REVIEW

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Chemical Biology of Peroxynitrite: Kinetics, Diffusion, and Radicals

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ABSTRACT Peroxynitrite is formed by the very fast reaction of nitric oxide and superoxide radicals, a reaction that kinetically competes with other routes that chemically consume or physically sequester the reagents. It can behave either as an endogenous cytotoxin toward host tissues or a cytotoxic effector molecule against invading pathogens, depending on the cellular source and pathophysiological setting. Peroxynitrite is in itself very reactive against a few specific targets that range from efficient detoxification systems, such as peroxiredoxins, to reactions eventually leading to enhanced radical formation (*e.g.*, nitrogen dioxide and carbonate radicals), such as the reaction with carbon dioxide. Thus, the chemical biology of peroxynitrite is dictated by the chemical kinetics of its formation and decay and by the diffusion across membranes of the species involved, including peroxynitrite itself. On the other hand, most durable traces of peroxynitrite passing (such as 3-nitrotyrosine) are derived from radicals formed from peroxynitrite by routes that represent extremely low-yield processes but that have potentially critical biological consequences. Here we have reviewed the chemical kinetics of peroxynitrite as a biochemical transient species in order to estimate its rates of formation and decay and then its steady-state concentration in different intra- or extracellular compartments, trying to provide a quantitative basis for its reactivity; additionally, we have considered diffusion across membranes to locate its possible effects. Finally, we have assessed the most successful attempts to intercept peroxynitrite by pharmacological intervention in their potential to increment the existing biological defenses that routinely deal with this cytotoxin.

Peroxynitrite is a biologically generated reactive species, the product of the coupling reaction of two free radicals, nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$), via the diffusion-controlled process

$$^{\bullet}NO_2 + O_2^{\bullet-} \rightarrow ONOO^- \quad (1)$$

(The term peroxynitrite is used to refer to the sum of peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH; $pK_a = 6.8$); IUPAC recommended names are oxoperoxonitrate and hydrogen oxoperoxonitrate, respectively. When required, the individual species will be specifically mentioned in the text.) This peroxide is continuously formed under basal metabolic conditions, but its biological actions become particularly notorious under enhanced cell/tissue rates of $O_2^{\bullet-}$ and/or $^{\bullet}NO$ generation as a result of its unique reactivity toward biomolecules and its tendency to initiate free radical processes. The chemical properties of peroxynitrite make it an important biological oxidant and, through this chemistry, a central pathogenic mediator in a variety of disease states including cardiovascular, neurodegenerative, and inflammatory disorders. Indeed, over the past decade peroxynitrite has been identified as a culprit in disease processes associated with the disruption of normal $^{\bullet}NO$ and redox metabolism. Recognizing the pathogenic role of peroxynitrite has not been an easy task because of its extremely short biological half-life (<10 ms), which results in a rather low steady-state concentration (nanomolar levels) and precludes its direct isolation and detection *in vivo*, and the fact that the biomarkers of oxidative damage left by peroxynitrite (in particular, protein tyrosine nitration) are indicative of but not entirely specific to this reactive species. A combination of methods that include the use of peroxynitrite-sensitive probes, modification of endogenous components by peroxynitrite-dependent reactions, and the

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utilization of pharmacological and genetic engineering approaches to interfere with peroxynitrite formation and reactions have assisted in the unraveling of it as a key player in numerous pathologies. More recently, strategies based on accumulated chemical biology knowledge have been directed to the design, development, and evaluation of molecules capable of catalytically decomposing peroxynitrite; the emerging successful molecules tested in disease conditions at the cell, organism, and animal level have opened avenues for treatment of peroxynitrite-mediated pathogenic processes. Importantly, peroxynitrite has been also identified as a key cytotoxic effector of immune system cells, notably macrophages, toward invading bacteria and parasites.

Many of the developments that have allowed characterizing the biological formation, actions and consequences of peroxynitrite have relied on several laborious studies dealing with the chemical biology of peroxynitrite, since it became first recognized as a biomolecule (*1*–*5*). The coexistence of both the anionic and protonated forms of peroxynitrite under physiological pH conditions, together with the fact that it can act as both one- and two-electron oxidant as well as decompose to secondary radicals, may imply that the chemical biology of this reactive species is intricate (*6*). However, as we will see, it is the availability of suitable targets in the different biological environments where it can be formed or located (*e.g.*, intramitochondrial, intracellular, or extracellular) that constitutes the fundamental factor critically influencing the biological fate of peroxynitrite, with both reactions and diffusion events through hydrophilic and hydrophobic biocompartments simultaneously taking place.

The content of this Review will concentrate on three key aspects concerning the chemical biology of peroxynitrite: (i) Despite its fast formation reaction rate, its biological formation is not granted *a priori* because of of the existence of strong competing processes over both $^{\bullet}NO$ and $O_2^{\bullet-}$. Moreover, the subcellular or extracellular sites of peroxynitrite formation will be determined by the location of its radical precursors and their differential diffusion properties. These particular points have been subject of much debate in the field and will be analyzed with a mechanistic view in this Review. The rational and experimental data that support peroxynitrite formation *in vivo* have been previously reviewed (*6*–*8*). (ii) The reactions of peroxynitrite with sensitive biological targets will be assessed to provide the chemical biology foundation that permits one to explain how peroxynitrite affects cell and tissue processes; indeed, peroxynitrite reactions can result in changes in cell viability including profound alterations of mitochondrial homeostasis, potentially leading to cell death. The biochemical mechanisms of peroxynitrite-mediated changes in cell and mitochondrial homeostasis and signaling of apoptotic and necrotic cell death have been reviewed elsewhere (*9, 10*). (iii) Finally, current chemical biology research and development strategies on compounds that pharmacologically interfere with peroxynitrite will be presented. The biological and pharmacological data demonstrating the protective effect of catalytic scavengers and molecules that interfere in peroxynitrite reaction pathways on a variety of disease models have been recently reviewed (*10*). Indeed, the information to be covered in this Review, including peroxynitrite kinetics and reaction mechanisms with target molecules, provides the foundation for the design and testing of anti-peroxynitrite compounds. In addition, physicochemical and redox properties of existing synthetic compounds that can readily react with peroxynitrite *in vitro* will be briefly analyzed in the context of their potential application to *in vivo* systems.

Peroxynitrite is an unstable metabolite, and as such its biochemistry is dictated by the kinetics of its formation and decay together with its diffusion. Although the vast majority of peroxynitrite formed ends up as nitrite, the radicals formed during some of the reactions are most likely to leave a trace of peroxynitrite's short-lived existence as more stable oxidized and nitrated biomolecules. The reaction kinetics of peroxynitrite formation and decay have come to be understood through *in vitro* experiments, whereas solid quantitative data for the estimation of peroxynitrite fluxes and steady-state concentrations in cell compartments have proven difficult to ob-

KEYWORDS

Carbonate radical ($CO_3^{\bullet-}$): Free radical formed by the reaction of peroxynitrite with carbon dioxide or by the oxidation of bicarbonate. It is a strong one-electron oxidant and does not form stable adducts.

Catalytic antioxidants: A series of synthetic molecules intended to catalyze the reduction or decomposition of biologically relevant reactive species, thereby providing protection against oxidative stress. This category of antioxidants includes metal porphyrins and mimics of superoxide dismutase and catalase activities.

Free radical: An atom or group of atoms that has an unpaired valence electron, which renders it extremely reactive in most cases. Unpaired-electron species containing transition metals are usually not considered free radicals.

Homolysis: The breaking of a bond in a compound that produces two fragments that are free radicals.

Nitric oxide (nitrogen monoxide, $^{\bullet}NO$): Small, nonpolar, and rather unreactive free radical formed enzymatically that has functions in neural transmission, vasorelaxation, platelet aggregation, and immune response. It reacts preferentially with other free radicals and heme iron.

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tain. Nevertheless, through the combined use of reporter reactions, pharmacological intervention, and knowledge of repair systems, the levels and relevance of this endogenous cytotoxin have become clearer.

Precursors and Formation. Evidence of the biological formation of peroxynitrite predates its identification as a relevant biochemical species, *i.e.*, reaction was observed without knowing the exact identity of the reactants; the effect of $O_2^{\bullet-}$ on the half-life of the at that point unidentified endothelium-derived relaxing factor was the first indirect clue of peroxynitrite relevance (*11, 12*). Soon afterward, $^{\bullet}NO$ was identified as a key component of the endothelium-derived relaxing factor and the reaction with $O_2^{\bullet-}$ was used as a pharmacological evidence of the identity (*13*). A few years later, peroxynitrite was proposed to be the intermediate formed from $^{\bullet}NO$ and $O_2^{\bullet-}$ and was also proposed to be responsible for most of the toxicity arising from these radicals (*1–3*).

The rate constant of reaction has been determined by several methodologies within the range $4-16 \times 10^9$ M^{-1} s^{-1} (*14–16*). This very large value explains how peroxynitrite can be formed at all considering that its precursors are very elusive species. Indeed, both $^{\bullet}NO$ and $O_2^{\bullet-}$ are free radicals and exist in low steady-state concentrations *in vivo* that are governed by very efficient disposal systems. $^{\bullet}NO$ is small, uncharged, and barely polar and thus freely diffusible across membranes from one compartment to another. This radical is rather unreactive but is readily depleted once it reaches a blood vessel where it is transformed to nitrate via its reaction with oxyhemoglobin (*17, 18*). Its partner in peroxynitrite formation, $O_2^{\bullet-}$, has a severely constrained diffusion due to its electric charge, and its concentration is maintained at a low level by the ultimate scavengers, the superoxide dismutases (SOD). These enzymes are extremely efficient (rate constant $>10^9$ M^{-1} s^{-1}), abundant (concentration >10 μM), and intracellularly ubiquitous. Nevertheless, if $O_2^{\bullet-}$ is formed extracellularly, it could reach higher steady-state concentrations since extracellular SOD is much less abundant.

The concentrations of $^{\bullet}NO$ and $O_2^{\bullet-}$ dictate the rate of peroxynitrite formation and are therefore a necessary starting point to ascertain its importance in various cellular scenarios. In this regard, a series of ingenious strategies have been devised to find out steady-state concentrations and rates of $O_2^{\bullet-}$ formation. We will focus on what we consider to be the two extreme scenarios for $O_2^{\bullet-}$ production, namely, basal mitochondrial formation and intraphagosomal formation in stimulated neutrophils.

Quijano *et al.* (*19*) quantitated intramitochondrial formation in bovine aortic endothelial cells by the rate of inactivation and reactivation of the $O_2^{\bullet-}$-sensitive enzyme aconitase. This work estimated a rate of 0.7 μM s^{-1} of $O_2^{\bullet-}$ in endothelial cell mitochondria under basal conditions. Considering MnSOD as the main target of $O_2^{\bullet-}$, using the reported [MnSOD] and rate constant of dismutation reaction, and equating formation and dismutation fluxes of $O_2^{\bullet-}$, one ends up with a steady-state concentration of 28 pM for the normoglycemic condition. This number increases ~9-fold ($[O_2^{\bullet-}]_{ss} = 250$ pM) for hyperglycemic cells (*19*). A number of assumptions were made in these calculations (*e.g.*, the mitochondrial volume is assumed to be 7% of cellular volume) that could alter the result, but even calculating with different assumptions we feel comfortable with a range of 10−50 pM for basal steady-state concentration of $O_2^{\bullet-}$.

At the other end of the scale, the maximal formation of $O_2^{\bullet-}$ happens in the intraphagosomal space of phagocytes. Using the oxygen consumption measured with neutrophils activated by phagocytosing opsonized polyacrylamide beads (*20*) and considering a phagosomal volume of 1.2 fL, Winterbourn *et al. (21)* have estimated a $O_2^{\bullet-}$ flux of 2.5 mM s^{-1}. Taking into account that the phagosome is a compartment practically devoid of endogenous SOD, $O_2^{\bullet-}$ decays via reaction with myeloperoxidase or by proton-catalyzed dismutation. Steady-state concentrations under these conditions were simulated, and reported values range from 10^{-5} to 10^{-4} M (*21*). Once again, assumptions were made to estimate this range. For instance the concentrations and availability of substrates to NADPH oxidase were not considered as limiting of the $O_2^{\bullet-}$ production. In the case of the phagocytosis of actual bacteria, SOD is known to exist in the periplasmic

KEYWORDS

Peroxiredoxins: Peroxidases characterized by a critical cysteine and the lack of metal cofactors. These enzymes catalyze the reduction of hydrogen peroxide (and other ROOH molecules) by two electrons using a second substrate that is usually a thiol-based reductant.

Peroxynitrous acid/peroxynitrite ($ONOOH/ONOO^-$): A peroxyacid and its conjugate base ($pK_a = 6.8$) are formed *in vivo* by the reaction of nitric oxide with superoxide. It is a strong oxidant and can yield free radicals upon reaction with carbon dioxide or slowly by homolysis of the acid.

Superoxide ($O_2^{\bullet-}$): Small and unstable free radical produced *in vivo* mainly by the one-electron reduction of oxygen. It can act as oxidant or reductant and dismutates spontaneously to oxygen and hydrogen peroxide even in the absence of catalysts.

Superoxide dismutase: Any of the enzymes that catalyze the dismutation of superoxide to oxygen and hydrogen peroxide. They are metalloenzymes containing copper, manganese, iron, or nickel and are characterized by their extreme catalytic efficiency and widespread abundance in aerobic organisms.

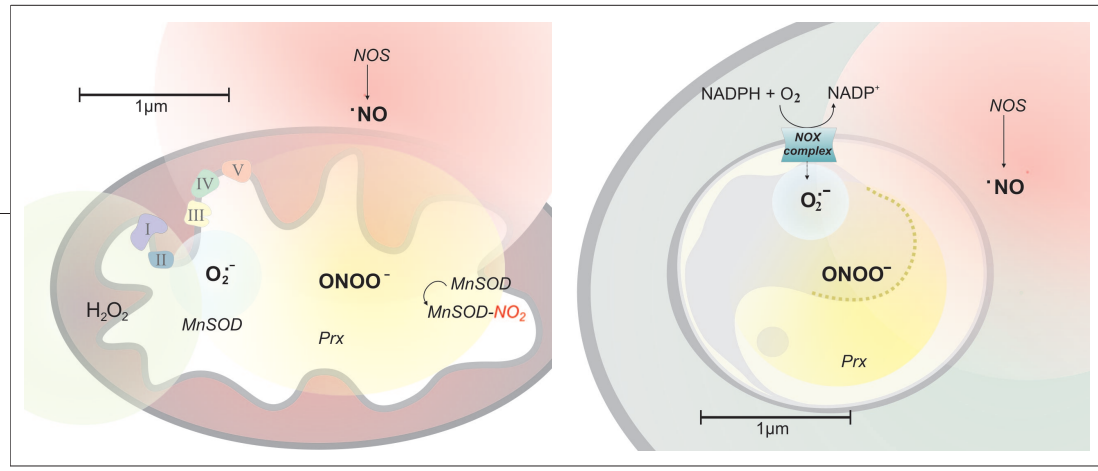


Figure 1. Peroxynitrite formation: $^{\bullet}NO$ searching for $O_2^{\bullet-}$. Two key subcellular compartments where peroxynitrite is generated are the mitochondria and the phagosome. In the first (left panel), $O_2^{\bullet-}$ is mainly formed toward the matrix *via* the one-electron reduction of molecular oxygen by electron transport chain components. The basal $O_2^{\bullet-}$ generation rates can be greatly enhanced under a variety of pathologically relevant conditions (*19, 101*), and $O_2^{\bullet-}$ is mainly detoxified by the diffusion-controlled dismutation reaction catalyzed by MnSOD to yield hydrogen peroxide. However, peroxynitrite will be formed in the presence of $^{\bullet}NO$ fluxes that can either arise by readily diffusing from extramitochondrial compartments or be formed locally by NOS-dependent (*102*) or independent-pathways (*103*). Peroxynitrite will be either detoxified by peroxiredoxin 3 and 5 or will trigger oxidative modification events intramitochondrially, such as the nitration and inactivation of MnSOD. In the right panel, peroxynitrite formation inside a phagosome occurs during phagocytosis of an invading microorganism (*e.g.*, bacteria, parasite) by a neutrophil or macrophage *via* the assembly and activation of NADPH oxidase (NOX), which generates $O_2^{\bullet-}$ in the lumen of the vacuole. If the immune cells were previously exposed to cytokine stimulation (*e.g.*, IFN-γ, TNF-α) over a period of over ~4 h, then the inducible isoform of NOS will be expressed to yield large levels of $^{\bullet}NO$ that will diffuse and reach the phagosome and subsequently react with $O_2^{\bullet-}$. Peroxynitrite and its secondary radicals can then react with the pathogen plasma membrane and intracellular components and act as a cytotoxic effector molecule. The bactericidal and parasiticidal potency of peroxynitrite can be partially or totally neutralized by effective antioxidant mechanisms in the pathogens, such as bacterial and parasitic peroxiredoxins (*31, 104*). For orientation, the average size of a mitochondria and phagocytic vacuole are indicated in micrometers and in the context of peroxynitrite diffusion distances.

space of many species and has been recognized as a virulence factor (*22, 23*). Inclusion of SOD in the phagosome model limits the steady-state concentrations of $O_2^{\bullet-}$; for instance, with 1 μM SOD a 10-fold reduction in $[O_2^{\bullet-}]_{ss}$ is estimated (*21*).

$^{\bullet}NO$ is mainly produced by nitric oxide synthases (NOS), a family of enzymes that include two (NOS1 and NOS3) constitutive and calcium-regulated isoforms and one inducible protein (NOS2) controlled by gene expression and typically expressed by immune system cells. One advantage over $O_2^{\bullet-}$ level estimation is that $^{\bullet}NO$ concentration can be measured directly using selective electrodes; such measurements give a range for basal perivascular $^{\bullet}NO$ of 200−1000 nM and have been recently reviewed (*24*).

Combining the basal values of $[^{\bullet}NO]_{ss}$ and $[O_2^{\bullet-}]_{ss}$ calculated for endothelial cells and the known second-order rate constant, a peroxynitrite formation flux in the range of 0.2−0.4 μM s^{-1} is obtained (a fraction of the 0.7 μM s^{-1} $O_2^{\bullet-}$ formed *vide supra*). This peroxynitrite flux might be overestimation since $[O_2^{\bullet-}]_{ss}$ is assumed constant and thus independent of peroxynitrite formation. Considering a direct competition between SOD and $^{\bullet}NO$ for the reaction with $O_2^{\bullet-}$ and taking into account the upper limit of $[^{\bullet}NO]_{ss}$, it can be calculated that 0.3 μM s^{-1} is the maximal rate of peroxynitrite formation under what we defined as basal conditions, *i.e.*, nonstressed mitochondria in cultured endothelial cells. We will see that this peroxynitrite flux can be much higher when other scenarios such as in inflammatory cells are considered.

It is difficult to visualize which of the two precursors is limiting for peroxynitrite formation, but since the reaction is constantly competing with other routes that consume or sequester $^{\bullet}NO$ and $O_2^{\bullet-}$, it is safe to assume that both are. Therefore, any increase in either $[^{\bullet}NO]_{ss}$ or $[O_2^{\bullet-}]_{ss}$ leads to an increase in peroxynitrite formation. Some time ago, experiments with *in vitro* nitration of tyrosine by fluxes of $^{\bullet}NO$ and $O_2^{\bullet-}$ (*25*) showed a bell-shaped profile when plotting the reaction yield *versus* the ratio of reactant fluxes. The maximum of the curve coincided with equimolar fluxes, and this is mainly due to the cross reactions of intermediates with excess radicals. From these results many researchers acquired the idea that equimolar fluxes are mandatory to observe peroxynitrite-related effects. Although it seems a common sense conclusion, equimolar fluxes imply maximal peroxynitrite formation only in simplified systems where there are no escape routes for the excess radicals. It has been shown (*26, 27*) that when the system complexity is increased by introducing compartmentalization and diverse competitive scavenging, the bell-

shaped curve is lost and both steady-state concentration and effects of peroxynitrite increase with the augmentation of either reactant flux, consistent with biological data (*7, 10*).

With this in mind, maximal production of $^{\bullet}NO$ and $O_2^{\bullet-}$, as happens in inflammation sites, implies maximal peroxynitrite formation and steady-state concentration. Although peak production of $^{\bullet}NO$ and $O_2^{\bullet-}$ could, in principle, be measured or estimated, it is very difficult to come by a plausible top rate of peroxynitrite formation. Some of the factors that hamper this calculation are as follows:

Simultaneity. Given the aforementioned efficient removal systems for both precursors, the temporal concurrence of $^{\bullet}NO$ and $O_2^{\bullet-}$ is mandatory.

Location. As the reactants differ greatly in their ability to cross membranes, it is expected that maximal peroxynitrite formation will occur preferentially in the sites of $O_2^{\bullet-}$ formation, for example, phagosomes and dysfunctional mitochondria (Figure 1).

Vectorial formation and diffusion. Concentration measurements by detector molecules in bulk solution tend to underestimate the rate of formation of reactive species in the surface of cells. For instance, our group has equaled fluxes of $^{\bullet}NO$ and $O_2^{\bullet-}$ produced by chemical and enzymatic means with those produced by activated macrophages (*28*). Nevertheless, in the latter case, the reactive species are formed and detected mainly in the vicinity of the cell monolayer, whereas in the former it happens throughout the solution. Conversely, when comparing the effects of homogeneous generation of peroxynitrite by chemical/enzymatic means on a target cell with those produced on the same cell upon phagocytosis and intraphagosomal formation of peroxynitrite, diffusion distance becomes of paramount importance, as seen in the case of cocultures of macrophages and *Trypanosoma cruzi* (*29*) (see Box 1). The spatial and temporal problem of $^{\bullet}NO$ and $O_2^{\bullet-}$ generation in cells has been mathematically modeled elsewhere (*30*).

Despite the previous limitations, with the measured $O_2^{\bullet-}$ superoxide fluxes, it is conceivable that intraphagosomal fluxes of peroxynitrite can be 2 or 3 orders of magnitude higher than those under basal mitochondrial conditions. These high fluxes could be sustained for up to 1 h, during which the killing of the phagocytosed cell should take place (*23, 29*). Bacterial and parasite resistance against peroxynitrite as a cytotoxic effector increase with periplasmic SOD in *Salmonella typhimurium* (*23*) and intracellular peroxiredoxins in *Trypanosoma cruzi* (*31*).

Reactions. Despite its complex and diverse reaction chemistry, the biological chemistry of peroxynitrite is relatively straightforward; as stated before, the fate of peroxynitrite will depend on reaction kinetics and diffusion, so different scenarios can be considered and the most likely outcome of peroxynitrite chemistry will be apparent. The relevance of peroxynitrite targets thus originates from their reaction kinetics, *i.e.*, the rate constant (k) of their reaction with peroxynitrite and their concentration ([T]) in the scenario in question. In fact, the product k[T] can be used to parametrize the reactivity toward that target in a homogeneous system. Considering these two parameters, only a few candidates for reaction appear to be noteworthy and can be short-listed as carbon dioxide, peroxiredoxins and a few other thiol proteins, glutathione peroxidase, and some hemeproteins (Figure 2). Of these relevant reactions, some participate in detoxification and some can lead to nitro-oxidative damage, as will be discussed below.

Carbon Dioxide.

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow 0.35(CO_3^{\bullet-} + {}^{\bullet}NO_2) + 0.65(CO_2 + NO_3^-) \quad (2)$$

The concentration of carbon dioxide is high ($\geq$1.3 mM) in most biological milieu, and it has a moderately high rate constant (5.8×10^4 M^{-1} s^{-1}, 37 °C (*32, 33*)). Both the rate constant and the concentration are pH-dependent, so a range of k[T] = 60−100 s^{-1} can be estimated to cover all conditions. It is important to remember that the reaction yields carbonate radical ($CO_3^{\bullet-}$) and nitrogen dioxide ($^{\bullet}NO_2$) (35% each) (*34, 35*), and therefore the reaction with CO_2 diverts peroxynitrite reactivity toward the formation of two new strong and short-lived oxidant radicals that have significantly different chemistries, targeting mainly on protein thiolates and aromatic residues (for a recent review, see ref 36), and even participate in DNA base modification (*37*). Given the ubiquity of CO_2, the potentially deleterious consequences of the formation of $CO_3^{\bullet-}$ and $^{\bullet}NO_2$, and the kinetic relevance of the reaction, we will use 60−100 s^{-1} as a benchmark to assess the relative importance of other biotargets.

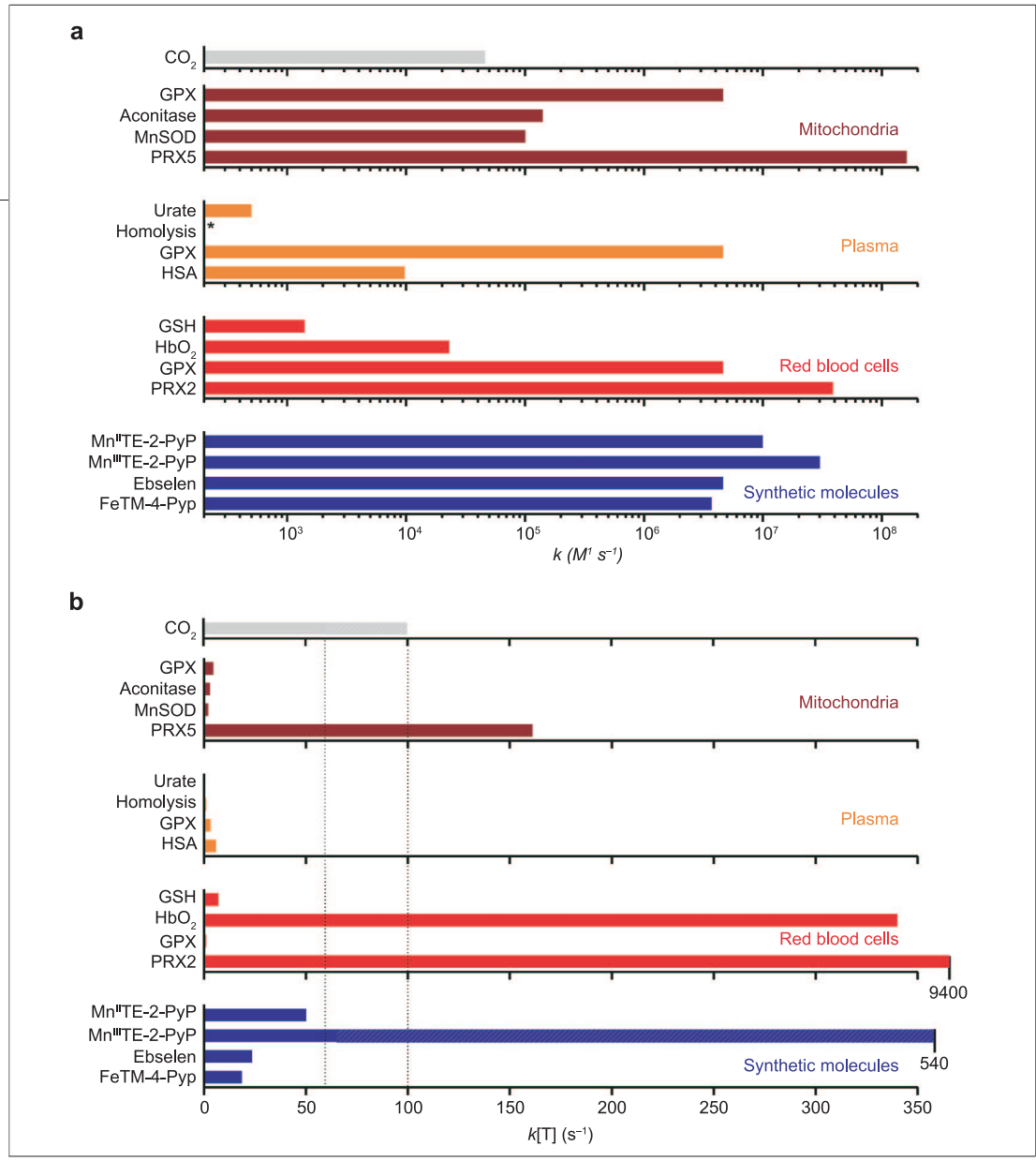


Figure 2. Peroxynitrite scavenging at a glance. Quantitative assessment of reactivity toward peroxynitrite is often presented through second-order rate constants (*k*); these are shown graphically in panel a for selected targets. More relevant to *in vivo* reactivity are the apparent pseudo-first-order constants obtained as *k* times the concentration of target, *k*[T] (panel b). These allow the comparison of reactivities as in simple competition kinetics. We have selected carbon dioxide's as the benchmark reactivity because of its ubiquity and reactivity and the oxidant character of the products. Allowing for the different pHs and [CO_2] encountered in cell compartments, 60−100 s^{-1} is established as a desirable starting range for a peroxynitrite scavenger to be competitive. All rate constants in panel a are at pH = 7.4 and either determined at 37 °C or extrapolated to that temperature for comparison assuming an activation energy of 10 kcal/mol. *In vivo* concentrations were obtained from the literature, in the case of synthetic molecules from pharmacokinetic studies (*3, 33, 38, 41, 45, 46, 59, 61, 77, 79, 81, 88−90, 109−112*). *Homolysis is a first-order reaction and thus cannot be plotted in this graph.

Peroxiredoxins (Prx).

$$ONOOH + PrxS^- \rightarrow NO_2^- + PrxSOH \qquad (3)$$

These thiol peroxidases have the highest rate constants, and some of them are very concentrated in some cellular compartments. In a way peroxiredoxins behave just as any thiol because in the first reaction of the catalysis the thiolate reduces peroxynitrous acid by two electrons, yielding nitrite and the corresponding sulfenic acid, but the enzymes are extremely fast with rate constants 10^3 to 10^5 times faster than those of low molecular weight thiols (*38*). For instance, the rate constant of peroxiredoxin 5 is 7×10^7 M^{-1} s^{-1} (pH 7.4, 25 °C (*39*)), so assuming any concentration above 1 μM yields a $k[T] > 70$ s^{-1}, *i.e.*, higher than that of CO_2. Peroxiredoxin 2 also has a large rate constant (1.7×10^7 M^{-1} s^{-1}, pH 7.4, 25 °C (*40*)) and is also very concentrated

(240 μM) in certain cell types, *e.g.*, erythrocytes (*41*), so $k[T] > 4000\ s^{-1}$ outcompetes carbon dioxide in these conditions. Peroxiredoxins are widespread in biological compartments and are located mainly in the cytosol, but they are found within mitochondria (*42*) and peroxisomes, associated with nuclei and membranes, and in at least one case, are exported (*43, 44*). Concentration and kinetics thus indicate that peroxiredoxins are the most efficient peroxynitrite scavengers known to date.

Glutathione Peroxidase (GPx).

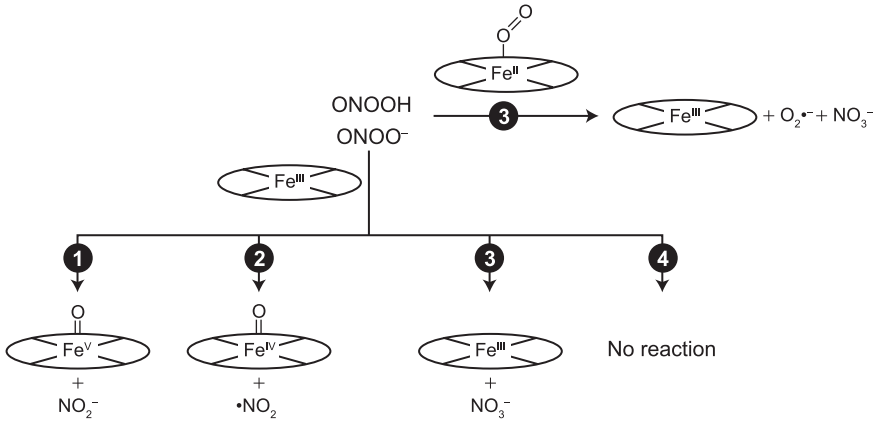
$$ONOOH + GPxSeH \rightarrow NO_2^- + PrxSeOH \quad (4)$$

The selenium-containing protein glutathione peroxidase also reacts quickly with peroxynitrite ($k = 8 \times 10^6\ M^{-1}\ s^{-1}$, pH 7.4, 25 °C (*45*)), and its concentration has been estimated as 2 μM in hepatocytes (*46*), yielding $k[T] > 16\ s^{-1}$, below that of CO_2 but still significant. Glutathione peroxidase also reduces peroxynitrite to nitrite catalytically at the expense of glutathione. In some situations, for instance, in erythrocytes, it can be observed that glutathione peroxidase does not furnish a detoxification route since the noncatalyzed reduction of peroxynitrite by glutathione (~5 mM, $k = 1.4 \times 10^3\ M^{-1}\ s^{-1}$ (*47*)) is a more significant reaction in terms of $k[T]$, given the low concentration of the enzyme (Figure 2).

Heme Proteins. Peroxynitrite reactions with heme proteins are diverse and hardly predictable. Some heme peroxidases count among the fastest reacting proteins, with rate constants $>10^6\ M^{-1}\ s^{-1}$ (*48–51*), but even in these cases of functionally related proteins there is variability in the reaction products. For instance, horseradish peroxidase and prostaglandin endoperoxide H synthase-1 are oxidized by two electrons, yielding peroxidase compound I and nitrite (route 1 in Scheme 1). On the other hand, myeloperoxidase and chloroperoxidase oxidations are one-electron reactions yielding peroxidase compound II and nitrogen dioxide (route 2), potentially leading to catalyzed tyrosine nitration (*52*). Some heme proteins catalyze the isomerization of peroxynitrite to nitrate (route 3) with rate constants on the order of $10^4\ M^{-1}\ s^{-1}$ (*53, 54*); this is the case of oxy- and methemoglobin and metmyoglobin. Finally, some heme proteins do not react with peroxynitrite at any detectable rate (route 4); examples are catalase (*48*) and oxidized (Fe^{III}) cytochrome *c* (*55*).

Consideration of the kinetic relevance of the reactions with heme peroxidases can be done as before. For instance, myeloperoxidase has one of the highest rate constants ($6.2 \times 10^6\ M^{-1}\ s^{-1}$ at 12 °C and pH 7.2 (*48*)), and its concentration is also elevated (500 μM) in neutrophils (*56*) which would represent a $k[T]$ of 3100 s^{-1}, thus making it an excellent potential target for peroxynitrite. Nevertheless, its range of action is limited to a few cell compartments, namely, the azurophil granules of neutrophils and monocytes and the endothelium and extracellular matrix at inflammation sites (*57*). Myeloperoxidase reaction produces compound II and $^{\bullet}NO_2$, and thus the reaction should be regarded as a diversion of the oxidant character, potentially catalyzing tyrosine nitration.

SCHEME 1. Reactions of peroxynitrite with heme proteins



Oxyhemoglobin also has a $k[T]$ larger than that of CO_2 (340 s^{-1} from $k = 1.7 \times 10^4$ M^{-1} s^{-1} at pH 7.4 and 37 °C and $[T] = 20$ mM), but as in the case of myeloperoxidase its potential as peroxynitrite scavenger is restricted by its location in red blood cells, where it is capable of isomerizing peroxynitrite to nitrate (*53*).

Less Relevant Reactions. Other potential targets are less efficient at reacting with peroxynitrite; for instance, glutathione, an often mentioned antioxidant, has a $k[T]$ of 7 s^{-1} at 37 °C and pH 7.4 (*47*), which represents a minor fraction of peroxynitrite reduction. Critical residues in enzymes inactivated by peroxynitrite such as aconitase and MnSOD have $k[T] < 3$ s^{-1} (*58, 59*), which means that they are fairly well protected from oxidation under normal mitochondrial conditions of catalytic antioxidants, such as peroxiredoxins (see Box 2). Finally, the proton-catalyzed homolysis of peroxynitrite is a first-order reaction that has the equivalent of $k[T] = 0.9$ s^{-1} (37 °C, pH 7.4), meaning that it represents a very minor route of peroxynitrite disappearance, still one that may be relevant in specific biocompartments triggering radical chain reactions (*vide infra*).

Steady-State Concentration. By using the above-discussed rates of formation and disappearance of peroxynitrite we can try to calculate a steady state, once again with a set of assumptions. The worst case scenario would be one of no catalytic reductants of peroxynitrite, and we will represent it as a CO_2-only consumption. Then, using the basal flux of 0.2 μM s^{-1} and the $k[T] = 60-100$ s^{-1} of CO_2, the steady-state concentration would be 2−3 nM and represents the upper limit expected for basal conditions. Any additional target would diminish this figure, for instance, by only considering 1 μM Prx5 the steady-state concentration decreases to 0.8 nM, and the intraerythrocytic steady-state concentration, considering Prx2 at 37 °C, plummets to less than 25 pM. A very recent article, modeling the kinetics and diffusion of oxidizing, nitrosating, and nitrating species derived from •NO, finds intracellular steady-state concentrations of peroxynitrite within the same range (*60*).

Homolysis and Other Radical-Forming Reactions. In view of the numerous reactive targets and their efficiency in reacting with peroxynitrite, it would appear that secondary reactions are irrelevant. So far we have not mentioned radical-dependent processes such as lipid oxidation and protein tyrosine nitration, often referred to as results of peroxynitrite chemistry (*2, 7*). Is it possible that these events are produced by peroxynitrite *in vivo*? The answer is definitely yes.

First, not all peroxynitrite reactions really "scavenge" it; as already mentioned, CO_2 and some heme peroxidases promote the formation of secondary oxidants and radicals.

Additionally, most "benign" targets of peroxynitrite (catalytic antioxidants such as Prxs and sacrificial reductants such as glutathione) are water-soluble and compartmentalized and cannot reach hydrophobic compartments, so they can be "left behind" by peroxynitrite crossing membranes with relative ease (*61–63*) (see Box 1). Then, in compartments of low antioxidant concentration such as extracellular fluids or hydrophobic compartments, the reaction with carbon dioxide and even the proton-catalyzed homolysis could prevail, leading to the formation of oxidizing radicals that produce the aforementioned damaging effects (*64*). The production of radical-derived products such as 3-nitrotyrosine and modified lipids can be considered as peroxynitrite-derived products through "indirect reactions" (*65*), but the truth is that once radical reactions start, it is both difficult and often useless assigning an originator or responsible molecule. As an example, the nitration and oxidation of the lipophilic tyrosine analog *N-tert*-butoxypyrocarbonate-L-tyrosine *tert*-butyl ester has been shown to happen in liposomes exposed to peroxynitrite (*66*). The yield of the reaction is dependent on the lipid composition and the oxygen concentration, because lipid peroxidation (which can be initiated by either hydroxyl radicals (•OH) or $^{\bullet}NO_2$) plays an amplifying role in the generation of radicals *via* oxygen-dependent propagation chain reactions. Then, oxidized and even the nitrated tyrosine analog can be regarded as peroxynitrite-derived, lipid peroxide-derived, oxygen-derived, or all of the above (*64*). In this context, it is important to remark that any of the radicals derived from peroxynitrite reactions can trigger chain reactions that could amplify the outcome and interplay with radicals derived from other processes. In this case, peroxynitrite would be contributing to, rather than causing, the formation of nitrated/oxidized biomolecules *in vivo*.

Products derived from secondary reactions such as 3-nitrotyrosine and oxidized/nitrated lipids are not quantitatively important since they represent a tiny fraction of the peroxynitrite formed and consumed (*26*). Their importance lies in their stability and the possibility to use them as the trail of peroxynitrite *in vivo*, but to

do so, it is important to make sure the proper controls are also performed since other mechanisms of oxidation/nitration could coexist with peroxynitrite *in vivo* (*7*). Additional to the reporter role of 3-nitrotyrosine, it has been proposed that the specific nitration of some proteins could have a role in the process of development of some diseases mainly through a gain-of-function process (*7, 67, 68*). The case of nitrated lipids has received increasing attention due to their anti-inflammatory potential (*69, 70*); however, neither their mode of formation by peroxynitrite nor the biological consequences have been fully defined (*71, 72*).

Reaction with Synthetic Scavengers. Many molecules have been claimed as peroxynitrite scavengers, most of them on the basis of being able to diminish or abolish the formation of peroxynitrite-derived products. As we already saw, the chemistry becomes complex after the formation of the first peroxynitrite-derived radicals, and there are many possibilities of blocking the formation of products (*e.g.*, 3-nitrotyrosine (*64*)) not directly related to peroxynitrite itself. For a distinction between direct peroxynitrite scavengers and compounds that intercept peroxynitrite-derived radicals (*e.g.*, nitroxides), see ref 10. Thus, heeding to the reaction kinetics, the most successful synthetic peroxynitrite scavengers belong to two chemical families: selenols and metal porphyrins. Three reactions are involved, and we will approach them chronologically, which also happens to be the inverse order of biological relevance.

One-Electron Reduction. Manganese porphyrins were found to catalyze peroxynitrite-mediated oxidations (*73, 74*), and this catalysis was found to divert, in the presence of the right reductants, peroxynitrite oxidation reactions to replenishable targets (*75, 76*). The catalytic cycle consists of the fast formation of an oxidized manganese complex that in turn oxidizes the available reductant and is exemplified in reactions and for manganese (III) *meso*-tetrakis((*N*-ethyl)pyridinium-2-yl)porphyrin (Mn^{III}TE-2-PyP).

$$Mn^{III}TM\text{-}2\text{-}PyP^{5+} + ONOO^- \rightarrow O{=}{=}Mn^{IV}TM\text{-}2\text{-}PyP^{4+} + {}^{\bullet}NO_2 \quad (5)$$

$$O{=}{=}Mn^{IV}TM\text{-}2\text{-}PyP^{4+} + RdH + H^+ \rightarrow Mn^{III}TM\text{-}2\text{-}PyP^{5+} + {}^{\bullet}RD + H_2O \quad (6)$$

The rate constant of reaction depends on the porphyrin and pH of the experiment and ranges from 10^5 to 10^7 M^{-1} s^{-1} at neutral pH (*77*); reaction would be rate limiting in most cases as the concentration of peroxynitrite is in the nanomolar range. The rate of reaction depends on the available reductants (Rd), and we have seen that urate and ascorbate are very effective in completing the catalytic cycle (*76*). One must bear in mind that the oxidized manganese complex is a strong oxidant, so reaction may take place with almost any reductant available, and particularly in controlled chemical systems, the cycle can be used to promote undesirable biochemical events such as DNA oxidation or tyrosine nitration (*73, 74*). Nevertheless, this is unlikely to happen *in vivo* where other potential reductants such as ascorbate, urate, and glutathione are more abundant and efficient (*76*). Iron porphyrins have been shown to catalyze the same reaction sequence (*78, 79*); however, the reactions are slower ($<10^7$ M^{-1} s^{-1}) and the kinetic characterization is less complete. The one-electron stoichiometry of these reactions implies that radicals are quantitatively formed, and this represents a potentially damaging mechanism depending on the efficiency of ${}^{\bullet}NO_2$ removal and on the nature of the ${}^{\bullet}$Rd formed. This mechanism has been shown to protect LDL *in vitro* against infused peroxynitrite only in the presence of urate (*80*). Going back to our simple competition model, a recent article studied the pharmacokinetics of one MnPorphyrin in mice (*81*) and determined plasma and organ concentration of the compound after a single ip administration of 10 mg kg^{-1}; the rate constant for reaction with this MnPorphyrin is 3×10^7 M^{-1} s^{-1}. Plasma concentration peaks at ~18 μM but drops sharply in 2 h, and hepatic concentrations reach ca. 6.5 μM in 8 h and remain above 1 μM for 1 week. Taking these values into account, $k[T]$ are >540 s^{-1} and >30 s^{-1} for plasma and liver, respectively.

Isomerization. Iron porphyrins were proposed to catalyze the isomerization of peroxynitrite to nitrate (*82*). The reaction is not simple, and different authors invoke different mechanisms (*78, 79*), always with rate constants around 10^6 M^{-1} s^{-1}. This reactivity would have to compete with the one-electron reduction mentioned in the previous paragraph and will only be observable in the absence of reductants. In fact, the isomerization pathway was deemed unlikely *in vivo* by its original proponents (*79*). Despite the fact that *in vivo* effects of FePorphyrins have been observed in a number of models and that most of the effects are routinely attributed to peroxynitrite scavenging, it is not clear whether isomeriza-

Box 1. Diffusion as a kinetic contender.

Diffusion across membranes can constitute an important kinetic determinant in peroxynitrite fate (*10, 30, 61–63*). First, the half-life of peroxynitrite consumption limits the distance a molecule can travel before decaying by chemical reaction. Using the values in Figure 2 to estimate half-lives and Fick's second law, it can be determined that peroxynitrite can traverse a mean distance of 3, 5.5, and 0.5 μm in mitochondria, blood plasma, and erythrocytes, respectively, during one-half-life. These numbers indicate that peroxynitrite formed in plasma is very likely to encounter an erythrocyte before decaying (*62*).

Another approach to the problem consists of comparing the encounter frequency of a small molecule with erythrocytes in whole blood, which can be calculated using the Smoluchowski approximation (*105*) as around 240 s^{-1}. Comparison of this value with the apparent first-order constant of peroxynitrite decay (~70 s^{-1} in plasma at 37 °C) indicates a 4:1 probability of encountering an erythrocyte before decaying. The membrane constitutes an additional hurdle, especially considering that peroxynitrite is partially ionized at pH 7.4, but equilibration time constants indicate that crossing the membrane is a rather fast process for small polar molecules such as ONOOH (91 s^{-1} for water (*106*)) and even for small anions such as $ONOO^-$ (12 s^{-1} for chloride (*107*)), which can cross membranes by passive diffusion or anion channels, respectively (*61*). Pondering all processes as a simple kinetic competition leads to the conclusion that ~27% of the peroxynitrite formed in plasma would end up being consumed inside red blood cells, consistent with experimental results obtained by our group (*108*). Once inside the erythrocyte, the high concentration of peroxiredoxin 2 and its rapid reaction will forestall peroxynitrite from diffusing out. Oxidation of hemoglobin and other targets will follow in case the peroxiredoxin reduction system fails or is exceeded.

The mitochondrial matrix escape through the inner membrane is more restricted. On one hand, the mitochondrial matrix is a smaller compartment and its large surface-to-volume ratio predicts rapid equilibration times across the membrane, but on the other hand, the inner mitochondrial membrane is less permeable and less abundant in anion channels than the erythrocyte. Additionally, scavenging of peroxynitrite is more efficient in the mitochondrial matrix than in plasma (Figure 2, panel b), further diminishing its possible release.

tion plays any role in these effects. Pharmaceutical development of FePorphyrins as drugs continues with interesting results (*83, 84*), but the kinetics of these catalysts is by no means completely understood. Isomerization is a very appealing reaction for getting rid of peroxynitrite as the product is relatively inert and there is no need of additional substrates to complete the cycle. Nevertheless, this reaction may not be at play in the case of FePorphyrins.

Two-Electron Reduction. Biological reduction of manganese porphyrins was proposed as early as 1994 in a seminal work by Fridovich's group (*85*). Reduction to Mn(II) was then assumed to play a part in the scavenging of $O_2^{\bullet-}$. Later on, our group and others (*86–88*) found that flavoenzymes were central to this reduction and mitochondria were a very suitable place to generate the Mn^{II}Porphyrin. Starting with the reduced porphyrin leads to a different reactivity toward peroxynitrite; reaction 7 is a two-electron process that avoids the formation of $^{\bullet}NO_2$, producing nitrite instead.

$$Mn^{II}TE\text{-}2\text{-}PyP^{4+} + ONOO^- \rightarrow O{=}{=}Mn^{IV}TE\text{-}2\text{-}PyP^{4+} + NO_2^- \quad (7)$$

Reduction to Mn(III) occurs as mentioned above in reaction while further reduction to Mn(II) uses a number of substrates in a reaction catalyzed by flavoenzymes such as succinate dehydrogenase, NADH dehydrogenase (of the mitochondrial electron transport chain), glucose oxidase, and xanthine oxidase (*88*). Reaction 7 has a rate constant $>10^7$ M^{-1} s^{-1} (*88*) (see Box 3 and Figure 3). Additionally, recent reports indicate that one particular MnPorphyrin is found in mouse heart mitochondria in a concentration of 5.1 μM up to 7 h after a single ip administration at 10 mg kg^{-1} (*89*). This would gather the catalyst and the reductants needed for catalysis in one of the main sites of oxidant formation.

Box 2. Mitochondrial peroxynitrite, checkpoints, and possible outcomes.

We have seen that under conditions of basal $^{\bullet}NO$ and mitochondrial $O_2^{\bullet-}$ generation, peroxynitrite is formed intramitochondrially (*9, 10*)(Figure 1). Intramitochondrial peroxynitrite is expected to be readily detoxified by peroxiredoxins 3 and 5, in a catalytic cycle that requires the rereduction of the enzyme by thioredoxin. Peroxiredoxin 5 is extremely efficient in its reaction (*38*), while peroxiredoxin 3 is highly abundant in the mitochondrial matrix (up to 60 μM in rat heart (*42, 113*)). MnSOD constitutes another defensive line, diverting $O_2^{\bullet-}$ to hydrogen peroxide and thus averting peroxynitrite formation. Both MnSOD and peroxiredoxins are *sine qua non* to organism viability; mice lacking MnSOD die within 2 weeks of birth (*114*), and mice whose peroxiredoxin catalysis is stopped by lack of reducing substrate fail to develop and die during embryogenesis (*115*). Peroxynitrite can inactivate MnSOD by nitration and oxidation (*116, 117*), and peroxiredoxins by oxidation of the critical cysteine to sulfinic/sulfonic acid (*31, 40*). If a peroxynitrite surge is able to overcome the protection provided by these enzymes, it will result in a less efficient elimination of mitochondrial-derived $O_2^{\bullet-}$, peroxynitrite, and hydrogen peroxide, which in turn will inactivate further Prx and MnSOD molecules, triggering a vicious cycle that culminates with large amounts of nitrated MnSOD and intramitochondrial nitro-oxidative stress (*118, 119*). This process can be considered as a prelude to cell death, as the loss of a central mitochondrial antioxidant and protective mechanism results in the oxidation of other mitochondrial protein and lipid components that can cause, depending on extent and velocity of the chemical modifications, either the cytosolic release of pro-apoptotic factors (*e.g.*, cytochrome *c*, Smac-diablo) or a bioenergetic collapse followed by a necrotic mode of death. In this context, overexpression of Prx 3 and MnSOD (*120–124*), as well as the more recent use of mitochondrial-targeted antioxidants, exerts strong protective effects against the toxicity of reactive oxygen and nitrogen species in various models of vascular and neuronal degeneration (*99, 100*). On the bright side, both MnSOD and Prx3 are induced by mild oxidative stress, so tissues can be preconditioned to stand further oxidative injury (*125*).

In terms of reactivity, k[T] is $>51\ s^{-1}$ for Mn^{III}TE-2-PyP in mouse heart mitochondria, considering that the exact rate constant is unknown and only its lower limit has been reported.

Another compound able to reduce peroxynitrite by two electrons with a significant rate is ebselen or 2-phenyl-1,2-benzisoselenazol-3-(2*H*)-one. This selenium compound mimics the reactivity of glutathione peroxidase, reacting rapidly with peroxynitrite ($k = 2 \times 10^6\ M^{-1}\ s^{-1}$, pH $\geq$ 8, 25 °C (*90*)) and being reduced back to its original form by glutathione. Experiments in clinical trials on ebselen have shown important plasma concentration of the drug ranging from 10 μM at 2 h after administration to a baseline level of 5.1 μM after 7 days oral administration of a daily dose of 400 mg (*91*). Under these conditions, k[T] is $10-20\ s^{-1}$. One point to consider is that plasma ebselen is mostly bound to albumin, forming a covalent selenosulfide bond that renders the selenol unavailable for reacting with peroxynitrite (*92, 93*).

Future of the Chemical Biology of Peroxynitrite. We think that great progress has been made in the understanding of the main chemical properties of peroxynitrite in biological systems, and its formation *in vivo* should be considered as an established phenomenon. However, we recognize that on occasion doubts arise regarding the biological relevance of peroxynitrite-dependent reactions, much of which is due to the difficulty of directly and unambiguously measuring its levels *in vivo*. Indeed, much of the evidence relies on indirect measurements, molecular footprints, and pharmacological interventions. Thus, there is an imperative need to develop peroxynitrite specific probes that (i) do not interfere significantly with redox events, (ii) yield products that can be measured with ease by most laboratories, and (iii) are of wide access for use to the research community. While a number of peroxynitrite-sensitive probes exist (*6*), they have limited specificity and are prone to artifacts (*94*), all of which require very careful controls and cautious interpretation of the data (*6*). Thus, there is an authentic need for new and better

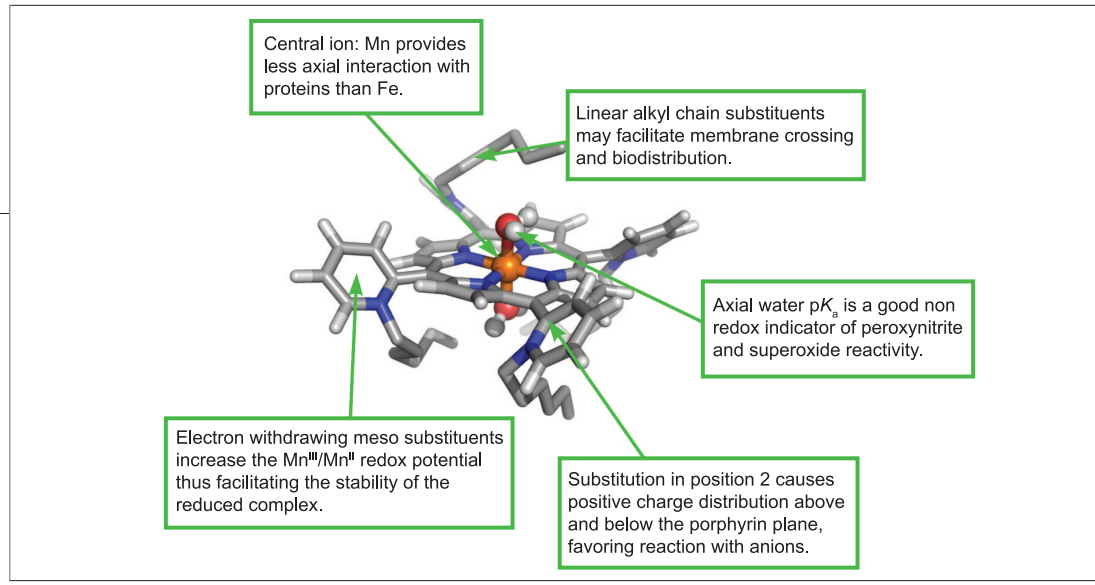


Figure 3. Anatomy of a promising reduction catalyst. Manganese(III) tetrakis(*N*-hexylpyridinium-2-yl)porphyrin (alkyl hydrogens omitted for clarity) reunites some of the best characteristics as SOD-mimic and peroxynitrite reduction catalyst: high reactivity ($k_{O_2^-} = 3 \times 10^7\ M^{-1}\ s^{-1}$; $k_{ONOO^-} = 1.3 \times 10^7\ M^{-1}\ s^{-1}$), high redox potential (0.314 V/ENH) facilitating enzymatic reduction to Mn(II), and good amphiphilic properties that make it a very promising catalytic antioxidant providing good protection at low doses in radioprotection and ischemia-reperfusion and blocking the development of morphine antinociceptive tolerance (*77, 88, 131–134*).

Box 3. Synthetic reductants: good, better, best.

The driving force behind MnPorphyrins as increasingly efficient peroxynitrite reductants originated in the search to enhance their catalytic efficiency as SOD models. In fact, a close relationship holds between SOD activity and the rate constant for reaction (*77*), reflecting that both reactions obey the same general properties. In the lower end of the correlation, some MnPorphyrins are poor peroxynitrite reductants but are inactive as SOD mimics, giving rise to some specificity (*126*).

The structural and electrostatic parameters that lead to the reactivity correlation can be summarized as (i) electrostatic facilitation by cationic substituents on the porphyrin (*127*) and (ii) asymmetric distribution of positive charges near the manganese ion (*76, 128*).

Experiments in bacterial culture have revealed additionally that MnPorphyrins may be less toxic than FePorphyrins (*129*). In addition to kinetic enhancement of peroxynitrite reduction, the attainability of the Mn(II) oxidation state is crucial for the two electron reduction of peroxynitrite via reaction 7, and thus a higher Mn(III)/Mn(II) redox potential is convenient in order to avoid $^{\bullet}NO_2$ formation (*88*). Finally, it has been put forward that modulation of the lipophilicity on the porphyrin substituents could boost the bioavailability and hence their observed antioxidant efficiency (*130, 131*), yet the methodology to assess the tissue and subcellular distribution of different MnPorphyrins is still in the development phase (*81*).

probes for peroxynitrite detection. In this regard, a few novel fluorogenic probes have been created recently by mechanism-based molecular design involving either aromatic nitration (*95*) or ketone oxidation (*96*) reactions and tested in cells. We see these developments (*95, 96*) as significant advances but realize and warn that a thorough characterization and validation at the (redox) biochemical and cellular level must be carried out before these probes can be widely applied. Additionally, it is likely that after the first generation of compounds is tested by different groups, optimization for more thorough biological uses (including the tissue level) will be required to improve sensitivity, decrease toxicity, and minimize diffusion of probe products across compartments. Unfortunately, the compounds are not readily available at present for further testing or use, and thus we cannot elaborate more about their real utility. At any rate, we hope that this new generation of probes will assist in moving the field forward and also stimulate other research groups to undertake the efforts for rational design and synthesis of much needed peroxynitrite probes.

Another important area is to delve further into the bioanalytical detection of peroxynitrite-derived products

formed endogenously. It is unlikely that one given product could be specifically attributed to peroxynitrite, but it is almost certain that if a group of modifications were measured in combination (*e.g.*, protein nitration and hydroxylation (*97*)), they may serve to assist in the identification of peroxynitrite as the proximal oxidant. There is a large area for defining footprints of peroxynitrite formation *in vivo* that has been very little investigated. This approach will be complementary to the one described above regarding the development of peroxynitrite probes.

The interplay between $O_2^{\bullet-}$ and $^{\bullet}NO$ can be considered as a "radical switch" that diverts the cytoprotective and cytoregulatory signal transducing action of nitric oxide toward oxidative and potentially cytotoxic pathways. It is important to recognize that in some cases involving low output $^{\bullet}NO$, such as that generated by eNOS, the reaction with $O_2^{\bullet-}$ will primarily have an effect through the decreased bioavailability of $^{\bullet}NO$ and not through peroxynitrite formation, which will be formed in low amounts and will be mainly detoxified by peroxiredoxins. On the other hand, if $O_2^{\bullet-}$ and $^{\bullet}NO$ levels are significantly enhanced and peroxynitrite formation increases, an initial effect may be related to oxidative redox signaling, one that has not been assessed in much detail so far and that may result in preconditioning responses. At even higher concentrations, peroxynitrite will become cytotoxic. The transition from nontoxic, to signaling, to toxic peroxynitrite levels should be much better defined, in particular, whether peroxynitrite at some concentrations can play salutary actions by triggering metabolic or genomic responses, *e.g.*, *via* thiol oxidation of key transcription factors or enzymes (*10, 98*).

The role of peroxynitrite in pathology relies in part on nitro-oxidative modifications that, at low levels, could generate a gain of toxic function in biomolecules and/or signaling cascades. This is becoming clear for a variety of proteins, in which for example tyrosine nitration triggers aberrant protein function including pro-oxidant (*e.g.*, cytochrome *c*), pro-apoptotic (*e.g.*, NGF), and pro-aggregant (*e.g.*, fibrinogen, α-synuclein) properties (*67, 135*). The identification of the molecular targets, the peroxynitrite-dependent modifications, their influence on metabolic and signaling pathways, and their pathophysiological impact awaits further investigation.

Finally, further developing and testing of peroxynitrite decomposition catalysts are needed for application in disease models and potentially in human pathologies. A key future approach is to assess and define the correlation between the effects of these molecules on specific cellular and metabolic processes and the clinical outcome. Molecules that react with and decompose peroxynitrite or peroxynitrite-derived radicals that can be directed to specific subcellular compartments where peroxynitrite is formed, such as mitochondria, can be a valid strategy in diseases where organelle dysfunction secondary to peroxynitrite formation plays a central pathogenic role (*99, 100*).

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