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The role of nitrite ion in phagocyte function—perspectives and puzzles

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Abstract

Macrophages and neutrophils are essential elements of host cellular defense systems that function, at least in part, by generating respiration-driven oxidative toxins in response to external stimuli. In both cells, encapsulation by phagocytosis provides a mechanism to direct the toxins against the microbes. The toxic chemicals formed by these two phagocytic cells differ markedly, as do the enzymatic catalysts that generate them. Nitrite ion is microbicidal under certain conditions, is generated by activated macrophages, and is present at elevated concentration levels at infection sites. In this review, we consider potential roles that nitrite might play in cellular disinfection by these phagocytes within the context of available experimental information. Although the suggested roles are plausible, based upon the chemical and biochemical reactivity of NO_2^- , studies to date provide little support for their implementation within phagosomes.

Keywords

Macrophages; Microbicidal reactions; Neutrophils; Oxidative toxins; Peroxidases/oxidases; Phagocytosis; Reactive nitrogen species; Reactive oxygen species

Oxidative vs. non-oxidative microbicidal mechanisms involving phagocyte NADPH oxidases

There is now abundant evidence at the organismal and cellular levels that susceptibilities of pathogenic microorganisms to different types of phagocytes can vary widely, as demonstrated by studies using metabolically deficient mice and/or microbes and *in vitro* viability assays utilizing isolated phagocytes challenged with diverse organisms [1–5]. There is also widespread recognition that multiple mechanisms of killing exist within individual cells, which include clearly distinguishable oxidative and non-oxidative processes [6–11]. However, the extent to which each of these processes contribute to the overall microbicidal function of the phagocytes, and indeed how they contribute, is uncertain and is a topic under active investigation. Within this context, an issue that has dominated recent discussions is the role of

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¹*Abbreviations used:* aa, arachidonic acid; cfu, colony-forming units; CGD, chronic granulomatous disease; COX, cyclooxygenase; DTPA, diethylenetriaminepentaacetic acid; EPO, eosinophil peroxidase; Hb, hemoglobin; 15-HPETE, 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid; iNOS, the inducible isoform of nitric oxide synthase; LOX, lipoxygenase; LPO, lactoperoxidase; Mb, myoglobin; MPO, myeloperoxidase; NOX, NADPH oxidase; 3-NT, 3-nitrotyrosine; RNS, reactive nitrogen species; ROS, reactive oxygen species; TMPD, N,N,N',N'-tetramethylphenylenediamine.

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phagocyte NADPH oxidases in cellular microbicidal mechanisms. Clinical data indicate that NADPH oxidase (NOX-2) of neutrophils is critical to prevention of chronic granulomatous disease (CGD), which is manifested as chronic, life-threatening infections [12,13]. Similarly, macrophages derived from knockout mice that are deficient in NOX activity also exhibit impaired capabilities to kill certain organisms [1–3,14]. Thus, NOX appears to play a pivotal role in the microbicidal action of both these phagocytes.

The roles of NOX-2 and myeloperoxidase (MPO) in neutrophils

The primary role of neutrophil NOX in microbicidal action has historically been ascribed to generation of $O_2^{\bullet-}$ and H_2O_2 for use as substrates in MPO-catalyzed oxidation of Cl^- to the potentially antimicrobial hypochlorous acid (HOCl) [12,15–17]. However, alternative mechanisms that are inherently non-oxidative in nature have recently been proposed wherein NOX-dependent stimulated respiration is used to polarize the phagosomal membrane, leading to a cascade of events that ultimately trigger activation of dormant microbicidal proteases [18,19]. In this scenario, the intraphagosomal role of MPO is relegated to that of a catalase, by which it is assumed to protect the proteases from NOX-generated H_2O_2 . Resolution of this issue is critical to evaluating potential nitrite involvement since intraphagosomal formation of reactive nitrogen species (RNS) relies upon the capacity for MPO to function as a peroxidase. Fortunately, recent studies have clearly established that phagocytosed bacteria undergo extensive chlorination in neutrophils [20,21], confirming earlier results using fluorescein-conjugated particles that bactericidal levels of HOCl can be generated within neutrophil phagosomes [22], at least in *ex vivo* assays. Quantitative arguments leading to these conclusions are outlined in a succeeding paragraph; the salient point, however, is that MPO does indeed function as a peroxidase within the phagosome using NOX-2 derived reactive oxygen species (ROS) as substrates. The question of RNS involvement in microbial killing by neutrophils therefore rests primarily upon the chemical reactivity of NO_2^- and constraints imposed by compartmentation within the phagosome.

The role of NOX-2 in macrophages

Unlike neutrophils, macrophages generally lack substantial MPO activity, but have the capacity to induce a highly active nitric oxide synthase (iNOS) following activation with bacterial lipopolysaccharide and inflammatory cytokines [23,24]. The capacity for these cells to generate both $O_2^{\bullet-}$ and $NO^{\bullet}$ has led to proposals that NADPH oxidase and iNOS work in concert to form peroxynitrite anion ($ONOO^-$) via radical-radical coupling [25–29]. This peroxide is a powerful oxidant [30] and is a source of secondary radicals, including $OH^{\bullet}$, $CO_3^{\bullet-}$, and $NO_2^{\bullet}$ [31–33]. All of these species have microbicidal [34–36] and parasitological [37,38] potential. Peroxynitrite formation by macrophages has been convincingly demonstrated in a laboratory setting using a transformed murine (J774) cell line. In these studies, the cells were first primed with γ -interferon and bacterial lipopolysaccharide to induce iNOS activity then, several hours later when the $NO^{\bullet}$ flux was maximal, subsequently stimulated with soluble or particulate agonists to activate NOX-2 [38]. Cells treated in this manner appear to produce near-quantitative yields of $ONOO^-$ [38] and have been shown to inhibit proliferation of the unicellular parasite, *Trypanosoma cruzi* [29]. Although these toxicity studies provided some evidence for possible independent roles for $O_2^{\bullet-}$ -derived ROS and $NO^{\bullet}$ -derived RNS, compelling evidence that $ONOO^-$ was a major toxin was obtained by demonstrating that bicarbonate in the buffer protected the trypanosomes from oxidative insult. In this case, CO_2 -mediated isomerization of $ONOO^-$ to unreactive NO_3^- [30] undoubtedly competed with reactions at the parasite, thereby minimizing oxidative damage; CO_2/HCO_3^- has no known influence on other recognized ROS or RNS.

Other studies, however, have raised doubt concerning the extent of macrophage-derived peroxynitrite formation in physiological environments. For example, genetically altered mice

lacking both NOX and iNOS activities are far more susceptible to spontaneous infection than either of the single-knockout litter mates [14]. These results imply that, rather than requiring coexpression of these activities to achieve protection, NOX and iNOS have essential microbicidal functions that are independent and at least partially redundant. Two specific issues have also developed from continuing studies on isolated macrophages. First, in contrast to $\text{NO}^\bullet$, the amount of $\text{O}_2^{\bullet-}$ generated by stimulated macrophages varies widely with cell line, and the highest reported value, for rat alveolar macrophages ($\sim 0.5 \text{ nmol O}_2^{\bullet-}/10^6 \text{ cells-min}$) [34], is nearly an order of magnitude less than stimulated rates measured for human neutrophils ($2\text{--}5 \text{ nmol O}_2^{\bullet-}/10^6 \text{ cells-min}$) [22,39]. Maximal rates of $\text{O}_2^{\bullet-}$ and $\text{NO}^\bullet$ formation reported for several mouse and rat cell lines are collected in Table 1; although iNOS-generated $\text{NO}^\bullet$ rates are very similar throughout the series, respiration-generated $\text{O}_2^{\bullet-}$ rates vary widely. For most of the cell lines, maximal rates of $\text{O}_2^{\bullet-}$ and $\text{NO}^\bullet$ formation are roughly equal, although the value for $\text{O}_2^{\bullet-}$ formation in RAW 264.7, another transformed murine macrophage cell, is exceptionally low, which must severely limit the amount of peroxynitrite that could conceivably be formed by it. This limitation is notable because this cell line is often used as a laboratory model representing natural macrophages. The second issue, applicable to all of the cell lines, is the large temporal mismatch between the periods in which synchronously stimulated macrophages generate detectable levels of $\text{O}_2^{\bullet-}$ and $\text{NO}^\bullet$. As demonstrated for RAW cells [40,41] and peritoneal macrophages [42], the NOX respiratory burst appears within a few minutes post-stimulation and appears to cease within an hour, but induction of detectable iNOS activity is delayed by several hours post-stimulation. Thus, for these cells, fluxes of $\text{O}_2^{\bullet-}$ and $\text{NO}^\bullet$ do not overlap significantly, precluding coupling between these radicals to form peroxynitrite. It is unclear whether this timing mismatch is consequential to physiological function, however. One expects that, unlike these laboratory conditions [40–42], macrophages at sites of infection would be nonsynchronously activated by cytokines and naturally “primed” by exposure to the activators prior to encountering particulate agonists.²

Alternatively, the primary purpose for NOX-2 in macrophages may not be to generate ONOO^- or other ROS for direct oxidative killing of ingested microbes, but could involve other inflammation-related functions, for example, redox signaling to induce cytokine production [43] or upregulation of other enzymes involved in host defense. In this sense, the role of NOX-2 could be regarded as non-oxidative. Consistent with this viewpoint, numerous NOX isoforms are now known, among which NOX-2 is one of the most widely distributed, even being found in nonphagocytic cells [44]. Other members of the NOX family carry out a wide range of cellular physiological functions unrelated to host defense [44].

Microbicidal potential of NO_2^-

There is considerable current interest in NO_2^- [45–47] and NO_3^- [48,49] as *in vivo* reservoirs of RNS and participation of so-called “nonenzymatic” reactions in microbial killing by NO_2^- have been proposed [50–51]. These reactions are thought to involve dehydration and chemical reduction of HNO_2 in acidic environments, ultimately leading to $\text{NO}^\bullet$ formation, although mechanistic details are lacking and no clear examples of this phenomenon have yet been presented (see, e.g., [52]). Although $\text{NO}^\bullet$ itself has only limited microbicidal potential, it is a precursor to both N_2O_3 , a powerful nitrosating agent, and $\text{NO}_2^\bullet$. Direct comparisons have shown that radiolytically-generated $\text{NO}_2^\bullet$ is as toxic as the strongly oxidizing carbonate radical ($\text{CO}_3^{\bullet-}$) toward bacterial suspensions of *Escherichia coli* [35]; $\text{CO}_3^{\bullet-}$ is likely to be a potent microbicide when formed in phagosomal compartments [53] and both radicals are generated

² These studies suggest a potentially useful laboratory approach to systematically investigating the roles of peroxynitrite versus other RNS in probing macrophage cellular reactions. Direct comparisons using RAW cells, where ONOO^- formation appears negligible, and primed J774 cells, where ONOO^- formation can be high, might allow assessment of the relative effectiveness of ONOO^- as a microbicidal agent in comparison to other $\text{NO}^\bullet$ -initiated reactions in cellular systems, particularly since the iNOS activities of the two transformed lines are comparable (Table 1).

in ONOO⁻-mediated reactions in physiological environments [31–33].³ Somewhat surprisingly, NO₂[•] exhibits negligible fungicidal activity toward *Saccharomyces cerevisiae*. Consequently, added NO₂⁻ effectively protected the yeast from killing by other oxidizing radicals when present in the radiolysis medium; under these conditions, NO₂⁻ rapidly scavenges the other radicals to generate NO₂[•]. Although virtually nothing is known about the actual pathophysiological mechanisms leading to cellular death by these oxidants, this result suggests that fundamentally different mechanisms exist for prokaryotes and simple eukaryotes.

When H₂O₂ is present, NO₂⁻ can participate in peroxidase reactions with heme proteins, including hemoglobin (Hb), myoglobin (Mb) [54], catalase [55], lactoperoxidase (LPO) [55, 56] and phagocyte peroxidases [55–58], each of which are enzymes found at sites of infection or in antimicrobial fluids. *In vitro* reactions of H₂O₂ with NO₂⁻ catalyzed by MPO, eosinophil peroxidase (EPO), and lactoperoxidase (LPO) have all been shown to be bactericidal toward *E. coli*, and enzymatic nitration of tyrosine to give 3-nitrotyrosine has been demonstrated for Mb [54], MPO, LPO, and horseradish peroxidase [56]. For MPO and EPO, mechanistic studies have demonstrated that NO₂⁻ reacts in sequential one-electron steps with compounds I and II, i.e., the reactions:

implicating formation of NO₂[•] as the immediate product [57–59]. Stoichiometric analyses of enzymatic intermediates eliminated the possibility of direct two-electron oxidation of NO₂⁻ by compound I to form either NO₃⁻ or ONOOH (analogous to Cl⁻ oxidation to HOCl). The rate constant for reaction with MPO compound II is relatively low, suggesting that reducing co-substrates might be required for enzyme cycling in physiological reactions [57]. In contrast, NO₂⁻ was found to react much more rapidly with EPO compound II; furthermore, modeling studies indicated that EPO-generated NO₂[•] could be an important bactericide under conditions existing at sites of infection [59].

Although reactions of Hb and Mb with NO₂⁻ are complex, particularly in aerobic media, recent studies [60] have delineated a series of reaction steps that define an anaerobic pathway for dehydration of nitrite ion to N₂O₃, as follows:

³Although the observations that CO₂ protects cells in suspension from the toxicity of ONOOH [29,36] and that CO₃^{•-} is potentially microbicidal [35] may seem paradoxical, there is a simple explanation which is based upon relative rate constants for the self-coupling and cross-coupling reactions of the radicals involved. CO₂ rapidly reacts with ONOO⁻ to generate CO₃^{•-} and NO₂[•], which then undergo an O(-1) transfer reaction at near-diffusion controlled rates leading to net isomerization of ONOO⁻ to NO₃⁻ with regeneration of CO₂ [35]. In contrast, rate constants for self-coupling reactions of CO₃^{•-} and NO₂[•] to give CO₂ + H₂O₂ and NO₂⁻ + NO₃⁻, respectively, are ~10²-fold lower. Consequently, in *in vitro* microbicidal assays where both radicals are simultaneously generated, most will annihilate each other before they are able to diffuse to the cell. When only one or the other of the radicals is generated, however, their lifetimes are sufficiently long that a significant fraction will reach and react with the cells. These qualitative predictions have been confirmed by kinetic simulations [35]. Radicals generated within phagosomes should have an entirely different reactivity pattern, however. In this case, the phagosomal dimensions greatly reduce diffusion lengths to the cells so that reaction with the cells is calculated to be extensive even in the presence of physiological levels of antioxidants and other reactive biomolecules [53].

This pathway, in which deoxyhemoglobin acts as a “nitrite dehydrase”, is co-catalytic in $\text{NO}^\bullet$. Consequently, unlike the NO_2^- -utilizing “peroxidase” pathway described above and all other recognized pathways for N_2O_3 formation, it does not require stoichiometric consumption of $\text{NO}^\bullet$, O_2 or ROS and might therefore be particularly effective in generating N_2O_3 from NO_2^- in relative anoxic environments such as presented by sites of infection within tissues.

Nitrite involvement in neutrophil reactions

Peroxidative activity of MPO is expressed within neutrophil phagosomes

Trapping studies from our laboratory utilizing fluorescein-conjugated polyacrylamide microspheres have demonstrated that microbicidal levels of HOCl can be generated within the phagosomes of isolated neutrophils during the “respiratory burst”, i.e., the period at which NOX is actively generating $\text{O}_2^{\bullet -}$ [22]. Specifically, $\sim 10^8$ molecules of HOCl were trapped as chlorofluoresceins by the phagocytosed probes, which is an amount equal to lethal doses for several nonvirulent strains of bacteria, as determined by viability assays using colony-forming units (cfu) as an index of cellular death [22,61,62]. In these studies, the total amount of HOCl trapped as chlorofluoresceins corresponded to the estimated amount of Cl^- introduced into the phagosome from the medium during phagocytosis,⁴ suggesting that Cl^- may have been the limiting reagent. Consistent with this proposal, the amount of HOCl trapped constituted only $\sim 10\%$ of the O_2 consumed in the respiratory burst. In contrast, as much as $\sim 40\%$ of the consumed O_2 has been trapped as taurine chloramine when soluble agonists were used to trigger extracellular release of MPO [63], and a recent kinetic model based upon independently determined reaction parameters has predicted that $\sim 90\%$ of the O_2 consumed will be converted to HOCl by MPO (if Cl^- is not limiting) [54]. Unlike fluorescein, which traps Cl^- , many of the more reactive biological targets for HOCl regenerate Cl^- as a product [15]; in these reactions, Cl^- can be viewed as a co-catalyst for MPO-mediated oxidations. Thus, while demonstrating that microbicidal amounts of HOCl are formed, the fluorescein trapping experiments may have greatly underestimated the actual amount of intraphagosomal HOCl produced by MPO-catalyzed reactions.

Two recent studies have utilized opsonized bacteria (*E. coli*, *Staphylococcus aureus*) containing ^{13}C -labeled tyrosine to probe the extent of their intraphagosomal reactions with HOCl [20,21]. In both cases, chlorination of bacterial protein was demonstrated by the appearance of chlorinated ^{13}C -tyrosine products in the mass spectra of protein digests from the recovered bacteria. In the study with *S. aureus*, it was also shown that $\sim 90\%$ of the total HOCl trapped had reacted with tyrosyl groups on the host protein; this distribution reflects the

⁴Based upon estimates from electron micrographs (M. Rozenberg-Arska, M. E. C. Salters, J. A. C. van Strijp, J. J. Geuze, J. Verhoef, Infect. Immun. 50 (1985) 852–859), the average diameter of a neutrophil phagosome is $\sim 1.5\ \mu\text{m}$. If one assumes spherical geometry, the corresponding volume of a phagosome containing a $1.0\ \mu\text{m}$ diameter particle is $\sim 1.2 \times 10^{-12}\ \text{mL}$; $\sim 10^8$ chlorine atoms were trapped by the particle [22]; this corresponds to $\sim 1.7 \times 10^{-13}\ \text{mmol Cl}^-$, requiring an apparent intraphagosomal concentration of $\sim 0.14\ \text{M}$.

much larger amount of host protein in the phagosome. Thus, it is evident from these studies as well that large amounts of HOCl are formed in MPO-catalyzed reactions within the phagosome. Additionally, if one accepts that the distribution of chlorotyrosines over bacterial and host protein accurately reflects the distribution over all HOCl-reactive target sites and that ~90% of the consumed O₂ leads to HOCl formation, as implied by the kinetic model [64], then the fraction of HOCl reacting with the bacteria constitutes a lethal dose. Specifically, ~2×10⁹ O₂ molecules are consumed per phagosome [22,64], leading to a predicted ~10⁸ molecules of HOCl reacting with entrapped bacteria, an amount identified as lethal by the cfu vs [HOCl] viability assays [39,61,62]. Thus, the various trapping experiments appear to be mutually consistent.

Nitrite modulation of chlorination by HOCl and MPO

The preceding studies were made using *in vitro* suspensions of neutrophils and bacteria or particulate probes which contained no nitrite in the medium. Nitrite ion could influence these reactions either by reacting directly with HOCl or acting as an alternate substrate for MPO. Direct reaction with HOCl leads to formation of nitryl chloride [65], i.e.,

which is capable of chlorinating and nitrating phenolic rings [66]. However, when present in the reaction media at concentration levels that would compete with the direct reaction between HOCl and fluorescein [56], NO₂[−] protected fluorescein and the fluorescein-conjugated microbeads from chlorination. This result indicates that NO₂Cl is less reactive than HOCl toward the target molecules. Nitrite also inhibited tyrosyl group chlorination by MPO-generated HOCl, as well as extracellular chlorination by stimulated neutrophils [67] and, when present in excess, completely protected *E. coli* from microbicidal levels of HOCl [55]. Furthermore, the rate constant for NO₂Cl formation is relatively low under physiological conditions ($k \leq 10^3 \text{ s}^{-1}$); since the biological milieu presents numerous target sites for HOCl that are more reactive than NO₂[−] [16,64,67], it is unlikely that significant amounts of NO₂Cl could even be formed within tissues by reaction of MPO-generated HOCl with free NO₂[−].

The other possibility, that NO₂[−] is a physiological substrate for MPO, has been probed by using fluorescein and fluorescein-conjugated microbeads. Fluorescein exhibits greater selectivity toward nitration over chlorination than phenolic groups in MPO-catalyzed *in vitro* reactions in media containing both NO₂[−] and Cl[−] (cf. [56] and [67]), and so should be a relatively sensitive probe for enzyme-catalyzed nitration. Indeed, *in vitro* competition studies have shown that MPO-catalyzed nitration of fluorescein can be detected in solutions containing 0.1 M Cl[−] with NO₂[−] concentrations as low as 40 μM [56]. In these studies, the yield of nitrofluorescein increased in a dose-dependent manner at the expense of chlorofluorescein, consistent with direct competition of the anions as substrates for MPO; at [NO₂[−]] ≈ 1 mM, the nitration yield was maximal and probe chlorination was nearly completely quenched. In similar competition studies, neutrophils stimulated with soluble agonists also catalyzed extracellular nitration of fluorescein bound to unopsonized microbeads [56]. A useful benchmark for these reactions is the physiologically relevant NO₂[−] concentration range, which for plasma is generally assumed to be ≤ 200 μM [67].

Phagocytosis prevents MPO-catalyzed nitration

Strikingly different results from the solution studies were obtained when opsonized beads were phagocytosed by the neutrophils [56]. In this case, chlorination yields were unaffected by NO₂[−] at concentration levels as high as 1 mM, and dropped sigmoidally thereafter with an apparent inhibition constant (K_i) of ~5 mM. At 100 mM NO₂[−], chlorination of the probe was

completely quenched, but under no conditions ($[\text{NO}_2^-] = 10 \mu\text{M} - 100 \text{ mM}$) was nitrofluorescein detected. The value of K_i is very nearly equal to the dissociation constant ($K_d \approx 2 \text{ mM}$) measured for axial ligation of ferric MPO [68], suggesting that inhibition of chlorination at the higher concentrations of nitrite were due to inactivation of MPO by coordination at the enzyme active site; this behavior is similar to the well-documented intraphagosomal inhibition by azide (N_3^-) ion, which is frequently used to model MPO deficiency in neutrophils [41]. Although several factors might have contributed to the absence of detectable nitration at the lower, physiologically relevant, NO_2^- concentrations, a dominant effect may simply be the topographic constraint imposed by phagocytosis. As previously noted, ⁴ very little extracellular fluid is carried into the neutrophil during particle phagocytosis; consequently, the total amount of NO_2^- introduced into the phagosome is very small, i.e., at 1 mM, less than 1% of the chloride ion. Therefore, unless NO_2^- is actively transported into the phagosome, it will be rapidly depleted before a significant amount of product can be formed. NOX is considered to function as an electrogenic electron transport chain that generates a negative electrochemical potential on the inward surface of the phagosomal membrane [18, 19,44]; consequently, it is unlikely that the phagosome could actively accumulate NO_2^- . Without pumping, the amount of entrapped NO_2^- would be too small to form detectable product.

This constraint holds as well for other anionic substrates for MPO which form microbicidal products, i.e., Br^- and SCN^- . The plasma concentration levels of these anions are very similar to that of NO_2^- [69]; consequently, they also would not be expected to contribute substantially to intraphagosomal microbicidal mechanisms. However, because plasma represents a large reservoir for these ions, they would not be stoichiometrically limited in extracellular reactions catalyzed by MPO and other peroxidases and (as has been discussed by others [57,59,67]) could function as precursors to microbicidal and inflammatory agents in that environment. In this context, it is interesting to note that NO_2^- is a relatively good substrate for EPO [59], whose physiological reactions appear to be directed primarily at helminthic parasites and other extracellular targets [70].

Nitrite involvement in macrophage reactions

Alternative microbicidal roles for iNOS

In instances where peroxynitrite involvement is minimal, macrophage iNOS might serve as a direct source of microbicidal RNS via formation of $\text{NO}^\bullet$. Alternatively, macrophage iNOS could elevate nitrite ion levels at infection sites to provide substrate for peroxidase-derived RNS. As exemplified by activated RAW cells, the high rate (Table 1) and relatively long duration (5–6 h) of iNOS-catalyzed formation of $\text{NO}^\bullet$ can lead to accumulation of a large amount of NO_2^- in the reaction medium [41].

Nitrite as a cyclooxygenase substrate

Following activation, RAW cells exhibit an interesting progression of induction of oxidizing capacity that extends over a period of 10–15 h post activation and includes (in order): NOX-catalyzed $\text{O}_2^{\bullet -}$ generation > generation of H_2O_2 by an unidentified mechanism > induction of iNOS activity > induction of cyclooxygenase (COX-2) activity [41]. As noted above, the NOX activity followed immediately upon stimulation and lasted less than an hour. At ~2 h post-activation, O_2 consumption increased approximately two-fold, accompanied by hydrogen peroxide formation and induction of peroxidase-like activity within the cell. From ~5–10 h post-activation, iNOS activity was detected, leading to accumulation of NO_2^- as the end-product of $\text{NO}_2^\bullet$ catabolism, and at ~10–20 h post-activation COX-2 activity was detected by activity assays using selective inhibitors. Induction of iNOS and COX-2 activities following phagocyte activation is well-documented [23,24,40,71–74], although NOX-independent

formation of H_2O_2 and the unidentified peroxidase activity have apparently not been previously recognized. In RAW cells, COX-2 undergoes autoinhibition that is accompanied by nitration of some of its tyrosyl groups [74], and evidence has been presented for more widespread tyrosyl nitration at approximately the same time post-activation [40]. These observations suggest that, like other peroxidases [55–59], COX-2 may be able to oxidize accumulated NO_2^- at its peroxidase site [75], generating $\text{NO}_2^\bullet$ via compounds I and II in sequential one-electron steps. As a working hypothesis, we have suggested that the observed activation pattern represents an orchestrated sequence of events leading to bactericidal action in which iNOS functions to supply NO_2^- and an oxygenase (possibly lipoxygenase (LOX)) functions to both activate COX-2 [75] and supply H_2O_2 or other peroxides as substrates for COX-generated $\text{NO}_2^\bullet$ [41], e.g., according to the following reaction scheme:

where aa = arachidonic acid. One should be aware, however, that the literature suggests a complex relationship for COX interactions with RNS and iNOS. Nitric oxide has been studied as a substrate for the HPETE peroxidase activity of the constitutive COX-1 isoform [76], although it was later found that $\text{NO}^\bullet$ also accelerated the inhibition of peroxynitrite-mediated inactivation of COX-1 [77]. Peroxynitrite has been implicated as both a COX-1 and COX-2 inhibitor through nitration of its active site tyrosyl radical (Tyr-385) [77–80], although at low concentrations it can stimulate cyclooxygenase activity by acting as a peroxide activator [78]. Physical association of iNOS and COX-2 leading to activation via S-nitrosylation has also been reported [81]. Although we have suggested that NO_2^- could be a substrate for COX-2, other researchers have been unable to obtain evidence for COX-1 peroxidase-mediated NO_2^- consumption using 15-HPETE as a substrate [77], an assay which relies on the coupled cyclooxygenase/peroxidase activities of COX, and NO_2^- has been shown to be a rather potent inhibitor of COX-2 cyclooxygenase activity, again through catalytic Tyr-385 nitration [74].

Comparisons of peroxidase-catalyzed $\text{NO}_2^\bullet$ -generating bactericidal systems

To test our hypothesis that COX-2 can function as a classical peroxidase toward NO_2^- , we have conducted several *in vitro* assays using the human recombinant enzyme. Results from these studies are given in Supplementary Material and summarized here. Nitrite ion modestly inhibited both the N,N,N',N'-tetramethylethylenediamine (TMPD) and guaiacol peroxidase activities of COX-2 in a dose-dependent manner, although concentrations of NO_2^- greater than 1 mM were required to observe an effect. Continuous consumption of NO_2^- was observed in a standard COX-2/hemin/ H_2O_2 / NO_2^- assay system over a 2 h period, but the consumption rate was not statistically different from that measured for hemin/ H_2O_2 / NO_2^- alone, i.e., without COX-2. (Hemin is required in these assays to prevent enzyme inactivation by loss of active site heme.) In contrast, two peroxidases known to use NO_2^- as a reductant, i.e., met-MB [54, 82] and MPO [55,56] gave extensive NO_2^- consumption under the assay conditions used in these studies. RNS production was detected as 3-nitrotyrosine (3-NT) in each of the MPO/ H_2O_2 / NO_2^- met-Mb/ H_2O_2 / NO_2^- , and COX-2/hemin/ H_2O_2 / NO_2^- assay systems, but not with hemin/ H_2O_2 / NO_2^- alone. In these studies, the yield of 3-NT depended upon the COX-2 concentration, consistent with an enzymatic mechanism involving COX-2 mediated formation of free $\text{NO}_2^\bullet$. The yields of 3-NT were quite modest when compared to the other peroxidase systems, however. The bactericidal potency of these NO_2^- -oxidizing peroxidase reactions was tested by measuring cell viabilities of *E. coli* suspended in the respective assay media. By analogy with the NO_2^- consumption and 3-NT production measurements discussed above, the

most lethal treatments were with met-Mb and MPO as catalysts. Treatment with hemin alone as catalyst gave a comparable decrease in cell viability. Addition of COX-2 to this microbicidal assay system led to concentration-dependent *protection* of the bacteria from killing. Although the basis for this effect is not presently understood, the cumulative results offer little support for our hypothesis that COX-2 could be directly involved in microbial killing within macrophages.

Alternative microbicidal mechanisms for iNOS-generated NO_2^-

Although COX-2 may not be involved in generating microbicidal amounts of RNS, it is possible that other macrophage peroxidases or heme proteins could serve this function. Alternatively, other mechanisms might be at play. For example, weakly acidic solutions containing both H_2O_2 and NO_2^- are bactericidal, although they are not under neutral conditions [36,55,83]. It has been suggested that toxicity arises from reaction between these reagents to form peroxynitrous acid [83] but, unless somehow catalyzed, this reaction is simply too slow above pH ~5 to generate much toxin [36,84]. For example, calculated reaction halftimes are $t_{1/2} \approx 12$ days at pH 5.0 and ~320 years at pH 7.0 for solutions containing 1 mM NO_2^- (and would be longer at lower NO_2^- concentrations); these values reflect the strong acid dependence of the reaction between H_2O_2 and NO_2^- [84]. The potential bactericidal role of ONOO^- in these solutions has been directly probed by comparing *in vitro* bactericidal assays in media that contained bicarbonate to media that were carbonate-free. Bicarbonate functions in these experiments to provide a reservoir of CO_2 which, as previously noted, catalyzes rapid isomerization of ONOO^- to NO_3^- [31]; in the dilute suspensions comprising the assay medium, bicarbonate thereby protects the bacteria from peroxynitrite-mediated killing. However, bicarbonate failed to protect *E. coli* from killing by H_2O_2 in media that might both favor peroxynitrite formation and could conceivably be achieved in physiological environments (pH 5, $[\text{NO}_2^-] = 20$ mM), excluding extracellularly generated ONOO^- as the bactericidal agent [36].³ At present, the microbial toxins and microbicidal mechanisms acting in these solutions are unknown.

Another issue bearing upon the antimicrobial action of macrophages is whether or not the phagosome undergoes acidification. Several studies have utilized fluorescein-conjugated particles, specifically, *S. aureus* [85,86] and polyacrylamide microbeads [41] to monitor changes in intraphagosomal pH following phagocytosis; these studies rely upon pH-dependent changes in the fluorescence emission intensity and/or spectral bandshapes of this dye that occur over the neutral-to-weakly acidic regime [87]. This method can be applied to probe the macrophage phagosome because independent studies have demonstrated that, unlike phagocytosis by neutrophils [22], the probe undergoes no detectable intraphagosomal chemical modification [41]. Following phagocytosis of opsonized beads by RAW cells, the intraphagosomal pH dropped transiently from ~7.2 to ~6.3 within the first two hours, then subsequently slowly increased, achieving levels as high as pH ~8 at 20 h post-phagocytosis [41]. Rapid acidification was also observed with mouse peritoneal macrophages challenged with fluorescein-conjugated *S. aureus*, which underwent changes to a steady-state level of pH ~6 within several minutes post-phagocytosis [85]; inhibition studies implicated a vacuole-type ATPase as the proton pump. These two sets of studies [41,85] appear to be mutually consistent, although it would have been helpful had the latter set been carried to longer times. This pH behavior underscores the profound differences in macrophage and neutrophil activation, the phagosome of the latter having been shown in several studies to undergo initial alkalinization on the time scale of microbial killing, followed by slow acidification to pH ~6 [88–90]. In any event, it appears that mechanisms of killing by H_2O_2 and NO_2^- occurring *in vitro* in acidic media are not expressed within either of these phagocytes because the requisite levels of acidification (pH 5) are not achieved on the respective microbicidal time scales. For the same reason, other “nonbiological” killing mechanisms involving NO_2^- , which require highly acidic

conditions, are highly unlikely to play a role in intraphagosomal killing. Interestingly, an interphagosomal transmembrane potential was not detectable in the macrophage studies [86], an observation which contrasts starkly with the behavior reported for neutrophils [18,19]. This difference might be ascribed to the much lower respiratory activity of macrophages, and could reflect differing primary functions for the two NOX-2 redox chains.

Conclusions

Evidence from diverse sources, including clinical studies, research on animal models, *in vitro* studies with isolated cells and their biological components, and investigations of underlying chemical reactions, strongly suggest that NO_2^- is not just a benign end-product of $\text{NO}^\bullet$ catabolism, but could be an active precursor to RNS within cells and tissues. This inference notwithstanding, the mechanisms by which NO_2^- might be involved remain obscure. Nitrite ion could serve as a precursor to microbicidal $\text{NO}_2^\bullet$ and N_2O_3 in extracellular fluids through the peroxidative and “nitrite reductase” activities of metalloproteins, and it is likely that these reactions make major contributions to RNS in these environments. However, in both neutrophils and macrophages, encapsulation by phagocytosis may effectively limit access of extracellular NO_2^- to enzymes that could form RNS. It is possible that this limitation is overcome in macrophages by generating nitrite intracellularly through reactions catalyzed by iNOS, although an enzymatic system that utilizes the accumulated NO_2^- has not been identified, and one likely candidate, COX-2, appears in initial studies not to be effective in generating microbicidal amounts of $\text{NO}_2^\bullet$, at least when isolated *ex vivo*. Nonenzymatic formation of RNS from NO_2^- in acidic solutions is also a subject of growing interest, although the phagosomes of both neutrophils and macrophages do not become sufficiently acidic to promote these reactions. Hypochlorous acid, generated from Cl^- by MPO-catalyzed peroxidation, remains most probable primary intraphagosomal oxidant in neutrophils, although NO_2^- is likely to be involved in MPO-mediated extracellular reactions associated with inflammation. Production of peroxynitrite by activated macrophages represents an alternative source of RNS to that of one-electron oxidation of NO_2^- , although there is considerable uncertainty within the scientific community regarding the extent to which ONOO^- is formed by these cells in physiological environments. Part of the confusion on this point may be simply due to experimental design. Most *in vitro* experiments have involved synchronous activation of the cells, which virtually ensures that fluxes of the peroxynitrite precursors, $\text{NO}^\bullet$ and $\text{O}_2^{\bullet -}$ will not overlap. This condition is quite unlike what is expected at infection sites, where a continuous migratory influx of phagocytic cells occurs over a relatively long time period. Studies that are designed to evaluate synergism in nonsynchronous and mixed populations of phagocytes may clarify the issue of peroxynitrite formation, and thereby help delineate the nature of macrophage-generated RNS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Hypothetic pathways for nitrite involvement in microbicidal action of phagocytes. Primary intraphagosomal microbicides are HOCl (for neutrophils) and either ONOO⁻ or peroxidase-generated NO₂[•] (for macrophages). Nitrite could be a precursor to extracellular NO₂[•] in reactions catalyzed by MPO or several other peroxidases and N₂O₃ from radical combination of NO₂[•] and macrophage-derived NO[•] or by heme protein catalyzed dehydration.

TABLE 1

Reported rates of superoxide and nitric oxide formation by activated macrophages.

Cell line	NO ^a (pmol mg ⁻¹ min ⁻¹)	O ₂ ^{•-} (pmol mg ⁻¹ min ⁻¹)	ref
RAW 264.7	~120	~13	[40]
Rat peritoneal	~219	~86	[42]
J774	200–300	300–400	[38]
Rat alveolar ^b	~100	~500	[34]

^a similar values for NO[•] formation have been reported in the earlier literature for cultured mouse peritoneal macrophages and RAW 264.7 cells ([24]; D. J. Stuehr, C. F. Nathan, J. Exp. Med. 169 (1989) 1543–1555; R. Iyengar, D. J. Stuehr, M. A. Marletta, Proc. Natl. Acad. Sci. USA 84 (1987) 6369–6373).

^b assumes a scaling factor of 1 mg/10⁶ cells.