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Review

Peroxynitrite: mediator of the toxic action of nitric oxide

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Peroxynitrite (oxoperoxonitrate(-1)), anion of peroxynitrous acid, is thought to mediate the toxic action of nitric oxide and superoxide anion. Peroxynitrite is formed in a fast reaction between these species, reacts with all classes of biomolecules, is cytotoxic, and is thought to be involved in many pathological phenomena. Its main reactions involve one- and two-electron oxidation and nitration. Protein nitration is often used as a footprint of peroxynitrite reactions *in vivo*. Nitration of tyrosine and of tyrosyl residues in proteins may be an important mechanism of derangement of biochemical signal transduction by this compound. However, apparently beneficial effects of peroxynitrite have also been described, among them formation of nitric oxide and nitric oxide donors in reactions of peroxynitrite with thiols and alcohols.

Two main roles have been ascribed to nitric oxide ($NO^{\cdot}$; name recommended by IUPAC: nitrogen monoxide). Apart from being an autocrine and paracrine mediator of homeostasis, nitric oxide has been found to inflict damage on important biomolecules and suggested to contribute to the cytocidal action of macrophages and to many pathological events [1–4]. The toxic effects of nitric oxide have often been ascribed to the presence of this molecule at relatively high concentrations as opposed to the signaling action of low concentrations of $NO^{\cdot}$. However, it seems more likely that the toxic action of nitric oxide is not a merely concentration effect but is mediated by other reactive species derived from $NO^{\cdot}$. The most extensively studied species of this type, believed presently to be the main mediator of the harmful actions of $NO^{\cdot}$, is peroxynitrite.

FORMATION OF PEROXYNITRITE

The reaction of nitric oxide with the superoxide radical anion leading to the formation of peroxynitrite

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

is very rapid and close to the diffusion limit ($k = (4.3–6.7) \times 10^9\ M^{-1}\ s^{-1}$) [5].

The rate constant for the reaction of superoxide with nitric oxide is even higher than that for the reaction of superoxide with superoxide dismutase ($2.3 \times 10^9\ M^{-1}\ s^{-1}$). Nevertheless, under

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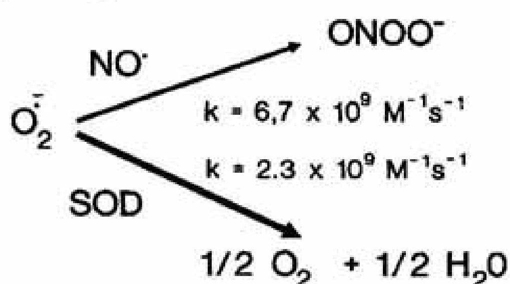
Abbreviations: ABTS, 2,2'-azino-bis(3-ethyl-1,2-dihydrobenzothiazoline-6-sulfonate; nNOS, neuronal nitric oxide synthase; PMN, polymorphonuclear leukocytes; SOD, superoxide dismutase.

physiological conditions superoxide is more likely to react with superoxide dismutase (SOD) than with $NO^{\cdot}$ (Scheme 1). The value of the superoxide target area (a measure of probability of reaction with the target, equal to the rate constant times concentration of the target) is $6.7 \times 10^2\ s^{-1}$ in the case of nitric oxide (assuming the $NO^{\cdot}$ concentration of 0.1 μM typical of its signaling function and the higher value of the rate constant reported) and $2.3 \times 10^4\ s^{-1}$ for SOD (assuming a typical intracellular SOD concentration of 10 μM). However, when the nitric oxide concentration reaches 10 μM in the vicinity of activated phagocytes [6], the target area for $NO^{\cdot}$ becomes $6.7 \times 10^4\ s^{-1}$, i.e. higher than that for SOD. Thus, nitric oxide may become the main target for the reactions of superoxide, provided the $NO^{\cdot}$ concentration is high enough.



Scheme 1. Two main reactions of superoxide: with superoxide dismutase (SOD) and with nitric oxide. The rate constant for the reaction with nitric oxide is higher but under physiological conditions superoxide reacts mainly with SOD which is present at higher concentrations.

The formation of peroxynitrite was reported to occur in many cell types including macrophages [7], neutrophils [8], Kupffer cells [9] and cultured endothelial cells [10]. Activated macrophages were estimated to produce 0.11 nmol peroxynitrite per million cells per minute which suggests that most of the nitric oxide produced by activated macrophages was converted to peroxynitrite [11]. Considering that approx. 10^7 alveolar macrophages reside in about 1 μl of rat lung epithelial lining fluid, it can be calculated that if all alveolar macrophages are activated in a rat lung, the local production of peroxynitrite can reach 1 mM/ /min. Inside of a phagocytic vesicle the rate of production of peroxynitrite may be even greater [12]. In the immunostimulated J774 macrophage cell line peroxynitrite production was about 5 pmol/(million cells × min) [13]. Activated human polymorphonuclear leukocytes were found to produce about 14 pmol peroxynitrite/(million cells × hour) [14]. Nitric oxide synthase can itself be a source of peroxynitrite as it releases superoxide under some circumstances. Purified neuronal nitric oxide synthase (nNOS) was demonstrated to produce superoxide at low L-arginine concentrations. Intracellular superoxide formation by the enzyme was observed in nNOS-transfected human kidney 293 cells upon L-arginine depletion [15]. This phenomenon may have pathophysiological relevance in such conditions as ischemia and wound healing when lack of perfusion could lead to tissue L-arginine depletion.

Under oxidative stress conditions formation of peroxynitrite may be further augmented due to upregulation of the inducible form of nitric oxide synthase by superoxide [16]. Enhanced peroxynitrite formation was suggested to occur in bovine lung endothelial cells in response to tumor necrosis factor α [17].

Cigarette smoke and other products of combustion are rich sources of peroxynitrite. Cigarette smoke contains large amounts of nitric oxide (about 10^{14} molecules per puff) and of superoxide anions, derived from redox cycling of semiquinones [18, 19].

Various reactions have been used for the preparation of peroxynitrite for research purposes, including (i) reaction of ozone with azide ions in alkaline medium, (ii) autoxidation of hydroxylamine in alkaline medium, (iii) reaction of nitrite with acidified hydrogen peroxide followed by quenching with an alkaline solution, (iv) reaction of hydrogen peroxide with 2-oxoethyl nitrite in alkaline medium, (v) reaction of nitric oxide with hydrogen peroxide in alkaline medium, (vi) reaction of nitric oxide with solid potassium superoxide, (vii) reaction of nitrous oxide with tetramethylammonium superoxide in liquid ammonia, (viii) UV photolysis of solid potassium nitrate, and (ix) reaction of hydrogen peroxide with isoamyl nitrite in a two-phase system ([20, 21] and references therein).

PROPERTIES AND REACTIONS OF PEROXYNITRITE

The peroxynitrite anion (name recommended by IUPAC: oxoperoxonitrate(-1)) is relatively

stable in basic solutions (pH> 12) and can be stored frozen for weeks. Its solutions have a yellow color; peroxynitrite concentrations are usually determined on the basis of absorbance at 302 nm (ε = 1670 M^{-1} cm^{-1}) [22]. At physiological pH peroxynitrite becomes protonated to give peroxynitrous acid (recommended name: hydrogen oxoperoxonitrite(-1))

$$H^+ + ONOO^- \Leftrightarrow ONOOH \qquad pK_a = 6.8\ [23] \quad (2)$$

which rearranges to form nitrate with a half-life near 1 s at neutral pH in the absence of oxidizable substrates (Scheme 2).

The rate constant for the decomposition of peroxynitrous acid was reported to be 0.64 s^{-1} at 37°C, pH 7.4, and to increase with decreasing pH to reach a maximum of 4.0 s^{-1} at pH 5.0 [23].

Compared to the anion, peroxynitrous acid is generally more reactive [24] and may be responsible for most of the cytotoxic reactions attributed to peroxynitrite. Its main reactions include one- and two-electron oxidations and nitration (Scheme 2). Unlike either of its precursors ($NO^·$ and $O_2^{\bar{·}}$), peroxynitrite is a strong oxidant and nitrating agent. Initially it was postulated that the O–O bond of the peroxynitrous acid can be cleaved, either homolytically

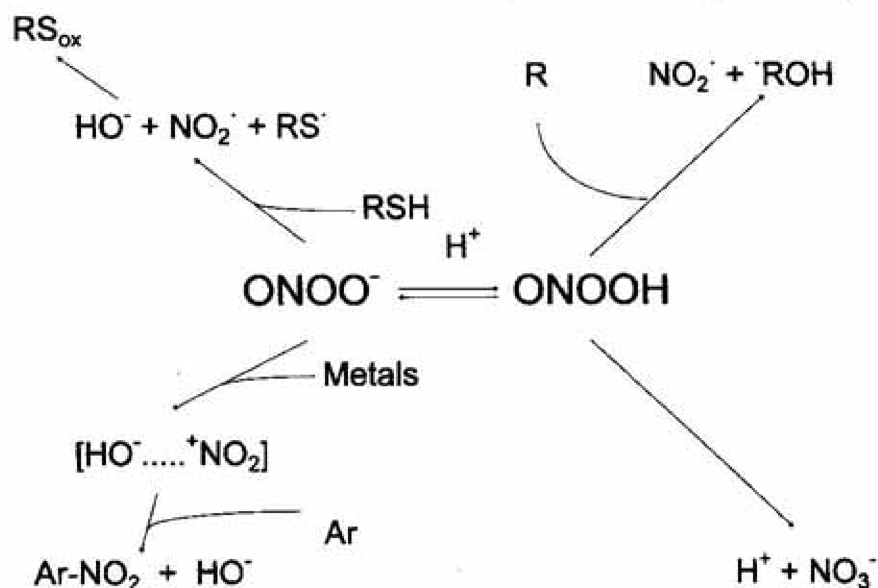
$$HOONO \Leftrightarrow HO^· + NO_2^· \qquad (3)$$

or heterolytically

$$HOONO \Leftrightarrow HO^- + NO_2^+ \qquad (4)$$

Reaction (3) yielding two strong oxidants, hydroxyl radical and nitrogen dioxide, would be responsible for oxidation while reaction (4) for generation of a strong nitration agent and nitration reactions of peroxynitrite. $HO^·$ is the strongest oxidant formed in biological systems; $NO_2^{\bar{·}}$ is also a potent oxidant capable of initiating lipid peroxidation and of nitrosylating aromatic amino acids. Metal ions appear to be necessary to catalyze the heterolytic cleavage of peroxynitrous acid (reaction 4) to form a nitronium-like species because the initial separation of charge requires a high activation energy [23].

In an apparent agreement with the postulated reaction (3), peroxynitrite initiates the polymerization of methyl acrylate, known to be initiated by free radicals, and oxidizes such substrates as dimethylsulfoxide, luminol, hydrogen peroxide and 2,2'-azino-bis(3-ethyl-1,2-dihydrobenzothiazoline-6-sulfonate) (ABTS) in reactions which are zero-order with respect to substrate, characteristic of reactions mediated by reactive radicals such as hydroxyl radical [26–28]. On the other hand, although peroxynitrite produces ethylene upon oxidation of methionine and 2-keto-4-thiomethylbutanoic acid, these reactions typical for the hydroxyl radical are not affected by hydroxyl radical scavengers such as benzoate, mannitol and dimethylsulfoxide [29]. Decomposition of peroxynitrite was reported to generate spin trap adducts with 5,5'-dimethyl-1-pyrroline-N-



Scheme 2. Main reactions of peroxynitrite.
Peroxynitrite can oxidize and nitrate substrates. Upon protonation peroxynitrous acid is formed which has oxidizing (and hydroxylating) properties, and isomerizes to nitric acid. R, substrate; RSH, oxidized substrate; R_{ox}, oxidized substrate; Ar, aromatic substrate. After [25].

oxide (DMPO) typical of ·OH radical [30]. However, this result cannot be used as a proof of ·OH formation during peroxynitrite decomposition since other mechanisms of formation of this adduct, not requiring the presence of ·OH, should be also considered. Other authors did not observe formation of the ·OH-type adduct on peroxynitrite decomposition in spin trapping experiments [31, 32]. Moreover, thermodynamic and kinetic arguments suggest that peroxynitrous acid cannot undergo homolysis to form HO· and $NO_2^{\cdot}$, especially if *trans*-peroxynitrous acid is the intermediate in this reaction [23, 30]. Such a reaction requires energy input of about 85 kJ/mol to occur [23] while the reverse reaction i.e.

$$NO_2^{\cdot} + {}^{\cdot}OH \rightarrow ONOOH \quad (5)$$

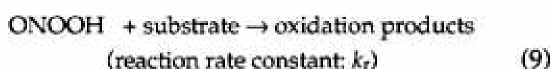
is known to proceed spontaneously with a rate constant of $4 \times 10^9\ M^{-1}\ s^{-1}$ [33].

It has therefore been postulated that the rearrangement of HOONO to nitric acid involves a high-energy intermediate HOONO*. This activated species is less reactive than the hydroxyl radical but more reactive than the ground-state peroxynitrous acid form. HOONO* reacts with substrates to form products that are similar to those formed by hydroxyl radical [27, 28]. According to this idea,

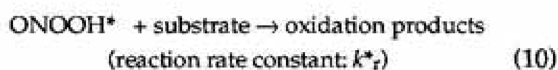
$$H^+ + ONOO^- \Leftrightarrow ONOOH \quad (6)$$

$$ONOOH \Leftrightarrow ONOOH^* \quad (7)$$

$$ONOOH^* \rightarrow NO_3^- + H^+ \quad (8)$$



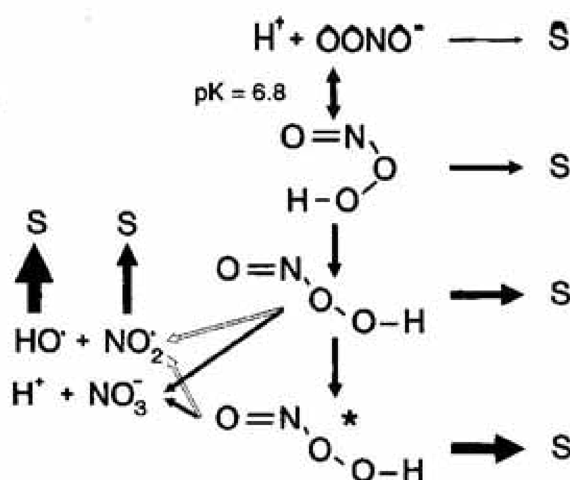
$$ONOOH + \text{substrate} \rightarrow \text{oxidation products (reaction rate constant: } k_r) \quad (9)$$



$$ONOOH^* + \text{substrate} \rightarrow \text{oxidation products (reaction rate constant: } k^*_r) \quad (10)$$

and $k^*_r >> k_r$. Ground-state peroxynitrous acid is in the *cis* conformation; most authors identify ONOOH* with *trans*-peroxynitrous acid which is a higher-energy conformation of this molecule, though some assume that it is a vibrationally excited form of *trans*-peroxynitrous acid [34]. This idea of peroxynitrite reactivity is nowadays generally but not unequivocally accepted [23, 34] (Scheme 3).

Peroxynitrous acid can oxidize substrates *via* the one-electron or two-electron pathways.



Scheme 3. Reaction mechanisms of peroxynitrite. Protonation of peroxynitrite yields ground-state (*cis*-) peroxynitrous acid which may isomerize to *trans*-peroxynitrous acid. Some authors assume that *trans*-peroxynitrous acid is the reactive intermediate while others identify the reactive intermediate (*) with vibrationally excited *trans*-peroxynitrous acid. *Trans*-peroxynitrous acid may isomerize to nitrate; some authors, as reviewed in [20, 23], believe it may decompose to hydroxyl radical and nitrogen dioxide (albeit with small yield; outlined arrows). All these forms can react with substrates (S). After [34].

Both respective redox potentials are high:
$E^{o\prime}$ (ONOOH, $2H^+/NO_2^{\cdot}$, H_2O) = 1.4 V
$E^{o\prime}$ (ONOOH, $2H^+/NO_2^-$, H_2O) = 0.99 V [23]. It has been suggested that ground-state HOONO and HOONO* react by different mechanisms: HOONO reacting with nucleophiles in a bimolecular (possibly SN_2) mechanism, while HOONO* reacts as a one-electron oxidant [29].

Interaction between bicarbonate, present in reaction media and peroxynitrite seems important for peroxynitrite reactivity. Bicarbonate protects *E. coli* cells against peroxynitrite toxicity [12] but enhances peroxynitrite-induced luminol chemiluminescence [27] and oxidation of ABTS as well as nitration of phenol [35] and tyrosine [36]. Apparently, the active species formed in the reaction between peroxynitrite and carbonate is more reactive and more short-lived than peroxynitrite i.e. it decomposes or reacts before reaching cellular target sites (especially the nucleus). This active species might be bicarbonate free radical formed in the reaction:

$$ONOO^- + HCO_3^- \rightarrow HCO_3^{\cdot} + NO_2^{\cdot} + HO^- \quad (11)$$

The bicarbonate radical is known to oxidize luminol and other aromatic and heterocyclic compounds. $ONOO^-$ may also peroxidize bi-

carbonate to peroxybicarbonate, a strong oxidizing species [27]. Another possibility is the generation of the unstable nitrosoperoxycarbonate anion adduct

$$CO_2 + O{=}NOO^- \rightarrow O{=}N\text{-}OOCO_2^- \qquad (12)$$

which can rearrange to give a nitrocarbonate anion $O_2N\text{-}OCO_2^-$. The nitrocarbonate anion was also suggested to be a secondary active species in peroxynitrite reactions [35]. Irrespective of the actual mechanism involved, the bicarbonate content in reaction media may be an important factor which should be taken into account when comparing data on peroxynitrite reactions.

The reaction of peroxynitrous acid with hydrogen peroxide proceeds probably *via* the one-electron pathway and results in liberation of oxygen [37]:

$$ONOOH + H_2O_2 \rightarrow O_2^{\cdot-} + H_2O + NO_2^{\cdot} + H^+ \qquad (13)$$

$$O_2^{\cdot-} + H^+ \Leftrightarrow HO_2^{\cdot} \qquad (14)$$

$$HO_2^{\cdot} + O_2^{\cdot-} + H_2O \Leftrightarrow H_2O_2 + O_2 + HO^- \qquad (5)$$

This reaction was reported to generate singlet oxygen [38].

Peroxynitrite can hydroxylate benzoic acid [39]. It also reacts with a wide range of phenolics including phenol, salicylate and tyrosine (but not *o*-tyrosine), and tyrosine residues in proteins to form nitrotyrosine derivatives [11, 40, 41]. Tryptophan can be also nitrated [42]. The simplest mechanism of this reaction would involve the heterolytic cleavage of peroxynitrous acid into nitronium ion and hydroxyl anion (reaction 4) and nitration of substrates by the nitronium cation [43]. However, nitration is catalyzed by complexes of transition metals (e.g. Fe^{3+}EDTA) so perhaps the actual nitrating agent is a metal-nitronium complex. A role of the product of the reaction between peroxynitrite and CO_2, $OONOCO_2^-$, as an intermediate has also been suggested [44]. Nitration of *p*-hydroxyphenylacetic acid at physiological pH belongs to faster peroxynitrite reactions; its rate constant is $5 \times 10^3\ M^{-1}\ s^{-1}$ at physiological pH. Superoxide dismutase is an efficient catalyst of nitration by peroxynitrite; the enzyme is not inactivated as a result of this reaction [43].

DETECTION OF PEROXYNITRITE

Various assays have been proposed to detect and quantify the production of peroxynitrite. Usually they work well in a test tube containing peroxynitrite as the main reactant. However, as the reactivity of peroxynitrite overlaps those of other oxygen reactive species, one should be careful in applying them to more complex systems.

The most common fluorimetric assay involves oxidation of nonfluorescent dihydrorhodamine 123 to fluorescent rhodamine 123 [45]. Neither superoxide nor nitric oxide alone significantly oxidize dihydrorhodamine 123 but this compound may be oxidized by hypochlorous acid, by hydrogen peroxide in a reaction catalyzed by heme proteins or by horseradish peroxidase alone. Peroxynitrite also oxidizes nonfluorescent 2',7'-dichlorofluorescin to fluorescent 2',7'-dichlorofluorescein [46].

Oxidation of luminol by peroxynitrite induces chemiluminescence which in carbonate buffer have a quantum yield of approx. 10^{-3} [27]. This may be a basis for an assay of peroxynitrite since neither superoxide nor nitric oxide alone induce luminol chemiluminescence. Peroxynitrite can also induce lucigenin chemiluminescence though with a yield equivalent to 25% of that of luminol.

A spectrophotometric assay for peroxynitrite based on the oxidation of *o*-phenylenediamine has also been proposed [46].

Another attitude, more useful in detection of peroxynitrite formation *in vivo*, consists in detection of nitration of protein tyrosine residues. Assuming that nitrogen dioxide is not likely to contribute significantly to protein nitrotyrosine formation *in vivo* (see below), protein nitration can be a useful footprint of peroxynitrite formation in the body and in cell cultures. While absorption spectroscopy is not sensitive and specific enough to detect low levels of nitration, gas chromatography and high performance liquid chromatography may be the methods of choice. Derivatization with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide allows for detection of nitrotyrosine and its major metabolites in plasma and urine.

Immunochemical methods employing polyclonal or monoclonal antibodies against nitrotyrosine allow to detect nitrated tyrosine residues in electrophoregrams or tissue sections [46, 47].

REACTIONS OF PEROXYNITRITE WITH BIOLOGICALLY RELEVANT COMPOUNDS

It has been proposed that superoxide acts as an inactivator of NO˙ since superoxide dismutase, decomposing the superoxide radical anion, prolongs the biological half-life of NO˙ [48–50]. On the other hand, NO˙ was reported to limit the cytotoxicity of superoxide [49, 51, 52]. It seems more important, however, that the reaction between NO˙ and superoxide yields peroxynitrite which, unlike any of its parent molecules, is a strong oxidant.

Peroxynitrite oxidizes a variety of substrates, among them biologically important molecules. The list of peroxynitrite-oxidized substances includes luminol [27], dihydrorhodamine 123, deoxyribose (to malondialdehyde), dimethylsulfoxide (DMSO, to formaldehyde) [26], 1,2-phenylenediamine, *o*-dianisidine [53], ascorbate, tocopherol [54, 55], lipids as well as protein and non-protein thiols.

Oxidation of methionine and methionine analog, 2-keto-4-thiomethylbutanoic acid by peroxynitrite proceeds by two competing mechanisms. Two-electron oxidation of methionine yields methionine sulfoxide while one-electron oxidation of either substrate gives ultimately ethylene [29]. One-electron oxidation by peroxynitrite leads to generation of free radicals. Free radicals formed in reactions of peroxynitrite with ascorbate, glutathione or other thiol compounds have been spin-trapped [56, 57]. Exposure of blood plasma to peroxynitrite induced ascorbyl free radical, albumin thiyl radical and uric acid-derived free radical [34].

The rate constant for the peroxynitrite reaction with ascorbate is 200 $M^{-1} s^{-1}$ at pH 5.0, and 235 $M^{-1} s^{-1}$ [6] and 236 $M^{-1} s^{-1}$ [28] at pH 7.4 at 25°C while the rate constants for reactions with thiols are much higher [23, 58]. At pH 7.4 and at 37°C the rate constants for the reaction of peroxynitrite with free cysteine and with the single thiol of bovine serum albumin (BSA) are 5900 $M^{-1} s^{-1}$ and 2600–2800 $M^{-1} s^{-1}$, respectively, and they are by three orders of magnitude higher than the corresponding rate constants for the reaction of hydrogen peroxide with sulfhydryls. Unlike hydrogen peroxide which oxidizes thiolate anion, peroxynitrite reacts preferentially with the undissociated form of the thiol group. Peroxynitrite oxidizes cysteine mainly to cystine and the thiol group of BSA to sulfenic acid (in about 50%) and also beyond this stage. Peroxynitrous acid is a less effective thiol-oxidizing agent than its anion. Transition metal anions do not play a significant role in the thiol oxidation by peroxynitrite [58].

Peroxynitrite can oxidize bovine serum albumin to give carbonyl groups reactive with dinitrophenylhydrazine [40]. Higher peroxynitrite concentrations were found to cause fragmentation of BSA [40] and of the tissue inhibitor of metalloproteinase-1 [59]. On exposure of sarcoplasmic reticulum vesicles to peroxynitrite thiol groups of Ca^{2+}-ATPase become oxidized, the oxidation being mostly reversible at low peroxynitrite concentrations. At higher concentrations (≥ 0.45 mM) peroxynitrite affects also other amino-acid residues (Met, Lys, Phe, Thr, Ser, Leu and Tyr) [60]. Peroxynitrite oxidizes the ferrous form of cytochrome *c* to the ferric form; the rate constant for this reaction being $2.3 \times 10^5 M^{-1} s^{-1}$ [61].

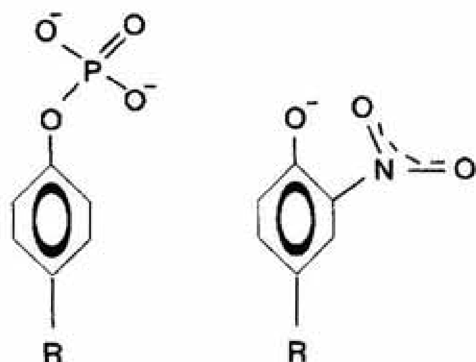
Peroxynitrite induces lipid peroxidation in soybean phosphatidylcholine liposomes [62, 63] and erythrocyte membranes [32]. This effect is apparently not critically dependent on transition metal ions since addition of Fe^{2+} or Fe^{3+} ions does not enhance the peroxidation. Moreover, peroxynitrite inactivates the pulmonary surfactant [64].

Oxidation reactions of peroxynitrite have been reported to be inhibited by such oxygen radical scavengers as ascorbate, cysteine, alpha-tocopherol, Trolox and a Mn-porphyrin derivative [16]. Desferrioxamine is a strong inhibitor of deoxyribose and DMSO oxidation and of lipid peroxidation by peroxynitrite in a manner not related to its iron-chelating ability since another iron chelator, diethylenetriaminepentaacetic acid (DTPA) is without effect [26, 62, 65].

Nitration of proteins and low-molecular mass phenolics is often assumed as a footprint of peroxynitrite *in vivo*. It is argumented that although other factors (e.g. nitrogen dioxide) are also nitrating agents, they cannot significantly

contribute to the *in vivo* formation of nitrotyrosine because of competing reactions [40].

Exposure of *E. coli* glutamine synthetase to peroxynitrite leads to tyrosine nitration and conversion of unadenylylated enzyme to a form similar to that formed by adenylylation while nitration of adenylylated enzyme leads to complete loss of its catalytic activity [44]. It appears that nitration of tyrosine residues can interfere with the regulatory functions of proteins that undergo phosphorylation or adenylylation in signal transduction cascades (Scheme 4). Indeed, nitration inhibits tyrosine phosphorylation of model peptide substrates catalyzed by protein kinases [44, 66, 67]. Nitration of one tyrosine residue per subunit in unadenylylated *E. coli* glutamine synthetase leads to changes in divalent cation requirements, pH-activity profile, affinity for ADP and susceptibility to feedback inhibition by end products. Nitration of one residue per subunit in the adenylylated enzyme leads to complete loss of its catalytic activity [44]. Similarly, nitration of tryptophan residues can affect protein structure and interfere with protein functions [42].



Scheme 4. Nitrotyrosine resembles phosphotyrosine and may block tyrosine phosphorylation and tyrosine phosphorylation-dependent signaling.

In human blood plasma proteins about 0.7% of tyrosine residues are nitrated. In polymorphonuclear leukocyte (PMN) proteins the fraction of nitrated tyrosine residues is higher (1.4%) which may be ascribed to peroxynitrite production in these cells. Activation of blood phagocytes with a phorbol ester increases the fraction of nitrated tyrosine residues up to 1.2% in blood plasma proteins and up to 2.1% in PMN proteins during 4-hour incubation of a blood sample [68].

Peroxynitrite inactivates the tissue inhibitor of metalloproteinase-1 [59], bovine aortic prostacyclin synthase [69] and erythrocyte membrane acetylcholinesterase [32]. Peroxynitrite is a more potent than NO˙ inhibitor of aconitase in which it oxidizes the [4Fe-4S] clusters [70, 71]. The rate constant for the inactivation of pig heart mitochondrial aconitase by $O_2^{\cdot-}$ (3.5×10^6 M^{-1} s^{-1}) is higher than that for peroxynitrite (1.4×10^6 M^{-1} s^{-1}) [71] but, nevertheless, aconitase inactivation can occur at biologically relevant concentrations of peroxynitrite. Among mitochondrial enzymes studied aconitase is the most sensitive to the action of peroxynitrite [71, 72].

Peroxynitrite modification of low-density lipoprotein increases electrophoretic mobility of the protein and converts it to a form recognized by the macrophage scavenger receptor [54, 55]. Peroxynitrite-modified BSA is preferentially degraded by blood cell lysates [66].

In the presence of a porphyrin complex of Mn(III) peroxynitrite induces extensive strand scission of plasmid DNA [73]. The reaction of guanine with peroxynitrite produces 8-nitroguanine; in contrast to tyrosine nitration, this reaction is not affected by ferric ions [74]. Another type of peroxynitrite reaction with nucleic acid constituents is the homolytic addition to the C4-C5 double bond of 2′-deoxyguanosine to form 4,5-dihydro-5-hydroxy-4-(nitrosooxy)-2′-deoxyguanosine [75]. The reaction of peroxynitrite with nucleosides and isolated DNA results also in oxidation of adenine and guanine moieties to form 8-oxo-7,8-dihydro-2′-deoxyguanosine, 2,2-diamino-4[(2-deoxy-β-D-*erythro*-pentofuranosyl)amino]-5-(2*H*)-oxazolone and 8-oxo-7,8-dihydro-2′-deoxyadenosine [76].

The biological significance of the peroxynitrite effects *in vitro* has been challenged because concentrations as high as 0.1–1 mM are usually used *in vitro* to produce measurable effects. However, one should take into account the rapid decomposition of peroxynitrite and compare its toxicity in terms of exposure expressed in units of time × concentration. The net exposure to peroxynitrite can be calculated from the pseudo-first-order kinetics of peroxynitrite decomposition. The concentration of peroxynitrite at time t is

$$[ONOO^-]_t = [ONOO^-]_0 \exp(-kt) \qquad (16)$$

where k is the rate constant for the decomposition of peroxynitrite. Integration of this equation with respect to time from zero to infinity gives exposure to peroxynitrite as

$$[ONOO^-]_t dt = -(1/k)\,[ONOO^-]_0 \exp(-kt) = \\ = (1/k)\,[ONOO^-]_0 \qquad (17)$$

which has the unit of time × concentration. It can be calculated that the exposure to e.g. a bolus of 250 μM peroxynitrite is equivalent to the exposure to a steady-state concentration of only 1 μM for 7 min [20].

CELLULAR EFFECTS OF PEROXYNITRITE

DNA single-strand breakage and "suicidal" activation of a repair enzyme poly-ADP ribosyltransferase can be induced by peroxynitrite (but not by superoxide or NO') in macrophages and aortic smooth muscle cells. These events result in depletion of NAD^+ and ATP and finally cell dysfunction and death [13, 45, 77]. Peroxynitrite inhibits mitochondrial respiration in J774 cells and rat aortic smooth muscle cells; it was suggested that endogenous peroxynitrite can suppress respiration of immunostimulated cells [77, 78]. Peroxynitrite strongly inhibits complex I- and complex II-dependent mitochondrial oxygen consumption and activities of succinate dehydrogenase and ATPase, without affecting complex IV-dependent respiration and cytochrome *c* oxidase activity [79]. It has been suggested that peroxynitrite formation may participate in the regulatory control of mitochondrial oxygen uptake [80] and be responsible for the electron transport inhibition during NO' challenge to target cells [79]. Exposure of neurons to peroxynitrite results in a decrease in activities of succinate-cytochrome *c* reductase and cytochrome *c* oxidase; NADH-ubiquinone-1 reductase is not affected [81].

Exposure of rat liver mitochondria to high doses of peroxynitrite induces depolarization and cyclosporin-A-sensitive calcium efflux which may be a crucial event in peroxynitrite-induced cell damage and death [82]. In another study, cyclosporine-inhibitable calcium release by mitochondria was found to be induced by peroxynitrite without affecting the mitochondrial membrane potential, in contrast to a similar effect induced by NO' which was accompanied by depolarization [83].

Peroxynitrite diminishes the ability of PC12 cells to synthesize 3,4-dihydroxyphenylalanine (DOPA), apparently due to inactivation of tyrosine hydroxylase [84]. Neurons were found to be more susceptible than astrocytes to the cytotoxic action of peroxynitrite [81].

Peroxynitrite also affects numerous membrane transporters: it decreases the Na^+ transport in rabbit alveolar type II cells by damaging apically located amiloride-sensitive Na^+ channels [85] and inhibits erythrocyte membrane Ca^{2+}-ATPase, Mg^{2+}-ATPase and (Na^+,K^+)-ATPase [32], rabbit muscle sarcoplasmic reticulum Ca^{2+}-ATPase [60], aminoisobutyrate transporter in cerebellar granule cells [86] and high affinity glutamate transporters from rat brain reconstituted in liposomes [87].

Exposure of cultured cardiac myocytes to peroxynitrite results in a decrease in spontaneous contraction of the cells followed by complete arrest of movement [88].

Peroxynitrite was demonstrated to be toxic to *Staphylococcus aureus* [89], *E. coli* [12] and *Trypanosoma cruzi* [90], and to *Leishmania amazonensis* promastigotes [25]. At 37°C and pH 7.4 the LD_{50} of peroxynitrite for *E. coli* was 250 μM [12]. Peroxynitrite was reported to be highly cytotoxic for cultured neurons [87]. Exposure of PC12 cells (a tumor cell line derived from rat pheochromocytoma cells) to large doses of peroxynitrite (> 2 mM) resulted in cell swelling and necrosis. Exposure of the cells to lower concentrations of peroxynitrite (EC_{50} = 850 μM) induced apoptosis [91]. Apoptosis of rat thymocytes was also observed after treatment with 20–200 μM peroxynitrite [92].

RELEVANCE OF PEROXYNITRITE TO MEDICINE

Peroxynitrite has been suggested to contribute to tissue damage in ischaemia-reperfusion injury [26, 93, 94], immuno-complex pulmonary dysfunction [95], pulmonary emphysema [96], neuroexcitotoxicity [97], familial amyotrophic lateral sclerosis [98], Parkinson's disease [93] and atherogenesis [99, 100]. One possible mechanism of peroxynitrite-related pathogenesis involves tyrosine nitration. As already

mentioned, nitrotyrosine cannot be phosphorylated by tyrosine kinases [101] and excessive tyrosine nitration can disturb critical signal transduction pathways, especially those dependent on most of growth factors. It has been proposed (though not proven) that mutant Cu,Zn-superoxide dismutases found in patients with familial amyotrophic lateral sclerosis have a greater ability to catalyze nitration by peroxynitrite of tyrosine residues on a key target in motor neurons [102, 103]. Interestingly, a defect of glutamate transport is typical of amyotrophic lateral sclerosis [87].

Nitrotyrosine was also detected in atherosclerotic plaques of human coronary vessels [76], in human acute lung injury [104, 105] and in blood serum and synovial fluid from patients with the inflammatory joint disease or rheumatoid arthritis but not from healthy donors [106]. In animal models, increased content of nitrotyrosine was found in lung macrophages [107] and in the aorta of rats injected with bacterial endotoxin [108]. Accumulation of nitrotyrosine residues found in rat skeletal muscle Ca^{2+}-ATPase during aging was ascribed to nitration by peroxynitrite [109].

Treatment with peroxynitrite causes a significant hyperresponsiveness of isolated guinea pig trachea to histamine and methacholine and epithelial damage of the trachea *in vivo* [110]. Intrarectal administration of peroxynitrite induces ulcerative colitis-like inflammation in the colon of rats [111]. Peroxynitrite is now considered to be the major compound responsible for tissue damage induced by endotoxic shock [112] and by inflammation [97, 102, 113], and therefore a contributor to the multistage carcinogenic process [74].

DEFENSE AGAINST PEROXYNITRITE

Evidence for the cytotoxic action of peroxynitrite leads to the question whether defense mechanisms against this agent exist. It seems that prevention of peroxynitrite formation may be an important function of superoxide dismutases [99].

Taking into account the low value of the rate constant for the reaction of peroxynitrite with ascorbate and the low content of ascorbate in blood plasma and many tissues, it is unlikely that ascorbate is a significant scavenger of peroxynitrite under physiological conditions [6]. It may become important in organs of high ascorbate content (e.g. 55 mg/100 g tissue in the adrenal glands) [28]. On the other hand, scavenging by thiols, first of all glutathione, may play a role (see also below) [56].

Peroxidases, in contrast to catalase, react with peroxynitrous acid with very high rate constants (myeloperoxidase: $2.0 \times 10^7\ M^{-1}\ s^{-1}$; horseradish peroxidase: $3.2 \times 10^6\ M^{-1}\ s^{-1}$) [114]. Ebselen (2-phenyl-1,2-benzisoselenazol-3(*2H*)-one), an antiinflammatory agent and glutathione peroxidase mimic, reacts with peroxynitrite also with a high rate constant of the order of $10^6\ M^{-1}\ s^{-1}$, yielding selenium oxide [115]. Selenium amino acids are more effective in protecting DNA against single-strand breaks than the respective sulfur amino acids [116], which may suggest a role for selenoproteins in the detoxification of peroxynitrite.

Although direct reactions of peroxynitrous acid with either nitric oxide or superoxide

$$ONOOH + O_2^{\cdot-} + H^+ \rightarrow NO_2^{\cdot} + O_2 + H_2O \quad (18)$$

$$ONOOH + NO^{\cdot} \rightarrow NO_2^{\cdot} + NO_2^{-} \quad (19)$$

have not been definitively demonstrated, they have been suggested to be thermodynamically possible [23]. Such reactions would explain the results of experiments in which the peroxynitrite-dependent effect was studied as a function of nitric oxide and superoxide concentrations in a system where peroxynitrite was formed in the reaction between these agents. Efficiency of peroxynitrite-dependent reactions was the highest at equimolar concentrations of $O_2^{\cdot-}$ and $NO^{\cdot}$, suggesting that an excess of either reactant could protect against peroxynitrite *in vivo* and could complicate measurements of peroxynitrite production [39]. Nitric oxide inhibits lipid peroxidation initiated by peroxynitrite [63]. These findings indicate that the complex relationships between peroxynitrite and other reactive oxygen and nitrogen species may determine the harmful effects of peroxynitrite. The common knowledge that small amounts of nitric oxide are beneficial while large amounts of $NO^{\cdot}$ are toxic may require revision since higher doses of nitric oxide may suppress peroxynitrite reactions. It becomes clear that both inhibitors of nitric oxide synthase and $NO^{\cdot}$ donors [117, 118] can reduce tissue damage by peroxynitrite: the former reduce formation of

peroxynitrite while the latter inhibit peroxynitrite reactivity [78].

POSSIBLE BENEFICIAL EFFECTS OF PEROXYNITRITE?

Though considered as a mediator of the toxic action of nitric oxide and superoxide, peroxynitrite can exhibit effects which, at least in some systems, can be seen as beneficial. Peroxynitrite causes aggregation of washed blood platelets; however, in the presence of blood plasma it not only does not show proaggregatory properties but acts as an inhibitor of platelet aggregation. This reversal of aggregatory effect of peroxynitrite can also be achieved in washed platelets by addition of serum albumin and glutathione, it is inhibited by hemoglobin and could be ascribed to formation of nitric oxide and *S*-nitrosoglutathione [119] or to nitrosylation of proteins [120]. Peroxynitrite causes relaxation of rabbit aortic strips in Krebs buffer containing glucose but not in a glucose-free buffer. Apparently, it reacts with sugars and other compounds containing alcohol groups to form $NO^{\cdot}$ or $NO^{\cdot}$ donors [121]. This reaction pathway may be important since $NO^{\cdot}$ itself is unable to form *S*-nitrosothiols in the absence of oxygen [50]. Peroxynitrite increases cyclic GMP level in endothelial cells and, in the presence of glutathione, stimulates guanylyl cyclase. This effect is due to conversion of about 1% of peroxynitrite to *S*-nitrosoglutathione [122]. Peroxynitrite inhibits agonist-stimulated Ca^{2+} efflux and Ca^{2+} release from internal stores in vascular endothelial cells [123]. This effect was interpreted in terms of normative modulation of Ca^{2+} signalling by peroxynitrite (though it may represent, instead, an early event in the process of cell injury by this compound). Some researchers are tempted to postulate that peroxynitrite may also play a role of a physiological mediator of vascular function [119–123]. Irrespective of whether this idea may be true, it should be taken in mind that the effects of peroxynitrite are critically dependent on the biological environment of its action and extrapolation of results of test-tube experiments in simple systems upon cells and organisms should be done cautiously.

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