Hypothesis

What nitrates tyrosine? Is nitrotyrosine specific as a biomarker of peroxynitrite formation in vivo?

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Abstract Peroxynitrite ($ONOO^-$) is a 'reactive nitrogen species' that can be formed (among other reactions) by combination of superoxide ($O_2^{\cdot -}$) and nitric oxide ($NO^{\cdot}$) radicals. It is being increasingly proposed as a contributor to tissue injury in several human diseases. The evidence presented for peroxynitrite participation usually includes the demonstration of increased nitrotyrosine levels in the injured tissue. Indeed, this is often the only evidence presented: the assumption is that formation of nitrotyrosine is a biomarker specifically diagnostic of $ONOO^-$ production. The present article examines this assumption and concludes that nitrotyrosine is a biomarker for 'nitrating species' rather than being specific for $ONOO^-$.

Key words: Peroxynitrite; Nitric oxide; Nitrotyrosine; Nitrogen dioxide; Hypochlorous acid; Reactive nitrogen species

1. Introduction

The free radical nitric oxide (nitrogen monoxide, $NO^{\cdot}$) is produced in vivo by constitutive and inducible nitric oxide synthase enzymes [1,2] and probably also by non-enzymic reactions [3,4] such as the reaction of nitrites with acid in the stomach [5]. Nitric oxide plays important physiological and pathological roles [1–7]. One of the mechanisms by which excess $NO^{\cdot}$ can injure tissues is by its rapid [8] reaction with superoxide radical ($O_2^{\cdot -}$) to give peroxynitrite, $ONOO^-$.

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

Peroxynitrite undergoes reaction with CO_2, protonation, isomerization and decomposition at physiological pH to give noxious products that deplete antioxidants and oxidize and nitrate lipids, proteins and DNA [6,9–19]. The detailed mechanisms by which $ONOO^-$ and species derived from it cause modification of biomolecules are still incompletely understood [6,9–21] but there is little doubt about the cytotoxicity associated with $ONOO^-$ formation at physiological pH [6,9,10,22–25].

Peroxynitrite has been suggested to be involved in the pathology of a wide range of diseases (Table 1). In all the cases listed in Table 1, part (or often all) of the evidence that implicates $ONOO^-$ is the detection of 3-nitrotyrosine (Fig. 1) in the injured tissues. Nitrotyrosine is generated when $ONOO^-$ is added to tyrosine itself, or to proteins containing tyrosine residues, under physiological conditions and the rate of nitration is usually elevated if transition metal ions or certain metalloproteins (e.g. CuZnSOD) are present [9,10,46–49]. The chemical identity of the nitrating species is uncertain [9–11,17,46–48,50]. Detection of 3-nitrotyrosine is most often achieved by antibody immunostaining of tissues [26,49], but HPLC-based [30,49,51,52,86] and GC/MS-based [27,53] techniques have also been described.

2. Is nitrotyrosine specific as a marker of peroxynitrite generation?

Peroxynitrous acid is known to be capable of leading to nitration of tyrosine. However, is the presence of nitrotyrosine a specific biomarker of $ONOO^-$ generation? The obvious question is whether other species can nitrate tyrosine. Let us examine some of the candidates.

2.1. Nitric oxide

There is no evidence that $NO^{\cdot}$ reacts with free tyrosine or tyrosyl residues within proteins. Such a reaction is unlikely given the generally poor reactivity of $NO^{\cdot}$ with non-radicals. However, $NO^{\cdot}$ reacts extremely fast with tyrosyl (tyr-$O^{\cdot}$) radicals, the second-order rate constant being greater than 10^9 M^{-1} s^{-1} [54]. It is possible that the addition products C-nitroso and/or O-nitrosotyrosine are reversibly formed (Fig. 1). Nitrosotyrosine could conceivably be converted to 3-nitrotyrosine by ROS generated at sites of inflammation [54]. Quenching of the active site tyrosyl radical of ribonucleotide reductase probably accounts for the inhibition of this enzyme by $NO^{\cdot}$ [55]. Tyrosyl radicals have been detected in a variety of other enzymes including cyclooxygenase, photosystem II of chloroplasts and in haem proteins after reaction with H_2O_2 [56–59]. Hence it is possible (but remains to be proved) that free radical attack upon tyrosine to generate Tyr–$O^{\cdot}$ followed by addition of $NO^{\cdot}$ and rearrangement *could* generate nitrotyrosine. Many free radicals can oxidize Tyr to Tyr-$O^{\cdot}$.

2.2. Nitrogen dioxide

The brown choking acidic free radical gas nitrogen dioxide ($NO_2^{\cdot}$) is a well-known toxin, formed by combustion of organic materials. It is an important constituent of polluted indoor and outdoor air [60] and cigarette smoke [61]. Exposure of tyrosine to $NO_2^{\cdot}$ in aqueous solution generates both nitrotyrosine and bityrosine [47,62,63] and $NO_2^{\cdot}$ can also nitrate tyrosine residues in proteins [47,62,63]. Even cigarette smoke nitrates tyrosine [61]. Indeed, $NO_2^{\cdot}$ production may be one mechanism accounting for tyrosine nitration on addition of $ONOO^-$ at physiological pH; production of bityrosine in the reaction mixtures is consistent with Tyr-$O^{\cdot}$ formation [47].

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Exposing animals to large amounts of $NO_2^{\cdot}$ gas could therefore lead to protein nitration. However, it seems that high levels of $NO_2^{\cdot}$ are needed to achieve tyrosine nitration, even in vitro [63]. Many of the human subjects and other animals used as healthy controls in the studies summarized in Table 1 lived in large industrial cities and would thus be breathing outdoor and indoor air containing some $NO_2^{\cdot}$ [60]. Presumably, some subjects were smokers. Yet tissues from healthy subjects rarely show much (if any) immunostaining for nitrotyrosine and levels of nitrotyrosine detected by GC/MS or HPLC are usually low, quoted as [30,64] less than 100 nM (or as $\leq$1 nitrated tyrosine per 10^6 residues [86]) in human body fluids. Hence normal atmospheric levels of $NO_2^{\cdot}$ (a few ppm at most) seem unlikely to confound nitrotyrosine measurements.

Nevertheless, the fast reaction of $NO_2^{\cdot}$ with tyrosine radicals generates nitrotyrosine (see below). $NO_2^{\cdot}$ can arise from $NO^{\cdot}$ oxidation (although this is a slow reaction), by oxidation of NO_2^- (Section 2.5) and perhaps from $ONOO^-$ at physiological pH.

2.3. Nitrous acid

Breakdown of $NO^{\cdot}$ in aqueous solution generates NO_2^- as the major end-product [65] whereas decomposition of $ONOO^-$ (via ONOOH) produces largely NO_3^- [9]. NO_2^- levels in healthy subjects have been reported as 0.5–13 μM in plasma, 15 μM in respiratory tract lining fluids, 30–210 μM in saliva, ~1 μM in CSF and 0.4–60 μM in gastric juice. Elevated levels have been reported in many human diseases [66–72], e.g. greater than 30 μM in serum of AIDS patients with pulmonary involvement [73]. Most NO_2^- generated from $NO^{\cdot}$ in vivo or absorbed from the gut is oxidized to NO_3^-, although NO_3^- can be re-reduced to give NO_2^- in the gut and in the oral cavity [74,75]. Exposure of NO_2^- to low pH generates oxides of nitrogen via formation and decomposition of nitrous acid, HNO_2. This could happen at the low pH of the human stomach [5] and might possibly occur in ischaemic tissues [4], and in the phagolysosome, but in most other tissues the pH probably never falls to a sufficiently low value to permit HNO_2 generation. It thus seems that HNO_2 formation would be an unlikely source of nitrating species in most tissues, but could contribute to events in the stomach (Table 1). Nevertheless, it must not be forgotten that the 'standard' laboratory preparation of peroxynitrite [9] involves initial reaction of HNO_2 with H_2O_2

$$HONO + HOOH \rightarrow HOONO + H_2O$$

Table 1
Some of the conditions in which the formation of peroxynitrite has been implicated as causing tissue injury

Condition	Reference
Atherosclerosis	[26,27] (but see [28])
Sporadic inclusion-body myositis	[29]
Rheumatoid arthritis	[30]
Inflammatory bowel disease	[31–33]
Neurodegenerative disease	[24,25,34,35]
Acute inflammation	[36]
Carbon monoxide toxicity	[37]
Adult respiratory distress syndrome	[38,39]
Skin inflammation	[40]
Gastritis (*H. pylori* infection)	[41]
Cystic fibrosis	[42]
Endotoxic shock	[43,44]
Ageing of skeletal muscle	[45]
Viral infection	[83]

Fig. 1. Structures of C-nitroso (A) and O-nitroso (B) tyrosines.

2.4. Nitryl (nitronium) chloride

Eiserich et al. [76], extending earlier work of Kono [77], showed that reaction of NO_2^- with hypochlorous acid (HOCl), generates a product that can nitrate tyrosine and other aromatic compounds. Detailed chemical characterization [76] strongly suggests that this compound is nitryl (nitronium) chloride, NO_2Cl. Hypochlorous acid is well known to be produced at sites of inflammation by activated neutrophils [78], where NO_2^- can be present in μM amounts (see above). Addition of HOCl [79,80] or of NO_2Cl [76] can lead to chlorination of tyrosine, generating 3-chlorotyrosine (Fig. 2). Although there is as yet no direct evidence for NO_2Cl production in vivo (HOCl generated in vivo can react with many molecular targets other than NO_2^- or tyrosine), the data in [76] suggest that caution should be exercised in attributing nitration to the effects of $ONOO^-$. Of course, $ONOO^-$ cannot chlorinate aromatic rings, so observation of nitration in the absence of chlorination would suggest that NO_2Cl is not involved.

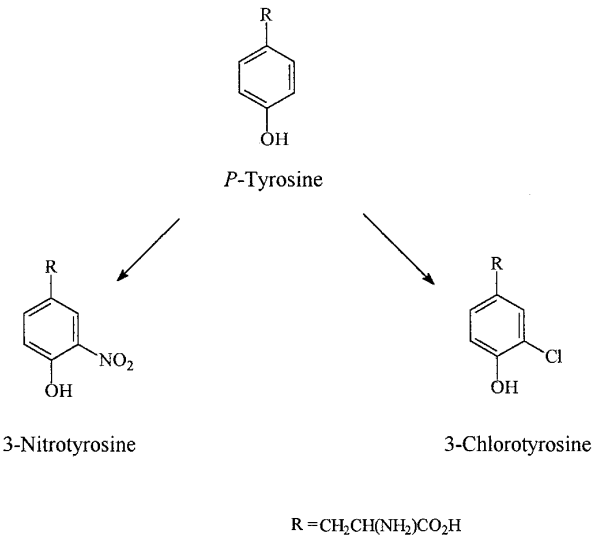

Fig. 2. Conversion of tyrosine to 3-nitrotyrosine and 3-chlorotyrosine.

2.5. Myeloperoxidase

Hypochlorous acid is generated by the H_2O_2-dependent oxidation of Cl^- by the haem-containing enzyme myeloperoxidase [78]

$$Cl^- + H_2O \xrightarrow{MPO} HOCl + HCl$$

Myeloperoxidase is a 'non-specific' peroxidase, capable of using H_2O_2 to oxidize a wide range of substrates. Van der Vliet et al. [81] showed that MPO (as well as lactoperoxidase and horseradish peroxidase) can oxidize NO_2^- in the presence of H_2O_2 into a species able to nitrate tyrosine. Given that peroxidases can catalyze single-electron oxidations, and that the product of one-electron oxidation of NO_2^- is $NO_2^{\cdot}$, it is possible that the nitrating species is $NO_2^{\cdot}$

$$HRP + H_2O_2 \rightarrow \text{compound I}$$

$$\text{compound I} + NO_2^- \rightarrow \text{compound II} + NO_2^{\cdot}$$

$$\text{compound II} + NO_2^- \rightarrow HRP + NO_2^{\cdot}$$

However, two-electron oxidation of NO_2^- to NO_2^+ is also conceivable. Formation of the nitrating species from NO_2^- still occurred in the presence of physiological (100 mM) levels of Cl^- [81]. Myeloperoxidase/H_2O_2 is capable of oxidizing tyrosine to tyrosyl radicals [84,85]. Such radicals could combine with $NO_2^{\cdot}$ to form nitrotyrosine, or with each other, generating dityrosine [81]. Indeed, any oxidizing species capable of oxidizing both tyrosine to tyrosyl radicals and NO_2^- to $NO_2^{\cdot}$ should lead to nitrotyrosine formation (Section 2.2): $ONOO^-$-derived species might generate nitrotyrosine in this way [47].

Again, there is no direct evidence that nitration of tyrosine residues by peroxidase/H_2O_2/NO_2^- systems happens in vivo. However, it is hard to rule out: nitration by this mechanism would not lead to simultaneous chlorination unless, of course, HOCl was being generated, in which case both HOCl itself and NO_2Cl could contribute to aromatic chlorination. Studies on myeloperoxidase-deficient patients or transgenic animal MPO 'knockouts' might provide useful insights.

3. Conclusion

On present evidence, it cannot be stated definitively that the formation of nitrotyrosine in vivo is due to $ONOO^-$. Conditions favouring formation of excess $O_2^{\cdot-}$ and $NO^{\cdot}$ are usually also those in which there is phagocyte recruitment and activation, and activated neutrophils are well-known to release MPO (indeed, levels of MPO in a tissue are often used as a marker of neutrophil infiltration [78]). It is probably safest to say that the detection of nitrotyrosine provides evidence for generation of *reactive nitrogen species* rather than specifically $ONOO^-$.

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