham, NC, USA

Abstract

The protected transport of nitric oxide (NO) by hemoglobin (Hb) links the metabolic activity of working tissue to the regulation of its local blood supply through hypoxic vasodilation. This physiologic mechanism is allosterically coupled to the O_2 saturation of Hb and involves the covalent binding of NO to a cysteine residue in the β-chain of Hb (Cys β93) to form *S*-nitrosohemoglobin (SNO-Hb). Subsequent S-transnitrosation, the transfer of NO groups to thiols on the RBC membrane and then in the plasma, preserves NO vasodilator activity for delivery to the vascular endothelium. This SNO-Hb paradigm provides insight into the respiratory cycle and a new therapeutic focus for diseases involving abnormal microcirculatory perfusion. In addition, the formation of *S*-nitrosothiols in other proteins may regulate an array of physiological functions.

Introduction

The discovery in the 1980s that nitric oxide (NO) is a biological signaling molecule, followed by nearly three decades of research in which a multiplicity of functions was identified for this ubiquitous molecule, has reawakened the search for mechanisms underlying a number of physiological processes and provided new ways of interpreting old observations. One of the novel concepts to emerge is that the ability of hemoglobin (Hb) to combine with NO—known long before NO was identified as an endogenous biomolecule—performs a vital physiological function. And this function, hypoxic vasodilation, has recently been the subject of intense interest, especially because it involves more than the release of dilator substances from the vessels themselves and does not depend directly on the presence of endothelial nitric oxide synthase (eNOS).

Corresponding author: Allen, B.W. (barry.w.allen@duke.edu)..

Matching perfusion with metabolism

In living organisms, the dynamic relationship between central control and peripheral autonomy of organ function is elegantly reflected in the physiological regulation of blood flow. When the full resources of an organism must be mobilized, for example during strenuous exercise, both central and local mechanisms provide a coordinated response in which cardiac output and systemic perfusion adjust to metabolic demand. However, when increased demand is confined to specific tissues, for example to a region of the brain or to the muscles of a limb, local vasodilation alone can enable sufficient increases in blood flow and O_2 extraction to meet the requirements of the working tissues. This process, the dilation of microvascular beds to supply more oxygen to specific groups of cells, is called hypoxic vasodilation (sometimes considered part of the autoregulation of blood flow) and excludes changes due to altered arterial pressure, overriding control by the central nervous system or the effect of circulating hormones.

Definitions, disparities and dichotomies

The recent history of this field has been characterized by a rapidly proliferating literature and conflicting interpretations. Some confusion, however, can be dispelled by the careful definition of physiological events. Accordingly, hypoxic vasodilation is a prompt vascular response to increased local demand for oxygen due to a change in metabolic activity in the absence of injury or disease. By contrast, post-ischemic or reactive hyperemia is a pathological response to an abnormal condition, for example transient oligemia or ischemia, that is manifest because local circulation is restored and often accompanied by tissue injury through the temporary deprivation of necessary metabolic substrates, tissue hypoxia and metabolite accumulation. And unlike hypoxic vasodilation, reactive hyperemia can decrease vascular resistance at a considerable distance from the ischemic site [1]. Conflation of hypoxic vasodilation with post-ischemic hyperemia was frequent in the earlier literature [2] and still occasionally occurs [3]. Also, hypoxic vasodilation should be distinguished from hypoxic potentiation of vasoactivity; for example, the vascular effects of some agents, including NO itself, are dependent on the partial pressure of oxygen (PO_2) and are not coupled to changes in Hb oxygen saturation. Yet another misinterpretation results from confusing hypoxic vasodilation with systemic vasodilation in alveolar hypoxia [4,5] because the former is a limited response to local metabolic conditions, whereas the latter is a response to a general limitation in O_2 availability —global hypoxemia. Although the fall in tissue PO_2 that occurs in hypoxemia recruits vasodilator mechanisms that are similar or related to those caused by an increased local metabolic demand for oxygen, the decrease in arterial O_2 saturation of Hb is not coupled to an increase in the rate of O_2 uptake. Furthermore, prolonged changes in metabolic rate, such as those caused by vigorous exercise, can also elicit multiple mechanisms of vasodilation.

Attempts to understand the role of NO in hypoxic vasodilation have been hindered by disparities in experimental results reported by different laboratories. For example, in a recent attempt to investigate hypoxic vasodilation, mice were genetically altered to prevent a reversible interaction between NO and Hb [6]. The paper reported that such an interaction was not required for hypoxic vasodilation because systemic blood pressure and time-to-fatigue in exercise had not significantly changed [6]. But these two physiological parameters are not related to hypoxic vasodilation. In other cases, misinterpretations occur when physiological differences in experimental preparations are not taken into consideration. For example, since the late 19th century it has been known that pulmonary vessels, unlike the systemic vasculature, do not dilate in response to local hypoxia—they constrict to prevent the gas in poorly ventilated alveoli from equilibrating with the pulmonary capillary blood and lowering the arterial PO_2 [7]. Nevertheless, isolated pulmonary vessels have occasionally been used to investigate hypoxic vasodilation [8], on which they shed no light.

Other difficulties arise from analytical procedures that alter or destroy evanescent biochemical species [9,10] or create conditions not found in living organisms. In addition, if endogenous substances are administered at supra-physiological levels, effects are elicited that are pharmacological rather than physiological [11]. This interpretation is corroborated when the effects of lower levels of these agents are too weak or too slow to account for authentic physiological responses [12]. Moreover, some cellular or molecular events fall outside the detection capabilities of certain techniques (for example, the venerable Griess reaction or tri-iodide chemiluminescence [9,13]) and require other, more appropriate, analytical methods [14–18]. Finally, multiple complementary mechanisms might be involved in hypoxic vasodilation, yet some authors claim that evidence for one pathway excludes others, thereby creating false dichotomies [19].

The objectives of this review are to lay out a conceptual framework within which data about the involvement of Hb and NO in hypoxic vasodilation can be evaluated, distinguish what is known from what is unknown, consider the clinical implications of this new knowledge and briefly look beyond this physiological mechanism to others that might employ similar chemistries for a range of different functions. (Please see Box 3, Outstanding Questions, at the end of this review.)

Concepts

Stimulus, sensor, signal and effector

Physiological signaling mechanisms evoke corrective responses to changes in homeostatic variables. Most such systems comprise, at a minimum, a stimulus, a sensor, a signal and an effector. However, any putative ordering of components and events may only offer a first-order understanding of what actually takes place at cellular and molecular levels because signaling pathways, like a *matryoshka* doll, often contain entities within entities. As for hypoxic vasodilation, it has long been known that the stimulus is hypoxia and the effector is vascular smooth muscle—but the sensor and the signal have been matters of speculation.

The stimulus

The hallmark of hypoxic vasodilation is local reduction in vascular resistance correlated with decreased oxygen saturation of Hb. This effect was reported in the late 1950s and early 1960s in the seminal studies of Arthur Guyton and colleagues, who concluded that local oxygen lack causes vasodilation by preventing the energy production needed to maintain basal vascular tone [20]. In other words, the postulated *sensor* is in the red blood cell (RBC) and the *signal* is decreased ATP production.

Guyton's idea was bolstered by the observation that mixed venous blood (hypoxic blood) caused prompt vasodilation when infused into normoxic tissues, but the same blood after passage through the lungs (normoxic blood) restored normal vascular caliber. This and other work clearly demonstrated that the vascular response is linked to HbO_2 saturation and is independent of tissue PO_2. However, it was thought that if a vasodilator in the infused venous blood were involved, it would have to be cleared or inactivated in the lungs. Because no endogenous agent was known to behave in this way, this explanation was rejected. This intriguing possibility—that an endogenous vasodilator could be inactivated in the lung—was conceived decades before it could be investigated rigorously.

The sensor

Recent data support the RBC—more specifically the Hb molecule—as the sensor that transduces the hypoxic stimulus into a signal as it undergoes allosteric transformation from the R-state (relaxed) to the T-state (tense), by releasing the dilator molecule NO or, more correctly,

a functional equivalent termed nitric oxide bioactivity. In the original proposal [21,22], NO bioactivity bound to a cysteine in the beta chain (Cys β93) of the Hb tetramer is released and subsequently carried in protected form as low molecular weight S-nitrosothiols (SNOs), which have substantially longer biological residence times than free NO and are not scavenged by Hb hemes [23,24]. For example, S-nitrosocysteine (CysNO) and S-nitrosoglutathione (GSNO) have biological activity in blood that unprotected NO lacks, and which they then convey to appropriate cellular targets.

In 2003, a different idea was proposed in which Hb would function as a `reductase' to transform nitrite (NO_2^-), which is not vasoactive at physiological concentrations, into NO [11]. More recently, others have proposed that eNOS and xanthine oxidoreductase (XOR) also reduce NO_2^- to NO in the RBCs. However, these proposals are problematic because XOR reduces NO_2^- only when PO_2 is extremely low [25] and NO_2^- levels are very high. And the hemes in Hb itself are well recognized and powerful scavengers of free NO [26]. In addition, the NO_2^- reductase activity is reportedly maximal at the P_{50} of Hb—when Hb is 50% saturated with O_2 [3]. However, this would be incompatible with optimal O_2 delivery because it would divert blood away from the most hypoxic tissues to those with higher PO_2. Nonetheless, for the SNO mechanism—the prototype for the involvement of Hb in the delivery of NO activity to hypoxic vascular beds—as well as for the NO_2^- reductase proposals, a common sensor (Hb) transduces similar signals from the hypoxic stimulus (Box 1).

Other hypotheses have been put forward that involve neither NO bioactivity, nor Hb nor the RBC [27]. For example, important work has shown that ATP, adenosine, vasoactive peptides, CO_2, H^+, K^+ and other endogenous substances are significant factors in the local regulation of perfusion [28–31], some of which might act in concert with NO-mediated pathways, whereas others might oppose or operate independently of NO. One or more of these other factors likely plays a part in hypoxic vasodilation; however, none alone explains the autoregulation of blood flow. The task of placing the assumed roles of these other dilators and their interrelationships into a unified mechanism is beyond the scope of this review; however, a recent survey of many of these control mechanisms is available [32]. There are also many vasoregulatory mechanisms that act on longer times scales than needed to accommodate moment-to-moment changes in metabolic demand; some of these more chronic adaptations involve remodeling of the vascular wall [33].

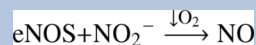
Box 1. Proposed mechanisms for hypoxic vasodilation

(1996 [21]) SNO-Hemoglobin Mechanism

$$Hb_r\,(SNO) \xrightarrow{\downarrow O_2} Hb_t + SNO$$
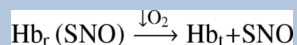
(2003 [11]) Hemoglobin as a Nitrite Reductase

$$Hb_r + NO_2^- \xrightarrow{\downarrow O_2} Hb_t + NO$$
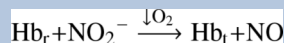
(2008 [25]) eNOS and XOR as Nitrite Reductases

$$eNOS + NO_2^- \xrightarrow{\downarrow O_2} NO$$

$$XOR + NO_2^- \xrightarrow{\downarrow\downarrow O_2} NO$$
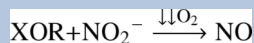
The signal

It is widely agreed that NO is the principal effector of local relaxation of vascular smooth muscle under hypoxic conditions and has the potential to act in the form of the free radical (NO) or as chemical species with the characteristics of nitrosyl (NO^+) or nitroxyl (NO^-) ions [34], or as SNOs [35–37]. However, two additional points must be made. First, the entry and egress of necessary chemical species across the red cell membrane must be compatible with the chemistries of Hb and NO. Second, because free NO has a short half-life in blood, perhaps only milliseconds [38], due to scavenging molecules in RBCs and plasma, some way to protect or preserve its vasodilator activity must be part of any cogent scheme.

The SNO mechanism avoids both these difficulties because free NO is not required. Instead, nitric oxide, as NO^+, is transferred from one low molecular weight thiol (R-SH) to another by S-transnitrosation (Box 2).
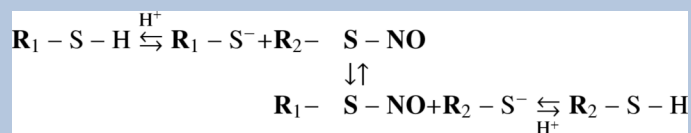
There are suitably distributed cytosolic, trans-membrane and plasma thiols to justify postulating a pool of donor and acceptor molecules, including the Hb β93 Cys, the highly conserved amino acid at position 93 in the beta chain of human Hb and that of all other mammals, birds and many other vertebrates [39]. Also, there are thiol-bearing transporters in the RBC membrane and low molecular weight thiols in the blood plasma that can be taken up, for instance, by amino acid transporters in the plasma membrane of the endothelial cell [40]. These molecules offer protected routes for NO bioactivity to reach the heme moiety of endothelial soluble guanylate cyclase (sGC) or calcium-dependent potassium channels in vascular smooth muscle, which can similarly be directly activated [41]. It has long been known that SNOs `are potent, bioactive compounds with NO-like effects', whose activity is independent of NO itself, as demonstrated by Mathews and Kerr in 1993 (cited in [42]) and confirmed more recently [43]. Furthermore, the kinetics of S-transnitrosation is much faster than the NO release from low molecular weight nitrosothiols such as GSNO and CysNO [44].

By contrast, the original NO_2^- reductase hypothesis leaves unspecified how NO bioactivity would escape the Hb molecule or the RBC, or survive long enough to reach its target in the endothelium. Physiological concentrations of NO_2^- do not in fact elicit prompt vasodilation when added to blood vessels *in vitro* [45,46] or infused into the forearm [11,47], but rather require biotransformation within the circulation to an active species [48]. In other words, NO_2^- does not meet the requirement of vasodilation at timescales commensurate with transit from arteries to veins. Moreover, some theoreticians have used mathematical modeling to explain how free NO could—like a broken-field runner—avoid rapid re-conversion to NO_2^- without the protection of a molecular carrier. Such conjectures include diffusion barriers interposed between NO and scavenger molecules, zones near vessel walls that for hydrodynamic reasons are free of RBCs and a favorable balance between NO production and consumption. Many of these proposals, some complex, are described in a recent review [38]. However, the challenge for all such model systems, no matter how comprehensive or plausible, is that the parameters needed to anchor calculations to actual physiological conditions are often unknown or difficult to quantify. Recently, the idea that physiological reduction of NO_2^- is one of the sources for SNO-Hb has been adapted to the concept of protected NO transport by SNOs in the revised hypothesis of van Faassen and colleagues: `Low molecular weight nitrosothiols may contribute to exportable vasodilatory activity' [49]. However, it is important

to understand that in this model the slow reduction of NO_2^- by deoxygenated hemes in Hb would occur in venous blood and could not be a direct effector of hypoxic vasodilation, which occurs in the arterioles.

Box 2. *S*-transnitrosation

Nitric Oxide, as NO^+, is transferred from one low-moleculer-weight thiol (R-SH) to another by S-transnitrosation.

$$R_1 - S - H \overset{H^+}{\leftrightarrows} R_1 - S^- + R_2 - \quad S - NO$$
$$\downarrow\uparrow$$
$$R_1 - \quad S - NO + R_2 - S^- \underset{H^+}{\leftrightarrows} R_2 - S - H$$

Box 3. Outstanding questions

• **How does NO bioactivity enter the erythrocyte?** The extent to which NO itself can diffuse across the RBC membrane is not clear. However, SNOs may be imported directly by mechanisms not yet identified. Alternatively, NO may be protectively escorted across the RBC membrane, for example by protein disulphide isomerase, or captured by hemoglobin associated with the RBC membrane, which then relocates to the cytosol. This is an area of active investigation.

• What is the role of nitrite (NO_2^-) or other higher oxides of nitrogen in the storage of nitric oxide bioactivity? It is clear that nitrite, even at pharmacological concentrations, does not induce RBC-mediated vasodilation rapidly enough to fulfill the physiological requirements of hypoxic vasodilation. Yet, it is possible that nitrite can, through slower processes, add to the store of intra- and extracellular SNOs and thus contribute indirectly to hypoxic vasodilation. However, any such biochemical pathways still need to be identified.

• How will the SNO-Hb paradigm be applied to the treatment of disease? There is no simple answer to this question, because the defects of NO transport and release by hemoglobin associated with different diseases have several different causes. For example in diabetes, the glycosylation of Hb may be the interfering circumstance, whereas in sickle-cell disease it is the aberrant structure of Hb itself-resulting from a genetic mutation, as well as a membrane processing defect resulting from membrane damage. However, it is clear that the paradigm opens new mechanistic fronts from which to attack these pathologies.

• How pervasive is the mechanism of *S*-nitrosylation in the regulation of protein function? Several such mechanisms have been identified involving proteins other than Hb, but this is a new field of inquiry and it remains to be seen if this process is as ubiquitous as, say, phosphorylation.

Integrating what we know

Physiological context

Many chemistries involving biological molecules can be implemented in the laboratory that are never found in living organisms. Thus, physiology has rules that are set by the organism under study, and include specific chemical, thermodynamic and temporal requirements. For any investigation of a physiological process such as hypoxic vasodilation, one must evaluate

experimental conditions against authentic physiological criteria and exclude trauma, disease or pharmacological effects.

So what do we know that is relevant to this process? We know that NO interacts with metalloproteins—especially those containing hemes—and with thiols. Hemes either transform NO into nitrate or promote its transformation into SNOs [50,51]. Nitrate lacks physiological vasodilator activity, whereas low molecular weight SNOs, such as CysNO, transduce NO-based signals largely by means of *S*-nitrosation [52]. Furthermore, SNOs retain vasodilatory activity in the blood, where newly developed assays have confirmed their ample presence [9, 16,18], whereas in animals *S*-nitrosation coordinates a multi-component physiological response to hypoxia [53–55]. We also know that RBCs act in hypoxic vasodilation through an NO-based mechanism that is independent of eNOS in the vasculature [29,45,56–58].

S-nitrosothiol-mediated vasodilation

Thiols have been identified as necessary for the action of nitrodilators for nearly three decades. For example, the potent and well known organic nitrodilator nitroglycerin requires thiol [59]. Plasma thiols do convey NO bioactivity in the mammalian circulation [60], and cytoplasmic entry of NO bioactivity may involve facilitation by protein disulphide isomerase (PDI) [61] on cell surfaces [62]. Finally, thiol modification, for example by N-ethylmalei-mide or diamide, inhibits guanylate cyclase as well as ion channels that mediate vasodilation [41,63].

Recently, additional confirmatory evidence was provided for *S*-nitrosothiol-mediated vasodilation by genetically-altered mice in which the β93 Cys on Hb was replaced by the amino acid alanine so that SNO-Hb could not be formed [6]. The investigators concluded that SNO Hb was not required for hypoxic vasodilation, but their findings of elevated levels of non-Hb SNOs and a nearly 10% increase in Hb concentrations, compensatory mechanisms for impaired O_2 delivery to tissues, are consistent with defective SNO-mediated hypoxic vasodilation [58].

How might SNO be transferred out of the RBCs, for example, from SNO-Hb? Three sets of observations provide clues. First, vasodilation evoked by native RBCs is dependent on the transfer of NO activity into the cell membrane and is potentiated by plasma thiols, such as Cys and N-acetylcysteine (NAC) [45,52,64]. Second, fresh RBCs export SNO, whereas ATP-depleted cells do not [21]. Third, deoxygenation of RBCs depletes SNO-Hb, resulting in the commensurate accumulation of both membrane and extra-cellular SNOs [15,55]. From these facts, two mechanisms can be inferred.

First, SNO-Hb binds Anion Exchanger 1 (AE1). By transnitrosation, the NO group then transfers to Cys in the N-cytoplasmic tail of AE1, leaving the free thiolate HbS^-. AE1 is the most abundant protein in the red cell membrane where it facilitates electroneutral anion exchange [65]. Thiol-coupled NO activity might also exit the RBC as GSNO, or related species, in an ATP-dependent mechanism [21]. In this case, the transporter seems to be an ATP-binding cassette (ABC) protein. This family of proteins uses energy derived from ATP to move various substrates across cell membranes. RBCs export glutathione disulfide and glutathione conjugates, as shown by Kondo and Beutler and others, by means of both high- and low-Km transporters [66]. These mechanisms are also potentiated by thiols [67]. It is plausible that these two transport pathways are linked; for example, SNO-AE1 in the membrane may be converted to GSNO since *S*-nitrosoglutathione reductase (GSNOR) knockout mice accumulate SNO-Hb at high levels [53]. What is unknown, however, is how NO bioactivity enters the RBC. Either SNOs are imported or the NO group is transferred across the membrane (for example, by PDI). It also possible that free NO is captured by membrane-associated Hb, which then relocates it to the cytosol. In the 1950s, Roughton and Comroe demonstrated that NO access by diffusion

is hindered by the erythrocyte membrane [68,69] and similar results have been reported more recently [70].

Nitrite reduction

The idea that NO_2^- is an abundant source of NO in the blood and that some blood components are capable of reducing it to NO, which then accounts for hypoxic vasodilation [71,72], was initially proposed as an alternative to the SNO paradigm because investigators using tri-iodide chemiluminescence did not detect circulating SNOs in quantities thought sufficient to account for hypoxic vasodilation. This led to the 2003 proposal that the NO_2^- reductase is Hb (Box 1) and that it releases NO under allosteric control as it makes the transition from the R- to the T-state [11,73].

Indeed, NO_2^- reduction does occur *in vivo*, especially at low pH, as in the digestive tract and perhaps in ischemic vessels, and a recent compilation of such reports provides a convenient entry into this literature [49]. For example, NO_2^--dependent vasodilation has been reported in the mouse, rat, dog, sheep, primate and human [11,74]. According to these studies, NO_2^- is consumed in A-V transit, consistent with conversion to NO or to nitrate [72]; NO_2^- at 40 μM promptly dilates isolated aortic rings in cell-free buffer at pH 6.6 [75]; vasodilation by inhaled NO is associated with increased plasma NO_2^- [76]; NO_2^- infused into the human forearm at 0.2 and 2.5 mM produces significant vasodilation associated with partial deoxygenation of Hb. Moreover, during exercise, vasodilation has been observed after five minutes at systemic NO_2^- concentrations of 16 μM [11]. That is, only high pharmacological concentrations of NO_2^- dilate vessels quickly (in seconds), whereas lower pharmacological concentrations (still many times physiological plasma levels) require biotransformation over minutes [77], thereby precluding direct involvement in hypoxic vasodilation. In addition, other investigators have found that SNOs are consumed in A-V transit, whereas NO_2^- levels actually increase [78]. These issues have yet to be resolved. In any case, the low pH levels that can occur in ischemic vascular beds *in vivo* might explain the observed NO_2^- depletion.

When Hb is exposed to NO_2^-, ferrous nitrosyl-hemoglobin (NO-Fe^{2+}Hb) is formed [79] and, hypothetically, free NO can be produced according to the following overall reaction [11]:

$$NO_2^- + HbFe^{2+} + H^+ \rightarrow HbFe^{3+} + NO + OH^-$$
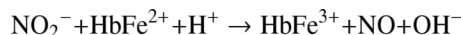
Moreover, because the formation of HbNO increases as Hb oxygen saturation drops, it was conjectured that circulating deoxyHb acts as a NO_2^- reductase in hypoxia. However, this reaction requires NO_2^- concentrations greater than that of Hb, which would be much higher than are found *in vivo*. Although NO can dissociate from NO-Fe^{2+}Hb, physiologically meaningful levels of free NO would be unsustainable in the face of the Hb concentrations present in the RBC. Indeed, massive infusions of NO_2^- are required to overcome the associated increases in NO-Fe^{2+}Hb and induce vasodilation in humans in hypoxia [4]. At physiological levels of NO_2^- (300 nM in humans), NO-Fe^{2+}Hb formation constitutes NO complexation [46,80], which must be overcome for NO bioactivity to increase.

A careful review of these measurements leaves nothing that demonstrates that NO_2^- reduction by Hb is necessary or sufficient for hypoxic vasodilation at physiological levels of NO_2^- (approximately 300 nM in humans, 200 nM in rats and 400 nM in mice [81]) or at the short timescales, seconds or less, required of a physiological response to increased metabolic demand [82]. More recent studies also support no role for either Fe^{2+}Hb or met-Hb(Fe^{III}) (methemoglobin) in the generation of free NO and further question the ability of NO_2^- reduction mediated by deoxy-Hb(Fe^{II}) to mediate physiological vasodilation. After mixing NO_2^- with deoxy-Hb(Fe^{II}) in solution, free NO does not accumulate in amounts significant

for vasoregulatory control, even after minutes at supra-physiological concentrations of NO_2^-. Under these conditions, NO_2^- reduction seems to be pre-empted by the autocapture of NO by deoxyHb, preventing the accumulation of free, unbound NO [83].

This insuperable difficulty has been acknowledged by some proponents of the NO_2^- hypothesis, who have noted that it is the `conversion of NO_2^- into longer-lived NO metabolites that accounts for most of the vasodilation' [49]. Infact, Hb(Fe^{III}) serves as a route to SNO-Hb production, and NO-Fe^{2+}Hb might also undergo oxidation-promoted transfer of NO to Cys β93 [84]. It is important to note that NO-Fe^{2+}Hb and metHb constitute micropopulations (99% of the Hb is found as HbO_2 and Fe^{2+}Hb) that can co-exist within the Hb tetramer to favor SNO formation [50].

SNO-Hb offers the more parsimonious explanation for what we know about Hb, NO and hypoxic vasodilation: (i) that unprotected NO is highly susceptible to degradation by Hb and other biochemical scavengers; (ii) that the conversion of free NO to nitrate is much faster than NO_2^- reduction to NO; and (iii) that the presence of thiols, both in the RBC membrane and the plasma, is important for hypoxic vasodilation. Careful measurements have confirmed the existence of heme-bound NO on deoxyhemoglobin, of SNO on oxygenated Hb and the release of vasodilator activity on transition from the R- to T-states [43,64]. This model, in which vasodilation is progressive across a physiological O_2 gradient and tracks the O_2 desaturation of Hb rather than tissue PO_2, replicates the signature feature of blood flow autoregulation described by early investigators and corroborated by more recent studies [43,45,85]. Finally, when Hb is reoxygenated in the pulmonary circulation, the vasoactive SNO on the Cys93 residue in the β-chains is no longer released—behaving just as the dilator that Guyton and colleagues had envisioned in 1962 (see Figure 1).

Implications for clinical medicine

The SNO-Hb paradigm expands our understanding of the respiratory cycle by identifying NO as a third respiratory gas and provides new insights into the pathophysiology of the many diseases in which microcirculatory blood flow is abnormal. Preliminary animal studies using the gas ethyl nitrite (ENO) [86,87], which when inhaled forms SNOs in the blood more readily than NO itself, might lead to methods for replenishing SNO-Hb. In a 2009 clinical trial, SNO-Hb is being measured before and after transfusion of RBCs [88], and the data are not yet available. In the future, this knowledge will be tested clinically as the handling of NO in the blood becomes a therapeutic target in the management of microcirculatory diseases and measurement of erythrocyte NO content becomes standard clinical practice.

Most vascular diseases involve abnormal regulation of blood vessel tone, and recent investigations of several important clinical conditions in which vascular function is impaired have implicated disordered delivery or release of NO bioactivity from the RBCs to microvessels in specific tissues. Diseases involving abnormalities in S-nitrosation include diabetes [89], sickle-cell anemia [90], congestive heart failure [91], sepsis [92], preeclampsia [93] and pulmonary hypertension [57]. In many of these states, circulating levels of SNO-Hb are low or undetectable, which implies that local control of the microcirculation is impaired. In other disorders, it is the presence of S-nitrosation that can be a factor in the disease processes, for example inducing pathological fission in cerebral mitochondria in Alzheimer's disease [94]. In addition, spontaneous SNO depletion in banked blood can increase the chance for complications following blood transfusions [95,96]. Therefore, an important goal of current research into the mechanisms by which Hb and SNOs mediate hypoxic vasodilation is to identify effective diagnostic strategies and therapeutic interventions for these pathological conditions.

S-nitrosylation: beyond hypoxic vasodilation

Both NO and thiols are ubiquitous constituents of mammalian cellular biology, and the roles of SNO proteins are not confined to hypoxic vasodilation. It has become increasingly apparent that the allosterically regulated covalent bonding of NO to thiols on other large molecules besides Hb is a versatile regulatory device that is vital to the functioning of many proteins, including ion channels, enzymes, transport and storage biomolecules, subcellular structural components, signaling molecules, clotting factors, proteases and kinases [97]. Because this posttranslational modification of proteins by SNO can be compared with phosphorylation and other similar processes—for example glycosylation, acylation, methylation and sumoylation—it has proven useful to identify this general process as S-nitrosylation [98]. Therefore, SNO-Hb-mediated hypoxic vasodilation is but the prototypical example of a rich biochemistry that has now been opened to investigation and awaits translation to clinical practice.

Acknowledgments

Glossary

β93 cysteine (Cys βb93), the thiol-containing amino acid occupying position 93 in the structure in the beta chain of normal human Hb and that of all other mammals and birds, as well as many other vertebrates [39]. The fact that this structural feature is so highly conserved across many species indicates that it has an important function.

Hypoxic vasodilation, the rapid dilation of microvascular beds in response to increased local metabolic demand, which is critically coupled to decreased Hb O_2 saturation and occurs in the time that blood transits a capillary bed from the arteries to the veins. This mechanism excludes changes because of altered arterial pressure, overriding control by the central nervous system, the effect of circulating hormones, prolonged hypoxia or ischemia.

Iron nitrosohemoglobin ($HbFe^{2+}$-NO), Hb that has a nitroso group (NO) coordinated to a heme and which has no vasodilator activity.

Microcirculation, the smallest functional unit of the circulation, consisting of an arteriole, the capillaries it supplies and the venule that drains them. The contractile state of smooth muscle fibers fine tunes the perfusion of individual microcirculatory units to match local metabolic demand.

Nitric oxide (NO), the endogenously synthesized free radical NO· that at low concentrations is an intercellular signaling molecule.

Nitric oxide synthase (NOS), the group of enzymes that synthesize NO from O_2 and the amino acid arginine, converting the latter into citrulline. The three known forms (isozymes) of this enzyme were named for the context in which they were originally discovered, neuronal NOS (nNOS or NOS I) in neuronal tissue, inducible NOS (iNOS or NOS II) in macrophages after a triggering event such as infection and endothelial NOS (eNOS or NOS III) in the vascular endothelium. However, all isoforms are widely distributed and their functions are diverse. Thus, for example, NO generated by any of the three isozymes can affect vascular function.

S-nitrosation and *S*-nitrosylation, these two terms refer to the covalent binding of a nitroso group (NO) to a thiol; however, the second is used more specifically in the context of posttranslational modification of proteins, cell-to-cell communication and intracellular signaling. The composite term, **S-nitros(yl)ation,** has come into use to more broadly refer to both sets of concepts.

S-nitrosothiols, organic compounds, ranging from low molecular weight species such as Cys or glutathione up to proteins such as Hb, which have a nitroso group covalently bound to the sulfur atom of a thiol; all have the general formula RSNO and are commonly referred to as SNOs.

S-nitrosohemoglobin (SNO-Hb), Hb that has a nitroso group bound to Cys in position 93 in a β-chain and which can transfer protected NO vasodilator activity to other thiols by S-transnitrosation.

Soluble guanylate cyclase (sGC), a heme-containing intracellular enzyme that is a receptor for NO that is present, for example, in endothelial cells. The binding of NO to sGC greatly increases the activity of the enzyme, which leads to cyclic guanosine monophosphate (cGMP) formation and the subsequent biochemical cascade that relaxes smooth muscle.

S-transnitrosation, the transfer of a nitroso group from one thiol to another, without the release of free NO.

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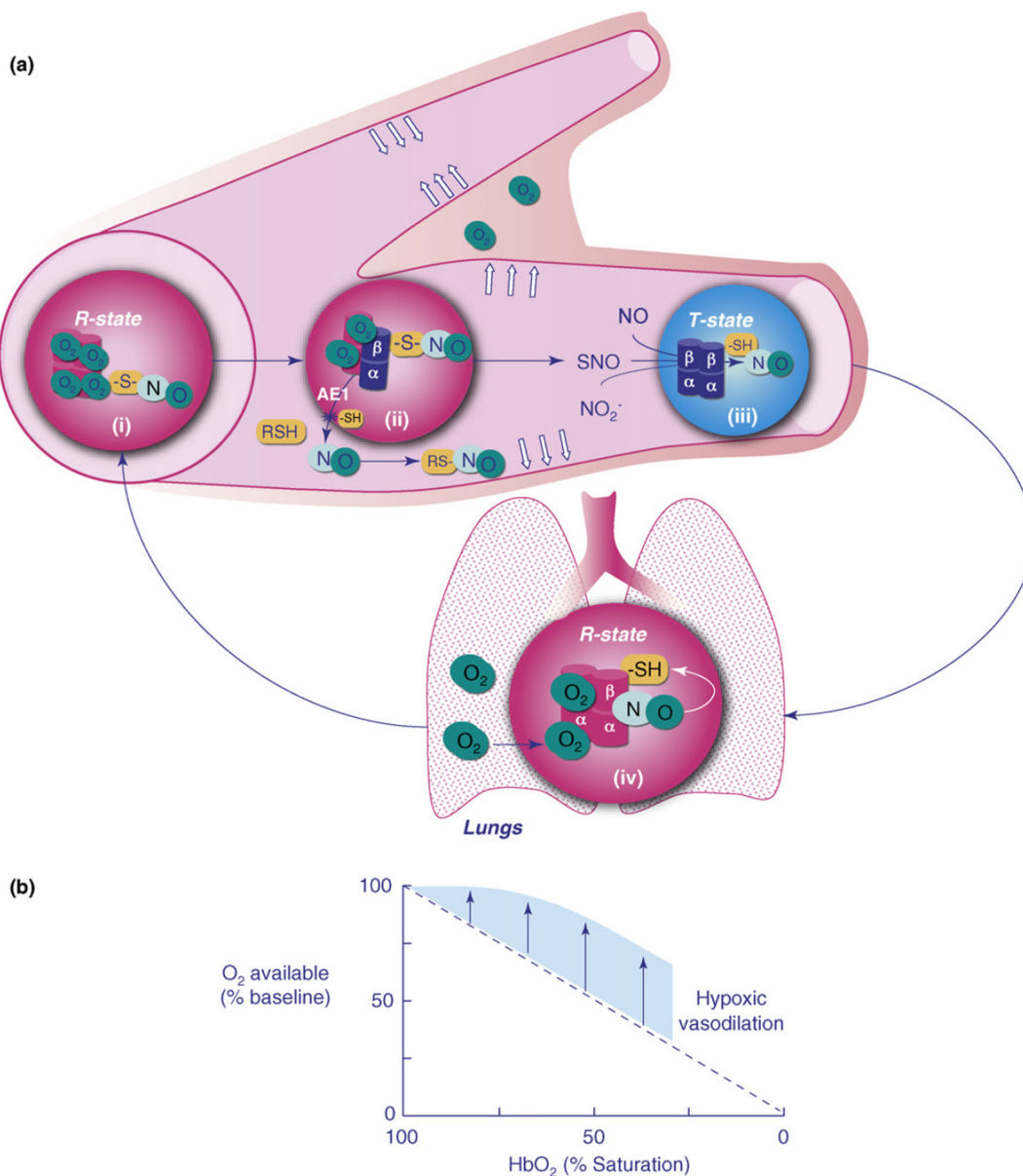
Figure 1.
Hypoxic vasodilation. (**a**) RBCs containing oxygenated Hb (**R-state**) approaching a microvascular branch point (**i**) are preferentially directed to a dilated capillary that supplies actively metabolizing tissue because desaturation of preceding RBCs (**ii**) has caused the allosteric release of NO bioactivity in the form of SNOs. RBCs containing deoxygenated Hb (**T-state**) enter the venous system where NO from several sources in the plasma as well as from SNO-Hb (thiol to heme transfer) recharge the HbFe- NO stores (**iii**) by binding hemes in the β-chains, forming metHb-containing hybrids (Fe^{III}/NO). Re-oxygenation in the **lungs** (inset, **iv**) displaces NO from the hemes to β93 cysteines, restoring the dilator capacity of the RBCs. AE1, Anion Exchanger 1. (**b**) The contribution of RBC-transported NO bioactivity is shown in the graph, in which the straight descending line reflects the availability of O_2 without vasodilation. The shaded area represents the increased O_2 extraction enabled by hypoxic vasodilation (data from [15,20,22,85]).