Metal-catalyzed protein tyrosine nitration in biological systems

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Protein tyrosine nitration is an oxidative postranslational modification that can affect protein structure and function. It is mediated *in vivo* by the production of nitric oxide-derived reactive nitrogen species (RNS), including peroxynitrite ($ONOO^-$) and nitrogen dioxide ($^\bullet NO_2$). Redox-active transition metals such as iron (Fe), copper (Cu), and manganese (Mn) can actively participate in the processes of tyrosine nitration in biological systems, as they catalyze the production of both reactive oxygen species and RNS, enhance nitration yields and provide site-specificity to this process. Early after the discovery that protein tyrosine nitration can occur under biologically relevant conditions, it was shown that some low molecular weight transition-metal centers and metalloproteins could promote peroxynitrite-dependent nitration. Later studies showed that nitration could be achieved by peroxynitrite-independent routes as well, depending on the transition metal-catalyzed oxidation of nitrite (NO_2^-) to $^\bullet NO_2$ in the presence of hydrogen peroxide. Processes like these can be achieved either by hemeperoxidase-dependent reactions or by ferrous and cuprous ions through Fenton-type chemistry. Besides the *in vitro* evidence, there are now several *in vivo* studies that support the close relationship between transition metal levels and protein tyrosine nitration. So, the contribution of transition metals to the levels of tyrosine nitrated proteins observed under basal conditions and, specially, in disease states related with high levels of these metal ions, seems to be quite clear. Altogether, current evidence unambiguously supports a central role of transition metals in determining the extent and selectivity of protein tyrosine nitration mediated both by peroxynitrite-dependent and independent mechanisms.

Keywords: Nitric oxide, Superoxide, Free radicals, Oxidative stress, Nitration, Transition metals, Hydrogen peroxide, Nitrogen dioxide

Introduction

Transition metals are essential components for all living organisms, as they participate in many key biological processes such as cellular respiration, gene transcription, and many enzymatic reactions. Their ability to oscillate between different redox states is the main feature that allows them to act as cofactors in most of these processes. In humans, the most abundant are iron (Fe), copper (Cu), and manganese (Mn); within the cells, they can be found in different forms: mainly, bound to proteins, but also in association with low molecular weight species and a small fraction as 'free' ions.[1,2] Due to their redox capability, under certain conditions these transition metals can catalyze the generation of reactive oxygen species (ROS) that may lead to oxidative damage of biomolecules, such as proteins and DNA. These ROS include superoxide radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^\bullet OH$).[3,4]

Nitric oxide ($^\bullet NO$) is an endogenously synthesized free radical with many physiological functions, like vasodilation and neurotransmission. However, in the presence of ROS and transition metals, it can give rise to several reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$) and nitrogen dioxide radical ($^\bullet NO_2$). These RNS can mediate the oxidation and nitration of different biomolecules, like the nitration of protein tyrosine (Tyr) residues to 3-nitrotyrosine (NO_2Tyr), an oxidative postranslational modification found in many pathological conditions.[5,6] Transition metals can also play an important role in this type of oxidative modification, although they are not required for it. In fact, the first protein identified to be nitrated *in vitro* by biologically relevant nitrating agents (i.e. $ONOO^-$) was a metalloprotein: Cu,Zn superoxide dismutase (SOD); the Cu^{2+} atom of the enzyme was show to be a key element in that process, considerably promoting NO_2Tyr

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formation.[7] Besides Cu,Zn SOD, those initial studies showed that low molecular weight transition metal complexes could also catalyze nitration of phenolics by $ONOO^-$.[8] As we will discuss below, transition metals can enhance and lead protein tyrosine nitration by several mechanisms and, so, they are undoubtedly necessary for explaining *in vivo* protein tyrosine nitration.

Biological formation of nitrating species

By the late 1980s, the role of the free radical $^{\bullet}NO$ as an important messenger in biological systems had been established.[9–11] Soon after that, it was shown that $^{\bullet}NO$ could also act as a cytotoxic effector molecule when it was overproduced; its capability to participate as a pathogenic mediator depends mainly on the formation of secondary intermediates such as $ONOO^-$ and $^{\bullet}NO_2$ that are considerably more reactive and toxic than $^{\bullet}NO$ itself. These RNS are produced from $^{\bullet}NO$ in the presence of other oxidant species like $O_2^{\bullet-}$, H_2O_2, and transition metal centers.[5]

Peroxynitrite is a short-lived oxidant species produced *in vivo* by the extremely fast reaction between $^{\bullet}NO$ and $O_2^{\bullet-}$ ($k \sim 10^{10}\ M^{-1}\ s^{-1}$). At physiological pH values, it can exist as the anionic form ($ONOO^-$) and as the peroxynitrous acid (ONOOH; pKa = 6.8); both species can participate directly in one- and two-electron oxidation reactions with biomolecules. The main targets for peroxynitrite in biological systems are thiols, carbon dioxide (CO_2) and transition metal centers ($Me^{n+}X$), all of which react directly with either ONOOH or $ONOO^-$ with second order rate constants ranging from $\sim 10^3$ to $\sim 10^7\ M^{-1}\ s^{-1}$.[12,13] In the absence of other targets, ONOOH decays by homolysis to nitrate (NO_3^-), producing also $^{\bullet}OH$ and $^{\bullet}NO_2$ with maximum yields of 30% in a first-order reaction that has a rate constant value of $0.9\ s^{-1}$ (37°C, pH 7.4).[13–15] The reaction between $ONOO^-$ and CO_2 and some reactions of $ONOO^-$ with transition metal centers also can produce secondary radicals. In the first case, the nucleophilic addition of $ONOO^-$ to CO_2 ($k = 5.8 \times 10^4\ M^{-1}\ s^{-1}$, 37°C) yields a nitrosoperoxocarboxylate adduct ($ONOOCO_2^-$) that undergoes a fast homolysis to $^{\bullet}NO_2$ and carbonate radicals ($CO_3^{\bullet-}$).[16,17] Reaction of $ONOO^-$ with transition metal centers (both from metalloproteins and from low molecular weight metal complexes) is more complex and can either be a one- or two-electron oxidation of the transition metal, in which $ONOO^-$ is reduced to $^{\bullet}NO_2$ or nitrite (NO_2^-), respectively. As a result of this reaction, the metal center is oxidized to a higher oxidation state oxo-metal complex ($O = Me^{(n+1)+}X$), a strongly oxidizing species.[5,17] The radical species formed from $ONOO^-$ direct reactions and its proton-catalyzed homolysis ($CO_3^{\bullet-}$, $^{\bullet}NO_2$, $O = Me^{(n+1)+}X$, and $^{\bullet}OH$) are all strong one-electron oxidants able to oxidize a great number of biomolecules and are responsible for many of the observed *in vitro* and *in vivo* peroxynitrite-dependent oxidations, like protein tyrosine nitration.[5,8,18,19] In contrast, the two-electron oxidation of low molecular weight and protein thiols by ONOOH represents a quite harmless way of $ONOO^-$ decomposition, as it generates the oxidized sulfenic acid from the thiol and NO_2^-.[20] The main fate of $ONOO^-$ in biological systems is summarized in Fig. 1.

Another RNS that accounts for much of $^{\bullet}NO$-mediated toxicity is $^{\bullet}NO_2$. This radical species undergoes a variety of reactions that include its recombination with other radicals, its addition to double bonds, electron transfer reactions and hydrogen atom abstractions from carbon–hydrogen bonds of unsaturated compounds, phenols and thiols. Of particular relevance are its recombination reactions with lipid- and protein-derived radicals that occur at near diffusion limited rates ($k > 10^9\ M^{-1}\ s^{-1}$) and produce nitrated lipids and proteins.[21] As explained before, many $ONOO^-$ decomposition pathways can produce $^{\bullet}NO_2$ *in vivo*; there are, however, other reactions that generate $^{\bullet}NO_2$ in biological systems. The most relevant is the one that involves the peroxidase-catalyzed oxidation of NO_2^-, which is of most relevance under inflammatory conditions. This one-electron oxidation of NO_2^- is catalyzed by several heme peroxidases (e.g. myeloperoxidase (MPO) and eosinophil peroxidase (EPO)) in the presence of H_2O_2 and begins with the reaction of the latter with the heme group located at the active site of the peroxidase in a two-electron oxidation (Eq. 1). This oxidation step produces an oxoferryl porphyrin π cation radical intermediate ($HP^{\bullet+}$-($Fe^{4+} = O$)), known as compound I, which is a strong one- and two-electron oxidant. One of the multiple targets of compound I is NO_2^-, which is oxidized by one electron to $^{\bullet}NO_2$; as a result, compound I is reduced to the corresponding oxoferryl intermediate (HP-($Fe^{4+} = O$)), known as compound II (Eq. 2). Compound II is also a strong one-electron oxidant that can oxidize NO_2^- to $^{\bullet}NO_2$ and regenerate the heme peroxidase to its ground state (Eq. 3); this reaction is, however, much slower than the one that implies compound I:[22]

$$HP-\left(Fe^{3+}\right)+H_2O_2 \rightarrow HP^{\bullet+}-\left(Fe^{4+}=O\right)+H_2O \quad (1)$$

$$HP^{\bullet+}-\left(Fe^{4+}=O\right)+NO_2^- \rightarrow HP-\left(Fe^{4+}=O\right)+{}^{\bullet}NO_2 \quad (2)$$

$$HP-\left(Fe^{4+}=O\right)+NO_2^- \rightarrow HP-\left(Fe^{3+}\right)+{}^{\bullet}NO_2 \quad (3)$$

Besides heme proteins, free hemin and other low molecular weight metal complexes have been shown to produce $^{\bullet}NO_2$ by this mechanism.[23] Finally, another

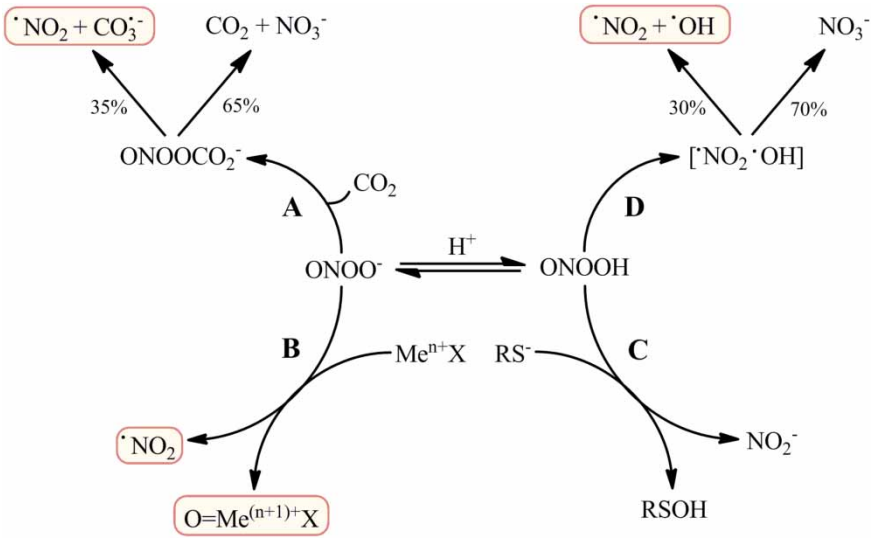

Figure 1 Principal fate of peroxynitrite in biological systems. Peroxynitrite anion ($ONOO^-$) and its acid form, peroxynitrous acid (ONOOH; pKa = 6.8), coexist at physiological pH values and can undergo direct reactions with several biomolecules, the most important of them being carbon dioxide (CO_2), thiols (RS^-) and transition metal centers ($Me^{n+}X$). Reaction of $ONOO^-$ with CO_2 (A) yields a nitrosoperoxocarboxylate adduct ($ONOOCO_2^-$), which homolyzes to nitrogen dioxide ($^{\bullet}NO_2$) and carbonate radicals ($CO_3^{\bullet-}$) with 35% yields. Some transition metal centers can reduce $ONOO^-$ by one electron (B), producing $^{\bullet}NO_2$ and a high oxidation state oxo-metal complex ($O=Me^{(n+1)+}X$). Thiols readily react with ONOOH in a two electron oxidation (C), in which the thiol is oxidized to sulfenic acid (RSOH), reducing ONOOH to nitrite (NO_2^-). Alternatively, ONOOH can undergo homolysis (D) to $^{\bullet}NO_2$ and hydroxyl radical ($^{\bullet}OH$) with maximum yields of 30%, the remaining decaying to nitrate (NO_3^-). The species highlighted are all strong one-electron oxidants that mediate much of the oxidative damage associated with peroxynitrite.

reaction that could generate $^{\bullet}NO_2$ *in vivo* is the one-electron oxidation of NO_2^- by $^{\bullet}OH$,[21] but this seems to be a minor route for the biological formation of $^{\bullet}NO_2$ due to the high reactivity and very low selectivity of $^{\bullet}OH$.

Biochemical mechanisms of tyrosine nitration

Under biological conditions, tyrosine nitration is mediated by free radical reactions in a two-step process: first, the phenolic ring of Tyr is oxidized to its one-electron oxidation product, the tyrosyl radical ($Tyr^{\bullet}$), and, then, the addition of $^{\bullet}NO_2$ to the $Tyr^{\bullet}$ produces the non-radical product NO_2Tyr. Many of the peroxynitrite-derived radicals mentioned earlier can act as the one electron oxidant responsible for producing the $Tyr^{\bullet}$: $CO_3^{\bullet-}$, $O=Me^{(n+1)+}X$ (both from metalloproteins or low molecular weight metal complexes), $^{\bullet}NO_2$ and $^{\bullet}OH$.[6,24] In hydrophobic environments, some of the intermediates of lipid peroxidation processes, like lipid peroxyl ($LOO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals, can also mediate the one-electron oxidation of Tyr, thereby contributing to protein tyrosine nitration in membranes and lipoproteins.[25,26]

Protein tyrosine nitration then requires a one-electron oxidant to oxidize Tyr to the $Tyr^{\bullet}$, and also $^{\bullet}NO_2$ to recombine with the $Tyr^{\bullet}$ formed; by several decay pathways, $ONOO^-$ produces both species, so it is one of the most relevant agents causing protein tyrosine nitration *in vivo*. Alternatively, tyrosine nitration can occur by the $^{\bullet}NO_2$ production catalyzed by heme peroxidases in the presence of H_2O_2 and NO_2^- that was described previously. This system also produces one-electron oxidants that can readily oxidize Tyr to $Tyr^{\bullet}$ (heme peroxidase compound I and II, $^{\bullet}NO_2$) and $^{\bullet}NO_2$ to generate the nitrated product.[6,24]

Tyrosine nitration can be achieved also by an $^{\bullet}NO_2^-$ independent route that implies the addition of $^{\bullet}NO$ to the $Tyr^{\bullet}$ instead of $^{\bullet}NO_2$, to yield 3-nitrosotyrosine. This intermediate can be further oxidized by two electrons to finally form NO_2Tyr in two steps of one-electron oxidation that can be mediated by heme peroxidases/H_2O_2.[27,28] This alternative mechanism may be of great relevance *in vivo* as it does not need $^{\bullet}NO_2$, which has an extremely short biological half-life compared to $^{\bullet}NO$.[29]

The free radical reactions that lead to the production of NO_2Tyr can also generate secondary products that are detected most of the time. Two of the most important of these products are 3,3′-dityrosine (DiTyr), which arises when two $Tyr^{\bullet}$ recombine, and 3-hydroxytyrosine (DOPA), produced mainly as a product of $^{\bullet}OH$ addition to Tyr and the subsequent loss of an electron of the adduct formed.[5,24] Fig. 2 shows the free radical mechanisms that lead to tyrosine nitration and the formation of secondary products. The rate constants for several reactions involved in peroxynitrite biochemistry and tyrosine nitration are shown in Table 1.

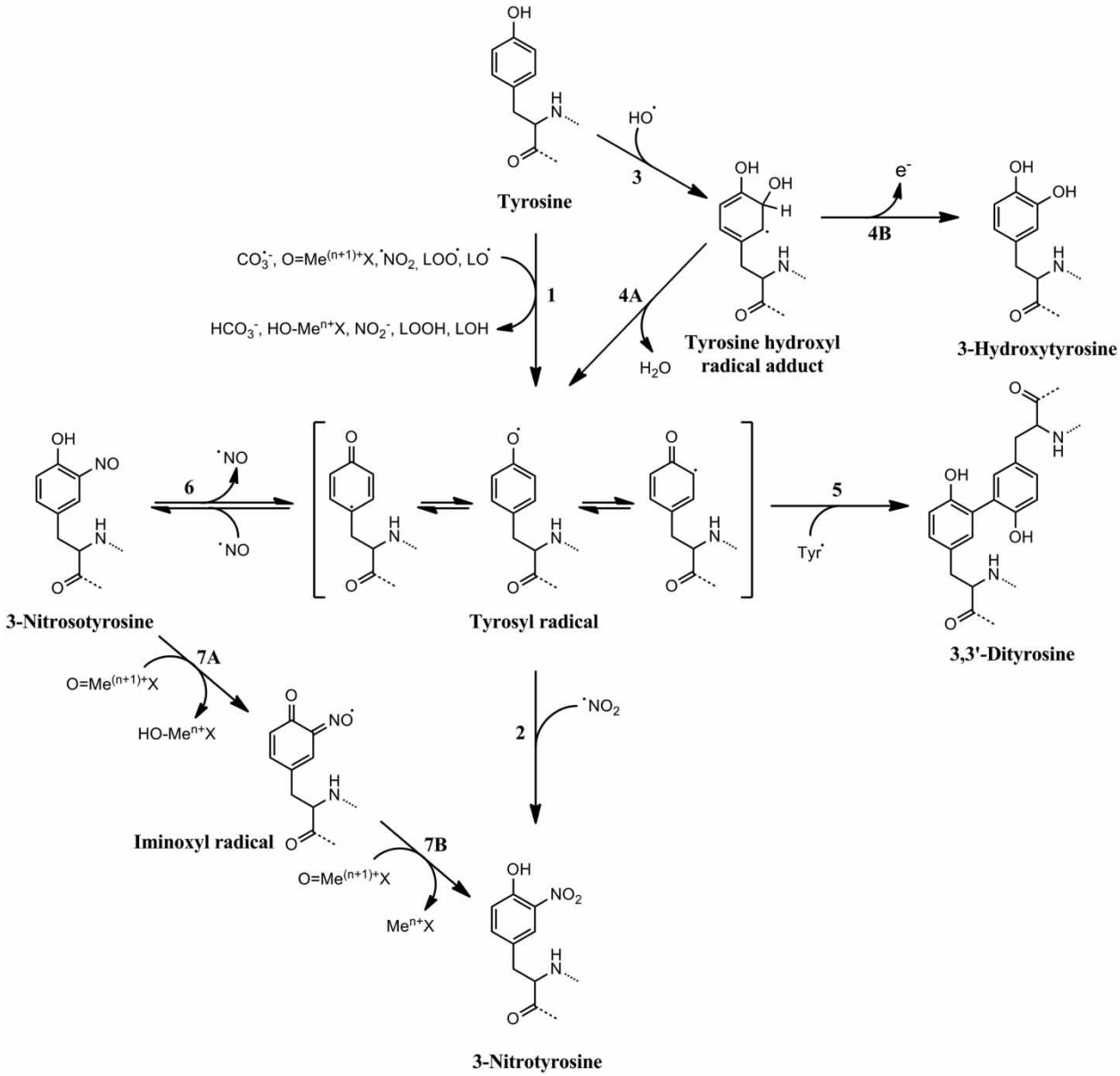

Figure 2 Free radical mechanisms of tyrosine nitration and oxidation. Several one-electron oxidants can oxidize tyrosine to tyrosyl radical (1), which can then react with nitrogen dioxide to produce 3-nitrotyrosine (2). Hydroxyl radical can add into the phenolic ring of tyrosine (3) yielding a tyrosine hydroxyl radical adduct that can dehydrate to tyrosyl radical (4A) or loose an electron to produce the stable product 3-hydroxytyrosine (4B). Tyrosyl radicals, besides reacting with $^{\bullet}NO_2$, can recombine between themselves to generate the oxidation product 3,3′-dityrosine (5). An alternative route to tyrosine nitration implies the reaction of tyrosyl radicals with nitric oxide (6) to yield 3-nitrosotyrosine. This intermediate can be then oxidized to 3-nitrotyrosine by two steps of one-electron oxidation mediated by oxo-metal complexes. The first oxidation produces an iminoxyl radical intermediate (7A) that is finally oxidized to 3-nitrotyrosine (7B). Modified from Ref. 5.

Initially, it was proposed that tyrosine nitration could be achieved by a free radical-independent mechanism (an electrophilic aromatic nitration) promoted by the reaction between $ONOO^-$ and transition metal centers.[5,7,8] In this mechanism, the transition metal center would promote the heterolytic cleavage of $ONOO^-$, producing the nitronium cation (NO_2^+) that could then attack the aromatic ring of tyrosine as a two-electron acceptor, yielding a nitroarenium ion intermediate that then evolves to 3-nitrotyrosine and a proton. In this case, there is no net redox change in the metal center.[5,7,8] However, this mechanism has not been confirmed in biological systems and the relevance of electrophilic aromatic nitration mediated by $ONOO^-$ and transition metal centers is undefined.

Transition metal center-mediated tyrosine nitration

Transition metals are not needed for peroxynitrite-mediated tyrosine nitration. In the absence of any direct targets, $ONOO^-$ finally decays by proton-catalyzed homolysis, producing both $^{\bullet}OH$ and $^{\bullet}NO_2$ that can oxidize and nitrate tyrosine. However, the yields

Table 1 Rate constants of some relevant reactions involved in peroxynitrite biochemistry and tyrosine nitration

Reaction	k (M^{-1} s^{-1})	Reference
$^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^-$	$0.4–1.6 \times 10^{10}$	30–32
$ONOO^- + CO_2 \rightarrow ONOOCO_2^-$	5.8×10^4	16,33
$ONOOH + Prx5(\text{-}S^-) \rightarrow NO_2^- + Prx5(\text{-}SOH)$	7×10^7	34
$ONOOH + GS^- \rightarrow NO_2^- + GSOH$	1.4×10^3	35
$ONOOH + MPO(Fe^{3+}) \rightarrow {}^{\bullet}NO_2 + MPO(Fe^{4+}=O)$	6.2×10^6	36
$ONOOH + Hb(Fe^{2+})O_2 \rightarrow Hb(Fe^{3+}) + O_2^{\bullet-} + NO_3^- + H^+$	1.04×10^4	37
$ONOO^- + Mn^{3+}TE\text{-}2\text{-}PyP \rightarrow {}^{\bullet}NO_2 + O=Mn^{4+}TE\text{-}2\text{-}PyP$	3.4×10^7	38
$MPO(P^{\bullet\pi+}Fe^{4+}=O) + NO_2^- \rightarrow MPO(PFe^{4+}=O) + {}^{\bullet}NO_2$	2.0×10^6	39
$^{\bullet}OH + NO_2^- \rightarrow {}^{\bullet}NO_2 + OH^-$	6.0×10^9	40
$Tyr + CO_3^{\bullet-} \rightarrow Tyr^{\bullet} + HCO_3^-$	4.5×10^7	41
$Tyr + {}^{\bullet}OH \rightarrow Tyr(OH)^{\bullet}$	1.2×10^{10}	42
$Tyr + {}^{\bullet}NO_2 \rightarrow Tyr^{\bullet} + NO_2^- + H^+$	3.2×10^5	43
$Tyr + MPO(Fe^{4+}=O) \rightarrow Tyr^{\bullet} + MPO(Fe^{3+}) + OH^-$	1.6×10^4	44
$Tyr + LOO^{\bullet} \rightarrow Tyr^{\bullet} + LOOH$	4.8×10^3	25
$Tyr + LO^{\bullet} \rightarrow Tyr^{\bullet} + LOH$	3.5×10^5	26
$Tyr^{\bullet} + {}^{\bullet}NO_2 \rightarrow NO_2Tyr$	3.0×10^9	43
$Tyr^{\bullet} + Tyr^{\bullet} \rightarrow DiTyr$	2.3×10^8	45

Experimental conditions used for rate constant determination (*e.g.* temperature, pH) can be found in each specific reference. The homolysis of ONOOH to yield $^{\bullet}OH$ and $^{\bullet}NO_2$ in a first order process is described in the text and shown in Fig. 1. The reactions of peroxynitrite with peroxiredoxins, glutathione and oxyhemoglobin inhibit tyrosine nitration by reduction or isomerization of peroxynitrite. Prx5, peroxiredoxin 5; GS^-, glutathione; MPO, myeloperoxidase; HbO_2, oxyhemoglobin; Mn^{3+}TE-2-PyP, manganese (III) *meso*-tetrakis ((*N*-ethyl)pyridinium-2-yl) porphyrin; $MPO(P^{\bullet\pi+}Fe^{4+}=O)$, myeloperoxidase compound I.

of NO_2Tyr formed under these conditions are usually low because no more than 30% of the $ONOO^-$ undergoes homolysis to $^{\bullet}OH$ and $^{\bullet}NO_2$, and also due to the extremely high reactivity of $^{\bullet}OH$ that allows it to participate in many other reactions that compete with tyrosine nitration. Several low molecular weight transition metal complexes and some metalloproteins have been shown to enhance notably tyrosine nitration by $ONOO^-$ *in vitro*.[8,19,46] The mechanism by which these metal complexes can promote $ONOO^-$-dependent tyrosine nitration implies the direct reaction between $ONOO^-$ and the transition metal center, which acts as a Lewis acid, to form a Lewis adduct. This adduct would then undergo a homolytic rupture to yield $^{\bullet}NO_2$ and the corresponding oxy radical of the Lewis acid, which finally rearranges to the respective oxo compound, via oxidation of the Lewis acid (Eq. 4; the charges of the species involved are not specified):[5,47]

$$ONOO^- + Me^{n+}X \rightarrow ONOO - Me^{n+}X \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}O - Me^{n+}X \rightarrow NO_2 + O = Me^{(n+1)+}X \quad (4)$$

As a result of this reaction, $^{\bullet}NO_2$ and oxo-metal complexes (e.g. $O=Fe^{4+}X$, $O=Mn^{4+}X$) are generated; these two species are able to oxidize and nitrate tyrosine residues. So, in the presence of transition metal centers (low molecular weight complexes or metalloproteins) that react with $ONOO^-$ by the described reaction, $ONOO^-$-dependent tyrosine nitration is mediated mainly by these two oxidants; NO_2Tyr yields are increased in this transition metal-dependent nitration largely by two factors. In the absence of transition metal complexes, $ONOO^-$ will decay by proton-catalyzed homolysis, generating a maximum of 30% of the oxidants that mediate tyrosine nitration ($^{\bullet}OH$ and $^{\bullet}NO_2$). However, in the presence of transition metal complexes that react with $ONOO^-$ at moderate rates, most of the $ONOO^-$ will be decomposed by reaction with the metal complex and a small fraction will undergo homolysis. Depending on some factors (e.g. rate constant of the reaction between the metal and $ONOO^-$, concentration of the metal complex), all of the $ONOO^-$ can decay through reaction with the metal complex, resulting in a generation of the secondary oxidants $^{\bullet}NO_2$ and $O=Me^{(n+1)+}X$ with a 100% yield with respect to the initial $ONOO^-$ concentration. Thus, the production of $ONOO^-$-derived secondary oxidants can reach much higher levels in the presence of some metal complexes and this will result in higher yields of tyrosine nitration. Besides this, nitration is enhanced also because of the higher selectivity of oxo-metal complexes to oxidize tyrosine residues than $^{\bullet}OH$; so, not only more oxidant is produced, but also a greater part of it may produce $Tyr^{\bullet}$ (Fig. 3).

Peroxynitrite-independent tyrosine nitration depends largely on the presence of transition metals. It can occur by two main pathways described previously that use H_2O_2 and NO_2^-: the peroxidase-dependent oxidation of NO_2^- to $^{\bullet}NO_2$ and the Fenton-dependent production of $^{\bullet}NO_2$ from NO_2^-. In the first case, the two-electron oxidation of a heme peroxidase by H_2O_2 produces the heme peroxidase compound I that can act as a one-electron oxidant in the two key reactions: the oxidation of Tyr to $Tyr^{\bullet}$ and the oxidation of NO_2^- to $^{\bullet}NO_2$. The one-electron reduction product of compound I, compound II, can also oxidize by one-electron Tyr and NO_2^-. So, by any combination of these reactions,

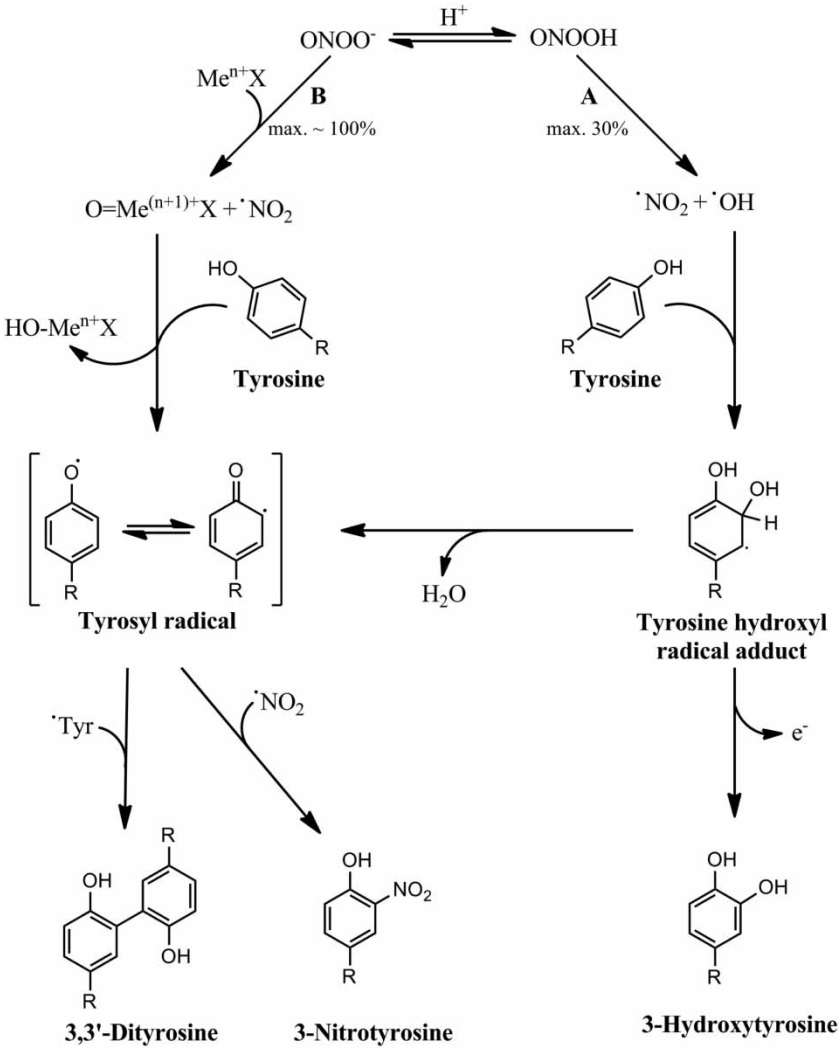

Figure 3 Peroxynitrite-dependent tyrosine nitration mediated by transition metal centers. In the absence of any direct target, peroxynitrous acid decays by homolysis producing nitrogen dioxide and hydroxyl radicals with maximum yields of 30% (A). These two radicals mediate tyrosine nitration and oxidation by a hydroxyl radical-dependent pathway that produces 3-nitrotyrosine in relatively low yields, as well as 3,3′-dityrosine and 3-hydroxytyrosine. However, in the presence of certain transition metal centers that react fast with $ONOO^-$, almost all of the peroxynitrite present can decompose by reacting with the metal complex (B), producing high levels of the oxidized oxo-metal complex and nitrogen dioxide. In this case, these two species mediate tyrosine nitration, which can occur now in higher yields. Also, high levels of 3,3′-dityrosine may be produced.

tyrosine nitration can be achieved, as both $Tyr^{\bullet}$ and $^{\bullet}NO_2$ are produced (Fig. 4).[22,48,49] This transition metal-catalyzed nitration is mediated by Fe^{3+} and Mn^{3+} atoms coordinated by tetrapyrroles – iron and manganese porphyrins – both free and protein-bound, that can be oxidized by two electrons by H_2O_2.[23,46,50]

In the ferrous state (Fe^{2+}), iron can also promote tyrosine nitration in the presence of H_2O_2 and NO_2^-, and in this case, the nature of the ligand group is not as relevant as for the mechanism mentioned before. This pathway to tyrosine nitration begins with the Fenton reaction, in which Fe^{2+} is oxidized by H_2O_2 to Fe^{3+}, producing a hydroxyl anion and $^{\bullet}OH$ (Eq. 5):

$$Fe^{2+}X + H_2O_2 \rightarrow Fe^{3+}X + OH^- + {}^{\bullet}OH \quad (5)$$

After its production, $^{\bullet}OH$ can oxidize by one electron both Tyr to $Tyr^{\bullet}$ and NO_2^- to $^{\bullet}NO_2$ that can finally recombine to yield NO_2Tyr (Eqs. 6–8). Clearly, the $^{\bullet}NO_2$ produced is also able to oxidize Tyr to $Tyr^{\bullet}$:

$$Tyr + {}^{\bullet}OH \rightarrow Tyr - OH^{\bullet} \rightarrow Tyr^{\bullet} + H_2O \quad (6)$$

$$NO_2^- + {}^{\bullet}OH \rightarrow {}^{\bullet}NO_2 + OH^- \quad (7)$$

$$^{\bullet}NO_2 + Tyr^{\bullet} \rightarrow NO_2Tyr \quad (8)$$

Besides iron, copper can also induce tyrosine nitration by this mechanism: Cu^+ atoms, coordinated by diverse ligands, participate as Fe^{2+} in the generation of $^{\bullet}OH$ (Eq. 9) that in the presence of NO_2^- can lead to tyrosine nitration:[23]

$$Cu^+X + H_2O_2 \rightarrow Cu^{2+}X + OH^- + {}^{\bullet}OH \quad (9)$$

Cupric ion-containing metal complexes ($Cu^{2+}X$) also

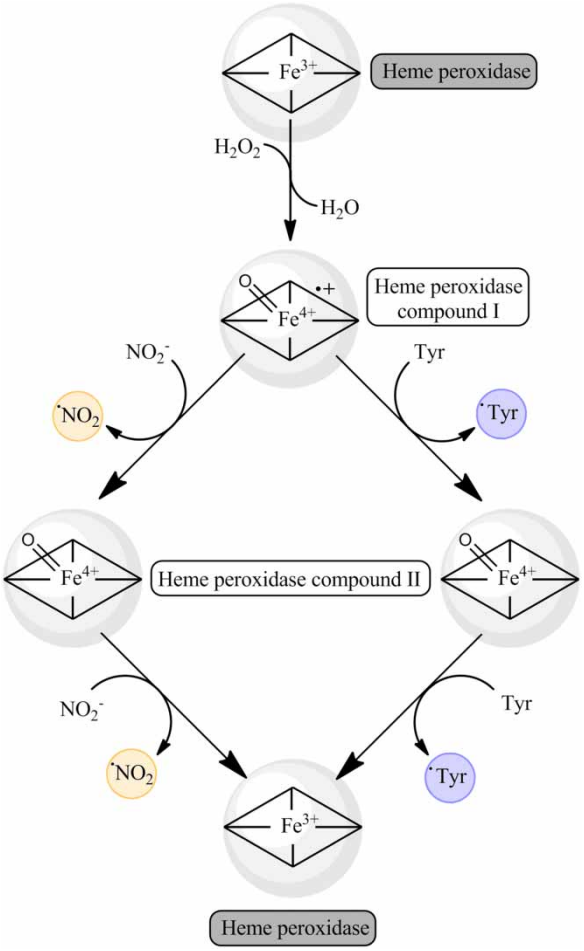

Figure 4 Heme peroxidase-dependent tyrosine nitration in the presence of H_2O_2 and NO_2^-. Heme peroxidases can reduce H_2O_2 by two electrons to H_2O, producing the oxidized compound I intermediate. This intermediate can oxidize by one-electron either tyrosine residues or nitrite, yielding tyrosyl radical or nitrogen dioxide, evolving to the compound II intermediate. Analogously, compound II can act also as a one-electron oxidant, able to oxidize tyrosine residues to tyrosyl radicals and NO_2^- to nitrogen dioxide. In this way, both tyrosyl radicals and nitrogen dioxide are produced and lead to 3-nitrotyrosine formation; also, 3,3′-dityrosine may be produced by the recombination of two tyrosyl radicals.

have been shown to mediate tyrosine nitration in the presence of H_2O_2 and NO_2^- by the same mechanism as Cu^+ complexes; however, the Cu^{2+} atom needs to be reduced first to Cu^+: this initial reduction can be promoted by hydroperoxide anions (HOO^-) that arise from H_2O_2 dissociation (Eq. 10):[23,51]

$$Cu^{2+}X + HOO^- \rightarrow (HOO^-) - Cu^{2+}X \rightarrow Cu^+X + O_2^{\bullet -} + H^+ \qquad (10)$$

Recently, a new mechanism was proposed to participate in copper-mediated tyrosine nitration. This novel mechanism is promoted by Cu^{2+} complexes in the presence of $^{\bullet}NO_2$ and implies the reduction of the Cu^{2+} atom to Cu^+ by $^{\bullet}NO_2$, which oxidizes to NO_2^+ and may promote tyrosine nitration by electrophilic aromatic nitration.[52] Further studies are needed to define the biological relevance of these reactions in copper-mediated tyrosine nitration.

Site-specificity in transition metal-catalyzed protein tyrosine nitration

Despite being a non-enzymatic amino acid modification, tyrosine nitration has shown to be a relatively selective process: not all proteins become nitrated and, among those proteins that undergo nitration, only one or two tyrosine residues are preferentially modified. The determinants of this selectivity are not fully established yet, but some relevant factors have been identified, like the protein structure, the nitration mechanism, and the environment where the protein is located.[24,29] A particular case where the selectivity of tyrosine nitration seems more clear is in transition metal-containing proteins that have tyrosine residues close to the metal center. Two well-described examples of this are the MnSOD and the prostacyclin synthase, where a particular tyrosine residue is nitrated by $ONOO^-$.[53,54] In proteins that lack redox-active transition-metal centers, tyrosine nitration is achieved by one-electron oxidants that arise, for example, by $ONOO^-$ decomposition in solution, that will preferentially react with solvent-exposed tyrosines. However, for several transition metal-containing proteins, nitration can occur through the direct reaction between $ONOO^-$ and the metal center that, as was described previously, may generate oxidizing and nitrating species *in situ* (e.g. oxo-metal center and $^{\bullet}NO_2$); these species may then promote the specific nitration of neighboring tyrosine residues. These metalloproteins may also catalyze their own site-specific nitration in the presence of H_2O_2 and NO_2^-, $^{\bullet}NO_2$, or $^{\bullet}NO$: the direct reaction between the oxidized metal center (e.g. Fe^{3+} or Mn^{3+}) and H_2O_2 would produce an oxo-metal compound that may oxidize nearby Tyr residues to Tyr$^{\bullet}$, which could then recombine with $^{\bullet}NO_2$ (that may also be produced by the oxo-metal compound catalyzed oxidation of NO_2^-) to produce NO_2Tyr. In the presence of $^{\bullet}NO$, the Tyr$^{\bullet}$ produced could also lead to tyrosine nitration through the formation of 3-nitrosotyrosine (NOTyr), which could be further oxidized to NO_2Tyr by the metalloprotein itself, in the presence of H_2O_2.[29]

The mechanisms of protein tyrosine nitration in controlled *in vitro* systems are well established nowadays; however, understanding properly how tyrosine nitration occurs *in vivo* is much more difficult, mainly because of the multiple targets and reactions that consume oxidizing and nitrating radicals by pathways different from tyrosine nitration, and also because of the presence of multiple biological reductants that could reduce tyrosyl radicals back to Tyr. When this is considered, site-specific nitration of

metalloproteins becomes a mechanism of greater relevance, as it generates nitrating and oxidizing intermediates in a different environment, much closer to the tyrosine residue that gets nitrated and not accessible to reductants and other competitors that may abolish nitration.[24]

Biological examples of metal-catalyzed tyrosine nitration

There are many studies that support the close relationship between transition metal levels and tyrosine nitration, both by *in vitro* and *in vivo* approaches. Free iron (Fe^{2+} and Fe^{3+}) and copper (Cu^{+} and Cu^{2+}) ions can promote protein tyrosine nitration in the presence of H_2O_2 and NO_2^- through Fenton-type chemistry.[23] This means that, under pathological conditions that involve high levels of these metal ions, there will be an increase in tyrosine nitration levels at least by this mechanism. Indeed, several studies have shown in some animal models of disease related with high levels of iron, how NO_2Tyr levels are significantly increased.[55–57]

Under inflammatory conditions, the peroxidase-catalyzed mechanism of nitration is of greater relevance, due to the release of leukocyte peroxidases, like MPO and EPO, involved in the production of oxidants that participate in the immune response against invading pathogens.[22,58,59] The reactive species generated by these heme peroxidases, besides acting as antimicrobial agents, can promote oxidative modifications of host biomolecules, like protein tyrosine nitration. Numerous studies have shown that the levels of protein-bound NO_2Tyr are greatly enhanced in inflammatory sites under certain disease states (e.g. atherosclerotic plaque), where MPO also is found.[60–63] In this case, not only the peroxidase/H_2O_2/NO_2^- system could be implied in nitration, but also $ONOO^-$-dependent nitration promoted by MPO might contribute to the high levels of NO_2Tyr detected.

Undoubtedly, one of the most relevant redox active transition metal complexes in biological systems is hemin (ferriprotoporphyrin IX). Under normal conditions, hemin is found as a prosthetic group in heme proteins, both in its reduced state (heme, Fe^{2+}-protoporphyrin IX) and in its oxidized state (hemin), where its reactivity is more or less controlled. However, under diverse pathological conditions, high amounts of hemin are released from their respective proteins, resulting in high levels of free hemin able to participate in multiple oxidative reactions. This situation can occur mainly in hemolytic diseases (e.g., hemolytic anemia, sickle cell disease and hemorrhage), where the abnormal intravascular hemolysis results in the massive release of hemin from red blood cell hemoglobin into the vascular space.[64–66]

Free hemin is highly toxic; much of its toxicity is given by its redox properties and its capacity to produce highly reactive species that mediate oxidative damage, mainly through its reaction with H_2O_2. Although under certain conditions hemin can catalyze H_2O_2 decomposition to H_2O and O_2 (catalase-like activity), in the presence of many different substrates the hemin/H_2O_2 system acts as powerful one- and two-electron oxidant able to oxidize a great number of biomolecules.[67,68] Protein oxidation and lipid peroxidation can be promoted by this system, among other modifications. Also, the reaction between hemin and lipid hydroperoxides produces secondary radical species that can lead to oxidative damage.[69] Besides this, as was mentioned previously, free hemin catalyzes protein tyrosine nitration by H_2O_2/NO_2^- and can also promote $ONOO^-$-dependent tyrosine nitration, enhancing greatly the levels of NO_2Tyr.[23,70] Indeed, it was observed that the levels of tyrosine nitrated proteins are much higher in aortas of animal models of hemolytic diseases with respect to normal ones, suggesting that the excess of hemin in the vasculature promotes tyrosine nitration, among other effects.[71] Due to its high hydrophobicity, released hemin tends to accumulate in cell membranes, so its effects would be more pronounced on membrane proteins and lipids.

Transition metal-dependent increase in tyrosine nitration can also occur as an unintended reaction by certain metal-based drugs: this is the case of synthetic metalloporphyrins of iron and manganese (FeP and MnP), a group of compounds that can catalytically decompose $ONOO^-$ and that have been used in numerous *in vivo* and *in vitro* studies to attenuate peroxynitrite-dependent cytotoxicity. Their ability to act as $ONOO^-$ decomposers relies on their quite fast reaction with $ONOO^-$ ($k \sim 10^5$–$10^7\ M^{-1}s^{-1}$ for Mn^{3+}/Fe^{3+}P).[12] Under normal cellular conditions, the compounds, administered as Mn^{3+}P, readily evolve to the Mn^{2+} state; this reduction can be achieved *in vivo* with relative ease by low molecular weight reductants, such as glutathione or ascorbate, or also enzymatically by a number of flavoenzymes including the mitochondrial electron transport chain. Then, the Mn^{2+}P can reduce $ONOO^-$ by two electrons, producing NO_2^- and O = Mn^{4+}P, in a reaction that is almost as fast as the one for the Mn^{3+}P and avoids $^{\bullet}NO_2$ formation.[12,13,72] However, under cellular oxidative stress conditions a larger fraction of both iron and manganese porphyrins can remain in the 3+ redox state and react with peroxynitrite in a one-electron reduction of $ONOO^-$, yielding $^{\bullet}NO_2$ and the oxo-metal 4+ state of the porphyrin (O = Fe/Mn^{4+}P), both strong oxidants. Although in a large number of studies in *in vivo* models these compounds have shown to protect against peroxynitrite dependent cytotoxicity,[12] *in vitro*

approaches showed early that Fe^{3+} and Mn^{3+} porphyrins can enhance peroxynitrite-mediated oxidations, like DNA strand breaks and tyrosine nitration.[46,73,74] It seems reasonable that these compounds starting in the 3+ state could promote tyrosine nitration by $ONOO^-$ as both $O=Fe/Mn^{4+}P$ and $^{\bullet}NO_2$ can oxidize and nitrate tyrosine residues. So, the effectiveness of Mn and Fe-porphyrins to neutralize deleterious actions of peroxynitrite depends on cell/tissue redox state and its use in subjects with critically low levels of intracellular reductants may result in more oxidative damage than protection.

Site-specific nitration of proteins directed by transition metal centers has been observed in diverse metalloproteins by *in vitro* approaches. For example, human MnSOD is nitrated by $ONOO^-$ specifically at tyrosine 34, which is located close to the Mn^{3+} atom.[75,76] MnSOD nitration is mediated by the direct reaction between the metal center and $ONOO^-$ that yields $Mn^{4+}=O$ and $^{\bullet}NO_2$.[53] Like MnSOD, prostaglandin H_2 synthase (PGHS) is also site-specifically nitrated by $ONOO^-$ at residue 385, in a process that depends on the heme group of the enzyme.[77] Another heme protein, prostacyclin synthase, undergoes site-specific nitration by $ONOO^-$ at tyrosine 430, a residue that lies near the heme group.[54] Interestingly, it was seen that PGHS can also be specifically nitrated at tyrosine 385 by a peroxynitrite-independent mechanism. During the enzyme turnover, a tyrosyl radical at Tyr 385 is generated; the trapping of this $Tyr^{\bullet}$ by $^{\bullet}NO$ can lead to NO_2Tyr through the oxidation of the nitrosotyrosine in the presence of more substrate.[27,78]

Thus, site-specific tyrosine nitration of metalloproteins might occur *in vivo* by diverse mechanisms and seems to be a quite relevant process in biological tyrosine nitration: metal-catalyzed protein tyrosine nitration can be achieved at considerable yields in vivo even in the presence of multiple targets that readily consume reactive oxygen/nitrogen species (Table 1).

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