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Peroxynitrite, a Stealthy Biological Oxidant*

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Peroxynitrite is the product of the diffusion-controlled reaction of nitric oxide and superoxide radicals. Peroxynitrite, a reactive short-lived peroxide with a pK_a of 6.8, is a good oxidant and nucleophile. It also yields secondary free radical intermediates such as nitrogen dioxide and carbonate radicals. Much of nitric oxide- and superoxide-dependent cytotoxicity resides on peroxynitrite, which affects mitochondrial function and triggers cell death via oxidation and nitration reactions. Peroxynitrite is an endogenous toxicant but is also a cytotoxic effector against invading pathogens. The biological chemistry of peroxynitrite is modulated by endogenous antioxidant mechanisms and neutralized by synthetic compounds with peroxynitrite-scavenging capacity.

Peroxynitrite: A Product from a Radical-Radical Combination Reaction

Free radicals typically react fast with each other via radical-radical coupling reactions. Indeed, radical combination reactions usually occur at near diffusion-controlled rates (1). This unique type of reaction is, in many cases, kinetically and thermodynamically favored by the fact that it results in the formation of a new chemical bond at the expense of the unpaired electrons of the precursors. There are many possible radical-radical combination reactions that can happen biologically, but low steady-state levels of intermediates and competing reactions usually limit reaction yields and quantitative relevance. A prime example of a relevant radical species produced at high rates biologically is represented by the superoxide radical anion ($O_2^{\bullet-}$), the product of the univalent reduction of molecular oxygen (2). $O_2^{\bullet-}$ is ubiquitous and continuously formed during normal cellular metabolism, with its production rates increasing severalfold during the disruption of cellular redox homeostasis and with inflammatory stimuli. Although excess $O_2^{\bullet-}$ production has been associated with oxidative damage, more controlled fluxes can lead to redox signaling (3).

The discovery of nitric oxide ($^{\bullet}NO$) as an enzymatically generated free radical was paralleled by the recognition that it could readily react with $O_2^{\bullet-}$ (4, 5). $^{\bullet}NO$ produced by a variety of nitric-oxide synthases (NOS)[2] participates as a mediator in the regulation of vascular tone, neurotransmission, and immunity, among other metabolic and cell signaling effects (6, 7). Thus, the reaction of $O_2^{\bullet-}$ with $^{\bullet}NO$ was first conceived as a mechanism of $^{\bullet}NO$ "inactivation" (8). Notably, the combination reaction leads to peroxynitrite (9, 10), a peroxy acid originally studied in the chemical literature as a strong oxidizing and nitrating compound (11, 12) (Equation 1).

$$O_2^{\bullet-} + {}^{\bullet}NO \rightarrow ONOO^- \quad \text{(Eq. 1)}$$

The reaction of $^{\bullet}NO$ with $O_2^{\bullet-}$ occurs biologically even in the presence of superoxide dismutase (SOD), indicating that it is extremely fast to outcompete the enzyme-catalyzed dismutation (Equation 2).

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad \text{(Eq. 2)}$$

Indeed, the formation of peroxynitrite occurs with a k_1 of $\sim 10^{10}$ $M^{-1}\,s^{-1}$, ~1 order of magnitude higher than that of enzymatic dismutation ($\sim 1–2 \times 10^9$ $M^{-1}\,s^{-1}$) (2, 13). Although SOD exists in cells in micromolar levels, $^{\bullet}NO$ concentrations can, in some cases, reach close to micromolar values. Thus, under appropriate conditions, the formation of peroxynitrite is the only known reaction for $O_2^{\bullet-}$ in biology that can be similar or even substantially faster than the dismutation reaction (*i.e.* Equation 3).

$$k_1 \times [{}^{\bullet}NO][O_2^{\bullet-}] \geq k_2 \times [SOD][O_2^{\bullet-}] \Rightarrow$$

$$k_1 \times [{}^{\bullet}NO] \geq k_2 \times [SOD] \quad \text{(Eq. 3)}$$

Although the proximal species formed from the $^{\bullet}NO$ plus $O_2^{\bullet-}$ reaction is peroxynitrite anion, the pK_a value of 6.8 and the rapid protonation imply that, under most biological conditions, $ONOO^-$ and ONOOH will both be present (13) (Equation 4).

$$ONOO^- + H^+ \rightleftharpoons ONOOH \quad \text{(Eq. 4)}$$

For instance, at pH 7.4, ~80% of peroxynitrite will be in the anionic form; conversely, at pH 6.2 (*e.g.* inside a macrophage phagocytic vacuole), up to 80% will be in the protonated form. The stability, reactivity, and capacity to permeate membranes of $ONOO^-$ and ONOOH are quite different (13, 14), and therefore, the biochemistry of peroxynitrite in biological systems is highly pH-dependent. This acid-base property of peroxynitrite contrasts with that of H_2O_2, which has a pK_a of ~11.6 and therefore is almost 100% protonated in the physiological pH range.

Early Evidence of the Oxidizing Capacity of Peroxynitrite in Biochemical Systems

As peroxide, the relatively labile O–O bond provides the possibility of homolysis to radicals (10, 12, 15, 16). Indeed, proto-

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[2] The abbreviations used are: NOS, nitric-oxide synthase(s); SOD, superoxide dismutase(s); MnP, Mn-porphyrin(s).

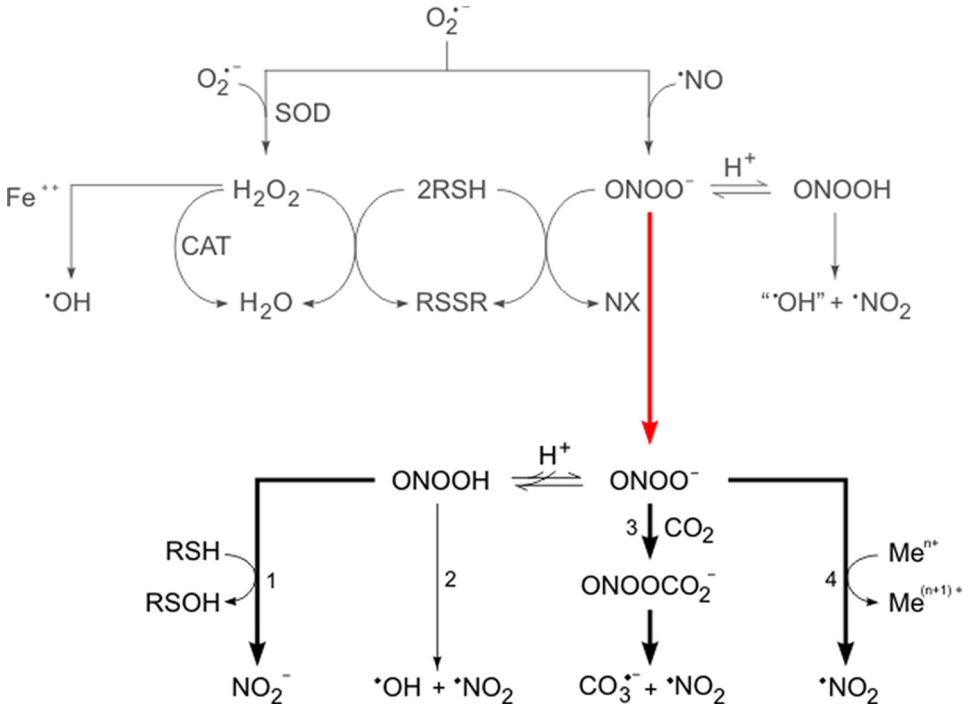

FIGURE 1. **Peroxynitrite as a mediator of superoxide radical and nitric oxide-dependent oxidative and cytotoxic processes.** The figure represents the evolution of our understanding of the biochemically relevant reactions in which peroxynitrite participates. It integrates the initial proposal (24) with what is currently known. *Upper*, shown in *gray* is a reproduction of the original scheme in which the commonly accepted mechanism of $O_2^{\bullet-}$-mediated oxidative damage was challenged with an alternative mechanism that considered the participation of •NO. The proposal indicated that the reaction of $O_2^{\bullet-}$ with •NO yielded peroxynitrite anion, which could then start oxidation reactions directly or via secondary radicals. The original scheme showed two main reactions for peroxynitrite, namely thiol oxidation and homolysis. The lack of absolute certainty at the time regarding the products of the decomposition of peroxynitrous acid led us to write the hydroxyl radical as "•OH". Similarly, NX indicated an uncharacterized nitrogen-containing product, later proved to be nitrite. The *red arrow* connects the early proposal with an updated scheme of the biological chemistry of peroxynitrite. *Lower*, shown in *black* are the main accepted reaction pathways of peroxynitrous acid and peroxynitrite anion, namely 1) two-electron oxidation of thiols, 2) homolysis, 3) nucleophilic addition to CO_2 and evolution to radicals, and 4) reaction with transition metal centers. The *width* of the *arrows* symbolizes the preferential routes of peroxynitrite consumption in biological systems, underscoring the fact that homolysis is a quantitatively minor process.

nation weakens the O–O bond in ONOOH and leads to homolytic cleavage to hydroxyl radicals (•OH) and nitrogen dioxide ($•NO_2$), two strongly oxidizing/hydroxylating and nitrating species, respectively (Equation 5).

$$ONOOH \rightarrow •NO_2 + •OH \qquad \text{(Eq. 5)}$$

The homolytic cleavage occurs with a k_4 of 4.5 s^{-1} at 37 °C, resulting in a half-life of 0.8 s in phosphate buffer at pH 7.4 (*i.e.* $k_{app} = 0.9\ s^{-1}$) (13). The recognition that the homolysis of ONOOH could yield •OH led to the postulation of a new biologically relevant mechanism of oxygen radical-mediated molecular damage, without the requirement of transition metal-dependent reactions. Indeed, until the emergence of peroxynitrite, much of $O_2^{\bullet-}$-dependent oxidative damage was postulated to occur via the Haber-Weiss cycle and/or the Fenton reaction (2), where ultimately •OH was formed (Equation 6).

$$H_2O_2 + Fe^{2+} \rightarrow •OH + OH^- + Fe^{3+} \qquad \text{(Eq. 6)}$$

In fact, $O_2^{\bullet-}$ is not a strong oxidant, and actually, it can also act as a reductant (17). Therefore, its *direct* toxicity is usually limited (*i.e.* oxidation and disruption of the iron-sulfur cluster in [4Fe-4S]-containing dehydratases) (2). Moreover, despite high formation rates (18), the steady-state levels of $O_2^{\bullet-}$ are always quite low due to the abundance and thorough distribution of SOD that promote its preferential dismutation to H_2O_2 (unless •NO is present). Thus, considerable $O_2^{\bullet-}$-dependent toxicity resides in the formation of secondary reactive species; these include H_2O_2 (Equation 6), peroxynitrite (Equation 1; to be analyzed in this minireview), and, possibly, reactive hydroperoxides formed by the fast reactions of $O_2^{\bullet-}$ with biomolecule-derived radicals (19, 20).

Similarly, although •NO was recognized early as a cytotoxic effector during cellular immune responses mediated by macrophages and neutrophils (21), the biological effects of •NO did not correlate well with its chemical reactivity, *i.e.* a relatively stable radical with modest redox properties (22). Thus, •NO-mediated toxicity was also further rationalized considering the generation of •NO-derived oxidants (23) such as peroxynitrite.

Soon after the proposal of the formation and homolysis of peroxynitrite in biological systems (10), it was reported that peroxynitrite could directly oxidize thiol groups at rates much faster than the homolytic cleavage (24). Overall, the initial observations (10, 24, 25) paved the way for a new paradigm of $O_2^{\bullet-}$- and •NO-mediated toxicity via peroxynitrite, which was schematized in JBC in 1991 (Fig. 1, *upper*) (24). The hypothesis led to predictions that could be tested experimentally, including how the inhibition of nitric oxide synthesis (*e.g.* using NOS inhibitors), the elimination of excess $O_2^{\bullet-}$ (*e.g.* overexpression of SOD), the catalytic decomposition of peroxynitrite, or the scavenging of peroxynitrite-derived radicals could influence oxidative processes and biological outcome. An updated version of the original proposal is shown in Fig. 1 (*lower*) and is analyzed below.

Key Aspects of Peroxynitrite Biological Chemistry: Redox Reactions and Nucleophilic Addition

Peroxynitrite is both an oxidant and nucleophile, and these two chemical properties dictate much of its biochemical actions *in vivo* (13). First, as an oxidant, it can promote one- and two-electron oxidations by direct reactions with biomolecular targets. Indeed, the redox potentials of peroxynitrite at pH 7 (E_0') for the $ONOO^-/{}^\bullet NO_2$ and $ONOO^-/NO_2^-$ pairs have been estimated as 1.4 and 1.2 V, respectively (26), supporting its performance as a good biological oxidant from a thermodynamic viewpoint (17).

A prime example of two-electron oxidations corresponds to the reaction of peroxynitrite with thiols, which yields the sulfenic acid derivative (and nitrite) (24, 27) (Equation 7).

$$ONOOH + RS^- \rightarrow NO_2^- + RSOH \quad \text{(Eq. 7)}$$

This reaction was originally described for cysteine and the single thiol group of albumin (Cys-34) and occurs with an apparent second-order rate constant of $>10^3\ M^{-1}\ s^{-1}$ at pH 7.4 and 37 °C (Table 1), ~3 orders of magnitude faster than the same reaction with H_2O_2. These data underscored the high chemical reactivity of peroxynitrite in biological systems (24). Nonetheless, peroxynitrite is less reactive toward thiols than other biologically relevant two-electron oxidants such as hypochlorous acid (HOCl) and hypobromous acid (HOBr), which react with cysteine and glutathione on the order of $10^7\ M^{-1}\ s^{-1}$ (28, 29). The reactivity of peroxynitrite with thiols is intermediary between that of H_2O_2 and that of hypohalous acids, resulting in a moderately strong and selective biological oxidant in a similar way to what has been proposed for thiol oxidation mediated by chloramines ($k \sim 100–200\ M^{-1}\ s^{-1}$) (28). Importantly, peroxynitrite can oxidize at even more remarkable rates some "fast reacting thiols," such as those present in mammalian and microbial peroxiredoxins (30). Indeed, peroxiredoxins react with peroxynitrite with constants on the order of 10^6–$10^7\ M^{-1}\ s^{-1}$ (Table 1) and represent a first line of enzymatic antioxidant defense against peroxynitrite.

Peroxynitrite also promotes one-electron oxidations directly (*e.g.* oxidation of cytochrome c^{2+} (31)) or secondarily through the homolysis of peroxynitrite. Indeed, ${}^\bullet OH$ is the most potent (and less selective) known biological oxidant and reacts with biomolecules at rates approaching the diffusion limit to cause hydroxylation or one-electron oxidation; ${}^\bullet NO_2$ is also a strong oxidant (32) and a key intermediate in nitration reactions (*i.e.* incorporation of a $–NO_2$ group). Although peroxynitrite homolysis is an interesting chemical process, its actual quantitative relevance at the biochemical level is less likely (13). Indeed, a lesson obtained from kinetic data is that the first-order rate constant of homolysis can hardly compete with other bimolecular reactions of peroxynitrite (Table 1). At most, homolysis represents a small percentage of the peroxynitrite-consuming reactions in living systems; nonetheless, homolysis generates reactive secondary radicals that initiate radical chain reactions such as lipid peroxidation and amplify the oxidation processes *in vitro* (25, 33, 34) and presumably *in vivo* (34).

As a nucleophile, a central reaction of peroxynitrite in biology is the addition of the anion to carbon dioxide (CO_2) to yield a nitrosoperoxocarboxylate adduct ($ONOOCO_2^-$) that undergoes a fast homolysis to ${}^\bullet NO_2$ and carbonate radicals ($CO_3^{\bullet-}$) (16, 35, 36) (Equation 8).

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow {}^\bullet NO_2 + CO_3^{\bullet-} \quad \text{(Eq. 8)}$$

This reaction is relevant because of the ubiquity of CO_2 in biological systems (*e.g.* 25 mM HCO_3^- is in equilibrium with ~1.3 mM CO_2 at pH 7.4) and its relatively high rate constant (Table 1). The "CO_2" reaction of peroxynitrite accounts for a substantial portion of its biological fate and chemistry (13). $CO_3^{\bullet-}$, rising from the reaction, is a good one-electron oxidant (32). Although the reaction of the HCO_3^-/CO_2 pair with peroxynitrite was inferred during studies of peroxynitrite-induced luminol chemiluminescence (37), direct EPR studies during continuous flow of peroxynitrite to carbonated phosphate buffers unambiguously revealed the formation of $CO_3^{\bullet-}$ (38). Thus, the formation and subsequent reactions of $CO_3^{\bullet-}$ and ${}^\bullet NO_2$ radicals are an integral part of the chemical biology of peroxynitrite. The nucleophilic addition of peroxynitrite anion to monocarbonyl- and dicarbonyl-containing compounds (*e.g.* glyoxal) (39) also occurs at significant rates; the adducts evolve to carbonyl-derived radical species and ${}^\bullet NO_2$ (secondarily, singlet oxygen is produced), although the biological significance of these processes is unknown.

Another type of reaction in which peroxynitrite can evolve to secondary oxidants involves its interaction with transition metal centers. These can be part of metalloproteins (*e.g.* Mn-SOD and hemeproteins) or metal complexes (*e.g.* Mn-porphyrins (MnP)). Transition metals may be regarded as Lewis acids, which can react with $ONOO^-$ to yield a Lewis adduct (13). Usually, the metal-based Lewis adducts undergo homolysis to yield ${}^\bullet NO_2$ and the corresponding oxyradical-metal complex, which rearranges to a strongly oxidizing oxo-metal complex (13, 23) (Equation 9).

$$ONOO^- + Me^n + X \rightarrow ONOO\text{-}Me^{n+}X \rightarrow {}^\bullet NO_2 + {}^\bullet O\text{-}Me^{n+}X \rightarrow {}^\bullet NO_2 + O{=}Me^{(n+1)+}X \quad \text{(Eq. 9)}$$

Peroxynitrite-mediated Protein Tyrosine Nitration

Peroxynitrite does not react directly with tyrosine (40). However, a recognized oxidative protein modification left by peroxynitrite *in vitro* and *in vivo* is the formation of 3-nitrotyrosine (23). Indeed, all of the secondary radicals arising from peroxynitrite (${}^\bullet OH$, $CO_3^{\bullet-}$, oxo-metal complexes, lipid peroxyl radicals (33), and ${}^\bullet NO_2$) promote protein tyrosine oxidation and nitration (41). The typical mechanism of tyrosine nitration in biological systems is a two-step radical process: a one-electron oxidant leading to the formation of a tyrosyl radical (Equation 10), which then combines at diffusion-controlled rates with ${}^\bullet NO_2$ to yield 3-nitrotyrosine (Equation 11) (42).

$$TyrH + CO_3^{\bullet-} \rightarrow Tyr^\bullet + HCO_3^- \quad \text{(Eq. 10)}$$

$$Tyr^\bullet + {}^\bullet NO_2 \rightarrow Tyr\text{-}NO_2 \quad \text{(Eq. 11)}$$

The nitration process started by oxo-metal complexes is particularly relevant in the *site-specific* tyrosine nitration of metallo-

TABLE 1

Kinetic aspects of peroxynitrite-mediated oxidations: selected reactions of biochemical relevance

The majority of the information highlights the reactivity of peroxynitrite with biomolecules and provides considerations about its quantitative relevance in biological systems. The last three listed compounds are examples of synthetic molecules utilized to either decompose or detect peroxynitrite. Prx, peroxiredoxin.

Reactant	k[a]	Process	Commentary
	$_{M}{}^{-1}{}_{S}{}^{-1}$		
Tyrosine	0	Tyr oxidation and nitration	There is no direct reaction (40). Tyr oxidation and nitration is accomplished by peroxynitrite-derived radicals (23).
Tryptophan	40	Trp oxidation and nitration	The direct reaction is rather slow and can cause Trp nitration (96).
Methionine	360	Methionine sulfoxide formation	It can account for enzyme inactivation (97). It is sometimes used to scavenge peroxynitrite in biochemical systems.
Uric acid	500	A variety of oxidation products can be formed, including allantoin, alloxan, and triuret (83, 85). The intermediate formation of uric acid-derived radicals may promote secondary oxidation reactions and products such as urate hydroperoxide (83, 98).	It is good inhibitor of peroxynitrite-dependent processes *in vitro* and *in vivo*. The direct reaction is relatively slow, so protection is ascribed to reaction with peroxynitrite-derived radicals. Uric acid is also a physiological substrate of myeloperoxidase (98) and may therefore interfere in heme peroxidase-dependent nitration reactions as well.
Glutathione[b]	1400	It evolves mainly to glutathione disulfide through the intermediacy of glutathione sulfenic acid (13). Glutathionyl radicals can be formed from peroxynitrite-derived radicals.	It is an endogenous compound that decomposes peroxynitrite. Considering a 5 mM intracellular concentration, the k[GSH][c] product results in a value of 7 s^{-1}, significantly faster that the rate constant of homolysis (0.9 s^{-1})[d] but much less than that of other direct reactions, so its direct reaction with peroxynitrite in biological systems is modest.
Cysteine[b]	5900	It evolves to cysteine disulfide (cystine) through the intermediacy of cysteine sulfenic acid (13, 24).	This was the first determination of a second-order rate constant of peroxynitrite reaction with a biomolecule. It provided the concept that direct reactions of peroxynitrite may be more relevant in biology than homolysis.
Human serum albumin	9700	About 40% of the direct reactivity is due to the reaction with the single thiol group (Cys-34) (40), leading to the sulfenic acid derivative.	A highly abundant plasma protein, it consumes a fraction of intravascular peroxynitrite but cannot outcompete the reaction with CO_2.
Oxyhemoglobin	2.3×10^4	It isomerizes peroxynitrite to nitrate (99).	It is relevant for peroxynitrite detoxification in red blood cells. At a concentration of 5 mM, k[oxy-Hb] = 340 s^{-1}, a remarkable velocity. However, peroxiredoxin-2 outcompetes oxyhemoglobin in peroxynitrite detoxification in the erythrocyte (13).
Mn-SOD	$>10^4$	The reaction of peroxynitrite anion with the Mn^{2+} atom produces enzyme nitration at Tyr-34 (43).	The nitration of the critical Tyr residue leads to enzyme inactivation. This process is largely observed *in vivo* under inflammatory conditions.
CO_2	5.8×10^4	Nucleophilic addition of peroxynitrite anion to CO_2 yields an unstable intermediate that undergoes homolysis (35, 36, 38).	This is a central reaction controlling peroxynitrite reactivity in biological system. A k[CO_2] value of ~60–100 s^{-1} has been established as a desirable starting range for a peroxynitrite scavenger to be competitive (13).
Aconitase[e]	1.4×10^5	Oxidation and disruption of the iron-sulfur cluster (57, 58)	A key reaction in mitochondria, aconitase inactivation slows down the Krebs cycle and causes iron release.
Peroxiredoxins	10^6–10^7	Fast reaction with the peroxidatic cysteine residue (30, 81)	Microbial and mammalian peroxiredoxins constitute a central catalytic mechanism for the detoxification peroxynitrite. The k[Prx] value ranges from $>10^2$ to 10^3 s^{-1} depending on cell types.
Ebselen	4.6×10^6	A synthetic seleno-containing compound that in the reduced state (selenol) undergoes two-electron oxidation, a reaction chemistry similar to that of thiols (100)	Ebselen readily decomposes peroxides and can create catalytic redox cycles at the expense of reducing compounds such as glutathione. It can be used pharmacologically to neutralize peroxynitrite (54).
MnP	$>10^7$	Mn^{2+} reduces peroxynitrite to nitrite and is catalytically recycled by endogenous reductants and the electron transport chain (89).	These compounds are used pharmacologically and can achieve 5–10 μM concentrations *in vivo*. Thus, with k[MnP] > 100 s^{-1}, they can effectively eliminate part of peroxynitrite (13).
Boronate-based compounds	$>10^6$	Peroxynitrite anion reacts directly via two-electron oxidation with boronate-based compounds to yield their corresponding hydroxyl derivatives (95). In the case of aryl boronates, the corresponding phenols are the major final products.	These compounds are a novel class of probes that can be utilized for peroxynitrite detection. They react with peroxynitrite at rates ~10^6 faster than hydrogen peroxide. The high rate constant and the lack of formation of probe-derived radical intermediates minimize secondary reactions and confounding results.

[a] Stopped-flow spectrophotometry has been utilized to determine the rate constants of peroxynitrite reaction with most compounds, taking advantage of the distinctive optical absorption of $ONOO^-$ at 302 nm (ϵ = 1670 M^{-1} cm^{-1}) as originally reported (24). Alternative approaches have been also used, with the application of competition kinetics with reference reactions of known rate constants (81).

[b] The actual reactants are peroxynitrous acid and the thiolate anion (Equation 7) (27); thus, the observed apparent reaction rate is strongly pH-dependent (24), with the thiol pK_{SH} representing a relevant variable. The table shows k_{app}, which is on the order of 10^3 M^{-1} s^{-1}; however, the actual (pH-independent) rate constant of the reaction is on the order of 10^5 M^{-1} s^1 (27, 30).

[c] The product of the second-order rate constant times the concentration of the reactant provides a pseudo-first-order rate constant in s^{-1} that allows ready comparison of the kinetic biological relevance among different peroxynitrite targets.

[d] In fact, the homolytic yields of $^{\bullet}NO_2$ and $^{\bullet}OH$ are ~30% of ONOOH due to "in cage" recombination of nascent radicals to nitrate (NO_3^-) before their diffusion to the bulk aqueous phase.

[e] Peroxynitrite also causes aconitase tyrosine nitration, but this is not related to the loss of activity, which is exclusively due to the oxidation of the [4Fe-4S] cluster (59).

proteins (41); in this case, the metal promotes the initial one-electron oxidation and enhances nitration yields in vicinal tyrosine residues (*e.g.* in Mn-SOD; see below) (43).

A long-lasting debate on the actual yields of peroxynitrite-mediated tyrosine nitration generated by variable fluxes of $O_2^{\cdot-}$ and $^{\cdot}NO$ (*i.e.* radical flux ratios $\neq 1$) has been largely clarified by considering factors such the actual steady-state of nitrating species, secondary radical processes over tyrosyl radicals, the presence of SOD, and the diffusion of excess $^{\cdot}NO$ across cellular compartments (44–46). Although originally described in biology to occur via peroxynitrite-dependent reactions (47, 48), protein tyrosine nitration can be also due to other $^{\cdot}NO$-mediated processes, most notably in heme peroxidase-catalyzed reactions (49–51). Nonetheless, peroxynitrite represents a substantial endogenous source of nitrating species (for a recent critical analysis, see Ref. 41 and references therein).

Nitric Oxide Interactions with Mitochondria and Peroxynitrite

At the cellular level, a prime *locus* related to the formation, reactions, and effects of peroxynitrite is represented by the mitochondrion (52). Indeed, mitochondria are central intracellular sources of $O_2^{\cdot-}$, and peroxynitrite formation is favored by the facile diffusion of $^{\cdot}NO$ from the cytosol (53, 54). Peroxynitrite reactions with mitochondrial components irreversibly affect the activity of electron transport chain complexes (complexes I and II) and ATPase, altering mitochondrial bioenergetics and calcium homeostasis and further promoting $O_2^{\cdot-}$ formation (54–56). In this scenario, mitochondria become a key cellular "sink" of $^{\cdot}NO$ and "source" of peroxynitrite.

Although the initial interactions of $^{\cdot}NO$ with mitochondria lead to relative reversible processes regulating respiration, membrane potential, and calcium homeostasis, the effects of peroxynitrite are harsher and typically promote to toxic events. Indeed, much of the initial evidence reporting long-lasting and toxic actions of $^{\cdot}NO$ on mitochondrial function was later demonstrated to be mainly peroxynitrite-dependent. For instance, its has been clarified that peroxynitrite is the main $^{\cdot}NO$-derived species responsible of the inactivation of mitochondrial aconitase (57–59), a [4Fe-4S] cluster-containing enzyme of the Krebs cycle (2). Disassembly of the cubane [4Fe-4S] cluster by peroxynitrite via oxidative attack leads to an inactive [3Fe-4S] enzyme (Equation 12).

$$[4Fe\text{-}4S]^{2+} + ONOO^- \rightarrow [3Fe\text{-}4S]^{1+} + Fe^{3+} + NO_2^- \quad \text{(Eq. 12)}$$

Iron released from aconitase can further propagate intramitochondrial oxidative damage by metal-mediated formation of oxidizing and nitrating species.

The relevance of the intramitochondrial formation of peroxynitrite is underscored by the established observation of mitochondrial protein nitration in pathological states and even under basal conditions (reviewed in Ref. 60). A key tyrosine-nitrated protein in mitochondria is Mn-SOD (61, 62), an essential antioxidant enzyme. Mn-SOD is found nitrated and inactivated *in vitro* and *in vivo* under conditions that facilitate intramitochondrial peroxynitrite formation (60). Not only is nitro-Mn-SOD a footprint of the process, but also its inactivation by nitration contributes to further amplify mitochondrial nitroxidative stress (54). Mn-SOD provides one of the few well established examples in which tyrosine nitration accounts for a physiologically relevant loss of function (*i.e.* the extent of nitration observed *in vivo* is sufficient to cause enough inactivation to alter metabolism) (23). Moreover, the nitration process leading to enzyme inactivation involves the site-specific manganese-catalyzed nitration of Tyr-34, the active site tyrosine lying only 5 Å from the manganese site) (43, 62, 63). Peroxynitrite anion enters the Mn-SOD active site through the same channel as $O_2^{\cdot-}$, reacts at relatively fast rates with manganese (approximately $>10^4\ M^{-1}\ s^{-1}$), and yields highly oxidant and nitrating species that cause nitration of Tyr-34. Once nitrated, the phenolic Tyr-34 hydroxyl group becomes partially deprotonated (*i.e.* its pK_a drops from ~10.3 to 7.3) (41), and therefore, the reaction of $O_2^{\cdot-}$ with manganese is hindered by both steric constraints and electrostatic repulsion (63, 64). The nitration of Mn-SOD Tyr-34 is strongly supportive of the intramitochondrial peroxynitrite formation (65, 66). Other oxidizing and nitrating species tend to react with tyrosines other than Tyr-34 and do not lead to enzyme inactivation.

Efforts have been also directed to understand how peroxynitrite-dependent nitration affects the redox properties of cytochrome *c*, an abundant mitochondrial protein and key partner of the electron transport chain (67). Recent evidence points to cytochrome *c* as an intramitochondrial heme peroxidase after a conformational change triggered by binding to cardiolipin and indicates that this process is related to apoptotic signaling (68). We have found that the preferential nitration of cytochrome by peroxynitrite-derived radicals in one of the solvent-exposed tyrosine residues (Tyr-74) leads to a conformational change that causes the displacement of the sixth ligand to the heme (Met-80) and a gain in peroxidase activity (67); the conformational change induced by nitration in cells also facilitates its translocation to the cytosol (even in non-apoptotic cells) (69). The biological significance of these events is currently under investigation.

Peroxynitrite-dependent Cytotoxicity

The reactions of peroxynitrite with biomolecules can led to cytotoxic events, which may result in apoptotic or necrotic cell death (54, 70, 71). The cytotoxic effects triggered by oxidation and nitration reactions usually involve the participation of a variety of effector molecules and processes. For example, peroxynitrite-mediated oxidation of mitochondrial membrane components facilitates the release of pro-apoptotic factors, whereas poly(ADP-ribose) polymerase activation secondary to DNA oxidative damage triggers necrosis (54). Although excess formation of peroxynitrite by mammalian tissues is deleterious in inflammatory and degenerative processes, it is also clear that our immune system can utilize peroxynitrite to repel microbial invasion (72). Indeed, whereas the shift in the signaling properties of $^{\cdot}NO$ toward oxidative pathways after reacting with $O_2^{\cdot-}$ is associated with pathology in the vascular and nervous systems, the antimicrobial activity of $^{\cdot}NO$ released by macrophages is largely dependent on the formation of peroxynitrite. Once formed, peroxynitrite can permeate cell membranes through

either anion channels or passive diffusion of the anionic and protonated forms, respectively (14), and over a biological half-life of ~5–20 ms, it promotes toxic effects locally at up to one- to two-cell diameters (~10 μm) from its site of formation.

Endogenous Toxicant—The participation of peroxynitrite in pathophysiological conditions has been recognized in cellular and animal disease models and in human pathology (54, 70). For example, peroxynitrite contributes to apoptotic neuronal death in a variety of acute injuries and neurodegenerative conditions (54, 73). Notably, it has just been reported that nitration of Hsp90 plays a central role in motor neuron death through activation of the Fas pathway (71). In the vascular wall, conditions such as hyperglycemia, atherosclerosis, and hypertension lead to enhanced $O_2^{\cdot-}$ formation, shorten the biological half-life of $^{\cdot}NO$, and cause endothelial dysfunction; the inexorable formation of peroxynitrite results in oxidation and nitration of biomolecules that may further contribute to vascular degeneration (74). Peroxynitrite also participates in vascular aging and protein tyrosine nitration of the vessel wall, including the selective nitration and inactivation of prostacyclin synthase (75). It is important to note that, during vascular inflammation, neutrophil degranulation of myeloperoxidase also contributes to protein tyrosine nitration processes by peroxynitrite-independent mechanisms (51, 74).

Cytotoxic Effector against Invading Pathogens—The cytotoxic properties of peroxynitrite can be also utilized by immune system cells to combat infecting microorganisms. Indeed, early experiments demonstrated the cytotoxic capacity of peroxynitrite toward *Escherichia coli* (76) and *Trypanosoma cruzi* (77), which was later extended to several other pathogens. For instance, peroxynitrite formation by macrophages represents a cytotoxic effector mechanism, which requires immunostimulation with cytokines that induce iNOS expression. Then, upon macrophage interaction with the microbial pathogen and association with a phagocytic process, the plasma membrane NADPH oxidase is activated for $O_2^{\cdot-}$ production (*i.e.* the respiratory burst): the simultaneous formation of $O_2^{\cdot-}$ and $^{\cdot}NO$ yields large levels of peroxynitrite in the phagosomal compartment over a 60–120-min period (72). For example, the intraphagosomal levels of peroxynitrite are sufficient for *T. cruzi* killing (*i.e.* the causative agent of Chagas disease). Nitrated and oxidized *T. cruzi* was evidenced inside the phagosome, confirming the diffusion of peroxynitrite from the macrophage to the pathogen (72). Instead, in other immune system cells such as neutrophils, alternative $^{\cdot}NO$-dependent mechanisms of cytotoxicity and pathogen protein tyrosine nitration operate, with the likely participation of myeloperoxidase-mediated reactions (51, 78, 79). The prolonged respiratory burst observed in macrophages (with respect to neutrophils) and the lack of significant myeloperoxidase activity (72) make the formation of peroxynitrite in immunostimulated macrophages a premier reaction pathway for the execution of $^{\cdot}NO$-derived cytotoxicity.

Modulation of the Redox Biochemistry of Peroxynitrite

The endogenous systems that cope with the toxic effects of peroxynitrite were established in initial work on microbial systems (80, 81). In fact, bacterial and parasitic peroxiredoxins were found to readily decompose peroxynitrite via a fast reaction with the "peroxidatic" cysteine residue of the enzyme active site (Equation 13),

$$\text{Prx-S}^- + \text{ONOOH} \rightarrow \text{Prx-SOH} + \text{NO}_2^- \qquad \text{(Eq. 13)}$$

where Prx is peroxiredoxin. This reaction was later shown in mammalian peroxiredoxin systems (13). The remarkable velocity for peroxiredoxin reactions with peroxynitrite (Table 1) extended earlier observations for H_2O_2 (30); the molecular determinants of such reactivity are under scrutiny but seem to depend of the stabilization of the enzyme-activated complex (30). The complete catalytic cycle to restore peroxiredoxin to the resting state is analyzed elsewhere (30). Due to the high concentration of peroxiredoxins, the fast rate constant, and its thorough distribution across various cellular compartments, it constitutes a prime endogenous antioxidant mechanism for the catalytic detoxification of peroxynitrite. In the case of microbial systems, peroxiredoxins have been recently revealed as virulence factors due to their capacity to detoxify peroxynitrite formed from macrophages both *in vitro* and *in vivo* (72, 82).

A large number of compounds have been used with pharmacological goals to cope with the toxic effects of peroxynitrite and peroxynitrite-derived radicals (54). A strong protector against peroxynitrite-mediated toxicity *in vitro* and *in vivo* is uric acid (54, 83–85), the end product of purine metabolism and an antioxidant compound in humans (86). The direct reaction of peroxynitrite with uric acid is rather slow to account for its protective effects (Table 1); thus, much of its effects may instead be due to the scavenging of peroxynitrite-derived radicals and the inhibition of tyrosine nitration reactions.

With respect to synthetic molecules that are effective against peroxynitrite *in vitro* and *in vivo*, MnP deserve special attention. This class of metal-based drugs, initially conceived as SOD mimics (87), readily reacts with peroxynitrite (13) and has been used to attenuate peroxynitrite-dependent cytotoxicity (54). Peroxynitrite can readily react with both the Mn^{2+} and Mn^{3+} states to yield nitrite or $^{\cdot}NO_2$, respectively. Although the MnP are typically administered in the 3+ state, the most effective and likely antioxidant mechanism in biological systems involves the 2+ state in a catalytic cycle as follows (Equations 14–17).

$$\text{Mn}^{3+} + \text{e}^- \rightarrow \text{Mn}^{2+} \qquad \text{(Eq. 14)}$$

$$\text{Mn}^{2+} + \text{ONOO}^- \rightarrow \text{Mn}^{4+}{=}\text{O} + \text{NO}_2^- \qquad \text{(Eq. 15)}$$

$$\text{Mn}^{4+}{=}\text{O} + \text{e}^- + 2\text{H}^+ \rightarrow \text{Mn}^{3+} + \text{H}_2\text{O} \qquad \text{(Eq. 16)}$$

$$\text{ONOO}^- + 2\text{e}^- + 2\text{H}^+ \xrightarrow{\text{MnP}} \text{NO}_2^- + \text{H}_2\text{O} \qquad \text{(Eq. 17)}$$

The reduction of the Mn^{3+}P is carried out by a variety of flavoenzymes, glutathione, and electron transport chain complexes. Then, Mn^{2+} readily reduces peroxynitrite anion to nitrite ($k \sim 10^6$–$10^7\ \text{M}^{-1}\ \text{s}^{-1}$); $Mn^{4+}{=}O$ is reduced back to the Mn^{3+} state by fast reactions with a variety of endogenous low molecular weight reductants, including ascorbate, glutathione, and uric acid (88, 89). Through the reactions shown in Equations 14–16, MnP can catalytically decompose peroxynitrite via a redox cycle of peroxynitrite reduction at the expense of

endogenous reducing equivalents. Notably, cationic MnP can accumulate in mitochondria to micromolar levels that can exert antioxidant activity (89, 90), and we have found that complexes I and II of the mitochondrial electron transport chain can readily provide the electrons for MnP reduction to the Mn^{2+} state under physiologically relevant oxygen concentrations (5–30 μM O_2) (91). Then, MnP protect mitochondria from peroxynitrite-mediated toxicity both *in vitro* and *in vivo*. Reduced MnP can also eliminate peroxynitrite-derived carbonate radicals (Table 1) (92). Even though MnP were initially conceived as SOD mimics, many of their protective antioxidant effects relate to peroxynitrite detoxification (93).

Another interesting strategy to connect the mechanisms of peroxynitrite-mediated toxicity with potential pharmacological applications is the use of cell-permeable tyrosine-containing peptides (73, 94). Tyrosine peptides do not react directly with peroxynitrite but interfere in the radical-dependent tyrosine nitration process and spare critical protein tyrosine residues. Although their precise mechanism of action in cells and *in vivo* remains to be established, intracellular delivery of tyrosine-containing peptides can protect cells from protein tyrosine nitration and death (73).

Concluding Remarks and Perspectives

Our current understanding of the biological chemistry of peroxynitrite provides a framework to understand the molecular mechanisms of oxidant-mediated cell and tissue injury in $^{\bullet}$NO-producing systems (Fig. 1). Although it is desirable to eliminate excess peroxynitrite to neutralize its toxicity in a variety of pathologies, peroxynitrite plays also a role as a strong antimicrobial agent, and therefore, tackling the peroxynitrite-detoxifying systems of microbes appears to be a good strategy for infection control. The elusive nature of peroxynitrite has made it difficult to determine its role as a key mediator in pathology, but the correct understanding of its redox biochemistry has greatly helped in the process (13). Moreover, the recent characterization of boronate-based probes that react quickly with peroxynitrite (95) (Table 1) provides possibilities for a more specific detection and even quantitation of peroxynitrite by bioanalytical and bioimaging techniques. Thus, although "stealthy" in nature, accumulated knowledge and new molecular tools are revealing peroxynitrite as a key redox mediator in pathological states and providing possible remedies for this furtive oxidant species.

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