Chapter 21

# Peroxynitrite Formation and Detection in Living Cells

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## ON THE GENERATION OF CELLULAR-DERIVED PEROXYNITRITE: TEMPORAL AND SPATIAL CONSTRAINTS

Peroxynitrite formation involves a radical-radical reaction between superoxide radical anion ($O_2^{\cdot-}$) and nitric oxide (·NO) (Eq. 21.1). This reaction occurs at diffusion-controlled rates ($\sim 10^{10}$ $M^{-1}$ $s^{-1}$) [1] and it is thermodynamically favored by the formation of a new chemical bond at the expense of two unpaired electrons combination [2]. Nevertheless, the radical nature of the reactants determines certain constraints for peroxynitrite synthesis in biological environments. Although both, $O_2^{\cdot-}$ and ·NO are formed physiologically in several tissues, the steady-state concentrations of both radicals remains low under basal conditions, mainly due to the presence of cellular targets and competing processes that limits their half-life and diffusion. Once formed, peroxynitrite acts as a powerful oxidant bearing an intricate chemistry, which may follow radical pathways or participate in direct reactions with different targets depending on the cellular environment. Peroxynitrite anion ($ONOO^-$) is in equilibrium with the protonated form, peroxynitrous acid, (ONOOH, p*K*a = 6.4–6.8 depending on the ionic strength) (Eq. 21.2). Given the different diffusional and chemical properties of the acid-base pair $ONOO^-$/ONOOH, local pH affects peroxynitrite reactivity (from now on, "peroxynitrite" will refer to both species $ONOO^-$ anion and ONOOH acid). Both species can promote direct oxidative modifications to biomolecules, particularly by reacting with transition metal centers, phenolic compounds, and thiols [3–7]. Many of the reactions of peroxynitrite with biological targets depend on the generation of secondary radicals by the proton-catalyzed decomposition of peroxynitrous acid giving hydroxyl (·OH) and nitrogen dioxide ($\cdot NO_2$) radicals with yields of 30% for both species (pH = 7.4 and 37°C; $k$ = 0.9 $s^{-1}$) [8,9] (Eq. 21.3).

$$O_2^{\cdot-} + \cdot NO \rightarrow ONOO^- \quad (21.1)$$

$$ONOO^- + H^+ \rightleftharpoons ONOOH \quad (21.2)$$

$$ONOOH \rightarrow \cdot OH + \cdot NO_2 \quad (21.3)$$

There are many examples of protein thiols that are oxidized by peroxynitrite leading to enzyme inactivation, such as, glyceraldehyde-3-phosphate dehydrogenase, aconitase, and mitochondrial creatine kinase among others [7,10–13]. The low molecular weight thiol glutathione (GSH) reaches millimolar concentrations within cells, and for some time it was considered a direct cellular scavenger of peroxynitrite. However, the reaction with GSH is not as fast to prevail over others reactions in vivo, being mainly a target for the secondary radicals of peroxynitrite [3,14]. Taking into consideration the relatively slow homolysis of ONOOH and given the abundance of other cellular targets for peroxynitrite, this turns out to be a minor route in biological systems [15]. Since peroxynitrite and its secondary radicals can initiate different paths capable of causing undesired biological oxidations, cells are equipped with efficient detoxifying tools to prevent peroxynitrite formation (e.g., superoxide dismutases, SODs) and/or to detoxify the oxidant once formed (1- and 2-Cysteine peroxiredoxins and glutathione peroxidase) [16,17]. $O_2^{\cdot-}$ dismutation to hydrogen peroxide ($H_2O_2$) and molecular oxygen ($O_2$), catalyzed by SODs is one of the fastest reactions known ($k \sim 10^9$ $M^{-1}$ $s^{-1}$) in cellular systems and determines the main fate of this radical in vivo [18]. This preferential reaction of $O_2^{\cdot-}$ with the ubiquitous and abundant SODs (intracellular concentration ~ 10 μM) in aerobic organisms keeps the intracellular concentration of $O_2^{\cdot-}$ at very low steady-state levels in the different cellular compartments (nM–pM range) [14,17,19]. However, ·NO represents one of the few biomolecules able to react fast enough with $O_2^{\cdot-}$ and produced at sufficient high rates to outcompete SODs

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dismutation. Indeed, it has been long established that peroxynitrite is formed in certain pathological scenarios, such as inflammation or altered cell homeostasis, when the steady-state concentration of the radical precursors $O_2^{\cdot-}$ and ·NO is augmented several folds. Temporal and spatial occurrence of $O_2^{\cdot-}$ and ·NO in the cellular milieu is mandatory for their reaction to take place. $O_2^{\cdot-}$ is a short-lived species with a net negative charge at intracellular pH (p*K*a = 4.8), which limits its diffusion across membranes [20]. On the other hand, ·NO is a small hydrophobic species capable of diffusing several cells diameters across lipid bilayers [21]. Given the differences in the diffusional properties of the reactants, it is expected that peroxynitrite formation will occur preferentially at the sites of $O_2^{\cdot-}$ generation (e.g., macrophage phagosomes, mitochondria matrix, and citosol) [17].

An important target for peroxynitrite reaction in the biological milieu is $CO_2$, present at high concentration, both intra- and extracellularly (1–2 mM). Its reaction with $ONOO^-$ ($k = 5.8 \times 10^4\ M^{-1}\ s^{-1}$) produces an unstable intermediate, nitrosoperoxocarboxylate ($ONOOCO_2^-$), which decomposes generating carbonate radical ($CO_3^{\cdot-}$) and $\cdot NO_2$ in approximately 35% yields [22]. This reaction diverts peroxynitrite reactivity with the formation of two potent short-lived oxidants that target principally protein thiols, aromatic compounds, and DNA [23–25]. Overall, peroxynitrite formation and reactivity in the cellular milieu will be highly influenced by spatial constraints, pH environment and the presence of different targets that may dictate the outcome from cell signaling to nitrooxidative stress conditions.

## FIRST REPORTS OF CELLULAR PEROXYNITRITE GENERATION

The first evidences of peroxynitrite formation in vivo arose from the study of "endothelial-derived relaxing factor" (EDRF). Before knowing the exact chemical nature of this vasodilating agent, researchers observed that the presence of SODs increased the half-life of the EDRF and protected cells from oxidative damage in a model of postischemic reperfusion vascular injury [26]. These observations led to the proposal that $O_2^{\cdot-}$ was an effective scavenger of this mediator and helped to identify ·NO as the chemical entity of EDRF [27–30]. In 1990, Beckman and coworkers, suggested for the first time, that the oxidative damage promoted by $O_2^{\cdot-}$ was due to the product of its reaction with ·NO, peroxynitrite. Until then, $O_2^{\cdot-}$ toxicity was attributed mainly to ·OH, which could be formed through iron-catalyzed Haber-Weiss reaction. However, the authors pointed out that the reaction between $O_2^{\cdot-}$ and ·NO was more likely to occur than the Haber-Weiss chemistry and provided a better explanation for the observed toxicity [9]. Accordingly, soon afterward Radi and coworkers demonstrated that peroxynitrite is able to oxidize thiols and initiate lipid peroxidation [6,7]. Furthermore, the authors established that direct bimolecular reactions with molecular targets, such as thiols are typically quantitatively much more important than the proton-catalyzed homolysis.

In those seminal works, the formation of peroxynitrite in biological systems was proposed for the first time, setting up the bases for the exploration on the relevance of the in vivo formation of this important oxidizing and nitrating agent [7,9]. From there on, many efforts were directed to identify possible scenarios where $O_2^{\cdot-}$, ·NO and thus peroxynitrite could be produced in different cellular conditions.

The known ability of professional phagocytes (macrophages and neutrophils) to synthesize both, $O_2^{\cdot-}$ and ·NO, and the early evidences showing that peroxynitrite exerted an important cytotoxic potential toward different microorganisms in vitro [31,32] led to propose that this oxidant could be a key effector-molecule of innate immune response. In 1992, Ischiropoulos and coworkers published one of the first evidences of peroxynitrite formation in macrophages, taking advantage of one of its most representative footprints, tyrosine nitration. By means of using the hydrophilic tyrosine analog 4-hydroxyphenylacetate (4-HPA) as a reporter, they observed a substantial nitration of this compound in activated rat alveolar macrophages, indicative of peroxynitrite formation. The incubation with the NOS inhibitor *N*-methyl-L-arginine abolished the 4-HPA nitration, revealing the involvement of the NOS-derived ·NO in this process [33,34]. From then on, the exploration of peroxynitrite generation was extended to other cellular models. In endothelial cells, peroxynitrite formation was evidenced following bradikynin and/or $Ca^{2+}$-ionophore stimulation (intracellular $Ca^{2+}$ influx and activation of endothelial, eNOS). These authors studied the effect of bradikynin on luminol and lucigenin chemiluminescence in the supernatant of endothelial cells, which promoted substantial light emission respect to nonstimulated cells. This light signal was inhibited by the NOS inhibitor L-nitro-arginine methyl ester (L-NAME), SOD, and uric acid, in agreement with peroxynitrite generation (the mechanism of uric acid inhibition will be discussed later in the chapter) [35].

Further investigations had focused on the consequences of peroxynitrite formation in vitro and in vivo. Importantly, peroxynitrite was proved to be detrimental to mitochondrial function, which provided insights on one of the mechanisms of peroxynitrite-derived cellular toxicity. Initial reports demonstrated that heart mitochondria were susceptible to peroxynitrite oxidative damage, leading to complex I (NADH-dehydrogenase) and complex II (succinate dehydrogenase) inactivation [36].

In an effort to determine the effects of endogenous-derived ·NO and $O_2^{\cdot-}$ production in macrophages, Zingarelli and coworkers studied the ·NO-dependent damage to DNA, and the concomitant poly-ADP ribose polymerase (PARP) activation, which depletes cells from $NAD^+$ altering cellular

energetic metabolism. The authors measured peroxynitrite formation following the oxidation of a redox sensitive probe, dihydrorhodamine 123 (DHR 123), in macrophages stimulated with lipopolysaccharide (LPS). Probe oxidation was susceptible to inhibition by *N*-methyl-L-arginine (iNOS inhibitor) and importantly, the presence of catalase had no significant effect, indicating that the fluorescent signal did not depend on $H_2O_2$. Time-course of peroxynitrite formation paralleled DNA strand-breaks generation and cell $NAD^+$ depletion in LPS-treated macrophages, which was not observed in macrophages forming only $O_2^{\bullet-}$ and/or ·NO alone. Therefore, it was concluded that endotoxin-related energetic failure was a consequence of peroxynitrite-mediated genetic damage and repair [37,38]. Peroxynitrite formation was also found to be related to the induction of programmed cell death by apoptosis in a rat motor neuron model. In 1998, Estevez and coworkers reported that embryonic motor neuron cell death induced by trophic factor deprivation was associated to an increase in protein 3-nitrotyrosine (vide infra). Treatment with manganese TBAP (a peroxynitrite decomposition catalyst) and a NOS inhibitor prevented from both nitration and apoptosis, supporting that peroxynitrite formation was sufficient to induce cell death in this model [39].

In this last quarter of century, large progress has been made regarding the biochemical properties, detection, and subcellular localization of this elusive oxidant in complex biological systems. Moreover, recent efforts have been directed toward the quantitation of peroxynitrite steady-state concentrations and fluxes under basal and cellular stress conditions.

## FIRST APPROXIMATIONS OF PRECURSOR RADICAL SPECIES AND PEROXYNITRITE FLUXES IN BIOLOGICAL SYSTEMS

The discovery of peroxynitrite as a novel biologically relevant cytotoxic agent opened the question of how much peroxynitrite is needed to exert a cytotoxic effect and how much is actually being produced by cells. In vitro bactericidal activity of peroxynitrite was studied by Beckman's group in 1992, using *Escherichia coli* ($10^6$ cfu/mL) and bolus addition of the oxidant with the determination of a lethal dose for the inhibition of 50% of bacterial growth ($LD_{50}$) of 250 μM [32]. Denicola and coworkers were interested in peroxynitrite toxicity toward the noninfective epimastigote stage of *Trypanosoma cruzi,* the causative agent of Chagas disease. In those experiments, a $LD_{50}$ of 500 μM was estimated when using growth inhibition in culture media, whereas an $LD_{50}$ of 150 μM was calculated when using thymidine-$[H]^3$ incorporation into parasite DNA [40]. Actually, this number is not as high as it may seem given the fast decomposition of peroxynitrite at pH 7.4 and 37°C. This is a major issue when measuring peroxynitrite toxicity, because "cell concentration" (number of cells per unit of volume) is determinant for peroxynitrite reactivity. The greater the concentration of cells in a given volume, the more peroxynitrite would react with targets, whereas in diluted cell suspensions most of peroxynitrite would homolyze before reaching any target. A thorough analysis of this matter was performed by Álvarez and coworkers, a decade after Denicola's work, where they analyze the effect of the distance between targets (i.e., *T. cruzi* epimastigotes), on peroxynitrite toxicity [41]. The authors pointed out that typical viability experiments set up an average distance between parasites of ~20 μm, while biologically relevant diffusion distances in the interaction between macrophages and *T. cruzi* are <1 μm. Taking this into account, authors determined an $LD_{50}$ for different cell concentrations. At higher cell concentrations (distances < 5 μm) peroxynitrite is extremely toxic ($LD_{50}$ = 0.3 fmol/*T. cruzi*) due to limited peroxynitrite homolysis before reacting with cells. Importantly, in such conditions $CO_2$ competition for peroxynitrite is also limited due to the abundance of targets cells [41]. Interestingly, the early observations of Denicola and coworkers stated an important experimental consideration: the same doses of peroxynitrite administered as a single bolus or as a continuous infusion flux led to different outcomes, and the latter was found to be the more cytotoxic condition. This observation could be explained but the fact that a single oxidant addition favors self-decomposition, diminishing the effective peroxynitrite concentration [40]. Importantly, peroxynitrite infusion as flux is more representative of formation kinetics in biological systems.

Many groups in the field have measured and reported rates of peroxynitrite formation in different cell types and through different experimental approaches. The variety of assay conditions, cell concentration, stimulus, and detection techniques employed hinders the comparison of the different peroxynitrite fluxes reported in the literature. However, the improvements regarding technologies and probes design allowed getting more precise quantitative data about oxidant formation in cells and tissues.

One of the first estimations of peroxynitrite formation rates came from the work of Ischiropoulos and coworkers, in which they reported an estimated flux of 0.11 nmol/$10^6$ cells/min produced by PMA-stimulated rat alveolar macrophages [42]. ·NO and $O_2^{\bullet-}$ were also measured and the reported formation rates were 0.10 and 0.12 nmol $10^6$ cells/min respectively, in agreement with the peroxynitrite fluxes calculated [34].

Different determinations of peroxynitrite rates formation are summarized in Table 21.1, obtained from different cellular models, stimulation protocols, and detection methods. In spite of the diversity of the experimental conditions, numbers are not so dissimilar. For example, macrophages from different origins and measured with a variety of probes presented similar fluxes of peroxynitrite formation

**TABLE 21.1 Estimation of Peroxynitrite Production Rates in Various Cell Types**

| Cell Type/Line | Stimulus | Peroxynitrite Production | Peroxynitrite Flux[a] | Detection Method |
|---|---|---|---|---|
| Primary rat alveolar macrophages [34] | PMA | Extracellular | 0.11 nmol/min/$10^6$ cell | HPA-nitration |
| J774.2 Macrophages [37] | LPS | Extracellular | ~0.0045 nmol/min/$10^6$ cell | DHR |
| J774A.1 Macrophages [64] | IFNγ-LPS, PMA | Extracellular | 0.13 nmol/min/$10^6$ cell[b] | $DCFH_2$ |
| J774A.1 Macrophages [155] | IFNγ-LPS, PMA | Extracellular | 0.06–0.12 nmol/min/$10^6$ cell | CBA |
| BAECs [83] | Ionomycin | Intracellular | 6 μM/min[d] | Fl-B |
| BAECs [17] | Basal | Mitochondria | 180 μM/min[d] | Theoretical[c] |
| J774A.1 Macrophages [83] | IFNγ-LPS, *T. cruzi* | Phagosome | 60 μM/min[e] | Fl-B |

[a]*Unit varies depending on the model.*
[b]*Estimated from data on [64] assuming 1 × $10^6$ cells ~ 1 mg protein.*
[c]*Estimated considering experimental $O_2^{\bullet-}$ data from [19] and steady state concentration of ·NO [17].*
[d]*Cell volume used to calculate peroxynitrite rate: 1232 $\mu m^3$ [156].*
[e]*Cell volume used to calculate peroxynitrite rate: 3 fL.*

in the range of ~0.1–0.13 nmol $10^6$ $min^{-1}$. Zingarelli and coworkers reported a considerably lower rate of peroxynitrite formation in J774.2 macrophages [37]. Nevertheless, different macrophage-cell lines may differ in peroxynitrite fluxes due to the limited production of one of the precursor radicals. For example, some variants of RAW 264 macrophages, in spite of presenting considerable ·NO production rates, they generate substantially less $O_2^{\bullet-}$ (and therefore less peroxynitrite) than J774 macrophages [43]. Moreover, there are differences in $O_2^{\bullet-}$ or ·NO production rates among different clones of the same parental line, as is the case for clone C3C generated from J774.16 by Tanaka and coworkers, which is unable to form $O_2^{\bullet-}$ [44]. Importantly, immunostimulated human macrophages usually yield significantly less ·NO than their murine counterparts, yet still at sufficient levels to cause protein tyrosine nitration and biological effects [45].

As expected, when peroxynitrite formation is confined to a subcellular compartment, such as the mitochondria or the phagosome, it can reach higher concentrations of those found in the supernatant of a monolayer cells (Table 21.1). In any case, it is important to bear in mind, that the high reactivity of peroxynitrite determines, that what is detected is only a fraction of what is actually formed, because the abundance of cellular targets that consumes part, if not the most, of the peroxynitrite formed [14,17].

## RELEVANT INTRACELLULAR SOURCES OF ·NO AND $O_2^{\bullet-}$ FOR PEROXYNITRITE FORMATION

The intracellular source of $O_2^{\bullet-}$ production and the concomitant ·NO generation by NOS stimulation are key factors that determine peroxynitrite formation in different subcellular compartments. $O_2^{\bullet-}$ is the product of the univalent reduction of $O_2$ and can be generated either enzymatically (i.e., NAD(P)H oxidases and xanthine oxidase) or as the result of the electron leakage of different enzymes, such as NOS [46,47], monooxygenases and NADH dehydrogenases [48], cyclooxygenases, lipoxygenases [49], and components of the mitochondrial electron transport chain [50,51].

Mitochondrial $O_2^{\bullet-}$ formation takes place mainly in Complex I, II, and III (ubiquinone-cyt *c* reductase), where partially reduced $O_2$-derived intermediates can escape from mitochondrial electron transport chain either to the mitochondrial matrix or to the intramitochondrial space and cytosol. Under normal basal conditions, it was estimated a mitochondrial $O_2^{\bullet-}$ steady-state concentration in endothelial cells of 10–50 pM, concentration that may increase ~9 times under pathological (hyperglycemic) conditions [17,19]. Importantly, the interaction of ·NO with mitochondrial-complex IV (terminal cytochrome oxidase) leads to the inhibition of mitochondrial respiration with an increase in $O_2^{\bullet-}$ generation in some components of the respiratory chain [52–54]. In this context, ·NO promotes mitochondrial-electron leakage leading to $O_2^{\bullet-}$ generation, and hence peroxynitrite formation within mitochondria. Peroxynitrite has been shown to inactivate most components of the mitochondrial respiratory chain including Complex I, II, III, and complex V (ATP synthase) through different mechanisms, involving sulfhydryl oxidation, tyrosine nitration, and disruption of iron-sulfur clusters in isolated mitochondria or cells initially exposed to reagent ·NO, S-nitrosothiols, or $ONOO^-$ [52,55,56]. Mitochondrial inactivation of respiratory complexes and redox dysfunction were also observed during excess endogenous formation of ·NO and $ONOO^-$ in cellular and animal models of disease [57–60]. Overall, the data support that peroxynitrite-dependent inhibition of mitochondrial complexes leads to an enhanced $O_2^{\bullet-}$ formation with the establishment of a positive-feedback loop for additional peroxynitrite generation causing mitochondrial oxidative damage and cell death.

Another major and direct enzymatic source of $O_2^{\bullet-}$ generation in living cells is the family of NAD(P)H oxidases

(Nox family), a group of enzymes that synthesize $O_2^{\cdot-}$ at expenses of NADPH and $O_2$. There are seven known Nox isoforms and they vary in function, cellular localization, and regulation [61]. The prototypic member of this family is Nox2 (NADPH oxidase), which is expressed mainly in professional phagocytes: macrophages, neutrophils, and dendritic cells. Its activity depends on the phagocytic stimuli after pathogen recognition, which triggers the assembly of the active enzyme complex at the plasma membrane following phosphorylation of key cytosolic counterparts [62,63]. The membrane invagination during phagocytosis results in an orientation of the $O_2^{\cdot-}$ production toward the vacuole lumen (extracellular space), aimed to cause oxidative damage to the internalized pathogen. Importantly, $O_2^{\cdot-}$ production by macrophage Nox2 is sustained during ~90 min [64]. If ·NO is being produce simultaneously, it could reach the phagosome compartment and react with $O_2^{\cdot-}$ yielding peroxynitrite and related nitrating species in the same site where the microorganism is being internalized [65,66]**.** This directionality in peroxynitrite delivery ensures maximal oxidant production toward the pathogen while limiting host oxidative cell damage.

Nitric oxide synthases (NOS) are responsible for ·NO formation in biological systems. The family of NOS, includes constitutive $Ca^{2+}$-regulated isoforms (endothelial, eNOS; neuronal, nNOS) which are ubiquitously expressed and the inducible $Ca^{2+}$-independent isoform (iNOS), controlled by gene expression in response to proinflammatory cytokines. Constitutively expressed NOS are activated upon increase in $Ca^{2+}$ concentration by coupling oxidase and reductase-domains through $Ca^{2+}$-calmodulin binding to the dimeric enzyme. In contrast, inducible NOS do not need $Ca^{2+}$ influx for activation and remains active several hours upon protein expression.

For both kind of isoforms, ·NO synthesis involves the $O_2$ reduction and incorporation into the guanidino group of L-arginine in the NOS-oxygenase domain, giving rise to ·NO and L-citrulline as the reaction products. NADPH-derived electrons flow from the NOS-reductase domain (of one monomer) to the heme iron in the oxygenase domain (of other monomer) allowing $O_2$ binding to form a transient ferrous–dioxygen complex [67]. The coupling of both domains is extremely important to the correct electron flux during reaction. The exact mechanism for correct domain coupling in constitutive NOS is still not well established but may involve availability of reduced cofactors (tetrahydrobiopterin, $BH_4$) and the presence of a Zn-cluster that orientates correctly both enzyme activities [68].

Classical iNOS was first described to be present mainly in professional phagocytes but this cytokine-inducible isoform was later on shown to be present in other cell types, such as cardiomyocytes [69]. The inducible isoform of professional phagocytes is specialized in producing large amounts of ·NO upon cytokine and pathogen-stimulation (mainly, IFN-γ and LPS). It has been thoroughly demonstrated that simultaneous iNOS activation and intraphagosomal $O_2^{\cdot-}$ formation in macrophages leads to peroxynitrite generation inside the phagocytic vacuole. This accounts for most of the macrophage cytotoxicity activity against many microorganisms, such as *T. cruzi* [41,66], *Mycobacterium tuberculosis* [70], and *Leishmania major* [71], among others.

Endothelial NOS (eNOS), modulates vascular tone, through ·NO-mediated activation of guanylate cyclase and consequent cGMP formation in the smooth muscle. Under certain conditions (e.g., reduced cofactor availability, Zn-thiolate cluster-oxidation), the eNOS uncouples NADPH-reduction from L-arginine oxidation, leading to the generation of $O_2^{\cdot-}$ formation at the reductase domain due to dissociation of the ferrous-dioxygen complex [13,68]. This "uncoupled" state of eNOS, leads to the simultaneous and spatial coexistence of $O_2^{\cdot-}$ and ·NO and thus peroxynitrite formation in the cell cytosol. $BH_4$ has been proven to be important in coupling NADPH consumption to L-arginine oxidation, and the concentration of this cofactor may modulate the $O_2^{\cdot-}$/·NO ratio. eNOS uncoupling has been associated with different disorders involving endothelial dysfunction, such as hypertension and atherosclerosis, among others [67]. Interestingly, endothelial cells exposed to "cigarette smoke extract" showed an increase in cytosolic $O_2^{\cdot-}$ production, which is insensitive to Nox inhibitors. $O_2^{\cdot-}$ generation in this cellular model was shown to be due to the eNOS uncoupling caused by peroxynitrite-dependent eNOS-Zn thiolate cluster oxidation. In this model, an early decrease in ·NO levels (owed to $O_2^{\cdot-}$ reaction and peroxynitrite generation), leads to further enzyme oxidation, closing a negative feedback loop. Therefore, eNOS uncoupling by peroxynitrite oxidation will result in the shift from ·NO-signal transduction to nitro-oxidative stress [72].

Interestingly, not only constitutive isoforms have been shown to form $O_2^{\cdot-}$, but also L-arginine depletion results in iNOS uncoupling in macrophages, leading to the formation of $O_2^{\cdot-}$. In this scenario of substrate shortage, iNOS produces both $O_2^{\cdot-}$ and ·NO with the concomitant formation of peroxynitrite. Interestingly, the killing capacity of macrophages against *E. coli* is improved upon L-arginine deprivation in addition to activation [73].

Owing to the implications of peroxynitrite generation in different diseases in the initiation or progression of pathology, its unequivocal detection and identification of cellular sources become relevant for appropriate pharmacological or genetic interventions.

## CHALLENGES FOR PEROXYNITRITE DETECTION: WHAT IS DETECTED IS A MINOR FRACTION OF WHAT IS FORMED

Peroxynitrite is both a strong oxidizing agent and a strong nucleophile, and these characteristics dictate much of its biochemical fates in vivo [74]. $ONOO^-$ is present in solution mainly in the *cis*-form and the oxidant reactivity is highly pH-dependent E°' ($ONOO^-$, $2H^+/\cdot NO_2$, $H_2O$) = 1.6 V at

pH = 7 [75,76]. Thus, peroxynitrite fate in cellular systems will depend on reaction kinetics, diffusion, and concentration of the different cellular targets that may be present in diverse scenarios. Peroxynitrite reactivity toward a cellular target will depend in two central parameters involving reaction kinetics ($k$, at biological relevant temperature 37°C) and target concentration [T]. The term $k$[T] is used to parameterize the reactivity of peroxynitrite toward a specific target [17]. Taking this into account, and knowing [T] at that specific cell condition, only a few biological targets are relevant for peroxynitrite reactivity including $CO_2$, peroxiredoxins, glutathione peroxidase, heme, and some thiol containing proteins [17]. From these targets, peroxiredoxins are the most efficient peroxynitrite scavengers known to date with a rate constant of $\sim 1–10 \times 10^7\ M^{-1}\ s^{-1}$ and highly concentrated in different cellular compartments [77]. Thus most of the endogenously produced peroxynitrite may be scavenged by the action of this peroxiredoxins. The antioxidant content of different cells types are not equal, with some having highly concentrated systems (e.g., in human erythrocytes, peroxiredoxin-2 is ca. 240 μM [78]) and others having a more modest antioxidant capacity (e.g., neurons). Moreover, the metabolic antioxidant capacity is not static, being regulated at the gene expression level by different prooxidant and proinflammatory stimuli, making the target concentration, specific for each cell type and cellular status. Overall, besides peroxynitrite instability (< 20 ms [17]), the precise determination of cellular peroxynitrite generation is challenging due to the variety of cellular targets concentration in the different cellular conditions and thus different $k$[T], this will be reflected in a minor oxidant detection and thus an underestimation of peroxynitrite production.

Many measurement strategies, based on different principles, have been developed for peroxynitrite detection, such as immune-spin trapping, chemiluminescent/fluorescent probes, electrochemical sensors, and even genetically-encoded probes (see next). Among these strategies, the probes that directly react with peroxynitrite are the most promising tools for the accurate oxidant detection that may allow investigators to understand its biological effects in vivo.

## PEROXYNITRITE DETECTION METHODS

It is important to mention that when assessing peroxynitrite generation in cells, it must be taken into evaluation the adequate experimental setups with the use of different pharmacological inhibitors for the unequivocal determination of its identity (e.g., use of NOS and Nox inhibitors and/or free radical scavengers). Furthermore, the use of some buffers or solvents may alter results diverting reactivity of peroxynitrite to other reactive-generated species with the probe biasing results. For instance, in HEPES (*N*-(2-hydroxyethyl)piperazine-*N*´-(2-ethanesulfonic acid) buffer the piperazine moiety reacts with $ONOO^-$ and/or $ClO^-$ yielding several by-products (e.g., chloramine-like compounds) [79,80], masking the reaction of peroxynitrite/HOCl toward the probe. Importantly, a widely use solvent is dimethyl sulfoxide (DMSO) where the sulfoxide functional group of DMSO is prone to be oxidized by $ClO^-$ yielding the corresponding sulfone [81,82]. Importantly, probes that react fast with peroxynitrite may also interfere in peroxynitrite-dependent processes if added in large amounts. Ideally, probe concentration should be as low as possible to minimize an additional route of peroxynitrite consumption (which may, otherwise, alter secondary intracellular processes), sampling only a minor fraction of total peroxynitrite [83]. A detailed description of the different methods actually used for the detection of peroxynitrite is analyzed in the following section with focus in advantages and constraints for their use in the detection of the peroxynitrite in biological systems.

### Peroxynitrite Footprints

The generation of peroxynitrite in biological milieu has been classically evidenced by detecting structural modifications that this oxidant, and its secondary radicals, promotes on the target molecules (footprint, Fig. 21.1). These modifications (oxidation and nitration) can be shaped by other oxidants and/or be removed by different repair and/or degradation pathways. The identification of specific and stable modifications caused by peroxynitrite are necessary to unequivocally detect this oxidant in cellular systems. Among these modifications, affecting amino acids, proteins, lipids, nitrogenous bases, and DNA, and even glycan oxidations, one of the most commonly used peroxynitrite biomarker is the nitration of tyrosine residues in proteins.

#### *Protein 3-nitrotyrosine*

Tyrosine nitration is a key biomarker of nitro-oxidative stress and is found in vitro and in vivo in different pathological conditions. This posttranslational modification depends on many factors concerning the chemical surroundings where tyrosine residue is located. Local charge and pH, presence of cysteine residues, metal centers nearby and hydrophobicity of the microenvironment, are all key modulators of tyrosine nitration [84–86].

The main mechanism of protein tyrosine nitration in vivo relies on free radical reactions. There is no direct bimolecular reaction of tyrosine with peroxynitrite but rather a reaction with peroxynitrite-derived radicals ($\cdot NO_2$ and $CO_3^{\cdot -}$). In the nitration process, oxidants derived from peroxynitrite react with the phenolic moiety leading to the formation of a radical semiquinone (tyrosyl radical; $\cdot Y$), which combines with $\cdot NO_2$ at diffusion-controlled rate to yield 3-nitrotyrosine (3-$NO_2$-Y). Both $CO_3^{\cdot -}$ and $\cdot NO_2$ can perform a one-electron abstraction to yield $\cdot Y$, therefore nitration by peroxynitrite requires the intermediate formation of secondary species.

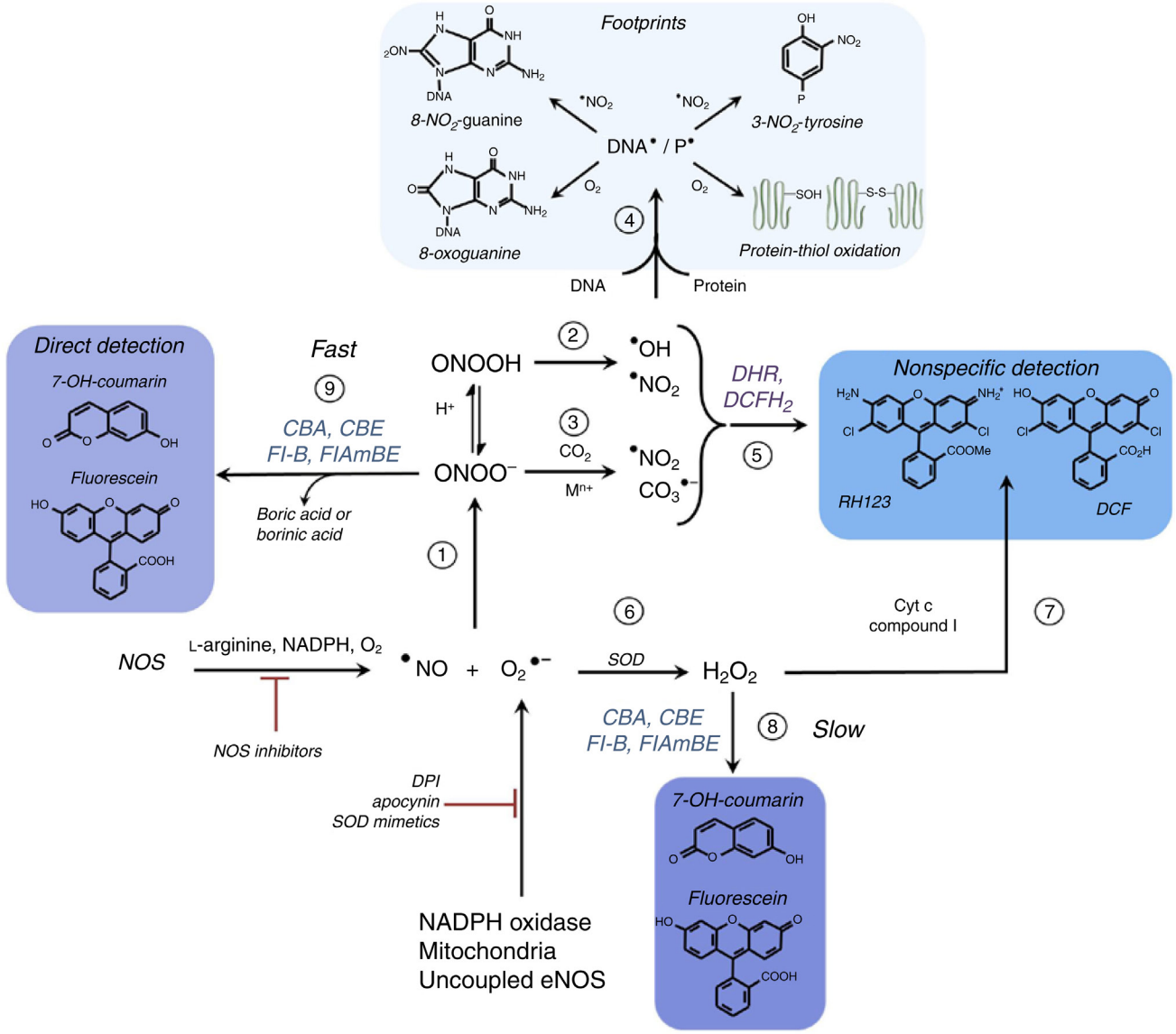


**FIGURE 21.1 Fates of peroxynitrite and detection strategies.** ① Peroxynitrite anion is formed from the diffusion-controlled radical combination between ·NO and $O_2^{\bullet-}$ ($k \sim 10^{10}\ M^{-1}\ s^{-1}$), which is in equilibrium with its conjugated acid, peroxynitrous acid. ② ONOOH could undergo homolysis to ·OH and ·$NO_2$ in 30% yield. ③ $CO_2$ is a biologically-relevant target for $ONOO^-$, giving the intermediate nitrosoperoxocarboxylate ($ONOOCO_2^-$), which decomposes to ·$NO_2$ and $CO_3^{\bullet-}$ in ~ 35% yield. Also, $ONOO^-$ promotes one-electron oxidation of metal centers ($M^{n+}$). ④ ·OH and ·$NO_2$ radicals react with biomolecules, generating protein/DNA centered radicals, which in the presence of ·$NO_2$ or $O_2$ leads to stable structural modifications. ⑤ Nonspecific redox probes (DHR, $DCFH_2$) are oxidized by peroxynitrite-derived radicals yielding fluorescent products. ⑥ Superoxide radical half-life is limited due to the SOD-catalyzed dismutation to $H_2O_2$. ⑦ $H_2O_2$ in the presence of peroxidases also mediates the one-electron oxidation of nonspecific redox probes. ⑧ Boronate-derived probes have a modest reactivity toward $H_2O_2$($k$~ 1–2 $M^{-1}\ s^{-1}$) yielding fluorescent derivatives; ⑨ in contrast, $ONOO^-$ reacts a million times faster ($k \sim 10^6\ M^{-1}s^{-1}$).

The electron-donor character of the phenolic hydroxyl group in tyrosine orientates *ortho* and *para* substitution in the aromatic ring. Since the *para* position is occupied in tyrosine, the only possible position of incorporation of the nitro group is the *ortho* position obtaining mainly *o*- or 3-$NO_2$-Y.

Since ·NO is unable of directly promoting nitration reactions, nitration was initially thought to constitute a specific footprint of peroxynitrite in biology [87]. However other mechanisms of nitration exist in biological milieu besides peroxynitrite that includes the highly nitrating species: ·$NO_2$. The major source of enzymatic-derived ·$NO_2$ is the $H_2O_2$-dependent oxidation of nitrite ($NO_2^-$) performed by myeloperoxidase (MPO) and eosinophil-peroxidase (EPO) [84]. Importantly, these alternative mechanisms of nitration appear to be more restricted than the nitration mediated by peroxynitrite since they may be limited to those tissue regions or cell compartments where MPO and EPO are present. Reaction of peroxynitrite with buried metal

centers located in the active site of proteins (Fe, Mn, and Cu) will also generate a highly oxidative species that could easily form site located ·Y with the concomitant generation of $\cdot NO_2$ at the local site [88].

The incorporation of a nitro group on the C-3 of the phenolic ring generates changes in key properties of the parent amino acid. A large decrease in the p*K*a of the OH group is observed (from ca. 10.0–10.3 to 7.2–7.5 for free tyrosine and 3-nitrotyrosine in water, respectively) [84]. The deprotonation of the phenol ring leads to the formation of a negative charge that can be delocalized into the aromatic ring. Since nitro is a strong electron withdrawing group and is in *ortho* position to the hydroxyl group, the negative charge can also delocalize into the oxygens of the nitro group generating more resonant structures, resulting in the stabilization of the negative charge. Thus weakening the O—H bond makes 3-$NO_2$-Y more acidic. In addition, the nitro group represents a bulky and hydrophobic substituent that may create local steric restrictions, triggering conformational changes, and preventing tyrosine phosphorylation [84].

While immunochemically based studies using antibodies against protein 3-nitrotyrosine has been useful for peroxynitrite detection and cellular/tissue localization of nitrated proteins, proteomic-based analyses have been fundamental to identify preferential protein targets of nitration, and bioanalytical methods have assisted quantitation of the extents of nitration [84]. There are many examples of proteins that are nitrated in vivo under nitro-oxidative stress and in many cases this modification causes a loss of function. Sometimes, protein $NO_2$-Y can result in a gain of function for the protein [89,90]. For instance, mitochondrial Mn-SOD is one of the first examples of nitrated proteins found in vivo in several pathological conditions, such as diabetes, inflammation, and aging [91]. It has been demonstrated that peroxynitrite-dependent nitration of one specific tyrosine (i.e., $Tyr^{34}$) leads to protein inactivation and eventually to disruption of redox-balance within the mitochondria [88]. Another important peroxynitrite target in mitochondria is cytochrome *c* (cyt *c*). Nitration of tyrosine 74 of cyt *c* results in an increased peroxidatic activity, amplifying the oxidative damage to mitochondria through the generation of more powerful oxidants [89]. The number of proteins found nitrated in vivo keeps evolving, and its effect on enzymatic activity and immunological signaling, is well established in many models [90]. Protein nitration has also been proposed to be a biological signaling mechanism. In this sense, it has been recently demonstrated that selective tyrosine nitration of Hsp-90 plays a causal role in the induction of cell death [92]. Although the use of 3-$NO_2$-Y detection as a biomarker of peroxynitrite (and other ·NO-derived oxidants, [90]) has been widely used, proper measurement and quantitation can be challenging and a series of experimental aspects must be carefully considered [93–95].

Protein tyrosine nitration is a low yield process, not all proteins become nitrated, and within a protein not all tyrosine residues, (only 1–5 over 10,000 tyrosine residues) may be nitrated. Therefore, the detection quantities of 3-$NO_2$-Y are too low to detect in some cases. This technique does not allow monitoring real-time formation of peroxynitrite, and as mentioned before 3-$NO_2$-Y formation can be also induced by other oxidant sources.

### Protein Carbonyls

Early studies suggested that another molecular modification by peroxynitrite is protein oxidation. Formation of protein carbonyls (C = O) in cells have been proposed to be a footprint of peroxynitrite reactions with proteins [96,97]. Nevertheless, later it was reported that barely detectable amounts of carbonyl groups are formed when pure proteins were treated with peroxynitrite at pH = 7.4 and physiological $CO_2$ concentrations, being unlikely that peroxynitrite contributes significantly to the increase in protein carbonyl derivatives [98]. This suggests that the presence of high levels of protein carbonyls in tissues might serve as a measure of oxidative damage caused by secondary oxidative processes triggered by peroxynitrite, but not by peroxynitrite itself [98,99].

### DNA Oxidation

Peroxynitrite is able to cause DNA strand breaks and also, it can react with DNA, resulting in both sugar and nucleus-base damage. Of the four DNA bases, guanine, with the lowest oxidation potential ($E^\circ$ = 0.81 V) [100] is the most reactive, and its reaction with peroxynitrite gives oxidation and nitration products, such as 8-oxoguanine and 8-nitroguanine [101] (Fig. 21.1). Other known oxidation products arising from the reaction of guanidine with peroxynitrite include oxazolone and its precursor, imidazolone. In addition, 8-oxoguanidine is more reactive toward peroxynitrite than guanidine, resulting in the formation of secondary oxidation products, such as spiroiminodihydantoin and guanidinohydantoin. Yun and coworkers studied the formation of these products from the treatment of calf thymus DNA with varying amounts of peroxynitrite in vitro. The identification and quantitation by LC-MS/MS, of DNA treated with peroxynitrite provided better understanding of the mechanism of formation of these lesions in DNA [102].

### Lipid Nitration

Peroxynitrite dependent nitration is not restricted to proteins or DNA, fatty acid present in membranes, and lipoproteins are also susceptible to this modification. Fatty acid nitration mechanism is not fully understood yet, but peroxynitrite is one of the proposed agents. There is evidence that nitro-fatty acids are formed in vivo in the picomolar range and its concentration increases during inflammatory processes. Nitrated lipids are found in plasma and other components present in the vascular compartment, and given their antiinflammatory properties, they are thought to have a protective

role against oxidative damage associated with inflammatory diseases [103–107].

### Glycan Oxidation

Glycosaminoglycans are a major component of the extracellular matrix, glycocalyx, and synovial fluid, and there are several studies about modifications of these structures that have been linked to multiple human pathologies. Of interest are oxidations found both in free glycosaminoglycans (e.g., hyaluronan) and as components of proteoglycans, (e.g., chondroitin sulfate, heparan sulfate). Davies and coworkers demonstrated that hyaluronan and chondroitin sulfate are extensively depolymerized by ·OH and $CO_3^{\cdot-}$, but not $\cdot NO_2$, which may be formed from peroxynitrite in in vitro systems of isolated glycosaminoglycans [108]. Furthermore, it has been shown that treatment of cell culture-derived matrix with peroxynitrite results in the release of matrix fragments that include both protein and carbohydrate components consistent with damage to proteoglycan.

Modifications in both the core protein structure, as evidenced by the formation of both 3-nitrotyrosine and protein carbonyls, and fragmentation of the proteoglycan were observed [109].

## Electrochemical Methods

The electrochemical sensors for extracellular peroxynitrite detection is based on the use of ultramicro or nanosensors to detect and quantify peroxynitrite in chemical and biological systems. The process involves an electron transfer between the analyte to the sensor and generation of an electrical current controlled by the diffusion of the analyte to the sensor described by Faraday's law. The faradic diffusion controlled current is proportional to the concentration of analyte (e.g., $ONOO^-$) and the selectivity of the sensors is based on specific redox potential of the analyte and on a rapid electron exchange between specific catalytic redox center of the sensor coating. Recently, the use of nanosensors for the in situ monitoring and measurement of the concentration ratio of ·NO and $ONOO^-$, [·NO]/[$ONOO^-$] as a marker of nitro-oxidative imbalance in human umbilical vein endothelial cells (HUVECs) and dysfunctional aorta tissue has been reported [110]. Moreover, this approach was used to measure peroxynitrite in oxidative stress bursts caused by mechanical stimulation at the single cell level [111]. An ultramicrosensor based on a conducting polymer-manganese ion complex was also reported for electrochemical detection of peroxynitrite, in PMA-stimulated YPEN-1 rat glioma cells during oxidative stress [112]. Other examples include, the amperometric ultramicrosensors for peroxynitrite detection released by myocardial cells upon ischemia/reperfusion injury [113]. Although interesting from a theoretical point of view, their practical use seems limited since the equipment availability may not be found in the majority of biochemical laboratories. Moreover, some considerations must be taken into account since the sensitivity and selectivity of nanosensors can be affected by the composition of buffers and cell culture media, temperature, electrical interference, vibration, and many other factors. Therefore, the sensors have to be calibrated frequently, before and after measurements with standard solutions [110]. The accurate positioning of sensors in relation to the cell membrane is the most important factor for reproducible measurements since peroxynitrite concentration decrease exponentially as a function of distance of the formation site.

Overall, this methodology may be sensitive for the extracellular detection of peroxynitrite; however, a more extensive characterization and independent validation is warranted in future studies, as there is yet little experience on their use.

## Fluorescent and Chemiluminescent Probes for Peroxynitrite Detection

Fluorescence detection methods are the most widely used and sensitive techniques for the measurement of intracellular oxidants generation. There are a large number of small organic molecules designed and synthesized to detect peroxynitrite. Most of them share the property of having weak fluorescence and increasing this property upon oxidation. There are basically two different mechanisms, either reacting directly with peroxynitrite, or with its derivative radicals (·OH, $\cdot NO_2$) [114,115]. Fluorescence intensity can be easily determined providing a simple and sensitive analytical method for detecting in real-time intracellular oxidants. Fluorescent probes can be classified in two main groups: (1) redox probes that yield a fluorescent end product (or chemiluminescent product according to the case) by a free radical mechanism, and (2) probes reacting by a different mechanism in which a nucleophilic attack by peroxynitrite to a particular functional group releases the masked fluorescence.

Luminescence measurements, especially fluorescence-based techniques, are advantageous and have become of routine practice in the field.

### Redox Probes

For the commonly used redox-fluorescent sensitive probes, that is, 2′,7′-dichlorodihydrofluorescein ($DCFH_2$), dihydrorhodamine (DHR-123), and hydroethidine (HE), as well as the chemiluminescent detector luminol, the most accepted mechanism at present, is the one-electron oxidation process that yields a radical intermediate, which is afterward oxidized to form highly resonant moieties responsible for the increase probe fluorescence. $DCFH_2$ and DHR-123 have similar structural features and are oxidized by a similar radical mechanism so the description of the oxidation process by peroxynitrite is analogous. It has been controversial in the literature that the detailed reaction mechanism of $DCFH_2$ with peroxynitrite and a direct reaction does not

seem possible, being ·OH, $\cdot NO_2$ and $CO_3^{\cdot-}$ the reactive species responsible of probe oxidation [116]. The lack of specificity of these probes is the greatest disadvantage since they are oxidized by numerous radical species or metal-dependent processes. ·OH radicals are very reactive toward aromatic compounds and it was reported a diffusion-controlled reaction between ·OH and $DCFH_2$ [117,118]. Thiyl radicals derived from the oxidation of glutathione can also oxidize $DCFH_2$ with a rate constant of $\sim 10^7 M^{-1} s^{-1}$ at pH 7.4 [119]. Neither ·NO nor $O_2^{\cdot-}$ are able to oxidize either probe at significant yields. However, ·NO and $O_2^{\cdot-}$ formation ratios different to one will result in peroxynitrite generation but also, in remaining quantities, of one of the precursor radical species. Both, excess of ·NO and/or $O_2^{\cdot-}$ may react with the radical anion intermediate of the probe ($DCFH^{\cdot-}$) in a termination reaction that gives a nonfluorescent product. Thus, the excess of radical production may result in the underestimation of peroxynitrite formation rates [74,118]. $DCFH_2$ and DHR are less suitable for reporting peroxynitrite formation in vivo and are certainly not specific for peroxynitrite but very useful in detecting unspecific increase generation of ROS/RNS in cells [116].

Another classic method for peroxynitrite detection that involves this type of mechanism are chemiluminescent probes, such as luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), one of the most studied chemiluminescent probes. Chemiluminescence is the emission of light (luminescence) as the result of a chemical reaction (e.g., oxidation), unlike fluorescence which is based on absorption of photons with the concomitant emission of light. It is essentially an oxyluminescence, since molecular oxygen is necessary for the reaction. Luminol is widely used for the detection of reactive oxygen species produced in biological systems. The general reaction mechanism of chemiexcitation involves two sequential steps of oxidation by appropriate oxidizing agents. The first oxidation could be initiated by a one-electron oxidation process (·OH or $CO_3^{\cdot-}$ but not $O_2^{\cdot-}$) to yield luminol radical (L·). In the second step of oxidation $O_2^{\cdot-}$ and/or $H_2O_2$ could react with the L· to yield an unstable cyclic endoperoxide intermediate. The endoperoxide cleaves spontaneously to excited 3-aminophtalate, which decays to ground state with the consequent emission of a blue light photon with a maximum at 425 nm. Early studies observed that peroxynitrite reacts with luminol yielding chemiluminescence being this luminescence greatly enhanced by bicarbonate ($HCO_3^-$) [120]. This mechanism of chemiexcitation by peroxynitrite in the presence of $HCO_3^-/CO_2$, involves the formation of $CO_3^{\cdot-}$ from the homolytic decomposition of $ONOOCO_2^-$, which is the responsible for the one-electron oxidation of luminol, initiating the pathway for light emission. This chemiluminescence is SOD-inhibitable, indicating that $O_2^{\cdot-}$ is a key intermediate for chemiexcitation. Neither ·NO nor $O_2^{\cdot-}$ alone are able to directly induce luminol chemiluminescence. In biological systems, either up- or downregulation of reactive oxygen species-dependent light emission may vary according to the relative amounts of ·NO, $O_2^{\cdot-}$, and peroxynitrite [121]. Nevertheless, ·NO can also limit oxidative processes due to its reactions with organic radicals, such as L· in a termination reaction that leads to the called "dark route" of luminol oxidation [121]. Chemiluminescence can be used to follow the time course of peroxynitrite formation and with highly sensitive photon counting techniques, peroxynitrite fluxes down to ~1 nM/min can be accurately detected [121]. However, the luminol reaction cannot identify the reactive species or provide mechanistic information. Apart from being nonspecific, there are numerous radical interactions that can influence the chemiluminescence response. Thus, the assay has little ability to discriminate between individual reactive oxygen species in biological systems being extremely prone to interferences [74,114,115].

### *Electrophilic Probes*

A group of small organic molecules proposed as fluorescent probes for the detection of peroxynitrite and other biological nucleophiles ($H_2O_2$, HOCl) share the main characteristics of reacting through a nucleophilic attack by the oxidant to an electrophilic functional group supported on the probe that releases a masked fluorescence. Several functional groups have been proposed as electrophilic centers, like activated ketones (in order to increase its electrophilic capacity). A group of probes called HKGreen- (HK: Hong Kong) named HKGreen-1 a trifluoro-ketone linked to dichlorofluorescein scaffold through an aryl ether linkage [122]; HKGreen-2, a trifluoro-ketone linked to BODIPY through phenol [123]; or HKGreen-3, trifluoro-ketone linked to rhodol scaffold [124], were developed for the detection of peroxynitrite. All of them can react with peroxynitrite by a mechanism where the ketone generates a dioxirane intermediate upon reaction with the oxidant, which oxidize the phenyl ring and release the fluorescent molecule [122–124].

The poor yield of the reaction of both HK-Green-1 and HK-Green-2 with peroxynitrite (21% yield based on 80% conversion and 52% yield based on 48% conversion, respectively) limits its ability to detect peroxynitrite in biological systems [122,123]. HKGreen-3 has an even lower yield, where the oxidation by peroxynitrite generates *N*-methylrhodol scaffold as the fluorescent product in 12% yield [124]. Although these last HK-Green probes showed the ability to detect peroxynitrite in cellular systems, cells were either exposed to exogenous fluxes of peroxynitrite generated by chemical sources (i.e., SIN-1 [122]) or peroxynitrite was generated by nonphysiological stimulation of cells (i.e., PMA, to stimulate NADPH oxidase) with immunostimulated macrophages for ·NO production with IFNγ/LPS [123,124]. The direct detection of endogenously cell-derived peroxynitrite was not evaluated with either

probe bringing out the question of whether they could be adequate for this end.

A new generation of HK-Green has been reported named HK-Green-4 (*N*-phenol-*N*-methylrhodol) for imaging peroxynitrite in cells and tissues [125]. The performance of HKGreen-4 was evaluated by confocal imaging of endogenously produced peroxynitrite in a model of immunostimulated macrophages (LPS/IFN-γ) challenged with heat-killed *E. coli*, where the cellular fluorescence gave an homogeneous and diffuse pattern. The use of Nox2 inhibitors, such as diphenyleneiodonium (DPI) and apocynin attenuated the cellular fluorescence, indicating that peroxynitrite is being generated in *E. coli*-challenged macrophages [125]. Yang and coworkers proposed that "$ONOO^-$ oxidizes HK-Green-4 through a direct two-electron oxidation of the electron-rich phenyl rings to form iminium ions that are further hydrolyzed to give the *N*-dearylation fluorescent product." The detailed mechanistic studies on the *N*-dearylation reactions of those compounds generated upon peroxynitrite reaction are currently under investigation as the authors claimed [125]. However, due to $ONOO^-$ chemical nature it is unlikely that it can act as an electrophilic center, and these activated aromatic compounds do not react directly with $ONOO^-$. The reaction is more likely to proceed via free radical mechanisms with secondary peroxynitrite-derived radicals (·OH, $\cdot NO_2$) being the responsible for probe oxidation giving a indirect reaction with peroxynitrite.

HPF (2-[6-(4′-hydroxy)phenoxy-3*H*-xanthen-3-on-9-yl] benzoic acid) and APF (2-[6-(4′-amino)phenoxy-3*H*-xanthen-3-on-9-yl]benzoic acid were developed as fluorescent probes to detect selectively "highly reactive oxygen species" (hROS), such as ·OH and/or hypochlorite anion ($ClO^-$) [126]. These probes also enhance fluorescence yields upon reaction with peroxynitrite. Since the reaction mechanism of HK-Green-4, HPF, and APF (analogous chemical structures) proceeds *via* an *O*- or *N*-dearylation yielding a fluorescent products and a quinone, they are more likely to be oxidized by ·OH radical derived from peroxynitrite rather than with $ONOO^-$ itself [127] as ·OH radical is a stronger oxidizing agent E° (·OH, $H^+/H_2O$) = 2.3 V at pH 7.4 [115]; E°' ($ONOO^-$, $2H^+/\cdot NO_2$) = 1.6 V [128]. Likely, these probes will be readily oxidized in Fenton-type reactions (e.g., ferrous iron reaction with $H_2O_2$ to yield ·OH) [129].

Another reported fluorescent probe suggested for peroxynitrite detection is Ds-DAB (*N*-(2-Aminophenyl)-5-(dimethylamino)-1-naphthalenesulfonic amide) [130]. In this probe, the supposed direct reaction with peroxynitrite releases the fluorescent dansyl-acid molecule. Nevertheless, it is reported that the fluorescence intensity emitted by the reaction of Ds-DAB with peroxynitrite in aqueous solution, reaches a plateau in ~20 s. This low rate confers a potential disadvantage for its use in cellular systems [130].

The introduction of organoboranes in the redox biology has been very important for the area. Interestingly, as early as in 1969, the possible reaction of peroxynitrite with borates had been suggested by Keith and Powell [131]. They mentioned the impossibility of working with borate buffers when measuring peroxynitrite due to its instability, proposing a direct reaction between them [131].

Structurally, boronic acids are trivalent boron-containing organic compounds that hold one alkyl or aryl substituent and two hydroxyl groups to fill the remaining valences on the boron atom. With only six valence electrons and a consequent deficiency of two electrons, the $sp^2$-hybridized boron atom possesses a vacant *p* orbital [132]. Replacement of the hydroxyl groups of boronic acids by alkoxy or aryloxy groups gives boronic esters. Thus, boronic acids and boronic esters functional groups are electrophilic centers and mild Lewis acids. There are increasing reports of boronated-base fluorescent probes for peroxynitrite detection, from small organic molecules [133,134] to even genetically encoded boronate-based green fluorescent protein (cpGFP) for the direct detection of peroxynitrite in mammalian cells [135]. Boronates, as electrophilic centers, are susceptible to nucleophilic attack by several nucleophiles like peroxides ($HOO^-$, $ROO^-$) or $ClO^-$ but the reaction with $ONOO^-$ is kinetically more favorable as was demonstrated in several reports [83,133,136].

Peroxynitrite reacts with most aryl-boronates nearly a million times faster than $H_2O_2$ ($k_{ONOO-} \sim 10^6\ M^{-1}\ s^{-1}$; $k_{H_2O_2} \sim 1–2\ M^{-1}\ s^{-1}$) [83,136] at physiological pH, in a rapid and direct reaction mechanism where the initial step involves the nucleophilic addition of $ONOO^-$ to the electrophilic boron atom, generating an anionic quaternary intermediate that suffer a subsequent heterolytic cleavage at the O—O bond and hydrolysis giving phenol and nitrite as the major, nonradical end products (approximately 85%–90% yield) [137]. The boronate based probes do not yield the latter products with one-electron oxidants, such as ·OH and $\cdot NO_2$ [138]. Boronate-based compounds have been particularly encouraging due to the straightforwardness and readiness of their reaction with peroxynitrite. Between them, fluorescein-dimethylamide boronate (FlAmBE) was reported as a fluorogenic probe derived from modified fluorescein attached to a pinacol boronic ester for monitoring peroxynitrite in biological systems [133]. An advantage of boronate derived-probes from fluorescein scaffolds is that their spectroscopic properties (FlAmBE: $\lambda_{ex}$ = 485 nm; $\lambda_{em}$ = 535 nm [133]) allow its use in common laboratory equipments for cellular assays, such as flow cytometry and epifluorescence microscopy, unlike other boronated reported probes like CBA (coumarin 7-boronic acid) [139] and CBE ($\lambda_{ex}$ = 332 nm; $\lambda_{em}$ = 470 nm) that limit its use for these techniques in many laboratories.

Recently, our group reported Fl-B (fluorescein-boronate) [83] where the pinacol boronic ester is covalently attached to the fluorescein core. Fl-B was successfully used for the direct detection and estimation of endogenously generated

peroxynitrite in normal endothelial cells, where endogenous peroxynitrite generation was detected upon eNOS activation, secondary to ionomycin-induced intracellular $Ca^{2+}$-influx and in macrophage phagosomes following infection with preloaded *T. cruzi* trypomastigotes (Table 21.1).

Another boronated-derived probe from fluorescein was recently reported named FBBE [4-(pinacol boronate) benzyl-derivative of fluorescein methyl ester] [140]. The oxidative conversion of FBBE to the fluorescent product (fluorescein methyl ester) is a two-step reaction. The reaction of an oxidant ($ONOO^-$, $ClO^-$, $HOO^-$) toward the boronate group leading to the corresponding phenol is the first step of this process. Second step is a slow quinone methide elimination leading to the formation of fluorescein methyl ester. Although the rate constant for the reaction of FBBE with peroxynitrite is high ($>10^5\ M^{-1}\ s^{-1}$), the buildup of the fluorescent product is relatively slow ($k = 0.09\ s^{-1}$), due to the sluggish para-quinone methide elimination [140]. This *O*-dearylation mechanism is more likely to be done by $^{\cdot}OH$ radical rather than peroxynitrite itself [126,127]. FBBE was used to study if the toxicity of doxorubicin (a quinone anthracycline antibiotic widely used as antineoplasic drug) in endothelium was associated with peroxynitrite generation. Endothelial cells (EAhy.926 human umbilical vein) were treated with doxorubicin for 24 h to induce reactive oxygen and nitrogen species and then FBBE oxidation was observed by fluorescent microscopy. To confirm the identity of the species responsible for FBBE oxidation, L-NAME and/or PEG-SOD (poly-ethylene glycol coupled-CuZnSOD) were used showing significant decrease in FBBE oxidation [140]. Albeit FBBE is useful in the direct detection of peroxynitrite, the inherent toxicity of the probe in cellular systems is high (<30 μM) when compared with other boronate-derived fluorescein probes (Fl-B; nontoxic in concentrations up to 1 mM for 24 h) [83] limiting its use in biological models.

Another boronate-based compound, reported recently by Kim and coworkers, has been used for the detection of peroxynitrite [141]. This arylboronate reacts with peroxynitrite to yield the corresponding phenol, which would undergo rapid elimination of *p*-quinomethane to produce phenolate and finally an intramolecular cyclization process to yield a highly fluorescent benzothiazolyl iminocoumarin derivative. The conversion of the arylboronate by peroxynitrite oxidation to the fluorescent product benzothiazolyl iminocoumarin has a reported low rate constant of 15,100 $M^{-1}\ min^{-1}$ for peroxynitrite [141] (equivalent to 252 $M^{-1}\ s^{-1}$), conferring a disadvantage for its use in cellular systems.

Even a novel genetically encoded probe for peroxynitrite detection based on a boronic acid-derived circularly permuted green fluorescent protein (cpGFP), have been developed [135]. Cells (HEK 293T) transiently transfected to express cpGFP and treated with exogenous peroxynitrite donor (SIN-1) showed fluorescence enhancement [135].

Importantly, in most cellular models in which boronate-based probes were assayed for peroxynitrite detection, experiments were done under nonphysiological conditions including: exogenous peroxynitrite addition (even authentic peroxynitrite and/or by SIN-1 peroxynitrite flux generation) and/or under exacerbated macrophage NOX2 stimulation (PMA in the presence of iNOS). Efficiency of the different probes for peroxynitrite detection in basal cellular conditions is only scarcely studied.

In this regard, Fl-B offers an advantage since it has been proven to be able to detect basal rates of peroxynitrite formation within cells. The intracellular peroxynitrite flux in ionomycin-stimulated BAECs was calculated as 0.6 μM/min showing the high capability of Fl-B for intracellular oxidant detection besides other cellular targets (T, targets; e.g., $CO_2$, peroxiredoxins, heme-containing proteins) [17]. Considering the overall rate of peroxynitrite reaction with the different cellular targets, the term $k$[T] is estimated to be between 300 and 500 $s^{-1}$ [14], therefore the *actual* intracellular flux of peroxynitrite in normal BAECs following eNOS activation is ~ 6 μM/min in agreement with previous theoretical calculations (Table 21.1) [83].

There is a large discussion about *specificity* of boronates in the detection of $H_2O_2$ versus peroxynitrite, and some authors argue that peroxynitrite is generated at much lower concentrations displaying markedly shorter biological half-live when compared to $H_2O_2$ that can offset the differences in the second-order rate constants [142]. However, it has been demonstrated by several authors that in cellular systems where $H_2O_2$ and peroxynitrite coexist, boronates will preferentially react with peroxynitrite [83,143]. $H_2O_2$ can freely diffuse in cellular systems where a battery of enzymes can lead to its detoxification (catalase and/or peroxidases), but most important, at physiological pHs only 0.005% of $H_2O_2$ is in the anion form of $HOO^-$ ($pKa_{H_2O_2} = 11.7$), while 83% of the peroxynitrite is in the anion form ($pKa_{ONOO^-} = 6.7$) [136] making $H_2O_2$ less reactive than peroxynitrite toward boronates.

## MODULATORS IN PEROXYNITRITE DETECTION

Pharmacological strategies are available and are very useful to study peroxynitrite generation in biological systems: modulation by NOS inhibitors and/or ·NO scavengers; inhibitors of $O_2^{\cdot-}$ formation (PEG-SODs and SODs mimetic; NOS inhibitors) and peroxynitrite decomposition catalysts and scavengers (boronates based compounds). The inhibition of ·NO formation is obtained by means of a wide variety of NOS inhibitors of various degrees of selectivity and potency over the three different isoforms of NOS (nNOS, iNOS, and eNOS) provide potential tools to define

the contribution of ·NO and ·NO-derived oxidants. Pharmacological inhibition of $O_2^{\cdot-}$ formation is more complex because the sources of $O_2^{\cdot-}$ are variable depending on cell types and metabolic status, and also because typically more than one cellular source contributes to overall cell $O_2^{\cdot-}$ formation. In the more straightforward case of $O_2^{\cdot-}$ formation via NADPH oxidase activation from macrophages and neutrophils, inhibition of this enzyme proves to be effective to inhibit peroxynitrite formation. The widespread used Nox inhibitor DPI also inhibit other flavin-containing enzymes (including the NOS reductase domain), depending on the concentrations used. In systems in which $O_2^{\cdot-}$ is principally formed due to the catalytic action of xanthine oxidase, enzyme inhibitors, such as allopurinol or oxypurinol may be useful [144].

Peroxynitrite or its secondary products can be readily decomposed by the use of decomposition catalysts and scavengers, in order to inhibit biological oxidations and/or nitrations and therefore protect from peroxynitrite-induced oxidative damage. However, to be effective in biological systems, both peroxynitrite decomposition catalysts and scavengers must be able to outcompete peroxynitrite reactions with other targets. Some compounds can catalyze peroxynitrite isomerization (e.g., iron porphyrins) or catalytically reduce peroxynitrite by redox cycles that consume endogenous reductants, such as thiols, ascorbate or urate (e.g., manganese-porphyrins, selenocompounds) [145,146]. Some compounds, such as the manganese porphyrins (Mntmpyp) can potentially have a dual action by acting both as SOD mimetics, as well as peroxynitrite decomposition catalysts. It is important to consider that these compounds can undergo secondary redox reactions under particular conditions. For instance, during the catalytic decomposition of peroxynitrite by manganese-porphyrins, cellular reductants, such as glutathione are consumed. Therefore, the inhibitory effects on peroxynitrite detection will be highly dependent on glutathione concentration in the cell [147].

Scavengers include a variety of endogenous or synthetic molecules that decompose peroxynitrite by oxidizing themselves. Endogenous scavengers that have been used (in addition to glutathione and cysteine) are methionine and uric acid. These compounds do not react fast with peroxynitrite but they can be added in relatively high concentrations and can react rapidly with the secondary intermediates of peroxynitrite reactions.

As example, uric acid has been evaluated in the treatment of an in vivo model of allergic encephalomyelitis (EAE) in the PLSJL strain of mice. Uric acid administration was found to have strong therapeutic effects in a dose-dependent fashion. Also, the incidence of multiple sclerosis and gout (hyperuricemic) revealed that these two diseases are almost mutually exclusive, raising the possibility that hyperuricemia may protect against multiple sclerosis [148]. Nevertheless, the therapeutic use of uric acid is controversial as the reaction of uric acid with reactive oxidants in vivo produce oxidation products that may mediate deleterious effects. As uric acid does not react fast with peroxynitrite, its effects rely on its reactions with peroxynitrite derived-radicals (·OH, $\cdot NO_2$), notably the potent inhibition of protein tyrosine nitration. Uric acid oxidation by peroxynitrite-derived species leads to the formation of intermediates that further can yield triuret (2,4-diimidotricarbonic diamide) [149]. Little is known about the potential in vivo effects of this metabolite. Some recent reports propose that triuret may contribute to electrolyte disorders [150], and it was even identified in significant amounts in patients with preeclampsia, whereas no triuret was produced in normal healthy volunteers [149].

Synthetic scavengers of peroxynitrite-derived radicals include hydroxamates and substituted guanidines. Desferrioxamine and other hydroxamates may be able to inhibit peroxynitrite-mediated oxidations and nitrations in vitro [151] and in vivo [152], leading to the formation of the corresponding nitroxide. Guanidines are also NOS inhibitors and may also provide a dual mechanism of protection against peroxynitrite-mediated modifications [153].

The reaction of peroxynitrite with $CO_2$ is critical because carbon dioxide is ubiquitous and highly concentrated in biological systems. Indeed, considerable levels of $CO_2$ are present on air-equilibrated solutions and buffer systems, depending on composition, ionic strength, and temperature. For instance, in air-equilibrated 50 mM phosphate buffer, pH 7.4, a typical value of contaminating carbon dioxide is in the order of 5–10 μM [154]. In addition, cells and tissues actively produce carbon dioxide and cell cultures are typically equilibrated with 5% $CO_2$ that translates to a dissolved concentration of 1.0–1.5 mM $CO_2$ in equilibrium with $HCO_3^-$ at pH 7.4. $CO_2$ might have the following consequences on peroxynitrite biochemistry: (1) decreases peroxynitrite half-life and therefore its diffusion distance, (2) decreases one-electron oxidations of probes, and (3) enhances nitration of phenolic compounds. Therefore, in biochemical and cellular systems the presence of $CO_2$ plays a critical role in modulating peroxynitrite biochemistry and affecting reaction yields of the different probes.

## CONCLUSIONS AND PERSPECTIVES

Since the initial reports proposing the cellular formation of peroxynitrite by cells under the simultaneous production of its precursor radicals ·NO and $O_2^{\cdot-}$, significant progress has been made in the more definitive and unambiguous detection of this short-lived oxidant. While there is no availability of a totally specific single method for detecting peroxynitrite, a combination of techniques together with the wise application of pharmacological or genetic modulations as well as changes in experimental variables, have provided solid information of peroxynitrite formation in cellular

systems. Sound experimental approaches for the detection of peroxynitrite and consistent interpretation of the results require a solid mechanistic and kinetic understanding of the chemical biology of this elusive oxidant species. Thus, while the detection of cell-derived peroxynitrite under basal and stressed conditions is not a trivial task, this review chapter provides evidence that it is an achievable goal. Emerging methods and probes for the cellular detection of peroxynitrite (e.g., boronate-based probes) are allowing to follow the formation of peroxynitrite overtime, and will likely soon permit to "visualize" preferential subcellular sites of generation as well as the quantitation of intra- and extracellular fluxes of peroxynitrite.

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