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Review

Nitric Oxide in the Vasculature: Where Does It Come From and Where Does It Go? A Quantitative Perspective

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ABSTRACT

Nitric oxide (NO) affects two key aspects of O_2 supply and demand: It regulates vascular tone and blood flow by activating soluble guanylate cyclase (sGC) in the vascular smooth muscle, and it controls mitochondrial O_2 consumption by inhibiting cytochrome *c* oxidase. However, significant gaps exist in our quantitative understanding of the regulation of NO production in the vascular region. Large apparent discrepancies exist among the published reports that have analyzed the various pathways in terms of the perivascular NO concentration, the efficacy of NO in causing vasodilation (EC_{50}), its efficacy in tissue respiration (IC_{50}), and the paracrine and endocrine NO release. In this study, we review the NO literature, analyzing NO levels on various scales, identifying and analyzing the discrepancies in the reported data, and proposing hypotheses that can potentially reconcile these discrepancies. Resolving these issues is highly relevant to improving our understanding of vascular biology and to developing pharmaceutical agents that target NO pathways, such as vasodilating drugs. *Antioxid. Redox Signal*. 10, 1185–1198.

INTRODUCTION

THE PAST DECADE has seen a wealth of research regarding nitric oxide (NO), a pivotal signaling molecule that regulates blood flow and tissue oxygenation. NO affects two key aspects of O_2 supply and demand: It regulates vascular tone and blood flow by activating soluble guanylate cyclase (sGC) in the vascular smooth muscle, and it controls mitochondrial O_2 consumption by inhibiting cytochrome *c* oxidase. Abnormalities in NO production and transport in vascular systems lead to numerous cardiovascular diseases, including hypertension, atherosclerosis, and angiogenesis-associated disorders (68, 73, 74). A recent review assessed the role of NO regulation of microvascular oxygenation (12).

Endogenous NO is derived from both enzymatic and nonenzymatic sources in and near the vasculature (Fig. 1). The enzymatic formation of NO is catalyzed by nitric oxide synthase (NOS) through a series of redox reactions. In this process, L-arginine is degraded to L-citrulline and NO in the presence of molecular oxygen and nicotinamide adenine dinucleotide phosphate (NADPH). To date, three isoforms of NOS have been definitively identified: neuronal NOS (NOS1 or nNOS), inducible NOS (NOS2 or iNOS), and endothelial NOS (NOS3 or eNOS). NOS1 and NOS3 are constitutive enzymes that are controlled by the availability of intracellular Ca^{2+}/calmodulin. NOS2 is an inducible enzyme that is controlled at the level of gene transcription and is expressed in the immune system in response to inflammatory or proinflammatory mediators. NO synthesized by NOS3 in the endothelium has been considered to be the major source of NO for regulating vasoactivity. NO could also be synthesized by mitochondrial nitric oxide synthase (mtNOS), but the existence of mtNOS is still under debate (55).

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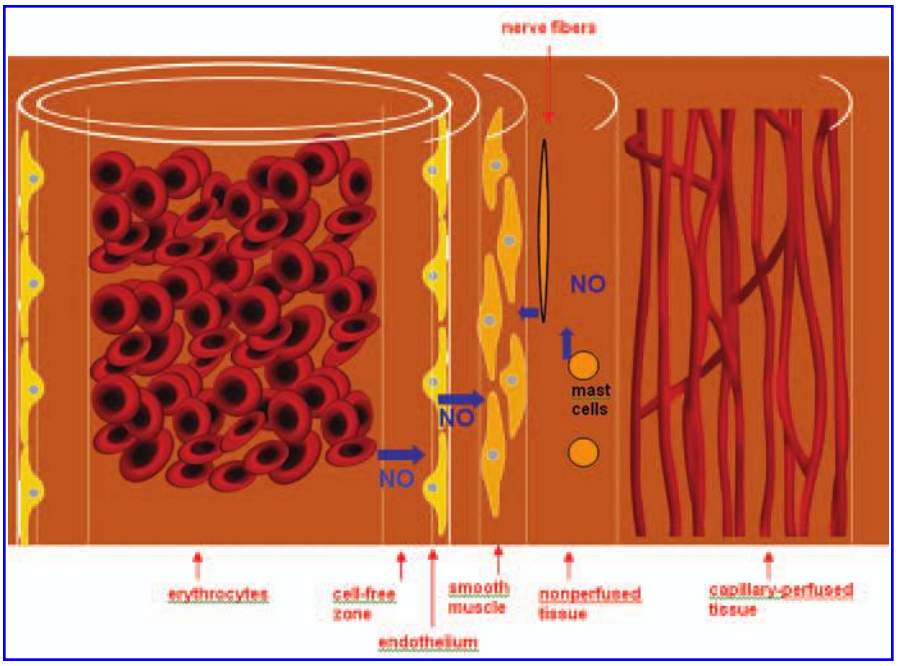

FIG. 1. The distribution of NO in the vasculature. The vasculature consists of the lumen containing erythrocytes, the erythrocyte-free zone resulting from blood flow, the endothelium, smooth muscle, the nonperfused region, and the capillary perfused region. (Adapted from ref. 104.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

In addition to the enzymatic formation of NO catalyzed by NOS, experimental evidence has shown that blood proteins can preserve and release NO bioactivity (64). Furthermore, the nitrite reservoir in the blood and extravascular tissue can be reduced to form free NO under hypoxic or low-pH conditions or both (24, 117). Once formed, NO can be consumed through reactions with a variety of species in the vasculature, such as hemoglobin (to form iron-nitrosyl-hemoglobin or nitrate), O_2, reactive oxygen species, reactive nitrogen species, sGC, and cytochrome *c* oxidase.

The interactions of NO with sGC and cytochrome *c* oxidase are the focus of this study. We have reviewed the literature and summarized the biochemical pathways that can lead to the release of NO, which can then regulate sGC and cytochrome *c* oxidase activities in the vasculature. Figure 2 outlines these pathways and the NO signaling activities in the immediate vicinity of the vascular wall.

With regard to the regulation of O_2 delivery, NO synthesized by NOS acts as a paracrine regulator of vascular tone, and NO released or converted by circulating blood proteins (*e.g.*, hemoglobin) acts as an endocrine regulator (57, 86). Autocrine regulation of protein *S*-nitrosylation has been recently observed inside endothelial cells (41). It has been well established that NO synthesized by NOS3 in the endothelium is the major contributor to the paracrine regulation of vascular tone and mitochondrial respiration. However, NO from endocrine sources and NOS isoforms other than NOS3 have also been shown to play important physiological roles. In the perivascular region, the inhibition of mitochondrial respiration also involves multiple NO sources. It would be extremely useful to determine quantitatively the contribution of NO from each pathway to its targets (sGC activation and cytochrome *c* oxidase inhibition) under various conditions, particularly normoxia and hypoxia, and to compare them with the NO-concentration measurements in the perivascular region. However, few studies have quantitatively reviewed the relations among these pathways (see Fig. 2) at the systems biology level.

In this review, we address the following quantitative questions about NO pathways: (a) How much NO is produced from each source in the vasculature, and how are these sources spatially distributed? (b) What is the EC_{50} for NO, the concentration required to achieve half-maximal vasodilation? (c) What is its IC_{50}, the concentration required to inhibit mitochondrial respiration by 50%? (d) Are these values in agreement with *in vivo* perivascular NO measurements obtained by using biosensors? (e) How do the amounts of NO from the paracrine and endocrine pathways compare? In our review of the literature, we found large apparent discrepancies among the published reports analyzing the various pathways in terms of perivascular NO concentration, EC_{50}, IC_{50}, and paracrine and endocrine NO release. Resolving these apparent discrepancies by obtaining reliable quantitative data will provide us with a better understanding of vascular biology and also contribute to the development of pharmaceutical agents that can target NO pathways, such as vasodilating drugs.

VARIATION IN PERIVASCULAR NO CONCENTRATION, C_{NO}

The concentration distribution of NO in the vascular smooth muscle and the perivascular region is a critical parameter for determining the degree of vascular relaxation and tissue oxygenation, and it has, therefore, been extensively studied by using various theoretical and experimental approaches. *In vivo* experimental measurements by using electrochemical approaches, which convert NO presence to electric current, generally report concentrations of perivascular NO (C_{NO}) on the order of several hundred nanomolar (100, 101, 108, 113); a summary of

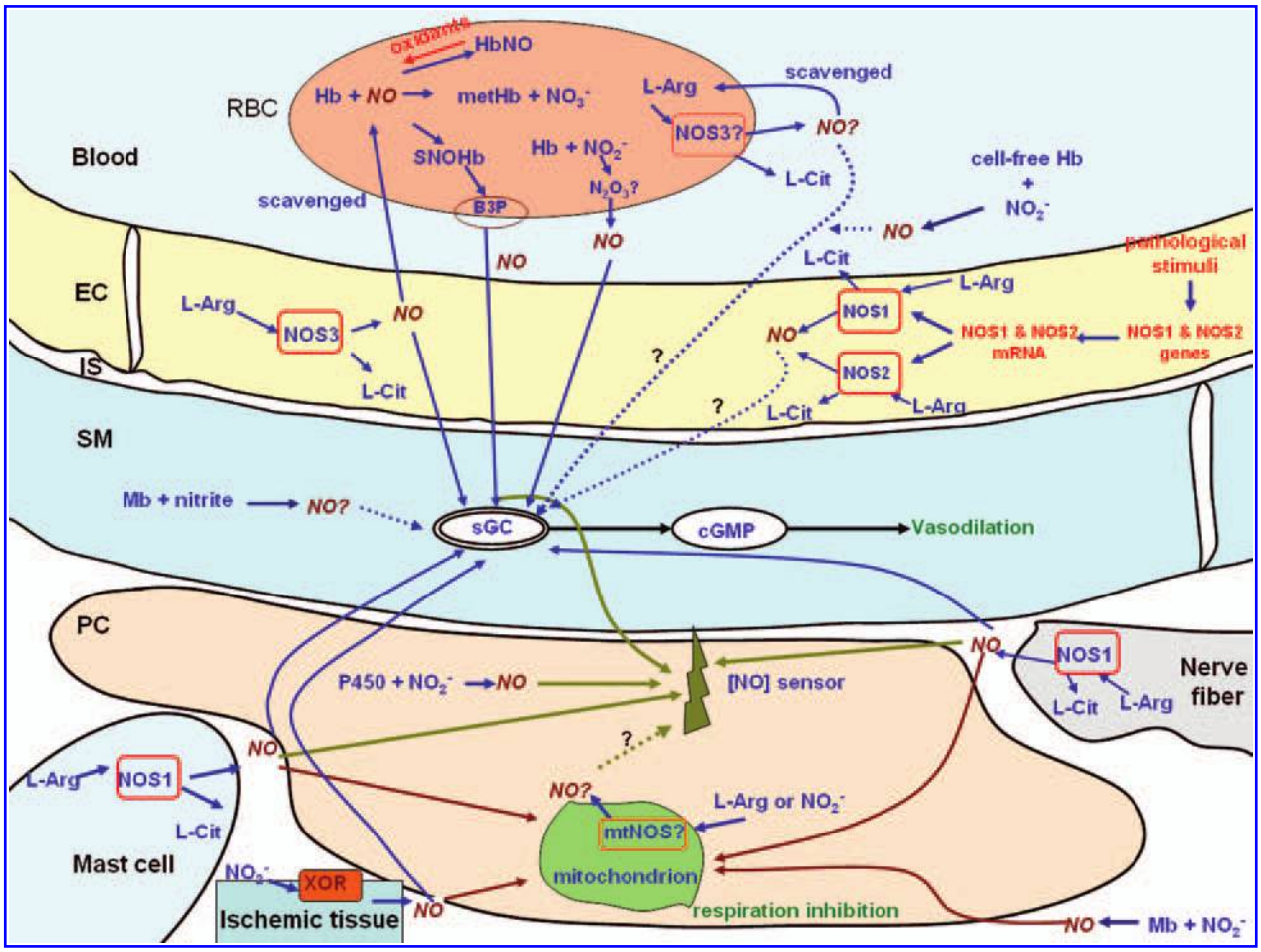

FIG. 2. Major pathways of NO release that can regulate the activities of sGC and cytochrome *c* oxidase in the immediate vicinity of the vascular wall. A variety of enzymatic and nonenzymatic sources of NO can contribute to the NO concentration measured in the perivascular region. [NO] sensor represents a device that can be placed in the perivascular region and measures NO concentration. NO sources by nitric oxide synthase include NOS3 in the endothelium and erythrocytes, NOS1 in the nerve fibers, mast cells, and other tissues, mtNOS in the mitochondria, and NOS2 in a variety of tissues under pathologic conditions. Xanthine oxidoreductase and cytochrome P450 reductase can reduce nitrite to NO, respectively. Nonenzymatic NO release can potentially come from *S*-nitrosylated blood proteins, nitrite reduction by heme-containing proteins, and iron-nitrosyl-hemoglobin, among other sources. See the text for the details of these pathways. EC, endothelial cell; IS, interstitial space between the endothelium and smooth muscle; PC, parenchymal cell; RBC, red blood cell; Hb, hemoglobin; Mb, myoglobin; B3P, band 3 protein; metHb, methemoglobin; NO_2^-, nitrite; NO_3^-, nitrate; L-arg, L-arginine; L-cit, L-citrulline; cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, 3′,5′-cyclic guanosine monophosphate; Mt, mitochondrion; XOR, xanthine oxidoreductase; P450, P450 reductase.

these data is given in Table 1. The reported values range from ~200 to 1,000 n*M* under control conditions. Zhou *et al.* (116) measured a level of 2.6 μM periarterial NO in spontaneously hypertensive rats (as compared with 1.1 μM in their control rats), a level that is, to our knowledge, the highest perivascular NO concentration reported thus far. Note that different NOS expression may be found and, thus, perivascular NO concentration distribution among different hierarchies of vasculature exist (49), but most experimental results on small arterioles (diameter, ~60 μm) point to several hundred nanomolar NO. If the NO is produced primarily in the endothelium, then, considering the geometric structure of the vasculature, it must first cross the vascular smooth muscle before reaching the perivascular region, where the measurements are conducted. Thus, the level of NO in the smooth muscle is presumably higher than, or no less than, that measured *in situ* (see Table 1). How the 200–1,000 n*M* (or even higher) concentrations of NO in the smooth muscle and perivascular region might regulate blood flow and tissue oxygenation is discussed later.

In addition to experimental approaches, *in silico* modeling has been used to predict the distribution and levels of NO. In 1994, Lancaster (57) constructed a computational model to simulate the diffusion and reaction of NO in a system mimicking the endothelium and its surrounding tissue. Vaughn *et al.* (107) formulated a mathematical model that included the geometric information of endothelial cells and also fitted the experimen-

TABLE 1. EXPERIMENTAL MEASUREMENTS OF PERIVASCULAR NO CONCENTRATIONS

Reference	*NO concentration*	*Animal model*	*Arteriolar size*	*Method*	*Experimental condition*
Tsai *et al.* (2006) (101)	200 n*M*	50- to 60-g male golden Syrian hamsters; retractor muscle	Diameter, 58 μm	Amperiometric biopolymer-coated carbon fiber microelectrodes	*In vivo*; superfused with salt solution
Tsai *et al.* (2006) (100)	600 n*M* 1,200 n*M*	55- to 60-g male golden Syrian hamsters; retractor muscle	Diameter, 50–60 μm	Amperiometric biopolymer-coated carbon fiber microelectrodes	*In vivo*; control samples, high-viscosity plasma
Vukosavljevic *et al.* (2006) (108)	310–388 n*M* for small, medium, and larger arterioles	Male Sprague-Dawley 200- to 300-g rats; exteriorized mesentery and intestine	Measured small, medium, and large arterioles	Recessed microelectrodes with Nafion membrane	*In vivo*
Zani and Bohlen (2005) (113)	545 ± 144 n*M*	Male Sprague-Dawley rats; intestine arterioles	Diameter, 53 μm	Recessed-tip glass microelectrode	*In vivo*
Nase *et al.* (2003) (71)	397 ± 26 n*M*	Male Sprague-Dawley rats; intestine arterioles	Diameter, 52 μm	Recessed-tip glass microelectrode	*In vivo*
Malinski *et al.* (1993) (65)	1,300 n*M*	Adult New Zealand rabbits; thoracic aorta	N/A	Porphyrinic electrochemical sensors	*Ex vivo*; stimulated by bradykinin

tal findings of Malinski *et al.* (65) to the model. With this model, Vaughn *et al.* estimated the NO production rate per unit area from the endothelium, assuming that all the measured NO was from the endothelial source. Several subsequent models also were based on the measurements of Malinski *et al.* (18) and the estimates of Vaughn *et al.* (20, 21, 29, 42, 47, 48, 105). Generally, these theoretic models predict an ~100-n*M* level of NO near an arteriole. Kavdia and Popel (46) and Lamkin-Kennard *et al.* (56) modeled the effect of other enzymatic NO production included in the simulation, assuming the nonendothelial NO production rates were comparable to that estimated for the endothelium by Vaughn *et al.* (107); in this analysis, several hundred nanomolar NO in the vascular bed was predicted.

Despite this success in measuring NO *in situ* and in modeling NO transport *in silico*, unresolved issues still concern the NO-concentration profile in the vasculature. First, the measured NO concentrations cover a large range, from 200 to >1,000 n*M* under control conditions. This ~800-n*M* variation is huge when compared with the potency of NO with regard to its targets (discussed later), suggesting that the physiological implications of this variation ought not to be ignored if we are to gain a quantitative understanding of the regulation of vasoactivity by NO. These large variations in experimental measurements may reflect either (a) a complex and dynamic distribution of specific NO sources or (b) limitations in the measurement method. Second, computational modeling has been limited in terms of considering a full scenario of NO production under physiologic conditions. Previous computational models have used a high rate of NO production by the endothelium, estimated by fitting the data for perivascular NO concentrations by using a mathematical model (107), with the assumption that NOS3-derived NO from the endothelium is the sole source in the vasculature. However, it has been recognized that a number of biochemical pathways can contribute to the NO presence in the perivascu-

lar region (see Fig. 2). For instance, microfluorographic and immunohistochemical analyses have indicated that strong expression of NOS1 exists around arterioles (38, 45, 98). In addition to the contributions from NOS1 and NOS3, other NOS isoforms in the vasculature, such as NOS2 or the putative mitochondrial nitric oxide synthase (mtNOS) (55), could contribute to the measured perivascular [NO]. Moreover, Kleinbongard *et al*. (51) reported the expression of functional NOS3 on the intraluminal erythrocyte membrane as another potential source of NO within the arteriole. This finding has been confirmed by others (109), but Hilarius *et al*. (36) reported finding little, if any, NOS3 from this source. Furthermore, the endocrine signaling from blood-borne NO bioactivity (*e.g*., *S*-nitrosohemoglobin and nitrite) has been proposed to be an important mechanism for matching blood flow to the metabolic activities of local tissues (24, 64, 117). However, little is known about NO production rates within specific cellular elements around an arteriole.

One may ask whether it is possible to predict the *in vivo* NO distribution by combining *in vitro* NO release studies and *in silico* simulations, because it is difficult to obtain NO release rates directly *in vivo*. However, the reported values for NO release from cultured endothelial cells cover a large range as well. Arnal *et al*. (4) measured the accumulation of nitrogen oxides (nitrite plus nitrate) from cultured bovine aortic endothelial cells. From their experiments, the basal NO production was calculated to be 0.04 μM/sec; the value reported by Kuchan and Frangos (54) from cultured human umbilical vein endothelial cells (HUVECs) was 0.21 μM/sec in the absence of a shear-stress stimulus and was 10.4 μM/sec when a shear stress of 6 dyn/cm^2 was applied. Hood *et al*. (37) measured the nitrite/nitrate accumulation over time from cultured HUVECs, and the NO production rate from their study was 0.24 μM/sec under control conditions. Moreover, it is possible that the *in vitro* measurements are complicated by the fact that other isoforms of NOS may be expressed in endothelial cells as a result of adaptive immune responses (25).

Although a variety of NO-sensing techniques, such as chemiluminescence, spectrophotometry, electron paramagnetic resonance (EPR), and electrochemical sensors (28, 79, 103, 110), are available, few are considered to be reliable *in vivo*, and fewer still permit wide-field temporal imaging. For instance, EPR spin-trapping and conventional fluorometric imaging suffer from low spatial resolution and nonspecific interactions that lead to unclear data (69). NO microelectrodes lack specificity as well. Diaminofluoresceins (DAF-2 and subsequent derivatives such as DAF-FM), a class of weakly fluorescent compounds that increase their fluorescence quantum efficiency by >100-fold after reacting with NO-derived species (*i.e*., nitrosonium ion), seem to allow temporal-spatial mapping of NO gradients in the microvasculature. However, their application to obtaining quantifiable data has been limited to *in vitro* measurements because of variations in the *in vivo* cellular topography and composition, leading to uneven dye absorption (or loading in the case of the diacetate form), nonuniform concentrations, and a poor correlation between NO levels and the increase in fluorescent intensity. In addition, these dyes are susceptible to nonspecific reactions with physiologic products such as peroxynitrite and ascorbic acid (114).

NO CONCENTRATION AND EC_{50}

In the vascular smooth muscle, NO activates sGC and causes vasodilation. It has been established that the activation of sGC by NO proceeds *via* a two-step mechanism: Initial NO binding to sGC partially activates this enzyme through the formation of a six-coordinate nitrosyl intermediate, and the subsequent conversion of this species to a five-coordinate nitroxyl complex occurs through NO-dependent or NO-independent pathways (104, 115). However, the EC_{50} for NO, the concentration required to achieve half-maximal sGC activation, has not yet been fully established. Clear answers to fill this gap in our knowledge are of great importance for the development of vasodilating drugs.

Studies addressing this problem have been conducted at both the molecular biology and physiology levels and by computational modeling. We have compiled the available information regarding the potency of NO in activating sGC or inducing vasodilation (Table 2). Several studies (5, 83, 95), including those that observed an interaction between NO and purified sGC, have shown that a high level of NO (250–1,600 nM) is required to produce half-maximal activity of the enzyme. Other experi-

TABLE 2. A SURVEY OF EC_{50} VALUES FOR THE POTENCY OF NO IN sGC ACTIVATION

Reference	*EC_{50} (nM)*	*Method*
Stone and Marletta (1996) (95)	250	*In vitro*; interaction of NO with purified sGC
Russwurm *et al*. (1998) (83)	690	*In vitro*; interaction of NO with purified sGC
Bellamy *et al*. (2000) (9)	20	*In vitro*; interaction of NO with purified sGC in cerebellar cells
Artz *et al*. (2001) (5)	1,600	*In vitro*; interaction of NO with purified sGC
Condorelli and George (2001) (23)	23	*In silico*; computational simulation of the kinetics of sGC activation by NO
Bellamy *et al*. (2002) (8)	3.9	*In vitro*; interaction of NO with sGC in brain tissue
Roy and Garthwaite (2006) (82)	10	*In vitro*; interaction of NO with sGC in cerebellar cells and platelets
Rodriguez-Juarez (2007) (81)	2.9	*In vitro*; interaction of NO with sGC in tetracycline-inducible HEK-293 cells
Yan *et al*. (2007) (112)	9.7	*Ex vivo*; apparent vasorelaxation of rat thoracic aorta in phosphate buffer

ments (9, 81, 82) that have characterized the NO–sGC interaction in the cellular environment or with the use of purified enzyme exposed to endogenous NO have yielded much smaller EC_{50} values (2.9–20 n*M*). Condorelli and George (23) conducted an *in silico* study by analyzing the kinetics of sGC and found the EC_{50} to be ~23 n*M*. Yan *et al*. (112) measured rat aortic relaxation as a function of NO concentration *ex vivo* and found that half-vessel relaxation could be induced by 9.7 n*M* NO in phosphate buffer. Also, as pointed out by these authors, because some of the 9.7 n*M* NO in the buffer must be consumed before reaching the target in smooth muscle, the real EC_{50} should be smaller than this value.

We have discussed the perivascular NO concentration measurements (see Table 1) that are in the range of 200–1,000 n*M*, or even higher. These studies have left us with an apparent paradox: On the one hand, the perivascular values for the concentration of NO as measured *in vivo* by NO microelectrodes have been generally reported as several hundred nanomolar under control conditions, and, in the interpretation of the results, most of the NO available to the smooth muscle has been attributed solely to endothelial sources; on the other hand, although variations are found in the EC_{50} values reported in the literature (see Table 2), studies in the cellular environment and using endogenous NO as the source usually point to levels <10 n*M* (81, 112). Furthermore, as suggested by Kollau *et al*. (53), concentrations <1 n*M* could trigger maximal vascular relaxation through a mechanism that does not require the full potential of 3′,5′-cyclic guanosine monophosphate (cGMP) accumulation achievable with full cyclase activation.

Thus, a review of the literature raises an important question: If the EC_{50} is indeed as low as the value quoted earlier (several nanomolar), and all the perivascular NO is derived from endothelium and is in the range of several hundred nanomolar, smooth muscle would be constantly relaxed. How, then, is the vasculature regulated? At present, this question remains unanswered. Although arteriolar dilation can be caused by a number of other factors (*e.g*., conducted responses and endothelium-derived hyperpolarizing factors), and vasodilation can be counteracted by constrictors (*e.g*., endothelin-1), a large apparent discrepancy exists between how much NO is present and how powerful it appears to be.

It should be noted that the NO-mediated regulation of sGC in the smooth muscle can proceed by both amplitude- and frequency-dependent mechanisms (90, 104). Tonic NO, reflecting a continuous low-level production of NO, forms a stable and low-activity sGC heme complex, whereas acute production of NO transiently and fully activates this NO-bound sGC (15). Transient NO release could represent a more efficient means of activating sGC, and it can increase cGMP formation severalfold. Thus, the frequency of NO bursts may limit cGMP formation and regulate vascular tone (104). Clearly, a study that will accurately determine the NO production from specific sources in the vasculature and assess its effect on sGC activation is desirable.

NO CONCENTRATION AND IC_{50}

In addition to its role in regulating vascular tone to increase O_2 delivery, NO competes with O_2 for binding to cytochrome *c* oxidase in the mitochondria of the extravascular tissue and causes a substantial inhibition of tissue respiration. As a result, the K_m value for tissue O_2 consumption is strongly dependent on the local NO concentration. The decreased oxygen consumption in the vicinity of the blood vessel can facilitate the delivery of the O_2 supply to more hypoxic tissue. Furthermore, the NO production (and thus NO distribution) is a function of P_{O_2}: enzymatic NO production is attenuated as the oxygen substrate supply decreases, whereas nonenzymatic NO production (*e.g*., reduction of extravascular nitrite under low pH and/or low O_2 conditions) is expected to increase (39, 80, 91). Thus, a mutual interdependence of NO release and O_2 delivery is found.

The interplay of NO and O_2 in the perivascular region is even more complicated because the IC_{50} of NO also depends on the O_2 concentration. The apparent K_m value of tissue respiration is a function not only of the NO concentration but also of the concentration of ambient O_2: Half-inhibition of tissue O_2 consumption in brown adipose tissue occurs at 364 n*M* NO with 180 μM O_2, 69 n*M* NO with 72 μM O_2, and 11 n*M* NO with 32 μM O_2, with respect to their values at 0 n*M* NO (52). Different values of IC_{50} have also been reported in other studies. Rodriguez-Juarez (81) and Bellamy *et al*. (8) reported IC_{50} values for NO of 141 n*M* in tetracycline-inducible HEK-293 cells and 120 n*M* in cerebellar cells, respectively, with 30 μM O_2. Basically, under different O_2 conditions, the inhibitory effect of the same amount of NO on cytochrome *c* oxidase is different (11, 58): Under hyperoxic conditions, cytochrome *c* oxidase activity is relatively insensitive to NO regulation, and its inhibition requires a high concentration of NO, far larger than that required to interact with sGC; under normoxic conditions, cytochrome *c* oxidase activity becomes more sensitive to this signaling molecule, but the IC_{50} is still fairly large; and under hypoxic conditions (such as tissue oxygenation being reduced to <30 μM), an even lower amount of NO can regulate mitochondrial respiration. It is worth noting that the tissue oxygen concentration, depending on the specific tissue and vascular bed, varies from ~35–120 μM (20–70 mm Hg) (10, 34, 72) and, thus, the hypoxic condition herein refers to the relative hypoxia at which an inadequate O_2 supply to tissues occurs.

It is important to note that the perivascular NO concentrations (200–1,000 n*M*, as mentioned earlier) are generally higher than the IC_{50} values, particularly at low P_{O_2} [*e.g*., 30 μM O_2, an oxygenation condition close to physiologic conditions in certain tissues (72)]. Whereas the discrepancy between the IC_{50} and C_{NO} is not as large as that for its EC_{50}, a C_{NO} of several-hundred nanomolar would still inhibit most of the cellular respiration and leave little room for any control of O_2 delivery through NO-dependent inhibition of mitochondrial respiration. This discrepancy at least exists in the region near the arteriole, where it is not perfused by erythrocyte-containing capillaries, which represent a significant NO sink and can cause a sharp decrease in NO availability. Thus, perhaps a heterogeneous distribution of NO in the microvasculature is determined by its specific sources and has a strong influence on the O_2-concentration distribution. It has recently been suggested that under hypoxic conditions, nitrite, which can induce vasodilation through a NO-generating mechanism reduced by deoxygenated hemoglobin (24) or independent of nitrite reductase activities (27), can also react with the myoglobin that is found in skeletal muscle and also in smooth muscle (77). This reaction

releases NO, which partially inhibits respiration in the adjacent tissue and ensures that more O_2 will be delivered to the more hypoxic tissue (88). Conversely, the cellular consumption of NO itself in the parenchymal tissue is a function of both NO and O_2 concentrations (99).

In addition to regulating respiration in the extravascular tissue, NO seems to be able to modulate tissue oxygenation by reducing O_2 consumption by the vessel walls (14, 87). According to some reports, large arteriolar transmural P_{O_2} gradients are attributable to the large amount of O_2 consumed by the microvascular wall, orders of magnitude greater than that consumed by the surrounding tissue (102). This high O_2-consumption rate can be regulated by NO: An increase in the local NO concentration can reduce the rate of O_2 consumption by the vascular wall, facilitating the delivery of O_2 to the surrounding hypoxic tissue; a decrease in the local NO concentration produces the opposite outcome. However, this hypothesis has been questioned by a number of studies. We and others have shown that it is unlikely that the thin endothelium has sufficient metabolic activity to consume three orders of magnitude more O_2 than the surrounding tissue (50, 106, 111). Also, recent P_{O_2}-profile measurements near arterioles in rat mesentery by using a scanning phosphorescence-quenching microscopy technique found no steep P_{O_2} gradient across the vascular wall (34). The authors of this study proposed that the measured higher-than-expected O_2 consumption by the endothelium (14, 87, 102) may be an artifact produced by the O_2 consumption by phosphorescence quenching in the interstitial space, where the consumed O_2 cannot be replenished as quickly as it is in the flowing blood.

As previously noted, NO regulates O_2 delivery through binding to its targets, sGC and cytochrome *c* oxidase. sGC is located in the vascular smooth muscle, whereas cytochrome *c* oxidase is located in the perivascular region. However, the sensitivities of these two targets to NO differ by 50-fold (IC_{50}: 141 n*M*; EC_{50}: 2.9 n*M*) at the 30-μ*M* O_2 level (81). Given these relatively low EC_{50} and high IC_{50} values, if all the measured NO in the perivascular region (see Table 1) were derived from the endothelium, the NO–sGC–cGMP pathway in smooth muscle would be saturated before NO from this source would exert any regulatory effect on the mitochondrial respiration in parenchymal cells. Thus, these findings add to the NO paradox and further encourage us to hypothesize that (a) multiple sources contribute to the NO in the perivascular region, and (b) a heterogeneous NO distribution is of importance in regulating microvascular oxygen delivery.

SOURCES OF VASCULAR AND PERIVASCULAR NO

Although the endothelium is considered the major NO-producing source for regulating vascular tone and tissue oxygenation (68), a number of sources of NO have been proposed, in addition to the endothelial source NOS3 in the arteriolar wall. Figure 2 and Table 3 present the results of our survey of the studies addressing possible NO sources, both enzymatic and nonenzymatic, in the vasculature and perivascular tissue. All of these sources could contribute to the measured NO concentration listed in Table 1.

In addition to the NOS3 in the endothelium, this form of the enzyme is also expressed in functional form in erythrocytes (51). Erythrocytes are rich in hemoglobin, which is a potent NO scavenger. The NO level resulting from intravascular nitric oxide is too low to have any physiological effect if free diffusion is the transport mechanism involved (61). It appears that most of the NO that is produced by this source through complex biochemical reactions involving multiple substrates and coenzymes would be immediately converted to end-metabolic products unless a protective mechanism existed to allow the NO that is formed to escape being scavenged. How much this source

Table 3. NO Sources in the Vasculature

Source	*Location*	*O_2 condition to function*
Hydroxylation of L-arginine by NOS1	Nerve fibers; mast cells (45)	Sensitive to O_2 (high K_m) (30, 96)
Hydroxylation of L-arginine by NOS2	Macrophages; aged endothelium; many tissues under inflammatory conditions (70, 84)	Sensitive to O_2 (relatively high K_m) (96)
Hydroxylation of L-arginine by NOS3	Vascular endothelium; erythrocytes (51, 68)	Insensitive to O_2 (low K_m) (78)
Nitrite reduction by NOS3	Endothelium (33)	NO released during anoxia (33)
mtNOS	Parenchymal tissues (55)	N/A
S-nitrosohemoglobin	Erythrocytes (64)	NO released during hypoxia (91)
S-nitroso-glutathione or S-nitrosoalbumin	Plasma (32)	NO released during hypoxia (32)
Nitrite reduction by hemoglobin	Erythrocytes, plasma (39)	NO released during hypoxia (39)
Nitrite reduction by myoglobin	Skeletal muscle and myocardial tissue (88)	NO released during hypoxia (88)
Nitrite reduction by xanthine oxidoreductase	Ischemic tissues (60, 62)	NO released under ischemic or anaerobic conditions (60, 62)
Nitrate and nitrite reductions by cytochrome P450 reductase and cytochrome P450	A variety of tissues (26, 59)	N/A

K_m refers to the Michaelis-Menten constant.

contributes to vasodilation and other physiological functions (*e.g*., regulating erythrocyte deformability) is not yet clear.

Recent microfluorographic and immunohistochemical analyses have provided a qualitative indication that in addition to NOS3, strong expression of NOS1 around arterioles exists: Kashiwagi *et al*. (45, 97) measured the NO distribution around the microvessels by using a fluorescence-detection technique coupled with specific NOS inhibitors and of NO produced by NOS1 in the nerve fibers and mast cells. This finding was also confirmed by their immunohistochemical staining to detect NOS isoforms in various tissues. Evidence also suggests that NOS1 can compensate for the loss of NOS3 in NOS3-knockout mice and thus induce a change in NOS expression. Studies in NOS3-knockout mice (38, 98) have shown that NOS1-derived NO and prostaglandins can maintain flow-induced vasodilation; immunohistochemical measurements also showed the presence of NOS1 in vascular tissue in wild-type mice and in the endothelium of the coronary arteries in NOS3-knockout mice. Mathematical models (46, 56) suggest that NOS1 (or NOS2) in the perivascular region can make a significant contribution to the perivascular NO distribution because it is located farther from the lumen than the NOS3 in the endothelium, thereby preventing the scavenging of the NO by hemoglobin. We have formulated a molecular-level theoretic/computational model based on the analysis of the biochemical pathways of NOS1 and NOS3 that has allowed us to quantify the NO production in the microvasculature. We have obtained paradoxic results, in that the NO derived from NOS3 could not account for the reported perivascular NO concentration (17, 18). The predicted NO release rates from NOS3 in the endothelium were in the range of 5–17 $\times$ 10^{-3} μM/sec (17), and those from NOS1 in the nerve fibers and mast cells in the perivascular region were in the range of 0.39–1.16 μM/sec (18); using these values gave NO concentrations at the arteriolar smooth muscle of ~0.1 and 5 n*M*, respectively. In our models, we specifically considered the catalytic activities (measured *in vitro*), amounts, and locations of NOS1 and NOS3.

These paradoxic predictions led us to investigate the amount of NO from each identified source and how each one specifically regulates its target (*i.e*., sGC and cytochrome *c* oxidase). However, a number of questions remain to be answered regarding the role of NOS1 in vasodilation. In NOS3-knockout animal models, hypertension or increased vascular tone has been observed, and the mechanotransduction-related production of NO (likely the results of an increasing influx of Ca^{2+} into the endothelial cells) and subsequent vasodilation of arterioles have also been documented in a variety of studies (6, 22, 75, 100). The conclusion that NOS1 is the major source of NO regulating vascular tone seems contradictory to those findings that closely link the regulation of vascular tone to NOS3 activity. Experiments featuring high-specificity approaches to real-time detection of NO-derived reactive species (such as 4,5-diaminofluorescein diacetate with a calibrated signal and the use of selective NOS inhibitors) would be very helpful in delineating the contribution of each NOS isoform.

The inducible form of NOS, or NOS2, can catalyze NO formation at a faster rate than can other NOS isoforms under the same conditions (96). NOS2 is expressed in macrophages and a variety of tissues under inflammatory conditions. Berkowitz and colleagues (84) also detected NOS2 expression in the endothelium of aged rats and showed that it serves as a source of *S*-nitrosylation for a variety of proteins (84). Moreover, the putative mtNOS could produce NO from the parenchymal cells, but, as discussed earlier, its existence is still under debate. Other enzymes that can produce NO include xanthine oxidoreductase in mammalian cells, which can reduce nitrite to NO *via* an acid-catalyzed mechanism, particularly in ischemic tissues (60, 62), and cytochrome P450 reductase and cytochrome P450, which can reduce nitrate to nitrite and nitrite to NO, respectively (26, 59).

Nonenzymatic NO production can also be a significant source of NO under certain conditions: *S*-nitrosylated blood proteins and peptides, such as hemoglobin and glutathione, are thought to serve as a storage pool for NO, and NO can be released when a reduction in P_{O_2} is sensed (91). Note that the SNOHb-induced vasodilation could be through an NO-independent mechanism (92). Also, NO can be stored in the form of circulating nitrite, which can be reduced to NO under low-pH conditions (117) by deoxygenated hemoglobin in the lumen (39), or by deoxygenated myoglobin in the tissue (88). It is interesting to note that nitrite-dependent hypoxic vasodilation can be independent of the NO-generating pathway by nitrite reductases (27). In addition to hemoglobin, NOS3, which normally requires O_2 as a substrate to produce NO, can react with nitrite under anoxic conditions, releasing NO (33). Furthermore, iron-nitrosyl-hemoglobin (HbNO), which is a relatively stable species as the product of NO and deoxyhemoglobin, can release the NO molecules at a significantly high rate in the presence of oxidants (89). Blood-borne NO bioactivity has been proposed to be a key mechanism for matching blood flow to the metabolic activity of local tissue.

Despite intense investigation of the endocrine NO reservoir as a vasodilator, little quantitative knowledge is available concerning the transport and concentration distribution from such a reservoir. This quantitative question has presented two conceptual problems: whether the NO from intraluminal sources is sufficient to induce vascular smooth muscle relaxation through the known NO–sGC–cGMP pathway, and whether it is comparable to the paracrine source of NO (61). Because of the complex biochemical reactions involved in NO release and the difficulty in discriminating the NO that might come from other enzymatic or nonenzymatic sources, no direct *in vivo* experimental measurements have been made of the amount of NO released from these pathways.

Computational modeling has been used to predict NO release from certain intraluminal pathways. By using a multicellular computational model simulating NO release from erythrocytes and assuming that a membrane-associated mechanism facilitates NO export out of these cells or a nonreactive intermediate species, we predicted that 0.25–6 p*M* or ~40 p*M* NO is delivered by intraerythrocytic *S*-nitrosohemoglobin (SNOHb) to vascular smooth muscle (19). The nitrite reduction by hemoglobin has also been quantified by modeling: Jeffers *et al*. (43) calculated that 0.08 p*M* NO is present in smooth muscle, assuming that free diffusion is the only transport mechanism. Our computational simulations confirmed a similar amount of NO delivery with free diffusion, whereas ~40–260 p*M* could be contributed by this source to smooth muscle, provided that a protected mechanism or a nonreactive intermediate species exists (16). The model predictions remain to be validated exper-

imentally when more-sophisticated approaches for detecting NO from different pathways become available.

Figure 2 shows the biochemical pathways that can lead to NO release in the vasculature and contribute to the NO-dependent regulation of sGC and cytochrome *c* oxidase activities. A quantitative analysis of the contribution made by each pathway is needed to address the complex and dynamic nature of NO signaling from a variety of specific sources, giving due consideration to their spatial distribution. A combination of experimental and computational approaches, including immunohistochemistry, *in situ* DAF-2T fluorescence for the detection of NO-derived reactive species, and computational analysis of biochemical pathways leading to NO release, could reveal with high resolution the spatial distribution of significant sources of NO.

PARACRINE OR ENDOCRINE REGULATION

Cytochrome *c* oxidase activity is regulated mainly through a paracrine mechanism: NO produced locally by various sources (except mtNOS) diffuses to the targets in the perivascular region and inhibits mitochondrial respiration. In addition, an autocrine regulation pathway may be involved if putative mtNOS does indeed exist.

The regulation of NO in vascular smooth muscle appears to be more complicated. Based on his computational analysis of NO diffusion and reactions, Lancaster (57) pointed out that the NO produced in endothelial cells primarily conducts paracrine signaling in the vasculature, and thus the spatial relations between the specific sites of NO formation and the neighboring targets of this NO are of great importance. Because of the close proximity of the endothelium to the blood vessel lumen, most of the NO that is locally produced by endothelium-based NOS3 was initially thought to be readily consumed because of the massive presence of hemoglobin in the lumen (*i.e.*, as a "cost" of paracrine signaling). Subsequent studies have shown, however, that the encapsulation of hemoglobin by the erythrocytic membrane and the existence of the cell-free region (shown in Fig. 1) in the blood can prevent, to a large extent, this NO from being consumed (13, 29, 67). Still, a large amount of NO derived from NOS3 is converted to inactive metabolic products (*e.g.*, nitrate) by hemoglobin through dioxygenation and iron-nitrosylation reactions (44).

In addition to its paracrine mechanism, NO seems to mediate endocrine signaling: NO bioactivity is preserved in other more-stable species that circulate in the blood and is released under conditions of hypoxia, when vasodilation is needed most. The SNOHb theory is one of the major hypotheses to account for the endocrine signaling: According to this hypothesis, in addition to reacting with the ferrous or ferric heme of hemoglobin, NO can be transferred from the heme iron of hemoglobin to the cysteine residue at position 93 on the β chain to form SNOHb, which is relatively stable and can convey NO bioactivity to hypoxic tissues (64). Other blood proteins or peptides that can be *S*-nitrosylated, such as glutathione, can potentially act as NO preservers as well (91, 93). As discussed earlier, NO delivered to smooth muscle through this pathway is calculated to be at the 0.25–6 p*M* or ~40 p*M* level, depending on the possible NO-transport mechanisms involved (19).

A competing hypothesis addresses the role of nitrite anion in endocrine signaling by NO. Nitrite, formed as one of metabolic products of enzymatic NO or obtained as a dietary supplement, is found nearly to double human forearm blood flow at a high dose (~200 μM) and to have an NO-dependent vasodilating effect at a near-physiologic concentration level (40). The molecular mechanism for this nitrite reduction involves the reducing activities of heme-containing globins, such hemoglobin or myoglobin, to release NO (39, 88). The resulting NO may be exported out of the lumen as the intermediate reaction product, likely in the form of nitrous anhydride (N_2O_3), as proposed by Robinson and Lancaster (80) and recently experimentally confirmed by Basu *et al*. (7). NO from the nitrite reservoir in the lumen is calculated to be ~40–260 p*M* under physiologic conditions, depending on the protected mechanisms of NO transport (16). Interestingly, the study by Angelo *et al*. (3) suggested a possible link between these two hypotheses: Hemoglobin could synthesize SNOHb, by using nitrite as a substrate (3). Furthermore, experimental evidence has shown that adenosine 5′-triphosphate (ATP) can be released from erythrocytes after O_2 reduction is sensed, leading to vasodilation produced by inducing a conducted vasomotor response or stimulating NOS catalytic activity (31, 94).

Thus, a growing body of evidence suggests that in addition to its paracrine regulation by NO that has been locally produced by NOS, vascular tone can be regulated through an endocrine effect produced by NO released from circulating erythrocytes after sensing low P_{O_2}. It is likely that paracrine NO is responsible for the basic homeostasis under resting conditions, whereas endocrine NO contributes to hypoxic vasodilation, when an increase in metabolic activity (such as exercise) consumes a large amount of O_2 and leads to a decrease in the tissue P_{O_2}. The change in O_2 level results in vasodilation, which increases the blood flow and ensures the delivery of the adequate O_2 to match the tissue's metabolic requirements.

How does the vasculature sense O_2, and what type of regulation becomes dominant when this process occurs? Although our understanding is still incomplete regarding the quantities of NO resulting from each specific pathway, it appears that local production of paracrine NO is higher than that of NO from endocrine sources in the lumen (18–20, 47, 101). It is reasonable to postulate that the paracrine regulation of vascular tone by NOS-derived NO is significantly attenuated under conditions of low O_2 tension, when NO from erythrocytes becomes an important source. The NO that is locally produced by NOS3 has been thought to act as a paracrine source to regulate the vascular tone; however, the K_m value of NOS3 for O_2 has been reported to be ~7 μM (78), a level much lower than the O_2 concentration in the precapillary arterioles (76). Thus, an apparent contradiction exists between endocrine NO acting as the major vascular relaxing factor under conditions of hypoxia and the low sensitivity of NOS3 to the change in O_2 concentration. One possible explanation for this paradox is that other isoforms of NOS with higher K_m values contribute significantly to this paracrine regulation and sense the change in ambient O_2 (18).

EXPERIMENTALLY MEASURED C_{NO} AND THEORETICAL MODELS

We have summarized the values for perivascular C_{NO} that have been measured or calculated by using various methods. The measured values are usually several hundred nanomolar, and the values predicted by mathematical models are close to these values when a high rate of NO release is used. This high rate of NO production by the endothelium has been estimated by fitting the data for perivascular NO concentrations by using a mathematical model (107), with the assumption that NOS3-derived NO from the endothelium is the sole source in the vasculature. This assumption may not be accurate, because multiple NO sources appear to be involved in the interaction of NO with sGC and cytochrome *c* oxidase. Thus, a modeling approach that analyzes NO release from specific pathways and their individual contributions to the perivascular C_{NO} could provide valuable information from another perspective.

We have formulated a molecularly based theoretical/computational model to quantify the NO production by NOS in the microvasculature (17, 18) (Fig. 3). The model is based on the analysis of the biochemical pathways of NOS1 and NOS3 that have been characterized *in vitro* (66, 85, 96). NOS goes through a five-electron transfer cycle and releases NO by using L-arginine, O_2, and NADPH as substrates and tetrahydrobiopterin (H_4B) as a cofactor. This pathway analysis of the catalytic activities of NOS resulted in a set of coupled ordinary differential equations to describe the change in the species concentrations. In our models, we specifically considered the catalytic activities, amounts, and locations of NOS1 and NOS3. Based on the topographic information on NOS isoforms revealed by fluorescent and immunohistochemical methods, we calculated the NO concentration distribution around an arteriole under various O_2 conditions. The model produced paradoxic results: The NO derived from NOS3 could not account for the reported perivascular NO concentration.

We then used our model to test the sensitivity of NO production to substrate availability, NOS3 concentration, and potential rate-limiting kinetic factors. The parameter sensitivity tests showed that only coordinated changes in multiple parameters could significantly increase the NO production from this source. However, it is unlikely that the kinetic parameters contain systematic errors, because they have been measured *in vitro* by multiple groups. The analysis also suggested that the predicted low level of NO production could be attributed to a low expression of NOS3 in the microvascular endothelial cells. This value has been measured by a few groups: Andersen *et al*. (2) obtained 2.5 ± 1.9 ng NOS3 from 10^6 HUVECs, which was equivalent to a 47-n*M* intracellular concentration when we considered 133 kDa as the molecular size of NOS3 and 400 μm^3 as the average volume of an endothelial cell (35). Experiments by Lubrano *et al*. (63) with HUVECs and human microvascular endothelial cells yielded NOS3 concentrations of 8 and 23 n*M*, respectively. Although these two experiments were not pri-

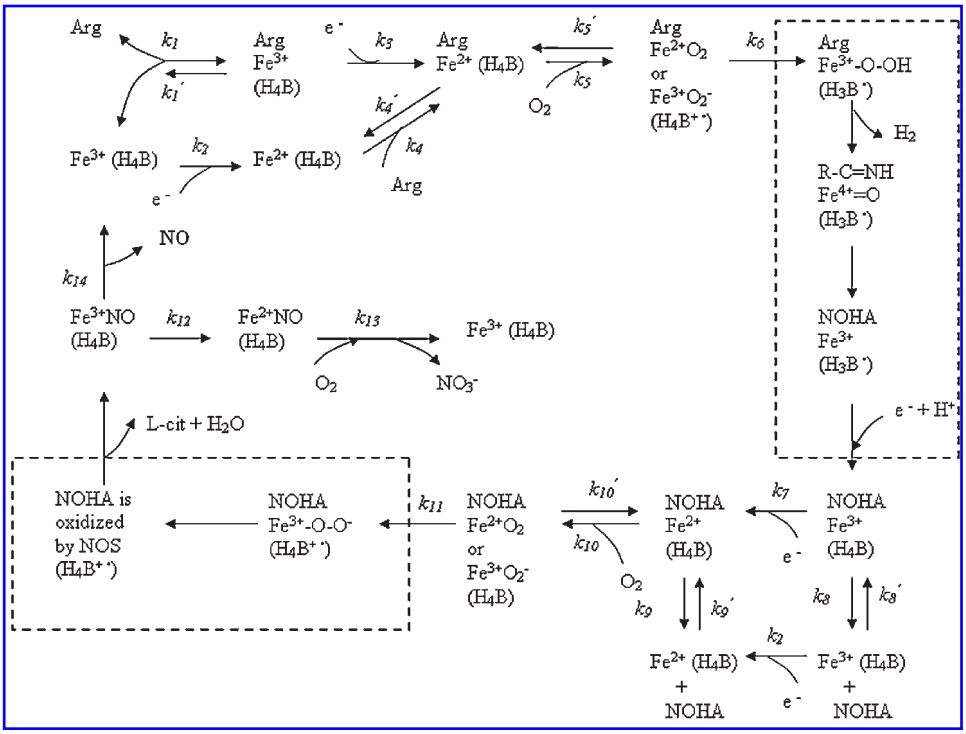

FIG. 3. Mechanism for the formation of NO *via* eNOS catalysis after the binding of the coenzyme tetrahydrobiopterin (H_4B). The heme iron (Fe) is the major catalytic site and represents NOS3 here. NOS3 undergoes a series of redox reactions. Arg represents L-arginine, and NOHA represents N^{ω}-hydroxyl-L-arginine. Parentheses around H_4B, $H_4B^{+\bullet}$, or $H_3B^{\bullet}$ mean that that species is bound to the enzyme. NO is released from $Fe^{3+}NO$. All three NOS isoforms share a similar catalytic mechanism. [Adapted from (66, 85).]

marily designed to quantify NOS3 expression in cells, they both point to an intracellular concentration of <50 n*M*. Also, their values are in good agreement with that from the manual for the NOS3 Quantikine Immunoassay Kit (R&D Systems, Minneapolis, MN), which quotes NOS3 concentrations of 5,137 pg/10^6 ECs (97 n*M*) for HUVECs and 1,396 pg/10^6 ECs (26 n*M*) for human microvascular endothelial cells. These low NOS3 expression values yield low NO production rates from the endothelium (17). Our model would require a 1,000-fold higher NOS3 concentration as an input to yield a NO production rate that would result in an NO concentration in the perivascular region (see Table 1). Here we used an average intracellular NOS3 concentration estimated from a large number (10^5 or 10^6) of endothelial cells. However, the distribution of NOS3 may be heterogeneous in different tissues or among endothelial cells from different vessels. Thus, detailed measurements of the NOS3 distribution among the different hierarchies of vasculature could provide a better understanding of NO production in different tissues.

We also applied the same biochemical-pathway analysis to examine the NO release by NOS1 and the contribution from this source to the perivascular NO concentration. Our model of NOS1 catalytic activity and its distribution show that mast cells and nerve fibers can significantly contribute to the perivascular C_{NO} (at levels of ~50 n*M*), but the predicted value still falls short of the experimentally measured values (18). This result points to a higher abundance of NOS1 or NOS3 and/or the existence of other enzymatic or nonenzymatic sources of NO in the microvasculature. Also, it should be noted that a limitation exists for using *in vitro* enzymatic parameters of NOS isoforms in theoretic modeling. NOS activities in intact cells could be different than those reported from the isolated enzyme *in vitro*. The posttranslational modifications or intracellular protein–protein interactions may have an important effect on regulatory control of NO generation, which requires further studies.

CONCLUSIONS

The relation between NO availability and the balance between O_2 supply and demand, especially under hypoxic conditions in precapillary regions, has been extensively studied in the past several years. However, significant gaps remain in our quantitative understanding of the regulation of NO production in the vascular region. Quantitative discrepancies in NO concentrations and activity have presented a conceptual problem in the biology of NO signaling. We have reviewed the NO literature, addressing the NO-concentration levels at various scales, we have identified and analyzed the discrepancies in the reported data, and we have proposed hypotheses that can potentially reconcile these seemingly contradictory data. Studies providing answers to the questions that remain will contribute to a better understanding of NO-production mechanisms and microvascular O_2 delivery, a major problem in cardiovascular physiology.

Discovering discrepancies between theory and experiment that cannot be reconciled, despite adjusting parameters within the physiologic range, is a powerful motivation for further studies and is the major value of theoretical models. Such discrepancies suggest either the existence of experimental artifacts or the need to formulate new hypotheses that form the foundation of the model. This approach is a paradigm for making new discoveries, and its value has been demonstrated time and time again in combined experimental and theoretic studies. We recognize that the modeling approaches that we used may be simplified and must be refined in the future when more-accurate measurements on certain parameters are available. However, the seemingly large discrepancies that have been identified, together with theoretic considerations, have led us to examine the NO sources and their potential heterogeneous distribution in the vasculature and their roles in regulating microvascular O_2 delivery.

ABBREVIATIONS

DAF, diaminofluorescein; EPR, electron paramagnetic resonance; H_4B, tetrahydrobiopterin; HUVEC, human umbilical vein endothelial cell; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; sGC, soluble guanylate cyclase; SNOHb, *S*-nitrosohemoglobin.

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