Reactive Oxygen Species and Neutrophil Function

Christine C. Winterbourn, Anthony J. Kettle, and Mark B. Hampton

Centre for Free Radical Research, Department of Pathology, University of Otago, Christchurch 8011, New Zealand; email: christine.winterbourn@otago.ac.nz, tony.kettle@otago.ac.nz, mark.hampton@otago.ac.nz

Annu. Rev. Biochem. 2016. 85:765–92

First published online as a Review in Advance on April 6, 2016

The *Annual Review of Biochemistry* is online at biochem.annualreviews.org

This article's doi: 10.1146/annurev-biochem-060815-014442

Keywords

superoxide, hydrogen peroxide, hypochlorous acid, hypothiocyanous acid, chloramine, myeloperoxidase, antimicrobial, inflammation, redox signaling, cell death

Abstract

Neutrophils are essential for killing bacteria and other microorganisms, and they also have a significant role in regulating the inflammatory response. Stimulated neutrophils activate their NADPH oxidase (NOX2) to generate large amounts of superoxide, which acts as a precursor of hydrogen peroxide and other reactive oxygen species that are generated by their heme enzyme myeloperoxidase. When neutrophils engulf bacteria they enclose them in small vesicles (phagosomes) into which superoxide is released by activated NOX2 on the internalized neutrophil membrane. The superoxide dismutates to hydrogen peroxide, which is used by myeloperoxidase to generate other oxidants, including the highly microbicidal species hypochlorous acid. NOX activation occurs at other sites in the cell, where it is considered to have a regulatory function. Neutrophils also release oxidants, which can modify extracellular targets and affect the function of neighboring cells. We discuss the identity and chemical properties of the specific oxidants produced by neutrophils in different situations, and what is known about oxidative mechanisms of microbial killing, inflammatory tissue damage, and signaling.

Contents

INTRODUCTION

Neutrophils are phagocytic white blood cells that are essential for effective innate immunity. They are recruited to sites of infection where they ingest and kill invading pathogens. They modulate the immune response and also contribute to ongoing inflammation in numerous diseases, including rheumatoid arthritis, lung diseases, sepsis, atherosclerosis, and cancer (1–3). One of the hallmarks associated with the antimicrobial and inflammatory actions of neutrophils is the activation of a powerful oxidative burst, during which large amounts of oxygen are consumed and converted to superoxide radicals (see **Table 1** for terminology). The enzyme system responsible is an NADPH oxidase complex that assembles in the membrane following activation. The superoxide dismutates to hydrogen peroxide, and in contrast to many other biological systems where superoxide and hydrogen peroxide are generated, neutrophils generate secondary oxidants via the activity of myeloperoxidase (MPO), an abundant peroxidase that constitutes approximately 5% of the protein in the cells. The production of these oxidants is critical for effective antimicrobial defense and is emerging as a regulator of other diverse neutrophil functions.

One of the challenges since the NADPH oxidase activity of neutrophils was first identified has been to dissect out how oxidant production contributes to their function and the mechanisms involved. This requires an understanding of when and where NADPH oxidase activation occurs, how MPO influences what oxidants are produced, and how the oxidants interact with the

Table 1 Neutrophil terminology

Term	Definition
Phagocytosis	Ingestion of microorganisms or other particulate material
Opsonization	Coating of microorganism with immunoglobulin or complement-derived factors to promote phagocytosis
Phagosomes (phagolysosomes)	Intracellular vacuoles containing ingested microorganisms into which NADPH oxidase products are directed and granule contents are released
Oxidative burst	Burst of oxygen consumption and superoxide production that occurs when the neutrophil NADPH oxidase is activated
Granules	Cytoplasmic vesicles containing a wide spectrum of antimicrobial and hydrolytic proteins that are released into the phagosome or to the exterior on activation of the neutrophil; myeloperoxidase is present in azurophil granules, whereas the membrane components of the NADPH oxidase are primarily associated with specific granules; also present are gelatinase and ficolin-containing granules and secretory vesicles
Phorbol myristate acetate (PMA)	Potent stimulus of neutrophil activation; although nonphysiological, it acts as an analog of diacylglycerol to activate the protein kinase C pathway
Opsonized zymosan	Opsonized yeast cell wall preparation that activates a robust oxidative burst
Formyl-Met-Leu-Phe	Formylated bacterial peptide that attracts neutrophils (chemotaxis) and induces a short oxidative burst
Priming	Interaction of the neutrophil with proinflammatory cytokines, endotoxin, or ionophores that elicits a preactivation state and greater NADPH oxidase activation in response to subsequent stimulation (for example, by particle ingestion)
Neutrophil extracellular traps (NETs)	Mesh-like structures containing chromatin and an array of predominantly granule proteins that are actively released by neutrophils and able to trap microorganisms
Chronic granulomatous disease (CGD)	An inherited condition in which one of the NOX2 subunits (usually X-linked $gp91^{phox}$) is absent or nonfunctional; associated with impaired microbicidal activity and recurrent infections

constituents of the neutrophil, the ingested microorganism, and with surrounding host tissue. In this article we address how the neutrophil produces oxidants, what species they are, and how they participate in microbial killing and other neutrophil functions.

NEUTROPHIL BIOLOGY: A BRIEF OVERVIEW

As described in detail elsewhere (1, 3), neutrophils are produced in the bone marrow, are released into circulation, where they live for about a day, and migrate to their site of action along a chemical gradient of chemokines released by infectious agents or other inflammatory cells. They contain a multilobed nucleus, few mitochondria, and cytoplasmic granules containing numerous proteolytic and hydrolytic enzymes (4). Although they undergo limited protein synthesis, they express membrane receptors for infection-related and inflammatory stimuli that, when activated, initiate rapid expression of predominantly cytokine genes (5). Their most obvious activity is the phagocytosis and subsequent killing of microorganisms in internal vacuoles called phagosomes. They also produce immunomodulatory molecules that influence the recruitment and behavior of other inflammatory cells and, eventually, signal processes leading to the resolution of inflammation. Another neutrophil activity, first described in 2004, is the extrusion of chromatin structures termed neutrophil extracellular traps (NETs), which have antimicrobial and inflammatory properties. Excessive activation or ineffectual clearance can lead to neutrophil lysis, tissue damage, and exacerbation of the inflammatory response.

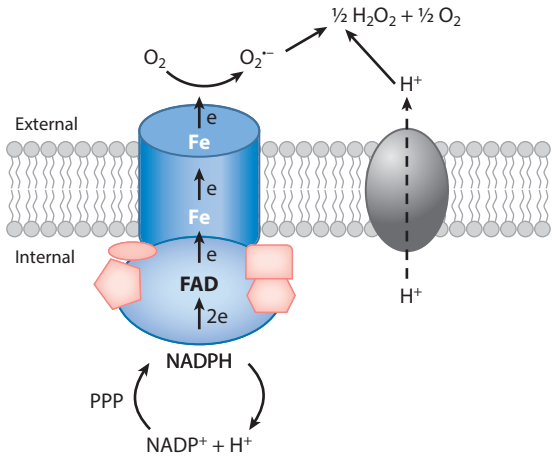

Figure 1

Membrane assembly of NOX2. NOX2 is a multicomponent electron-transfer complex. The catalytic subunit gp91^{phox} comprises a membrane-spanning domain containing two stacked b-type cytochromes and an associated p22 subunit (*darker blue*) and a FAD-containing cytoplasmic domain (*light blue*). As described in detail elsewhere (7, 9–11), activation of the neutrophil leads to a series of phosphorylations that recruit cytoplasmic regulatory subunits (*pink*) to the cytoplasmic domain to give enzymatically active NOX2. Electrons are transferred singly to oxygen through a heme relay from FAD, which then undergoes two-electron reduction by NADPH. $NADP^+$ is recycled to NADPH through the pentose phosphate pathway (PPP). The charge is balanced primarily through proton channels (*gray*). Note that electron flow is directional, from the cytoplasm to the external surface of the membrane.

NOX2 AND SUPEROXIDE GENERATION

Properties of Neutrophil NOX2

Stimulated neutrophils are the most intense physiological producers of superoxide radicals through the activation of the NADPH oxidase complex NOX2. In response to phagocytosis, immune stimuli, or bacterial components (6, 7), this complex assembles in the membrane and catalyzes the reduction of oxygen by a single electron, with the electrons shuttling through the complex from NADPH (**Figure 1**). Oxygen consumption by neutrophils can increase to 100-fold of basal metabolic activity. Neutrophils and other professional phagocytes were the first cells shown to exhibit NADPH oxidase activity. The enzyme complex was originally named phagocyte oxidase, and it was thought to be unique for killing microorganisms. However, the neutrophil enzyme is now known to be one of a widely distributed family of NOX isoforms and (or, more correctly, its catalytic subunit gp91^{phox}) is referred to as NOX2. In other cells, NOXs are activated in response to a wide variety of receptor ligands, and their main function is proposed to be the transmission of redox signals (8). As well as its established involvement in antimicrobial activity, other cell signaling functions of the neutrophil NOX2 are becoming apparent.

The enzymology of NOX2 has been widely studied, both in isolation and in the neutrophil (reviewed in 7, 9–11). On activation, there is rapid electron transfer from NADPH through the complex to oxygen. The neutrophil can maintain maximal activity down to a dissolved oxygen concentration of approximately 20 μM (10% of air saturation) (12), and except under severe hypoxia, activity is more likely to be limited by the rate of reduction by NADPH. Based on measurements of the maximum rate of electron transfer in the isolated reconstituted enzyme, it

has been calculated that at full capacity NOX2 would oxidize all of the NADPH in the neutrophil (approximately 50 μM) in less than 1 second (6). Early work showed that the regeneration of NADPH occurs via the pentose phosphate pathway, with 20% of normal activity sufficient to maintain an adequate NADPH supply (13).

An important property of NOXs is that electron flow is directional, from NADPH in the cytosol to oxygen on the external surface of the membrane (**Figure 1**). Superoxide will, therefore, be released extracellularly when NOX2 is on the cell surface, but when activity is localized to internal membranes, such as phagosomes, it will be released into the enclosed vesicle. The directional nature of NOX2 activity also creates a charge imbalance that must be counteracted to prevent depolarization of the membrane and the shutdown of activity (6). Also, protons are produced in the cytosol and consumed in the external compartment (for example, the phagosome) through the dismutation of superoxide (**Figure 1**). Both situations are largely overcome by a balancing flow of protons transported by voltage-gated proton channels, primarily VSOP/HV1, which are activated in parallel with the oxidase (14). The pH of the phagosome is regulated by these activities. In contrast to the phagosomes of macrophages, in which pH drops following particle ingestion, neutrophil phagosomes remain alkaline during the period that the oxidase is active. Until recently, their pH has been accepted to lie between 7.5 and 8. However, in a 2015 study using a probe that is more sensitive at higher pH, an average pH closer to 9 was measured in individual phagosomes (15).

Sites of NOX2 Activation

To understand how neutrophils use superoxide and the other products of NOX2, it is important to know where and when the enzyme complex is activated. Detecting NOX activity is not straightforward, especially when it occurs at intracellular sites, as the oxidant products are short lived and often not accessible to detectors (reviewed in 16). One approach, as illustrated in **Figure 2*a***, is to show the sites of assembly of the NOX2 subunits by immunocytochemistry. Direct evidence of activation can be obtained from oxygen consumption measurements, although they do not provide information on location and may not detect low-level activity. Extracellular superoxide and hydrogen peroxide can be measured quantitatively by using specific detection systems, such as cytochrome *c* or Amplex Red (16). However, detecting intracellular NOX2 activity is more problematic and, at best, is qualitative rather than quantitative. The most definitive evidence of intracellular localization comes from electron microscopic studies that used the deposition of cerium peroxide to detect hydrogen peroxide and diaminobenzidine–Mn^{2+} for superoxide (17, 18). Numerous investigators have used fluorescent or luminescent probes to study NOX activation, including dihydrodichlorofluorescein, dihydrorhodamine, and isoluminol. These are all oxidized by a radical mechanism involving peroxidase activity, and they are open to artifact and misinterpretation (reviewed in 19, 20). In neutrophils, peroxidase activity comes from MPO, and provided this is appreciated and the conditions are well controlled, useful information can be obtained (21, 22). As discussed for hypochlorous acid in the section Oxidant Production and Reactions in the Phagosome, more specific probes are becoming available, with the potential to provide much needed quantitative information.

NOX2 is strongly activated when neutrophils undergo phagocytosis. The majority of this activity is localized to the phagosomal membrane, thus directing superoxide into the phagosomal space. When a neutrophil recognizes particles for ingestion, the plasma membrane invaginates, forming, first, a cup and, then, an enclosed phagosome (**Figure 2*a***). This occurs extremely rapidly (in approximately 45 s) and the NOX2 subunits assemble before the phagosome is fully formed (23). Intracellular granules fuse with the developing phagosome, releasing MPO and a host of digestive enzymes. The majority of $gp91^{phox}$ is associated with specific granule membranes, with

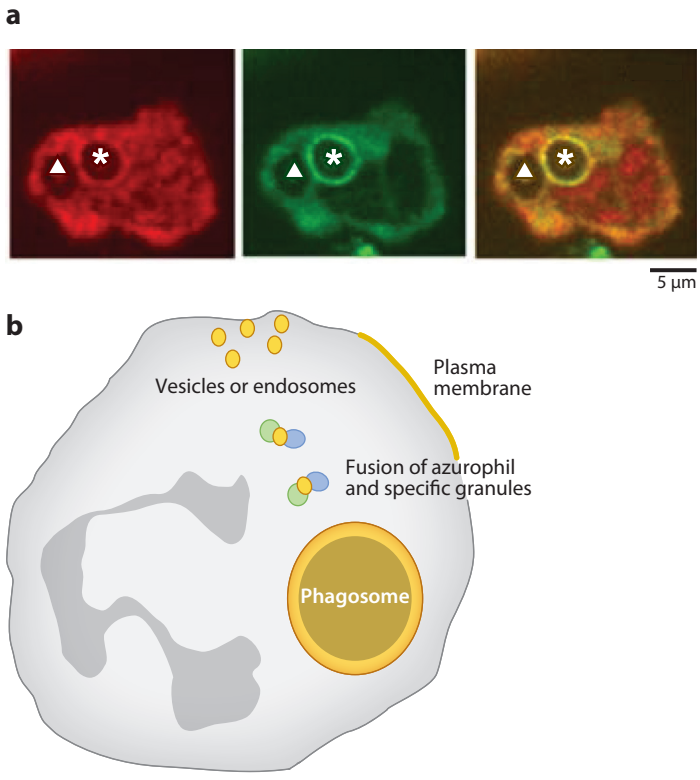

Figure 2

(*a*) Neutrophil following phagocytosis of zymosan particle showing accumulation of p67 (*green*) and p47 subunits (*red*) of NOX2 on the phagosomal membrane and in the merged image (*yellow*). The asterisk represents the positive, and the triangle the negative, phagosome for NOX2 assembly. Each zymosan particle fills its phagosomal space. Panel *a* reproduced from Reference 23 with permission. (*b*) Neutrophil showing sites (*gold*) where NOX2 activity has been observed following stimulation. See text for details.

the remainder on the plasma membrane, so active NOX2 incorporates into the phagosome as the granule and plasma membranes fuse. Localization of NOX2 has been seen by immunostaining of the subunits (**Figure 2*a***), electron microscopy, and cerium peroxide detection (18), and in studies using ingested fluorescent probes (24, 25).

As shown diagrammatically in **Figure 2*b***, lesser amounts of NOX2 activation occur at other intracellular sites and on the cell surface following phagocytosis (18, 21, 22, 26). For example, NOX2 assembly has been detected in the vicinity of phagosomes, apparently associated with fusion between azurophil and specific granules (27). It is unclear whether this is a step that precedes integration with the phagosome or whether it represents an independent oxidative event. Electron microscopic studies have shown peroxide production at vesicular or endosomal sites that are apparently unrelated to antimicrobial activity (18).

NOX2 is also activated by soluble stimuli without phagosome formation. With phorbol myristate acetate (PMA), a potent stimulus that is widely used experimentally (**Table 1**), most activity is at the cell surface, but some occurs in intracellular vesicles (18, 22, 28). The latter sites have not been well characterized, but electron micrographs have shown a granular appearance of peroxide deposits associated with intracellular vesicles or endosomes, which may fuse with the plasma membrane (17, 18). Interaction with the bacterial peptide formyl-Met-Leu-Phe induces NOX2

activation and superoxide release only on the plasma membrane. NOX2 activation associated with other receptor-mediated processes has also been observed. Although less is known about the sites and consequences, there is increasing interest in determining whether this represents redox signaling activity (21, 22, 29).

Differences in the mechanism of NOX2 activation at different sites have been observed. For example, one of the regulatory subunits, p40, is required for activation at intracellular sites but not on the cell surface (30), and it appears that only intracellular activation is inhibited by the phosphoinositide 3-kinase inhibitor wortmannin (31). The duration of NOX activity also varies depending on the stimulus (6). With PMA, activity can be detected for up to 2 h. The timing during phagocytosis is more difficult to measure, as not all of the particles are taken up at the same time. However, when synchronized uptake was achieved with yeast particles, superoxide production peaked at 10–15 min, then gradually declined over a similar period (32). Likewise, when individual phagosomes were followed using fluorescent probes, oxidant production was maximal for up to 8 min following ingestion, then tailed off by 15 min (33, 34). Little is known about activation at nonphagosomal intracellular sites. Termination appears to involve phosphorylation steps rather than self-regulation by oxidative inactivation (6).

THE REACTIVE OXYGEN SPECIES OF NEUTROPHILS

Neutrophils have the potential to generate almost the whole spectrum of radical and nonradical oxidants that are relevant to redox biology. It is common to refer to these as reactive oxygen species, or ROS. This can be a useful and convenient collective term. However, it is also widely misunderstood in the literature where ROS is often assumed to be a single, poorly defined entity that produces the same outcome regardless of which species are involved. This usage is not helpful, especially when trying to understand mechanisms, as the different species can have quite different reactivities. The most relevant oxidants produced by the neutrophil, apart from superoxide and hydrogen peroxide, are those generated by MPO. MPO uses hydrogen peroxide to oxidize a wide range of substrates to reactive products. These include nonradical oxidants, the main ones being hypochlorous and hypothiocyanous acids, as well as radicals produced from organic and inorganic substrates. Which of these reactive species is produced depends on whether MPO is present and the relative availability of its substrates, and this will differ depending on where NOX2 activation occurs.

Table 2 summarizes the chemistry of major neutrophil oxidants, and **Figure 3** gives a pictorial representation of the relative reactivities of the nonradical oxidants with different substrates based on measured rate constants. Major differences are apparent. For hydrogen peroxide, apart from dedicated thiol and selenoperoxidases, reactions with thiol groups are slow and less favorable than reactions with transition metal centers. Notably, the latter are one-electron reactions and lead into radical chemistry. Hypochlorous acid reacts preferentially with thiols and methionine (Met) residues, with amines reacting more slowly. The chloramines produced by hypochlorous acid and amines also show selectivity for sulfur centers, although they are less reactive. Hypothiocyanous acid reacts almost exclusively with thiols. Other relevant features that distinguish between oxidants include whether they react by a one- or two-electron mechanism, as only the former initiate radical reactions such as lipid peroxidation, and whether they are restricted by permeability barriers.

ENZYMOLOGY OF MYELOPEROXIDASE

Hydrogen peroxide reacts with the ferric form of MPO to give a strongly oxidizing and short-lived intermediate, Compound I. The reaction of Compound I with a halide completes a halogenation cycle (**Figure 4**) in which the halide is oxidized by two electrons to the corresponding hypohalous

Table 2 Chemical reactivity of major neutrophil oxidants[a]

Oxidant	Description
Superoxide ($O_2^{\bullet -}$)	Weak one-electron oxidant and moderate reductant Mostly ionized at neutral pH (pK_a 4.8) Protonated form ($HO_2^{\bullet}$) is more oxidizing Low membrane permeability Fastest reactions with iron–sulfur centers, NO, and other radicals Radical addition reactions give hydroperoxides Slow reaction with thiols
Hydrogen peroxide (H_2O_2)	Strong two-electron oxidant, but high activation energy means few biological substrates Fastest reactions with thiol and selenoperoxidases Other thiols much less reactive; reaction is with thiolate Reacts with transition metal centers to generate hydroxyl radicals or initiate radical reactions Membrane permeable, preferentially through aquaporins
Hypochlorous acid (HOCl)	Strong two-electron oxidant with wide substrate reactivity HOCl more reactive than OCl^- (pK_a 7.4), and membrane permeable Fastest reactions with cysteine and methionine residues Ionized (low pK_a) thiols are more reactive Thiol oxidation products include disulfides and higher oxidation products Minor reactions include chlorination of tyrosine residues (to give 3-chlorotyrosine) and nucleotides, and chlorohydrin formation on unsaturated lipids and NAD
Chloramines (R-NHCl)	Generated from HOCl and amine groups on amino acids or proteins $R\text{-}NH_2 + HOCl \rightarrow R\text{-}NHCl + H_2O$ Two electron oxidants, weaker than HOCl and more selective for sulfur centers Low pK_a thiols are more reactive; little oxidation beyond disulfides Break down slowly to give aldehydes and NH_3 Can be broken down by transition metals to radicals Reaction with a second HOCl gives more reactive dichloramines
Hypothiocyanite ($OSCN^-$)	Predominant form of hypothiocyanous acid (pKa 5.3) at neutral pH Biological reactions almost entirely with cysteine residues to give disulfides; low pK_a thiols are more reactive $R\text{-}S^- + HOSCN \rightarrow R\text{-}SSCN\ (+ OH^-) + RS^- \rightarrow RSSR + SCN^-$ No reaction with methionine
Organic radicals	Formed from MPO and many substrates Initiate chain reactions, such as lipid peroxidation Depending on reduction potential, vary from highly reactive (e.g., NO_2) to almost inert (ascorbyl)

Abbreviations: MPO, myeloperoxidase; NAD, nicotinamide adenine dinucleotide; pK_a, logarithmic acid dissociation constant.
[a]For more extensive coverage, see References 71, 132, and 154. For references on the reactivity of MPO products, see References 53, 122, 123, and 155–159.

acid. MPO is unique among mammalian peroxidases in having a sufficiently high reduction potential (1.16 V) to oxidize chloride to hypochlorous acid (35). It also oxidizes bromide, iodide, and the pseudohalide thiocyanate. Thiocyanate is more reactive than chloride, but the greater abundance of chloride means that it is usually the preferred physiological substrate (36).

MPO also performs a peroxidase cycle in which Compounds I and II oxidize the substrate to its radical in single-electron steps (**Figure 4**). Compound I generally reacts faster, and the Compound II reaction limits the rate at which the enzyme turns over. Physiological substrates include tyrosine, ascorbate, urate, serotonin, and nitrite (reviewed in 37, 38). MPO generates

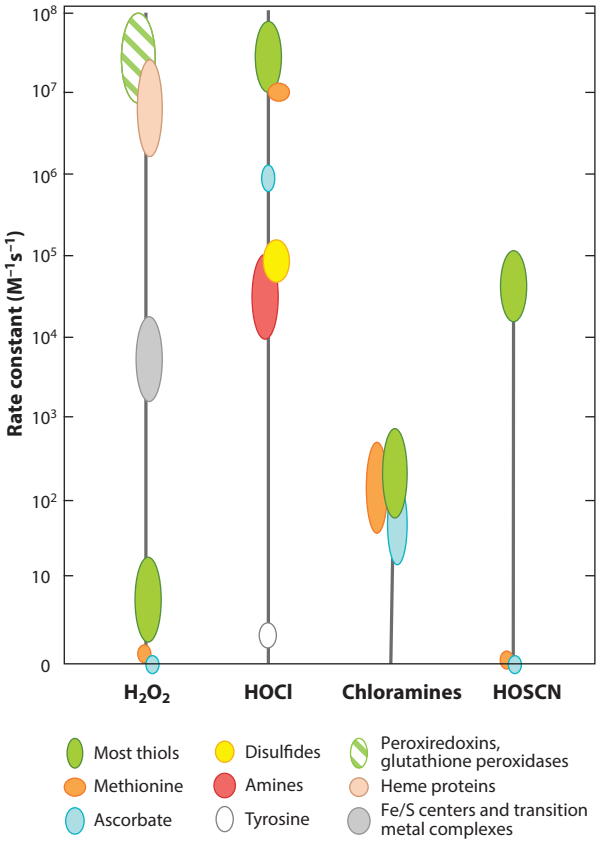

Figure 3

Pictorial representation of the relative reactivities of hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), chloramines, and hypothiocyanous acid (HOSCN) with different substrates based on measured rate constants. Note the log scale. Absolute values for the rate constants are given in References 122, 123, 154, 155, 160, and 161.

radicals from many drugs and xenobiotics, particularly phenols and aromatic amines, and this may contribute to their toxic and immunogenic effects (39, 40).

MPO activity is regulated by the reactions of the redox intermediates with superoxide (41). The product with ferric MPO is an oxy–heme complex, Compound III (**Figure 4**). Compound III is formed when MPO is exposed to superoxide in the presence of chloride, and this is the predominant form in the neutrophil phagosome. It can be recycled by ascorbate and by superoxide (41, 42), the latter reaction being required to maintain MPO activity in the phagosome. As the products of the cycling of Compound III by superoxide are hydrogen peroxide and oxygen, MPO functions as a superoxide dismutase (SOD), although the slow reaction with Compound III makes it 10,000 times less effective than specialized SODs. Superoxide reacts rapidly with both Compound I and Compound II (41) and can, therefore, complete a catalytic cycle in which hydrogen peroxide oxidizes two superoxides to oxygen (**Figure 4*b***). This activity is inhibited by chloride, which competes for Compound I and, as discussed in the next section, is likely to be relevant in the phagosome if the chloride concentration is low. Superoxide can also overcome the inhibition of halogenation activity by substrates that react well with Compound I but poorly with Compound II

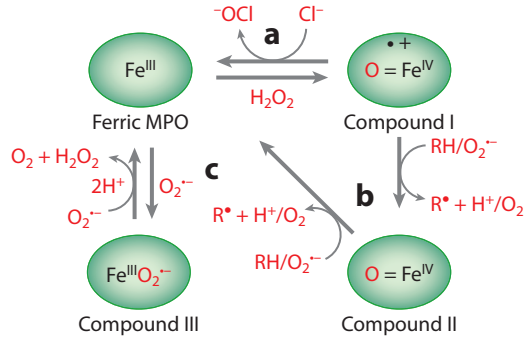

Figure 4

Reactions of myeloperoxidase (MPO). Native ferric MPO reacts with hydrogen peroxide (H_2O_2) to form Compound I. (*a*) In the halogenation cycle, Compound I oxidizes chloride (Cl^-) and other halides in a two-electron step to hypohalites. (*b*) In the peroxidase cycle, Compounds I and II oxidize organic substrates (RH) to radicals ($R^{\bullet}$) by removal of a single electron. Compounds I and II also oxidize superoxide to molecular oxygen. (*c*) Ferric MPO dismutates superoxide via Compound III to form H_2O_2 and molecular oxygen. The redox states of the heme iron are shown. These mechanisms are described in more detail elsewhere (37, 38, 41, 42).

(37). Compound II accumulates under these conditions, but can be reduced by superoxide back into the halogenation cycle.

Hydrogen peroxide reduces Compound I of MPO, liberating molecular oxygen. In combination with its reaction with the ferric enzyme, this represents catalase activity, although in most circumstances this reaction is minor due to competing reactions with chloride or other substrates.

OXIDANT PRODUCTION AND REACTIONS IN THE PHAGOSOME

The conditions under which NOX2 and MPO function dictate which oxidants are produced. For example, for neutrophils in plasma, extrapolation from experimental studies predicts that when NOX2 is activated on the external membrane accompanied by the release of MPO, the majority of the superoxide should dismutate and the hydrogen peroxide react with MPO. With typical plasma concentrations of 140 mM chloride and 30 μM thiocyanate, and a 730-fold-higher specificity constant for the latter (36), about 80% of the product would be expected to be hypochlorous acid, the remainder being hypothiocyanite and approximately 10% radical products, due to the oxidation of urate and other peroxidase substrates (43).

The conditions in the phagosome are radically different from those in the cell's surroundings or used experimentally. As is evident from **Figure 2** and shown diagrammatically in **Figure 5**, there is extremely close contact between the ingested particle and the phagosome membrane, providing only a narrow space into which superoxide is released. Little external medium is taken up, and the release of granule contents results in high concentrations of proteins, including millimolar MPO. As calculated from oxygen consumption measurements, superoxide is generated at several millimolar per second (44). These extraordinary conditions have a number of important implications. MPO will turn over at an extremely high rate and oxidize the limited amounts of substrates present in the small volume unless they are replenished. Chloride is likely to be the main substrate, as the much lower concentrations of thiocyanate and peroxidase substrates should be rapidly oxidized and account for only a tiny fraction of the hydrogen peroxide generated. Although MPO can oxidize nitrite to the strongly oxidizing and nitrating nitrogen dioxide radical,

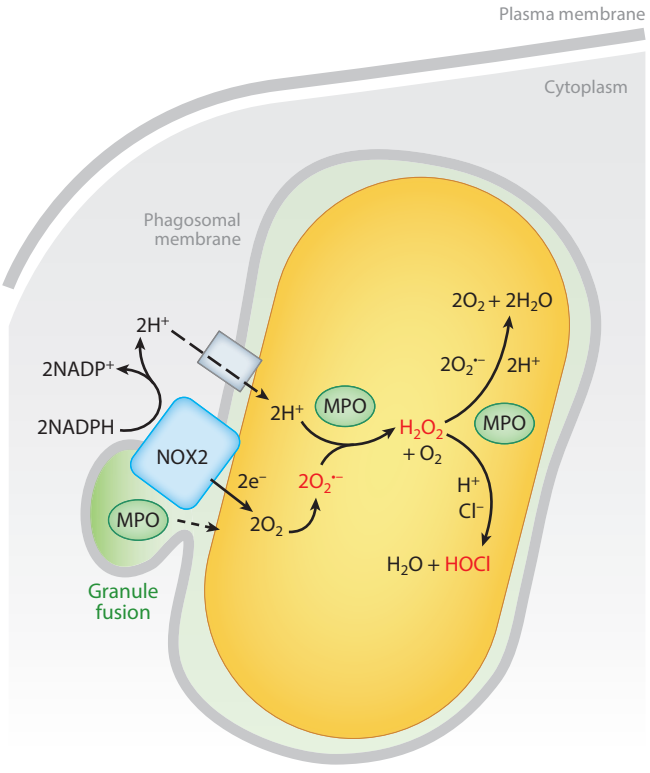

Figure 5

Portion of neutrophil showing ingested bacterium (*yellow*) within a phagosome, activated NOX2 on the membrane, and fusion of a granule with the release of contents (*green*). The main redox reactions are shown. Superoxide ($O_2^{\bullet-}$) is released into the narrow space between the membrane and bacterium. The charge is balanced through proton channels. $O_2^{\bullet-}$ dismutation [mainly catalyzed by myeloperoxidase (MPO)] gives hydrogen peroxide (H_2O_2) as a substrate for MPO, which is released from azurophil granules. Competition between chloride (Cl^-) and $O_2^{\bullet-}$ regulates hypochlorous acid (HOCl) production.

very little nitration was detected inside neutrophil phagosomes, even in the presence of high nitrite (45). Nevertheless, lipid peroxidation has been observed when neutrophils ingest bacteria (46, 47), indicating that radical reactions do take place.

It is not feasible to sustain experimentally the high superoxide generation rates of the phagosome or to study the reactions of MPO at millimolar concentrations. However, modeling phagosomal conditions using known morphological and biochemical characteristics of the neutrophil and the kinetic properties of MPO provides insights into what reactions are likely to take place (41, 44). It shows that even though superoxide and hydrogen peroxide are formed extremely rapidly, hydrogen peroxide is consumed by MPO as fast as it is generated to give a steady-state concentration in the micromolar range (**Figure 6*a***). Most of the superoxide undergoes MPO-dependent dismutation via Compound III cycling, but it stabilizes at a high concentration of approximately 20 μM. If the initial chloride concentration, estimated at 70 mM (48), can be maintained, MPO should convert the majority of the hydrogen peroxide to hypochlorous acid. However, with a

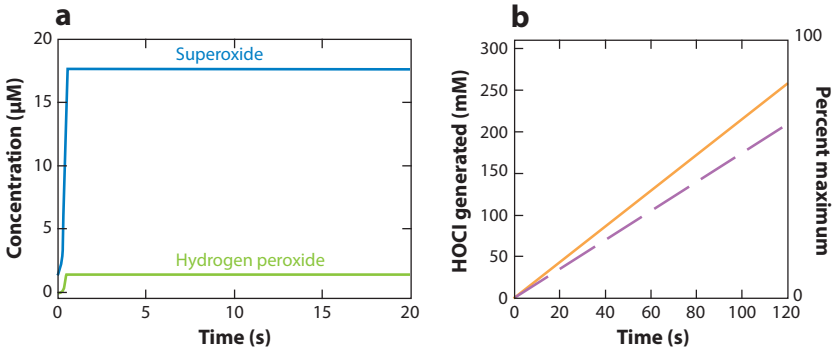

Figure 6

Simulations of (*a*) steady-state superoxide and hydrogen peroxide concentrations and (*b*) hypochlorous acid production in the neutrophil phagosome (41, 44). The solid line represents 70 mM and the dashed line represents 20 mM chloride.

lower chloride concentration, superoxide is able to compete for Compound I (**Figure 5**) and hypochlorous acid production declines (**Figure 6*b***).

Therefore, the ability of the neutrophil to keep generating hypochlorous acid in its phagosomes should depend on how rapidly chloride can be replenished. Painter & Wang (48) measured about 70 mM chloride in phagosomes using a chloride-sensitive probe attached to beads and showed that chloride could enter from the cytoplasm, in part through the cystic fibrosis transmembrane conductance regulator (49). However, MPO was inhibited, so they did not address whether influx could keep up with hypochlorous acid formation.

Although the modeling is useful, it gives a simplistic representation of the phagosomal environment as a homogeneous solution, whereas granule fusion occurs progressively at discrete sites and superoxide is generated on the membrane. Also, it is apparent that not all phagosomes express active oxidase. Li and coworkers (23) detected NOX2 activation on only about half of the phagosomes formed when neutrophils ingested zymosan–immunoglobulin G complexes (**Figure 2**), and we observed that a similar proportion of phagosomes gave a positive response to a fluorescent probe for hypochlorous acid (A. Albrett, unpublished observation). The implications of this heterogeneity are not understood. Also, modeling has been performed with kinetic data obtained at pH 7–7.8 and the effects of a higher phagosomal pH (15) need to be assessed.

There is substantial experimental evidence that hypochlorous acid is produced in neutrophil phagosomes (41, 50). This includes the detection of 3-chlorotyrosine in phagocytosed bacteria (51, 52) and in phagosomal proteins isolated after particle ingestion (53). MPO-dependent oxidation of Met residues in ingested *Escherichia coli* (54) and the chlorination of probes bound to phagocytosed particles (34, 45) have also been observed. Others have used real-time imaging with fluorescent probes to show localized hypochlorous acid production in individual phagosomes (24, 25). Recently, there has been an explosion in probe development, with nine new probes for hypochlorous acid reported in 2014 alone. In many cases, specificity and kinetic properties need further characterization, but they have the potential to further characterize oxidant activity.

A further conclusion from modeling phagosomal targets is that much of the hypochlorous acid would react with proteins close to the site of generation before reaching the microorganism (44). This conclusion is supported by experimental findings of more tyrosine chlorination in phagosomal proteins than proteins derived from ingested *Staphylococcus aureus* (51, 53). Based on reactivity (**Table 2**), chlorotyrosine would be a minor product, so this implies that Met and

cysteine (Cys) residues in neutrophil proteins would be more extensively oxidized, as well as amino groups, to give chloramines. Although hydroxyl radicals and singlet oxygen can theoretically be formed from hypochlorous acid and superoxide or hydrogen peroxide, these should not be favored due to competing reactions (44).

OXIDANTS AND ANTIMICROBIAL ACTIVITY

To fulfill their fundamental role, neutrophils must kill ingested microbes. This killing occurs within phagosomes, and it involves a battery of oxidative and nonoxidative antimicrobial mechanisms (50, 55, 56). Although not required for some organisms, oxidative killing is essential for broad spectrum antimicrobial defense. Evidence for this comes largely from the persistent infections associated with chronic granulomatous disease (CGD) (3, 57), a condition in which NOX2 activity is lacking (**Table 1**). Although defective killing cannot account for all of the problems associated with CGD, isolated neutrophils that lack NOX2 activity kill a wide range of microorganisms poorly, and this is a major contributor to the disease (56). The question is, which of the oxidants generated as a result of NOX2 and MPO activity have microbicidal activity? We discuss the evidence for the most likely contenders: superoxide, hydrogen peroxide, hypochlorous acid, and chloramines.

Hypochlorous Acid

Low concentrations of hypochlorous acid kill a wide spectrum of microorganisms, and as recently reviewed (41, 58), the killing of many species of bacteria by isolated neutrophils is strongly impaired when MPO is absent. Although this does not necessarily exclude other peroxidase-dependent mechanisms operating in the neutrophil, the general consensus is that the amount of hypochlorous acid generated is sufficient to kill the ingested microbes (45, 50, 58). Rosen and coworkers (54) found the extent of MPO-dependent Met oxidation in ingested *E. coli* to be similar to that in isolated bacteria killed with hypochlorous acid, and others have shown that bacterial killing declines in parallel with hypochlorous acid production when neutrophils are placed in a low-chloride environment (53, 59, 60).

However, there are some puzzling observations. Even though it seems counterintuitive, the results of modeling of the phagosome imply that much of the hypochlorous acid would react with proteins close to the site of generation before reaching the microorganism (44). Furthermore, measurements showing more extensive tyrosine chlorination in phagosomal proteins than in proteins from ingested *S. aureus* (51, 53) imply that the former are exposed to more hypochlorous acid. One possible explanation is that killing occurs indirectly via chloramines. Based on the reactivity of hypochlorous acid (**Figure 3**), the chlorination of tyrosine residues would be accompanied by substantially more oxidation of amino groups to chloramines. Although protein chloramines are not bactericidal, smaller chloramines, such as ammonia chloramine, that penetrate cells are cytotoxic (61). Another mechanism for increasing the effectiveness of hypochlorous acid would be for MPO to bind to the surface of the microbe. This occurs with some bacteria (62), and although only a small proportion of the total phagosomal MPO should be bound (41), it could direct some of the hypochlorous acid at its target.

For such a potent biocide, the mechanism of toxicity of hypochlorous acid is not fully defined. Early studies showed a loss of inner membrane-localized functions related to energy metabolism and the loss of ATP (50, 63, and references therein). Based on reactivity (**Table 2**), Met and Cys should be likely targets. The greater sensitivity to hypochlorous acid of *E. coli* that lack methionine sulfoxide reductases points to the importance of Met oxidation (54). Bacterial Cys-containing

peptides, including glutathione (GSH), bacillithiol, and mycothiol, form mixed disulfides and provide protection when the bacteria are treated with hypochlorous acid (64, 65), but specific thiol modifications associated with toxicity have not been identified.

Superoxide

Evidence that the other oxidants are effective microbicides is much more limited. Superoxide has limited reactivity, and the conventional view is to consider it merely as a precursor of hydrogen peroxide and other oxidants. However, this may not be correct. In 1975, Johnston and coworkers (66) observed that ingestion of SOD along with *E. coli* or *S. aureus* inhibited killing. Subsequently (67), a more modest effect with SOD bound to *S. aureus* was seen and attributed to superoxide-enhancing MPO activity. *Salmonella typhimurium*, which contains a periplasmic SOD and survives when ingested by macrophages, is more susceptible to being killed by these cells when its SOD is absent (68, 69). This could not be an effect of MPO (which was not present in the macrophages), and in view of the poor penetrance of superoxide into cells, toxicity has been proposed to be due to an attack on an unidentified membrane target. *S. typhimurium* does not survive in neutrophils, and this has been attributed to killing by MPO-derived hypochlorous acid (70).

A direct microbicidal effect of superoxide might be feasible in MPO-deficient neutrophils. Dismutation of superoxide in the phagosome will be much slower without MPO, and concentrations could reach 100 μM (44). However, no mechanism is obvious because known targets for superoxide, such as iron–sulfur proteins (71), are intracellular and not accessible. If nitric oxide were generated concurrently, it would be expected to react rapidly with superoxide to give peroxynitrite, which is both a strong oxidant and microbicidal (72). Nitric oxide production by neutrophils has been observed but, at least for humans, requires stimulation with proinflammatory cytokines, and it is still not clear whether both radicals are produced at the same time. If so, a microbicidal role for peroxynitrite is feasible, especially in MPO deficiency.

Hydrogen Peroxide

In sufficient concentrations, hydrogen peroxide is bactericidal. Toxicity involves reaction with intracellular iron–sulfur centers to release iron, and subsequent generation of hydroxyl and other reactive radical species via the Fenton reaction (71). Bacteria generate hydrogen peroxide endogenously (at an estimated rate of 10 μM/s in *E. coli*) but, through a combination of catalase and the peroxiredoxin AhpC, maintain an intracellular concentration of approximately 20 nM (73). These enzymes enable the bacteria to survive exposure of up to millimolar concentrations of hydrogen peroxide, and both enzymes must be knocked out to achieve sensitivity in the micromolar range (73). In addition, hydrogen peroxide reacts with the oxyR regulon in many bacterial species to upregulate the expression of antioxidant and repair proteins and to protect against toxicity. The activation of oxyR requires at least a 10-fold increase in intracellular hydrogen peroxide concentration, still well below the toxic range. The hydrogen peroxide in neutrophil phagosomes (predicted in **Figure 6*a*** to be micromolar) was sufficient to activate oxyR in ingested in *E. coli* (74). However, the deletion of oxyR had no impact on survival when *S. typhimurium* was ingested by macrophages (70), from which the authors concluded that uninduced bacterial defenses are adequate to handle phagosomal hydrogen peroxide without it reaching the toxic range. The implication for the neutrophil is that even in MPO deficiency, where modeling predicts a hydrogen peroxide concentration of approximately 30 μM (44), it still may be insufficient to kill.

Electrogenic Effects

Segal and coworkers (15, 75) have proposed an alternative mechanism for the role of NOX2 in microbial killing. They propose that it acts as transmembrane electron transporter to bring counter ions into the phagosome to facilitate nonoxidative killing, that superoxide production is incidental to oxygen acting as an electron acceptor, and the role of MPO is to break down the oxidants produced. Although this proposal does not accommodate the body of reported evidence for oxidant-mediated killing, the electrogenic effect could have consequences, such as providing a driving force for secondary transport into negatively charged vesicles (76, 77).

Overall Assessment

To sum up, hypochlorous acid is the fastest acting and most potent antimicrobial oxidant produced by neutrophils, and the bulk of evidence points to it being primarily responsible for oxidative killing. As discussed in more detail elsewhere (58), it has the credentials to be the frontline defender in the neutrophil armory. There remains a conundrum, however. Although neutrophils from individuals with MPO deficiency kill many microorganisms poorly, in contrast to CGD, MPO deficiency is relatively common and only rarely associated with serious infection (56, 58). It is possible that there are NOX2-dependent backup mechanisms that perform adequately under most circumstances. The evidence that these involve hydrogen peroxide or superoxide is not particularly strong, and their nature continues to be debated (15, 41, 50, 55, 75, 78). Alternatively, the more severe phenotype associated with CGD may be a combination of the microbicidal defect and the dysfunctional regulation of the inflammatory response. Further investigation is clearly needed.

BACTERIAL RESPONSES TO NEUTROPHIL OXIDANTS

Microorganisms do not surrender and accept their fate when ingested by neutrophils. Many pathogens have evolved mechanisms to combat or evade the neutrophil attack, some by targeting oxidative mechanisms. For example *Salmonella* secrete a protein that prevents the assembly and activation of NOX2 (79). Some bacteria export SOD into their periplasm, apparently as a survival mechanism (69, 80). It has also been proposed that the golden carotenoid of *S. aureus*, staphyloxanthin, is a virulence factor that acts as an oxidant scavenger (81), but this is in doubt because the virulence of a nonpigmented clone was not augmented when staphyloxanthin synthesis was introduced (82).

Many bacteria actively respond to protect themselves against oxidative injury. One strategy is to limit damage to existing constituents by upregulating genes for protective or repair enzymes. An example already discussed is the activation of the transcription factor oxyR by hydrogen peroxide (74). Sublethal hypochlorous acid and chloramines also induce changes in bacteria that enhance survival when exposed to higher doses. One well-characterized mechanism that has been observed in *E. coli* is activation of the chaperone protein HSP33 (83). HSP33 is a monomer containing four zinc coordinated Cys residues. These are readily oxidized to disulfides by hypochlorous acid, causing the release of zinc, unfolding, and dimerization to give an active holdase. HSP33 is then able to protect against the aggregation of other proteins that become oxidized at higher hypochlorous acid exposure (84).

Sublethal hypochlorous acid also activates transcription factors that upregulate the expression of survival genes and enable the bacteria to withstand higher doses (85). The broadly conserved transcriptional repressor NemR is selectively (although not exclusively) inactivated by

hypochlorous acid and chloramines. This occurs through the oxidation of a Cys residue, which is postulated to form a sulfenamide linkage with a neighboring lysine and, thus, induce a conformational change (86). The *E. coli* transcriptional regulators RclR and HypT are more specific for hypochlorous acid (87, 88). Cys oxidation is involved in RclR activation, whereas the sensitive sites in HypT appear to be Met residues. Although the nature of the protective mechanisms induced by hypochlorous acid still need to be identified and it is yet to be demonstrated whether these responses occur in phagocytosed bacteria, they could be useful markers for these species in the phagosome, as well as promising targets for antimicrobial therapy.

INTRACELLULAR OXIDANT PRODUCTION AND CELL SIGNALING

As described above, NOX2 is activated in neutrophils in response to soluble stimuli and at nonphagosomal intracellular sites where it is unlikely to be involved in antimicrobial activity. This lower-level activity is more typical of the NOX activity associated with receptor-mediated signaling pathways in other cells (8), and there is growing interest in NOX2-dependent signaling in neutrophils (7, 29). In other cells, activation generally takes place in endosomes, caveolae, or membrane invaginations, and although detailed mechanisms have not been well established, in many cases signal transmission involves the oxidation of selected thiol proteins in phosphorylation pathways. Signaling in the neutrophil is still relatively unexplored, with little known about the extent of NOX activation at different sites, which oxidants are produced, and how they react. However, there are a few well-validated examples where an intracellular signaling function seems likely.

One is associated with neutrophil migration down a gradient of the chemotactic peptide formyl-Met-Leu-Phe. This directional movement is associated with increased phosphorylation and the accumulation of phosphatidylinositol (3,4,5) triphosphate at the leading edge (89). It depends on colocalized activation of NOX2, and it has been proposed to involve oxidative inhibition of the phosphatase PTEN (90). Priming neutrophils to enhance their response to a full stimulus (**Table 1**) is another situation in which NOX2 is likely to act as a signal. Traditionally, priming has not been considered to activate the oxidase. However, Lamb et al. (26) have shown that endotoxin does cause low-level intracellular activation in a secretory vesicle–endosomal compartment. This has the hallmarks of a NOX-mediated redox signaling pathway, although downstream oxidative reactions have not been characterized. Neutrophil autophagy associated with antibacterial defense also appears to be redox-regulated through NOX2 activation (91, 92).

Neutrophil Extracellular Traps

The discovery of NET formation is the most intriguing feature of neutrophils to emerge in recent years. NETs consist of networks of nuclear chromatin and associated proteins, primarily of granule origin (93). They are expelled from the cells in response to various stimuli, including PMA, bacteria, and crystals of urate or cholesterol (94, 95). Although much of the evidence is still inferential, they are proposed to have a positive function in host defense by trapping and aiding in the removal of bacteria (96). However, NETs are implicated in an ever-increasing range of inflammatory conditions in which they are postulated to have deleterious effects (2, 97).

In spite of the intense interest, surprisingly little is known about the molecular processes leading to NET formation and release. With most in vitro stimuli, they are formed 2–4 hours after stimulation of the neutrophils. Morphological changes, histone deimination, elastase mobilization, and chromatin condensation precede NET release (98–100), and autophagy has been implicated (91, 101). With almost all stimuli, NOX2 activity is required; NETs are not formed in CGD neutrophils or when NOX2 is inhibited (102–104). However, it is not known which oxidants

are involved, where and when they are produced, or what they react with. Superoxide, hydrogen peroxide, singlet oxygen, and hypochlorous acid have all been implicated (102, 103, 105), but many of these conclusions have relied on adding large doses of oxidant, or nonspecific or inappropriate inhibitors (102). Even in well-performed studies, the effects of oxidant scavengers have been modest or equivocal. Studies using inhibitors and MPO-deficient neutrophils have shown a dependency on MPO with PMA and some bacterial species (100, 104–107) but not with others (104). Further studies are required to establish how oxidants mediate this important neutrophil activity. However, the limited impact of extracellular scavengers, plus the induction of NETs by agents that cause modest oxidant production (94), raises the possibility that the process involves signaling by intracellular, nonphagosomal NOX activity (102, 108).

Neutrophil Oxidants and Cell Death

Neutrophils in the bone marrow are exposed to survival factors that prevent them from undergoing apoptosis. Upon release into circulation, a spontaneous apoptosis program is activated, leading to the externalization of eat-me signals and removal by macrophages in the spleen and liver (109). This apoptosis is mediated through a conventional caspase-dependent pathway and occurs independently of NOX2 activation (110). In contrast, neutrophils at inflammatory sites are exposed to host and pathogen-derived factors that delay spontaneous apoptosis, enabling them to participate fully in the inflammatory process (109). The mode of death will influence the outcome of inflammation. Ingestion and clearance of the neutrophil before the discharge of intracellular contents are typically associated with an anti-inflammatory phenotype and the resolution of inflammation, whereas cell lysis will amplify the inflammatory response. Lysis may be valuable during severe infection, but will exacerbate tissue injury when the underlying infection is difficult to clear.

How neutrophils die following phagocytosis has not been well characterized, although NOX2 activation is involved (111). Neutrophils undergo a form of regulated cell death but not typical apoptosis. Morphological changes to the nucleus have been described as apoptotic (112), but this interpretation is complicated by the extensive intracellular rearrangements that occur as a consequence of ingesting large numbers of bacteria. Phosphatidylserine exposure is associated with subsequent uptake by macrophages and requires NOX2 activity (111). Hydrogen peroxide, as a bolus or generated continuously by glucose oxidase, is sufficient to trigger phosphatidylserine exposure in resting neutrophils (111). A potential mechanism is oxidation of the aminophospholipid translocase that transports phosphatidylserine to the inner leaflet of the plasma membrane to maintain asymmetry. This enzyme contains oxidant-sensitive Cys residues, modification of which inhibits activity (113). Alternatively, the initial drop in cytosolic pH caused by NOX2 activation could activate ion channels and a Na^+–H^+ exchanger in the phagosomal membrane. Activation of a Na^+–H^+ exchanger triggers phosphatidylserine exposure in platelets (114).

Oxidative inactivation of caspases has been observed following neutrophil phagocytosis (115), although there has been a contrary report (116). This suggests that caspase-independent cell death can occur (110). One possibility is that the cells undergo an alternative form of regulated cell death, such as necroptosis, which occurs only when caspases are inactivated (117). The relevance of this pathway to neutrophils is not certain, as one study showed no effect of a necroptosis inhibitor on cell death (101) and another showed inhibition (118). The high dose used in the latter study may have caused off-target effects. Alternatively, neutrophil death may be a passive necrotic process associated with inhibition of the enzymes responsible for maintaining membrane integrity. Further research is required to determine the mechanism of cell death in activated neutrophils and which oxidative reactions are involved.

REACTIONS OF RELEASED OXIDANTS

From a biochemical perspective, there are two main requirements for assessing the impact of neutrophil oxidants: specific biomarkers for detecting oxidant levels in clinical studies and characterization of the reactions that alter molecular or cellular function. Several biomarkers are available, the most specific to the neutrophil being products of hypochlorous acid (reviewed in 119). These include 3-chlorotyrosine and chlorohydrins formed from plasmalogens and other unsaturated lipids, which have the advantage of incorporating chlorine, and glutathione sulfonamide, a higher-oxidation product of GSH. Although these are only minor products of hypochlorous acid, they are detectable by sensitive mass spectrometry methods. These and other oxidative markers have been widely used to investigate numerous diseases. We will not try to cover this vast literature, but note that [as reviewed by Davies et al. (38)] elevated levels have frequently been seen and often correlate with disease severity. However, few studies have demonstrated a causal relationship.

In describing the reactions of neutrophil oxidants, we have focused on hypochlorous acid as the major oxidant that is characteristic of these cells. It should be noted that hypochlorous acid can be produced not only by activated neutrophils themselves but also from MPO that is released from the cells into plasma or inflammatory fluids as a result of NET formation, incomplete phagocytosis, or necrosis. For example, hypochlorous acid is produced when hydrogen peroxide is added to NETs and provides most of their bactericidal activity (120), and MPO is active when adhered to endothelium and in atherosclerotic lesions (121).

Almost every biological molecule that has been studied has been shown to react with hypochlorous acid (122, 123). Most of this evidence comes from studies with pure compounds and includes modification of DNA bases, membrane disruption, protein cross-linking, and inactivation of enzymes or ion channels through modification of Cys or Met residues. However, not every reaction that takes place in isolation will necessarily occur in a complex biological milieu. Relative reaction rates (**Figure 3**), abundance, and proximity to the site of production will determine the significant targets.

Modification of Extracellular Biomolecules

Reactions of hypochlorous acid with numerous components of body fluids or the extracellular matrix have been studied (122, 123). One of the first functional effects to be identified was inhibition of the antiprotease activity of the plasma protein α_1-antitrypsin through the oxidation of Met residues. α_2-Macroglobulin is also inhibited but gains chaperone activity for misfolded proteins (124). Low-density lipoprotein becomes recognizable by scavenger receptors (125); high-density lipoprotein becomes less effective at transporting cholesterol out of macrophages (126); extracellular matrix constituents become less cell-adhesive (127); the metalloproteinase responsible for cleaving von Willebrand factor, ADAMTS, is inactivated (128); and the prevalent neutrophil protein calprotectin loses metal-binding affinity (129). In most cases, Met or Cys oxidation has been characterized, and some of the proteins become cross-linked, but often the critical oxidative modifications have not been characterized. Further investigation is needed to assess which of these reactions takes place in more complex physiological environments.

Effects on Neighboring Cells

Many of the inflammatory effects of neutrophil oxidants result from their interactions with neighboring cells, with high oxidant exposure causing cytotoxicity and sublethal doses having more subtle effects. Within the vasculature, surrounding red blood cells can scavenge the hydrogen peroxide generated by adjacent neutrophils through a combination of catalase and peroxiredoxin

activity. The detection of red cell peroxiredoxin oxidation in an endotoxin-stressed mouse model is evidence that this occurs in vivo (130). It also implies that the hydrogen peroxide could, in theory, react with other cells in the vicinity. Sublethal hydrogen peroxide has been shown to regulate numerous cell signaling pathways, including stress responses, shifts in metabolic activity, and phosphorylation cascades that lead to the production of inflammatory mediators, proliferation, growth arrest, or cell death (reviewed in 131, 132). Little is known about whether neutrophil hydrogen peroxide initiates these changes in a physiological setting or whether its effects are indirectly mediated via MPO.

High concentrations of hypochlorous acid lyse cells. As recently reviewed (133), sublethal doses cause a variety of biochemical changes, including activation of signaling pathways that could exacerbate or dampen an inflammatory response. As expected, GSH and protein thiols are major cellular targets. GAPDH oxidation is one of the most sensitive changes and could be metabolically significant, as it would divert glucose utilization to the pentose phosphate pathway (134). Cell permeable chloramines exert similar effects to hypochlorous acid, and the effects of hypochlorous acid on cells in culture media are due mainly to chloramines generated from amines in the media (135). Signaling responses to both oxidants include activation of the Nrf2 stress response and MAP kinase pathways (136, 137). They also inhibit NF-κB activation by oxidizing a Met residue in IκB and preventing its degradation (138, 139), thereby inhibiting cellular expression of adhesion molecules and inflammatory cytokines (140, 141). Again, the physiological relevance of these changes during inflammation is yet to be established.

Oxidation of GSH and multiple thiol enzymes, including GAPDH, protein tyrosine phosphatases, and caspases, has been observed in cells treated with hypothiocyanite (133, 142, 143). The low cytotoxicity of hypothiocyanite has led to a proposal that a shift from hypochlorous acid to hypothiocyanite production by MPO could provide a protective advantage to exposed cells (144). However, it remains uncertain whether hypothiocyanite is pro- or anti-inflammatory (145). It is reduced by thioredoxin reductase, which could provide antioxidant protection (146), but it also upregulates inflammatory mediators via the MAP kinase and NF-κB pathways (147) and activates a caspase-independent form of cell death (142, 143).

INHIBITION OF NEUTROPHIL OXIDANT PRODUCTION

Although NOX2 activity and oxidant generation are essential for the microbicidal activity of neutrophils, limiting the effects of oxidants could be advantageous in situations of prolonged activation and inflammation. One way of accomplishing this would be to scavenge the oxidants with antioxidants. This strategy has been feasible in some experimental studies. However, it is seldom a practical therapeutic option because the scavenger concentrations required to outcompete physiological targets tend to be prohibitively high.

Another approach would be to inhibit NOX2. Inhibitors have been employed in numerous experimental studies, but the search for therapeutic agents still has to overcome major obstacles regarding specificity and potency (148). Diphenylene iodonium fully inhibits superoxide production by all NOX isoforms and has been the most widely used experimentally. A significant limitation is that it also inhibits other flavoproteins. Although its interaction with the heme groups in the NOXs give them a lower 50% inhibitory concentration ($IC_{50} = 1$ μM) than other flavoproteins (148, 149), low NOX selectivity and off-target effects make diphenylene iodonium an unsuitable starting point for drug development.

Apocynin inhibits the assembly of the NOX complex. It has been used in vitro as a NOX inhibitor and shown to have anti-inflammatory effects in several animal models (148). However,

mechanistic studies have indicated that it requires activation by MPO to inhibit NOX (150), and it is unclear whether NOX2 inhibition is responsible for its anti-inflammatory effects.

Several specific peptide inhibitors, including NOX2ds-tat, which blocks interaction between the gp91 and p47 subunits, have shown good specificity and potency for inhibiting superoxide production by NOX2. However, peptides are difficult candidates for drug development. Several high-throughput screens of nonpeptide drug libraries have produced promising hits for general NOX inhibition but, as yet, no specific NOX2 inhibitor (148).

Although the potential for treating inflammatory diseases justifies the continued search for NOX2 inhibitors, there are potential risks of increased susceptibility to infection and proinflammatory phenotypes, as has been seen in humans and mice lacking NOX2 activity. For a more focused approach, there are advantages in targeting MPO. This would impact mainly on neutrophils (and monocytic cells) and limit production of their strongest oxidants, such as hypochlorous acid. Numerous compounds that inhibit MPO have been identified, but no drugs are available.

MPO inhibitors fall into four classes: competitive substrates, suicide substrates, those that bind tightly to the ferric enzyme, and those that convert the enzyme to an inactive redox intermediate (125). The fourth class is less effective because endogenous substrates, including superoxide, recycle the enzyme back to its active form. Acetaminophen at pharmacological doses outcompetes chloride for oxidation and inhibits in this way (151). It is oxidized by a radical mechanism to aminoquinine, which is potentially undesirable, but the normally safe profile of acetaminophen suggests that it could be useful during acute inflammation.

2-Thioxanthines and related compounds are suicide substrates for MPO (152). Their oxidation products covalently bind to its heme groups so that it cannot oxidize chloride. They have clinical potential because they undergo limited oxidation to radicals, but their potency is limited because they must compete with endogenous substrates for oxidation.

Compounds that stop MPO reacting with hydrogen peroxide by binding tightly to the active sites have the greatest potential as inhibitors of the enzyme because they do not need to compete against high concentrations of endogenous substrates, and they are not metabolized to radical species. Aromatic hydroxamates bind extremely tightly but, unfortunately, are unstable under physiological conditions (153). However, exploring this strategy has promise for developing drugs that block the unwanted action of MPO-derived oxidants.

CONCLUSIONS AND FUTURE DIRECTIONS

The enzymatic activities of the NOX2 and MPO systems have been well characterized, and we have a good understanding of which reactive oxidants they are capable of producing. These enzymatic activities are important for neutrophils to kill microorganisms efficiently, and it has become appreciated that they have a range of other cellular functions. However, a lot of phenomenology surrounds the oxidative mechanisms involved in these processes. To gain greater mechanistic understanding, more biochemical studies are needed that identify the specific oxidants produced under different conditions and at different sites and to characterize their molecular targets. A number of questions need to be addressed. There is still uncertainty about the oxidative mechanisms in the phagosome that are responsible for microbicidal activity in the presence and absence of MPO, the role of reactive chlorine species, and whether ingested bacteria sense and upregulate defenses against these reactive oxidants. Regarding the signaling functions of NOX2, there is little or no information on which oxidants are responsible and the nature of their interactions with the regulatory pathways. Finally, neutrophil oxidants modify numerous biomolecules and cellular functions, but we need to know which of these occur physiologically and contribute to the pathology of inflammation.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENT

The work of the authors on this topic has been supported by the Health Research Council of New Zealand.

LITERATURE CITED

1. Nathan C. 2006. Neutrophils and immunity: challenges and opportunities. *Nat. Rev. Immunol.* 6:173–82
2. Kolaczkowska E, Kubes P. 2013. Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* 13:159–75
3. Rigby KM, DeLeo FR. 2012. Neutrophils in innate host defense against *Staphylococcus aureus* infections. *Semin. Immunopathol.* 34:237–59
4. Nauseef WM, Borregaard N. 2014. Neutrophils at work. *Nat. Immunol.* 15:602–11
5. Tecchio C, Micheletti A, Cassatella MA. 2014. Neutrophil-derived cytokines: facts beyond expression. *Front. Immunol.* 5:508
6. Decoursey TE, Ligeti E. 2005. Regulation and termination of NADPH oxidase activity. *Cell. Mol. Life Sci.* 62:2173–93
7. Nunes P, Demaurex N, Dinauer MC. 2013. Regulation of the NADPH oxidase and associated ion fluxes during phagocytosis. *Traffic* 14:1118–31
8. Lambeth JD, Neish AS. 2014. Nox enzymes and new thinking on reactive oxygen: a double-edged sword revisited. *Annu. Rev. Pathol. Mech. Dis.* 9:119–45
9. Leto TL, Morand S, Hurt D, Ueyama T. 2009. Targeting and regulation of reactive oxygen species generation by Nox family NADPH oxidases. *Antioxid. Redox Signal.* 11:2607–19
10. Nauseef WM. 2008. Nox enzymes in immune cells. *Semin. Immunopathol.* 30:195–208
11. Cross AR, Segal AW. 2004. The NADPH oxidase of professional phagocytes—prototype of the NOX electron transport chain systems. *Biochim. Biophys. Acta* 1657:1–22
12. Gabig TG, Bearman SI, Babior BM. 1979. Effects of oxygen tension and pH on the respiratory burst of human neutrophils. *Blood* 53:1133–39
13. Baehner RL, Johnston RB Jr., Nathan DG. 1972. Comparative study of the metabolic and bactericidal characteristics of severely glucose-6-phosphate dehydrogenase-deficient polymorphonuclear leukocytes and leukocytes from children with chronic granulomatous disease. *J. Reticuloendothel. Soc.* 12:150–69
14. DeCoursey TE. 2010. Voltage-gated proton channels find their dream job managing the respiratory burst in phagocytes. *Physiology* 25:27–40
15. Levine AP, Duchen MR, de Villiers S, Rich PR, Segal AW. 2015. Alkalinity of neutrophil phagocytic vacuoles is modulated by HVCN1 and has consequences for myeloperoxidase activity. *PLOS ONE* 10:e0125906
16. Nauseef WM. 2014. Detection of superoxide anion and hydrogen peroxide production by cellular NADPH oxidases. *Biochim. Biophys. Acta* 1840:757–67
17. Kobayashi T, Robinson JM, Seguchi H. 1998. Identification of intracellular sites of superoxide production in stimulated neutrophils. *J. Cell Sci.* 111:81–91
18. Robinson JM. 2008. Reactive oxygen species in phagocytic leukocytes. *Histochem. Cell Biol.* 130:281–97
19. Winterbourn CC. 2014. The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells. *Biochim. Biophys. Acta* 1840:730–38
20. Kalyanaraman B, Darley-Usmar V, Davies KJ, Dennery PA, Forman HJ, et al. 2012. Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations. *Free Radic. Biol. Med.* 52:1–6

21. Bjorkman L, Dahlgren C, Karlsson A, Brown KL, Bylund J. 2008. Phagocyte-derived reactive oxygen species as suppressors of inflammatory disease. *Arthritis Rheumatol.* 58:2931–35
22. Bylund J, Brown KL, Movitz C, Dahlgren C, Karlsson A. 2010. Intracellular generation of superoxide by the phagocyte NADPH oxidase: how, where, and what for? *Free Radic. Biol. Med.* 49:1834–45
23. Li XJ, Tian W, Stull ND, Grinstein S, Atkinson S, et al. 2009. A fluorescently tagged C-terminal fragment of $p47^{phox}$ detects NADPH oxidase dynamics during phagocytosis. *Mol. Biol. Cell* 20:1520–32
24. Kenmoku S, Urano Y, Kojima H, Nagano T. 2007. Development of a highly specific rhodamine-based fluorescence probe for hypochlorous acid and its application to real-time imaging of phagocytosis. *J. Am. Chem. Soc.* 129:7313–18
25. Chen X, Lee KA, Ha EM, Lee KM, Seo YY, et al. 2011. A specific and sensitive method for detection of hypochlorous acid for the imaging of microbe-induced HOCl production. *Chem. Commun.* 47:4373–75
26. Lamb FS, Hook JS, Hilkin BM, Huber JN, Volk AP, et al. 2012. Endotoxin priming of neutrophils requires endocytosis and NADPH oxidase-dependent endosomal reactive oxygen species. *J. Biol. Chem.* 287:12395–404
27. Anderson KE, Chessa TA, Davidson K, Henderson RB, Walker S, et al. 2010. PtdIns3P and Rac direct the assembly of the NADPH oxidase on a novel, pre-phagosomal compartment during FcR-mediated phagocytosis in primary mouse neutrophils. *Blood* 116:4978–89
28. Lundqvist H, Follin P, Khalfan L, Dahlgren C. 1996. Phorbol myristate acetate-induced NADPH oxidase activity in human neutrophils: only half the story has been told. *J. Leukoc. Biol.* 59:270–79
29. Dupre-Crochet S, Erard M, Nubetae O. 2013. ROS production in phagocytes: why, when, and where? *J. Leukoc. Biol.* 94:657–70
30. Matute JD, Arias AA, Wright NA, Wrobel I, Waterhouse CC, et al. 2009. A new genetic subgroup of chronic granulomatous disease with autosomal recessive mutations in $p40^{phox}$ and selective defects in neutrophil NADPH oxidase activity. *Blood* 114:3309–15
31. Karlsson A, Nixon JB, McPhail LC. 2000. Phorbol myristate acetate induces neutrophil NADPH-oxidase activity by two separate signal transduction pathways: dependent or independent of phosphatidylinositol 3-kinase. *J. Leukoc. Biol.* 67:396–404
32. Granfeldt D, Dahlgren C. 2001. An intact cytoskeleton is required for prolonged respiratory burst activity during neutrophil phagocytosis. *Inflammation* 25:165–69
33. Li Q, Spencer NY, Oakley FD, Buettner GR, Engelhardt JF. 2009. Endosomal Nox2 facilitates redox-dependent induction of NF-κB by TNF-α. *Antioxid. Redox Signal.* 11:1249–63
34. Tlili A, Dupre-Crochet S, Erard M, Nusse O. 2011. Kinetic analysis of phagosomal production of reactive oxygen species. *Free Radic. Biol. Med.* 50:438–47
35. Arnhold J, Furtmuller PG, Regelsberger G, Obinger C. 2001. Redox properties of the couple compound I/native enzyme of myeloperoxidase and eosinophil peroxidase. *Eur. J. Biochem.* 268:5142–48
36. van Dalen CJ, Whitehouse MW, Winterbourn CC, Kettle AJ. 1997. Thiocyanate and chloride as competing substrates for myeloperoxidase. *Biochem. J.* 327:487–92
37. Kettle AJ, Winterbourn CC. 1997. Myeloperoxidase: a key regulator of neutrophil oxidant production. *Redox Rep.* 3:3–15
38. Davies MJ, Hawkins CL, Pattison DI, Rees MD. 2008. Mammalian heme peroxidases: from molecular mechanisms to health implications. *Antioxid. Redox Signal.* 10:1199–234
39. O'Brien PJ. 1988. Radical formation during the peroxidase catalysed metabolism of carcinogens and xenobiotics: the reactivity of these radicals with GSH, DNA, and unsaturated lipid. *Free Radic. Biol. Med.* 4:169–84
40. Jiang X, Khursigara G, Rubin RL. 1994. Transformation of lupus-inducing drugs to cytotoxic products by activated neutrophils. *Science* 266:810–13
41. Winterbourn CC, Kettle AJ. 2013. Redox reactions and microbial killing in the neutrophil phagosome. *Antioxid. Redox Signal.* 18:642–60
42. Kettle AJ, Anderson RF, Hampton MB, Winterbourn CC. 2007. Reactions of superoxide with myeloperoxidase. *Biochemistry* 46:4888–97
43. Meotti FC, Jameson GN, Turner R, Harwood DT, Stockwell S, et al. 2011. Urate as a physiological substrate for myeloperoxidase: implications for hyperuricemia and inflammation. *J. Biol. Chem.* 286:12901–11

44. Winterbourn CC, Hampton MB, Livesey JH, Kettle AJ. 2006. Modeling the reactions of superoxide and myeloperoxidase in the neutrophil phagosome: implications for microbial killing. *J. Biol. Chem.* 281:39860–69
45. Jiang Q, Hurst JK. 1997. Relative chlorinating, nitrating, and oxidizing capabilities of neutrophils determined with phagocytosable probes. *J. Biol. Chem.* 272:32767–72
46. Quinn MT, Linner JG, Siemsen D, Dratz EA, Buescher ES, et al. 1995. Immunocytochemical detection of lipid peroxidation in phagosomes of human neutrophils: correlation with expression of flavocytochrome *b*. *J. Leukoc. Biol.* 57:415–21
47. Wilkie-Grantham RP, Magon NJ, Harwood DT, Kettle AJ, Vissers MC, et al. 2015. Myeloperoxidase-dependent lipid peroxidation promotes the oxidative modification of cytosolic proteins in phagocytic neutrophils. *J. Biol. Chem.* 290:9896–905
48. Painter RG, Wang G. 2006. Direct measurement of free chloride concentrations in the phagolysosomes of human neutrophils. *Anal. Chem.* 78:3133–37
49. Painter RG, Marrero L, Lombard GA, Valentine VG, Nauseef WM, et al. 2010. CFTR-mediated halide transport in phagosomes of human neutrophils. *J. Leukoc. Biol.* 87:933–42
50. Hurst JK. 2012. What really happens in the neutrophil phagosome? *Free Radic. Biol. Med.* 53:508–20
51. Chapman ALP, Hampton MB, Senthilmohan R, Winterbourn CC, Kettle AJ. 2002. Chlorination of bacterial and neutrophil proteins during phagocytosis and killing of *Staphylococcus aureus*. *J. Biol. Chem.* 277:9757–62
52. Painter RG, Valentine VG, Lanson NA Jr., Leidal K, Zhang Q, et al. 2006. CFTR expression in human neutrophils and the phagolysosomal chlorination defect in cystic fibrosis. *Biochemistry* 45:10260–69
53. Green JN, Kettle AJ, Winterbourn CC. 2014. Protein chlorination in neutrophil phagosomes and correlation with bacterial killing. *Free Radic. Biol. Med.* 77:49–56
54. Rosen H, Klebanoff SJ, Wang Y, Brot N, Heinecke JW, et al. 2009. Methionine oxidation contributes to bacterial killing by the myeloperoxidase system of neutrophils. *PNAS* 106:18686–91
55. Nauseef WM. 2007. How human neutrophils kill and degrade microbes: an integrated view. *Immunol. Rev.* 219:88–102
56. Klebanoff SJ. 2005. Myeloperoxidase: friend and foe. *J. Leukoc. Biol.* 77:598–625
57. Segal BH, Leto TL, Gallin JI, Malech HL, Holland SM. 2000. Genetic, biochemical, and clinical features of chronic granulomatous disease. *Medicine* 79:170–200
58. Klebanoff SJ, Kettle AJ, Rosen H, Winterbourn CC, Nauseef WM. 2013. Myeloperoxidase: a front-line defender against phagocytosed microorganisms. *J. Leukoc. Biol.* 93:185–98
59. Painter RG, Bonvillain RW, Valentine VG, Lombard GA, LaPlace SG, et al. 2008. The role of chloride anion and CFTR in killing of *Pseudomonas aeruginosa* by normal and CF neutrophils. *J. Leukoc. Biol.* 83:1345–53
60. Ng HP, Zhou Y, Song K, Hodges CA, Drumm ML, et al. 2014. Neutrophil-mediated phagocytic host defense defect in myeloid Cftr-inactivated mice. *PLOS ONE* 9:e106813
61. Grisham MB, Jefferson MM, Melton DF, Thomas EL. 1984. Chlorination of endogenous amines by isolated neutrophils: ammonia-dependent bactericidal, cytotoxic, and cytolytic activities of the chloramines. *J. Biol. Chem.* 259:10404–13
62. Allen RC, Stephens JT Jr. 2011. Myeloperoxidase selectively binds and selectively kills microbes. *Infect. Immun.* 79:474–85
63. Barrette WCJ, Hannum DM, Wheeler WD, Hurst JC. 1989. General mechanism for the bacterial toxicity of hypochlorous acid: abolition of ATP production. *Biochemistry* 28:9172–78
64. Loi VV, Rossius M, Antelmann H. 2015. Redox regulation by reversible protein *S*-thiolation in bacteria. *Front. Microbiol.* 6:187
65. Chi BK, Roberts AA, Huyen TT, Basell K, Becher D, et al. 2013. *S*-bacillithiolation protects conserved and essential proteins against hypochlorite stress in *Firmicutes* bacteria. *Antioxid. Redox Signal.* 18:1273–95
66. Johnston RBJ, Keele BBJ, Misra HP, Lehmeyer JE, Webb LS, et al. 1975. The role of superoxide anion generation in phagocytic bactericidal activity: studies with normal and chronic granulomatous disease leukocytes. *J. Clin. Investig.* 55:1357–72
67. Hampton MB, Kettle AJ, Winterbourn CC. 1996. Involvement of superoxide and myeloperoxidase in oxygen-dependent killing of *Staphylococcus aureus* by neutrophils. *Infect. Immun.* 64:3512–17

68. De Groote MA, Ochsner UA, Shiloh MU, Nathan C, McCord JM, et al. 1997. Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH-oxidase and nitric oxide synthase. *PNAS* 94:13997–4001
69. Craig M, Slauch JM. 2009. Phagocytic superoxide specifically damages an extracytoplasmic target to inhibit or kill *Salmonella*. *PLOS ONE* 4:e4975
70. Burton NA, Schurmann N, Casse O, Steeb AK, Claudi B, et al. 2014. Disparate impact of oxidative host defenses determines the fate of *Salmonella* during systemic infection in mice. *Cell Host Microbe* 15:72–83
71. Imlay JA. 2003. Pathways of oxidative damage. *Annu. Rev. Microbiol.* 57:395–418
72. Fang FC. 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat. Rev. Microbiol.* 2:820–32
73. Mishra S, Imlay J. 2012. Why do bacteria use so many enzymes to scavenge hydrogen peroxide? *Arch. Biochem. Biophys.* 525:145–60
74. Staudinger BJ, Oberdoerster MA, Lewis PJ, Rosen H. 2002. mRNA expression profiles for *Escherichia coli* ingested by normal and phagocyte oxidase-deficient human neutrophils. *J. Clin. Investig.* 110:1151–63
75. Segal AW. 2005. How neutrophils kill microbes. *Annu. Rev. Immunol.* 23:197–223
76. Rada BK, Geiszt M, Kaldi K, Timar C, Ligeti E. 2004. Dual role of phagocytic NADPH oxidase in bacterial killing. *Blood* 104:2947–53
77. Lamb FS, Moreland JG, Miller FJ Jr. 2009. Electrophysiology of reactive oxygen production in signaling endosomes. *Antioxid. Redox Signal.* 11:1335–47
78. Slauch JM. 2011. How does the oxidative burst of macrophages kill bacteria? Still an open question. *Mol. Microbiol.* 80:580–83
79. Vazquez-Torres A, Xu Y, Jones-Carson J, Holden DW, Lucia SM, et al. 2000. *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science* 287:1655–58
80. Beaman BL, Black CM, Doughty F, Beaman L. 1985. Role of superoxide dismutase and catalase as determinants of pathogenicity of *Nocardia asteroides*: importance in resistance to microbicidal activities of human polymorphonuclear neutrophils. *Infect. Immun.* 47:135–41
81. Liu CI, Liu GY, Song Y, Yin F, Hensler ME, et al. 2008. A cholesterol biosynthesis inhibitor blocks *Staphylococcus aureus* virulence. *Science* 319:1391–94
82. Tong SY, Sharma-Kuinkel BK, Thaden JT, Whitney AR, Yang SJ, et al. 2013. Virulence of endemic nonpigmented northern Australian *Staphylococcus aureus* clone (clonal complex 75, *S. argenteus*) is not augmented by staphyloxanthin. *J. Infect. Dis.* 208:520–27
83. Jakob U, Muse W, Eser M, Bardwell JC. 1999. Chaperone activity with a redox switch. *Cell* 96:341–52
84. Winter J, Ilbert M, Graf PC, Ozcelik D, Jakob U. 2008. Bleach activates a redox-regulated chaperone by oxidative protein unfolding. *Cell* 135:691–701
85. Gray MJ, Wholey WY, Jakob U. 2013. Bacterial responses to reactive chlorine species. *Annu. Rev. Microbiol.* 67:141–60
86. Gray MJ, Li Y, Leichert LI, Xu Z, Jakob U. 2015. Does the transcription factor NemR use a regulatory sulfenamide bond to sense bleach? *Antioxid. Redox Signal.* 23:747–54
87. Parker BW, Schwessinger EA, Jakob U, Gray MJ. 2013. The RclR protein is a reactive chlorine-specific transcription factor in *Escherichia coli*. *J. Biol. Chem.* 288:32574–84
88. Drazic A, Miura H, Peschek J, Le Y, Bach NC, et al. 2013. Methionine oxidation activates a transcription factor in response to oxidative stress. *PNAS* 110:9493–98
89. Mazaki Y, Hashimoto S, Tsujimura T, Morishige M, Hashimoto A, et al. 2006. Neutrophil direction sensing and superoxide production linked by the GTPase-activating protein GIT2. *Nat. Immunol.* 7:724–31
90. Kuiper JW, Sun C, Magalhaes MA, Glogauer M. 2011. Rac regulates $PtdInsP_3$ signaling and the chemotactic compass through a redox-mediated feedback loop. *Blood* 118:6164–71
91. Chargui A, El May MV. 2014. Autophagy mediates neutrophil responses to bacterial infection. *APMIS* 122:1047–58
92. Huang J, Canadien V, Lam GY, Steinberg BE, Dinauer MC, et al. 2009. Activation of antibacterial autophagy by NADPH oxidases. *PNAS* 106:6226–31

93. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, et al. 2004. Neutrophil extracellular traps kill bacteria. *Science* 303:1532–35
94. Guimaraes-Costa AB, Nascimento MT, Wardini AB, Pinto-da-Silva LH, Saraiva EM. 2012. ETosis: a microbicidal mechanism beyond cell death. *J. Parasitol. Res.* 2012:929743
95. Urban C, Zychlinsky A. 2007. Netting bacteria in sepsis. *Nat. Med.* 13:403–4
96. Brinkmann V, Zychlinsky A. 2007. Beneficial suicide: why neutrophils die to make NETs. *Nat. Rev. Microbiol.* 5:577–82
97. Cools-Lartigue J, Spicer J, Najmeh S, Ferri L. 2014. Neutrophil extracellular traps in cancer progression. *Cell. Mol. Life Sci.* 71:4179–94
98. Brinkmann V, Zychlinsky A. 2012. Neutrophil extracellular traps: Is immunity the second function of chromatin? *J. Cell Biol.* 198:773–83
99. Neeli I, Khan SN, Radic M. 2008. Histone deimination as a response to inflammatory stimuli in neutrophils. *J. Immunol.* 180:1895–902
100. Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. 2010. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J. Cell Biol.* 191:677–91
101. Remijsen Q, Vanden Berghe T, Wirawan E, Asselbergh B, Parthoens E, et al. 2011. Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Res.* 21:290–304
102. Parker H, Winterbourn CC. 2013. Reactive oxidants and myeloperoxidase and their involvement in neutrophil extracellular traps. *Front. Immunol.* 3:424
103. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, et al. 2007. Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* 176:231–41
104. Parker H, Dragunow M, Hampton MB, Kettle AJ, Winterbourn CC. 2012. Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. *J. Leukoc. Biol.* 92:841–49
105. Palmer LJ, Cooper PR, Ling MR, Wright HJ, Huissoon A, et al. 2012. Hypochlorous acid regulates neutrophil extracellular trap release in humans. *Clin. Exp. Immunol.* 167:261–68
106. Metzler KD, Fuchs TA, Nauseef WM, Reumaux D, Roesler J, et al. 2011. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood* 117:953–59
107. Metzler KD, Goosmann C, Lubojemska A, Zychlinsky A, Papayannopoulos V. 2014. A myeloperoxidase-containing complex regulates neutrophil elastase release and actin dynamics during NETosis. *Cell Rep.* 8:883–96
108. Bjornsdottir H, Welin A, Michaelsson E, Osla V, Berg S, et al. 2015. Neutrophil NET formation is regulated from the inside by myeloperoxidase-processed reactive oxygen species. *Free Radic. Biol. Med.* 89:1024–35
109. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, et al. 2010. Neutrophil kinetics in health and disease. *Trends Immunol.* 31:318–24
110. Fadeel B, Åhlin A, Henter J, Orrenius S, Hampton MB. 1998. Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species. *Blood* 92:4808–18
111. Hampton MB, Keenan JI, Vissers MCM, Winterbourn CC. 2002. Oxidant-mediated phosphatidylserine exposure and macrophage uptake of activated neutrophils: possible impairment in chronic granulomatous disease. *J. Leukoc. Biol.* 71:775–81
112. Watson RW, Redmond HP, Wang JH, Condron C, Bouchier-Hayes D. 1996. Neutrophils undergo apoptosis following ingestion of *Escherichia coli*. *J. Immunol.* 156:3986–92
113. Tyurina YY, Basova LV, Konduru NV, Tyurin VA, Potapovich AI, et al. 2007. Nitrosative stress inhibits the aminophospholipid translocase resulting in phosphatidylserine externalization and macrophage engulfment: implications for the resolution of inflammation. *J. Biol. Chem.* 282:8498–509
114. Bucki R, Pastore JJ, Giraud F, Janmey PA, Sulpice JC. 2006. Involvement of the Na^+/H^+ exchanger in membrane phosphatidylserine exposure during human platelet activation. *Biochim. Biophys. Acta* 1761:195–204
115. Wilkie RP, Vissers MC, Dragunow M, Hampton MB. 2007. A functional NADPH oxidase prevents caspase involvement in the clearance of phagocytic neutrophils. *Infect. Immun.* 75:3256–63

116. Zhang B, Hirahashi J, Cullere X, Mayadas TN. 2003. Elucidation of molecular events leading to neutrophil apoptosis following phagocytosis: cross-talk between caspase 8, reactive oxygen species, and MAPK/ERK activation. *J. Biol. Chem.* 278:28443–54
117. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. 2010. Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat. Rev. Mol. Cell Biol.* 11:700–14
118. Greenlee-Wacker MC, Rigby KM, Kobayashi SD, Porter AR, DeLeo FR, et al. 2014. Phagocytosis of *Staphylococcus aureus* by human neutrophils prevents macrophage efferocytosis and induces programmed necrosis. *J. Immunol.* 192:4709–17
119. Kettle AJ, Albrett AM, Chapman AL, Dickerhof N, Forbes LV, et al. 2014. Measuring chlorine bleach in biology and medicine. *Biochim. Biophys. Acta* 1840:781–93
120. Parker H, Albrett AM, Kettle AJ, Winterbourn CC. 2012. Myeloperoxidase associated with neutrophil extracellular traps is active and mediates bacterial killing in the presence of hydrogen peroxide. *J. Leukoc. Biol.* 91:369–76
121. Lau D, Baldus S. 2006. Myeloperoxidase and its contributory role in inflammatory vascular disease. *Pharmacol. Ther.* 111:16–26
122. Pattison DI, Davies MJ. 2006. Reactions of myeloperoxidase-derived oxidants with biological substrates: gaining chemical insight into human inflammatory diseases. *Curr. Med. Chem.* 13:3271–90
123. Pattison DI, Davies MJ, Hawkins CL. 2012. Reactions and reactivity of myeloperoxidase-derived oxidants: differential biological effects of hypochlorous and hypothiocyanous acids. *Free Radic. Res.* 46:975–95
124. Wyatt AR, Kumita JR, Mifsud RW, Gooden CA, Wilson MR, et al. 2014. Hypochlorite-induced structural modifications enhance the chaperone activity of human α_2-macroglobulin. *PNAS* 111:E2081–90
125. Malle E, Marsche G, Arnhold J, Davies MJ. 2006. Modification of low-density lipoprotein by myeloperoxidase-derived oxidants and reagent hypochlorous acid. *Biochim. Biophys. Acta* 1761:392–415
126. Shao B, Tang C, Heinecke JW, Oram JF. 2010. Oxidation of apolipoprotein A-I by myeloperoxidase impairs the initial interactions with ABCA1 required for signaling and cholesterol export. *J. Lipid Res.* 51:1849–58
127. Vissers MCM, Thomas C. 1997. Hypochlorous acid disrupts the adhesive properties of subendothelial matrix. *Free Radic. Biol. Med.* 23:401–11
128. Wang Y, Chen J, Ling M, Lopez JA, Chung DW, et al. 2015. Hypochlorous acid generated by neutrophils inactivates ADAMTS13: an oxidative mechanism for regulating ADAMTS13 proteolytic activity during inflammation. *J. Biol. Chem.* 290:1422–31
129. Magon NJ, Turner R, Gearry RB, Hampton MB, Sly PD, et al. 2015. Oxidation of calprotectin by hypochlorous acid prevents chelation of essential metal ions and allows bacterial growth: relevance to infections in cystic fibrosis. *Free Radic. Biol. Med.* 86:133–44
130. Bayer SB, Maghzal G, Stocker R, Hampton MB, Winterbourn CC. 2013. Neutrophil-mediated oxidation of erythrocyte peroxiredoxin 2 as a potential marker of oxidative stress in inflammation. *FASEB J.* 27:3315–22
131. Holmstrom KM, Finkel T. 2014. Cellular mechanisms and physiological consequences of redox-dependent signalling. *Nat. Rev. Mol. Cell Biol.* 15:411–21
132. Winterbourn CC. 2013. The biological chemistry of hydrogen peroxide. *Methods Enzymol.* 528:3–25
133. Rayner BS, Love DT, Hawkins CL. 2014. Comparative reactivity of myeloperoxidase-derived oxidants with mammalian cells. *Free Radic. Biol. Med.* 71:240–55
134. Dick TP, Ralser M. 2015. Metabolic remodeling in times of stress: Who shoots faster than his shadow? *Mol. Cell* 59:519–21
135. Peskin AV, Midwinter RG, Harwood DT, Winterbourn CC. 2005. Chlorine transfer between glycine, taurine, and histamine: reaction rates and impact on cellular reactivity. *Free Radic. Biol. Med.* 38:397–405
136. Pi J, Zhang Q, Woods CG, Wong V, Collins S, et al. 2008. Activation of Nrf2-mediated oxidative stress response in macrophages by hypochlorous acid. *Toxicol. Appl. Pharmacol.* 226:236–43

137. Midwinter RG, Peskin AV, Vissers MCM, Winterbourn CC. 2004. Extracellular oxidation by taurine chloramine activates ERK via the epidermal growth factor receptor. *J. Biol. Chem.* 279:32205–11
138. Kanayama A, Inoue J, Sugita-Konishi Y, Shimizu M, Miyamoto Y. 2002. Oxidation of IκBα at methionine 45 is one cause of taurine chloramine-induced inhibition of NF-κB activation. *J. Biol. Chem.* 277:24049–56
139. Midwinter RG, Cheah FC, Moskovitz J, Vissers MC, Winterbourn CC. 2006. IκB is a sensitive target for oxidation by cell-permeable chloramines: inhibition of NF-κB activity by glycine chloramine through methionine oxidation. *Biochem. J.* 396:71–78
140. Park E, Jia J, Quinn MR, Schuller-Levis G. 2002. Taurine chloramine inhibits lymphocyte proliferation and decreases cytokine production in activated human leukocytes. *Clin. Immunol.* 102:179–84
141. Chorazy M, Kontny E, Marcinkiewicz J, Maslinski W. 2002. Taurine chloramine modulates cytokine production by human peripheral blood mononuclear cells. *Amino Acids* 23:407–13
142. Lloyd MM, Grima MA, Rayner BS, Hadfield KA, Davies MJ, et al. 2013. Comparative reactivity of the myeloperoxidase-derived oxidants hypochlorous acid and hypothiocyanous acid with human coronary artery endothelial cells. *Free Radic. Biol. Med.* 65:1352–62
143. Bozonet SM, Scott-Thomas AP, Nagy P, Vissers MC. 2010. Hypothiocyanous acid is a potent inhibitor of apoptosis and caspase 3 activation in endothelial cells. *Free Radic. Biol. Med.* 49:1054–63
144. Chandler JD, Day BJ. 2015. Biochemical mechanisms and therapeutic potential of pseudohalide thiocyanate in human health. *Free Radic. Res.* 49:695–710
145. Barrett TJ, Hawkins CL. 2012. Hypothiocyanous acid: benign or deadly? *Chem. Res. Toxicol.* 25:263–73
146. Chandler JD, Nichols DP, Nick JA, Hondal RJ, Day BJ. 2013. Selective metabolism of hypothiocyanous acid by mammalian thioredoxin reductase promotes lung innate immunity and antioxidant defense. *J. Biol. Chem.* 288:18421–28
147. Wang JG, Mahmud SA, Nguyen J, Slungaard A. 2006. Thiocyanate-dependent induction of endothelial cell adhesion molecule expression by phagocyte peroxidases: a novel HOSCN-specific oxidant mechanism to amplify inflammation. *J. Immunol.* 177:8714–22
148. Diebold BA, Smith SM, Li Y, Lambeth JD. 2015. NOX2 as a target for drug development: indications, possible complications, and progress. *Antioxid. Redox Signal.* 23:375–405
149. Doussiere J, Gaillard J, Vignais PV. 1999. The heme component of the neutrophil NADPH oxidase complex is a target for aryliodonium compounds. *Biochemistry* 38:3694–703
150. Kanegae MP, Condino-Neto A, Pedroza LA, de Almeida AC, Rehder J, et al. 2010. Diapocynin versus apocynin as pretranscriptional inhibitors of NADPH oxidase and cytokine production by peripheral blood mononuclear cells. *Biochem. Biophys. Res. Commun.* 393:551–54
151. Koelsch M, Mallak R, Graham GG, Kajer T, Milligan MK, et al. 2010. Acetaminophen (paracetamol) inhibits myeloperoxidase-catalyzed oxidant production and biological damage at therapeutically achievable concentrations. *Biochem. Pharmacol.* 79:1156–64
152. Tiden AK, Sjogren T, Svesson M, Bernlind A, Senthilmohan R, et al. 2011. 2-Thioxanthines are suicide inhibitors of myeloperoxidase that block oxidative stress during inflammation. *J. Biol. Chem.* 286:37578–89
153. Forbes LV, Sjogren T, Auchere F, Jenkins DW, Thong B, et al. 2013. Potent reversible inhibition of myeloperoxidase by aromatic hydroxamates. *J. Biol. Chem.* 288:36636–47
154. Winterbourn CC. 2008. Reconciling the chemistry and biology of reactive oxygen species. *Nat. Chem. Biol.* 4:278–86
155. Peskin AV, Winterbourn CC. 2001. Kinetics of the reactions of hypochlorous acid and amino acid chloramines with thiols, methionine, and ascorbate. *Free Radic. Biol. Med.* 30:572–79
156. Winterbourn CC, Kettle AJ. 2000. Biomarkers of myeloperoxidase-derived hypochlorous acid. *Free Radic. Biol. Med.* 29:403–9
157. Harwood DT, Kettle AJ, Winterbourn CC. 2006. Production of glutathione sulfonamide and dehydroglutathione from GSH by myeloperoxidase-derived oxidants and detection using a novel LC-MS/MS method. *Biochem. J.* 399:161–68
158. Fu X, Mueller DM, Heinecke JW. 2002. Generation of intramolecular and intermolecular sulfenamides, sulfinamides, and sulfonamides by hypochlorous acid: a potential pathway for oxidative cross-linking of low-density lipoprotein by myeloperoxidase. *Biochemistry* 41:1293–301

159. Peskin AV, Turner R, Maghzal GJ, Winterbourn CC, Kettle AJ. 2009. Oxidation of methionine to dehydromethionine by reactive halogen species generated by neutrophils. *Biochemistry* 48:10175–82
160. Winterbourn CC, Metodiewa D. 1999. Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. *Free Radic. Biol. Med.* 27:322–28
161. Storkey C, Davies MJ, Pattison DI. 2014. Reevaluation of the rate constants for the reaction of hypochlorous acid (HOCl) with cysteine, methionine, and peptide derivatives using a new competition kinetic approach. *Free Radic. Biol. Med.* 73:60–66