Connecting the Chemical and Biological Properties of Nitric Oxide

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Connecting the Chemical and Biological Properties of Nitric Oxide

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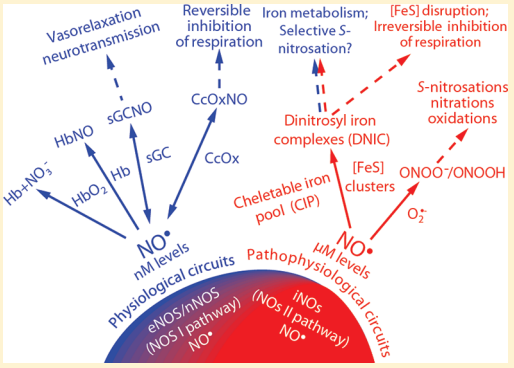
ABSTRACT: Nitric oxide ($NO^{\bullet}$; nitrogen monoxide) is known to be a critical regulator of cell and tissue function through mechanisms that utilize its unique physicochemical properties as a small and uncharged free radical with limited reactivity. Here, the basic chemistry and biochemistry of $NO^{\bullet}$ are summarized through the description of its chemical reactivity, biological sources, physiological and pathophysiological levels, and cellular transport. The complexity of the interactions of $NO^{\bullet}$ with biotargets, which vary from irreversible second-order reactions to reversible formation of nonreactive and reactive nitrosyl complexes, is noted. Emphasis is placed on the kinetics and physiological consequences of the reactions of $NO^{\bullet}$ with its better characterized biotargets. These targets are soluble guanylate cyclase (sCG), oxyhemoglobin/hemoglobin (HbO_2/Hb) and cytochrome *c* oxidase (CcOx), all of which are ferrous heme proteins that react with $NO^{\bullet}$ with second-order rate constants approaching the diffusion limit (k_{on} approximately 10^7 to 10^8 M^{-1} s^{-1}). Likewise, the biotarget responsible for the most described pathophysiological actions of $NO^{\bullet}$ is the superoxide anion radical ($O_2^{\bullet-}$), which reacts with $NO^{\bullet}$ in a diffusion-controlled process (*k* approximately 10^{10} M^{-1} s^{-1}). The reactions of $NO^{\bullet}$ with proteins containing iron−sulfur clusters ([FeS]) remain little studied and the reported rate constants of the first steps of these reactions are considerable (*k* approximately 10^5 M^{-1} s^{-1}). Not surprisingly, the interactions of proteins containing iron−sulfur clusters with $NO^{\bullet}$ remain ambiguous and have been associated with both physiological and pathophysiological effects. Overall, it is emphasized that any claimed biological action of $NO^{\bullet}$ should be connected with its interaction with kinetically relevant biotargets. Although reactivity toward biotargets is only one of the factors contributing to cellular and tissue responses mediated by short-lived species, such as $NO^{\bullet}$ and other oxygen-derived species, it is a critical factor. Therefore, taking reactivity into account is important to advancing our knowledge on redox signaling mechanisms.



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1. INTRODUCTION

The free radical nitrogen monoxide (nitric oxide, $NO^{\bullet}$) controls fundamental biological functions.[1] This statement is obvious today but when the connection between the endothelium derived relaxing factor[2] and $NO^{\bullet}$ was formally made in the late 20th century,[3,4] it was a surprise because radicals were considered to be toxic reactive species. $NO^{\bullet}$ is unique[5] as a second messenger not only because it is a free radical but also because it is a gaseous, inorganic, uncharged diatomic molecule that is so structurally simple that it cannot conceivably be recognized by proteins through the weak

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intermolecular forces that typify the recognition of other second messengers. Instead, NO• signals primarily by binding to the ferrous heme of the enzyme soluble guanylate cyclase (sGC), promoting its activation.[6,7] Thus, the major advance from the discovery of the signaling functions of NO• was not the identification of a new second messenger but of a new class of messengers that control biological processes by an entirely novel mechanism.[5] The repercussions and implications of these findings in several different fields ranging from chemistry, biology, pharmacology, and medicine were recognized with the Nobel Prize in Medicine and Physiology awarded to Furchgott, Ignarro, and Murad in 1998.

Also crucial to promoting paradigmatic changes in the area relating free radicals to physiology in the past decade of the 20th century was the discovery that hydrogen peroxide (H_2O_2) can act as a second messenger for receptor agonists, such as growth factors and hormones.[8] For decades, free radical research in biology focused on understanding the properties of molecular oxygen (O_2) causing its propensity to produce free radicals and oxidants as metabolites, and elucidating how proteins, lipids, and DNA are damaged by them, resulting in cellular injury and disease. However, it became clear that free radicals, such as NO•, and the so-called reactive oxygen species, such as H_2O_2, were also essential to the homeostasis of cells and organisms.[9] These discoveries prompted the concept of redox signaling, which involves cellular signal transduction networks that rely on reactive species as messengers.[10−16] By exerting second messenger effects, reactive species can regulate major physiological and pathophysiological circuits. The knowledge of redox signaling mechanisms, however, is currently developing.

Biological mediators usually interact rapidly and reversibly with a small and specific set of proteins/enzymes, and, thus, selectivity is a fundamental principle of biological signaling. Not surprisingly, both H_2O_2 and NO• have limited and specific reactivity with biological targets. The accumulated evidence indicates that H_2O_2 mediates signaling through the reversible oxidation of catalytic and structural cysteine residues,[10−16] whereas NO• signals mainly by the binding to and activation of sGC, in a process that does not involve redox change.[17] Nevertheless, NO•, O_2, and oxygen metabolites are interrelated physiologically, and many of the physiological consequences of NO• production are associated with this fact. Thus, to advance our understanding of the physiological and pathophysiological circuits mediated by free radicals and oxidants, it is important to limit, as much as possible, the use of general terms such as reactive oxygen species (ROS) and reactive nitrogen species (RNS).[15,17] In fact, it is necessary to recognize the individuality of each of these species with respect to their reactivity toward biological targets and diffusion into tissue and cellular compartments. Additionally, the pathways regulating their production, the concentrations they can attain under different physiological conditions, and their interactions with each other should be taken into consideration.

Here, the basic chemistry and biochemistry of NO• are summarized and linked with its biological actions. The loose connections will be noted to emphasize the need for interdisciplinary approaches to unravel the physiological and pathophysiological circuits mediated by free radicals and oxidants.[10−12,14−16,18]

2. BASIC PROPERTIES OF NITRIC OXIDE RELEVANT TO BIOLOGY

2.1. Chemical Bonding. A closer look into the nature of the chemical bonds of the NO• molecule is relevant for understanding its physiological functions (Figure 1). The

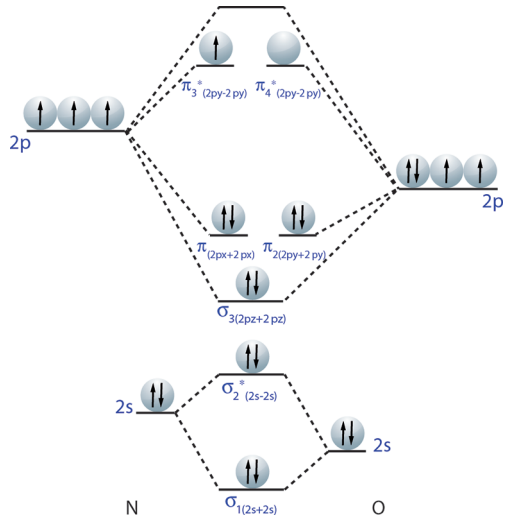

Figure 1. Qualitative energy level and molecular orbital diagram for NO•.

difference in the electronegativities of the oxygen and nitrogen atoms, which is represented by the asymmetry of the diagram in Figure 1, results in molecular orbitals with distinct nitrogen and oxygen contributions. The bonding molecular orbitals belong predominantly to oxygen, as the oxygen wave functions contribute more than those of nitrogen to these orbitals, while all antibonding molecular orbitals belong predominantly to nitrogen. This simple bonding model shows the radical nature of NO• and a bond order of 2 1/2, which is consistent with its intermediary bond length (1.15 Å) between those of N_2 (1.06 Å) and O_2 (1.18 Å).

The two lowest sigma molecular orbitals (σ_1 and σ_2*), which result from the combination of the 2s atomic orbitals, have no net contribution to bonding; both can be considered essentially nonbonding orbitals. The more energetic antibonding σ_2* is represented as the nitrogen nonbonding electron pair, and its σ nature, symmetry, and energy govern the basic aspects of NO• chemistry. The unpaired electron occupies a π* antibonding orbital polarized toward nitrogen, explaining why recombination reactions with radicals and transition metal ions occur exclusively through the nitrogen atom. This polarization also explains the selectivity of NO• reactions. Thus, the notation recommended by IUPAC for radicals[19] with a superscript dot to the right to represent the unpaired electron can be confusing in the case of NO•.

Despite the fact that NO• is the most studied metabolite of molecular nitrogen (N_2), it is important to note that the number of redox metabolites that can be produced from N_2 is greater than those produced from O_2 (Figure 2). Because the nitrogen atom has five electrons in the outermost shell (Figure 1), it can formally donate up to five electrons to the electronegative oxygen atom to form various oxyanions. Alternatively, the nitrogen atom can formally gain up to three electrons from less electronegative atoms, such as carbon and hydrogen to fill its outermost shell with eight electrons. All of

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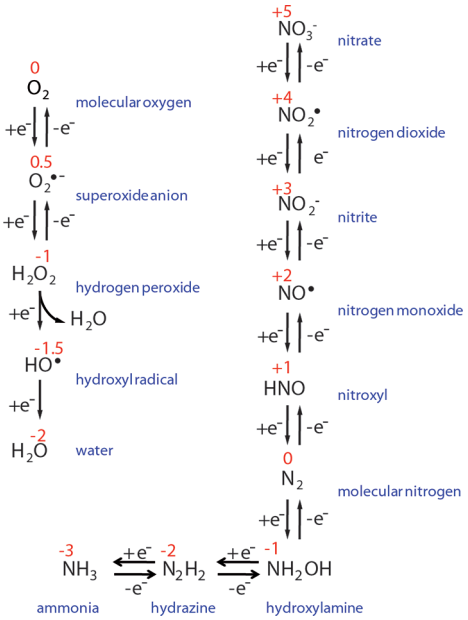

Figure 2. Comparison of the metabolites of O_2 and N_2 with respect to the oxidation state of the central atom. The equations were not balanced for clarity.

these possibilities explain the number and versatility of nitrogen metabolites (Figure 2), some of which are mainly produced by microorganisms and plants.

2.2. Fundamental Chemical Reactivity of NO•. The HOMO (highest occupied molecular orbital) of radicals is half-filled and receives the special name single occupied molecular orbital (SOMO). The SOMO is used in radical recombination reactions and represents the orbital in which an electron is removed or added in redox reactions. Therefore, the nature, symmetry, and particularly the energy of the SOMO are critical to radical reactivity. A convenient quantitative parameter for the energy of the SOMO is the redox potential of the half reaction, such as the one- electron reduction of NO• (eq 1).

$$\mathrm{NO^{\bullet} + e^{-} \rightarrow {}^{3}NO^{-}}\ \varepsilon^{0'} = -0.76\ \mathrm{V\ vs\ NHE\ (1\ mol\ L^{-1}\ nitric\ oxide)} \quad (1)$$

Numerous and contradictory values for the potential of NO• reduction have been reported, ranging from positive +0.4[20−22] to negative −1.0 V[23,24] values, based on a variety of techniques and procedures. More recently, an authoritative paper estimated the redox potential for the one-electron reduction of NO• to be approximately −0.80 V vs NHE (1 mol L^{-1} nitric oxide) by a combination of electrochemistry and quantum mechanics calculations.[25] This latter value is more consistent than those previously reported with the resistance of NO• to chemical reduction. At physiological concentrations of NO•, which are estimated to be within low pM to high nM, the redox potential is shifted to even more negative ranges. Therefore, with such a high negative redox potential, the direct reduction of NO• is biologically inaccessible.[25] The product of one-electron reduction of NO• is a triplet ($^3NO^-$), but because a pK_a of 11.6 ± 3.4 was calculated for this species from the −0.76 V redox potential,[25] the half reaction should be written with the proton coupled at neutral pH, and the relevant species is actually the singlet HNO. The production of the singlet $^1NO^-$ is energetically less favorable ($\varepsilon^{0'}$ = −1.7 V vs NHE, 1 mol L^{-1} nitric oxide)[25] because the spin pairing involves a higher Coulombic repulsion of two equally charged species occupying the same orbital and is of no biological significance. The direct oxidation of NO• is also difficult under biological conditions because a redox potential of +1.2 V vs NHE has been estimated in aqueous solutions.[24,26] At the low physiological concentrations of NO•, this redox potential is even more positive. Nevertheless, micromolar concentrations of NO• have been shown to be oxidized in vitro by compounds I and II of heme peroxidases, such as horseradish peroxidase[27] and myeloperoxidase.[28] The mechanism proposed was the one-electron oxidation of NO• to NO^+ followed by its hydrolysis to nitrite (NO_2^-).[27,28] The oxidation of NO• by myeloperoxidase may become relevant in inflammatory settings in which the concentrations of myeloperoxidase are high, and iNOS is expressed (see also below).

$$\mathrm{NO^{+} + e^{-} \rightarrow NO^{\bullet}}\ E^{0'} = +1.2\ \mathrm{V\ vs\ NHE\ (1\ mol\ L^{-1}\ nitric\ oxide)} \quad (2)$$

In conclusion, NO• is a poor oxidant and a poor reducing agent under physiological conditions. In addition, NO• is an uncharged molecule and too weak of a Lewis base to act as a nucleophilic agent. Spin pairing with other species containing unpaired electrons, such as other radicals and transition metal ions, provides the only known rapid reactions of NO• with biological compounds/intermediates. Therefore, despite being a free radical, NO• reacts with only a few targets in cells, and this preference or selectivity was very important for the evolution of NO• into a biological mediator.

The basic and most biologically important reaction of NO• with transition metal ions is a simple association, as illustrated by eq 3, which is generally referred to as nitrosylation. NO• is a common ligand for metals, and its complexes are known for all transition metals.[29−35] This reactivity is a consequence of the favorable symmetry and energetic interactions of metal d orbitals and NO• orbitals that often result in multiple, strong M-NO bonds.[30−35] NO• usually has a higher affinity for a given metal than O_2 and CO (carbon monoxide) and has a higher affinity for iron(II) than for iron(III).

$$\mathrm{M + NO^{\bullet} \rightleftarrows M\text{-}NO} \quad (3)$$

Although the nitrosylation reaction is apparently simple, the effective overlap and extension of the interactions of the metal and NO• orbitals (electron sharing or transfer) depends to a large extent on the energy of the metal d orbitals. This orbital energy, in turn, depends on the identity and oxidation state of the metal, as well as on specific characteristics of the complex, such as its geometry, additional ligands, and coordination number.[29−36] These factors determine the strength of the M-NO bond and the reactivity and kinetics of the nitrosyl complex.[29−36] For example, the Fe−N−O bond angles in nitrosyl species vary from a linear unit with the formal oxidation state of Fe^{2+}-NO^+ to a bent unit with the formal oxidation state of Fe-NO^- (with the intermediacy of Fe-NO•).[31,32,35] The formal charge on NO indicates that bound NO behaves as an eletrophile or a nucleophile.[30] Thus, although direct redox reactions of NO• to produce NO^+ and HNO are likely to be rare under physiological conditions, metal/NO• interactions occur with substantial electron transfer from the metal to NO• or vice versa, conferring a certain degree of electrophilicity or nucleophilicity to the bound NO. This metal effect determines the reactivity of the bound NO and might be relevant to the formation of *S*-nitrosothiols,[37−39] which have emerged as

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potential mediators of NO• signaling.[40,41] In addition, bound NO^+ usually delabilizes,[42] while bound NO• (or NO^-) labilizes the M-L bond of the ligand *trans*-positioned to NO• in the complex. Therefore, iron-NO bonding determines the reactivity and kinetics of metal nitrosyl complexes in levels ranging from the metal substitutional properties to the reactivity of bound NO• and other ligands.

2.3. Biological Transport of NO•. As an uncharged and almost nonpolar molecule, NO• is reasonably soluble in water (1.9 mM at 1 atm and 298 K)[43] and about 10 times more soluble in hydrophobic solvents.[44] Therefore, the hydrophobic double layer of biological membranes does not represent a barrier to NO•, which is transported by simple diffusion. This type of transport confers unique biological properties to NO• among messengers: (i) it easily crosses membranes and does not require specialized membrane transporters; (ii) it is not confined to the cells or cell compartments that produce it, acting in an autocrine and paracrine fashion; and (iii) there will be a net movement of NO• from producing cells, where it is more concentrated, to neighboring cells because simple diffusion is a random process driven by a spatial concentration gradient. The principle of molecular free diffusion has been addressed previously for NO•[45,46] and can be mathematically described by the Einsten−Smoluchowski equation (eq 4), where Δx is the average distance that NO• travels, t is the time, and D is the diffusion coefficient.

$$\Delta x = \sqrt{Dt} \tag{4}$$

As a small uncharged molecule, NO• has a large D (3300 μm^2 s^{-1}),[47,48] which is certainly important to specific biological functions requiring paracrine action, such as smooth muscle cell relaxation and neurotransmission. The actual radial distribution and reach of NO• from its source also depends on its rate of production and consumption, an issue that has been addressed through computer simulation.[45,46,49]

3. BIOLOGICAL SOURCES AND LEVELS OF NO•

3.1. Biological Sources. The sources of NO• in plants and microorganisms remain unclear[50−52] and will not be addressed here. In mammals, NO• is mainly produced from the oxidation of the amino acid L-arginine in a highly controlled process catalyzed by specialized nitric oxide synthases (NOS).[52−54] All three known NOS isoforms are homodimers, each containing two complementary domains fused into a single polypeptide chain.[52−54] The C-terminal domain is a reductase that uses electrons from NADPH to reduce the heme iron in the N-terminal domain through the intermediacy of the prosthetic groups FAD and FMN, in that order. The N-terminal catalytic domain shares structural and functional similarities with cytochrome P450 because it binds a heme prosthetic group with a thiolate as the sixth ligand and uses molecular oxygen to oxidize the guanidinium nitrogen atom of L-arginine to produce NO• and L-citrulline. The catalytic domain also binds tetrahydrobiopterin (BH_4), which is believed to provide a rapid and coupled proton/electron transfer for oxygen activation.[52−54] Indeed, it has been shown that the depletion of or damage to tetrahydrobiopterin can switch NOS from NO• production to superoxide radical anion ($O_2^{\bullet-}$) generation.[55,56]

There are two broad classes of NOS. NOS I enzymes include endothelial NOS (eNOS) and neuronal NOS (nNOS), which are constitutive enzymes and are regulated by calcium through physical interaction with calcium-bound calmodulin.[53,54] These enzymes produce low amounts of NO• upon physical or chemical stimulus and are usually associated with physiological functions. The other class, NOS II, consists of the inducible NOS (iNOS), which is calcium-independent because it is permanently bound to calmodulin. This enzyme is transcriptionally regulated and expressed in macrophages, producing large amounts of NO• usually associated with infectious and inflammatory conditions[53,54] (Figure 3). The constitutive

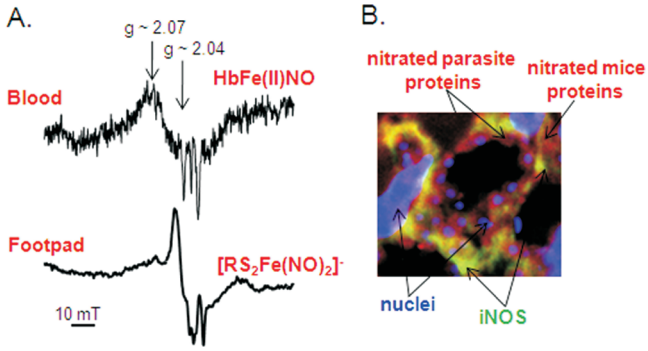

Figure 3. NO• production by C57BL/6 mice after 6 weeks of *L. amazonensis* infection at which time parasites started dying and the infection became controlled. (A) Representative low temperature EPR spectra of blood and footpads from mice showing the characteristic spectra of HbFe(II)NO and dinitrosyl iron complexes (DNIC), respectively. Adapted with permission from ref 77. Copyright 1998 American Society for Microbiology. (B) Confocal microscopy of mouse footpad emphasizing a phagolysosome containing parasites showing extensive protein nitration (3-nitrotyrosine stains red); parasite and host nuclei (blue staining) and iNOS (green staining); representative data are from ref 78.

enzymes, eNOS and nNOS, have quite different dependencies on O_2, with apparent K_m values for O_2 of 2−4 μM and above 300 μM, respectively.[57] In the brain, where the mean $[O_2]$ is approximately 20 μM,[58] the rate of NO• production is proportional to the O_2 concentrations, while in the endothelium, the rate of production is essentially O_2-insensitive (mean arterial $[O_2]$ approximately 150 μM).[59] iNOS has a K_m for O_2 of 130 μM[57] and should show partial dependency on O_2. The production of NO• in mammals through pathways that are independent of the NOS enzymes is receiving increasing attention in the literature because of the potential therapeutic implications. This interest was triggered by the discovery that nitrite (NO_2^-, pK_a = 3.4), which was considered to be a final and inactive NO• metabolite, can be reduced back to NO•, particularly at low oxygen concentrations, by several transition metal-containing proteins, such as xanthine oxidase[60−62] and deoxymyoglobin/deoxyhemoglobin.[63,64] The physiological relevance of NO_2^- as a source of NO• has been questioned because of the very low rate constant of NO_2^- reduction by xanthine oxidase[62] and deoxyhemoglobin[63−66] and because of the low physiological concentrations of NO_2^-. However, this route may be a relevant source of NO• under hypoxic conditions. Moreover, the therapeutic potential of NO_2^- has been demonstrated in several studies, justifying the need for a better understanding of its reduction in vivo (see, for instance, ref 67).

3.2. Physiological and Pathophysiological Levels. Although NO• concentrations can be measured directly with selective electrodes in cells and single cell experiments,[47] the values attained in different tissues remain debatable, and different values have been predicted by a variety of computer models.[45,46,49,68−71] These models differ mainly in the chosen

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geometry and critical parameters, such as the rate of NO• production[72] and consumption, which are usually set to fit the experimental value of approximately 1 μM that has been determined in single cell experiments.[47] A widely accepted range for the physiological concentration of NO• is 1 nM to 100 nM, although molecular biology tools associated with fluorescence techniques (sGC, cGMP, and GFP coupled biosensor)[73−75] suggest a significantly lower range, from 100 pM to 5 nM.[76] Under infectious and inflammatory conditions, the NO• concentration increases by several orders of magnitude because of the expression of iNOS in the cells of the immune system. This increase permits the detection of instantaneous concentrations of NO• in the biological fluids and tissues of experimental animals by electron paramagnetic resonance (EPR), the sensitivity of which is in the micromolar range. For instance, Figure 3A shows the corresponding EPR spectra of nitrosyl hemoglobin (HbFe(II)NO) (approximately 6 μM) and paramagnetic dinitrosyl iron complexes (approximately 8 nmol cm^{-3} tissue) present in the blood and footpad, respectively, of mice infected with the parasite *Leishmania amazonensis* after 6 weeks of infection.[77,78] Usually, sustained NO• production through the expression of iNOS leads to the accumulation of oxidized, nitrated, and nitrosated biomolecules in cells and tissues.[18,78−82] Therefore, under these conditions, the actions of NO• are considered to have been diverted from physiological to pathophysiological circuits (Figure 3B).

The rates of NO• production in vivo by humans and experimental animals have also been measured by many investigators using diverse methodologies that have rendered variable rate values. A meta-analysis of the studies available on PubMed that measure in vivo NO• synthesis in healthy humans using stable isotopic methods, which is considered to be the gold standard methodology, was recently published.[83] This analysis showed that the rate value obtained was influenced by the choice of the protocol with a large difference obtained within and between protocols. Considering the average of all values reported in the literature, the average rate of NO• synthesis in healthy human adults was calculated to be 0.63 ± 0.45 μmol kg^{-1} h^{-1}.[83] Under pathophysiological conditions, the rate of NO• production was linked with the etiopathogenesis of the disease, with increased immune and inflammatory responses triggering greater rates of NO• production. In the case of animal models, the physiological production of NO• in rats and humans was comparable (0.55 ± 0.05 versus 0.38 ± 0.06 μmol kg^{-1} h^{-1}) but considerably lower than that of mice (7.68 ± 1.47 μmol kg^{-1} h^{-1}).[83−85] Therefore, human infectious and inflammatory diseases may not be accurately reproduced by mouse models.

The above summary indicates that both the physiological and pathophysiological levels of NO• as well as its rate of synthesis can be estimated with a certain degree of confidence but that further refinement of the available methodologies is required to achieve more accurate determinations.

4. BIOLOGICAL TARGETS AND CONSEQUENCES OF NO• PRODUCTION

4.1. Targets, Kinetics, and Consequences. Similar to other radicals and oxidants, the physiological and pathophysiological actions of NO• depend on its interactions and reactions with biotargets, which compete for NO• in the crowded physiological environment. The relevance of the target will be determined by its concentration in the specific environment and by the chemical properties of NO• and the target, which define the kinetics, that is, the rate constant by which NO• reacts with the target. In contrast to peroxynitrite and H_2O_2, which typically react with their biotargets by irreversible second-order reactions,[15,16,18] the kinetics of NO• reactions vary considerably and are more complex. Some reactions of NO• are irreversible second-order reactions, such as that with oxyhemoglobin (HbFe(II)O_2) (eq 5), while others are reversible, such as that of the nitrosylation of methemoglobin (HbFe(III)) (eq 6)[86] and of ferrous heme sGC (Figure 4).

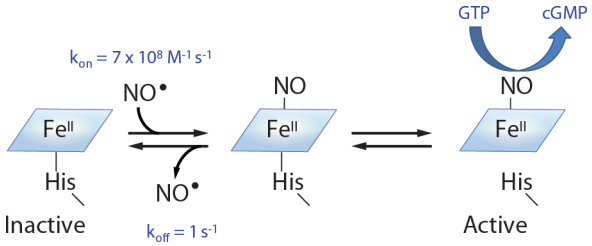

Figure 4. Classical single site model of sGC activation by NO•. This view is currently being substituted by more complex models to account for the properties of the pentacoordinated-NO complex, which is strongly affected by the availability of ATP, the GTP substrate, or excess NO• (see text).

Other reactions of NO• are reversible but proceed through an intermediate nitrosyl complex that might itself be reactive, as suggested for the heme-assisted *S*-nitrosation of a proximal thiolate in the *Cimex lectularius* nitrophorin (Np), which is responsible for NO• storage and delivery in this insect (eq 7).[87] In addition, other NO• reactions are overall irreversible third-order reactions, such as that of NO• autoxidation (eq 8).

$$NO^{\bullet} + HbFe(II)O_2 \xrightarrow{k_{av}=3.7\times10^{7}\ M^{-1}s^{-1}} HbFe(III) + NO_3^- \quad (5)$$

$$NO^{\bullet} + HbFe(III) \underset{k_{offav}=1.0\ s^{-1}}{\overset{k_{onav}=1.0\times10^{3}\ M^{-1}s^{-1}}{\rightleftharpoons}} HbFe(III)NO \quad (6)$$

$$NO^{\bullet} + NpFe(III)SR \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} NpFe(III)NOSR + NO^{\bullet} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} NpFe(II)NO + RSNO \quad (7)$$

$$4NO^{\bullet} + O_2 + 2H_2O \xrightarrow{k=2.4\times10^{6}\ M^{-2}s^{-1}} 4H^+ + 4NO_2^- \quad (8)$$

The complexity of NO• reactions with biotargets is paramount to its physiological actions. The reversible reactions of NO• are those likely to participate in signaling processes, the amplitude and duration of which depend on the characteristics and concentrations of NO• and its specific targets, and on the presence of nearby competitors/modulators (see below; Figures 4 and 5). A role for the reversible reactions of NO• proceeding through a reactive nitrosyl intermediate complex in signaling processes remains under scrutiny. The rate of these processes also depends on the concentration/proximity of the attacked nucleophile that may (eq 7) or may not be a residue of the targeted protein. If the rate-determining step is the attack of the nucleophile by bound NO, the overall process is independent of NO• concentration opening the possibility of a signal that persists after NO• decay.

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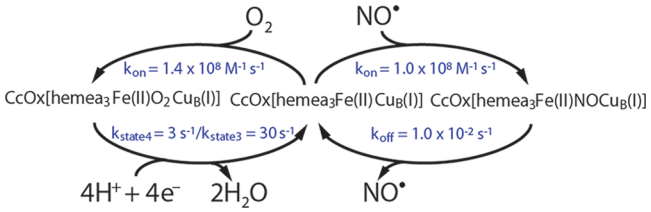

Figure 5. Competitive model for the inhibition of mitochondrial respiration by NO•. O_2 and NO• compete for the pentacoordinated heme a_3 Cu_B center of CcOx (CcOx[$heme_{a3}Fe(II)Cu_B(I)$]) with similar second-order rate constants. Because of the usually higher concentration, O_2 outcompetes NO• under most physiological conditions. Dissociation of NO• and reduction of O_2 restore the dinuclear center, which is maintained in a steady-state concentration. Since NO• dissociation is slower than oxygen reduction independently of the mitochondrial state, the concentration of nitrosyl CcOx (CcOx[$heme_{a3}Fe(II)NOCu_B(I)$]) slowly increases resulting in the inhibition of respiration (see text).

Despite the variability of NO• reactions (eqs 5−8), the fraction of NO• reacting with a specific target in a given environment is initially (t approximately 0) proportional to the value of the product of the second-order rate constant of their reaction and the concentration of that target, that is, $k_{on} \times$ [target] (eq 9). This equation can be employed as a general guide for ranking NO• targets and may be useful to connect physiological actions of NO• to specific targets. It is important to mention that the kinetics of the reactions of NO• with potentially important biotargets, such as proteins containing iron sulfur centers, remain largely uncharacterized (see below). In addition, it is not trivial to assess target concentrations in physiological environments or the influence of media heterogenicity on reaction kinetics. Even so, the better-known targets of NO• in vivo are those that react with it with second-order rate constants approaching the diffusion limit (10^7−10^{10} M^{-1} s^{-1}), as will become evident below.

$$\frac{-d[NO^\bullet]}{dt} = [NO^\bullet](k_{on1}[target_1] + k_{on2}[target_2] + k_{on}[target_3] + ...) \quad (9)$$

4.2. Ferrous Hemeproteins as Targets. As previously discussed, biological compounds containing unpaired electrons are the only species known to react rapidly with NO•. Among them, ferrous heme proteins, such as sGC, hemoglobin, and cytochrome c oxidase (CcOx), are noteworthy because they led to some of the best-understood physiological effects of NO•. The resulting effect depends on the specificity of the reaction of NO• with each of these heme proteins and differs because the reactivity of each protein is modulated by the protein environment, the type of heme coordination, and the oxidation state of the iron, among other factors. For instance, sGC binds extremely rapidly to NO• but does not bind the competitor O_2, in contrast with most ferrous heme proteins.

sGC is a 72 kDa α/β heterodimer, and only the β chain N-terminal region binds heme, whereas the C-terminal segments of both units compose the GTP cyclase catalytic domain.[5,88] The sGC pentacoordinated ferrous heme reacts with NO• with a bimolecular rate constant approaching the diffusion limit (k = 7.0×10^8 $M^{-1}s^{-1}$).[89] This binding causes the dissociation of the imidazolic nitrogen of His105,[90] which is trans-positioned to NO•, resulting in the formation of the pentacoordinated nitrosyl heme sGC.[91,92] This coordination presumably induces a conformational change in sGC that propagates to the catalytic subunit and increases by several hundred times the rate of the conversion of guanosine 5′-triphosphate (GTP) into the second messenger cyclic guanosine monophosphate (cGMP), by which NO• exerts its classical signaling function (Figure 4). In cells, sGC is rapidly deactivated upon NO• removal (NOS deactivation), a feature that could be explained by the rapid NO• dissociation (k_{off} = 1 s^{-1}).[93] However, this finding has been difficult to reproduce with the purified enzyme, which presents a very slow NO• dissociation. Indeed, recent studies with purified sGC show that this dissociation and, thus, sGC activity may be regulated by the availability of ATP and GTP.[94−97] Consequently, the classical single site model of sSG activation by NO• (Figure 4) is being replaced by more complex models to account for the properties of the pentacoordinated-NO complex.[94−98] In addition to the rapid interaction of the pentacoordinated ferrous heme of sGC with NO•, there are other factors that control sCG sensitization and desensitization. Thus, further studies are required to understand the mechanistic details by which sCG translates NO• into vasodilation.

The major sink of NO• in vivo is hemoglobin, the tetrameric $2\alpha/2\beta$ hemeprotein that is the major protein in the blood of mammals (approximately 10 mM in whole blood). Both oxyhemoglobin ($HbFe(II)O_2$) (eq 5) and deoxyhemoglobin (HbFe(II)) (eq 10) react very rapidly with NO• but in different ways.[86] The nitrosylation of the heme occurs in the case of HbFe(II) leading to HbFe(II)NO complexes, the dissociation of which is so slow that the overall process can be considered irreversible (eq 10). Indeed, while the dissociation of NO• from HbFe(II) occurs in the hour time scale, the dissociation of O_2 from $HbFe(II)O_2$ occurs in the millisecond time scale (k_{offav}= 35 s^{-1}). Not surprisingly, HbFe(II)NO can be detected in the mixture of venous and arterial blood drawn from the orbital plexus of mice carrying a footpad infection (Figure 3A).[77,78] The kinetic constants for hemoglobin are taken as the average and abbreviated as k_{av} because of the allosteric behavior of this protein.

$$NO^\bullet + HbFe(II) \underset{k_{offav}=5.0\times10^{-5}\ s^{-1}}{\overset{k_{onav}=2.0\times10^{7}\ M^{-1}s^{-1}}{\rightleftharpoons}} HbFe(II)NO \quad (10)$$

In the case of $HbFe(II)O_2$, the reaction with NO• is irreversible, and both NO• and heme are oxidized at the expense of molecular oxygen (eq 5). Because both atoms of O_2 are incorporated into nitrate (NO_3^-), this process is called NO• dioxygenation,[99,100] but its mechanism has not been fully elucidated. Early evidence indicated the transient formation of a bound peroxynitrite intermediate ($HbFe(III)ONOO^-$).[100,101] More recent studies with metmyoglobin and peroxynitrite indicated that an analogous transient complex, MbFe(III)-$ONOO^-$, undergoes O−O bond cleavage producing the caged pair [MbFe(IV)=O and $NO_2^\bullet$], which either collapses to the ferric protein form and NO_3^- or dissociates to the protein ferryl form (MbFe(IV)=O) and nitrogen dioxide ($NO_2^\bullet$).[102,103] The overall yield of $NO_2^\bullet$ is about 10%, and therefore, the reaction of $HbFe(II)O_2$ or of $MbFe(II)O_2$ with NO• functions primarily as a NO• sink. Indeed, if eq 9 is applied to estimate the consumption of 1 nM NO• by 10 mM $HbFe(II)O_2$ at a specific point of a vessel due to the constant flow of red blood cells, the value obtained is 370 μM s^{-1}. This astonishing value initially raised skepticism about the identity of EDRF as NO•,[49] but the concern was dispelled by the demonstration of the existence of

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retardation mechanisms for NO• consumption by red blood cells in vivo, such as an erythrocyte free zone in capillaries under a laminar flow regimen.[104−108] Despite the retardation mechanisms, the consumption of NO• by red cells is still very rapid and, importantly, is always occurring. Because no functional cell in tissues is more than 100−200 μm away from a blood vessel, these reactions are a powerful and unstoppable sink for NO• in vivo, which determine its dynamics and bioavailability.

Another important target of NO• is cytochrome *c* oxidase (CcOx), the terminal complex of the mitochondrial electron transport chain. CcOx catalyzes the four-electron reduction of molecular oxygen to water by a complex multistep mechanism.[109] By interacting with CcOx, NO• inhibits mitochondrial respiration reversibly (inhibition increases with NO• and decreases with O_2 concentrations) and potently (inhibition occurs at high O_2/NO• ratios).[110−113] It has also been shown that state 3 mitochondria (CcOx high turnover; rapid electron transfer and O_2 reduction) are more sensitive than state 4 mitochondria (CcOx low turnover; slower electron transfer and O_2 reduction) to NO• inhibition.[114,115] Of the four CcOx metal prosthetic groups, the only one known to react rapidly and reversibly with both O_2 and NO• is the ferrous heme a_3 of the fully reduced binuclear catalytic center composed of heme a_3 and Cu_B (CcOx[hemea$_3$Fe(II)Cu$_B$(I)]), which is the likely NO• target.[110−113] Therefore, O_2 and NO• compete for the available pentacoordinated CcOx[hemea$_3$Fe(II)Cu$_B$(I)] center. NO• binding is a nonconsuming reversible reaction, whereas O_2 binding is followed by electron entry and O_2 reduction to water (Figure 5). Both processes restore and maintain a steady-state concentration of the CcOx[hemea$_3$Fe(II)Cu$_B$(I)] center available for the binding of O_2 or NO•. Because of the similar rate constants[116,117] and the usually higher physiological concentration, O_2 outcompetes NO• under most conditions (Figure 5). Still, a percentage of the CcOx[hemea$_3$Fe(II)-Cu$_B$(I)] center, which is given by the [O_2]/[NO•] ratio, becomes nitrosylated at each CcOx turnover. Because the reduction of O_2 is more rapid than NO• dissociation regardless of the mitochondrial state (Figure 5), the nitrosyl CcOx (CcOx[hemea$_3$Fe(II)NOCu$_B$(I)]) gradually accumulates up to its equilibrium concentration, which is determined by NO• concentration, resulting in respiration inhibition.[118,119] State 3 mitochondria are more sensitive than state 4 simply because they restore the reduced dinuclear center more rapidly, providing a higher CcOxhemea$_3$Fe(II)Cu$_B$(I) steady state concentration for nitrosylation. Therefore, all the basic features of the inhibition of mitochondrial respiration by NO• are explained by the model schematized in Figure 5.[118,119]

This competitive inhibition of respiration by NO• probably dominates the scenario under most dynamic concentrations of NO• and O_2 and mitochondrial states expected in vivo.[118,119] However, it has been shown that NO• reacts fairly rapidly (*k* approximately 10^5 M^{-1} s^{-1}) with several oxidized CcOx intermediates to produce bound NO_2^-,[120−122] which then slowly dissociates from ferric heme a_3 or cupric Cu_B, giving origin to an uncompetitive component of the NO• inhibition of respiration. Because NO• intercepts oxidized CcOx intermediates, these reactions increase in importance with high O_2 (which is necessary to generate the NO•-derived intermediates) and very low CcOx turnover (which provides more time for NO• to react with CcOx intermediates).[123] Interestingly, these reactions consume both O_2 and NO•. Whether they are relevant to cellular NO• catabolism is not known, but they may constitute one of the routes by which NO• is metabolized to NO_2^-, a process that remains poorly understood (see also below). The modulation of mitochondrial respiration by NO• has been considered a physiological process, although this designation remains controversial. Some critics of physiological effects argue that the significant inhibition of CcOx requires levels of NO• that are higher than the accepted physiological range (1−100 nM). Under hypoxia, in which the consumption of NO• is lowered and the competition between NO• and O_2 for CcOx is decreased, the interaction between CcOx and NO• is expected to increase. Similarly, a more significant fraction of active CcOx heme a_3 may be nitrosylated in CcOx deficiency diseases and in chronic inflammatory/infectious conditions because of the increased NO• levels (Figure 3).

4.3. Ferric Heme Proteins as Targets. The interactions of NO• with heme proteins in the iron(III) state are usually much slower than its interactions with ferrous heme proteins as observed for methemoglobin (eq 6) and ferric cytochrome *c* (eq 11),[124] and these proteins are unlikely to be important direct targets of NO•.

$$\mathrm{NO^{\bullet}} + \mathrm{CitcFe(III)} \underset{k_{offav}=8.7\times10^{-2}\ s^{-1}}{\overset{k_{onav}=1.3\times10^{3}\ M^{-1}s^{-1}}{\rightleftharpoons}} \mathrm{CitcFe(III)NO} \quad (11)$$

However, NO• coordinated to ferric heme carries a significant positive character and is susceptible to nucleoplilic attack (eq 7). In addition, the higher oxidation states produced during the turnover of ferric heme peroxidases with H_2O_2, such as compounds I and II, oxidize NO• to NO_2^- with second-order rate constants in the range of 10^4−10^5 M^{-1} s^{-1}.[27,28] Similar intermediates may be produced from CcOx turnover and be responsible for the mitochondrial oxidation of NO• to NO_2^- observed under very high O_2 concentration and very low CcOx turnover. These processes are more likely to occur under conditions of pathophysiological NO• levels.

4.4. Nonheme Iron Sites as Targets. Proteins containing iron−sulfur clusters are among the metalloproteins that are targeted by NO•. Because these proteins perform a variety of functions in fundamental processes such as energy metabolism, gene expression, and iron homeostasis, their interactions with NO• may have profound physiological consequences.[125] Iron−sulfur clusters appear with several different configurations of iron and sulfide, such as [2Fe-2S], [3Fe-4S], and [4Fe-4S], but the iron remains in a conserved microtetrahedral geometry bound to two sulfides and two other identical ligands, which are usually protein cysteine thiolates.[125] The unsaturated tetrahedral coordination sphere of the iron in the clusters leaves room for additional ligands permitting interactions with NO•. Indeed, iron−sulfur clusters of any configuration are known to react rapidly with NO•, although these reactions remain poorly understood, and their products have not been fully characterized. Until recently, it was generally accepted that the products were paramagnetic dinitrosyl iron complexes (DNIC) because these complexes have been universally detected in cells and tissues under conditions of high NO• production (see, for instance, Figure 3). However, recent results have challenged this view. For instance, it has been shown that a considerable portion (50−75%) of the cellular iron source for DNIC is actually the chelatable iron pool (CIP).[126,127] Additionally, recent studies with purified iron−sulfur cluster proteins have confirmed that the S = 1/2 paramagnetic DNIC accounts for only a minor fraction of the products of NO• attack.[128,129] The main products identified

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were Roussińs red ester and Roussińs black salt, both of which are diamagnetic dinitrosyl complexes (exemplified in Figure 6).

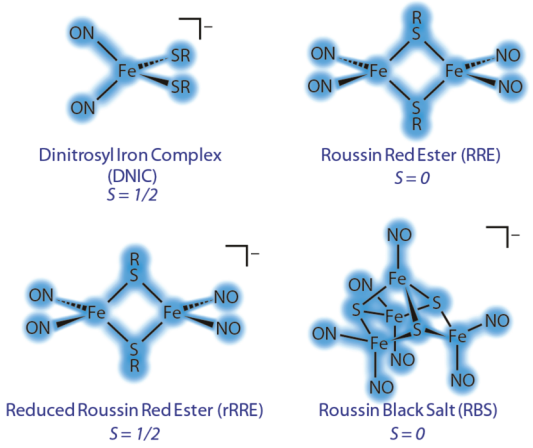

Figure 6. Generic structures of paramagnetic and diamagnetic dinitrosyl iron complexes (DNIC). DNIC are formed primarily from the chelatable iron pool (CIP), while the diamagnetic RRE and RBS have been shown to be the main products of the reactions of proteins containing iron−sulfur clusters with NO•.

There are few detailed kinetic studies of the reaction of NO• with iron−sulfur proteins, and the results reveal complex behavior.[128,129] The reaction of the [4Fe-4S] iron−sulfur clusters of WhiB and WhiD shows multiphase kinetics indicative of multistep consecutive reactions with at least three intermediates.[128] The rate of each kinetic phase follows a first-order dependence on NO•. The apparent second-order rate constant value for the first step is high (approximately 10^5 $M^{-1}s^{-1}$), but those of the subsequent steps are progressively lower (eq 12).[128] Similarly, the kinetics of the reaction of the *Pseudomonas* sp. Rieske center [2Fe-2S] with NO• show multiphase behavior.[129] Therefore, the kinetics and products of the reaction with NO• appear to be independent of the cluster type.[128,129] These studies did not identify the several possible intermediates of the reactions, although a mononitrosyl species with a pentacoordinated iron was characterized for the reaction of NO• with the Rieske center.[129] Additionally, the reversibility of each step has not been addressed, and this behavior may be crucial to establishing the role these reactions may have in signaling mechanisms.

$$8NO^{\bullet} + P[4Fe\text{-}4S] \overset{(1)}{\rightarrow} \overset{(2)}{\rightarrow} \overset{(3)}{\rightarrow} \overset{(4)}{\rightarrow} 2[Fe^{I}(Cys)_2(NO)_4] + S^{2-} + 3S^0$$

$$\text{WhiD} \quad k(1) = 6.5 \times 10^5\ M^{-1}s^{-1}$$

$$k(2) = 2.1 \times 10^4\ M^{-1}s^{-1}$$

$$k(3) = 5.3 \times 10^3\ M^{-1}s^{-1}$$

$$k(4) = 1.0 \times 10^3\ M^{-1}s^{-1} \qquad (12)$$

The demonstration that the major source of the DNIC detected in cells and tissues is the intracellular chelatable iron pool (CIP)[126,127] has added new prospects for unraveling the consequences of the interactions of NO• with iron−sulfur clusters and complexes. The CIP represents a small (0.2−3%) proportion of total cellular iron and is defined as the cellular source of iron that can be complexed with strong chelating compounds. As a source of iron, the CIP iron pool reacts rapidly with NO• and is primarily converted to paramagnetic protein-bound DNIC, as shown by different laboratories in cell culture experiments.[39,126,127,130−132] Under short NO• exposures, 1 h or less, the amount of DNIC formed approaches, but never exceeds, the amount of CIP.[126] Long exposures, however, result in DNIC in amounts higher than the CIP, suggesting other cellular iron sources may form complexes or are mobilized and then form DNIC.[127] These sources are likely to include iron−sulfur clusters attacked by NO• or by its metabolites, such as peroxynitrite.[133,134] Because the CIP is not a defined chemical species, there are no kinetic data available, but indirect evidence points to a rapid reaction with NO•.[126,127] In addition, it is clear that formation of these complexes requires the CIP and free NO•[126,127,131,132] and that DNIC are universally detectable in cells and tissues exposed to exogenous or endogenous NO• (Figure 3).[77,78,135] The formation of DNIC is practically insensitive to the NO• and O_2 concentrations and the complexes accumulate to levels that are much higher than those of 3-nitrotyrosine, nitrolipids, and *S*-nitrosothiols in cells chronically exposed to NO• .[127] This accumulation probably results from the stability of the DNIC.[136,137] Although the physiological consequences of DNIC formation have not been fully explored, it is likely to impact iron homeostasis by decreasing the CIP.[138] Additionally, dinitrosyl complexes have been suggested to mediate the formation of cellular *S*-nitrosothiols.[39] This suggestion is particularly relevant because the nitrosation of protein thiol groups is a ubiquitous posttranslational protein modification that has been extensively studied in the context of cell toxicity and cell signaling.[40,41]The mechanisms by which *S*-nitrosothiols are produced in vivo, however, remain debatable. Until recently, the proposed mechanisms involved the redox reaction of thiols with metalloproteins or with NO• metabolites, such as N_2O_3 and NO_2• (Figure 7).[37−41,139−141] Although all of these

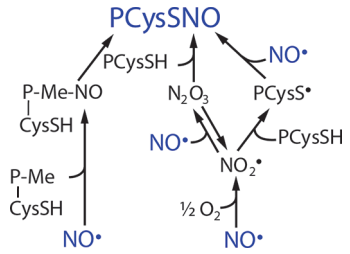

Figure 7. Schematic representation of the possible mechanisms for the formation of *S*-nitrosothiols (*S*-nitrosation mechanisms). Protein-metal dependent *S*-nitrosation is the only route that might be selective. The radical route shown as resulting from NO• autoxidation can also result from radicals produced from peroxynitrite (see Figure 8). The radical route is most likely nonselective and connected to pathophysiology. In the case of oxidation of GSH by radicals, the production of *S*-nitrosoglutathione may result, which could nitrosate proteins by *trans S*-nitrosation.

mechanisms may operate in vivo,[142] protein transnitrosation by DNIC produced from the CIP is attractive from a signaling perspective because it can occur under very low O_2 concentrations and may be catalytic and selective.[39]

4.5. Reaction with Molecular Oxygen. O_2 has two unpaired electrons in the ground state, which makes it a triplet molecule and reactive toward other species containing unpaired electrons, including NO•. The overall reaction of NO• with O_2

in aqueous buffer produces NO_2^- (eq 8) by a multistep, complex reaction that is not completely understood (eqs 13–15).[143−148]

$$NO^\bullet + O_2 \xrightarrow{k=2.4\times10^6\ M^{-2}s^{-1}} 2NO_2^\bullet \quad (13)$$

$$NO^\bullet + NO_2^\bullet \rightleftharpoons N_2O_3 \quad (14)$$

($K=2.6\times10^6\ M^{-1}$)

$$N_2O_3 + H_2O \xrightarrow{k_{pi}=8.3\times10^5\ s^{-1}} 2NO_2^- + 2H^+ \quad (15)$$

The reactive N_2O_3 (dinitrogen trioxide) and $NO_2^\bullet$ (nitrogen dioxide) are produced as intermediates and may participate in *S*-nitrosation (Figure 7) in addition to other deleterious reactions. N_2O_3 also participates in *N*-nitrosation reactions and is considered an important mediator in DNA base deamination.[149] $NO_2^\bullet$ is a moderately oxidant free radical (E^0 = 0.99 V) that can oxidize and nitrate proteins, lipids and DNA.[79] All of these reactions are likely to be favored in hydrophobic environments, such as those found in membranes and in the hydrophobic core of proteins, because both $NO^\bullet$ and O_2 accumulate in these environments, causing a concentration-related 30-fold acceleration of the autoxidation rate (eq 13).[148] Although $NO_2^\bullet$ and N_2O_3 can eventually be produced from $NO^\bullet$ autoxidation in hydrophobic environments, it is difficult to consider that the overall process is the one responsible for the well-established metabolism of $NO^\bullet$ to NO_2^-. Indeed, the rate of $NO^\bullet$ disappearance by autoxidation (eq 13) is given by a third-order rate law (eq 16) and will be too slow at physiological concentrations of $NO^\bullet$ to account for the rate of $NO^\bullet$ consumption in vivo.[148]

$$\frac{-d[NO^\bullet]}{dt} = 4k[NO^\bullet]^2[O_2] \quad (16)$$

For instance, given the k value of the autoxidation reaction ($k = 2 \times 10^6\ M^{-2}\ s^{-1}$) and assuming the physiological concentration of O_2 to be 50 μM, it is calculated that $NO^\bullet$ would disappear with a half-life of 2.8×10^3 and 2.8 h at concentrations of 1 nM and 1 μM, respectively. Therefore, the routes that rapidly metabolize approximately 40% of $NO^\bullet$ to NO_2^- remain under scrutiny. It is likely that CcOx,[120−122] heme peroxidases,[27,28] and other heme proteins[149−151] may be the major players in cells whereas ceruloplasmin[152] may operate extracellularly. This aspect should be clarified in the near future.

4.6. Reaction with Radicals Resulting in Antioxidant and Pro-Oxidant Activities. Radical−radical reactions occur at near diffusion-controlled rates (k approximately $10^9\ M^{-1}s^{-1}$), and not surprisingly, $NO^\bullet$ reacts extremely rapidly with other radicals. All radicals that are produced under biological conditions are more reactive than $NO^\bullet$ toward biotargets because they are much stronger oxidants. In fact, the oxidizing strength of a radical is given by its one-electron reduction potential (Table 1)[9,15,153,154] because of the low activation energy of radical reactions. Therefore, reactive radicals are likely to react preferentially with biotargets, which are present at higher concentrations, than with the usually low levels of $NO^\bullet$. However, the reaction of $NO^\bullet$ with biotarget-derived radicals has the important consequence of endowing $NO^\bullet$ with antioxidant properties. Indeed, $NO^\bullet$ has been shown to be a potent inhibitor of both lipid peroxidation[155,156] and protein oxidation[157] by recombination reactions with lipid- and protein-derived radicals, which terminate radical chain reactions (see, for instance, eq 17).

Table 1. Reduction Potential of Selected Radicals

radical[a]	reduction potential ($E^{o\prime}$, V)
$NO^\bullet/{}^3NO^-$	−0.80
$ONOO^\bullet/ONOO^-$	0.40
$O_2^{\bullet-}, 2H^+/H_2O_2$	0.94
$HO_2^{\bullet-}, H^+/H_2O_2$	1.06
$ROO^\bullet, H^+/ROOH$	1.00
$NO_2^\bullet/NO_2^-$	1.04
$RO^\bullet, H^+/ROH$	1.60
$CO_3^{\bullet-}, H^+/HCO_3^-$	1.78
$HO^\bullet, H^+/H_2O$	2.31

[a]Data for the one-electron reduction potential collected from ref 153 except for $NO^\bullet$,[25] $CO_3^{\bullet-}$,[79] and $ONOO^\bullet$.[154]

$$NO^\bullet + LipidO^\bullet \xrightarrow{k_{av}\sim10^9\ M^{-1}s^{-1}} LipidONO \quad (17)$$

In contrast, the reaction of $NO^\bullet$ with the superoxide radical anion ($O_2^{\bullet-}$) greatly increases the oxidant potential of both $NO^\bullet$ and $O_2^{\bullet-}$, as discussed below.

4.7. Reaction with the Superoxide Radical Anion and Peroxynitrite Production. The diffusion-controlled reaction of $NO^\bullet$ with $O_2^{\bullet-}$ is important because it may limit $NO^\bullet$ availability under certain conditions, in addition to producing peroxynitrite ($ONOO^-/ONOOH$) (eq 18),[158] an inorganic peroxide with unusual properties.

$$NO^\bullet + O_2^{\bullet-} \xrightarrow{k=(0.4-1.9)10^9\ M^{-1}s^{-1}} ONOO^- \quad (18)$$

Compared with other peroxides, peroxynitrite has a low pK_a (6.8) and is present as a mixture of the anion and the acid ($ONOO^-/ONOOH$) at most physiological pHs. Additionally, in contrast with other peroxides, peroxynitrite is prone to proton- and carbon dioxide-catalyzed homolysis that generate potent one-electron oxidants, such as the hydroxyl radical ($HO^\bullet$), nitrogen dioxide ($NO_2^\bullet$), and the carbonate radical ($CO_3^{\bullet-}$) (Figure 8; Table 1).[159,160] The reaction with CO_2 and,

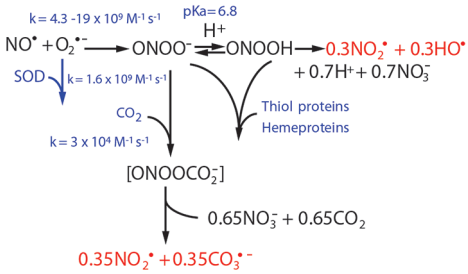

Figure 8. Schematic representation of the major reactions involved in the production and fates of peroxynitrite under physiological conditions. The details are discussed in the text.

thus, the production of $NO_2^\bullet$ and $CO_3^{\bullet-}$ radicals is an important consequence of peroxynitrite production in vivo because of the high concentration of CO_2 in biological fluids (approximately 1.3 mM in equilibrium with HCO_3^-) and the considerable second-order rate constant of the reaction between CO_2 and peroxynitrite ($3 \times 10^4\ M^{-1}\ s^{-1}$).[161] The production of $HO^\bullet$ from peroxynitrite is less likely because of competing reactions, including those with heme proteins and thiol proteins. In these reactions, peroxynitrite acts as a two-electron oxidant although there are some exceptions in the case of heme proteins.[18] The particularly high second-order rate

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constant of the reaction of peroxynitrite with thiol proteins from the peroxiredoxin family (Prx) (10^5–10^8 M^{-1} s^{-1}) argues for a role of these proteins in peroxynitrite detoxification.[18]

The chemical biology of peroxynitrite acting either as an endogenous cytotoxin toward host tissues or a cytotoxic effector molecule against invading pathogens was critically reviewed recently.[18] It is worth emphasizing that peroxynitrite production is favored under conditions of high $NO^{\bullet}$ and $O_2^{\bullet-}$ production to overcompete the rapid $O_2^{\bullet-}$ dismutation catalyzed by the ubiquitous superoxide dismutases (SOD) (Figure 8). This scenario is present under infectious and/or inflammatory conditions as a result of the expression of iNOS and the mounting of NADPH oxidases (Nox) (Figure 3). If the reaction of peroxynitrite with the ubiquitous CO_2 overcomes its reaction with other targets, the net result will be the transformation of a poorly reactive radical ($NO^{\bullet}$) into a moderate ($NO_2^{\bullet}$) and a strong one-electron oxidant ($CO_3^{\bullet-}$) (Table 1), which are able to oxidize, nitrate and, eventually, nitrosate biomolecules through radical mechanisms (Figure 7). The biological chemistry of $NO_2^{\bullet}$ and $CO_3^{\bullet-}$ was reviewed relatively recently.[79,162] A relevant addition to the previously reported properties of $CO_3^{\bullet-}$ is its ability to cause protein–protein[163] and DNA–DNA cross-links.[164] Therefore, it is not surprising that infectious and inflammatory conditions are typically accompanied by tissue damage. On the contrary, the routes responsible for transforming $NO^{\bullet}$ into reactive oxidants provide new targets to explore pharmacological interventions aiming to handle the harmful effects of chronic inflammatory conditions.[18,81,82,165]

5. CONCLUSIONS

The physicochemical properties of $NO^{\bullet}$ as a small, uncharged and poorly reactive free radical that reacts rapidly only with biotargets containing unpaired electrons permitted its evolution as a biological mediator. By summarizing the characteristics and consequences of the reactions of $NO^{\bullet}$ with known biotargets, we aimed to emphasize the usefulness of kinetics as a tool to unravel the physiological and pathophysiological actions of species that are short-lived under physiological conditions, such as radicals and other oxygen-derived species.[9,14,15,18] In contrast to the latter, however, the reactions of $NO^{\bullet}$ with biotargets vary from typically irreversible second-order reactions to the formation of reactive and nonreactive metal nitrosyl complexes (eqs 5–7). These characteristics add layers of complexity and regulatory possibilities to the interactions of $NO^{\bullet}$ with biotargets. For instance, the fate of the pentacoordinated sGCNO complex is strongly affected by the availability of ATP, GTP, or excess $NO^{\bullet}$ to control sGC activation and deactivation.[94,95,97,98] Nevertheless, signaling through sGC nitrosylation is a reversible process that is dependent on the available $NO^{\bullet}$ concentration (Figure 4). Similarly, the inhibition of mitochondrial respiration through CcOx nitrosylation is reversible and dependent on $NO^{\bullet}$ concentration (Figure 5). In contrast, $NO^{\bullet}$ actions that occur through reactive nitrosyl intermediates (eq 7) are likely to be insensitive to the concentration of $NO^{\bullet}$ and to persist after its decay. Such a mechanism may operate in the case of nitrosothiol-mediated signaling.[39] These diverse possibilities do not change the fact that any claimed biological action of $NO^{\bullet}$ should be connected to its interaction with a kinetically relevant target. Accordingly, the biotargets responsible for the best-known physiological effects of $NO^{\bullet}$, sGC, HbO_2, and CcOx are ferrous heme proteins that react with $NO^{\bullet}$ with second-order rate constants approaching the diffusion limit (k_{on} approximately 10^7 to 10^8 M^{-1} s^{-1}). Likewise, the biotarget responsible for the most described pathophysiological actions of $NO^{\bullet}$ is $O_2^{\bullet-}$, which reacts with $NO^{\bullet}$ in a diffusion-controlled process (k approximately 10^9 to 10^{10} M^{-1} s^{-1}) (eq 18). However, there are very few studies of the kinetics of the reactions of $NO^{\bullet}$ with proteins containing iron–sulfur clusters (eq 12).[128,129] The determined second-order rate constant of the first step of these reactions is considerable (k approximately 10^5 M^{-1} s^{-1}), indicating that iron–sulfur clusters are important $NO^{\bullet}$ targets. However, little is known about the reversibility of the steps of these interactions, a property crucial to disclose their physiological roles. Not surprisingly, the interactions of proteins containing iron–sulfur clusters with $NO^{\bullet}$ remain ambiguous and have been associated with both physiological and pathophysiological effects. Therefore, more kinetic studies with these proteins are warranted. The structural and kinetic characterization of the chelatable iron pool (CIP) is also crucial because it is the major source of the paramagnetic dinitrosyl iron complexes (DNIC) universally detected in cells and tissues exposed to exogenous or endogenous $NO^{\bullet}$.[126,127,131,132]

The reactivity of radicals and oxidants toward biotargets still receives limited attention in the redox literature. Certainly many other factors are important for triggering a cellular response, such as radical fluxes, radical diffusion into tissue and cellular compartments, and in situ available targets. However, the reactivity of the radical or oxidant is crucial in triggering enzymatic cascades, the consequences of which may include modulation of the enzyme that generates the radical or oxidant and, thus, of its flux. Further advances in our understanding of how these inter-related processes translate into physiological and pathophysiological responses will require interdisciplinary approaches combining system biology with rigorous chemical and biological studies.

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Notes

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ABBREVIATIONS

CIP, chelatable iron pool; DNIC, paramagnetic dinitrosyl iron complexes; EPR, electron paramagnetic resonance; [FeS], iron sulfur clusters; Hb, Hb(Fe(II), hemoglobin; HbO_2, HbFe(II)-O_2, oxyhemoglobin; HbFe(III), methemoglobin; MbFe(IV)=O, ferryl myoglobin; CitcFe(III), ferric cytochrome *c*; NHE, normal hydrogen electrode; NOS, nitric oxide syntases; Nox,

NADPH oxidases; peroxynitrite, the sum of peroxynitrite anion ($ONOO^-$, oxoperoxonitrate (−1)) and peroxynitrous acid (ONOOH, hydrogen oxoperoxonitrate) unless specified; sGC, soluble guanylate cyclase; ROS, reactive oxygen species; RNS, reactive nitrogen species; cGMP, cyclic guanosine monophosphate; GTP, guanosine triphosphate; GFP, green fluorescent protein; CcOx, cytochrome oxidase; CcOx[$heme_{a3}$Fe(II)Cu_B(I)], fully reduced cytochrome *c* oxidase; CcOx[hemea3Fe(II)NOCuB(I)], nitrosyl cytochrome *c* oxidase

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