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Forum Review

Peroxynitrite Detoxification and Its Biologic Implications

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Abstract

Peroxynitrite is a cytotoxic oxidant formed *in vivo* from the diffusional–controlled reaction between nitric oxide and superoxide radicals. Increased peroxynitrite formation has been related to the pathogenesis of multiple diseases, thus underlining the importance of understanding the mechanisms of its detoxification. In nature, different enzymatic routes for peroxynitrite decomposition have evolved. Among them, peroxiredoxins catalytically reduce peroxynitrite *in vitro*; modulation of their expression affects peroxynitrite-mediated cytotoxicity, and their content changes in pathologic conditions associated with increased peroxynitrite formation *in vivo,* thus indicating a physiologic role of these enzymes in peroxynitrite reduction. Selenium-containing glutathione peroxidase also catalyzes peroxynitrite reduction, but its role *in vivo* is still a matter of debate. In selected cellular systems, heme proteins also play a role in peroxynitrite detoxification, such as its isomerization by oxyhemoglobin in red blood cells. Moreover, different pharmacologic approaches have been used to decrease the toxicity related to peroxynitrite formation. Manganese or iron porphyrins catalyze peroxynitrite decomposition, and their protective role *in vivo* has been confirmed in biologic systems. Glutathione peroxidase mimetics also rapidly reduce peroxynitrite, but their biologic role is less well established. Flavonoids, nitroxides, and tyrosine-containing peptides decreased peroxynitrite-mediated toxicity under different conditions, but their mechanism of action is indirect. *Antioxid. Redox Signal*. 10, 1607–1619.

Peroxynitrite[a], formed *in vivo* by the diffusion-controlled reaction between nitric oxide (·NO) and superoxide ($O_2{\cdot}^-$) radicals (9, 59, 100), is an oxidant species implicated in the development of a wide variety of pathologic conditions, including diabetes (116, 146), neurodegenerative disorders (64, 120), and cardiovascular diseases (10, 12, 80, 86). Because of its cytotoxic actions, peroxynitrite formation by inflammatory cells like activated macrophages can participate in infectious disease control (28, 66, 68). More recently, the role of peroxynitrite in cell signaling has been the subject of intensive research (25, 58, 69, 70). In selected cellular compartments, a 50- to 100-μM/min peroxynitrite formation rate has been estimated (2). In the absence of direct targets for peroxynitrite reactivity, ONOOH decays (k_a = 0.9 per second at pH 7.4 and 37°C) by homolysis of its peroxo bond, leading to the formation of hydroxyl (·OH) and nitrogen dioxide ($\cdot NO_2$) radicals in 30% yields (67, 77). However, this is obviously an artificial situation: in most biologic media, peroxynitrite decays by direct reactions with different targets, resulting in a half-life in the millisecond range and a steady-state concentration in the nanomolar range. Peroxynitrous acid can passively diffuse through membranes, and some anion channels also allow peroxynitrite anion to permeate across them (27, 72), influencing surrounding cells within 5–20 μm (one or two cell diameters).

Peroxynitrite exerts its cytotoxic effects by the oxidative modification of the different macromolecules present in

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[a]The term peroxynitrite is used to refer to the sum of peroxynitrite anion ($ONOO^-$) and its conjugated form, peroxynitrous acid (ONOOH) [pK_a = 6.8 (97)]. IUPAC-recommended names for $ONOO^-$ and ONOOH are oxoperoxonitrate (1-) and hydrogen oxoperoxynitrate, respectively.

cells[b] by one or more than one of the following mechanisms: (a) it can directly react with cellular targets, including critical residues as well as prosthetic groups in different proteins, leading to protein function modification; (b) its reaction with CO_2 [$k = 5.8 \times 10^4\ M^{-1}s^{-1}$ at 37°C, pH independent (29,131)], present in biologic media at millimolar concentrations, leads to the formation of up to 35% $\cdot NO_2$ and carbonate radical ($CO_3\cdot^-$) (112). These are potent one-electron oxidants that can, in turn, lead to nitration of different phenolic compounds by a radical route; (c) in selected hydrophobic compartments, peroxynitrous acid could homolyze to ·OH and $\cdot NO_2$, thus contributing to peroxynitrite-dependent oxidations (7); (d) products formed from peroxynitrite-mediated direct or indirect reactions are frequently not inert but can be involved in further oxidative (and nitrative) chemistry.

In light of the different potential cytotoxic effects mediated by peroxynitrite, the understanding of the molecular basis of its detoxification, either by naturally occurring compounds or by pharmacologic agents, seems to be a priority. As an example, different pathogenic microorganisms can live and even proliferate inside the oxidative environment of the phagosome of activated macrophages, where they can be exposed to relatively high fluxes of peroxynitrite. Knowing the molecular mechanisms responsible for peroxynitrite detoxification in those pathogens could help to find a rational drug design that could inhibit proteins naturally involved in peroxynitrite reduction. In addition, pharmacologic agents with peroxynitrite-detoxification activity could be useful in the treatment of those diseases in which peroxynitrite formation has been shown to play a pathogenic role (115).

A compound active in peroxynitrite decomposition should fulfill several characteristics: (a) it should react with peroxynitrite rapidly, to trap the oxidant before it can react with other naturally occurring targets; (b) it should be present at sufficiently high concentrations in cellular compartments, because preferential targets for peroxynitrite reactivity are dictated by the value of "apparent rate constants," which can be calculated by multiplying the second-order rate constant for the reaction times target concentration; (c) it should act catalytically (*i.e.*, its oxidized form should be rapidly re-reduced by naturally occurring compounds, so as not to be consumed during the reaction and in some cases, to avoid further damage derived from oxidized form of these compounds); and (d) products formed from peroxynitrite decomposition should not be toxic by themselves. In this respect, those compounds leading either to peroxynitrite isomerization to nitrate or to its two-electron reduction to nitrite seem to be much more convenient than those leading to peroxynitrite one-electron reduction to $\cdot NO_2$ (Fig. 1).

Herein, we review the main mechanisms by which peroxynitrite detoxification can be achieved in biologic systems that have been described in the literature. First, we summarize the mechanisms that have evolved in nature, and then, we briefly describe the main pharmacologic strategies developed for that purpose.

Physiologic Routes for Peroxynitrite Reduction

Different proteins have been reported to reduce peroxynitrite catalytically. We focus mainly on peroxiredoxins (Prxs), whose reactivity with peroxynitrite has been extensively studied and whose importance for peroxynitrite

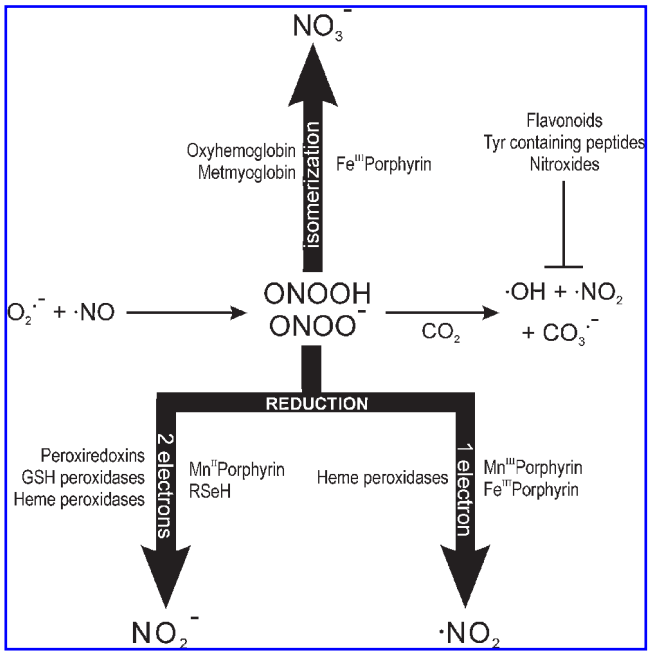

FIG. 1. Different routes leading to peroxynitrite detoxification. Peroxynitrite detoxification can proceed either by isomerization to nitrate or reduction. Isomerization to nitrate is catalyzed by some heme proteins, like oxyhemoglobin and metmyoglobin, and by synthetic Fe^{III}Porphyrins. Reduction can involve either two or one electron processes. Enzymes such as peroxiredoxins, glutathione peroxidase, and some heme peroxidases (see Table 2) catalyze the two-electron reduction of peroxynitrite to nitrite, and this reaction is also catalyzed by some Mn^{II}Porphyrins and by synthetic selenols like ebselen. Reduction by one-electron yielding nitrogen dioxide is catalyzed by some heme peroxidases (see Table 2) or by synthetic Mn^{III} and Fe^{III} porphyrins. Other compounds, like flavonoids, tyrosine-containing peptides and tempol can contribute to lessen peroxynitrite-mediated damage by reacting with peroxynitrite-derived radicals. In the case of tempol, the product formed by its one-electron oxidation oxidizes peroxynitrite and nitric oxide is formed, thus redirecting peroxynitrite reactivity toward nitrosation. Many of these compounds can also decrease peroxynitrite formation rate by diminishing the steady-state concentrations of its radical precursors nitric oxide (·NO) and superoxide anion ($O_2\cdot^-$), but these actions are beyond the scope of this review.

[b]Regarding peroxynitrite-mediated protein modifications, which in turn could result in changes in protein function, direct reactions involve mainly two-electron oxidation of cysteine residues, as well as prosthetic groups modifications. Other amino acid residues in proteins react too slowly with peroxynitrite to represent an important fate for direct peroxynitrite reactivity *in vivo* (1). This oxidant can also modify proteins by indirect mechanisms, including tyrosine or tryptophan nitration, and one-electron oxidation of different amino acids (98). Indirect peroxynitrite-mediated reactions can also lead to lipid peroxidation to give hydroxy and peroxy fatty acid derivatives, conjugated dienes, and malondialdehyde (99), as well as the more recently characterized nitrolipids (103, 123), and transformation of DNA due to deoxyribose oxidation and nitrogenous base modifications (19). Although initially thought to be exclusively as toxic or pathogenic actions of peroxynitrite, some of these reactions are now considered to be implicated in physiologic redox-signaling processes.

Table 1. Different Classes of Peroxiredoxins Catalyze Peroxynitrite Reduction

Peroxiredoxin	*Class*	*k* ($M^{-1}s^{-1}$)	C_pK_a	*Natural reductant*	*Reference*
M. tuberculosis TPx	Atypical 2-Cys	1.5×10^7	N.R.	Thioredoxin B and C	52
M. tuberculosis AhpC*	Typical 2-Cys	1.3×10^6	<5	Thioredoxin C, dihydrolipoamide-AhpD	16
T. brucei cytosolic TXNPx	Typical 2-Cys	9×10^5	N.R.	Tryparedoxin	128
T. cruzi cytosolic TXNPx	Typical 2-Cys	8×10^5			
P. falciparum TPx1	Typical 2-Cys	1×10^6	N.R.	Thioredoxin	83
Human PRDX5	Atypical 2-Cys	$7 \times 10^{7\dagger}$		Thioredoxin 1 and 2	31
		1.2×10^8	5.2		129
Yeast TSA1	Typical 2-Cys	7.4×10^5	5.7	Thioredoxin	85
TSA2		5.1×10^5	6.3		

Reported rate constants at pH 7.4 unless otherwise indicated.

N.R., not reported.

Bovine Prx 6, which is a 1-Cys Prx, has also been reported to rapidly react with peroxynitrite, although the rate constant of this reaction was not determined (92). Postulated physiological reductants for this enzyme include ascorbate as well as glutathione after heterodimerization with glutathione transferase (81).

*At pH 6.8. The reaction between peroxynitrite and AhpC from other bacteria has also been reported, and reported rate constants were very similar. Unlike *M. tuberculosis*, many bacteria contain AhpF which is the electron donor for AhpC.

†At pH 7.8.

detoxification in biologic systems has been well documented. Other proteins that have been also reported to reduce peroxynitrite catalytically are mentioned at the end of this section.

Peroxiredoxins catalyze peroxynitrite reduction to nitrite

Peroxiredoxins are a group of thiol-containing proteins that catalyze hydrogen peroxide, organic hydroperoxide, and peroxynitrite reduction by an enzymatic substitution (ping-pong) mechanism (50, 95, 101, 138). According to the number of cysteine residues required for catalysis, they are classified into one- and two-cysteine Prxs. The first step in the catalytic cycle is common for all Prxs and consists on oxidizing substrate–mediated two-electron oxidation of Prx peroxidatic cysteine residue (C_P), usually deprotonated at physiologic pH, to form a sulfenic acid derivative. One-Cys Prxs are directly reduced by reducing substrates, whose identity is still a matter of debate, candidates include ascorbate (81) and different thiol-containing compounds. In two-Cys Prxs, the sulfenic acid formed in C_P further reacts with a second cysteine residue, the resolving one (C_R), present either in the same protein subunit (atypical 2-Cys Prxs) or in another inversely oriented subunit (typical 2-Cys Prxs), to form an intramolecular or an intermolecular disulfide bridge, respectively. The disulfide is then reduced by the reducing substrate, which is most frequently the small protein thioredoxin (or a thioredoxin-related protein), which in turn is reduced by thioredoxin reductase at NADPH expense (95). The conserved active site of Prxs is composed of a catalytic triad, including the deprotonated C_P, which is hydrogen bonded to a threonine (or less frequently serine) residue, and adjacent to a basic arginine residue, all of which are required for enzymatic activity, and are considered to be responsible for thiolate stabilization in the active site (38).

The first evidence on Prxs catalyzing peroxynitrite reduction to nitrite was reported by Bryk *et al*. in 2000 (16), and was related to alkyl hydroperoxide reductase C (AhpC), a typical 2-Cys Prx present in bacteria, including the intracellular pathogen *Mycobacterium tuberculosis*. The enzyme, with a peroxidatic thiol pK_a of <5 (16), had been previously suggested as a virulence factor in this bacterium (49, 73, 136), reacted rapidly with peroxynitrite ($1 \times 10^6\ M^{-1}s^{-1}$ at pH 6.8 and room temperature) (16), and is reduced by *Mycobacterium tuberculosis* thioredoxin B and C (52), as well as by other cellular enzymatic systems (17). After that, several other Prxs were reported to react with peroxynitrite with similar, or even higher second-order rate constants. This included members of the 1-Cys, atypical and typical 2-Cys classes of Prxs. As shown in Table 1, reported reactivities for the reaction between peroxynitrite and Prxs are strikingly high. The rate constants of peroxynitrite reactions with thiols are related to thiol pK_a. At physiologic pH, reactivities are higher for those thiols having the lower thiol pK_a, consistent with the generally accepted mechanisms of the reaction, in which the reactive species are peroxynitrous acid and thiolate anions (126).

Conversely, intrinsic thiolate reactivities are higher for those thiols having the highest pK_a, consistent with those thiols being better nucleophiles (129). From Brønsted plots, a positive nucleophilic factor of 0.4 has been reported for peroxynitrite oxidation of low-molecular-weight thiols, but it also applies for some protein thiols such as the single thiol group in human serum albumin. However, the reaction between peroxynitrite and the C_P in peroxiredoxins does not fit those Brønsted plots, indicating that protein factors other than thiol pK_a lead to a decrease in the activation energy of the oxidation process, indicating that the enzyme is specialized toward peroxynitrite (and probably other peroxide) detoxification (129).

However, as indicated earlier, to compare theoretically the preferential targets for peroxynitrite reactivity in a cellular system, one should know not only the bimolecular rate constant values of the different reactions but also the concentration of targets. Limited information is available concerning different Prx concentrations in cellular compartments. However, for those peroxiredoxins for which these data do exist (and considering that the oxidation step in catalysis is

the rate-limiting one, and all Prx is active), apparent rates for peroxynitrite reduction can be calculated.

Similar calculations can be made for other important cellular peroxynitrite targets, such as reduced GSH and CO_2, which react slower with peroxynitrite but can reach millimolar concentrations in cellular compartments. These data could help to understand the importance of the different targets of peroxynitrite, including Prxs, such as the fate of its reactivity.[c]

The situation *in vivo* is much more complex than this approximation: first, we should know thioredoxin (or other reducing substrate) concentration as well as the rate constant of thioredoxin-mediated Prx reduction so as to know whether and under which conditions the reductive part of the catalytic cycle is rate limiting. In the case of 2-Cys Prxs, the rate constant of disulfide bridge formation should also be taken into account. Moreover, Prxs catalyze only one step of a series of reactions leading to peroxide reduction, so rate constants and reactant concentrations of all the other steps involved in the system (generally NADPH-dependent thioredoxin reduction catalyzed by thioredoxin reductase) should be known to have a complete view of the system.

Finally, Prxs are known to be oxidatively inactivated by different oxidants (13, 134), and mechanisms of enzymatic reactivation of these enzymes have been described (11, 101). In many cases, *in vivo* formation of peroxynitrite is accompanied by other peroxide formation, which could lead to Prx oxidative inactivation. Moreover, peroxynitrite itself could lead to Prx overoxidation, as was described for *Trypanosoma cruzi* tryparedoxin peroxidase (TPXNPx) (93), but this reaction has not been kinetically characterized.

The importance of peroxiredoxins for peroxynitrite detoxification has been confirmed in different cellular systems, by modulating the levels of their expression:

- Yeast with no functional TSA1 and TSA2 were hypersensitive to peroxynitrite, and expression of these PRDXs was stimulated by cell exposition to sublethal doses of peroxynitrite (137).
- *Mycobacterium tuberculosis* AhpC knock-out mutants showed increased susceptibility to peroxynitrite and decreased survival in macrophages (73).
- Functional complementation in yeast revealed a protective role of chloroplast 2-Cys Prxs against reactive nitrogen species (105).
- Mitochondrial Prx 3 protects hippocampal neurons from excitotoxic injury *in vivo* (47).
- Overexpression of cytosolic TPXNPx in *L. chagasi* parasites enhanced survival when exposed to exogenous peroxynitrite or to macrophages (6).
- Overexpression of cytosolic or mitochondrial TPXNPx in *Trypanosoma cruzi* enhanced survival when exposed to either exogenous or endogenous peroxynitrite and improved invasion to macrophages (93).

The expression of different peroxiredoxins is also intrinsically modulated in several pathophysiologic conditions including inflammation (26, 140). In astrocytes from multiple sclerosis lesions in human brain, Prx 5 expression is strikingly high, particularly in chronic lesions. This Prx, which is present in different cellular compartments including mitochondria, has the highest reactivity toward peroxynitrite reported so far (Table 1), and it has been suggested that its upregulation represents a prolonged response to long-term oxidative damage of the central nervous system, likely involving peroxynitrite generation (51). The authors also suggest that similar increases of Prxs could occur in other inflammatory neurodegenerative disorders. Accordingly, motor neuron death in SOD1-associated familial amyotrophic lateral sclerosis cases has been attributed (at least partially) to nerve growth factor-p75 neurotrophin receptor–related superoxide anion and peroxynitrite formation (89). Because nuclear factor erythroid 2–related factor 2 (Nrf2) expression increases in nerve growth factor–induced motor neuron apoptosis (90), and Prx 1 is one of the antioxidant enzymes under Nrf2 control (57), it is probable that the modulation of this Prx content could play a relevant pathogenic role in this form of the disease. However, the possible peroxynitrite-reductase activity of Prx 1 has not been investigated.

In addition to this intrinsic modulation of peroxiredoxin content, which would be indicative of the biologic relevance of these enzymes in reactive oxygen and nitrogen species detoxification, the pharmacologic modulation of peroxiredoxin expression in target cells has been successfully used to increase radiosensitivity of selective cancer cells (142).

Reduction of peroxynitrite by glutathione peroxidase (GPx)

The tetrameric enzyme selenium-containing glutathione peroxidase (GPx) (from bovine erythrocytes, a type I GPx), in its reduced form, reacts with peroxynitrite with a rate constant of 8×10^6 $M^{-1}s^{-1}$ at pH 7.4 and 25°C (15). This is a catalytic two-electron peroxynitrite reduction to nitrite (although nitrite has been reported to be formed in only ~50% yields), and the oxidized form of the enzyme is reduced by the highly abundant glutathione, which is in turn maintained at the reduced state by glutathione reductase at NADPH expense (43). The concentration of glutathione peroxidases varies between cellular types,[d] but a value of ~2 μM has been reported in some tissues, and therefore, an apparent k can be calculated as 16 per second at 25°C, and probably twice as high at 37°C (127), indicating that this enzyme should be regarded as an important target for peroxynitrite reactivity at least in tissues with higher glutathione peroxidase expression. *In vivo* evidence supporting the protective effect of GPx on peroxynitrite-mediated cytotoxicity exists, although it is scarce. Deficiency of glutathione peroxidase-1

[c]Apparent rate constant for the reaction between peroxynitrite and some cellular targets for peroxynitrite reactivity at pH 7.4 and 37°C:

a) peroxynitrite and $CO_2 = k_{CO2} \times [CO_2] = 4.6 \times 10^4$ $M^{-1}s^{-1} \times 1.3 \times 10^{-3}$ $M = 60$ per second
b) peroxynitrite and Prx 5 $= k_{Prx5} \times$ [Prx5] $= 1.2 \times 10^8$ $M^{-1}s^{-1} \times 1 \times 10^{-6}$ $M = 120$ per second
c) peroxynitrite and GSH $= k_{GSH} \times$ [GSH] $= 1{,}350$ $M^{-1}s^{-1} \times 5 \times 10^{-3}$ $M = 6.8$ per second

Thus, it could be expected that in those cells expressing Prx 5, its reaction with peroxynitrite would be an important route for peroxynitrite detoxification. Other Prxs react with peroxynitrite at lower rates, but this fact is frequently compensated for by higher intracellular concentrations (127).

[d]Estimated cellular concentrations of GPx vary from 0.1 to 0.2 μM in a glioma cell line and red blood cells, respectively (69–71) to 5.8 μM for rat liver cytosolic GPx-1 (114).

Table 2. Reactions Between Peroxynitrite and Selected Heme-Containing Proteins

Heme protein	*k* ($M^{-1}s^{-1}$)	*Conditions*	*Products*	*Reference*
Myeloperoxidase	2×10^7	12°C, pH independent	Compound II and $\cdot NO_2$	39
Lactoperoxidase	3.3×10^5	25°C, pH 7.4	Compound II and $\cdot NO_2$	39
Horseradish peroxidase	3.2×10^6	25°C, pH independent	Compound I and NO_2^-	39
Fe^{II} cytocrome c	2.3×10^5	25°C, pH independent	Fe^{III} cytocrome c and $\cdot NO_2$	118
Oxyhemoglobin	1×10^4	25°C, pH 7.4	Methemoglobin, $O_2.^-$ and NO_3^-	102
Metmyoglobin	7.7×10^4	20°C, pH 7.0	Metmyoglobin and NO_3^-	48
M. tuberculosis catalase-peroxidase	1.4×10^5	37°C, pH 7.4	Compound II and $\cdot NO_2$ (most likely)	135
Chloroperoxidase	3.8×10^6	23°C, pH 7.1	Compound II and $\cdot NO_2$	42
PGHS-1	1.7×10^7	8°C, pH 7	Compound I and NO_2^-	125
iNOSoxy	2.2×10^5	pH 7.4	Compound II-like heme and $\cdot NO_2$	71
Cytochrome *c* oxidase	$> \sim 10^6$	RT, pH 7.4	Two-electron oxidation and NO_2^-	88

RT, room temperature.

has been shown to accelerate the progression of atherosclerosis in apolipoprotein E–deficient mice, and increased tyrosine nitration (as marker of peroxynitrite production), mostly in and around macrophage-derived foam cells, was detected in those animals, suggesting that peroxynitrite might be involved in the evolution of the atherosclerotic lesions and that GPx-1 contributes to its detoxification in this model system (121). However, it has also been reported that whereas glutathione peroxidase 1 protects against apoptosis induced by superoxide, the enzyme promoted apoptosis induced by peroxynitrite in murine hepatocytes (40), and therefore, the biologic role of GPx in peroxynitrite decomposition is still a matter of debate and requires further investigation.

Other selenium-containing proteins have been reported to contribute to peroxynitrite reduction (109, 122). However, kinetic data for those reactions are not available, and most studies rely on the protective effect of the proteins on peroxynitrite-dependent target oxidations, which are often not directly performed by peroxynitrite itself, therefore complicating the interpretation of the experimental data. The roles of other types of selenium-containing or non–selenium-containing GPx in peroxynitrite reduction have not been addressed.

Peroxynitrite decomposition by heme proteins

Peroxynitrite reacts with heme proteins, by mechanisms and at rates that differ depending on the protein. Efficiency of heme proteins in reacting with peroxynitrite is relative, because although some of the reported rate constants are quite high, those proteins are usually localized at specific compartments, transforming their reactions with peroxynitrite into events of local importance. Mechanisms of reduction of peroxynitrite-oxidized heme proteins usually cannot be regarded as means of catalytic peroxynitrite detoxification. Moreover, in many cases, including the neutrophil myeloperoxidase, highly abundant at sites of acute inflammation, peroxynitrite reduction leads to the formation ferryl species as well as $\cdot NO_2$, two oxidant species (Table 2) (39)

$$MPO\ (Fe^{III}) + ONOOH \rightarrow \text{compound II}\ (Fe^{IV}{=}O) + \cdot NO_2 \quad (1)$$

The most accepted mechanism of this reaction (as well as for other peroxynitrite-mediated peroxidase oxidation to compound II) involves the initial formation of a complex between peroxynitrite and the enzyme as the rate-limiting step in the reaction (41, 42).

In the case of hemoglobin, its reaction with peroxynitrite leads mainly to its isomerization to nitrate

$$HbFe^{II}O_2 + ONOOH \rightarrow HbFe^{III} + O_2\cdot^- + NO_3^- \quad (2)$$

Taking into account the relatively high rate constant for this reaction as well as its millimolar intracellular concentration, oxyhemoglobin should be considered an important route of peroxynitrite decay in these cells (102). Similar reactions with other heme-containing proteins have been proposed to occur in defined cell types (48).

Although reduced cytochrome oxidase has been shown to catalyze peroxynitrite reduction *in vitro*, the rate constant for this reaction (Table 2) is about two orders of magnitude slower than that with oxygen, and therefore, its importance *in vivo* is uncertain under most physiologic conditions (88).

Peroxynitrite Detoxification by Drugs

Different pharmacologic approaches have been used in an attempt to decrease peroxynitrite-mediated cytotoxicity in those conditions in which increased peroxynitrite formation is considered to perform a pathogenic role.

Manganese or iron porphyrins

Metal porphyrins are redox catalysts widely used in synthetic organic chemistry (78). In particular, manganese and iron porphyrins found their way into biologic applications in the search for catalysts able to dismute superoxide in the early 1980s (87, 91). Reactions with other biologically relevant reactive species were encountered later, and reactions with peroxynitrite began to be considered of importance in the mid 1990s. Most water-soluble manganese and iron porphyrins are stable in air-equilibrated aqueous solution with the metal in oxidation state III, and this naturally first led to study the reactions of Mn^{III} and Fe^{III} porphyrins with peroxynitrite. It was noticed early that Mn^{III}Porphyrins enhanced peroxynitrite-mediated oxidations such as DNA strand breaks and tyrosine nitration (21, 34, 45).

Table 3. Rate Constants for the Reduction of Peroxynitrite by Selected Metal Porphyrins

*Porphyrin**	k ($M^{-1}s^{-1}$)	*Reference*
Mn^{III}TCPP	1×10^5	37
Mn^{III}TSPP	3.6×10^5	37
Mn^{III}TM-4-PyP	4.0×10^6	36
Mn^{III}TM-2-PyP	3.1×10^7	37
Mn^{II}TM-4-PyP	$>5.0 \times 10^6$	†
Mn^{II}TM-2-PyP	$>1.0 \times 10^7$	35
Fe^{III}TMPS	6.5×10^5	113
Fe^{III}TM-4-PyP	2.2×10^6	113
FP15	3.2×10^6	116

All data at 37°C and pH 7.4.

*Ligand names: TCPP, meso tetrakis (4-carboxylatophenyl) porphyrin; TSPP, meso tetrakis (4-sulfonatophenyl) porphyrin; TM-4-PyP, meso tetrakis ((N-methyl)pyridinium-4-yl) porphyrin; TE-2-PyP, meso tetrakis ((N-ethyl)pyridinium-2-yl) porphyrin; TMPS, meso tetrakis (2,4,6-trimethyl-3,5-sulfonatophenyl) porphyrin, FP15 is iron(III) meso tetrakis (N-triethylene glycol monomethyl ether) pyridinium-2-yl porphyrin.

†Ferrer-Sueta G., unpublished results.

Mn^{III} and Fe^{III} porphyrins effectively react with peroxynitrite in a reaction that, strictly speaking, results in the reduction of peroxynitrite, but increased oxidation is nevertheless observed. The reason lies in the yield and selectivity of the reaction products. The reaction of Mn^{III}Porphyrins with peroxynitrite produces the corresponding oxo manganese(IV) complex and $\cdot NO_2$:

$$Mn^{III}Porphyrin + ONOO^- \rightarrow O{=}Mn^{IV}Porphyrin + {}^{\cdot}NO_2 \quad (3)$$

Reaction (3) produces two strong oxidants, just as the proton or carbon dioxide catalyzed homolyzes, but it is quantitative in contrast with the 30–35% yield of radicals previously mentioned. Additionally, the reactivity of $O{=}Mn^{IV}$Porphyrin is notoriously different from that of $\cdot OH$ or $CO_3\cdot^-$, so it can be construed that Mn^{III}Porphyrin can divert rather than prevent oxidative damage arising from peroxynitrite. If this diversion consumes an easily replenishable or otherwise expendable reductant and spares a more important target, then the observed outcome would be protection. The reaction is made catalytic by a reduction step that reforms the initial Mn^{III}Porphyrin.

$$O{=}Mn^{IV}Porphyrin + RedH \rightarrow Mn^{III}Porphyrin + Red^{\cdot} \quad (4)$$

This catalytic cycle has been studied (36, 63) and can be quite efficient if the proper reductant is present. In addition, the reactivity of different Mn^{III}Porphyrin toward peroxynitrite is diverse, with rate constants ranging from 10^5 to 10^7 $M^{-1}s^{-1}$ at 37°C (36, 37). The combined Mn^{III}Porphyrin with reductants has been used in *ex vivo* experiments studying the oxidation of low-density lipoprotein (124).

Despite the high rates of peroxynitrite reduction that can be achieved by using the fastest studied Mn^{III}Porphyrin [$k = 3.1 \times 10^7$ $M^{-1}s^{-1}$ (37)] coupled to reduction by urate ($k = 1 \times 10^6$ $M^{-1}s^{-1}$), it must be kept in mind that $\cdot NO_2$ and urate-derived radicals are also produced during the catalytic cycle, which will provide adequate protection only to species resistant to these radicals.

Fe^{III}Porphyrins also are able to reduce peroxynitrite in a reaction analogous to Eq. 3, the rate constants of the studied porphyrins are somewhat lower than those for manganese, ranging from 10^5 to 10^6 $M^{-1}s^{-1}$ at 37°C (54, 113). Also like the manganese complexes, the oxidized oxo-Fe^{IV} intermediate can be reduced back, completing a catalytic cycle. The reductive step is mentioned in the literature as made possible by ascorbate, with rate constants ranging from 10^5 to 10^7 $M^{-1}s^{-1}$ (54, 63); no data have been presented for other reductants.

In general, Fe^{III}Porphyrins have been regarded more as catalyzing the isomerization of peroxynitrite to nitrate, and their potentially important role as peroxynitrite reduction catalysts has not been described in detail. Remarkably, the isomerization of peroxynitrite is reportedly detectable only when peroxynitrite is in excess over Fe^{III}Porphyrins (63), which rules out its importance *in vivo*, where this condition is extremely unlikely.

An interesting variation of the peroxynitrite reduction is achieved with porphyrins with manganese in an oxidation state (II); this reaction occurs with the exchange of two electrons, avoiding the formation of $\cdot NO_2$:

$$Mn^{II}Porphyrin + ONOO^- \rightleftharpoons ONOO - Mn^{II}Porphyrin \rightarrow O{=}Mn^{IV}Porphyrin + NO_2^- \quad (5)$$

Reaction (5) is at least as fast as reaction (3) for the three Mn^{II}Porphyrins studied so far. The actual rate constants have not been determined because the reaction occurs with formation of an intermediate, and the second step becomes rate limiting at relatively low peroxynitrite concentrations. Nevertheless, a lower limit of 10^7 $M^{-1}s^{-1}$ has been put forward (35).

One point in favor of the biologic relevance of Mn^{II}Porphyrins instead of Mn^{III}Porphyrins as peroxynitrite reductants is that some of the MnPorphyrins can be reduced *in vivo* with relative ease either by low-molecular-weight reductants, such as glutathione (32) or ascorbate (110), or enzymatically by a number of flavoenzymes, including components of the mitochondrial electron-transport chain (32, 35, 56).

It is extremely important, when considering metal porphyrins as potential peroxynitrite reductants, that not all of them are equally fast in their redox reactions. In this regard, both reactions of the catalytic cycle must be considered. If a $Mn^{III}/O{=}Mn^{IV}$ cycle is chosen, then the reduction of peroxynitrite is very sensitive to the identity of the porphyrin (see Table 3), whereas reduction of $O{=}Mn^{IV}$Porphyrin is almost independent if urate or glutathione are used as reductants (36). Conversely, if the cycle considered involves $Mn^{II}/O{=}Mn^{IV}$, the reduction step from Mn^{III} to Mn^{II} seems to be much more influenced by both the porphyrin and the reductant. For instance, succinate dehydrogenase/succinate reduce sMn^{III}TE-2-PyP 10 times faster than Mn^{III}TM-4-PyP, whereas with xanthine oxidase/hypoxanthine, the ratio of $t_{1/2}$ of reduction is 1:1.5:1,000 for Mn^{III}TE-2-PyP, Mn^{III}TM-4-PyP, and Mn^{III}TSPP, respectively; finally, reduction by submitochondrial particles by using NADH is 100 times faster for Mn^{III}TE-2-PyP than for Mn^{III}TSPP. In summary, the trend follows the redox potential of the Mn^{III}/Mn^{II} couple (8), and the higher-potential couples are reduced faster, but the ratios of rates are also very dependent on the reductant used.

In terms of kinetic competition, the fastest-reacting Mn^{III}-Porphyrins seem able to provide noticeable reduction of peroxynitrite, as they have rate constants comparable to those of Prxs and have been found at significant concentrations in mitochondria. In particular, Mn^{III}TE-2-PyP has been found

in the mitochondrial fraction of cultured cells treated with 200 μM for 90 min; the reported concentration (2.5 ng/mg protein) can be calculated as ~16 μM, assuming a mitochondrial volume of 1.2 μl/mg protein (65). Furthermore, in mice, the mitochondrial fraction of heart tissue contained 5 μM Mn^{III}TE-2-PyP 4 or 7 h after one i.p. injection containing 10 mg/kg of the porphyrin (111). The concentration and rate constants for Mn^{III}TE-2-PyP would thus indicate that the porphyrin would be providing a significant acceleration in the reduction of peroxynitrite, an apparent k between 150 and 460 per second. Conversely, Mn^{III}TCPP was also used and quantitated in the same cell-culture experiment and reached similar micromolar concentrations after treatment with 100 μM (65), but could not efficiently compete with other biologic reductants of peroxynitrite, as its k_{app} would be ~1 per second.

Remarkably, Mn^{III}TCPP is one of the porphyrins most widely and successfully used in models of protection against oxidative stress (24), but has proven to be the slowest in its reaction with peroxynitrite or superoxide. Additionally, no biologic reductant has been reported to be able to reduce this compound. It is then possible that some other yet-unknown mechanisms might be at play for the biologic activity of this and other porphyrins.

The general question about the mechanism by which metal porphyrins exert protection in a number of models is not fully answered, but kinetic studies support catalysis of peroxynitrite reduction as an important contribution in the case of MnTE-2-PyP and compounds of similar reactivity (Table 3).

Other mechanisms, not strictly related to catalytic scavenging of reactive species, have been proposed and begun to be explored, like inactivation of HIF1 (79), inhibition of NF-κB binding to DNA (130), and inactivation of AP1 (145). Some studies even show some nonredox mechanisms that may be at play in certain models in which metal porphyrins have been used successfully, including the induction of heme oxygenase (61), modulation of Ca^{2+} metabolism (117) and modulation of protein expression (104).

Organoselenium compounds

The design and application of antioxidant catalysts that mimic the catalytic activity of the selenoenzyme glutathione peroxidase started before the discovery of its peroxynitrite-reduction effects, and was related to the known role of the different isoenzymes in hydroperoxide detoxification. In 1996, it was reported that ebselen [2-phenyl-1,2-benzoisoselenazol-3(2H)-one], a lipid-soluble seleno-organic compound that exhibits glutathione peroxidase–like activity *in vitro*, reacted with peroxynitrite (14, 108, 109) with a second-order rate constant of 2×10^6 $M^{-1}s^{-1}$ (74, 75). The compound catalytically reduces peroxynitrite to nitrite, and the corresponding selenoxide is formed, which is then reduced either by glutathione or by the selenoprotein thioredoxin reductase (4) ($k > 2 \times 10^7$ $M^{-1}s^{-1}$) (144) at NADPH expense. Ebselen exhibits antiinflammatory activity in a number of experimental models, some of which have been ascribed to peroxynitrite formation (18, 55, 139), and has been developed for clinical use (phase III). As an example, the compound reduced nitration and restored voltage-gated potassium channel function in small coronary arteries of diabetic rats, which exhibit enhanced peroxynitrite formation (18). However, it has been also reported that in cellular systems, ebselen is present as thiol adducts and thus loses its high reactivity toward peroxynitrite (23). Moreover, several antiinflammatory actions of ebselen, independent of its glutathione-peroxidase–like activity, have been described (96, 107).

Other organoselenium compounds were also reported to react with peroxynitrite, although kinetic determinations for those reactions are frequently incomplete (14, 22). For instance, bis(2-aminophenyl)-diselenide and ebselen had very similar protective effects against peroxynitrite-induced oxidative/nitrative damage to human plasma proteins and lipids (84).

Flavonoids, tyrosine- containing peptides and nitroxides: indirect contribution to peroxynitrite detoxification

It has been reported that several flavonoids inhibit different peroxynitrite-dependent target oxidation processes (3, 5, 46, 76, 106, 132, 133). However, they do not increase the peroxynitrite decomposition rate, indicating that they do not react directly with peroxynitrite (at least for those flavonoids whose reactivity with peroxynitrite has been kinetically addressed) (119). Indeed, some confusion exists in the literature, because from competitive kinetics analysis, a direct fast reactivity between peroxynitrite and some flavonoids has been reported (60), which probably reflects the ability of flavonoids to react with peroxynitrite-derived radicals. In many cases, the peroxynitrite-scavenger activity of flavonoids has been addressed by their inhibitor effects on tyrosine nitration. However, tyrosine nitration is not produced directly by peroxynitrite, but by peroxynitrite-derived radicals, either in the presence or absence of CO_2, or by direct one-electron reduction of peroxynitrite by metalloproteins, forming an oxidized form of the metal, which promotes the one-electron oxidation of tyrosine that recombines with $\cdot NO_2$. Accordingly, $CO_3\cdot^-$ and $\cdot NO_2$ have been shown to react very rapidly (~10^8 $M^{-1}s^{-1}$) with different flavonoids (143), which can also reduce oxidized hemeperoxidases (62), therefore providing a mechanism of inhibition of indirect peroxynitrite-dependent reactions either *in vitro* or *in vivo*.[e] Flavonoids have also been reported to inhibit PMN NADPH oxidase, thus reducing peroxynitrite formation rates at sites of inflammation by inhibiting the formation of one of its precursors, superoxide radical (94). In addition, many of the antiinflammatory actions of flavonoids are related to modulation of transcription factors and subsequent increase in antioxidant defenses, as well as the inhibition of protein kinases involved in signal transduction [review in (44)].

Tyrosine-containing peptides have been proven to inhibit apoptosis and nitrotyrosine formation in primary motor neuron cultures exposed to exogenous peroxynitrite or trophic-factor deprivation and subsequent endogenous peroxynitrite formation. However, and in a similar way as flavonoids, the

[e]It should be taken into account that both $CO_3\cdot^-$ and $\cdot NO_2$ rapidly react with other compounds like glutathione and several amino acidic residues in proteins, naturally present at high concentrations (> mM) in different cellular compartments, and thus kinetic considerations indicate that scavenging of those radicals by either flavonoids, peptides, or nitroxides is not very likely under most physiologic conditions.

peptides did not react directly with peroxynitrite, and their protective actions have been ascribed to their capacity to scavenge peroxynitrite-derived radicals (141). Protection afforded by membrane-permeable nitroxide radical Tempol (4-hydroxy-2,2,6,6,tetramethyl-1-piperidinyloxy) in animal or cellular models of oxidative stress involving peroxynitrite formation are also related to the ability of this compound to trap $CO_3\cdot^-$ and $\cdot NO_2$[6] and subsequent reactions that lead to an increase in protein cysteine nitrosation (33). Other compounds, such as mitochondrial-targeted ubiquinol (53) and carboxy-proxyl (30), may also exert part of their antioxidant and cytoprotective actions via scavenging of peroxynitrite-derived radicals (20).

Concluding Remarks

Multiple physiologic routes for peroxynitrite detoxification have been reported to occur in biologic systems. Among them, *in vitro* experiments indicated that the ubiquitous antioxidant enzymes Prxs catalytically reduce peroxynitrite at thioredoxin (or other reducing substrate) expense. Moreover, modulation of Prx content in cellular systems affected peroxynitrite-mediated cytotoxicity, and Prx expression was reported to change in pathologic conditions associated with increased peroxynitrite formation, thus indicating a physiologic role for these enzymes in peroxynitrite reduction. GPx has also been reported to catalyze peroxynitrite reduction, but its role *in vivo* is still a matter of debate. Other proteins, like hemoglobin or myoglobin, may represent important routes for peroxynitrite decomposition in selected cellular systems. Moreover, pharmacologic approaches to reduce peroxynitrite-mediated toxicity have been successfully used. In the case of manganese or iron porphyrins, the mechanism of their reaction with peroxynitrite and subsequent reduction by endogenous reductants has been extensively studied, and their protective role *in vivo* confirmed in multiple biologic systems. In the case of ebselen, its role in protecting against peroxynitrite-mediated cytotoxicity is less well established. Flavonoids, nitroxides, and tyrosine-containing peptides have also been demonstrated to reduce peroxynitrite-mediated toxicity under different conditions, but they do not react directly with peroxynitrite, and their mechanism of action is at present not completely resolved.

In most cellular types, mitochondria represent a main site of superoxide anion and hence, peroxynitrite formation. Moreover, mitochondrial damage with increased peroxynitrite generation have been shown to contribute to the pathology of many diseases, including neurodegenerative disorders, diabetes, and ischemia–reperfusion injury [for a review, see (115)]. Thus, raising the expression levels of mitochondrial enzymes with peroxynitrite reductase activity or targeting antioxidant compounds with peroxynitrite-detoxification capabilities toward mitochondria would be potential therapies for those pathologies. Indeed, several mitochondria-targeted compounds like manganese porphyrins, nitroxides, and quinones have been developed and successfully used in animal models of pathologies associated with oxidative stress (82). However, unambiguously to determine whether the protective effect observed in some of those models is related to peroxynitrite detoxification requires further investigation.

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Abbreviations

AhpC, alkyl hydroperoxide reductase C; $CO_3\cdot^-$, carbonate radical; ebselen, 2-phenyl-1,2-benzoisoselenazol-3(2H)-one; GPx, glutathione peroxidase; Mn^{III}TCPP, manganese(III) meso tetrakis (4-carboxylatophenyl) porphyrins; Mn^{III}TE-2-PyP, manganese(III) meso tetrakis [(*N*-ethyl)pyridinium-2-yl] porphyrin; Mn^{III}TM-4-PyP, manganese(III) meso tetrakis [(*N*-methyl)pyridinium-4-yl] porphyrin; Mn^{III}TSPP, manganese(III) meso tetrakis (4-sulfonatophenyl) porphyrin; Nrf2, nuclear factor erythroid 2–related factor 2; ·NO, nitric oxide; $\cdot NO_2$, nitrogen dioxide; NO_2^-, nitrite; NO_3^-, nitrate; ·OH, hydroxyl radical; $ONOO^-$, peroxynitrite anion; ONOOH, peroxynitrous acid; Prx, peroxiredoxin; $O_2\cdot^-$, superoxide radical; Tempol, 4-hydroxy-2,2,6,6,tetramethyl-1-piperidinyloxy; TXNPx, tryparedoxin peroxidase.

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