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Review

Kinetic and mechanistic considerations to assess the biological fate of peroxynitrite☆

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ABSTRACT

Background: Peroxynitrite, the product of the reaction between superoxide radicals and nitric oxide, is an elusive oxidant with a short half-life and a low steady-state concentration in biological systems; it promotes nitroxidative damage.
Scope of review: We will consider kinetic and mechanistic aspects that allow rationalizing the biological fate of peroxynitrite from data obtained by a combination of methods that include fast kinetic techniques, electron paramagnetic resonance and kinetic simulations. In addition, we provide a quantitative analysis of peroxynitrite production rates and conceivable steady–state levels in living systems.
Major conclusions: The preferential reactions of peroxynitrite *in vivo* include those with carbon dioxide, thiols and metalloproteins; its homolysis represents only <1% of its fate. To note, carbon dioxide accounts for a significant fraction of peroxynitrite consumption leading to the formation of strong one-electron oxidants, carbonate radicals and nitrogen dioxide. On the other hand, peroxynitrite is rapidly reduced by peroxiredoxins, which represent efficient thiol-based peroxynitrite detoxification systems. Glutathione, present at mM concentration in cells and frequently considered a direct scavenger of peroxynitrite, does not react sufficiently fast with it *in vivo*; glutathione mainly inhibits peroxynitrite-dependent processes by reactions with secondary radicals. The detection of protein 3-nitrotyrosine, a molecular footprint, can demonstrate peroxynitrite formation *in vivo*. Basal peroxynitrite formation rates in cells can be estimated in the order of 0.1 to 0.5 μM s^{-1} and its steady-state concentration at ~1 nM.
General significance: The analysis provides a handle to predict the preferential fate and steady-state levels of peroxynitrite in living systems. This is useful to understand pathophysiological aspects and pharmacological prospects connected to peroxynitrite. This article is part of a Special Issue entitled Current methods to study reactive oxygen species - pros and cons and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

1. Peroxynitrite biochemistry

1.1. Peroxynitrite formation pathways

The reaction between the free radicals nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$) leads to the diffusion-controlled formation of peroxynitrite,[1] a potent oxidizing and nitrating agent formed *in vivo* [1].

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

Nitric oxide is a relatively stable and mildly reactive free radical mainly generated enzymatically from L-arginine, NADPH and oxygen in a reaction catalyzed by several isoforms of nitric oxide synthase (NOS). Nitric oxide is a ubiquitous intracellular messenger, which mediates multiple physiological processes, including regulation of blood pressure, neurotransmission, immune response and platelet aggregation [2–4]. Its consumption in biological systems is determined by reactions preferentially with other paramagnetic species, such as

Abbreviations: $ABTS^{2-}$, 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid); DTPA, diethylenetriaminepentaacetic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; MPO, myeloperoxidase; EPO, eosinophil peroxidase; NT, 3-nitrotyrosine; NOS, nitric oxide synthase; SOD, superoxide dismutase; GSH, glutathione; Prx, peroxiredoxin

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[1] The term peroxynitrite is used to refer to the sum of peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH). IUPAC recommended names are oxoperoxonitrate (1−) and hydrogen oxoperoxonitrate, respectively.

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organic radicals (tyrosyl and peroxyl radicals) or transition metal centers, most remarkably with oxyhemoglobin, which represents an important fate of $^{\bullet}NO$ in the vasculature [5,6]. Another relevant reaction responsible for $^{\bullet}NO$ depletion is with $O_2^{\bullet-}$ to yield peroxynitrite [2,4,7,8]. The sources of $O_2^{\bullet-}$, the one electron reduction product of molecular dioxygen, include enzymes such as NAD(P)H oxidase, xanthine oxidase and uncoupled NOS, the electron leakage in the mitochondrial respiratory chain, and through redox cycling of xenobiotics, among other possible mechanisms. $O_2^{\bullet-}$ can react with iron sulfur clusters, transition metal centers and thiols but the main route for its consumption in biological systems is the reaction with superoxide dismutases (SOD), which are extremely efficient in catalyzing $O_2^{\bullet-}$ dismutation to hydrogen peroxide (H_2O_2) and dioxygen (rate constant $> 10^9\ M^{-1}\ s^{-1}$) [9–11]. This preferential reaction with SODs, which are abundant (concentration > 10 μM) and ubiquitous enzymes, keeps the concentration of $O_2^{\bullet-}$ at a low steady-state level (10^{-9}–10^{-12} M range) [12–14]. However, this value can increase several-fold under conditions of altered cellular homeostasis and during inflammatory processes.

An early evidence of the biological formation of peroxynitrite was obtained in experiments that showed that SOD prolonged the half-life and biological effects of the $^{\bullet}NO$ [15,16]. The rate constant of $O_2^{\bullet-}$ reaction with $^{\bullet}NO$ to form peroxynitrite (Eq. (1)), has been reported within the range of $(4–16) \times 10^9\ M^{-1}\ s^{-1}$ [17–20], which is a higher value than that of the reaction with Mn or Cu,ZnSOD ($1–2 \times 10^9\ M^{-1}\ s^{-1}$) [10,11]. Therefore, $^{\bullet}NO$ can be produced at a sufficient concentration and react fast enough to outcompete SOD for its reaction with $O_2^{\bullet-}$. In fact, it can be estimated that in the presence of physiological concentrations of SOD ~10 to 20 μM, $^{\bullet}NO$ produced at micromolar concentrations will be able to outcompete SOD and trap a significant fraction of the $O_2^{\bullet-}$ formed.

Nitric oxide is a hydrophobic uncharged radical which can easily permeate cell membranes and diffuse outside the cell, while $O_2^{\bullet-}$ is much more short-lived and due to its anionic character ($pK_a \sim 4.8$) has restricted diffusion across biomembranes. For this, peroxynitrite formation, which requires the simultaneous generation of both radicals, will be spatially circumscribed to the sites of $O_2^{\bullet-}$ formation. The steady-state concentrations of $^{\bullet}NO$ and $O_2^{\bullet-}$ also command the rate of peroxynitrite formation. Considering a cellular scenario where diverse competitive scavenging occurs, particularly direct competition between SOD and $^{\bullet}NO$ for the reaction with $O_2^{\bullet-}$, from a combination of experimental values and the known rate constants it was estimated that the maximal rate of peroxynitrite formation in endothelial cell mitochondria under basal metabolic conditions is 0.3 μM s^{-1} [21,22]. In this sense, from a reported kinetic model that included the effects of multiple cellular targets, intracellularly steady-state concentrations of peroxynitrite in the nanomolar range were estimated [21,23]. Even though the levels of $^{\bullet}NO$ and $O_2^{\bullet-}$ *in vivo* are regulated efficiently by the scavengers and disposal systems, the concentration could increase several-fold under altered cellular homeostasis and therefore influence the rate of peroxynitrite formation. Thus, peroxynitrite flux can be increased when other scenarios are considered, such as in inflammatory cells, where both $^{\bullet}NO$ and $O_2^{\bullet-}$ production rates are largely enhanced. For example, in selected cellular compartments such as the phagosome, fluxes of peroxynitrite produced by immuno-stimulated (*i.e.* cytokine exposure leading to iNOS expression) and activated (*i.e.* trigger of the respiratory burst) macrophages were estimated as ~0.83–1.66 μM s^{-1} in the murine cell line J774A.1 [24].

1.2. Physicochemical properties of peroxynitrite

Peroxynitrite is more reactive than its precursors $^{\bullet}NO$ and $O_2^{\bullet-}$. With one- and two-electron reduction potentials of $E^{\circ\prime}(ONOO^-, 2H^+/{}^{\bullet}NO_2, H_2O) = +1.4$ V and $E^{\circ\prime}(ONOO^-, 2H^+/NO_2^-, H_2O) = +1.2$ V, respectively [25,26], peroxynitrite is a relatively strong biological oxidant and nitrating agent able to react with a wide range of biomolecules. The anionic form of peroxynitrite ($ONOO^-$) exists in equilibrium with its conjugated acidic form (ONOOH) ($pK_a \sim 6.8$, Eq. (2)). Thus, under biological conditions both species will be present at ratios depending on the local pH. For example, at the physiological pH of 7.4, peroxynitrite anion will be present in a proportion of 80%.

$$ONOOH \rightleftharpoons ONOO^- + H^+ \quad \left(K_a = 1.58 \times 10^{-7} M^{-1}\right) \tag{2}$$

The coexistence of the anionic and protonated forms of peroxynitrite is also relevant since they have different reactivity [21] and diffusional [27,28] properties.[2]

The anionic form of peroxynitrite displays a moderate absorbance near the ultraviolet region. The absorption spectrum in aqueous alkaline solution consists of a single band with a maximum centered at 302 nm, which has been used to quantify peroxynitrite and follow its reactions, using the reported extinction coefficient ($\varepsilon_{302} = 1670\ M^{-1}\ cm^{-1}$) [30].

2. Kinetics and reaction mechanisms of peroxynitrite

Peroxynitrite biochemistry is dictated by the kinetics of its formation and decay together with its partial limitation to diffuse through biological membranes: most importantly the availability of targets in different cell compartments critically modulates its biological fate. In biological systems, peroxynitrite promotes the oxidative modification of target molecules by different types of reactions involving: a) peroxynitrite-derived radicals from the homolytic cleavage of peroxynitrous acid or secondary to the reaction with carbon dioxide or b) by direct oxidation reactions.

2.1. Peroxynitrite reactivity

2.1.1. Homolytic cleavage of ONOOH

In the absence of direct targets peroxynitrite anion is relatively stable. However, peroxynitrous acid decays rapidly by homolysis of its peroxo bond ($k = 0.9\ s^{-1}$ at 37 °C and $0.26\ s^{-1}$ at 25 °C and pH 7.4) leading to the formation of nitrogen dioxide ($^{\bullet}NO_2$) and hydroxyl radicals ($^{\bullet}OH$) in ~30% yield whereas the rest of peroxynitrous acid directly isomerizes to nitrate (NO_3^-) [31–33]. Hydroxyl radical is a much stronger oxidant than $^{\bullet}NO_2$, however it reacts very rapidly with most biomolecules ($\sim 10^9\ M^{-1}\ s^{-1}$) in a non-selective manner, with addition reactions predominating over one-electron abstractions. In contrast, $^{\bullet}NO_2$ reacts at slower rates but represents a more selective one-electron oxidant. Thus, these peroxynitrite-derived radicals ($^{\bullet}OH$ and $^{\bullet}NO_2$) can mediate several reactions that may lead to the oxidation or nitration of different targets, such as tyrosine nitration and lipid peroxidation.

Considering the relative slowness of ONOOH homolysis compared to the reaction of peroxynitrite with multiple cellular targets that react directly with relatively high rate constants (*vide infra*), it can be estimated that in biological systems, most peroxynitrite formed will be consumed by direct reactions, with <1% evolving to $^{\bullet}NO_2$ and $^{\bullet}OH$, so the homolytic route is just a modest component of peroxynitrite reactivity in the aqueous compartment [34]. However, the homolytic decomposition of ONOOH may be a relevant process in hydrophobic compartments initiating radical-dependent processes such as lipid peroxidation and even protein and lipid nitration [35–39].

[2] Peroxynitrous acid can cross biological membranes through the lipid bilayer by passive diffusion, whereas the anionic form can penetrate cells through anion channels [27,28]. Despite the short biological half-life of peroxynitrite at physiological pH (~10 ms, [28]), due to a multiplicity of reactions with biotargets, the ability to cross cell membranes implies that peroxynitrite generated by a cellular source could influence surrounding target cells within one or two cell diameters (~5–10 μm) [29]. In fact, considering the peroxynitrite targets in different compartments, it has been estimated that it can traverse a mean distance of 0.5, 3 and 5.5 μm in erythrocytes, mitochondria and blood plasma, respectively, during one half-life [21].

2.1.2. Direct reactions

Peroxynitrite anion and peroxynitrous acid react directly with different biomolecules by one- or two-electron oxidation reactions. For example, low molecular weight or protein thiols, selenium compounds, metal centers and carbon dioxide (CO_2), constitute the main reported targets of peroxynitrite *in vivo*, with second-order rate constants in the range of ~10^3 to 10^7 M^{-1} s^{-1} [40,41]. Those reactions with endogenous components that modulate peroxynitrite reactivity and its possible fates in a cellular system will be described in detail.

2.1.2.1. Carbon dioxide. One of the most biologically relevant reactions of peroxynitrite is its fast reaction with CO_2 (in equilibrium with bicarbonate anion), which is present at relatively high concentrations (≥1.3 mM) in biological systems. The nucleophilic addition of $ONOO^-$ to CO_2, with a second-order rate constant of 4.6×10^4 M^{-1} s^{-1} (at pH 7.4 and 37 °C), yields a transient nitroso-peroxocarbonate ($ONOOCO_2^-$) which rapidly decomposes homolytically to carbonate radical ($CO_3^{\bullet-}$) and $^{\bullet}NO_2$ in ~34% yields, with the remaining yielding carbon dioxide and nitrate [42–45]. With reduction potentials of $E^{\circ\prime}(CO_3^{\bullet-}, H^+/HCO_3^-) = +1.78$ V and $E^{\circ\prime}(^{\bullet}NO_2/NO_2^-) = +0.99$ V, $CO_3^{\bullet-}$ is a relatively strong one-electron oxidant whereas $^{\bullet}NO_2$ is a more moderate oxidant and also a nitrating agent [46–49]. Thus, CO_2 instead of being a "scavenger" of peroxynitrite, rather redirects its reactivity promoting the formation of two new strong and short-lived one-electron oxidants that can lead to nitro-oxidative events, targeting mainly towards amino acids in proteins (most notably tryptophan, tyrosine, cysteine, methionine and histidine residues) [50]. Considering the ubiquity of CO_2, its reactivity with peroxynitrite and the potentially oxidant character of the products, CO_2 can be used as a hinge of peroxynitrite reactivity. Carbon dioxide outcompetes with other biological targets for the direct reaction with peroxynitrite and could inhibit their two-electron oxidation. However, most of the targets would be oxidized by one-electron by the peroxynitrite-derived radicals. As a consequence, depending on the target concentration and on the second-order rate constant of its reaction with peroxynitrite, CO_2 would divert target oxidation from two to one-electron mechanisms particularly at neutral pH. Based on the rate constant ($k = 4.6 \times 10^4$ M^{-1} s^{-1}, at pH 7.4 and 37 °C) and CO_2 concentrations found in cell compartments ([T] ~ 1.3 mM), the product k[T] ~ 60 s^{-1} can be used to parameterize the reactivity of peroxynitrite toward CO_2 and as a benchmark to assess the relative importance of other biotargets for a peroxynitrite scavenger to be competitive, as will be described later.

2.1.2.2. Thiols. Thiols are preferential targets of reactive species. The apparent second-order rate constants of peroxynitrite with cysteine, glutathione (GSH), homocysteine and the single thiol group of albumin (Cys34) are ~10^3 M^{-1} s^{-1} at pH 7.4 and 37 °C, about three orders of magnitude faster than the corresponding reactions with hydrogen peroxide [8,47,51]. Moreover, rate constants in the order of 10^6–10^7 M^{-1} s^{-1} have been reported for the reaction of peroxynitrite with very reactive thiols in proteins, such as those present in peroxiredoxins (Prx), which constitute an efficient key detoxification system of peroxynitrite [21,52–54].

The direct two-electron oxidation of thiols (RSH) by peroxynitrite results in the formation of nitrite and sulfenic acid (RSOH) as intermediate, which reacts with another thiol forming the corresponding disulfide (RSSR). The mechanism involves the nucleophilic attack of the thiolate on one of the peroxidic oxygens of peroxynitrous acid, with nitrite as leaving group [51]. Alternatively, the one-electron oxidation of thiols by the radicals derived from peroxynitrite homolysis in the absence or presence of CO_2, results in a sulfur-centered radical (thiyl radical, $RS^{\bullet}$), which is highly reactive and can recombine to form disulfide bridges (RSSR) or react with a thiolate to yield a disulfide radical anion ($RSSR^{\bullet-}$) which in the presence of oxygen can promote the formation of disulfide and $O_2^{\bullet-}$. Alternatively, $RS^{\bullet}$ can initiate an oxygen-dependent chain reaction to produce a number of secondary radicals, including thiyl peroxyl radical ($RSOO^{\bullet}$) and sulfinyl radical ($RSO^{\bullet}$) which can finally yield sulfenic acid [46,55,56].

In order to kinetically substantiate these peroxynitrite-mediated direct *versus* radical pathways, computer-assisted simulations of peroxynitrite reaction with GSH were performed according to the reported rate constants shown in Table 1. As observed in Fig. 1A, at low GSH concentrations, the homolysis of peroxynitrite predominates (0.9 s^{-1}, pH 7.4, 37 C) yielding $OH^{\bullet}$ and $^{\bullet}NO_2$ which can oxidize GSH in a one-electron process, leading to the formation of glutathionyl radical ($GS^{\bullet}$). When the concentration of GSH increases, the direct reaction of peroxynitrite as a two-electron oxidation process becomes more significant. In addition, peroxynitrite-mediated oxidation of low molecular weight thiols is associated with oxygen consumption, in agreement with previous reported observations [46,55,57]. In this sense, from the computer-simulated profiles of the total oxygen consumption *versus* increasing GSH concentrations, a biphasic profile was obtained (Fig. 1B). This oxygen consumption pattern that quantitatively reproduced the reported experimental results obtained from the peroxynitrite-dependent cysteine oxidation, is also consistent with the two competing pathways participating in the oxidation of GSH by peroxynitrite [55]. At low GSH concentrations (≤0.8 mM), the total consumption increases since the radicals derived from peroxynitrite homolysis can oxidize GSH in a one-electron process, leading to the formation of $GS^{\bullet}$, which is

Table 1
Reactions participating in the aerobic oxidation of GSH by peroxynitrite that were used in the simulation of Fig. 1.

Reaction[a]	Rate constant	Reference
Reactions involved in peroxynitrite decay		
$ONOOH \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}OH$	0.27 s^{-1} [b]	[47]
$ONOOH \rightarrow NO_3^- + H^+$	0.63 s^{-1} [b]	[47]
$^{\bullet}OH + NO_2^- \rightarrow {}^{\bullet}NO_2 + OH^-$	6×10^9 M^{-1} s^{-1}	[58]
$2\,{}^{\bullet}NO_2 \rightleftharpoons N_2O_4$	4.5×10^8 M^{-1} s^{-1} (f) [c];	[58]
	6.9×10^3 s^{-1} (r)	
$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + H^+$	1×10^3 s^{-1}	[162]
$^{\bullet}NO_2 + O_2^{\bullet-} \rightleftharpoons O_2NOO^-$	4.5×10^9 M^{-1} s^{-1} (f);	[44]
	1.1 s^{-1} (r)	[163]
$O_2NOO^- \rightarrow O_2 + NO_2^-$	1.3 s^{-1}	[44]
		[163]
$2\,O_2^{\bullet-} + 2\,H^+ \rightarrow O_2 + H_2O_2$	2×10^5 M^{-1} s^{-1}	[164]
$ONOO^- + CO_2 \rightarrow {}^{\bullet}NO_2 + CO_3^{\bullet-}$	1.6×10^4 M^{-1} s^{-1} [d]	[43]
$ONOO^- + CO_2 \rightarrow NO_3^- + CO_2$	3.0×10^4 M^{-1} s^{-1} [d]	[43]
$CO_3^{\bullet-} + {}^{\bullet}NO_2 \rightarrow NO_2^- + CO_2$	1×10^9 M^{-1} s^{-1}	[58]
$CO_3^{\bullet-} + O_2^{\bullet-} \rightarrow CO_3^{2-} + O_2$	6.5×10^8 M^{-1} s^{-1}	[58]
Additional reactions in the presence of GSH		
$GSH + ONOOH \rightarrow GSOH + NO_2^-$	1.35×10^3 M^{-1} s^{-1}	[47,51]
$GSOH + GSH \rightarrow GSSG + OH^-$	1×10^5 M^{-1} s^{-1}	[165]
$GSH + H_2O_2 \rightarrow GSOH + OH^-$	0.87 M^{-1} s^{-1}	[166]
$GSH + {}^{\bullet}NO_2 \rightarrow GS^{\bullet} + NO_2^- + H^+$	2×10^7 M^{-1} s^{-1}	[75]
$GSH + {}^{\bullet}OH \rightarrow GS^{\bullet} + H_2O$	2.3×10^{10} M^{-1} s^{-1}	[58]
$GSH + O_2^{\bullet-} \rightarrow GS^{\bullet} + H_2O_2$	200 M^{-1} s^{-1} [f]	[167]
$GSH + CO_3^{\bullet-} \rightarrow GS^{\bullet} + CO_3^{2-}$	5.3×10^6 M^{-1} s^{-1}	[58]
$GS^{\bullet} + GS^{\bullet} \rightarrow GSSG$	7.5×10^8 M^{-1} s^{-1}	[168]
$GS^{\bullet} + GSH \rightleftharpoons GSSG^{\bullet-}$	1×10^8 M^{-1} s^{-1} (f);	[169]
	2.5×10^5 s^{-1} (r)	
$GS^{\bullet} + O_2 \rightarrow GSOO^{\bullet}$	2×10^9 M^{-1} s^{-1}	[170]
$GS^{\bullet} + {}^{\bullet}NO \rightarrow GSNO$	1×10^9 M^{-1} s^{-1} [e]	[171]
$GS^{\bullet} + {}^{\bullet}NO_2 \rightarrow GSNO_2$	3×10^9 M^{-1} s^{-1} [e]	[171]
$GSSG^{\bullet-} + O_2 \rightarrow GSSG + O_2^{\bullet-}$	1.6×10^8 M^{-1} s^{-1}	[172]
$GSOO^{\bullet} + GSH \rightarrow + GSO^{\bullet} + GSOH$	2×10^6 M^{-1} s^{-1}	[173]

[a] Reactions were considered at neutral pH and 37 °C when data were available.
[b] Calculated considering the rate constant for peroxynitrous acid decay (0.9 s^{-1} at pH 7.4) [47] and the radical yield (30%) [32,33].
[c] (f) and (r) represent forward and reverse kinetic constants, respectively.
[d] Calculated considering the rate constant for the reaction of $ONOO^-$ with CO_2 ($k = 4.6 \times 10^4$ M^{-1} s^{-1}) [43] and the radical yields (34 and 66%) [45,171,174].
[e] The rate constants for the recombination reaction of $GS^{\bullet}$ with $^{\bullet}NO$ and $^{\bullet}NO_2$ were estimated to be similar to that of the recombination reaction of $^{\bullet}NO$ and $^{\bullet}NO_2$ with tyrosyl radicals respectively, as reported previously [45,171,174].

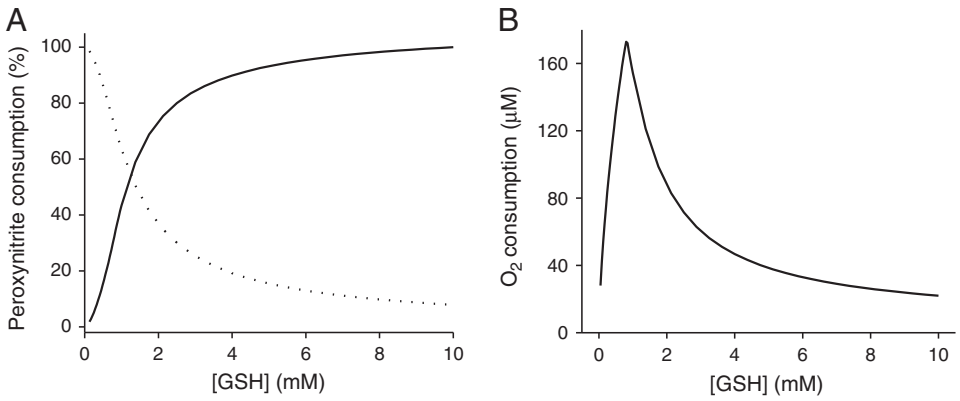

Fig. 1. Computer-assisted simulations of the peroxynitrite-mediated decay in the presence of GSH. (A) Simulated profile of peroxynitrite (0.5 mM) decay by direct reaction with GSH (0.05–10 mM) (solid line) *versus* homolysis to free radicals (dotted line). (B) Simulated profile of oxygen consumption against GSH concentration. The initial oxygen concentration was 0.217 mM (oxygen solubility at 37 °C). As the concentration of GSH increases, peroxynitrite decay by the direct two-electron oxidation process becomes more significant, instead of its decay through homolysis, which predominates at low GSH concentrations. Computer-assisted kinetic simulations were performed according to the reactions shown in Table 1 with the software Gepasi [161].

capable of reacting with oxygen triggering an oxygen-dependent radical chain reaction that could amplify the one-electron oxidation pathway. Indeed, $^{\bullet}OH$ reacts with GSH leading to $GS^{\bullet}$ with a reported rate constant of 2.3×10^{10} M^{-1} s^{-1} [58]. On the other hand, at high GSH concentrations (>0.8 mM), oxygen consumption decreases due to the preferential direct reaction with peroxynitrite. In fact, the observed decrease in oxygen consumption at high GSH concentrations disappeared when the direct reaction was not included in the simulation. In addition, the simulation also demonstrates that the reaction that most contributes to oxygen consumption is that of $GS^{\bullet}$ with oxygen, given that deletion of this reaction dropped oxygen consumption by one order of magnitude.

2.1.2.3. Metal centers. Peroxynitrite can also directly oxidize transition metal centers of proteins, particularly heme and non-heme iron, copper and manganese ions, with higher rate constants ranging from ~10^4 to 10^7 M^{-1} s^{-1}. Depending on the transition metal-containing protein, the interactions with peroxynitrite undergo different mechanisms. In the case of heme proteins, peroxynitrite reacting as a one-electron oxidant yields nitrite (NO_2^-) while reacting through a two-electron process yields $^{\bullet}NO_2$ in addition to the oxidized metal center (Fig. 3). In addition, some metal complexes can catalyze the isomerization of peroxynitrite to nitrate (NO_3^-). In the case of the hemeproteins myeloperoxidase (MPO) and cytochrome P450, the one-electron reaction with peroxynitrite leads to the formation of $^{\bullet}NO_2$ and ferryl-oxo compounds [59–61], which are strong secondary oxidizing species that can be reduced by appropriate reductants such as GSH or ascorbic acid, yielding nitrite and regenerating the metal center, or on the other hand they can react with a critical amino acid nearby and lead to a loss of the protein function. For example, the reaction of peroxynitrite with the Cu,Zn and Mn centers of superoxide dismutase yields the oxidant species oxo-copper and oxo-manganese, in addition with $^{\bullet}NO_2$ that potentially lead to metal-catalyzed histidine oxidation and tyrosine nitration, respectively [14,62,63].

2.2. How to experimentally disclose the preferential reactions of peroxynitrite in living systems

Kinetic studies can enable us to understand and differentiate between the different peroxynitrite-oxidation pathways. For instance, when peroxynitrite reacts directly with a target molecule in an overall second-order process, the reaction mechanism is first order in peroxynitrite and first order in the target so the apparent rate constant of peroxynitrite decomposition increases linearly with target concentration. In order to study the reaction between peroxynitrite and glutathione, the kinetics of peroxynitrite decay can be measured through stopped-flow spectrophotometry at increasing GSH concentrations. As shown in Fig. 2, the decay of peroxynitrite at 302 nm followed exponential functions and the observed pseudo-first order rate constants (k_{obs}) increased linearly with GSH concentrations, confirming a direct reaction. From the slope of the plot, the apparent second-order rate constant was determined as $(1.65 \pm 0.01) \times 10^3$ M^{-1} s^{-1} at pH 7.35 and 37 °C, similar to the reported value of 1.35×10^3 M^{-1} s^{-1} [47,51]. In the other pathway, the target molecule does not react directly with peroxynitrite, but can be modified through species derived from peroxynitrous acid homolysis ($^{\bullet}NO_2$ and $^{\bullet}OH$). Since the formation of the radicals would be the rate-limiting step ($k = 0.9$ s^{-1} at 37 °C), this process is first order in peroxynitrite and zero order in the target. This is the case of the reaction between tyrosine with peroxynitrite, where there is no direct bimolecular reaction, as evidenced from the fact that tyrosine does not increase the rate of peroxynitrite decomposition (Fig. 2). Nevertheless, tyrosine can be modified through the intermediate formation of peroxynitrite-derived radicals [64]. It is important to note that plots of the apparent second-order rate constants as a function of pH, as well as oxygen consumption studies and computer-assisted simulation of the reactions involved can also give important information about the reaction mechanisms and product yields.

A helpful example to analyze is the case of desferrioxamine, an iron chelating agent capable of inhibiting peroxynitrite-mediated oxidations by mechanisms *independent* of metal chelation [38,65,66]. It has been determined that it does not react directly with peroxynitrite, since as was reported previously, it did not increase the rate of peroxynitrite decomposition followed at 302 nm by stopped-flow spectrophotometry. The lack of a direct reaction was also supported by the fact that the direct two-electron oxidation of GSH by peroxynitrite was unaffected in the presence of desferrioxamine. Instead, desferrioxamine has been shown to inhibit peroxynitrite-dependent oxidation and nitration by reacting with $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ radicals [66]. In fact, in addition to direct $^{\bullet}NO_2$ scavenging, it has been also reported that desferrioxamine can reduce tyrosyl radical ($Tyr^{\bullet}$) intermediate and thus inhibits the radical–radical addition step that leads to 3-nitrotyrosine (NT) formation [66,67]. Thus, desferrioxamine can be used to define between the radical and non-radical mechanisms by which peroxynitrite produces oxidative modifications in biochemical systems.

In some cases, molecules that directly react with peroxynitrite (*e.g.* thiols), can also be oxidized by the radicals derived from peroxynitrite homolysis, $^{\bullet}NO_2$ and $^{\bullet}OH$. In order to evaluate the incidence of radical species in the oxidative modifications, it is highly recommended to use radical scavengers that react with

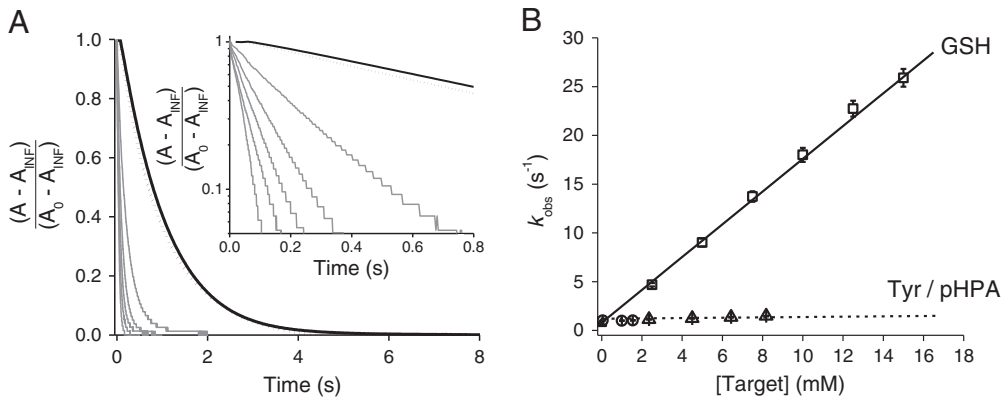

Fig. 2. Kinetics of peroxynitrite reaction with GSH and tyrosine. (A) Kinetic traces of peroxynitrite decomposition in the presence of glutathione and tyrosine. Peroxynitrite (56 μM) was mixed with phosphate buffer (0.1 M, pH 7.35, 0.1 mM DTPA) at 37 °C, alone (black line) and in the presence of 1.5 mM tyrosine (dash line) or in the presence of increasing GSH concentrations (from right to left: 2.5, 5, 7.5, 10 and 15 mM, gray lines). Please note that the plot intercepts the *y*-axis at ~0.9 s^{-1}, a value corresponding to the peroxynitrite homolysis at 37 °C. *Inset*: Logarithmic plot of the stopped-flow kinetic traces at 302 nm up to 0.8 s. A is the absorbance at time t, and A_0 and A_{INF} are the initial and final values, respectively. (B) The observed rate constants (k_{obs}) determined from the fit of the decay of peroxynitrite at 302 nm to a single exponential function, are shown as a function of target concentrations. In one pathway, peroxynitrite reacts directly with GSH in an overall second-order process (squares). In the other pathway, peroxynitrite first decomposes into nitrogen dioxide and hydroxyl radicals, which are the ultimate oxidants, in a process that is zero order in tyrosine (circles). For tyrosine concentrations >1.5 mM, we utilized the hydrophilic tyrosine analog p-hydroxyphenylacetic acid (pHPA) (triangles) due to the limit of solubility of tyrosine in aqueous solution. Each data point represents the mean ± standard deviation of $n \geq 7$ determinations.

peroxynitrite-derived radicals but not directly with peroxynitrite itself. For example, the target molecule can be exposed to peroxynitrite in the presence of mannitol, a known hydroxyl radical scavenger, or to p-hydroxyphenylacetic acid (pHPA), a soluble tyrosine analog that reacts with both $^{\bullet}NO_2$ and $^{\bullet}OH$ radicals. If mannitol has no effect while pHPA partially protects the target molecule from the oxidative modification, the result will suggest that $^{\bullet}NO_2$ radical may contribute to the observed effect. In addition, EPR spin-trapping can also be used to discriminate between radical and non-radical mechanisms. For instance, the inhibition of product yields in the presence of spin traps can provide evidence for the participation of free radicals in product formation.

In order to rationalize the biological fate of peroxynitrite and its derived radicals, it is necessary to understand the kinetics and characterize the reaction mechanisms with endogenous components. In this regard, in the next sections we review different experimental and computational approaches with illustrative examples taken primarily from our own laboratories, which, in addition to a theoretical analysis, provide information about the biological reactivity of peroxynitrite. The review will also include the analysis of the formation of 3-nitrotyrosine in proteins, a key "footprint" of peroxynitrite *in vitro* and *in vivo*. Finally, we provide a quantitative estimation of peroxynitrite steady-state levels and its modulation in living systems.

3. Methods to study the reactivity of peroxynitrite

Peroxynitrite is a transient species with a short biological half-life and low steady-state concentration, which makes its direct detection *in vivo* problematic. Thus, in order to infer the formation of peroxynitrite and its possible fates in biological systems, it is relevant to understand and characterize the kinetics of its reactions with biomolecules. Herein, we briefly summarize different approaches to address the biochemical reactions of peroxynitrite and its derived radicals.

3.1. Stopped-flow rapid mixing

To study the kinetics of peroxynitrite reactions in a time scale of milliseconds, special fast techniques such as stopped-flow spectrophotometry, should be used. Up to date, the rate constant and reaction mechanisms of peroxynitrite reactions with several target biomolecules have been studied through stopped-flow experiments [40,64,68,69]. Two kinds of approaches can be designed: (a) direct studies by following peroxynitrite disappearance using both integral or initial rate approach [8,64]; and (b) indirectly by competition with the reaction of a known rate constant.

3.1.1. Direct measurements

The rate of peroxynitrite decomposition, in the presence or in the absence of the target molecules, can be conveniently followed at 302 nm, where the peroxynitrite anionic form absorbs [8,30]. If the target molecule is a protein, in order to avoid interferences from its absorption, the peroxynitrite decomposition rate can also be determined at 310 nm ($\varepsilon = 1600\ M^{-1}\ cm^{-1}$) [70]. The usual direct approach is through an integral rate method, under pseudo-first order conditions using 10-fold and increasing concentrations of the target molecule with respect to peroxynitrite. Under these conditions, peroxynitrite decomposition follows an exponential function *versus* time, and the observed rate constant (k_{obs}) is determined through the fit of the kinetic trace to this function (as shown for example with GSH in Fig. 2). The apparent second-order rate constant of the reaction can be obtained from the slope of k_{obs} *versus* the molecule concentration. In some circumstances it is not possible to achieve pseudo-first order conditions in stopped-flow experiments since the limiting availability of the target molecule does not permit an excess over peroxynitrite concentration and the fact that peroxynitrite concentrations should not be much less than 0.05 mM in order to detect a variation of absorbance ≥0.08. In these cases, initial rate kinetic approaches should be more conveniently used with target molecule concentrations similar or lower to those of peroxynitrite. It is recommendable to acquire half of the experimental data points (~200 absorbance measurements) during the initial part of the reaction enough for the fast reaction between peroxynitrite and the target molecule to be completed (typically the first 0.2 s), and the other half points should be obtained until more than 99.9% of the peroxynitrite had decomposed (0.2–10 s). The initial slopes (dA/dt) are obtained for the linear decrease in absorbance at 302 nm (0.1–0.2 s), and the observed rate constant can be determined as:

$$k_{obs} = \frac{-(dA/dt)}{\left(A_o - A_f\right)} \quad (3)$$

where A_o and A_f are the initial and final absorbance measurements, as reported previously [14,64]. The second-order rate constant for the reaction can be calculated from the slope of the plot of k_{obs} *versus* target concentrations. In order to be sure that the initial rates are being measured, it is important to check that the concentration of reagents remains constant (or less than 10% are consumed) during the time chosen for initial rate measurements. As control, in the absence of the target molecule, the rate constants of peroxynitrite decomposition determined from the initial rate method should compare well with the values determined from the integral rate method.

The reaction of peroxynitrite and the thiol groups of cysteine and bovine serum albumin was the first reaction of peroxynitrite with a biomolecule studied by stopped-flow [8]. Since then many direct reactions of peroxynitrite with different molecules have been addressed kinetically by stopped-flow measurements, with reported rate constants ranging from 10^2 to 10^8 M^{-1} s^{-1}. In addition to follow the peroxinitrite decomposition by the decrease of its absorbance, a fluorimetric approach can also be used. For example, the kinetics of human peroxiredoxin 5 reaction with peroxynitrite has been studied taking advantage of the single tryptophan (Trp84) differential fluorescence emission under the reduced and oxidized forms of the protein [53]. This, has the benefit of being a very sensitive method which allows the measurement of rate constants in the range of ~10^7 M^{-1} s^{-1}.

3.1.2. Competition approaches

When the reactivity of peroxynitrite with target molecules is too fast to allow determination of the precise rate constants by direct stopped-flow techniques, or if the reaction cannot be monitored directly due to the low sensitivity in the absorbance or fluorescence emission change of the reactants or products, the rate constant can be studied using a simple competition analysis. The competing reactions between a characterized and an unknown reaction can be used to determine the unknown rate constant with peroxynitrite. When reactions with a common reagent yield different products, it can be shown that the ratio of these products will be proportional to the relative rate constants of the reactions [71], as described by the following equations (Eqs. (4) and (5)):

$$\begin{aligned} A_1 + B &\xrightarrow{k_1} P_1 \\ A_2 + B &\xrightarrow{k_2} P_2 \end{aligned} \tag{4}$$

$$\frac{[P_1]}{[P_2]} = \frac{k_1[A_1]}{k_2[A_2]} \tag{5}$$

Therefore, the ratio of the rate constants can be deduced from the ratio of products formed (Eq. (5)). Thus, knowing one of the rate constants in addition to the initial concentration of reactants and the final concentration of at least one product, the value of the second-order rate constant can be obtained. A_1 and A_2 can be used in 10-fold excess relative to B, thus providing pseudo-first order conditions. In this case, it can be shown that:

$$\frac{F}{1-F} = \frac{k_2[A_2]_0}{k_1[A_1]_0} \tag{6}$$

where F represents the fraction of P_1 formation inhibited in the presence of a competing scavenger $[A_2]_0$. Rearranging the Eq. (6), plots of $[F / (1 - F)](k_1[A_1]_0)$ *vs.* $[A_2]_0$ can be drawn to obtain k_2 as the slope. Under experimental conditions where no large excess of competitive reactants can be used, the following relationship should be applied (Eq. (7)):

$$\frac{k_1}{k_2} = \frac{\ln\left\{\frac{[A_1]_0}{[A_1]_0 - [P_1]_\infty}\right\}}{\ln\left\{\frac{[A_2]_0}{[A_2]_0 - [P_2]_\infty}\right\}} \tag{7}$$

When designing the competition experimental proceedings it is advisable to have an estimate of the magnitude of the rate constant and thus choose the competing molecule accordingly. In addition it is important to control that there is no cross-reaction between the product and one of the competing reagents since this may alter the results and lead to an underestimation of the rate constant of interest.

The competition approach has been applied several times in the literature. For example, competition with a synthetic Mn^{III}Porphyrin constitutes the first antecedent in the study of peroxynitrite reaction with peroxiredoxins [70,72]. Mn^{III}Porphyrins are rapidly oxidized by peroxynitrite to the O═Mn^{IV} derivative at rate constants comparable to those of peroxiredoxins ($k = 10^5$ to 10^7 M^{-1} s^{-1}, at pH 7.4 and 37 °C) in a process that can be conveniently followed at the Soret band as the decay of absorbance in the 450–470 nm region [73]. Additionally, a heme peroxidase such as horseradish peroxidase (HRP) can be used as a competing target for peroxynitrite, as reported to determine the second-order rate constant of the reaction of thioredoxin peroxidases from *S. cerevisiae* (Tsa1 and Tsa2) with peroxynitrite [74]. HRP reacts fast with peroxynitrite ($k = 1.02 \times 10^6$ M^{-1} s^{-1}, at pH 7.4 and 25 °C) [60] to produce compound I, and its spectral change can be followed at 398 nm. It is important to consider that other heme peroxidases such as MPO are known to react with peroxynitrite at higher rate constants producing compound II and $^{\bullet}NO_2$, and this secondary-reactive species may react with one of the competing reagents. For example, $^{\bullet}NO_2$ is known to react fast with thiols, with second-order rate constants reported in the range of 10^7 M^{-1} s^{-1} [75] and thus lead to misinterpretations of the results.

3.2. Pulse radiolysis

Reactions of radicals with target molecules, or radical–radical reactions most often proceed with rate constants >10^7 M^{-1} s^{-1}, which yield half-lives of the reactions <ms. Since these times are considerably shorter than those detectable by rapid-mixing techniques such as stopped-flow spectrophotometry, pulse radiolysis results as the more suitable methodology for the direct observation of radicals (*e.g.* the peroxynitrite-derived $^{\bullet}OH$, $^{\bullet}NO_2$ and $CO_3^{\bullet-}$), and determination of their fast kinetic rate constants with target molecules [76].

This technique provides the possibility of generating one of the reacting species (radicals or radical ions), by exposure of the sample to a short pulse of high-energy radiation, which would initiate chemical processes and can be followed by measuring a physical property of either one of the reacting species or products, such as absorbance as a function of time [77]. In this sense, $CO_3^{\bullet-}$ can be generated by radiolysis of N_2O-saturated solutions containing bicarbonate (HCO_3^-)/carbonate (CO_3^{2-}). It should be noted that the reaction of $^{\bullet}OH$ with bicarbonate has a low rate constant compared with carbonate [78], therefore $CO_3^{\bullet-}$ formation would be favored in alkaline conditions under which most of this type of experiments are carried out. The reaction of $CO_3^{\bullet-}$ with target molecules can be monitored directly following its decay at 600 nm ($\varepsilon = 1850$ M^{-1} cm^{-1} M^{-1} cm^{-1}) [79,80]. For example, the reported rate constant of $CO_3^{\bullet-}$ reaction with desferrioxamine was measured by fitting first-order exponential decays of $CO_3^{\bullet-}$ in the presence of increasing desferrioxamine concentrations, leading to the formation of desferrioxamine nitroxide radical with a second-order rate constant of 1.7×10^9 M^{-1} s^{-1} at pH 7.5 [66]. This rate constant is close to the diffusion-controlled limit, 300 and 40 times higher than the corresponding rate constants of $CO_3^{\bullet-}$ with glutathione and tyrosine, respectively [81]. The data with desferrioxamine support that in biochemical systems it can inhibit peroxynitrite-mediated oxidative processes by direct scavenging of its derived radicals. When the participating species (radicals or the target molecules) cannot be detected directly by spectrophotometry, the reaction can be monitored using a reporter molecule with an intense absorption spectrum. This reference compound can be used as a competing agent for the determination of the rate constant of a

radical with the target molecule. For example, the kinetics of the reaction between $^{\bullet}NO_2$ and GSH, with a reported rate constant of $2 \times 10^7\ M^{-1}\ s^{-1}$ at pH 7.4, was determined using (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{2-}$) as a competing compound, whose oxidation leads to the formation of the intense chromophore $ABTS^{\bullet-}$ ($\varepsilon_{645nm} = 13{,}400\ M^{-1}\ cm^{-1}$) [75].

3.3. Electron paramagnetic resonance (EPR)

EPR spectroscopy is a widely used methodology to detect and identify free radicals, with the possibility of unraveling oxidative mechanisms, and also of discriminating radical from non-radical mechanisms [82,83]. In addition, in combination with analytical methods it allows the identification of the protein residues which are the target of oxidants. Relevantly, by the use of direct EPR during the continuous flow of peroxynitrite to carbonated phosphate buffers, the formation of $CO_3^{\bullet-}$ was unequivocally demonstrated [42]. Since only a few radicals or protein-radicals have been shown to be stable enough to be detectable by direct EPR under aerobic conditions at room temperature, most EPR studies have been performed by rapid freeze-quench EPR and by EPR spin-trapping [83,84]. The spin-trapping approach has been extensively used to study peroxynitrite decomposition pathways and characterize the reactions of its derived radicals with target molecules. For example, the formation of $^{\bullet}OH$ can be detected using the spin trap DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide) [31], a cell permeable hydrophilic nitrone, that yields a more stable spin trap-OH adduct [85]. DMPO has also been shown to react with thiyl radicals (*e.g.* with $Cys^{\bullet}$ and $GS^{\bullet}$) [46] and with $CO_3^{\bullet-}$ [86]; the formation of $CO_3^{\bullet-}$ by peroxynitrite in a cellular system consisting of activated/immuno-stimulated macrophages was confirmed by EPR spin trapping [86]. If peroxynitrite-derived radicals react with a protein and generate a protein radical, the EPR signal of the protein-spin trap adduct looks as a broad spectrum, characteristic of relatively immobilized nitroxide. For example, in the study of the copper–zinc superoxide dismutase (Cu,ZnSOD) inactivation by peroxynitrite, the addition of the nitroso spin trap 2-methyl-2-nitrosopropane (MNP) led to the EPR detection of an anisotropic signal typical of an immobilized protein radical adduct. Partial proteolysis by treatment with pronase, which increases the rotational dynamics of free radical adduct and improves its detection, revealed a nearly isotropic signal consistent with the formation of a histidinyl radical in the active site of the enzyme [62]. It must be taken into account that a major problem with several spin traps in biological systems is that the spin adduct (nitroxide derivative) can be oxidized to a nitrone derivative or can also be reduced to the corresponding hydroxylamine, thus decreasing its half-life and precluding EPR detection. Immunospintrapping is a novel alternative based on the association of spin trapping with immunochemical detection. Interestingly, protein $Tyr^{\bullet}$ formation in cells and tissues undergoing nitroxidative stress can be detected using polyclonal antibodies that bind to the protein nitrone adduct of the spin trap DMPO [87–90]. This method has also been applied for western blotting and immunochemistry *in vitro* and *in vivo* to assist in the identification of peroxynitrite as a mediator of protein oxidative damage [91].

4. Peroxynitrite footprints

4.1. Protein tyrosine nitration

Peroxynitrite promotes oxidative modifications in target biomolecules such as DNA, lipids and proteins and most notably, leads to several modifications in protein amino acids. Herein we will focus on protein tyrosine nitration mediated by peroxynitrite and derived-oxidants [92,93]. Protein tyrosine nitration is a post-translational modification mediated by nitric oxide-derived oxidants such as peroxynitrite and represents the substitution of hydrogen by a nitro group ($-NO_2$) in the position 3 of the phenolic ring, yielding 3-nitrotyrosine (NT). Important work performed by Sokolovsky and co-workers in the early sixties demonstrated that tyrosine nitration mediated by nitrating agents such as tetranitromethane (TNM) resulted in dramatic changes either in function and structure of isolated proteins [94]. More recent data confirmed that protein tyrosine nitration leads to functional alterations in a variety of proteins *in vivo* [95]. Indeed, the biological relevance of protein tyrosine nitration was definitely established in the early nineties, after the recognition of nitric oxide-derived oxidant formation, and the implication of these free radical species in several pathophysiological conditions [7,8,38,96].

Beckman and co-workers, demonstrated for the first time the presence of NT in atherosclerotic plaques of samples from coronary arteries of patients with cardiovascular disease confirming that nitric oxide-derived oxidants, such as peroxynitrite are formed during human atherosclerosis, and may be responsible for the pathogenesis [97]. The detection of NT was made immunochemically using anti-protein 3-nitrotyrosine antibodies, a sensitive method that was improved and extensively used afterwards [98]. In addition, bioanalytical methods have been developed for quantitation of NT levels and have been reviewed recently elsewhere [99].

The increased levels of NT with respect to the basal levels in normal conditions, have been considered a footprint of nitro-oxidative damage *in vivo*, being revealed as a strong biomarker and predictor of disease onset and progression [100]. Indeed, protein tyrosine nitration has been established both *in vivo* and in animal models in several diseases such as cardiovascular disease, atherosclerosis, ischemia–reperfusion, and stroke [101–104]; in neurodegenerative processes such as Parkinson and Alzheimer [105–108] and in diabetes and inflammation among others [105,106].

Herein, we will analyze the biochemistry of tyrosine nitration reactions that take place by the action of peroxynitrite and hemeperoxidases, and the physicochemical factors that can modulate these pathways under biological conditions.

Though initially the formation of NT was considered a footprint of peroxynitrite formation, now we know that tyrosine nitration can occur biologically by a variety of routes, mainly, dependent on peroxynitrite and hemeperoxidases such as MPO[3] or eosinophil peroxidase (EPO), however both mechanisms are based in free radical chemistry (Fig. 3) (*i.e.* there is no direct reaction of tyrosine with peroxynitrite [64,109], Fig. 2).

The formation of NT requires the intermediate formation of the transient tyrosyl radical ($^{\bullet}Tyr$) followed by the diffusion-controlled reaction with $^{\bullet}NO_2$. The one-electron oxidation of tyrosine to $^{\bullet}Tyr$ can be achieved by a number of oxidants such as the peroxynitrite-derived radicals $^{\bullet}NO_2$ ($k = 3.2 \times 10^5\ M^{-1}\ s^{-1}$, pH 7.5) [110], $^{\bullet}OH$ ($k = 1.3 \times 10^{10}\ M^{-1}\ s^{-1}$) [111] and $CO_3^{\bullet-}$, ($k = 4.5 \times 10^7\ M^{-1}\ s^{-1}$) [81] and the myeloperoxidase-derived compounds I ($k = 2.93 \times 10^4\ M^{-1}\ s^{-1}$) [112] or II ($k = 1.57 \times 10^4\ M^{-1}\ s^{-1}$) [113].

More recently we have demonstrated that lipid-derived alkoxyl model radicals ($LO^{\bullet}$) ($k = 5 \times 10^5\ M^{-1}\ s^{-1}$) [114] and peroxyl ($LOO^{\bullet}$) ($k = 4.5 \times 10^3\ M^{-1}\ s^{-1}$) [115] radicals can also promote the one-electron oxidation of tyrosine to $^{\bullet}Tyr$. This has particular relevance in membranes and lipoproteins, where an important amount of unsaturated fatty acids undergo lipid peroxidation reactions. Peroxynitrite-derived radicals (*i.e.* $^{\bullet}OH$) are able to promote the abstraction of hydrogen from an unsaturated fatty acid, in the initial reaction of the lipid peroxidation chain. Once lipid peroxyl radicals are formed, nitration pathways may be fueled in membranes by the oxidation of tyrosine, in a connection reaction between tyrosine nitration and lipid peroxidation defined recently [114,115].

[3] In the case of MPO-catalyzed nitration, the enzyme reacts with hydrogen peroxide, yielding compound I, which in turn can oxidize nitrite (NO_2^-) to $^{\bullet}NO_2$, and tyrosine to $^{\bullet}Tyr$ radical (Fig. 3), and then NT can be formed. Kinetic data indicate that nitrite is oxidized faster than tyrosine by compound I and tyrosine is oxidized faster by compound II [111].

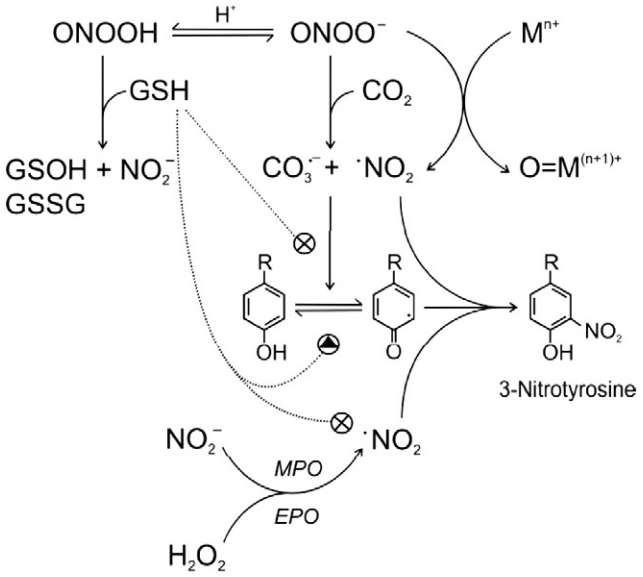

Fig. 3. Biochemical mechanisms of peroxynitrite reaction with GSH, CO_2 and tyrosine nitration. Peroxynitrite anion ($ONOO^-$), formed from the diffusion-controlled reaction between $^{\bullet}NO$ and $O_2^{\bullet-}$, is in equilibrium with peroxynitrous acid (ONOOH), which can oxidize glutathione (GSH) to the corresponding sulfenic (GSOH) and disulfide (GSSG) derivatives, yielding also nitrite (NO_2^-). A fundamental reaction of $ONOO^-$ in biological systems is its fast reaction with carbon dioxide (CO_2, in equilibrium with physiological levels of bicarbonate anion), which leads to the formation of the one-electron oxidants carbonate ($CO_3^{\bullet-}$) and nitrogen dioxide ($^{\bullet}NO_2$) radicals in ~35% yield while the remaining 65% evolves to CO_2 and NO_3^-. Additionally, $ONOO^-$ may react with transition metal centers (M^{n+}), yielding $^{\bullet}NO_2$ and the corresponding oxo-metal complex ($O=M^{(n+1)+}$). Tyrosine nitration involves the intermediate formation of the tyrosyl–phenoxyl radical ($Tyr^{\bullet}$) by one-electron oxidation of tyrosine followed by the combination of the phenoxyl radical with $^{\bullet}NO_2$ leading to the formation of 3-nitrotyrosine. $Tyr^{\bullet}$ can be reduced back to tyrosine in the presence of numerous reductants, such as GSH, in competition with the nitration reaction. Additionally, GSH is an excellent scavenger of $^{\bullet}NO_2$. The enzymes myeloperoxidase and eosinophil peroxidase (MPO and EPO, respectively) can promote, in the presence of hydrogen peroxide (H_2O_2), the one-electron oxidation of NO_2^- to $^{\bullet}NO_2$ and participates in a peroxynitrite-independent pathway of tyrosine nitration.

In addition to NT, other products can be formed from $^{\bullet}Tyr$. 3,3′-Dityrosine is formed by the combination of two tyrosyl radicals, or the hydroxylated derivative, 3,4-dihidroxyphenylalanine, (DOPA) by the addition of $^{\bullet}OH$, [35] and tyrosine hydroperoxide by the addition of $O_2^{\bullet-}$ [116]. Finally, the reaction between $^{\bullet}Tyr$ and $^{\bullet}NO$ yields 3-nitroso tyrosine, which in the presence of further oxidation reactions can evolve to the intermediate formation of iminoxyl radical and then NT as a final product [92].

Depending on the products that are formed, MPO and peroxynitrite routes could be discriminated. The colocalization of 3-hydroxytyrosine and NT was once considered to be a useful tool to discriminate between peroxynitrite and other nitrating species [117], but it was later on discovered that 3-hydroxytyrosine formation can be also accomplished by hemeperoxidase-dependent reactions [118]. On the other hand, the concomitant presence of 3-chloro or 3-bromotyrosine with NT is indicative of the participation of MPO and EPO, respectively in the nitration process [92,119], which can be further complemented by immunochemical detection of the hemeperoxidases.

In addition, peroxynitrite-mediated nitration pathways in cells and animals can be disclosed genetically or pharmacologically by the overexpression of enzymes that catalytically decompose peroxynitrite and $O_2^{\bullet-}$ such as peroxiredoxins and SOD (promoting its detoxification), or by using knock-outs of particular enzymes (*i.e.* MPO). Indeed, in important work by Brennan and coworkers in MPO knock out mice, it was revealed that NT formation in acute inflammation models is largely MPO-dependent [119], consistent with the influx and degranulation of activated neutrophils in the inflammatory sites. MPO-mediated nitration pathways can be further distinguished, by using specific MPO-inhibitors [120] such as alkyl indole derivatives [121].

4.2. Factors that influence protein tyrosine nitration yields and selectivity: the inhibitory effect of glutathione

Protein tyrosine nitration is found in basal levels in normal tissues or cells, however yields are low, mainly due to competing reactions which may consume the nitrating agents (*e.g.* glutathione readily consumes $^{\bullet}NO_2$), the action of several enzymes which attenuate oxidant formation (*i.e.* peroxiredoxins and SOD) or by the repair of $^{\bullet}Tyr$, either by reductants such as glutathione or ascorbic acid, or by intramolecular electron transfer processes [122,123] (Fig. 3). In spite of these considerations, some proteins are preferentially nitrated. Several physicochemical factors control the selectivity of protein tyrosine nitration such as protein abundance and structure, the nitration mechanism and the environment where the tyrosine residue is located [35]. It is clear that within a protein, not all tyrosine residues become nitrated and it is not easy to predict which are the conditions required for the preferential reaction. Mapping the nitration sites within a protein may assist in the understanding of the nitration mechanism, and disclose the action of different oxidants [93]. A notable example of this contention, is the case of MnSOD, a critical mitochondrial enzyme, where the Mn-catalyzed nitration of one particular tyrosine residue (out of a total of nine tyrosine residues) by peroxynitrite (Tyr 34) [14] is responsible for its inactivation [63,124]. This site-specificity is indicative of peroxynitrite formation in mitochondria and can be used to discriminate from other nitrating agents [93]. Moreover, it has been recently demonstrated that peroxynitrite promotes tyrosine nitration of up to five (of 24) tyrosine residues in Hsp90; interestingly, among those residues, nitration of a single tyrosine (either Tyr 33 or Tyr 56) on Hsp90 is sufficient to induce motor neuron cell death [125].

Several endogenous agents can modulate protein tyrosine nitration *in vivo*, and in this sense the role of GSH will be analyzed (Fig. 3). Glutathione, present at intracellular mM levels, is a strong endogenous inhibitor of protein tyrosine nitration. However, the mechanism of inhibition deserves a subtle analysis. First, glutathione reacts directly with peroxynitrite, but this reaction is not very fast ($k = 1.35 \times 10^3\ M^{-1}\ s^{-1}$), constituting a modest route of decay biologically (*vide infra*). Relevantly, GSH is an important scavenger of $^{\bullet}NO_2$ ($k = 2 \times 10^7\ M^{-1}\ s^{-1}$), probably being the most relevant reaction *in vivo* for the inhibition of tyrosine nitration [75]. In addition, GSH also readily reacts with $CO_3^{\bullet-}$ and other one-electron oxidants and it can also reduce $^{\bullet}Tyr$ back to tyrosine, as additional contributory mechanisms. By pulse radiolysis experiments, we were able to measure a rate constant for the reaction between $^{\bullet}Tyr$ and GSH ($k = 2 \times 10^6\ M^{-1}\ s^{-1}$) [122], however this repair reaction is close to equilibrium (repair equilibrium constant ~ 1), and consequently, an efficient reduction of $^{\bullet}Tyr$ by GSH requires the removal of thiyl radicals, to allow the repair reaction (in cells, other reductants, such as ascorbic acid repair $^{\bullet}Tyr$ radicals faster than GSH [122]). Computer assisted simulations [126] indicate that mM glutathione concentrations inhibit peroxynitrite-dependent tyrosine nitration yields by several orders of magnitude [126]. In spite of this restriction, it was found that for a given GSH concentration, NT levels correlate well with increased peroxynitrite formation rates.

Protein tyrosine nitration would be promoted in compartments where glutathione is scarce (*e.g.* extracellular), or under conditions of decreased GSH concentrations arising in pathological situations associated with oxidative stress [127]. The critical role of GSH in modulating peroxynitrite reactivity can be evidenced in GSH-depleted cells by treatment with buthionine sulfoximine, which has been shown to increase the detection of intracellular nitration [128–130]. In addition, cultured astrocytes and neurons depleted of GSH have enhanced cell susceptibility to peroxynitrite-mediated damage and tyrosine nitration possibly contributing to the development of neurodegenerative diseases such

as amyothrophic lateral sclerosis (ALS) and Parkinson's disease [128–130].

5. Modulation of the steady-state concentration of peroxynitrite

The steady-state concentration of peroxynitrite in biological compartments is low due to its rapid decay *via* a variety of processes; these include the reaction with CO_2, one- and two-electron oxidations, isomerization and homolysis.

The overall equation rate is given by:

$$\frac{d[ONOO^-]}{dt} = k_f[^{\bullet}NO][O_2^{\bullet -}] - k_H[ONOO^-] - k_T[T][ONOO^-] \quad (8)$$

where k_f represents the rate constant of peroxynitrite formation, and k_H and k_T the rate constants of peroxynitrite decay by homolysis or by reaction with target molecules, respectively. The term $k_T[T]$ represents the overall rate of peroxynitrite reaction with different cellular components, which is determined by the product of a "global" rate constant and target(s) concentration, and can be used to parameterize and compare theoretically the reactivity in a homogeneous system. Formally, $k_T[T]$ is better expressed by:

$$k_T[T] = \left(k_m[m] + k_{GSH}[GSH] + k_{CO_2}[CO_2] + k_{Prx}[Prx] + \ldots k_n[n]\right) = \sum_{i=m}^{n} k_i[T_i] \quad (9)$$

In the steady-state condition:

$$\frac{d[ONOO^-]}{dt} = 0$$

$$k_f[^{\bullet}NO][O_2^{\bullet -}] = (k_H + k_T[T])[ONOO^-]_{ss}$$

Then,

$$[ONOO^-]_{ss} = \frac{k_f[^{\bullet}NO][O_2^{\bullet -}]}{(k_H + k_T[T])} \quad (10)$$

Since the proton-catalyzed homolysis of peroxynitrite represents a very minor route of peroxynitrite disappearance ($k_H = 0.9\ s^{-1}$), we can assume that $k_H \ll k_T[T]$, thus:

$$[ONOO^-]_{ss} = \frac{k_f[^{\bullet}NO][O_2^{\bullet -}]}{(k_T[T])} \quad (11)$$

As an example, we can study the situation in the cytosol, where changes in concentration of key endogenous modulators such as CO_2, peroxiredoxins, other thiol proteins and some hemeproteins, will significantly modify the rate of peroxynitrite disappearance and thus its steady state concentration. For instance, knowing the bimolecular rate constant values (k) of the different reactions and the concentration of targets ([T]) in the cytosolic compartment, we can estimate a value of $k[T] \sim 330\ s^{-1}$[4]. Considering the intracellular steady-state values of $[^{\bullet}NO]_{ss}$ and $[O_2^{\bullet -}]_{ss}$ reported for endothelial cells, a peroxynitrite formation flux under basal metabolic conditions can be estimated as $0.3\ \mu M\ s^{-1}$ and the biological steady-state of peroxynitrite would be ~1 nM [21–23]. Recently, a kinetic model that included the effects of multiple cellular targets estimated an intracellular steady-state concentration of peroxynitrite within the same range [21,23].

For a constant value of peroxynitrite formation rates, the increase or decrease in the $k_T[T]$ term of Eq. (11), will significantly impact on the $[ONOO^-]_{ss}$. In general, endogenous compounds that would influence the $[ONOO^-]_{ss}$ should result in $k_T[T] \geq 60\ s^{-1}$ (higher than that of CO_2) to have a significant effect. For example, doubling Prx 5 concentration from 1 to 2 μM, represents a $k_T[T]$ value of ~280 s^{-1}, which would imply a decrease in the steady-state peroxynitrite concentration to 0.6 nM. Considering certain cell types such as erythrocytes where Prx 2 is also very concentrated (240 μM), with a rate constant of $1.7 \times 10^7\ M^{-1}\ s^{-1}$ at pH 7.4 and 25 °C [131], a $k_T[T] > 4000\ s^{-1}$ implies that $[ONOO^-]_{ss}$ falls to 36 pM at 37 °C [21]. Oxyhemoglobin, which also has a $k_T[T]$ value larger than that of CO_2, (340 s^{-1}, from $k = 1.7 \times 10^4\ M^{-1}\ s^{-1}$ at pH 7.4, 37 °C and concentration of 20 mM, [90]) will significantly drop the $[ONOO^-]_{ss}$, but its potential activity as a peroxynitrite scavenger, catalyzing its isomerization to nitrite, is restricted by its location in the red blood cells. An interesting lesson learned during the accumulation of kinetic data on peroxynitrite biochemistry, was the modification of an initial idea pointing oxyhemoglobin as the main peroxynitrite "sink" in the erythrocyte [28]. Indeed, in spite of the high $k_T[T]$ value for oxy-hemoglobin, it was more recently found that the Prx 2 reaction is much faster, and the principal decay mechanism of peroxynitrite in red blood cell (340 s^{-1} *vs.* >4000 s^{-1}, respectively).

On the other hand, there are a series of endogenous molecules that modulate the biological chemistry of peroxynitrite but that do not significantly alter the $k_T[T]$ term of the equation. For example, GSH has a $k_T[T]$ value of 7 s^{-1} (at 37 °C and pH 7.4), representing a minor fraction of peroxynitrite reduction. Thus, even doubling the concentration, GSH is not able to outcompete other targets such as CO_2 or Prx and will not directly affect much the $[ONOO^-]_{ss}$. In addition, other low molecular antioxidants, such as ascorbate and uric acid present in cell systems at concentrations of 0.5 and 0.1 mM, respectively, react relatively slowly with peroxynitrite ($k \sim 10^2\ M^{-1}\ s^{-1}$) [132,133], with $k_T[T] < 0.1\ s^{-1}$. Therefore, GSH and other low molecular weight antioxidants by themselves appear to be inefficient direct peroxynitrite scavengers, although their role on the modulation of peroxynitrite reactivity is increasingly attributed in the scavenging of its secondary-derived radicals, in the repair reactions or in the recycling of appropriate scavengers. For example, most of the protective effects of uric acid against peroxynitrite-mediated toxicity *in vitro* and *in vivo* have been attributed to the scavenging of peroxynitrite-derived radicals and the inhibition of tyrosine nitration reactions [133–135]. In fact, uric acid reacts fast with $^{\bullet}OH$ and $^{\bullet}NO_2$, with rate constants of $1.0 \times 10^9\ M^{-1}\ s^{-1}$ and $1.8 \times 10^7\ M^{-1}\ s^{-1}$, respectively [136,137] and with $CO_3^{\bullet -}$ it has been estimated at a value of $2.9 \times 10^8\ M^{-1}\ s^{-1}$ [50].

These general concepts also apply for the pharmacological modulation of the $[ONOO^-]_{ss}$, for example using the synthetic peroxynitrite scavengers metal porphyrins and selenols. Manganese porphyrins (MnPorphyrins), initially conceived as superoxide dismutase-mimics, can readily react with peroxynitrite in the Mn^{+2} and Mn^{+3} states to yield nitrite or $^{\bullet}NO_2$, respectively [69,138]. The one-electron oxidation of Mn^{+3} implies the formation of two strong oxidants manganese (IV) complexes and $^{\bullet}NO_2$, which represents a potentially damaging mechanism. Although MnPorphyrins are typically administered in the +3 state, they can be reduced *in vivo* by low-molecular weight reductants (glutathione or ascorbate), or enzymatically by a number of flavoenzymes including the electron transport chain complexes, even under physiological oxygen tensions [139,140]. Then Mn^{+2}Porphyrins reduce peroxynitrite anion to nitrite (rate constants $\geq 10^7\ M^{-1}\ s^{-1}$) in a two-electron oxidation process [141]. Through these mechanisms, MnPorphyrins can catalytically decompose peroxynitrite at the expense of endogenous reducing equivalents and avoid the formation of $^{\bullet}NO_2$. Even though the uptake and subcellular distribution of MnPorphyrins

[4] Apparent rate constant for the reaction between peroxynitrite and cytosolic targets at pH 7.4 and 37 °C. Concentrations and rate constants were assumed, respectively: carbon dioxide: 1.3 mM, $k = 4.6 \times 10^4\ M^{-1}\ s^{-1}$ (60 s^{-1}); peroxiredoxin 5: 1 μM, $k = 1.4 \times 10^8\ M^{-1}\ s^{-1}$ at 37 °C (140 s^{-1}); glutathione: 5 mM, $k = 1.35 \times 10^3\ M^{-1}\ s^{-1}$ (7 s^{-1}); other proteins: 15 mM, $k = 5 \times 10^3\ M^{-1}\ s^{-1}$ (75 s^{-1}); metal and selenium-containing proteins: 0.5 mM, $k = 1 \times 10^5\ M^{-1}\ s^{-1}$ (50 s^{-1}) [107].

are poorly characterized, they can accumulate to micromolar levels in mitochondria. For example, recent reports indicate that one particular cationic MnPorphyrin can accumulate in mouse heart mitochondria in a concentration of 5.1 μM, 7 h after a single i.p. administration at 10 mg kg^{-1} [21,142]. Thus, a $k_T[T]$ value $\geq 51\ s^{-1}$ would be significant to lower the $[ONOO^-]_{ss}$ and site-specifically protect mitochondria from peroxynitrite-mediated damage. Similar considerations may be apply to the synthetic compound ebselen [2-phenyl-1,2-benzisoselenazol-3-(2H)one], a lipid soluble selenium compound that exhibits glutathione peroxidase-like activity, which reduce peroxynitrite by two-electrons with a significant rate constant of $2 \times 10^6\ M^{-1}\ s^{-1}$[143]. Experimental models after drug administration have determined ebselen plasma concentrations in the range of 5–10 μM, which represents a $k_T[T]$ value ~ 10–20 s^{-1} and could account for the reported protective effects of ebselen in several models of inflammation and reperfusion injury mediated by peroxynitrite [21,144–146]. Other organo selenium compounds were also reported to react with peroxynitrite. For instance, diphenyl diselenide (PhSe) protected endothelial cells from peroxynitrite-dependent apoptosis and protein tyrosine nitration [147].[5]

There are other synthetic compounds with potential pharmacological applications that do not react directly with peroxynitrite. Therefore they do not affect $[ONOO^-]_{ss}$ and their protective actions have been attributed to their capacity to scavenge peroxynitrite-derived radicals. For example, tyrosine-containing peptides have been proven to inhibit apoptosis and NT formation in motor neuron cultures exposed to exogenous and endogenous peroxynitrite [149,150]. Other compounds, such as the membrane-permeable nitroxide radical tempol (4-hydroxy-2,2,6,6, tetramethyl-1-piperidinyloxy), desferrioxamine, and mitochondrial-targeted ubiquinol, may also exert part of their antioxidant and cytoprotective effects *via* scavenging of peroxynitrite-derived radicals [66,151,152].

Under conditions of excess peroxynitrite formation, another alternative is to lower $[ONOO^-]_{ss}$ by the decrease of the formation rates $k_f[^{\bullet}NO][O_2^{\bullet -}]$. This could be accomplished by decreasing $^{\bullet}NO$ and $O_2^{\bullet -}$ levels either by inhibiting their formation (*e.g.* by NOS and NADPH inhibitors [153–155]), or by enhancing their consumption, for example using $^{\bullet}NO$ scavengers such as phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) [156], or enhancing $O_2^{\bullet -}$ elimination *via* supplementation with cell-permeable SOD mimics or by overexpression of cytosolic or mitochondrial SOD [157–159].

On an opposite scenario, the cytotoxic properties of peroxynitrite can also be used by the immune system to combat microbial invasion [21]. For example, it has been demonstrated that internalization of *Trypanosoma cruzi* by immunostimulated macrophages triggers the assembly of the NADPH oxidase complex and together with iNOS-derived $^{\bullet}NO$, generates intraphagosomal peroxynitrite levels, which mediate microbial killing [160]. Importantly, peroxynitrite-mediated killing in *T. cruzi* is neutralized in parasite strains containing high peroxiredoxin levels, which readily cope with peroxynitrite. Thus, in some stage of infection processes it may be timely to promote peroxynitrite formation (or decrease its decay) in order to increase $[ONOO^-]_{ss}$ as a cytotoxic effector.

Therefore, the kinetic analysis provided in this section and summarized in Eq. (11) provides a conceptual and quantitative handle to better predict the preferential fate and steady-state levels of peroxynitrite in living systems.

[5] Kinetic considerations are essential to develop and test novel probes for peroxynitrite detection in biological systems. In particular, boronate-based compounds readily react with peroxynitrite ($k > 10^6\ M^{-1}\ s^{-1}$) and are being successfully used for its detection and quantitation [148].

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