REACTIVE OXYGEN AND NITROGEN SPECIES AND ADULT RESPIRATORY DISTRESS SYNDROME (ARDS): NEW MECHANISMS TO BE CONSIDERED

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1. INTRODUCTION

The adult respiratory distress syndrome (ARDS) represents a common response of the lung to a variety of different and often unrelated insults, most frequently sepsis, trauma, aspiration and shock. Important consequences are the priming and activation of many components of the inflammatory-immune system accompanied by alterations in the permeability of endothelial and epithelial cell membrane barriers manifest by accumulations of inflammatory pulmonary edema fluid accompanied by phagocyte infiltrations in the interstitial and alveolar compartments of the lung. The net physiologic result is severe hypoxemic acute respiratory failure due to lung ventilation-perfusion mismatching and extensive intrapulmonary shunts. As with inflammatory-immune activation states involving other systems (e.g., rheumatoid arthritis in joints), reactive oxygen species (ROS) and reactive nitrogen species (RNS) are likely to play a significant role in the pathobiology of the lung injury seen in ARDS (Louie et al., 1997; Forni et al., 1997).

There are numerous potential sources for the generation of ROS and RNS in the pathobiology of acute lung injury, not the least of which are the high concentrations of oxygen and of nitric oxide (NO•) which are often administered to subsets of patients with ARDS (Mankelow et al., 1997; Troncy et al., 1997). Some of these potential pathways are listed in Table 1. In the present paper we will discuss possible oxidative mechanisms involving phagocytes, focusing on pathways related to myeloperoxidase, ROS and RNS.

Table 1. Possible Pathways for ROS/RNS Generation in ARDS

- Xanthine oxidase
- Mitochondrial respiration
- Phagocyte/NADPH oxidase/peroxidase
- Non-phagocytic NADPH-oxidases/peroxidases
- Lipoxygenases
- NO• administration
- P-450 systems
- Catecholamine oxidation
- NOS pathways
- Proinflammatory cytokines
- Redox-cycling metals
- O_2 toxicity

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2. PHAGOCYTES AND ARDS

Although phagocytes are critical for our survival, it has been long recognized that migration and activation of polymorphonuclear neutrophils (PMNs) in the lungs is almost surely a major contributing factor to the acute lung injury that occurs in patients with ARDS (Boxer *et al.*, 1990; Repine and Beehler, 1991). This argument is buttressed by observations that PMN depletion prevents animal models of ARDS (Heflin and Brigham, 1981), that PMNs and their oxidative products contribute to endothelial cell injury in vitro and lung injury in vivo (Shasby *et al.*, 1983); and that PMNs and their products accumulate in the lungs of patients with ARDS (Weiland *et al.*, 1986).

However, PMN migration alone may not injure the lungs of normal humans. For example, activation and priming of their proteolytic and oxidant injury mechanisms may be required (Martin *et al.*, 1989, 1991; Martin, 1997). The multitude of cytokines found to be present in ARDS (Chollet-Martin et al., 1992) would be expected to further potentiate PMN activation, thereby increasing their production of reactive oxidant species . . . even the circulating PMNs have been found to be activated in patients with ARDS (Zimmerman *et al.*, 1983; Martin *et al.*, 1991; Chollet-Martin *et al.*, 1992).

Myeloperoxidase represents an important phagocytic enzyme, more plentiful in PMNs than monocytes and macrophages (Odeberg et al., 1974; Bos et al., 1978; Kettle & Winterbourn, 1997), and has been heavily used as a biomonitor of PMN traffic in numerous organs undergoing various stages of cell injury and related inflammatory-immune system activations including ARDS (Fantone & Ward, 1985; Weiland *et al.*, 1986; Denis *et al.*, 1994; Shayeritz *et al.*, 1995; Sinclair *et al.*, 1995; Okabayashi *et al.*, 1996; Koh *et al.*, 1996; Kushimoto *et al.*, 1996), as depicted in Table 2.

Table 2: Lung Myeloperoxidase in ARDS

- A monitor of PMN traffic
- Elevated in following ARDS models:
  - Endotoxin, sepsis, IP zymosan
  - Hemorrhagic shock, gut ischemia, pancreatitis
  - IL-1, TNF, Complement (C') and PMN-activation
  - Transplant and cardiopulmonary bypass

3. OXIDES OF NITROGEN AND THE LUNG

Nitric oxide (NO•) is now well-recognized for its participation in diverse biological processes in nearly all aspects of life (Moncada and Higgs, 1991), including in inflammatory-immune processes in the lung (Gaston et al, 1994). Multiple lung cells synthesize NO• under both physiological and pathophysiological settings (Barnes and Belvisi, 1993), utilizing a family of enzymes termed NO• synthases, which use arginine as their substrate. NO• can travel significant distances to reach target cells neighboring the NO• generating cells (Lancaster, 1994; Malinski and Taha, 1992). Along this migration, NO• can interact with other oxidative molecules, including molecular O_2, to form higher nitrogen oxides (e.g. NO_2•, N_2O_3) which can either react with other biomolecules (e.g., thiols, amines) or simply hydrolyze to form NO_2^- and NO_3^-. Furthermore, and importantly for the context of this paper, NO• and its metabolites (e.g. NO_2^- specifically), themselves generated in increased amounts at inflammatory-immune reaction sites, can react with several phagocyte-derived oxidants, to form more reactive RNS (e.g., Koppenol, 1994). The extent of either of these reactions

depends on the microenvironmental conditions under which NO• is released, and the concentration of the other bioreactants present.

Concerning the interactive role of NO• with other active pro-oxidant species, most recent research has focused emphasis on NO• reaction with $O_2^{\bullet -}$, to yield the powerful oxidant species peroxynitrite ($ONOO^-$) (Beckman, 1995, 1996). Based primarily on the nearly diffusion limited reaction kinetics of this reaction (Huie and Padmaja, 1993), it is predicted that $ONOO^-$ is generated whenever $O_2^{\bullet -}$ and NO• are produced simultaneously. The potential *in vivo* importance of such a reaction is supported by findings that superoxide dismutase (SOD) prolongs the biological half-life of NO• and increases its biological actions, putatively by lowering $O_2^{\bullet -}$ levels and minimizing degradation of NO• via reaction with $O_2^{\bullet -}$ (e.g., Gryglewski et al, 1986).

A second argument for *in vivo* generation of $ONOO^-$ (especially during inflammatory conditions) is detection of 3-nitrotyrosine in proteins from a large number of diseased or inflamed tissues, often in conjunction with induction of iNOS and increased production of NO• (Beckman, 1995, 1996; Halliwell, 1997). As NO• itself is unable to nitrate tyrosine residues (e.g., Eiserich et al., 1995), more reactive NO•-derived nitrogen oxides (NO_2•, $ONOO^-$) are thought responsible for tyrosine nitration, and based on kinetic considerations, it is commonly assumed that 3-nitrotyrosine *in vivo* is caused by $ONOO^-$. Using largely immuno-histochemical techniques, 3-nitrotyrosine has also been detected at sites of inflammatory-immune processes in the lung (Saleh et al, 1997), including ARDS (Haddad et al, 1994; Kooy et al, 1995). However, the pathophysicological importance of 3-nitrotyrosine formation and the precise oxidative and nitrosative mechanisms involved in its formation are relatively unknown. These issues will be the focus of the remainder of this paper.

4. TYROSINE NITRATION VIA RADICAL MECHANISMS

Chemical studies of tyrosine nitration by $ONOO^-$ have indicated that nitration can be promoted in the presence of superoxide dismutase or Fe(III)EDTA, as well as heme peroxidases (Beckman *et al.*, 1992; Sampson *et al.*, 1996), presumably by formation of an NO_2^+- like intermediate, which is known to be capable of nitrating aromatic rings by electrophilic aromatic substitution (Olah *et al.*, 1989). However, another major product of tyrosine oxidation by $ONOO^-$ is 3,3'-dityrosine, indicative of formation of intermediate tyrosyl radicals (van der Vliet et al., 1995). Hence, nitration of tyrosine by $ONOO^-$ appears to occur via a one-electron mechanism, involving initial formation of tyrosyl radical and $NO_2^{\bullet}$, and dityrosine and 3-nitrotyrosine are subsequently formed by radical combination reactions (e.g., Prutz *et al.*, 1985; Lymar *et al.*, 1996), as illustrated in reactions 1-4.

$ONOOH + Tyr\text{-}OH$	$\rightarrow$	$Tyr\text{-}O^{\bullet} + NO_2^{\bullet} + H_2O$	(1)
$NO_2^{\bullet} + Tyr\text{-}OH$	$\rightarrow$	$Tyr\text{-}O^{\bullet} + NO_2^- + H^+$	(2)
$NO_2^{\bullet} + Tyr\text{-}O^{\bullet}$	$\rightarrow$	3-nitrotyrosine	(3)
$2\ Tyr\text{-}O^{\bullet}$	$\rightarrow$	3,3'-dityrosine	(4)

Reaction (2) becomes more favorable over reaction (3) when tyrosine concentrations are relatively high, resulting in relatively more dityrosine formation and less nitration, as was indeed observed in studies with tyrosine in solution (van der Vliet *et al,* 1995), as well as in studies in freshly obtained human plasma. In the latter case, $ONOO^-$-induced nitration and dimerization of free or protein-associated tyrosine was markedly affected when plasma was supplemented with free tyrosine. The overall formation of 3-nitrotyrosine was decreased, whereas more dityrosine was formed (unpublished results), consistent with a radical reaction mechanism (reactions 1-4). Formation of tyrosyl radicals in plasma by $ONOO^-$ has recently

been detected by ESR (Pietraforte and Minetti, 1997), which gives further support to this nitration mechanism.

It has become clear that tyrosine nitration by NO_2^+ in aqueous systems also occurs by a one-electron mechanism via intermediate tyrosyl radicals, rather than via direct electrophilic substitution. However, free NO_2^+ is extremely unstable in aqueous solution and hydrolizes rapidly to nitrate (NO_3^-), and is thus unlikely to be involved in nitration reactions in vivo (van der Vliet *et al,* 1996; 1997). Both tyrosine nitration and dimerization by $ONOO^-$ are enhanced in the presence of bicarbonate (van der Vliet *et al,* 1994; Lymar *et al.,* 1996; Gow *et al.*, 1996; Lemercier *et al.*, 1997), which was discovered to be due to reaction of $ONOO^-$ with CO_2 to form $ONOOCO_2^-$, which appears more efficient as a nitrating species (Lymar *et al.,* 1996). Collectively, irrespective of the nature of the nitrating species (ONOOH, $NO_2^{\bullet}$ or NO_2^+), the mechanism of tyrosine nitration appears to involve intermediate formation of tyrosyl radicals, and 3,3'-dityrosine is formed as an additional product.

5. ALL THAT NITRATES IS NOT PEROXYNITRITE

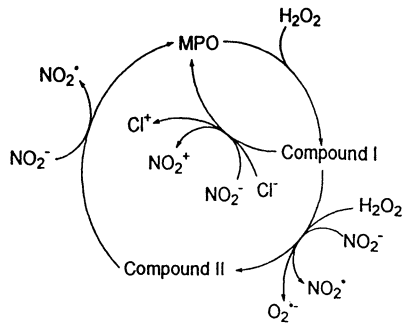
As tyrosine nitration is radical-mediated, involving formation of tyrosyl radicals and $NO_2^{\bullet}$, formation of these intermediates from other sources would be expected to contribute to tyrosine nitration in biological systems. Indeed, several such mechanisms can be envisioned to occur *in vivo*, especially during inflammatory-immune processes. $NO_2^{\bullet}$ can be generated via autoxidation of NO•. This reaction is slow, especially at physiological NO• levels, hence formation of $NO_2^{\bullet}$ by NO• autoxidation is expected to be minimal (e.g., Beckman and Koppenol, 1996).

Additionally, $NO_2^{\bullet}$ can be generated via one-electron oxidation of NO_2^-, and various biological oxidants can be expected to promote such reactions. For instance, it has long been recognized that heme peroxidases or pseudoperoxidases, such as methemoglobin or metmyoglobin, are able to oxidize NO_2^- in the presence of H_2O_2, and this has been postulated to occur via one-electron mechanisms (van der Vliet et al., 1997 and refs therein).

Although $NO_2^{\bullet}$ is capable of directly nitrating tyrosine residues in proteins (Prütz et al., 1985), this process is considered relatively inefficient, as two $NO_2^{\bullet}$ molecules are necessary to nitrate one tyrosine residue. However, tyrosyl radicals can also be generated by various mechanisms, including peroxidases (Kettle and Winterbourn, 1997), and radical combination between tyrosyl radicals and $NO_2^{\bullet}$ is very rapid, yielding 3-nitrotyrosine.

Our recent studies have shown that NO_2^- can be oxidized by various heme peroxidases, including horseradish peroxidase, myeloperoxidase (MPO), and lactoperoxidase (LPO), in the presence of hydrogen peroxide (H_2O_2), to most likely form NO_2•, and was found capable of promoting tyrosine nitration, which may be relevant during inflammatory processes (van der Vliet et al., 1997). The physiological importance of such mechanisms depends on whether NO_2^- is a competitive substrate for MPO or other peroxidases *in vivo*. Phenolic nitration by MPO-catalyzed NO_2^- oxidation was found to be only partially inhibited by chloride (Cl^-), the presumed major physiological substrate for MPO, and low concentrations of NO_2^- (2-10 μM) were in fact demonstrated to **catalyze** MPO-mediated oxidation of Cl^-, indicated by increased chlorination of aromatic substrates, and simultaneously cause aromatic nitration. The observed enhanced MPO-mediated Cl^--oxidation by NO_2^- was similar to that observed by other reductants, such as ascorbate or 5-amino salicylate (Bolscher et al., 1984; Zuurbier et al., 1990), and can be attributed to reduction of MPO compound II, which is inactive with respect to Cl^--oxidation, thereby recycling MPO (Fig. 1).

Fig. 1



Peroxidase-catalyzed oxidation of NO_2^-, as indicated by phenolic nitration, could also be detected in the presence of thiocyanate (SCN^-), an alternative physiological substrate for mammalian peroxidases, suggesting that NO_2^- may act as a pathophysiological substrate for the MPO (and perhaps other peroxidases). Thus, formation of $NO_2^{\bullet}$ via peroxidase-catalyzed oxidation of NO_2^- may provide an additional pathway contributing to **aromatic nitration *in vivo*.**

Interestingly, 3-nitrotyrosine and large amounts of active MPO have both been detected in atherosclerotic lesions (Beckman et al, 1994; Buttery et al, 1996; Daugherty et al, 1994), in joints of patients with rheumatoid arthritis (Farrell et al, 1992; Kaur and Halliwell, 1994), as well as in the lungs of patients with acute pulmonary inflammation (Haddad et al, 1994; Kooy et al, 1995), a condition characterized by activation and lung infiltration of PMNs as well as increased production of NO• and NO_2^- (e.g., Hunt et al, 1995; Kharitonov et al, 1996). Plasma nitrotyrosine levels have been noted to be elevated in septic shock (Fukuyama *et al.*, 1997), a condition known to be often associated with ARDS. However, it should be also noted that studies performed on inducible NO synthase-deficient mice suggest a significant role for augmented NO• levels in sepsis as being important as a homeostatic regulator in PMN activation and recruitment to endothelial surfaces (Hickey *et al.*, 1997).

Formation of Novel Nitrating and Chlorinating Intermediates During Reaction of NO_2^- with HOCl/OCl^-

One of the most potent and plentiful oxidants produced by phagocytes is hypochlorous acid (HOCl/OCl^-), which is formed via MPO-catalyzed oxidation of Cl^- (Weiss et al, 1983). As NO• is also present in increased quantities at sites of inflammatory-immune reactions, interactions between NO•-derived RNS with the inflammatory oxidant HOCl/OCl^- can be expected to occur under inflammatory conditions. Results from our laboratory have indicated that NO_2^-, the major metabolite of NO• in extravascular fluids, reacts with HOCl/OCl^- to form nitrate (NO_3^-) via intermediate formation of nitryl chloride ($ClNO_2$) and/or chlorine nitrite (ClONO). These intermediates are powerful nitrating and chlorinating species, hence, formation of $ClNO_2$/ClONO by this reaction may represent a previously unrecognized mechanism of inflammation-mediated biological damage, and offer an additional or alternative mechanism of tyrosine nitration independent of $ONOO^-$ formation (Eiserich et al., 1996). This argument is strengthened by the recent finding that hypochlorous acid and nitrate cause oxidative modification and nitration of human lipoproteins (Panasenko et al, 1997).

Normally, NO_2^- is present at levels of 0.5-3.6 μM in plasma (Leone et al, 1994; Ueda et al, 1995), ~15 μM in respiratory tract lining fluids (Gaston et al, 1993), 30-210 μM in saliva, and 0.4-60 μM in gastric juice, but extracellular NO_2^- levels are markedly increased during inflammatory processes, reflecting increased NO• production. For instance, increased NO_2^- levels have been detected in synovial fluids of patients with rheumatoid arthritis (Farrell

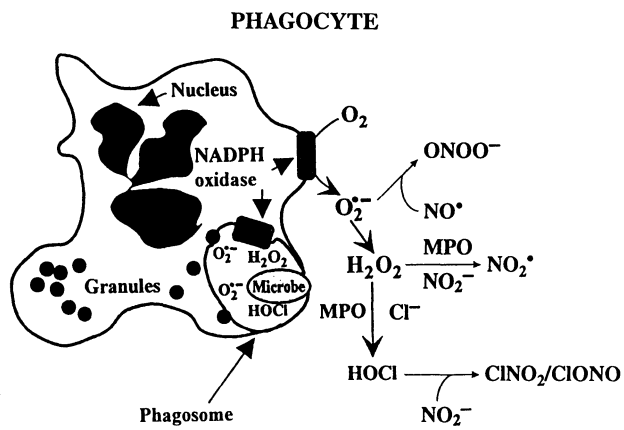
et al, 1992), and serum NO_2^- levels of 36 μM have been reported in human immunodeficiency virus-infected patients with interstitial pneumonia (Torre et al, 1996), dramatically higher than normal serum NO_2^- levels. Increased NO_2^- levels have also been detected in condensed exhalates from patients with asthma compared with those of healthy subjects (Hunt et al, 1995), consistent with increases in expired NO• by asthmatics compared with healthy control subjects (Kharitonov et al, 1996). Nitrite levels are reportedly very high in respiratory tract surfaces (Goviadaraju et al, 1997), far exceeding plasma levels (e.g., possibly 1000x higher in the rat), presumably because epithelial cells generate NO• and the airway surface does not have hemoglobin degradation systems oxidizing NO• (or nitrite) to nitrate (NO_3^-). [However, it has been shown that most inhaled NO• results in $*NO_3^-$, as detected in blood and in urine (Westfelt et al, 1995).]

Do Human PMNs Utilize these Pathways?

We have obtained recent evidence indicating that activated human PMNs can utilize these above-described MPO-dependent pathways to form both nitrating and chlorinating intermediates (Eiserich et al., 1997). Addition of NO_2^- (1-50 μM) to PMA-stimulated PMN was found to cause nitration of phenolic substrates and enhance PMN-mediated chlorination reactions. The enhanced chlorination can be explained by: i) NO_2^--mediated recycling of inactive MPO Compound II to the native ferric enzyme; and ii) the ability of NO_2^- to compete with taurine released from PMN for reaction with HOCl/OCl^- to form a more potent electrophilic chlorinating intermediate ($ClONO/ClNO_2$). Furthermore, we have obtained evidence that exposure of activated PMN to pathophysiologic fluxes of NO• resulted in nitration and chlorination reactions that in some conditions were **dependent** on active MPO, rather than formation of $ONOO^-$. Under identical conditions, addition of $^{15}NO_2^-$ led to ^{15}N enrichment of nitrated phenolic substrates, unequivocally confirming contribution of NO_2^- in PMN-mediated reaction pathways.

In summary, formation of NO•-derived reactive nitrogen species that are capable of inducing aromatic nitration appears to occur by multiple mechanisms, especially during inflammation and PMN activation, as schematically depicted below:

Fig. 2



There is likewise an accumulating documentation of increased formation of 3-chlorotyrosine in tissues subjected to chronic inflammatory-immune reactions (Halliwell, 1997), and indeed in some of the same tissues where increased nitrotyrosine has been found (e.g., Beckman et al, 1994; Hazell et al., 1996; Hazen et al, 1997a). With increased sensitivity of techniques designed to quantitate chlorotyrosine (Hazen et al, 1997b), it can be expected that chlorotyrosine will be found in many tissues where elevated nitrotyrosine has been found.

6. POSSIBLE CONSEQUENCES OF THESE IRREVERSIBLE TYROSINE MODIFICATIONS

Although there is ample evidence for the formation of 3-nitrotyrosine (and 3-chlorotyrosine or 3,3'-dityrosine) in a number of inflammatory diseases, the potential contribution of these modifications to the development of tissue injury has received less documentation. Nitration of tyrosine residues using the NO_2^+-donor tetranitromethane has been used extensively to investigate the location and essentiality of tyrosine residues in a large number of proteins (e.g., Riordan and Vallee, 1972; Mierzwa and Chan, 1987; Haddad *et al.*, 1996), and has indicated that nitration of tyrosine residues is often associated with a loss of either enzyme or protein function. Increasingly, investigators are using peroxynitrite to selectively inactivate proteins via tyrosine nitration mechanisms (Zou et al., 1997). Furthermore, studies with isolated tyrosine kinase systems have indicated that nitration of critical tyrosine residues in tyrosine kinase substrates causes inhibition of tyrosine phosphorylation (Martin *et al.*, 1990; Kong *et al.*, 1996; Gow *et al.*, 1996). Hence, formation of reactive nitrogen species may importantly affect signaling pathways involving (receptor) tyrosine kinases, however, this possibility has not yet been convincingly documented in intact cellular systems.

Cytoskeletal proteins such as actin or neurofilaments may represent important targets for tyrosine nitration reactions, as they are abundant proteins and contain several tyrosine residues which appear to be involved in structural assembly of these proteins. Chemical nitration of actin or neurofilaments has been demonstrated to disrupt assembly of these proteins, and modification of only a few subunits appears necessary to cause disruption of a structure involving thousands of subunits (Beckman, 1996 and refs. therein). Although extensive tyrosine nitration has been shown to occur in the myocardium during inflammatory forms of myocarditis (Kooy *et al.*, 1997), it is still speculative whether or not the nitration plays a pathologic role in mediating the myocardial dysfunction.

More relevant to ARDS, several studies have indicated that $ONOO^-$ or related reactive nitrogen species are capable of nitrating tyrosine residues in surfactant proteins, and nitration of tyrosine residues in surfactant protein A has been causatively linked to decreases in its ability to aggregate lipids or decreases in binding to mannose receptors. Hence, such modification may disturb functions of SPA by diminishing its function to lowering alveolar surface tension (Zhu et al., 1996; Haddad et al., 1996), or by compromising its function to facilitate phagocytic uptake and killing of bacteria.

Similar to nitration, tyrosine chlorination or dimerization may affect cellular pathways involving critical tyrosine residues. As techniques to measure and characterize these compounds become increasingly available (Shigenenaga et al., 1997; Leeuwenburgh et al., 1997a, 1997b; Yi et al., 1997), their role in tissue pathobiology will become apparant. It has been recently described that 3-nitro-tyrosine attenuates hemodynamic responses to adrenoreceptor agonists and to angiotensin II (Kooy and Lewis, 1996).

7. REMAINING IMPORTANT QUESTIONS

One of the implications of our recent findings is that NO_2^- may not be a stiochiometric marker of NO• production by phagocytes or at sites of inflammation, as it is potentially removed by reaction with inflammatory oxidants. Determination of NO• production in tissues and fluids of patients with acute and chronic inflammation, or from isolated phagocytes as measured by NO_2^- may likely be an underestimate, and should include analysis of both NO_2^- and NO_3^-. Moreover, these NO_2^- -oxidation mechanisms may also modulate PMN function or affect PMN-dependent tissue injury. For instance, NO_2^- has been shown to inhibit the

bactericidal activity of $HOCl/OCl^-$, proposedly by direct reaction of these two species (Kono, 1995; Klebanoff, 1993). The reaction product $ClNO_2/ClONO$, although a strongly oxidizing species and potentially an antimicrobial agent in its own right, is a short-lived intermediate and appears less efficient in bacterial killing compared to HOCl in bacterial suspensions.

However, it is difficult to extrapolate such findings to the situation present in the phagolysosome, where oxidant reactions with bacterial constituents are less likely to be limited by chemical stability or diffusion. Moreover, NO_2^- may catalytically enhance $MPO/H_2O_2/Cl^-$ -dependent bacterial killing, via the mechanism depicted in Fig. 1. Furthermore, peroxidases have been found capable of converting NO_2^- itself into a bactericidal agent, presumably NO_2• (Klebanoff, 1995; Kono, 1995). It remains unclear, therefore, to what extent NO• or NO_2^- affect PMN function with respect to bactericidal activity. Interestingly, cytokine-stimulated human PMNs were found to contain increased levels of iNOS, which was co-localized with MPO in primary granules and tyrosine nitration could be detected around ingested bacteria (Evans et al, 1996), which is likely to involve MPO-mediated pathways.

Taken together, augmented NO• generation at inflammatory-immune activation sites may generate an expanding number of potential nitrosating and/or nitrating species and potentially play a role in augmenting the production of chlorinating species, which collectively could result in (ir)reversible modifications in proteins, lipids or nucleic acids. Although irreversible tyrosine modifications may affect various cellular processes, the relevance of such modifications to the pathobiology of ARDS or other localized or systemic inflammatory-immune processes still needs to be established.

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