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Commentary

Even the casual reader of current papers on the biogenesis of reactive nitrogen species (RNS) and their role in oxidative stress will recognize that controversies abound. These controversies encompass interrelated questions ranging from basic descriptions of the chemistry of the putative oxidants to discussions of the biological milieu in which RNS might accumulate and the pathophysiological roles they might play. As is often the case in new fields, seemingly straightforward interpretations of fundamental discoveries involving RNS metabolism have grown equivocal in the face of continuing investigations. Thus, the discovery that proteins at sites of cellular damage in diseases associated with oxidative stress bear heavily nitrated tyrosyl groups (1) gave initial support to Beckman et al.'s suggestion (2) that the powerful oxidant peroxynitrite (ONOO⁻) could be a major causative agent of the diseases. A circumstantial link between the two could be drawn because nitrotyrosine is found primarily in respiring tissues that might form ONOO⁻ by simultaneous generation of precursor nitric oxide (NO[•]) and superoxide (O₂^{•-}) radicals; ONOO⁻ is well known to nitrate phenolic compounds, including the tyrosine ring, under physiological conditions. In subsequent work, it has often been assumed, erroneously, that nitrotyrosine is a specific marker for peroxynitrite. As shown in Figure 1, however, there are several plausible alternative biochemical origins. Radical doubts Significant participation of ONOO⁻ in biological nitration has [...]

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Even the casual reader of current papers on the biogenesis of reactive nitrogen species (RNS) and their role in oxidative stress will recognize that controversies abound. These controversies encompass interrelated questions ranging from basic descriptions of the chemistry of the putative oxidants to discussions of the biological milieu in which RNS might accumulate and the pathophysiological roles they might play. As is often the case in new fields, seemingly straightforward interpretations of fundamental discoveries involving RNS metabolism have grown equivocal in the face of continuing investigations. Thus, the discovery that proteins at sites of cellular damage in diseases associated with oxidative stress bear heavily nitrated tyrosyl groups (1) gave initial support to Beckman et al.'s suggestion (2) that the powerful oxidant peroxynitrite (ONOO⁻) could be a major causative agent of the diseases. A circumstantial link between the two could be drawn because nitrotyrosine is found primarily in respiring tissues that might form ONOO⁻ by simultaneous generation of precursor nitric oxide (NO[•]) and superoxide (O₂^{•-}) radicals; ONOO⁻ is well known to nitrate phenolic compounds, including the tyrosine ring, under physiological conditions. In subsequent work, it has often been assumed, erroneously, that nitrotyrosine is a specific marker for peroxynitrite. As shown in Figure 1, however, there are several plausible alternative biochemical origins.

Radical doubts

Significant participation of ONOO⁻ in biological nitration has recently been questioned on two grounds. First, yields of nitrotyrosine are very low in *in vitro* reactions when ONOO⁻ is slowly infused into the reaction environment or slowly generated from

chemical precursors. Second, nitration and chemical oxidations in biomimetic reactions are efficient only when fluxes of the precursor radicals, NO[•] and O₂^{•-}, are equal (3, 4) – a condition not likely to be met in respiring tissues. Although the dependence of yield upon ONOO⁻ formation rate appears to have puzzled some investigators, leading to a spate of papers on this topic, the mechanistic principles governing this behavior were clearly delineated in an early kinetic study (5). The peroxynitrite anion itself is unreactive toward tyrosine, but protonation to the conjugate acid (ONOOH) or Lewis adduct formation with CO₂ to give ONOOCO₂⁻ generates biologically relevant tyrosine-nitrating agents (Figure 1). These reactions are initiated by one-electron oxidation of tyrosine to the tyrosyl radical, in contrast to the more typical electrophilic aromatic substitution processes common in organic syntheses. Also generated is NO₂[•], whose fate is dictated by the relative concentrations of reaction partners. When tyrosine is in large excess, NO₂[•] will preferentially react with it to

form a second tyrosyl radical, giving dityrosine as the ultimate oxidation product. When tyrosine is not in large excess, NO₂[•] will efficiently couple with the tyrosyl radical, yielding nitrotyrosine as the predominant product. Thus, the critical reaction parameter is the ratio of tyrosyl radical to tyrosine. When the ONOO⁻ concentration is kept low, the steady-state concentration of tyrosyl radical is also low, so that nitrotyrosine formation is suppressed in favor of dityrosine.

Whether the concerns about low nitrotyrosine yields weaken the case for ONOO⁻ as a source of nitrotyrosine *in vivo* is unclear. Since the relative concentrations of cellular targets for NO₂[•] are unknown, it is particularly inappropriate in this case to extrapolate *in vitro* results to biological reactions. Furthermore, not all tyrosyl groups in biological environments are equally susceptible to nitration. Metalloproteins target specific tyrosyl groups within their active sites for nitration, and various tyrosyl groups in proteins that do not contain metal ion cofactors also

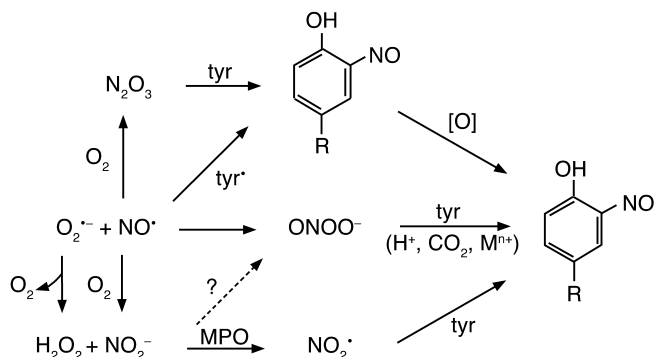


Figure 1

Representative pathways for biological formation of 3-nitrotyrosine. This product has been widely assumed to arise from the interaction of secondary oxidants derived from peroxynitrite (ONOO⁻) with tyrosine-containing proteins. However, several alternative pathways exist, including one mediated by the enzyme myeloperoxidase (MPO), that may be important in respiring tissues. tyr, tyrosine; tyr[•], tyrosyl radical.

display a wide range of selectivity toward ONOO⁻ (6). Additionally, nitration of lipophilic tyrosyl-containing derivatives in artificial bilayer membranes is strongly favored over dityrosine formation, an effect that can be attributed to the considerably lower lateral diffusion rates of the tyrosyl compounds than of NO₂[•] within the ordered hydrocarbon domain of the membrane (7). Similar kinetic effects would be anticipated to favor protein nitration over tyrosyl radical coupling within the biological milieu.

The requirement for equal in vivo fluxes of NO[•] with O₂⁻ constitutes a more serious challenge to the notion that ONOO⁻ is a major source of oxidative damage in respiring tissues. Both O₂⁻ and NO[•] can protect tissues by scavenging intermediary oxidizing radicals generated by ONOO⁻ (3, 4), thereby minimizing coupling of the tyrosyl radical to NO₂[•] and oxidation of other cellular biological targets. Murine macrophages have long been considered capable of generating NO[•] and O₂⁻ but are thought to lack substantial peroxidase activity; as such, they are particularly suited for investigation of this issue. Mayer and coworkers recently demonstrated modest nitration of tyrosyl proteins in an immortalized murine macrophage cell line (RAW 264.7) following induction of NO synthase (iNOS) activity (8). However, nitration became appreciable only 15 hours after immunological stimulation, a point at which both cellular O₂⁻ and NO[•] production had essentially ceased. Since ONOO⁻ does not persist under physiological conditions, it seems highly unlikely that peroxyxynitrite could have been the nitrating agent. Furthermore, the opportunity for significant intracellular ONOO⁻ formation in the first place seems remote, since the conditions of Mayer and coworkers' experiment allow for very little temporal overlap between the generation of O₂⁻ (and H₂O₂) by stimulated respiration and the activation of iNOS. Thus, the synchronously activated cells never exhibited the large, equal fluxes of the precursor radicals necessary for ONOO⁻ formation. Inhibition studies suggested that the nitration mechanism within the RAW cells may have involved a peroxidase.

These basic observations have now been duplicated in our laboratory with both RAW cells and rat peritoneal exudates, using particle-conjugated reactive dyes as probes of intraphagosomal oxidative capabilities. This is all very surprising since murine macrophages are widely considered to be quintessential generators of peroxyxynitrite. What seems more likely now is that if phagocytes are to generate substantial amounts of ONOO⁻, it will be done through synergistic production of O₂⁻ and NO[•] by mixed populations of cells or by nonsynchronous cells that are in different phases of activation.

Myeloperoxidase and nitrotyrosine formation

The most widely discussed alternative mechanism for in vivo tyrosine nitration involves myeloperoxidase-catalyzed (MPO-catalyzed) oxidation of NO₂⁻, a catabolic end-product of NO[•] that accumulates at infection sites. The importance of MPO-mediated reactions in intraphagosomal oxidations of neutrophils has been clearly demonstrated using both enzyme-conjugated bacteria (9) and fluorescent dyes (10) as probes. Nitration of bacteria within the phagosomes of iNOS-expressing human neutrophils has also been demonstrated (11). Chlorotyrosine is thought to be a specific marker for MPO, since other mammalian peroxidases cannot effectively oxidize Cl⁻; its accumulation within human atherosclerotic plaques has correspondingly been attributed to participation of recruited neutrophils and monocytes in oxidative damage (12).

Probing the molecular origins of oxidative stress in vivo

The use of genetically altered laboratory animals offers a direct means to evaluate these alternative hypotheses in living systems and to probe aspects of the pathology of oxidative stress that are not readily amenable to other types of investigation. A particularly compelling illustration of the power of the approach is the study reported by Heinecke and associates in this issue of the *JCI* (13). They found that nitrotyrosine levels in peritoneal fluids from MPO-deficient mice infected with *Klebsiella pneumoniae* are markedly reduced relative to those

from wild-type mice — this despite the induction of iNOS in both strains and a comparable accumulation of NO₂⁻. By simultaneously measuring chlorotyrosine and nitrotyrosine in these fluids, the authors showed that active MPO is present in the peritoneum of the infected wild-type mice but not in the MPO knockout mice; specifically, the yields of the two tyrosine derivatives were comparable in the wild-type mice, but chlorotyrosine was completely lacking in the MPO knockouts. Since the tyrosine-nitrating and -chlorinating activities of MPO should be roughly comparable under the prevailing in vivo conditions, it follows that the large increase in nitrotyrosine yields measured in normal mice most likely arose by MPO catalysis, the unattractive alternative being that MPO somehow enabled expression of another as-yet unidentified nitration mechanism. Very similar conclusions have been reached by Hazen and collaborators, who compared nitrotyrosine accumulation in several acute inflammatory models that made use of MPO knockout and eosinophil peroxidase knockout mice (14).

Other studies using knockouts to examine the role of MPO in tissue damage have not been so readily interpretable. In an earlier *JCI* publication, Heinecke and associates reported that LDL receptor- and MPO-deficient double-knockout mice were more susceptible to atherosclerosis than were littermates whose neutrophils contained normal MPO activity (15). Similarly, Takizawa and coworkers have recently reported that ischemia/reperfusion-induced cerebral damage was greater in MPO knockout than in wild-type mice and was accompanied by increased levels of nitration of protein tyrosyls (16). In both studies, MPO was apparently not present at the nitration sites. Numerous potential explanations (summarized in ref. 15) can be imagined for the apparent protection by MPO, although the actual mechanisms remain to be determined. An even more provocative study has involved use of mice deficient in neutrophil granule proteases, but with apparently normal oxidative capacities (17). These protease-deficient mice were unusually susceptible to infection by *Staphylococcus aureus* and *Candida albicans*, which was

reflected in inefficient killing of these organisms by their isolated neutrophils. The authors of this study suggest that proteases are the central agents of microbicidal action, the primary purpose of respiratory activation being to electrogenically increase the intraphagosomal ionicity, thereby electrostatically triggering release of the proteases from inhibitory complexes with acid proteoglycans. MPO in this model is assigned the role of protecting the proteases from inactivation by H₂O₂ via catalatic degradation to O₂ and H₂O₂.

The present study (13) provides a clear illustration of another critical factor that is becoming evident through the use of genetically modified animals, namely, strain-selective differential responses of the host organism to microbes. The nitrite accumulation that followed injection of *K. pneumoniae* did not occur when cecal ligation and puncture was used to infect the peritoneum with enteric bacteria, nor was there any evidence of enhanced tyrosine nitration by MPO attending this injury. Model-dependent responses were also observed by Hazen and colleagues (14) and in the

protease-knockout (17) study. Overall, experiments such as these with knockout animals portend that our current models of oxidative stress, which have necessarily been based upon long extrapolations of ex vivo results to living systems, will prove naive.

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