Peroxynitrite biochemistry: formation, reactions and detection

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Peroxynitrite is a powerful and cytotoxic oxidant formed by the diffusion-limited reaction between $^{\cdot}NO$ and $O_2^{\cdot-}$. Peroxynitrite reactions can be classified into a) direct reactions, including those with thiols, transition metal centers and CO_2, that account for more than 95 % peroxynitrite reactivity in biology, and b) the hydroxyl radical/nitrogen dioxide pathway, which arises from homolysis of peroxynitrous acid. Different methods have been utilized to expose biochemical or cellular systems to peroxynitrite including a) addition of authentic peroxynitrite, either as a bolus or a continuous infusion, and b) generation of fluxes of the radical precursors $^{\cdot}NO$ and $O_2^{\cdot-}$. Reaction yields and product distributions are sometimes difficult to interpret, mainly due to the multiple possible and competing radical interactions. Peroxynitrite detection relies on a) the detection of footprints, and b) the modification of probes. The lack of total specificity of the methods in use makes obligatory the use of a combination of them and/or pharmacological approaches in order to unambiguously unravel peroxynitrite formation either in biochemical or biological systems.

Introduction

Peroxynitrite[1] is a powerful oxidant produced in biological systems from superoxide ($O_2^{\cdot-}$) and nitric oxide ($^{\cdot}NO$). Peroxynitrite has been implicated in a host of disease states, including neurodegenerative disorders [1], chronic inflammation and autoimmune diseases [2,3], ischemia-reperfusion injury [4] and septic shock [5].

The chemistry of peroxynitrite is complex and highly pH-dependent [6,7]. Not only peroxynitrite anion ($ONOO^-$) and its conjugated acid, peroxynitrous acid (ONOOH) ($pK_a = 6.8$) are potent oxidants by themselves [8,9] but also they can lead to the production of secondary reactive species. Moreover, peroxynitrite is capable of nitrating aromatics [10] in a process that can be enhanced by metal centres and CO_2 [11].

Despite of being a reactive and short-lived species, which precludes its direct isolation or measurement, peroxynitrite detection and quantitation can be achieved with the use of detector molecules as well as molecular footprints that take advantage of oxidation and nitration reactions. A good detector molecule should be sensitive and outcompete the multiple reactions that peroxynitrite can undergo in biological systems. Moreover, both detector molecules and footprints should be specific, in order to discriminate between the reactions of peroxynitrite from those of other species, including its precursors $^{\cdot}NO$ and $O_2^{\cdot-}$, and other $^{\cdot}NO$ or $O_2^{\cdot-}$-derived oxidants. None of the detection systems used until now fulfil all these requisites, and a subtle knowledge of the biological chemistry of peroxynitrite is required in order to choose and utilise the best approach in different biochemical or biological systems.

Peroxynitrite formation

Although several possible routes lead to peroxynitrite formation, the main one in biology arises from the radical-radical combination reaction of $^{\cdot}NO$[12] and $O_2^{\cdot-}$ [13,14]

$$^{\cdot}NO + O_2^{\cdot-} \rightarrow ONOO^- \qquad (1)$$

This is a diffusion-controlled reaction with a second order rate constant (k_2) that has been independently determined as 4.3, 6.7 and 19×10^9 $M^{-1}.s^{-1}$ [15-17]. Considering an average value of 10×10^9 $M^{-1}\,s^{-1}$, this rate constant is ~ 5-10 times greater than that for the reaction of $O_2^{\cdot-}$ with Cu-Zn or Mn-superoxide dismutase (SOD)(2×10^9 $M^{-1}.s^{-1}$ and 1.9×10^9 $M^{-1}.s^{-1}$ respectively). Therefore, depending on $^{\cdot}NO$ and SOD concentrations, $^{\cdot}NO$ can outcompete the reaction of SOD with $O_2^{\cdot-}$, leading to $ONOO^-$ formation. Due to the more limited diffusion and shorter half-life of $O_2^{\cdot-}$ respect to $^{\cdot}NO$ [18-20], peroxynitrite is mainly formed closer to $O_2^{\cdot-}$ generation sites, under conditions such as inflammatory states, in which both the formation rates of $^{\cdot}NO$ and $O_2^{\cdot-}$ increase.

[1] The term peroxynitrite refers to the sum of peroxynitrite anion (ONOO-) and peroxynitrous acid (ONOOH). IUPAC recommended names for peroxynitrite anion, peroxynitrous acid, nitroso-peroxocarboxylate ($ONOOCO_2^-$) and nitric oxide are oxoperoxynitrate (1-), hydrogen oxoperoxynitrate, 1-carboxylato-2-nitrosodioxidane and nitrogen monoxide respectively.

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An alternative pathway to peroxynitrite may involve the production of nitroxyl anion (NO^-)[21-24]. NO^- ($pK_a = 4.7$) is a short lived species, which can decompose *via* dimerization to nitrous oxide ($1.8 \times 10^9\ M^{-1}\cdot s^{-1}$) [25], or react with multiple targets including thiols [26] and metal centres [27]. The reaction of NO^- with molecular oxygen (O_2) can produce peroxynitrite (eq. 2) [28-30], with a proposed second order rate constant of $5.7 \times 10^7\ M^{-1}.s^{-1}$ [31].

$$NO^- + O_2 \rightarrow ONOO^- \quad (2)$$

Finally, the NO-dependent oxidation of hemoproteins such as oxy-hemoglobin [32] and oxy-myoglobin [33] can also lead to peroxynitrite formation (eq. 3).

$$NO + [Hb^{2+}\text{-}O_2 \leftrightarrow Hb^{3+}\text{-}O_2^{\cdot-}] \rightarrow Hb^{3+}\text{-}ONOO^- \rightarrow Hb^{3+} + NO_3^- \quad (3)$$

The pK_a of peroxynitrous acid is 6.8 [8], and therefore at physiological pH both the anion and the hydrogenated forms are present, each with its own reactivity and diffusional property [34].

Peroxynitrite reactivity

Clasification of peroxynitrite reactions

Direct reactions

Both $ONOO^-$ and ONOOH can react directly with different targets. These are bimolecular reactions that are frequently studied by stopped-flow techniques, that follow peroxynitrite disappearance at 302 nm ($\varepsilon = 1670\ M^{-1}\cdot cm^{-1}$).

$$-d\,[ONOO^-]\,/dt = k_2\,[ONOO^-]\,[target] \quad (4)$$

Some biologically-relevant second order rate constants are summarised in table I. In order to consider which would be the prevalent reactions for peroxynitrite in biological systems, the second order rate constant and the concentration of the target should be taken into account. Thus, a particularly important reaction occurs with protein and low-molecular weight thiols such as glutathione and cysteine, which have moderate second order rate constants but high intracellular concentration (*e.g.* 5-10 mM glutathione).

Peroxynitrite reacts with carbon dioxide (CO_2) [35] with a second order rate constant of $5.7 \times 10^4\ M^{-1}.s^{-1}$ at 37 ºC [11], yielding an adduct, the nitroso-peroxocarboxylate anion ($ONOOCO_2^-$). As both the intracellular and extracellular CO_2 concentration are high (1-2 mM), this reaction represents another major route for peroxynitrite reactivity *in vivo*. $ONOOCO_2^-$ homolyses readily ($t_{1/2} < 1$ ms) [36] to carbonate radical ($CO_3^{\cdot-}$), and $^{\cdot}NO_2$ in ~ 30-35 % yields [37,38] (Fig. 1). $CO_3^{\cdot-}$ is a good one-electron oxidant ($E^{o\prime}_{CO_3^{\cdot-}/CO_3^{2-}} = 1.50$ V), while $^{\cdot}NO_2$ is both an oxidising ($E^{o\prime}_{^{\cdot}NO_2/NO^{2-}} = 1.04$ V) and nitrating agent, and therefore peroxynitrite decomposition in presence of CO_2 leads to secondary oxidation events.

Table I: Apparent second order rate constants ($k_{2\ app}$) for some reactions of peroxynitrite.

Compound	$k_{2\ app}$ ($M^{-1}.\ s^{-1}$)	*Reference*
$^{\cdot}OH$	4.8×10^9 [c]	Goldstein *et al.,* 1998 [43]
N_2O_3	3.1×10^8 [a]	Goldstein *et al.,* 1999 [44]
$CO_3^{\cdot-}$	7.7×10^6 [c]	Goldstein *et al.,* 1998 [43]
CO_2	4×10^4 [b]	Denicola *et al.,* 1996 [11]
Glutathione peroxidase	8×10^6 [a]	Briviba *et al.,* 1998 [106]
Aconitase	1.4×10^5 [a]	Castro *et al.,* 1994 [107]
Cytochrome c^{2+}	1.3×10^4 [a]	Thomson *et al.,* 1995 [78]
Oxyhemoglobin	1×10^4 [a]	Denicola *et al.,* 1998 [71]
Human serum albumin	9.7×10^3 [b]	Alvarez *et al.,* 1999 [108]
Glutathione	1.35×10^3 [b]	Koppenol *et al.,* 1992 [9]
Cysteine	5×10^3 [b]	Radi *et al.,* 1991 [8]
Methionine	1.8×10^2 [a]	Pryor *et al.,* 1994 [40]
Tryptophan	37 [b]	Alvarez *et al.,* 1996 [109]
Uric acid	1.5×10^2 [b]	Squadrito *et al.,* 2000 [101]
Ascorbate	42 [a]	Squadrito *et al.,* 1995 [100]
Ebselen	1.6×10^6 [a]	Masumoto *et al.,* 1996 [110]

Apparent rate constant (pH-dependent) for some of peroxynitrite bimolecular reactions [a] at pH 7.4 and 25 °C, [b] at pH 7.4 and 37 °C, and [c] at alkaline pH and ambient temperature.

Peroxynitrite can directly perform one- ($E^{o\prime}_{ONOO^-/NO_2} = 1.4$ V) or two-electron oxidation reactions ($E^{o\prime}_{ONOO^-/NO_2^-} = 1.2$ V) [9]. Examples of direct two electron oxidation reactions are those with thiols [8], seleno-compounds [39], methionine [40] and oxyhemoglobin [41]. Instead, peroxynitrite oxidizes cytochrome c^{2+} and Ni^{II}-cyclam by one electron, leading to cytochrome c^{3+} and Ni^{III}- cyclam [42], respectively. Peroxynitrite itself can also be oxidized ($E^{o\prime}ONOO^{\cdot}/ONOO^- = 0.8$ V) [43] by different reactive species such as N_2O_3 [44], $^{\cdot}OH$ and $CO_3^{\cdot-}$ [43] (Tab. I), but the contribution from these reactions to the overall peroxynitrite decomposition is predicted to be marginal under most biological conditions, due to the low steady-state concentration of the reactants.

The hydroxyl radical/nitrogen dioxide pathway

In the absence of direct targets peroxynitrous acid decomposes with a half-life of less than 1 second at physiological pH and 37 ºC, being the main product of decomposition nitrate. The mechanism of decomposition involves the homolytic cleavage of its peroxo-bond producing hydroxyl radical (OH) and nitrogen dioxide (NO_2) in a solvent cage from which ~ 30 % of the radicals escape and can be trapped at high target concentration, while the rest recombine to produce nitrate (Fig. 1)[45,46]. The reactions depending on the products of homolysis are first order in peroxynitrite and zero order in target, i.e the presence of the target does not affect the rate of peroxynitrite decay.

$$-d\,[ONOO^-]\,/\,dt = k\,[ONOO^-] \quad (5)$$

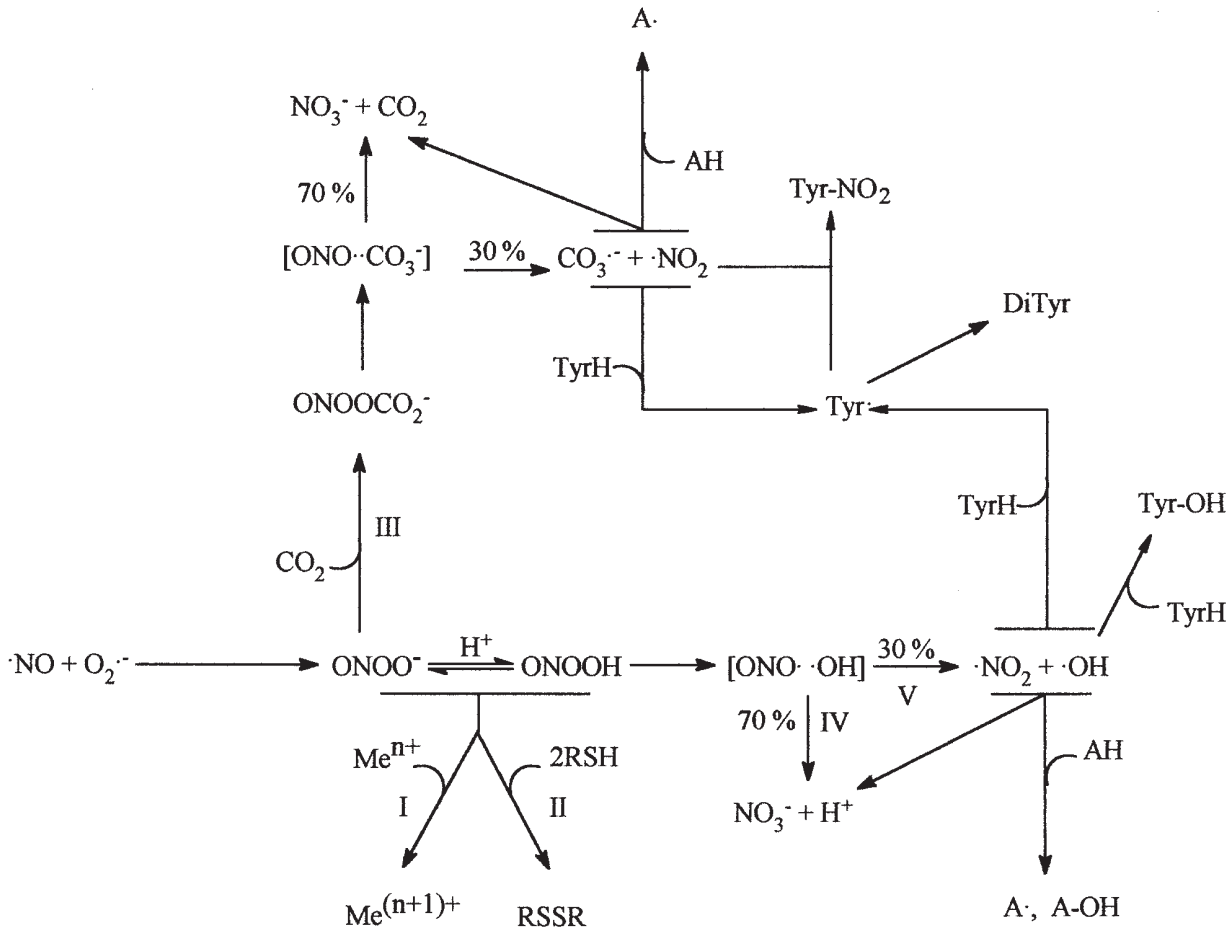

Figure 1. Peroxynitrite reactivity.

$ONOO^-$ is formed from the reaction of $^{\cdot}NO$ and $O_2^{\cdot -}$. Alternatively, the reaction of NO^- and O_2 can lead to $ONOO^-$ formation. Pathways I and II shows that either $ONOO^-$ and its conjugated acid, ONOOH (pK_a = 6.8) can participate in bimolecular reactions such as the one-electron oxidation of transition metal ion complexes (Me^{n+} to $Me^{(n+1)+}$) and the two-electron oxidation of thiols (RSH) to disulfides (RSSR). The direct reaction of $ONOO^-$ with CO_2 (III) leads to the formation of $ONOOCO_2^-$, whose homolysis renders free $CO_3^{\cdot -}$ and $^{\cdot}NO_2$ in 30 % yields. Both radicals can participate in one-electron oxidations of biomolecules (AH). In particular, the one-electron oxidation of tyrosine (TyrH) yields tyrosyl radical ($Tyr^{\cdot}$) which can either dimerize to dityrosine (DiTyr) or recombine with $^{\cdot}NO_2$ to produce 3-nitro-tyrosine (Tyr-NO_2). ONOOH escaping from direct reactions decomposes to NO_3^- (IV), or produces free $^{\cdot}NO_2$ and $^{\cdot}OH$ (V) in 30% yields, that can participate in oxidative and nitration reactions; the addition of $^{\cdot}OH$ to different biomolecules (AH, TyrH) leads to the formation of hydroxylated products (A-OH, Tyr-OH).

Therefore, peroxynitrite can oxidize biomolecules either directly or by producing secondary oxidants such as $CO_3^{\cdot -}$, $^{\cdot}NO_2$ and $^{\cdot}OH$, which are involved in one-electron oxidation reactions. In biological systems, where the presence of direct target is ubiquitous, most peroxynitrite (> 95 %) will be consumed by direct reactions, mainly with thiols, transition metal centers and CO_2 (Fig. 1). It is important to note that direct reactions can lead to secondary oxidative events, such as the formation of $CO_3^{\cdot -}$.

Peroxynitrite-dependent nitrations

Peroxynitrite nitrates aromatic and aliphatic residues. It nitrates free or protein-bound tyrosine to form the stable product 3-nitro-tyrosine by addition of a nitro group to the 3-position adjacent to the hydroxyl group of tyrosine. There are several reports indicating that peroxynitrite-dependent protein nitration may compromise its function [47,48]. In the absence of catalyzers, the rate of peroxynitrite-dependent tyrosine nitration has a zero order dependence on the amino acid concentration, suggesting an indirect mechanism depending on $^{\cdot}OH$ and $^{\cdot}NO_2$. In this mechanism, tyrosine reacts first with $^{\cdot}OH$ or $^{\cdot}NO_2$ yielding tyrosyl radical, which recombines with $^{\cdot}NO_2$ to produce 3-nitro-tyrosine. Also 3,3'-dityrosine (Dityr) is formed from the dimerization of tyrosyl radicals [49]. As the addition reaction of $^{\cdot}OH$ on tyrosine predominates over the one-electron abstraction, 3-hydroxytyrosine (Tyr-OH)(L-dopa) is formed as well,

especially at acidic pH [50]. The reactions involved in the nitration process and their rate constants at physiological pH are the following:

$$\text{ONOOH} \rightarrow 0.3\ {}^{\cdot}\text{OH} + 0.3\ {}^{\cdot}\text{NO}_2 + 0.7\ \text{NO}_3^- + 0.7\ \text{H}^+ \quad k_6 = 0.4\ \text{s}^{-1} \tag{6}$$

$$\text{TyrH} + {}^{\cdot}\text{NO}_2 \rightarrow \text{Tyr}^{\cdot} + \text{NO}_2^- \quad k_7 = 3.2 \times 10^5\ \text{M}^{-1}.\ \text{s}^{-1}\ [51] \tag{7}$$

$$\text{TyrH} + {}^{\cdot}\text{OH} \rightarrow 0.95\ \text{Tyr-OH}^{\cdot} + 0.05\ \text{Tyr}^{\cdot} \quad k_8 = 1.4 \times 10^{10}\ \text{M}^{-1}.\ \text{s}^{-1}\ [52] \tag{8}$$

$$\text{Tyr}^{\cdot} + {}^{\cdot}\text{NO}_2 \rightarrow \text{Tyr-NO}_2 \quad k_9 = 3 \times 10^9\ \text{M}^{-1}.\ \text{s}^{-1}\ [51] \tag{9}$$

$$\text{Tyr}^{\cdot} + \text{Tyr}^{\cdot} \rightarrow \text{Dityr} \quad 2k_{10} = 4.5 \times 10^8\ \text{M}^{-1}.\ \text{s}^{-1}\ [53] \tag{10}$$

$$2\ \text{Tyr-OH}^{\cdot} \rightarrow \text{Tyr-OH} + \text{products} \quad k_{11} = 3 \times 10^8\ \text{M}^{-1}.\ \text{s}^{-1}\ [52] \tag{11}$$

$$\text{Tyr-OH}^{\cdot} \rightarrow \text{Tyr}^{\cdot} + \text{H}_2\text{O} \quad k_{12} = 1.8 \times 10^8\ \text{M}^{-1}\ .\ \text{s}^{-1} \tag{12}$$

Nitration yields are close to 6 % at pH 7.4 and 37 ºC, but vary depending on several factors including pH and presence of scavengers or catalysers, such as transition metal ions and CO_2. In the presence of 1 mM CO_2, nitration occurs via the one-electron oxidation of tyrosine by ${CO_3}^{\cdot -}$ (k = 4.5 $\times 10^7\ \text{M}^{-1}\cdot\text{s}^{-1}$ [54]).

$$\text{TyrH} + {CO_3}^{\cdot -} \rightarrow \text{Tyr}^{\cdot} + \text{HCO}_3^- \tag{13}$$

followed by the recombination of tyrosyl radical with ${}^{\cdot}NO_2$ [36] (eq. 9). Nitration yields increase to ~ 15 % due to the more efficient formation of tyrosyl radicals by ${CO_3}^{\cdot -}$ compared to ${}^{\cdot}OH$; dimerization also occurs, but hydroxylation is inhibited [55].

Low molecular-mass transition metals, metalloporphyrins [56], hemeperoxidases [57] and both cooper-zinc and manganese superoxide dismutase [10] also increase nitration rates and yields. A possible methal-mediated mechanism of nitration is through the formation of a nitronium (NO_2^+) – like intermediate that can nitrate aromatic rings by electrophilic aromatic substitution [58]. Peroxynitrite can also oxidize transition metal ions to form oxo-ferryl species, which in turn oxidizes tyrosine to tyrosyl-radical that combines with ${}^{\cdot}NO_2$ to form 3-nitro-tyrosine. Although 3-nitro-tyrosine is a stable product and it is considered to be long-lived in tissues, recent reports suggest the presence of a 3-nitro-tyrosine denitrase activity in plasma and some tissues [59,60], which would be capable of reverse protein nitration. The denitration of proteins could permit the restoration of their function, but awaits further confirmation.

3-Nitro-tyrosine formation is not totally specific for peroxynitrite. Alternative biological pathways for phenolic nitration exist: a) tyrosine reactions with ${}^{\cdot}NO_2$ [61], b) oxidation of nitrosotyrosine [62], formed by the reaction of ${}^{\cdot}NO$ and tyrosyl radical, c) the reaction of tyrosine with nitryl choride ($Cl\text{-}NO_2$) [63], formed from the reaction of nitrite with hypochlorous acid, d) heme peroxidase (*e.g.* myeloperoxidase and eosinophil peroxidase)-catalyzed oxidation of tyrosine and nitrite [64,65]. Discrimination of the nitration pathway involved could be achieved by measuring accompanying modifications such as co-localization of 3-hydroxy-tyrosine and 3-nitro-tyrosine during peroxynitrite-dependent nitration [55], whereas in myeloperoxidase-dependent nitration 3-chlorotyrosine and 3,5-dichlorotyrosine should also be present [66].

Authentic peroxynitrite versus peroxynitrite generated by fluxes of ${}^{\cdot}NO$ and ${O_2}^{\cdot -}$

Although the main biological source of peroxynitrite is the reaction between ${}^{\cdot}NO$ and ${O_2}^{\cdot -}$, most of our knowledge about peroxynitrite biochemistry relies on experiments that have utilised authentic peroxynitrite, *i.e.* alkaline stock solutions of peroxynitrite, instead of its generation by fluxes of ${}^{\cdot}NO$ and ${O_2}^{\cdot -}$. Moreover, experiments are frequently performed adding peroxynitrite as a bolus to the targets under study, instead of the more physiological continuous infusion of peroxynitrite. As already mentioned, peroxynitrite reactions frequently involve a variety of radical intermediates which can often react with each other as well as with the radicals precursors of peroxynitrite. In order to minimise these interactions, fluxes of ${}^{\cdot}NO$ and ${O_2}^{\cdot -}$ should be equal, so that they preferentially react with each other instead of interfering with the process under study [67]. An additional complicating factor arises from contaminants carried from the synthesis of peroxynitrite, such as nitrite, which reacts with several radicals involved in the nitration process. Moreover, when generating peroxynitrite by fluxes of ${}^{\cdot}NO$ and ${O_2}^{\cdot -}$, the source of the radicals should be carefully chosen: as an illustrative example xanthine oxidase has been frequently used to generate ${O_2}^{\cdot -}$, although several reports indicate that it is inactivated by peroxynitrite [68]; also, the use of xanthine as substrate leads to the formation of uric acid, a well known inhibitor of peroxynitrite-mediated oxidations [69]. All these factors should be taken into account when studying the reaction of peroxynitrite with different targets. In particular, the *in vitro* nitration yield of tyrosine at pH 7.4 obtained by simultaneous equimolar fluxes of ${}^{\cdot}NO$ and ${O_2}^{\cdot -}$ is lower than that obtained upon bolus addition of authentic peroxynitrite. Indeed, nitration yields depend on peroxynitrite steady state concentrations, since tyrosine exposed to low concentrations of peroxynitrite ($\leq$ 5 µM) yields dityrosine as the main product whereas 3-nitro-tyrosine predominates at higher concentrations of peroxynitrite [70]. Moreover, nitration yields by authentic peroxynitrite in the presence of CO_2 are much higher by bolus addition than continuous infusion [54]. Continuous infusion of peroxynitrite generates a lower concentration of ${}^{\cdot}NO_2$ and tyrosyl radicals, allowing tyrosine to compete with tyrosyl radical for ${}^{\cdot}NO_2$ (reaction 7 *versus* reaction 9), thus increasing the formation of tyrosyl radical at expense of ${}^{\cdot}NO_2$ and lowering the ratio between nitration yield and dimerization yield. These findings are potentially relevant when studying nitration yields in reporter molecules by biologically produced ${}^{\cdot}NO$ and ${O_2}^{\cdot -}$.

Peroxynitrite diffusion

Cell compartimentation is an important determinant of the biological reactivity of peroxynitrite since it may limit its ability to reach specific biological targets. Both peroxynitrous acid and peroxynitrite anion are able to cross biological membrane [71]. Peroxynitrous acid freely diffuse across membranes [71,72] while peroxynitrite anion diffuses through anionic channels [71]. It has been estimated that the diffusion distances of peroxynitrite in biological systems are in the order of 5-20 µm and shown that peroxynitrite formed in one compartment could react with biomolecules that are in another compartment. As a consequence, footprints of peroxynitrite could be found at a certain distance from its site of formation. Compartimentation should always be taken into account when detecting or quantitating peroxynitrite by cellular systems, and makes obligatory the study of probe permeation through membranes in each particular system.

Table II. Effect of bicarbonate on peroxynitrite-dependent DHR oxidation.

Addition	*DHR oxidation* ([a]µM or [b]µM/min)		*DHR oxidation yield* (%)	
	$-HCO_3^-$	$+HCO_3^-$	$-HCO_3^-$	$+HCO_3^-$
$ONOO^-$ (bolus)[a]	3.0 ± 0.3	0.6 ± 0.1	30	6
$ONOO^-$ (Cont. Inf)[b]	0.37 ± 0.06	0.07 ± 0.01	37	7
NOC-7 + XO[b]	0.30 ± 0.01	0.31 ± 0.01	30	31
SIN-1[b]	0.40 ± 0.01	0.42 ± 0.02		

DHR (50 µM) was exposed to peroxynitrite either as a a) bolus addition of authentic peroxynitrite (10 µM), b) continuous infusion of authentic peroxynitrite (1 µM/min), c) fluxes of $^{\cdot}NO$ (1 µM/min) generated by NOC-7 (3 µM) and $O_2^{\cdot -}$ (1 µM/min) from lumazine (100 µM) plus xanthine oxidase, and d) fluxes of $^{\cdot}NO$ and $O_2^{\cdot -}$ generated form SIN-1 (2 mM, ca. 1,2 µM/min peroxynitrite estimated from either oxygen consumption or cytochrome c oxidation assays), in 100 mM potassium phosphate buffer pH 7.4, 25 °C for 10 min in the presence or absence of 25 mM HCO_3^-.

Detection of peroxynitrite

Peroxynitrite detection relies on a) the modification of exogenously added molecules, that are called probes for peroxynitrite and b) the detection of endogenous biomolecules modified by a peroxynitrite-dependent reaction, *i.e.* footprints. As none of them are totally specific for peroxynitrite, additional criteria must be fulfilled in order to detect unambiguously peroxynitrite formation in biological systems. Whereas probes are frequently used to detect or quantitate peroxynitrite in biochemical or cellular systems, footprints are utilised either in cells or tissues.

Probes for peroxynitrite

Oxidation of fluorescent probes

Peroxynitrite oxidizes 2',7'-dichlorofluorescin (DCFH) [73,74] and dihydrorhodamine 123 (DHR) [75,77] to the highly fluorescent products dicholorofluorescein (DCF) (λ_{ex} = 500 nm, λ_{em} = 523 nm) and rhodamine (RH) (λ_{ex} = 500 nm, λ_{em} = 536 nm), respectively. This techniques allow the detection of submicromolar levels of peroxynitrite, *e.g.* 50 nM. Both DCFH and DHR can enter cells, and therefore can be used to detect either intracellular or extracelular formation of peroxynitrite. The mechanism of DCFH and DHR oxidation by peroxynitrite is still unknown: formation of the fluorescent products requires a 2-electron oxidation but radicals are formed as intermediates. The fact that $^{\cdot}OH$ scavengers are unable to protect probes from oxidation suggests that peroxynitrite itself may be involved in the process, but a direct measurement of the reaction is at the moment lacking. Oxidation yields are in the range of 33-38 % of peroxynitrite added for DCFH and 30-40 % for DHR, but are highly influenced by conditions such as pH, buffer composition, and the presence of other targets for peroxynitrite [75]. Oxidation yields remain the same when peroxynitrite is generated by equal fluxes of $^{\cdot}NO$ and $O_2^{\cdot -}$, but decrease when the ratio between fluxes is different to one, due to secondary reactions with radical intermediates (semiquinone radicals) formed by the one electron oxidation of the probes [73]. Another potential confounding factor is that the semiquinone radicals formed by the one electron oxidation of the probes could produce $O_2^{\cdot -}$ by reaction with O_2, and could contribute to artifactual peroxynitrite formation in systems that produce $^{\cdot}NO$ [76,77].

DCFH and DHR oxidation is not specific for peroxynitrite since other oxidants such as $^{\cdot}OH$, hipochlorous acid, oxo-iron complexes, and to a marginal extent $^{\cdot}NO_2$, are able to oxidize the probes. On the other hand, thiols and urate, which compete for peroxynitrite, decrease peroxynitrite-dependent oxidation of the probes. The oxidation of both probes provide information about the rate of peroxynitrite production *in vitro*, but their use *in vivo* is subject to several potential pitfalls arising from the lack of specificity, the fact that *in vivo* peroxynitrite is generated by fluxes of $^{\cdot}NO$ and $O_2^{\cdot -}$ that are likely to be not equal and the presence of other targets for peroxynitrite including CO_2. Table II shows the effect of CO_2 on DHR oxidation by peroxynitrite: whereas CO_2 inhibits DHR oxidation by authentic peroxynitrite, it has no effect on DHR oxidation rates by fluxes of $^{\cdot}NO$ plus $O_2^{\cdot -}$, generated either by SIN-1 (3-Morpholinosydnonimine), that generates equimolar fluxes of $O_2^{\cdot -}$ and $^{\cdot}NO$, or by NOC-7 (3-(hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propanamine), a $^{\cdot}NO$ donor, plus xanthine oxidase using lumazine as substrate. The explanation for this observation deserves further investigation, but probably relies on the multiple possible radical interactions that arise when generating peroxynitrite by fluxes.

Oxidation of chromophores: the example of cytochrome c^{2+}

Several chromophores have been utilised to detect or measure peroxynitrite formation. Among them, an illustrative

Table III. Cytochrome c^{2+} oxidation by fluxes of $^{\cdot}NO$ and $O_2^{\cdot -}$.

Condition	*Cytochrome c^{2+} oxidation (μM/min)*
SNAP alone	0.52 ± 0.07
+XO	
No addition	2.71 ± 0.04
+ SOD (25 μM)	0.29 ± 0.01
+ Mannitol (20 mM)	2.76 ± 0.04
+ DMSO (50 mM)	2.21 ± 0.13
+ GSH (7.5 mM)	0.13 ± 0.06
+ Methionine (20 mM)	0.27 ± 0.01

Effect of different scavengers on the oxidation of cytochrome c^{2+} (40 μM) by S-nitroso N-acetylpenicillamine (SNAP) (3 μM $^{\cdot}NO$/min), plus 150 μM hypoxanthine and 3 mU/ml xanthine oxidase (3 μM $O_2^{\cdot -}$/min), in 100 mM potassium phosphate plus 0.1 mM dtpa, pH 7.4.

example is the peroxynitrite-dependent oxidation of cytochrome c^{2+}: Peroxynitrous acid reacts with cytochrome c^{2+} with a second order rate constant of 1.32×10^4 $M^{-1}.s^{-1}$ at pH 7.4 25 ºC [78]. However, cytochrome c^{2+} oxidation is not specific for peroxynitrite since other oxidants can do it: H_2O_2 –dependent oxidation should be ruled out by the addition of catalase, and the reaction of $^{\cdot}NO$ with cytochrome c^{2+} leads to the formation of cytochrome c-nitrosyl complexes, which have characteristic spectrum. Oxidation yields depend on cytochrome c^{2+} concentration: at 50 μM cytochrome c^{2+} ~ 50 % oxidation yield are obtained. Table III shows that whereas S-nitroso N-acetyl penicillamine (SNAP), producing 3 μM/min $^{\cdot}NO$, slowly oxidizes cytochrome c^{2+}, the addition of xanthine oxidase plus hypoxanthine increases cytochrome c^{2+} oxidation rate, an effect that is dependent of $O_2^{\cdot -}$ as shown by the inhibition afforded by SOD. Peroxynitrite scavengers such as methionine and glutathione decrease cytochrome c^{2+} oxidation rate whereas $^{\cdot}OH$-scavengers like DMSO and mannitol have no effect, in agreement with a direct reaction between peroxynitrite and cytochrome c^{2+}.

While $O_2^{\cdot -}$ can reduce cytochrome c^{3+}, this process is negligible under initial conditions in which 100 % of the cytochrome is in the reduced form.

EPR spectroscopy

As peroxynitrite is able to participate in one electron-oxidation reactions, either directly or by producing secondary oxidants, it often leads to free radical intermediates that could be detected by EPR techniques [79]. Most free radicals formed in solution are not stable enough to be detected by direct EPR spectroscopy, and therefore other techniques such as spin trapping, which uses a diamagnetic molecule that reacts with a short-lived free radical to produce a more persistent one, allows their detection [80,81]. For instance, $^{\cdot}OH$, thiyl radicals and carbon-centered radicals have been detected using the spin trap DMPO (5,5-dimethylpyrroline N-oxide) during peroxynitrite decomposition in the absence or presence of target molecules [82]. Also, formation of ascorbyl and desferrioxamine-derived radicals has been detected by direct EPR after peroxynitrite reactions.

Chemiluminescence

Luminol (5-amino-2, 3-dihydro-1, 4-phtalazinedione)-derived chemiluminescence is frequently used to study the formation of reactive species in biological systems. The mechanism of chemiexcitation depends on the one or two electron oxidation of luminol to yield luminol radical or luminol diazoquinone, respectively, which by reacting with $O_2^{\cdot -}$ or H_2O_2 yield an unstable endoperoxide, that decomposes to an electronically excited state of aminophtalate whose decayment to ground state emit light. Peroxynitrite can induce luminol chemiluminescence [83], especially in the presence of CO_2, by forming $CO_3^{\cdot -}$ that oxidises luminol to luminol radical, therefore starting the sequence of reactions that leads to light emission. This method is highly sensitive: with appropriate intruments peroxynitrite fluxes down to 1 nM/min can be accurately detected. However, it is not specific, since different reactive species such as $O_2^{\cdot -}$, $^{\cdot}OH$, H_2O_2, HOCl and peroxynitrite can lead to the light–emitting species of luminol. On the other hand, $^{\cdot}NO$ can direct the pathway to a dark route, by termination reaction with luminol radicals. Therefore, quantum yields from authentic peroxynitrite are higher than those obtained with equimolar fluxes of $^{\cdot}NO$ and $O_2^{\cdot -}$ [84].

Probe nitration

Among the different aromatic compounds that peroxynitrite is able to nitrate, tyrosine and its more soluble analog p-hydroxyphenylacetic acid (p-HPA) are frequently chosen as probes for peroxynitrite formation. Nitro-phenolics typically have a pKa close to 7.5 and can be directly assessed spectrophotometrically (ε_{360} = 2790 $M^{-1}.cm^{-1}$ and ε_{430} = 4400 $M^{-1}.cm^{-1}$ for 3-nitro-tyrosine at low and alkaline pH respectively) with a sensitivity limit of ~ 1 μM. The presence of other compounds that absorb in the same region may interfere with this technique [85].

By using HPLC techniques, frequently reverse phase, a more sensitive and specific detection of nitrophenolics (but also of parental phenolics as well as products of its oxidation such as dimers and hydroxylated products) can be achieved. Detection systems include UV-visible, fluorescence, electrochemistry and mass spectrometry.

a) UV-visible detection: using acidified samples, every phenolic absorbs at 280 nm, whereas at 365 nm only nitrophenolics can be detected: thus, simultaneous detection at 280 and 365 nm is recommended [85].

b) Fluorescence detection: 3-nitro-tyrosine is not fluorescent, but it can be reduced to aminotyrosine which fluoresces (λ_{ex} = 277, λ_{em} = 308 and 350 nm at pH 3-3.5), thus enhancing the sensitivity and the specificity with respect to direct detection of 3-nitro-tyrosine.

c) Electrochemical detection: 3-nitro-tyrosine can be measured by oxidation at high positive voltage (850 mV), with a reported detection limit of < 1 pmol. By transforming 3-nitro-tyrosine into aminotyrosine [86] a lower oxidative voltage (70 mV) is required, that results in a better signal to noise ratio.

d) A highly specific and sensitive approach, which is increasingly used and developed is the combination of gas chromatography (GC) and mass spectrometry (MS) techniques to quantitate 3-nitro-tyrosine. The detection limit of this methodology is below the fentomol range [87]. The use of GC with tandem MS provides accurate structural information and is the method of choice to unambigously define and measure the presence of 3-nitro-tyrosine in complex biological samples [88]. However, the use of this method is limited by the access to costly equipment and experimental complexities.

Footprints

Nitration of endogenous components of cells and tissues, mainly proteins, is frequently used as footprints of peroxynitrite [89]. Nitrated proteins are detected either by protein hydrolysis followed by detection of 3-nitro-tyrosine by the separation and detection methods already described, or by immunochemical methods.

Protein hydrolysis can be achieved chemically (most frequently by acid hydrolysis [90] although alkaline hydrolysis has also been used [91]) or by protease treatment (*e.g.* pronase) [90]. The techniques are subject of potential pitfalls, as the artifactual nitration by NO_2^- at low pH in the case of acid hydrolysis, or autoproteolysis and increase of tyrosine concentration in the case of enzymatic treatment.

Immunochemical methods rely on the detection of 3-nitro-tyrosine by specific monoclonal or polyclonal antibodies and are widely used to unravel the *in vivo* formation of peroxynitrite. However, studies concerning the fine structure of the epitope recognised by these antibodies are lacking. The fact that 3-nitro-tyrosine-containing peptides are more efficient to displace the antibody binding from nitrated proteins than 3-nitro-tyrosine indicates that other aminoacids participate in the epitope that recognises the antibody. This aminoacids may differ from protein to protein therefore resulting in variable antibody specificity and avidity for different nitrated proteins. In addition, the position of 3-nitro-tyrosine in the protein should influence the binding, and for example, some internal 3-nitro-tyrosine moieties may not be detected. Taking in account these considerations it can be advanced that polyclonal antibodies will be more sensitive than monoclonal to detect protein-bound 3-nitro-tyrosine. In spite that the specificity of the available antibodies has been proven, it is recommended to confirm their specificity for nitrated proteins in the samples under study by displacing the antibody binding with 3-nitro-tyrosine or 3-nitro-tyrosine containing peptides, and by reducing the 3-nitro-tyrosine moieties to 3-aminotirosine with sodium dithionite [92,93].

Immunochemical techniques for detection and quantitation or semi-quantitation of 3-nitro-tyrosine-containing proteins such as ELISA or western-blots have been utilised. ELISA is suitable for detection and measure 3-nitro-tyrosine in biological fluids and proteins and levels of 3-nitro-tyrosine in the range of fmol can be detected if using appropriate concentration of anti-nitro-tyrosine polyclonal antibody and sensitive chemiluminescence-coupled techniques. Depending on the sample, different approaches can be performed: a simple ELISA for single proteins or a capture or competitive ELISA for more complex samples [94,95]. Western-blot is a descriptive method that allows semi-quantitative analysis when digital imaging techniques are applied. It can be used to detect protein-bound 3-nitro-tyrosine residues in single proteins, biological fluids and protein extracts from cells or tissues [71,96,97]. Immunoreactive proteins can be further characterised by their molecular weight, specific recognition by a second western blot developed with antibodies directed against specific proteins or preferably, after immunoprecipitation [2]. *In situ* localisation of nitrated proteins is performed by immunocyto- and histochemical techniques. Immunofluorescence has been applied to detect 3-nitro-tyrosine in degenerating motoneurons *in vitro* [98]. We successfully applied flow cytometry to detect membrane-bound or cytoplasmatic nitrated proteins in peripheral blood mononuclear cells [93]. Immunohistochemistry has been widely used to detect 3-nitro-tyrosine in different human and animal tissues (for a review see [99]) and it permits localization of 3-nitro-tyrosine in specific areas, cells and sub-cellular compartments.

Pharmacological criteria

The lack of complete specificity that characterise all the methods for peroxynitrite detection can be frequently solved by pharmacological approaches (Tab. IV):

a) Inhibition of the production of the precursors $^{\cdot}NO$ or $O_2^{\cdot -}$ should result in lower levels of peroxynitrite detected.

b) Use of $^{\cdot}NO$ or $O_2^{\cdot -}$ scavengers should avoid their interactions and therefore inhibit peroxynitrite formation.

c) Peroxynitrite scavengers could also be used, but special care should be taken regarding their mechanism of action. Both uric acid and ascorbate react slowly with peroxynitrite [100] and cannot compete well with the direct reactions of peroxynitrite. However, they could interfere with peroxynitrite detection by different possible mechanisms: a) by scavenging $^{\cdot}OH$, b) by reacting with $CO_3^{\cdot -}$ and $^{\cdot}NO_2$ [101,102] and c) by reducing back radical intermediates formed by peroxynitrite or peroxynitrite-derived oxidants. On the contrary, other scavengers such as thiols and methionine [40], react bimolecularly with peroxynitrite therefore inhibiting either its direct or indirect reactions.

d) Certain molecules collectively known as decomposition catalysts promote peroxynitrite decomposition to nitrate or

Table IV. Some of the pharmacological tools available to unravel peroxynitrite formation in biochemical or biological systems.

Action	*Compound*	*Reference*
Inhibitors of ˙NO synthesis	L-NAME [a]	Griffith *et al.,* 1996 [111]
	L-NMMA [b]	
	7-NI [c]	Moore *et al.,* 1996 [112]
˙NO scavengers	Carboxy-PTIO[d]	Hooper *et al.,* 1997 [113]
SOD-mimics	Metalloporphyrins	Pasternack *et al.,* 1979 [104]
Peroxynitrite scavengers	RSH	Radi *et al.,* 1991 [8]
	Uric acid	Squadrito *et al.,* 2000 [101]
	Methionine	Pryor *et al.,* 1994 [40]
Peroxynitrite decomposition catalyst	Metalloporphyrins	Ferrer-Sueta *et al.,* 1999 [103]
	Seleno-compounds	Arteel *et al.,* 1999 [105]

[a] N^G-nitro-L-arginine methyl ester
[b] N^G-mono-methyl-L-arginine
[c] 7-nitroindazole
[d] Carboxy-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide

its reduction to nitrite at the expense of reducing equivalents. In the case of metalloporphyrins, they react with peroxynitrite very fast to form NO_2 and an oxo-Fe(IV) or oxo-Mn(IV) intermediate, that in the prescence of reductants such as ascorbate and glutathione is reduced to the original (III) species [56,103]. As the intermediates species formed in the reaction possess their own reactivity, and some metalloporphyrins are known to catalyse the dismutation of $O_2^{\cdot -}$ [104], the effect of metalloporphyrins on peroxynitrite detection is sometimes difficult to interpret. In addition, seleno-containing compounds catalyse peroxynitrite reduction and recycle in the presence of thiols [105].

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